Ficus septica plant extracts for treating Dengue virus *in vitro*

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ABSTRACT

Dengue virus types 1-4 (DENV-1-4) are positive-strand RNA viruses with an envelope that belongs to the *Flaviviridae*. DENV infection threatens human health worldwide. However, other than supportive treatments, no specific therapy is available for the infection. In order to discover novel medicine against DENV, we tested 59 crude extracts, without cytotoxicity, from 23 plants *in vitro*; immunofluorescence assay revealed that the methanol extracts of fruit, heartwood, leaves and stem from *Ficus septica* Burm. f. had a promising anti-DENV-1 and DENV-2 effect. However, infection with the non-envelope *picornavirus*, Aichi virus, was not inhibited by treatment with *F. septica* extracts. *F. septica* may be a candidate antiviral drug against an enveloped virus such as DENV.

Subjects Virology, Drugs and Devices
Keywords *Ficus septica*, Dengue virus, Aichi virus, Crude extract

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INTRODUCTION

Dengue fever is an acute infectious disease caused by dengue virus (DENV), which is transmitted by mosquitoes to humans; about 50 million people are infected per year worldwide (*Guzman et al., 2010*). According to different serotypes of the virus, four types (DENV types 1-4) are divided. Each type has the ability to cause disease. DENV infection causes varying degrees of disease manifestation, such as self-limited febrile dengue fever, skin rash or drowsiness, agitation, liver enlargement, or dengue hemorrhagic fever (DHF) and even death. A second DENV infection may lead a life-threatening dengue shock syndrome (DSS) (*Abel, Liautaud & Cabie, 2012*; *Kyle & Harris, 2008*; *Martina, Koraka &*

Osterhaus, 2009). Currently, no specific therapy is available for the infection other than supportive treatments (Guzman et al., 2010).

The identification and use of medicinal plants for treatment of various diseases has been done throughout human history. Certain medicinal plants also show antiviral activity, such as *Carissa edulis* Vahl against herpes simplex virus, *Geranium sanguineum* L. against influenza virus A, *Boehmeria nivea* L. against hepatitis B virus, *Saxifraga melanocentra* Engl. & Irmsch. against hepatitis C virus, *Lycoris radiata* (L'Hér.) Herb. against severe acute respiratory syndrome-associated coronavirus and *Phyllanthus amarus* Schum. & Thonn. against HIV (Mukhtar et al., 2008). In addition, the neem (*Azadirachta indica* A. Juss.) showed potential inhibition of DENV-2 replication (Parida et al., 2002). Thus, discovering a novel antiviral medicine from medical plants would be a promising strategy.

In this study, we collected 23 plants from among Taiwanese folk medicinal plants for screening anti-DENV herbs. We also investigated the antiviral effect on Aichi virus (AiV), a pathogenic *picornavirus* that causes gastroenteritis. Among these plants, we found that *F. septica* Burm. f. could be a potential medicinal plant against DENV.

MATERIALS AND METHODS

Virus and cell line

We used local Taiwanese strains of DENV-1 766733A and DENV-2 PL046 (Genbank accession no. AJ968413.1) isolated from patients with dengue fever (Lin et al., 1998). The viruses were propagated in mosquito cell line C6/36 (ATCC: CRL-1660) grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS). The human Aichi virus (AiV) was isolated from a newborn with diarrhea in Taiwan and propagated in Vero cells (ATCC: CCL-81) (Chen et al., 2013). Vero cell, A549 human lung epithelial carcinoma cells (ATCC: CCL-185) and Huh7.5 human hematoma cells (ATCC® PTA-8561™) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA). HepG2 human hepatocellular carcinoma cells (ATCC® HB-8065™) and WS1 human fetal skin normal fibroblasts (BCRC: 60300) were cultured in MEM medium supplemented with 10% FBS and non-essential amino acids (NEAA; Gibco, Thermo Fisher, Waltham, MA, USA).

