



Original Article

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Antiviral activity of *Sonchus arvensis* L. extracts on dengue virus replicationNurin Naziha Zulkefle¹, Nurhafiza Zainal^{1✉}, Dwi Kusuma Wahyuni^{2✉}, Mahmood Syed³, Kim Ling Chin^{1,4✉}¹Department of Medical Microbiology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia²Department of Biology, Faculty of Science and Technology, Universitas Airlangga Surabaya, Surabaya, East Java, Indonesia³Department of Pharmaceutical Technology, Faculty of Pharmacy, Universiti Malaya, Kuala Lumpur, Malaysia⁴Institute for Advanced Studies, Universiti Malaya, Kuala Lumpur, Malaysia

ABSTRACT

Objective: To investigate the antiviral potential of *Sonchus arvensis* L. extracts against dengue virus (DENV)-1 and DENV-2 *in vitro*, and elucidate its underlying antiviral mechanisms.

Methods: The cytotoxicity of the ethanol, ethyl acetate, and *n*-hexane extracts of *Sonchus arvensis* L. was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, followed by post-treatment antiviral assays. Multiple treatment regimens were conducted to assess antiviral effects at different stages of the DENV replication cycle. qRT-PCR analysis was performed to evaluate the effects of the ethanol extract on genes associated with host innate immune and inflammatory responses.

Results: The CC₅₀ values of the ethanol, ethyl acetate, and *n*-hexane extracts were 391.5 µg/mL, 104.9 µg/mL, and 409.2 µg/mL, respectively. The ethanol and ethyl acetate extracts reduced DENV-1 and DENV-2 titers by >90% at 30–50 µg/mL, while the *n*-hexane extract showed comparable activity against DENV-2. The ethanol extract at 30 µg/mL demonstrated potent multi-stage antiviral activity, including direct virucidal, prophylactic, anti-adsorption, and anti-internalization effects. qRT-PCR analysis revealed no significant change in *IFN-β* expression but marked suppression of *NF-κB*, *TNF-α*, and *IL-6* expression.

Conclusions: *Sonchus arvensis* L. ethanol extracts demonstrate potent multi-stage antiviral activity against DENV *in vitro*, highlighting its ability to inhibit viral entry, reduce viral replication, and attenuate excessive inflammatory responses.

KEYWORDS: Antiviral; Dengue; Dengue virus; *Sonchus arvensis* L.; Anti-inflammatory; NF-κB

1. Introduction

Dengue has re-emerged as a global public health threat, particularly impacting populations in tropical and subtropical regions. It remains the most prevalent and significant arboviral disease in humans, with an estimated 40% of people worldwide at risk of infection annually. The widespread burden of dengue is driven primarily by rapid urbanization, increased international travel, and climate change,

Summary

Question: Can *Sonchus arvensis* L. extracts inhibit dengue virus (DENV) replication, and what mechanisms underlie their antiviral activities?

Findings: *Sonchus arvensis* L. ethanol extracts demonstrated potent anti-DENV activity, achieving more than 90% inhibition of viral replication. Mechanistic assays indicated that the extracts interfered with multiple stages of the viral life cycle and suppressed the gene expression of pro-inflammatory cytokines, including *NF-κB*, *TNF-α*, and *IL-6*, without affecting *IFN-β* expression.

Meaning: *Sonchus arvensis* L. shows potential as an anti-dengue candidate. Its antiviral activity may be associated with inhibition of multiple stages of DENV infection and modulation of pro-inflammatory cytokines.

✉To whom correspondence may be addressed. E-mail: kimling@um.edu.my (KL. Chin); nurhafizazainal@um.edu.my (N. Zainal); dwi-k-w@fst.ac.id (DK. Wahyuni).

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which collectively accelerate the transmission and geographic expansion of both the virus and its vector into previously unaffected regions, leading to substantial economic and public health challenges worldwide[1].

Dengue fever is caused by infection with dengue virus (DENV), an enveloped, positive-sense single-stranded RNA virus belonging to the genus *Flavivirus* within the family Flaviviridae. The virus comprises four antigenically distinct serotypes (DENV-1 to DENV-4), which are differentiated based on amino acid sequence variability and genotypic characteristics[2]. Clinically, DENV infection ranges from asymptomatic cases to severe and potentially fatal disease manifestations. Early symptoms, including fever and headache, are non-specific and often overlap with other flaviviral infections such as Japanese encephalitis virus and Zika virus, complicating early clinical diagnosis.

Spanning approximately 11 kb in length, the DENV genome encodes a single polyprotein, which is subsequently cleaved into three structural proteins [capsid (C), pre-membrane/membrane (prM) and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The structural proteins are involved in virion assembly and host cell entry, with the E protein mediating receptor binding and membrane fusion, whereas the non-structural proteins play critical roles in viral replication, polyprotein processing, and evasion of host immune responses[2,3]. The viral life cycle begins with an early phase comprising attachment of the E protein to host cell surface receptors. These receptors can be broadly classified into two distinct molecular groups: glycosaminoglycan, particularly heparan sulfate; and protein receptors with different molecular masses, including cluster of differentiation 14-associated protein, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, heat shock proteins 70 and 90. Interactions between the E protein and these receptors determine the efficiency of viral attachment, cellular entry, and tissue tropism. Following attachment and internalization occurring within the first 2.5 hours post-infection, the virus enters a late phase of intracellular replication and assembly within the cytoplasm, leading to the release of progeny virions at approximately 12 to 24 hours post-infection, depending on the cell type[2–4].

The immunopathology of DENV infection is characterized by a dysregulated host immune response. Upon infection, DENV is recognized by pattern recognition receptors, which activate two key innate immune signaling pathways: type I interferon (IFN-I) and nuclear factor kappa B (NF- κ B). The IFN-I pathway mediates the early antiviral response, leading to interferon production and induction of interferon-stimulated genes that establish an antiviral state in infected and neighbouring cells[5]. Meanwhile, as a key regulator of inflammation, NF- κ B controls the expression of numerous cytokines and chemokines involved in the innate immune response. However, DENV can exploit NF- κ B signaling to drive overproduction of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), contributing

to the cytokine storm that causes severe dengue complications, including vascular leakage, hemorrhagic diathesis, and organ dysfunction[6–8].