Plant materials

All plants were purchased from a traditional herb shop or a Chinese medicinal herb shop in Taiwan. The plants were identified by one of the co-authors, Dr. Wei-Yu Lin (October, 2008 to May, 2014). Those plants are *Alisma orientalis* (Sam.) Juz., *Asparagus cochinchinensis* (Lour.) Merr., *Broussonetia papyrifera* (L.) L'Herit. *ex* Vent., *Catharanthus roseus* (L.) G. Don, *Clausena excavate* Burm. f., *Cinnamomum insulari-montanum* Hayata, *Cornus officinalis* Torr. ex Dur., *Euonymus japonicas* Thunb., *Elaeocarpus sylvestris* (Lour.) Poir., *Fraxinus griffithii* C. B. Clarke, *Ficus septica, Ficus sarmentosa* B. Ham. *ex* J. E. Sm. var. *henryi* (King *ex* D. Oliver) Corner, *Garcinia subelliptica* Merr., *Lumnitzera racemosa* Willd., *Litchi chinensis* Sonn., *Phytolacca americana* L., *Pueraria lobata* (Willd.) Ohwi ssp. *thomsonii* (Benth.) Ohashi & Tateishi, *Sida acuta* Burm. f., *Sambucus chinensis* Lindl., *Scrophularia ningpoensis* Hemsl, *Saurauia tristyla* var. *oldhamii*

(Hemsl.) Finet & Gagnep., *Tribulus terrestris* L., *Xanthium sibiricum* Patrin ex Widder and *Strophanthus divaricatus* (Lour.) Hook. Edt Arn. We included material from the whole plant, root, leaves, stem, fruit, pericarp, root bark, flower or heartwood.

Plant extracts

Materials of plant species were ground, extracted with the indicated solvent for one week. The extracts were concentrated under vacuum. Plant materials of the species no. 13 (leaves) was subjected to an additional one-step extraction with ethyl acetate (EtOAc) and filtered and dried as described before. The volumes (5 ml) of solvents were used per gram of plant material.

Cell proliferation assay

WST-1 assay (Roche, Basel, Switzerland) was used to monitor cell proliferation (*Chou et al., 2014*); cells were trypsinized and resuspended in culture medium, then plated at 5×10^3 cells per well in 96-well plates and incubated overnight. After plant extracts treatment for 48 h, the cells were incubated with 10 µl WST-1 reagent for 2 h. The cell viability was quantified by multi-well spectrophotometry (Anthos, Biochrom, Cambridge, UK). The absorbance at 450 nm was monitored, and the reference wavelength was set at 620 nm.

Treatment

In the extracts screening of DENV inhibition, the cells (5×10^3 cells) were treated with plant extracts with serial dilution dose of 100, 50, 25, 12.5, 6.25, 3.125 or 1.56 µg/ml or DMSO solvent control for 3 h. Then, these cells were infected by DENV-1, DENV-2 and AiV infection (multiplicity of infection [MOI] $= 2.5$). After 2 h virus adsorption, the medium mixture was replaced by fresh growth medium. At 42 h after infection, the virus-infected cells were analyzed by immunofluorescence assay. In another experiment, the viral stocks of DENV-1, DENV-2 and AiV were preincubated with a series of dilution doses of leaf methanol extract of *F. septica* (FS-(L)-M) for 1 h at room temperature. The mixture of virus plus the plant extract was then used to infect A549 cells.

Immunofluorescence assay

Immunofluorescence assay was conducted to determine the DENV and AiV infectivity as we previously described (*Chen et al., 2013*; *Wang et al., 2015*). In brief, cells were fixed with 4% paraformaldehyde for 30 min, then permeabilized with 0.5% Triton X-100 for 10 min. After two washes with phosphate buffered saline (PBS), cells were blocked with 10% skim milk in PBS. The cells infected with DENV or AiV were detected by antibody against NS3 (Yao-Houng, Biotechnology, Taipei) or anti-AiV VP1 antibody followed by IRDye 800 CM goat anti-mouse or -rabbit IgG (Li-Cor, Lincoln, NE, USA) or Alexa fluor 488 conjugated anti-mouse IgG (Thermo Fisher, Waltham, MA, USA) . The fluorescence intensity was measured and quantified by using the Li-Cor odyssey CLx imaging system or fluorescence microscopy (Zeiss, AX10).

Statistical analysis

Significant differences between groups were analyzed by 2 tailed Student *t* test with the software GraphPad Prism 6 (La Jolla, CA, USA). Data are presented as mean ± SD.

$P < 0.05$ was considered statistically significant. The statistical datasets are showed in the supplementary information.

RESULTS AND DISCUSSION

We aimed to reveal a medicinal plant candidate against DENV. We extracted 70 different crude compounds from materials of 24 plants. DENV-caused respiratory disease was revealed (*Rodrigues et al., 2014*; *Wang et al., 2007*), in addition, our previous study showed that the lung carcinoma A549 cells were well susceptible target cells for DENV, these cells have been used in the study model of viral-host interaction. Thus, A549 cells were applied as the screening model in this study (*Chang, Liao & Lin, 2006*). The cytotoxic effect of the extracts was evaluated in lung carcinoma A549 cells by WST-1 cell proliferation assay. Except *S. divaricatus,* extracts from other 23 plants revealed no cytotoxicity effect at the maximum tested concentration, 100 μg/ml. Therefore, *S. divaricatus* extracts were excluded in the antiviral screening (Supplementary information, Table S1).

The immunofluorescence results indicated that the *F. septica* materials root bark acetone (FS-(RB)-A) and fruit methanol extracts (FS-(Fr)-M), heartwood methanol extract (FS-(HW)-M), leaf acetone and methanol extracts (FS-(L)-A, FS-(L)-M), and stem methanol extract (FS-(S)-M) significantly inhibited DENV-2 infection, with IC_{50} from 3.05 ± 0.75 to 37.46 ± 12.3 μg/ml (Table 1). In addition, leaf extracts of *F. sarmentosa* var. *henryi* showed an anti-DENV-2 effect, with IC_{50} 72.04 ± 14.5 μg/ml, which was higher than for the extracts of *F. septica* (Table 1).

In addition to DENV-2, the DENV-1 was inhibited by FS-(L)-M in A549 cells ($IC_{50} = 28 \pm 10.4$ μg/ml); however, the AiV infection was not affected by FS-(L)-M treatment (Figs. 1A and 1B). DENV infection-mediated liver disorder was reported (*Samanta & Sharma, 2015*; *Tristao-Sa et al., 2012*), therefore, the *F. septica* against DENV1 and DENV-2 but not AiV were also confirmed in the hematoma cell lines, HepG2 and Huh7.5 cells (Figs. 1C and 1E). Interestingly, FS-(L)-M showed more potent anti-DENV-1 and DENV-2 effect in HepG2 cells ($IC_{50} = 10.1 \pm 2.4$ μg/ml and 12.2 ± 2.1 μg/ml, respectively) then in Huh7.5 cells ($IC_{50} = 39.8 \pm 6.9$ μg/ml and 21.9 ± 3.9 μg/ml, respectively) (Figs. 1D and 1F). Moreover, skin normal fibroblasts (*Wang et al., 2015*), WS1 cells were used as the non-cancerous cells for testing the anti-viral effect of FS-(L)-M. The similar results showed that the FS-(L)-M inhibited DENV-1 and DENV-2 in WS1 cells with IC_{50} 13.3 ± 2.6 μg/ml and 10.6 ± 1.1 μg/ml, respectively (Figs. 1G and 1H). A higher dose of *F. septica* (IC_{50} 41.1 ± 6.7 μg/ml) against AiV was determined in WS1 cells, which was not showed in other tested cells types. This data implicated a cell type-specific manner of AiV inhibition by *F. septica*. However, the precise mechanism remains to be further explored.

In order to understand whether *F. septica* leaf extracted with methanol, FS-(L)-M, directly inactivated DENV, we preincubated viral stocks of DENV-1, DENV-2 and AiV with a series of dilution doses of FS-(L)-M for 1 h at room temperature. After virus adsorption, the mixture of virus plus the plant extract was replaced by fresh growth medium. At 42 h after infection, the virus-infected cells were analyzed by immunofluorescence assay

Table 1 The IC_{50} of plant crude extracts against Dengue virus type 2 infection.