Given the significant disease burden and the complex host-pathogen interactions underlying dengue pathogenesis, effective preventive and therapeutic strategies remain a global priority. Although licensed dengue vaccines such as Qdenga® and Dengvaxia® have demonstrated effectiveness against all four DENV serotypes, their widespread implementation remains limited by safety concerns and variable effectiveness across different populations. In particular, the risk of antibody-dependent enhancement, a phenomenon in which sub-neutralizing antibodies worsen disease severity upon heterologous secondary infection, continues to pose challenges to vaccine deployment and long-term dengue control strategies[9]. Furthermore, the absence of a Food and Drug Administration-approved antiviral drug that is effective against all serotypes with an acceptable safety profile remains a critical gap in dengue management[10]. Despite extensive vector control, dengue incidence remains persistently high, highlighting the inadequacy of prevention-focused strategies and reinforcing the critical need for novel antiviral agents.

One such alternative for treating viral infections is the utilization of potential medicinal plants, which have demonstrated diverse antiviral mechanisms and represent a promising source of natural therapeutic agents. Plant extracts, in particular, offer a rich reservoir of novel bioactive compounds with potential medicinal properties that can be exploited for the development of synthetic drugs with enhanced efficacy and reduced toxicity[11]. Owing to their lower toxicity, minimal side effects, and cost-effectiveness, plant extracts have been extensively screened for antiviral activity. Notably, numerous studies have reported promising anti-DENV activity demonstrated by various bioactive-enriched extracts from medicinal plants, including *Cocculus hirsutus*, *Cissampelos pareira*, *Carica papaya*, and *Glycyrrhiza glabra*[12]. Therefore, plant-derived antivirals hold strong potential as alternative treatment strategies for dengue infection.

Among the various traditional medicinal plants, *Sonchus arvensis* (*S. arvensis*) L., a highly invasive species native to Europe, northern Africa, and Asia, is traditionally used to treat ailments such as malaria and kidney stones[13]. Recent research has increasingly recognized its pharmacological properties, including anti-diabetic[14], anti-inflammatory[15], and antiviral activities[16]. Notably, *S. arvensis* L. has shown potential antiviral effects against SARS-CoV-2 *in silico*, which is attributed to the presence of dual inhibitors, α -amyrin and β -amyrin[16]. Despite its extensive medicinal use, the antiviral activity of *S. arvensis* L. against DENV has not been investigated, and its mechanism of action in inhibiting viral replication has yet to be elucidated. Given the absence of specific antiviral therapies for dengue and the urgent need for effective treatment options, this study aimed to investigate the *in vitro* antiviral effects of *S. arvensis* L. extracts against DENV-1 and DENV-2 infections in Huh7 hepatocellular cells and to elucidate the underlying mechanisms of

viral inhibition. In particular, this study focused on the IFN- β and NF- κ B signaling pathways, which play central roles in antiviral defense and DENV-induced immunopathology, making them key targets for understanding host-virus interactions. Overall, this work seeks to provide novel insights into the antiviral potential of *S. arvensis* L. and its relevance as a source of plant-derived therapeutic agents for dengue infection.

2. Methods

2.1. Cells and viruses

In this study, two cell lines were utilized: human hepatocellular carcinoma cells (Huh7; iCell h080) were maintained for cell cytotoxicity and antiviral assays, whereas African green monkey kidney cells (Vero; ATCC CCL-81) were used for the propagation and titration of DENV viruses. The liver is one of the primary targets of DENV infection and is the most common organ involved in the disease[17]. Huh7 cells were used to conduct the cytotoxicity and antiviral assays. On the other hand, Vero cells were used due to their high susceptibility to DENV infection and support efficient DENV replication. Both Huh7 and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), L-glutamine growth medium (Gibco, NY, USA), and penicillin-streptomycin (Gibco, NY, USA) under standard conditions. Two DENV serotypes were used in this study: DENV-1, a clinical isolate obtained from the WHO Collaborating Center at the University of Malaya, and DENV-2 strain PL046. These DENV serotypes were selected for this study due to their predominant circulation and epidemiological significance in recent years. Both DENV-1 and DENV-2 were propagated in a monolayer of Vero cells. The virus-containing supernatant was harvested, centrifuged for 5 min at 1600 $\times g$, and sterile-filtered (0.22 μ m filter) prior to being aliquoted into the screw cap tubes. The harvested supernatant was titrated *via* a focus-forming assay (FFA) and kept at -80°C until further use.

2.2. *S. arvensis* L. extracts

Leaves of *S. arvensis* L. were used as the plant material for extract preparation. Three leaf extracts prepared using different extraction solvents (ethanol, ethyl acetate and *n*-hexane) were kindly provided by Department of Biology, Faculty of Science and Technology, Universitas Airlangga Surabaya. Each dried crude extract (10 mg) was completely dissolved in 1 mL of dimethyl sulfoxide (DMSO; Macklin Biochemical, Shanghai, China) to prepare stock solutions at a concentration of 10 mg/mL. The stock solutions were then kept at -20°C until further use. The working concentrations were achieved by diluting the stock solutions in 2% heat-inactivated FBS-DMEM.

2.3. FFA

FFA was employed for quantifying the infectious virus particles[18]. Serially diluted virus supernatant was added to confluent Vero cells, followed by virus adsorption on a rocker at room temperature for 80 min. Subsequently, the supernatants from each well were discarded and overlaid with medium containing 2% carboxymethylcellulose and 2% FBS-DMEM for 96 h at 37°C until viral foci formed. Following incubation, 4% paraformaldehyde was added to fix the cells. After 30 min, the cells were rinsed and permeabilized with 1% Igepal for 15 min at room temperature. Then, 3% skim milk was added to block non-specific binding for 2 h at room temperature, followed by incubation of the cells with DENV-positive human serum (primary antibody; 1:500) for 80 min, before the addition of peroxidase-conjugated goat anti-human-IgG (secondary antibody; 1:250) (Sigma, USA) for 80 min at 37°C . Metal-enhanced diaminobenzidine was added to the immunostained cells to visualize the viral foci. The viral titers were calculated by counting the number of virus foci that appeared and represented as focus-forming units per mL (FFU/mL).

2.4. Cytotoxicity assay (MTS assay)

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (Promega, Madison, WI) was employed to determine the non-cytotoxic concentration range of *S. arvensis* L. extracts for subsequent antiviral evaluations. Monolayer confluent Huh7 cells in a 96-well plate were treated with various doses of different types of *S. arvensis* L. extracts (ethanol, ethyl acetate, *n*-hexane) up to 1000 $\mu\text{g/mL}$. Following incubation with extracts for 72 h, 20 μL of MTS solution was added directly into the well. The absorbance was determined after 2 h of incubation with an Infinite F50 microplate reader (Tecan, Männedorf, Switzerland) at $\lambda = 490$ nm. The half-maximal cytotoxic concentration (CC_{50}) and maximum non-toxic dose (MNTD) values were calculated using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

2.5. Post-infection treatment

Monolayer Huh7 cells in a 24-well plate were rinsed with $1\times$ PBS, followed by infection with DENV-1 or DENV-2 at a multiplicity of infection (MOI) of 0.5. After infection, the cells were either mock-treated or subjected to increasing doses of ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. extracts (10, 30, and 50 $\mu\text{g/mL}$). The concentration range of the extracts was selected based on non-toxic levels established in prior cytotoxicity assays. Viral titers were assessed using FFA after 48 h of incubation. The ratio of the CC_{50} to the inhibitory concentration 50% (IC_{50}) was used to calculate the selectivity index (SI). Antiviral efficiency of the extracts was calculated in percentage using the formula: $(\text{Virus titer}_{\text{Virus control group}} - \text{Virus titer}_{\text{Treated group}}) / \text{Virus titer}_{\text{Virus control group}} \times 100$.

2.6. Virucidal assay

The purpose of this assay is to determine if the extract can directly inactivate or neutralize extracellular virus particles. To evaluate the virucidal effect of *S. arvensis* L. on DENV, 30 µg/mL of *S. arvensis* L. ethanol extract was pre-incubated with the virus inoculum (DENV-1/DENV-2 at an MOI of 0.5) for 1 h. Then, confluent Huh7 cells were infected or mock-infected with the virus-extract mixture at 37°C before being discarded and supplemented with fresh DMEM containing 2% FBS. Supernatants were harvested for virus titer determination using FFA as previously described.

2.7. Prophylactic assay

The prophylactic effects of 30 µg/mL of *S. arvensis* L. ethanol extract were assessed based on a modified protocol[19]. This assay evaluates whether pre-treating host cells with the extract can induce a protective state that prevents the virus from successfully establishing an infection upon subsequent exposure. Huh7 cells were pre-treated with 30 µg/mL of *S. arvensis* L. ethanol extract for 5 h, washed with 1×PBS, and infected with either DENV serotypes (MOI 0.5) at 37°C. After unbound viruses were removed, the cells were incubated in media with 2% FBS under standard conditions for 48 h. Supernatants were collected for virus titration as previously described.

2.8. Anti-adsorption assay

The inhibitory effect of the extract on the initial attachment and binding of the virus to receptors on the host cell was assessed through the anti-adsorption assay with a modified protocol[19]. The virus inoculum (DENV-1/DENV-2) at an MOI of 0.5, prepared in culture medium supplemented with or without 30 µg/mL of *S. arvensis* L. ethanol extract, was added onto Huh7 cells. After the cells were incubated for 90 min at 4°C, they were subsequently rinsed with PBS and incubated with DMEM for 48 h before being subjected to FFA for virus quantification.

2.9. Virus internalization inhibition

To assess whether the extract blocks DENV internalization into host cells after attachment, 30 µg/mL of *S. arvensis* L. ethanol extract was subjected to a virus internalization assay following the methodology previously described by Mohd *et al.*[20]. Huh7 cells were pre-chilled at 4°C for 1 h, followed by infection with virus solutions (DENV-1/DENV-2 at an MOI of 0.5) at 4°C. After removing unbound virus with PBS, the cells were incubated with media containing 30 µg/mL of *S. arvensis* L. ethanol extract, or control media for 1 h at 37°C, rinsed and subsequently treated briefly with citrate buffer for 2 min. Following incubation, fresh media were added to the cells for 48 h at 37°C. The supernatants were

collected for estimation of virus titers as previously described.

2.10. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

qRT-PCR was performed to quantify the gene expression of host innate antiviral in mock-treated or treated DENV-infected Huh7 cells. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, USA), following the manufacturer's protocol. The extracted RNA served as the template for one-step qRT-PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) and the SensiFAST SYBR Hi-ROX One-Step Kit (Bioline, Boston, MA, USA). For viral RNA quantification, 2 µL of RNA was used with serotype-specific primers targeting DENV-1 and DENV-2, respectively. Direct amplification of DENV-1 genome was performed by targeting a 625-base pair (bp) region within the structural E protein gene, corresponding to nucleotides 1056 to 1680 of the reference polyprotein coding sequence. On the other hand, direct amplification of the DENV-2 genome targeted a 455-bp region encompassing the 5' untranslated region (5' UTR) and the proximal region of the C protein gene.

To elucidate the antiviral mechanism of 30 µg/mL of *S. arvensis* L. ethanol extract on the expression of innate antiviral genes, primers targeting human interferon-beta (*IFN-β*), *NF-κB*, *TNF-α*, and *IL-6* were used (Integrated DNA Technologies, San Diego, CA). The cycling parameters were optimized as follows: 10 min of reverse transcription at 45°C, 2 min of polymerase denaturation at 95°C, 10 s of annealing at 60°C, and 5 s of elongation at 72°C. Gene expression levels were presented as fold induction relative to the control group and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which served as an internal reference gene (Integrated DNA Technologies, San Diego, CA). The primers targeting *DENV-1*, *DENV-2*, *IFN-β*, *NF-κB*, *IL-6*, and *TNF-α* employed in this study are listed in Table 1. Each sample was analyzed in duplicate from three independent experiment.

Table 1. Primer sequences used for qRT-PCR.

Gene	Primers (5'→3')
<i>DENV-1</i>	Forward: TGGACATTGAACCTCTGAAGACGG Reverse: TCCTGCTTCTTTCATGAGCTG
<i>DENV-2</i>	Forward: CGTGGACCGACAAAGACAGA Reverse: TGGTTCTCCGTTACGTGTGG
<i>IFN-β</i>	Forward: TAGCACTGGCTGGAATGAGA Reverse: TCCTTGGCCTTCAGGTAATG
<i>NF-κB</i>	Forward: ATGTGGAGACATTGAGCAGC Reverse: CCTGGTCTGTGTAGCCATT
<i>TNF-α</i>	Forward: CCCATGTTGTAGCAAACCCTC Reverse: GCTGGTTATCTCTCAGCTCCAC
<i>IL-6</i>	Forward: CTATGAACTCCTTCTCCACAAGCGCCTT Reverse: GGGGCGGCTACATCTTTGGAATCTT
<i>GAPDH</i>	Forward: CAGCCTCAAGATCATCAGCA Reverse: TGTGGTCATGAGTCCTTCCA

2.11. Statistical analysis

Data analysis, including absorbance measurements, virus titer reduction, and gene expression levels, was performed with GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA). Non-linear regression, one-way or two-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* tests were conducted for statistical evaluation. The data are expressed as mean $\pm$ SEM. Differences between the groups will be considered significant when a *P* value is less than 0.05.