No.	Botanical name	Part of plant	Extract	Abbreviation of crude extract	IC_{50} (μg/ml) of viral inhibition	*P* value (IC_{50} vs. Ctrl)*
1	*Alisma orientalis* (Sam.) Juz.	Whole plant	Methanol	Ao-(WP)-M	>100	
2	*Asparagus cochinchinensis* (Lour.) Merr.	Root	Methanol	ACM (R)-M	>100	
3	*Broussonetia papyrifera* (L.) L'Herit. *ex* Vent.	Leaves	Methanol	BP (L)-M	>100	
4	*Catharanthus roseus* (L.) G. Don	Whole plant	Methanol	CaR-(WP)-M	>100	
5	*Clausena excavata* Burm. f.	Leaves	Methanol	Ce-(L)-M	>100	
6	*Cinnamomum insulari-montanum* Hayata	Leaves	Methanol	CiM-(L)-M	>100	
7	*Cornus officinalis* Torr. ex Dur.	Whole plant	Acetone	CO-(WP)-A	>100	
		Whole plant	Methanol	CO-(WP)-M	>100	
8	*Euonymus japonicus* Thunb.	Leaves	Acetone	EJa-L-A	>100	
9	*Elaeocarpus sylvestris* (Lour.) Poir.	Leaves	Acetone	ES-(L)-A	>100	
		Leaves	Chloroform	ES-(L)-C	>100	
		Leaves	Methanol	ES-(L)-M	>100	
		Stem	Methanol	ES-(S)-M	>100	
10	*Fraxinus griffithii* C. B. Clarke	Leaves	Acetone	FG-(L)-A	>100	
		Leaves	Chloroform	FG-(L)-C	>100	
		Leaves	Methanol	FG-(L)-M	>100	
11	*Ficus septica* Burm. f.	Root Bark	Acetone	FS-(RB)-A	**3.05 ± 0.75**	**<0.001**
		Leaves	Methanol-Ethyl acetate	FS-(L)-M-ET	**24.62 ± 4.04**	**<0.001**
		Fruit	Methanol	FS-(F)-M	**37.46 ± 12.3**	**<0.001**
		Heartwood	Methanol	FS-(HW)-M	**24.07 ± 13.18**	**<0.001**
		Leaves	Acetone	FS-(L)-A	**25.58 ± 9.13**	**<0.001**
		Leaves	Chloroform	FS-(L)-C	>100	
		Leaves	Methanol	FS-(L)-M	**18.37 ± 10.6**	**<0.001**
		Stem	Methanol	FS-(S)-M	**35.64 ± 21.2**	**<0.001**
12	*Ficus sarmentosa* B. Ham. *ex* J. E. Sm. var. *henryi* (King *ex* D. Oliver) Corner	Leaves	Acetone	FSVH-(L)-A	72.04 ± 14.5	<0.05
		Leaves	Chloroform	FSVH-(L)-C	>100	
		Leaves	Methanol	FSVH-(L)-M	>100	
		Stem	Methanol	FSVH-(S)-M	>100	
13	*Garcinia subelliptica* Merr.	Flower	Methanol	GS-(F)-M	>100	
14	*Lumnitzera racemosa* Willd.	Leaves	Methanol	Lr-(L)-M	>100	

(*continued on next page*)

Table 1 (*continued*)

No.	Botanical name	Part of plant	Extract	Abbreviation of crude extract	IC_{50} (μg/ml) of viral inhibition	*P* value (IC_{50} vs. Ctrl)*
15	*Litchi chinensis* Sonn.	Leaves	Acetone	LC-(L)-A	>100	
		Leaves	Chloroform	LC-(L)-C	>100	
		Leaves	Methanol	LC-(L)-M	>100	
		Stem	Acetone	LC-(S)-A	>100	
		Stem	Chloorform	LC-(S)-C	>100	
		Stem	Methanol	LC-(S)-M	>100	
		Fruit	Acetone	LC-(FR)-A	>100	
		Pericarp	Acetone	LC-(Peri)-A	>100	
		Pericarp	Methanol	LC-(Peri)-M	>100	
16	*Phytolacca americana* L.	Whole plant	Acetone	PA-(WP)-A	>100	
		Whole plant	Chloroform	PA-(WP)-C	>100	
		Whole plant	Methanol	PA-(WP)-M	>100	
17	*Pueraria lobata* (Willd.) Ohwi ssp. *thomsonii* (Benth.) Ohashi & Tateishi	Whole plant	Methanol	PL -(WP)-M	>100	
18	*Sida acuta* Burm. f.	Whole plant	Methanol	Sa-(WP)-M	>100	
19	*Sambucus chinensis* Lindl.	Whole plant	Acetone	Scl-(WP)-A	>100	
		Whole plant	Chloroform	Scl-(WP)-C	>100	
		Whole plant	Methanol	Scl-(WP)-M	>100	
20	*Scrophularia ningpoensis* Hemsl	Whole plant	Methanol	SN-(WP)-M	>100	
21	*Saurauia tristyla* var. *oldhamii* (Hemsl.) Finet & Gagnep.	Leaves	Chloroform	STV-(L)-C	>100	
		Leaves	Methanol	STV-(L)-M	>100	
		Leaves	Acetone	STV-(L)-A	>100	
22	*Tribulus terrestris* L.	Fruit	Acetone	TT-(Fr)-A	>100	
		Fruit	Methanol	TT-(Fr)-M	>100	
		Fruit	Chloroform	TT-(Fr)-C	>100	
		Fruit	Methanol	TT-(Fr)-M	>100	
		Whole plant	Acetone	TT-(WP)-A	>100	
		Whole plant	Chloroform	TT-(WP)-C	>100	
		Whole plant	Methanol	TT-(WP)-M	>100	
23	*Xanthium sibiricum* Patrin ex Widder	Fruit	Chloroform	XS-(Fr)-C	>100	
		Fruit	Methanol	XS-(Fr)-M	>100	