3. Results

3.1. Cytotoxicity effects of *S. arvensis* L. extracts on Huh7 cells

The potential cytotoxic effects of ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. on Huh7 cells were evaluated using the MTS cytotoxicity assay (Figure 1). The cells were treated with increasing concentrations (6.25 μ g/mL to 1000 μ g/mL) of ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. Among the extracts, the *n*-hexane extract exhibited the lowest cytotoxicity with a CC_{50} of (409.20 $\pm$ 42.49) μ g/mL, while the ethyl acetate extract had the highest cytotoxic activity with a CC_{50} of (104.90 $\pm$ 2.36) μ g/mL. The ethanol extract showed moderate cytotoxicity to Huh7 cells with a CC_{50} of (391.50 $\pm$ 13.40) μ g/mL. Cell viability remained above 80% after treatment with ethanol and *n*-hexane extracts at concentrations up to 200 μ g/mL. In contrast, the ethyl acetate extract decreased cell viability below 80% at concentrations exceeding 60 μ g/mL. Based on these results, concentrations of 10 μ g/mL, 30 μ g/mL, and 50 μ g/mL, which demonstrated minimal cytotoxicity, were selected for subsequent post-treatment antiviral assays against DENV-1 and DENV-2.

3.2. Antiviral effects of *S. arvensis* L. extracts against DENV-1 and DENV-2 (Post-treatment)

To determine the antiviral effects of ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. against DENV-1 and DENV-2, post-infection treatment was employed. Huh7 cells were treated with different non-toxic concentrations (10 μ g/mL, 30 μ g/mL, and 50 μ g/mL) of extracts following infection with DENV-1 and DENV-2 at an MOI of 0.5, respectively. Mock-treated DENV-infected Huh7 cells were used as the virus control. As shown in Figure 2, post-treatment with ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. significantly inhibited replication of both DENV-1 and DENV-2 in a dose-dependent manner. Specifically, treatment with the ethanol extract reduced the virus titer by 86.3% ($P < 0.001$) at 10 μ g/mL, 100.0% ($P < 0.001$) at 30 μ g/mL, and 100.0% ($P < 0.001$) at 50 μ g/mL compared to the control cells (Figure 2A and 2B). Similarly, in comparison with mock-treated DENV-2-infected cells, the infectious virus titers in the presence of 10 μ g/mL, 30 μ g/mL, and 50 μ g/mL of *S. arvensis* L. ethanol extract were decreased by 84.3% ($P < 0.001$), 96.3% ($P < 0.001$), and 99.8% ($P < 0.001$) (Figure 2C and 2D). For the ethyl acetate extract, significant DENV-1 reductions of 96.1% ($P < 0.001$), 100.0% ($P < 0.001$) and 100.0% ($P < 0.001$) (Figure 2A and 2B) and DENV-2 reductions of 93.5% ($P < 0.001$), 100.0% ($P < 0.001$) and 100% ($P < 0.001$) were observed at respective concentrations of 10 μ g/mL, 30 μ g/mL and 50 μ g/mL (Figure 2C and 2D). Additionally, treatment with *n*-hexane at concentrations 10 μ g/mL, 30 μ g/mL and 50 μ g/mL achieved a reduction of DENV-1 viral yield by 5.6%, 75.6% ($P < 0.001$) and 87.2% ($P < 0.001$) (Figure 2A and 2B) and a reduction of DENV-2 viral yield by 83.0% ($P < 0.001$), 97.8% ($P < 0.001$), 99.8% ($P < 0.001$) (Figure 2C and 2D). To further quantify antiviral potency, the IC_{50} value for each *S. arvensis* L. extract was determined against both DENV-1 and DENV-2 serotypes. The IC_{50} value of each *S. arvensis* L. extract against the DENV-1, ranked by antiviral efficacy, was 6.38 μ g/mL for the ethyl acetate extract, 9.15 μ g/mL for the ethanol

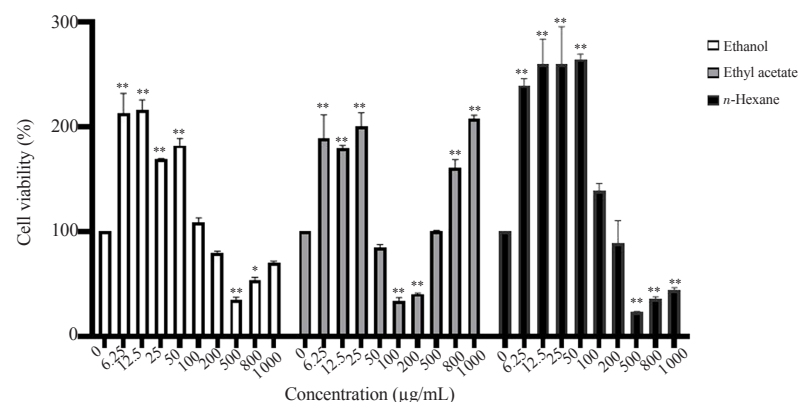


Figure 1. Cytotoxic effects of *Sonchus arvensis* (*S. arvensis*) L. ethanol, ethyl acetate and *n*-hexane extracts on Huh7 cells. Huh7 cells were treated with different concentrations of each extract up to 1000 μ g/mL for 72 h by MTS assay. The data are expressed as mean $\pm$ SEM of three independent experiments and analyzed using one-way ANOVA. * $P < 0.05$ and ** $P < 0.001$ compared with control cells.

extract, and 29.02 µg/mL for the *n*-hexane extract. For DENV-2, the IC₅₀ value was 5.38 µg/mL for the ethanol extract, 5.73 µg/mL for the *n*-hexane extract, and 7.03 µg/mL for the ethyl acetate extract. Among the tested extracts, the ethanol extract exhibited the highest SI against DENV-2 (SI = 72.77), followed by DENV-1 (SI = 40.15), indicating strong antiviral efficacy with low cytotoxicity. The ethyl acetate extract also showed favorable selectivity, with an SI value of 16.44 for DENV-1 and 14.92 for DENV-2. In contrast, the *n*-hexane extract had a relatively high SI against DENV-2 (SI = 71.41) but a lower SI against DENV-1 (SI = 14.08), suggesting serotype-dependent activity. These results demonstrate significant post-treatment reductions in viral titers for both viruses with increasing concentrations of ethanol, ethyl acetate, and *n*-hexane extracts, respectively, indicating that *S. arvensis* L. extracts possess promising

anti-DENV activity. Therefore, the ethanol extract at 30 µg/mL was selected for subsequent in-depth antiviral evaluation due to its potent antiviral activity with minimal cytotoxicity.

3.3. Virucidal activity of *S. arvensis* L. ethanol extract against extracellular DENV particles

To investigate whether the antiviral activity of 30 µg/mL of *S. arvensis* L. ethanol extract directly targets extracellular DENV particles, DENV-1 and DENV-2 at an MOI of 0.5 were pre-incubated with the extract for 1 h before infection. After 48 hours post-infection, infected cell culture supernatants were collected and subjected to virus titration using FFA. As shown in Figure 3, pre-incubation of DENV-1 with 30 µg/mL of *S. arvensis* L. ethanol

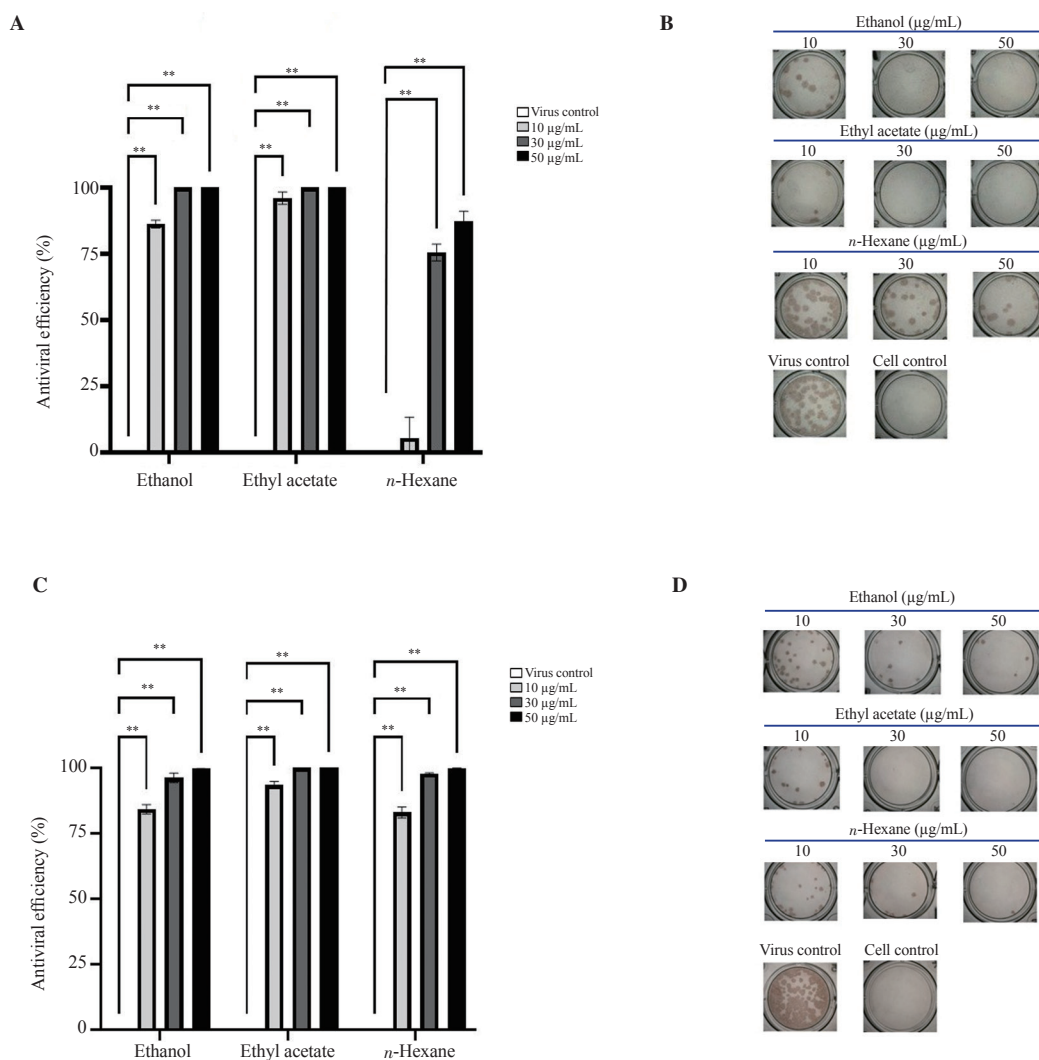


Figure 2. Antiviral efficiency of *S. arvensis* L. ethanol, ethyl acetate, and *n*-hexane extracts against (A, B) DENV-1 and (C, D) DENV-2 infection at a multiplicity of infection of 0.5. Huh7 cells were treated with different concentrations (10, 30, and 50 µg/mL) of *S. arvensis* L. extracts under post-infection treatment conditions for 48 h. The virus was titrated using a focus-forming assay. The data are expressed as mean ± SEM of three independent experiments and analyzed using two-way ANOVA. Statistical significance between groups was denoted as ***P* < 0.001.