Notes.

*$P < 0.05$ estimated by 2-tailed Student *t* test (IC_{50} vs. Control).

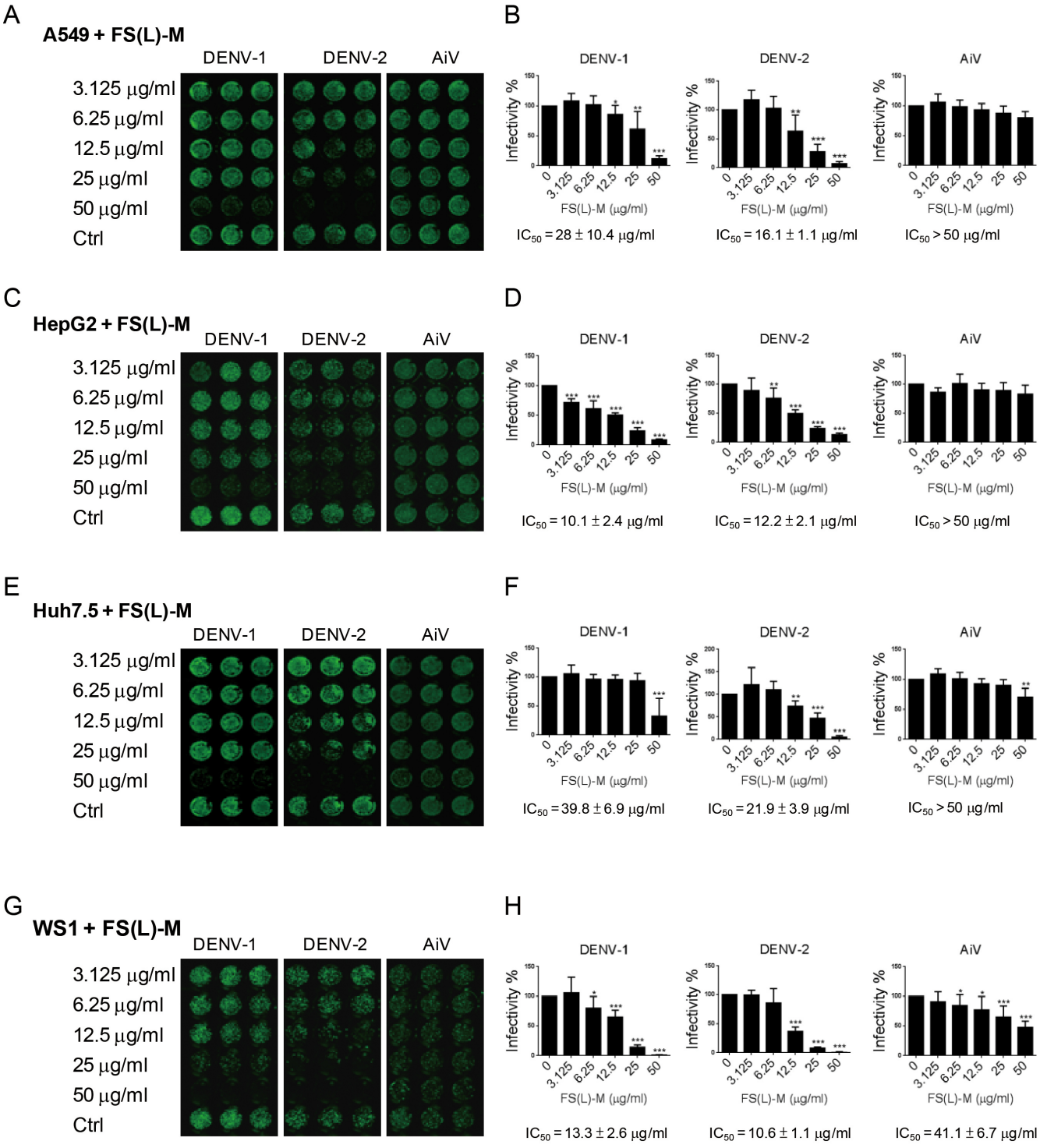

Figure 1 ***Ficus septica* leaf methanol extract inhibits DENV infection in various cell types.** (A, C, E and G) A549, HepG2, Huh7.5 and WS1 cells (3×10^4) were incubated with various doses of *F. septica* leaf methanol extract (FS-(L)-M, 3.125∼50 μg/ml) or DMSO solvent control (Ctrl) for 3 h before dengue virus type I and type II (DENV-1, DENV-2) and Aichi virus (AiV) infection at MOI = 2.5. After 2 h of virus adsorption and 42 h incubation, the immunofluorescence assay was performed to detect the viral infected cells. (B, D, F and G) Fluorescence intensity was measured and quantified by the Li-Cor odyssey CLx imaging system. The IC_{50} of FS-(L)-M on virus inhibition was indicated. The data are mean ± SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed Student t test.