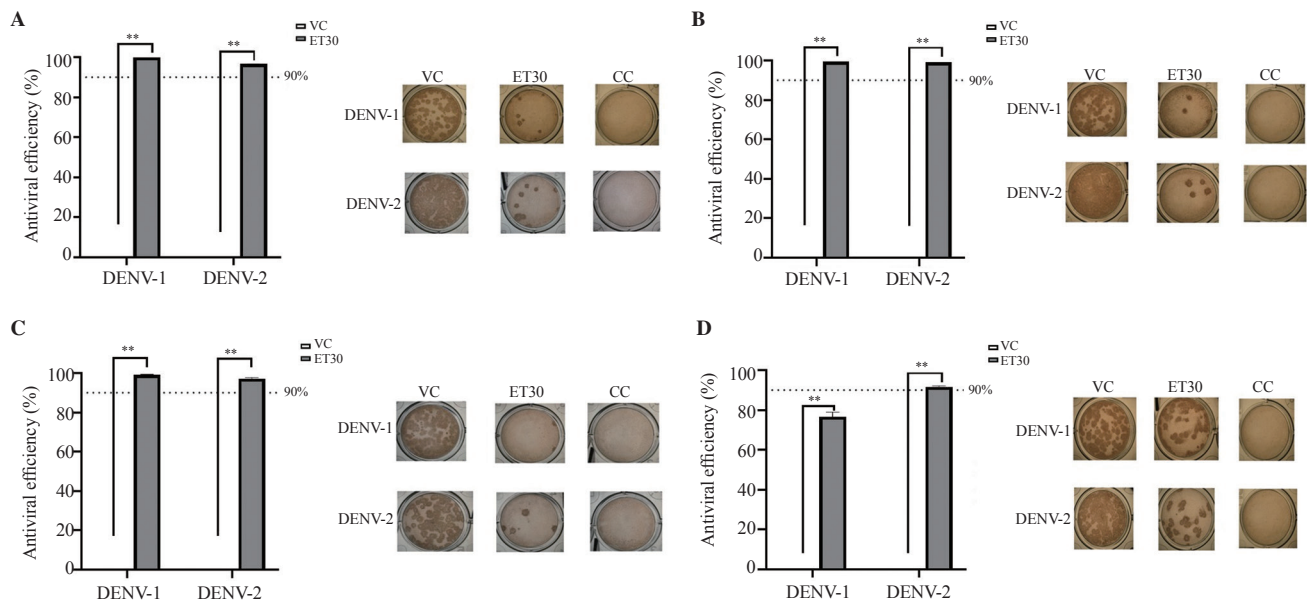


Figure 3. Inhibitory effects of 30 µg/mL of *S. arvensis* L. ethanol extract at different stages of viral replication cycle at a multiplicity of infection of 0.5. (A) Virucidal activity of 30 µg/mL of *S. arvensis* L. ethanol extract against extracellular DENV-1 and DENV-2 particles. (B) Prophylactic activity of the extract against DENV-1 and DENV-2 replication. (C) Anti-adsorption activity of the extract against DENV-1 and DENV-2 replication. (D) Anti-virus internalization activity of the extract at the entry stage of DENV replication. Focus-forming assays were performed using the harvested supernatants to determine the virus titers. The data are expressed as mean $\pm$ SEM of three independent experiments and analyzed using two-way ANOVA. Statistical significance between groups was denoted as $^{**}P < 0.001$. VC: virus control; ET30: 30 µg/mL of *S. arvensis* L. ethanol extract; CC: cell control.

extract resulted in a 99.89% inhibition ($P < 0.001$) of viral titer compared to the virus control. Similarly, the extract demonstrated relatively strong antiviral activity against DENV-2 replication with 96.11% ($P < 0.001$) antiviral efficiency (Figure 3A). These findings indicate that 30 µg/mL of *S. arvensis* L. ethanol extract exerts direct antiviral effects on extracellular DENV-1 and DENV-2 particles, suggesting its potential as a virucidal agent capable of neutralizing the virus before cell entry.

3.4. Prophylactic effects of *S. arvensis* L. ethanol extract against DENV infection

S. arvensis L. ethanol extract at 30 µg/mL demonstrated significant prophylactic antiviral activity against both DENV-1 and DENV-2 in infected Huh7 cells, with 99.4% ($P < 0.001$) and 99.0% ($P < 0.001$) viral foci reduction, respectively (Figure 3B). These findings highlight the promising potential of *S. arvensis* L. ethanol extract as a prophylactic agent against DENV infection.

3.5. Anti-adsorption effects of *S. arvensis* L. ethanol extract against the attachment of DENV

Given that 30 µg/mL of *S. arvensis* L. ethanol extract could directly induce virucidal and prophylactic effects on DENV-1 and DENV-2 particles, we further examined whether it could inhibit DENV binding to the host cells. An anti-adsorption assay was performed

with the extract during virus adsorption at 4°C. As shown in Figure 3C, the extract significantly inhibited the attachment and adsorption of DENV-1 and DENV-2 to Huh7 cells, achieving reductions up to 99.07% ($P < 0.001$) and 97.22% ($P < 0.001$), respectively, in comparison with the virus-infected cells, implying a significant decrease in virus entry following treatment.

3.6. Anti-virus internalization activity of *S. arvensis* L. ethanol extract at the entry stage of DENV

In the viral internalization inhibition assay, where viral binding was allowed before treatment, treatment with 30 µg/mL of *S. arvensis* L. ethanol extract significantly reduced DENV-1 titers by approximately 76.52% compared to virus control cells ($P < 0.001$) (Figure 3D). Similarly, the extract also demonstrated higher potent activity against DENV-2 internalization in Huh7 cells, achieving approximately 91.57% reduction in virus titer ($P < 0.001$; Figure 3D). Taken together, these results suggested that *S. arvensis* L. ethanol extract not only disrupts viral entry processes post-binding but may also act directly on virus particles already attached to the cell surface, thereby effectively preventing uptake into host cells.

3.7. Effects of *S. arvensis* L. ethanol extract on the host IFN response following DENV infection

To explore the potential mechanisms underlying its antiviral

activity, the effect of 30 µg/mL of *S. arvensis* L. ethanol extract on the host IFN response was assessed after post-treatment of DENV-1- and DENV-2-infected Huh7 cells. Type-1 IFN, specifically IFN-β, was evaluated in mock- and *S. arvensis* extract-treated cells using qRT-PCR (Figure 4).

Our results demonstrated that IFN-β mRNA expression was elevated in the virus control cells compared to uninfected cell controls, with a 9.23-fold increase for DENV-1 and a 47.69-fold increase for DENV-2 ($P < 0.001$). However, treatment with 30 µg/mL of *S. arvensis* L. ethanol extract induced no significant effect on the gene expression of IFN-β in DENV-infected cells compared to the virus control. These findings indicate that the antiviral activity of the extract following infection is unlikely to be mediated through the induction of IFN-β, suggesting other mechanisms may contribute to its activity.

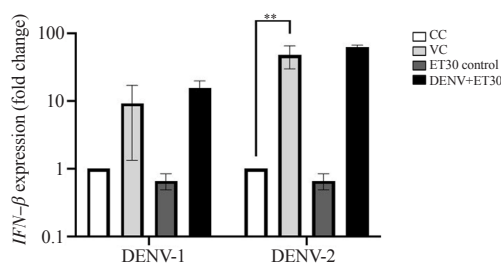


Figure 4. Effects of *S. arvensis* L. ethanol extract on IFN-β gene expression in DENV-infected Huh7 cells. Huh7 cells were mock-treated or treated with 30 µg/mL of *S. arvensis* L. ethanol extract following DENV-1 and DENV-2 infection, respectively, at a multiplicity of infection of 0.5. After 48 h, mRNA levels of IFN-β were then quantified using qRT-PCR and normalized to *GAPDH*. The data are expressed as mean ± SEM of three independent experiments and analyzed using two-way ANOVA. Statistical significance between groups was denoted as ** $P < 0.001$. CC: cell control; VC: virus control; ET30 control: 30 µg/mL of *S. arvensis* L. ethanol extract without infection.

3.8. Effects of *S. arvensis* L. ethanol extract on the inflammatory response following DENV infection

In addition to evaluating IFN expression, we also examined whether post-treatment with 30 µg/mL of *S. arvensis* L. ethanol extract modulates the inflammatory response. Gene expression levels of several key pro-inflammatory cytokines, including *NF-κB*, *IL-6*, and *TNF-α*, were quantified in DENV-1- and DENV-2-infected cells in the presence or absence of the extract using qRT-PCR (Figure 5). Our results revealed DENV infection significantly elevated *NF-κB* expression (DENV-1: 6.13-fold, $P < 0.001$; DENV-2: 1.88-fold, $P < 0.05$) compared to cell controls (Figure 5A). Similarly, *IL-6* (DENV-1: 4.31-fold, $P < 0.001$; DENV-2: 9.03-fold, $P < 0.001$) and *TNF-α* (DENV-1: 210.76-fold, $P < 0.001$; DENV-2: 57.71-fold, $P < 0.001$) were markedly elevated (Figure 5B and C). In comparison to virus control cells, post-treatment with 30 µg/mL of *S. arvensis* L. ethanol extract significantly reduced the expression of these genes in infected cells. *NF-κB* levels were notably downregulated (DENV-1: 2.20-fold reduction, $P < 0.001$; DENV-2: 1.08-fold reduction, $P < 0.001$), as were *IL-6* (DENV-1: 1.32-fold reduction, $P < 0.001$; DENV-2: 1.90-fold reduction, $P < 0.001$) and *TNF-α* (DENV-1: 69.38-fold reduction, $P < 0.001$; DENV-2: 11.57-fold reduction, $P < 0.05$) (Figure 5). Collectively, these results indicate strong inhibitory effects of 30 µg/mL of *S. arvensis* L. ethanol extract on DENV-induced inflammatory responses following infection, suggesting that its antiviral activity may be mediated through the modulation of inflammation-associated signaling pathways.

4. Discussion

Dengue infection is a major global public health menace. While licensed dengue vaccines exist, their overall effectiveness remains

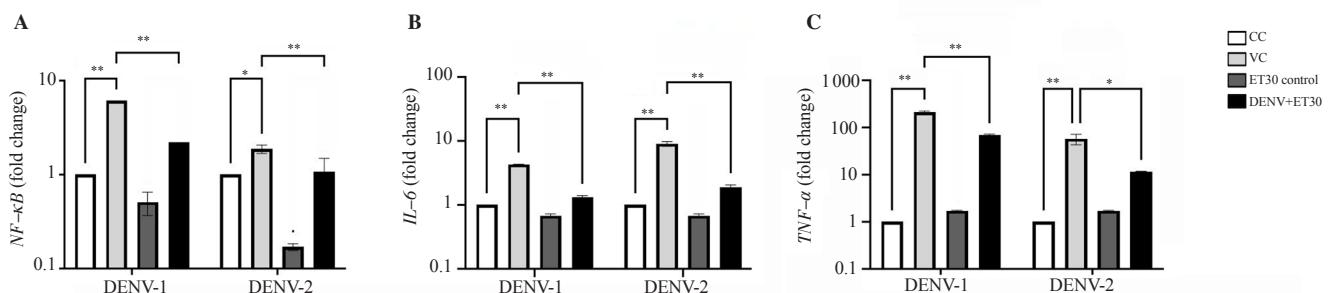


Figure 5. Effects of *S. arvensis* L. ethanol extract on *NF-κB*, *IL-6*, and *TNF-α* gene expression in DENV-infected Huh7 cells. Huh7 cells were mock-treated or treated with 30 µg/mL of *S. arvensis* L. ethanol extract following infection with DENV-1 or DENV-2 at a multiplicity of infection of 0.5. After 48 h, the gene expressions of (A) *NF-κB*, (B) *IL-6*, and (C) *TNF-α* were quantified by qRT-PCR and normalized to *GAPDH*. The data are expressed as mean ± SEM of three independent experiments and analyzed using two-way ANOVA. Statistical significance between groups was denoted as * $P < 0.05$ and ** $P < 0.001$.

limited due to variable protection across serotypes, age groups, and pre-existing immunity, as well as the potential risk of adverse events in certain populations. Moreover, the absence of approved antiviral drugs and the increasing incidence of dengue worldwide underscore the urgent need for alternative intervention strategies. For centuries, medicinal plants have been utilized in the treatment of both infectious and non-infectious diseases, including viral infections. Given that many antiviral agents and synthetic drugs have originated from natural sources, ethnopharmacology and traditional medicine are increasingly recognized as valuable platforms for novel bioactive compounds. Building on this premise, the present study aims to explore *S. arvensis* L., a medicinal plant native to Malaysia, as a potential anti-DENV agent. This plant is selected based on its documented traditional medicinal uses and emerging evidence of various pharmacological properties, including antimicrobial and antiviral potentials[21].

One concern of the study was the potential cytotoxicity of *S. arvensis* L. extracts to the cell line used in the antiviral assays. To address this concern, Huh7 cells were treated with varying concentrations of the different *S. arvensis* L. extracts to assess their cytotoxicity effects using the MTS assay. This colorimetric assay quantitatively measures cell viability based on the activity of mitochondrial dehydrogenase enzymes in metabolically active cells, which reduce the soluble tetrazolium compound into a coloured formazan product[22]. Interestingly, treatment with *S. arvensis* extracts showed apparent cell viability exceeding 100%, particularly at low to moderate concentrations. This is likely to reflect increased metabolic activity rather than cell proliferation, as MTS assays primarily assess mitochondrial function. Phenolics and flavonoids in the extracts may

enhance cellular redox activity or interact with tetrazolium salts, leading to elevated readings[23]. Overall, the results demonstrate metabolic stimulation and cytocompatibility, with increasing cytotoxic effects observed at increasing concentrations. In general, cytotoxicity levels can be interpreted based on established CC_{50} thresholds: very strong ($CC_{50} < 10 \mu\text{g/mL}$), strong ($CC_{50} 10\text{--}100 \mu\text{g/mL}$), moderate ($CC_{50} 100\text{--}500 \mu\text{g/mL}$), and minimal ($CC_{50} > 500 \mu\text{g/mL}$)[24]. Accordingly, the findings of the MTS assay showed that the calculated CC_{50} values of ethanol, ethyl acetate, and *n*-hexane extracts of *S. arvensis* L. in Huh7 cells were $391.5 \mu\text{g/mL}$, $104.9 \mu\text{g/mL}$, and $409.2 \mu\text{g/mL}$, respectively, indicating a moderate cytotoxicity toward Huh7 cells. These values differed markedly from a prior study[25], where the CC_{50} values were $(778.77 \pm 10.53) \mu\text{g/mL}$ (ethanol), $(437.39 \pm 7.46) \mu\text{g/mL}$ (ethyl acetate), and $(1420.88 \pm 20.88) \mu\text{g/mL}$ (*n*-hexane), respectively. Nevertheless, both studies demonstrate a similar cytotoxicity ranking among the *n*-hexane, ethanol, and ethyl acetate, being from least to most cytotoxic. The observed discrepancies in the CC_{50} values may be attributed to differences in assay duration: the present work used a 72 h incubation compared to 48 h in the earlier study. Longer exposure time may result in lower CC_{50} values, as cumulative cellular damage increases with prolonged treatment[26]. Consistent with these results, MNTD results showed that ethanol and *n*-hexane extracts maintained $>80\%$ cell viability at concentrations up to $200 \mu\text{g/mL}$ following 72 hours of exposure, whereas the ethyl acetate extract exhibited a lower MNTD80 value of $63.27 \mu\text{g/mL}$. Therefore, these concentration ranges were subsequently used to guide the selection of non-toxic doses for evaluating the antiviral potential of the extracts.