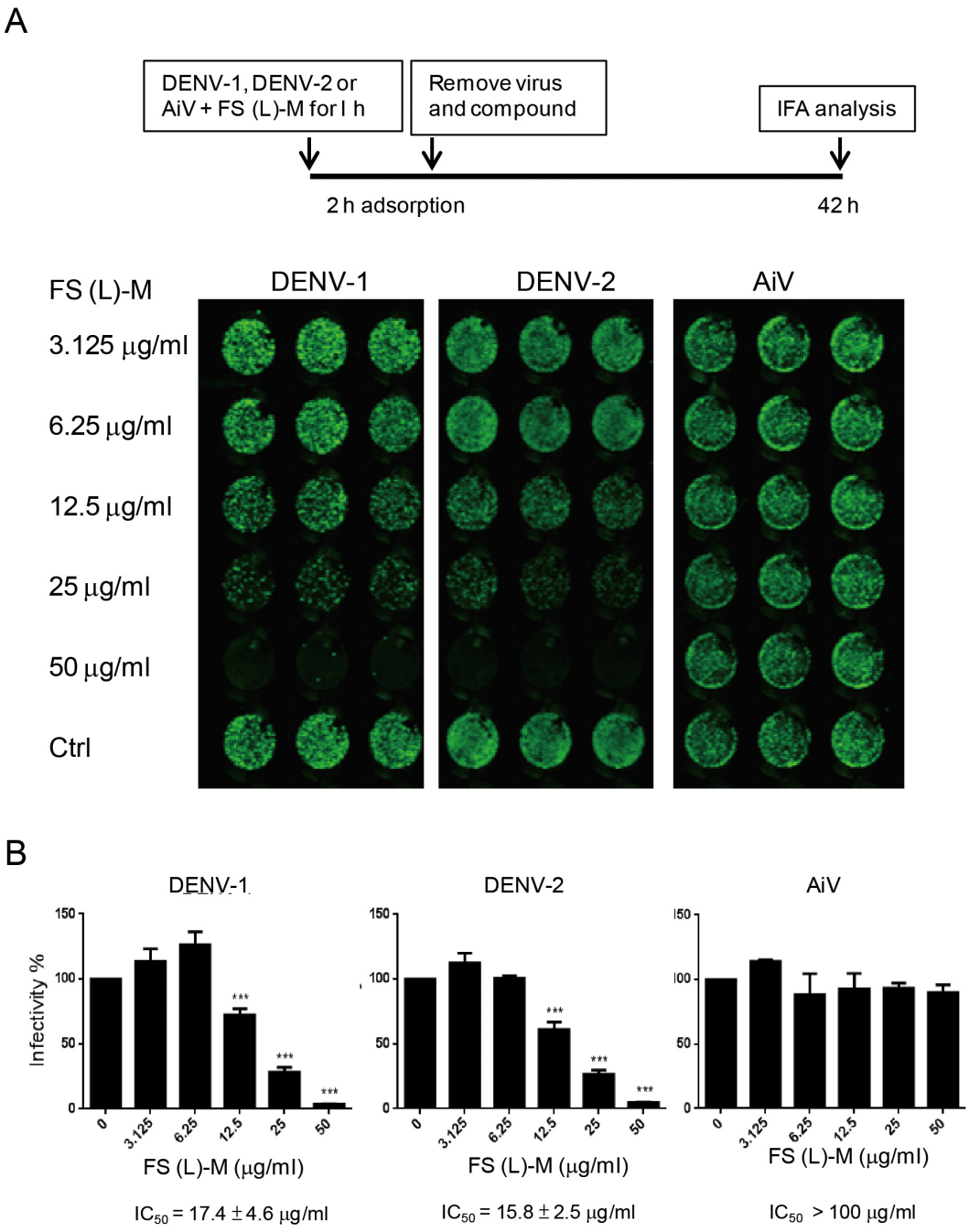

Figure 2 Leaf methanol extract of *Ficus septica* inhibits enveloped viral infection. (A) DENV-1, DENV-2 and AiV viral stocks were incubated with various doses of FS-(L)-M for 1 h at room temperature before used to infect A549 cells (3×10^4) at MOI = 2.5. After 2 h of virus adsorption, the virus–compound mixture medium was replaced by fresh growth medium for further 42 h incubation. DENV- or AiV-infected cells were detected by immunofluorescence assay. (B) Fluorescence intensity was measured and quantified by the Li-Cor odyssey CLx imaging system. The data are mean ± SD from three independent experiments. *** $P < 0.001$ by two-tailed Student t test.

(Fig. 2A). FS-(L)-M significantly inhibited DENV-1 ($IC_{50} = 17.4 \pm 4.6$ μg/ml) and DENV-2 ($IC_{50} = 15.8 \pm 2.5$ μg/ml) but not AiV (Fig. 2B). Thus, FS-(L)-M directly impaired enveloped viral particles but not non-enveloped virus.

In this study, *F. septica* extracts had a promising anti-DENV-1 and -DENV-2 effect. Nevertheless, the non-enveloped *picornavirus* AiV was not efficiently inhibited by *F. septica* extract. Thus, *F. septica* would be a possible antiviral drug candidate against enveloped virus, such as DENV.

F. septica, a member of the family *Moraceae*, is widely distributed in the tropic and subtropic regions of the Western Pacific area (*Weekly Asahi Encyclopedia, 1995*). In Papua New Guinea, this plant has been used as a medicine to treat illnesses such as cold, fever, gastralgia and fungal and bacterial disease (*Holdsworth, Hurley & Rayner, 1980*). Several bioactive compounds from *F. septica* identified include phenanthroindolizidine and aminocarophenone- and pyrrolidine-type alkaloids (*Damu et al., 2005*; *Damu et al., 2009*; *Ueda, Takagi & Shin-ya, 2009*). Among them, compounds of ficuseptine, 4,6-*bis*-(4-methoxyphenyl)-1,2,3-trihydroindolizidinium chloride and antofine isolated from methanolic extracts of *F. septica* leaves showed strong antibacterial and antifungal activities (*Baumgartner et al., 1990*). In addition, some alkaloids, including dehydrotylophorine, dehydroantofine and tylophoridicine, isolated from methanolic extracts of *F. septica* twigs showed antimalarial activity (*Kubo et al., 2016*).

Here, we reveal a new bioactivity of *F. septica* against dengue virus. Importantly, an enveloped virus but not non-enveloped virus is sensitive to the extract pretreatment, which suggests that certain compounds of *F. septica* might disrupt the DENV envelope structure or interfere with DENV contacting cells. Moreover, the anti-DENV effect of *F. septica* was demonstrated in the lung and liver cell types with clinical relevant.

F. septica may be a promising medical plant against DENV. The *F. septica* materials root bark acetone and leaf methanol extracts showed the best anti-DENV efficacy, further identification of the antiviral compounds from these two parts of *F. septica* would be important for drug development.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Nan-Chieh Huang performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Wan-Ting Hung and Wei-Lun Tsai performed the experiments, analyzed the data, reviewed drafts of the paper.
- Feng-Yi Lai, Jih-Jung Chen and Wei-Yu Lin contributed reagents/materials/analysis tools.
- You-Sheng Lin and Mei-Shu Huang performed the experiments, analyzed the data, prepared figures and/or tables.
- Jing-Ru Weng and Tsung-Hsien Chang conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

The raw data for Table 1, Figure 1, and Figure 2 has been supplied as Supplementary Files. The raw datasets are included in the Material and Methods section of the manuscript.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3448#supplemental-information.

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