The antiviral efficacy of *S. arvensis* L. ethanol, ethyl acetate,

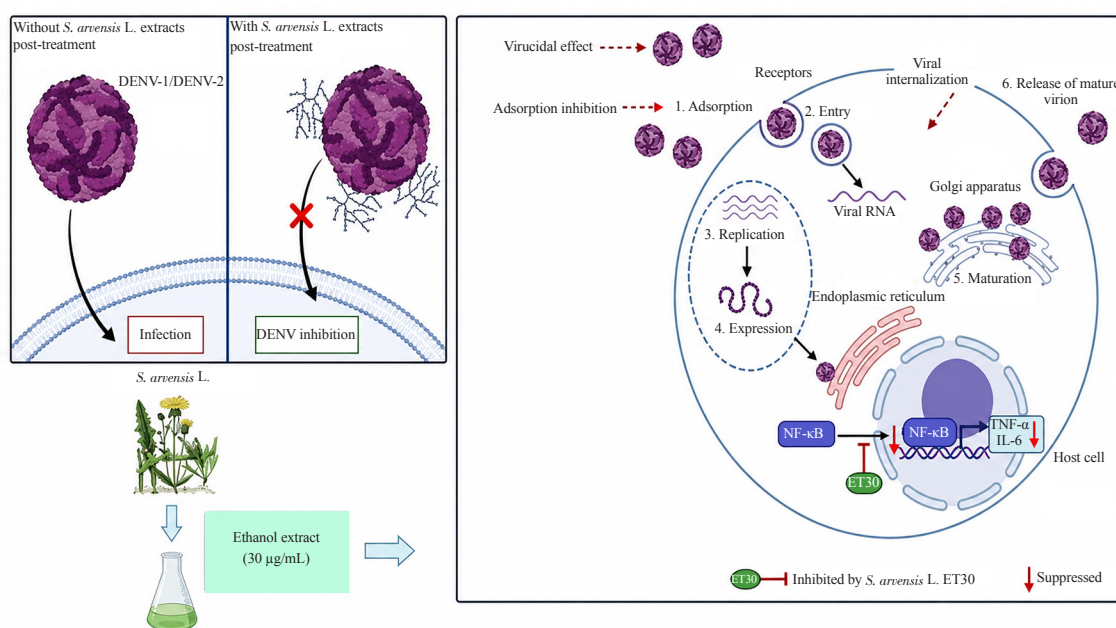


Figure 6. Proposed model of the antiviral mechanisms of *S. arvensis* L. extracts against DENV-1 and DENV-2.

and *n*-hexane extracts was evaluated by determining their IC₅₀ values against different DENV serotypes, DENV-1 and DENV-2, under post-treatment conditions. The extracts exhibited significant and dose-dependent antiviral activity within the non-cytotoxic concentration range (10, 30, and 50 µg/mL) except for *n*-hexane at 10 µg/mL in DENV-1-infected cells. The substantially lower inhibition observed at 10 µg/mL (5.6%) compared with 30 µg/mL (75.6%) may be attributed to a concentration-dependent threshold effect of the bioactive constituents present in the *n*-hexane fraction. As the *n*-hexane fraction comprises a complex mixture of lipophilic compounds, its antiviral activity is likely mediated through the additive or synergistic effects of multiple constituents. At 10 µg/mL, the concentration of individual bioactive compounds may be insufficient to reach the minimum effective level required to significantly disrupt dengue virus replication, resulting in only marginal inhibition. In contrast, increasing the concentration to 30 µg/mL likely provides sufficient bioactive density for these constituents to exceed the antiviral threshold, leading to a marked reduction in viral titers. The SI, a measure of antiviral potency relative to cytotoxicity, is generally considered promising when ≥ 10 , whereas values near or below 1 indicate potential cytotoxicity and limited therapeutic potential[27]. Our results demonstrated that all three extracts possessed relatively high SI values, ranging from 14.08 to 72.77, suggesting that *S. arvensis* L. extracts are potentially effective and safe candidates for DENV-1 and DENV-2 treatment. Therefore, ethanol extract at a non-toxic concentration of 30 µg/mL was selected for the subsequent mechanistic antiviral assays owing to its superior SI relative to the *n*-hexane and ethyl acetate extracts. Furthermore, the selection of ethanol extract is also supported by its high extraction efficiency and superior bioavailability of phytochemicals. Phytochemical analyses have demonstrated that the ethanol extract of *S. arvensis* L. contains a diverse range of compounds, including flavonoids, alkaloids, terpenoids, saponins, and polyphenols, while *n*-hexane extract yields the least amount of bioactive compounds (flavonoids, alkaloids, and terpenoids) due to its non-polar nature[25]. These findings align with previous studies showing that *n*-hexane is less effective in extracting polar compounds like phenolics and flavonoids[28] while ethanol, a polar solvent capable of dissolving both polar and non-polar compounds, makes it versatile in extracting diverse phytochemicals[29]. Notably, ethanol is extensively used in various industries, including pharmaceuticals, cosmetics, and food, due to its low toxicity and favourable safety profile[30]. In addition, it has been designated as a generally recognized safe substance by the Food and Drug Administration[31].

The antiviral mechanisms of plant-derived compounds involve interfering with viral replication and modulating the host immune response. To further elucidate the antiviral action of *S. arvensis* L., 30 µg/mL of *S. arvensis* L. ethanol extract was evaluated using various treatment regimens targeting different phases of the DENV life

cycle. The present findings indicated significant suppression of early-stage viral replication in both DENV-1 and DENV-2. The virucidal assay demonstrated that 30 µg/mL of *S. arvensis* L. ethanol extract significantly inhibits extracellular DENV particles, which could potentially limit viral dissemination during the viremic phase and reduce transmission to mosquito vectors[20,32]. Moreover, remarkable prophylactic activity possessed by the extract against DENV-1 and DENV-2 implies the potential benefits in dengue-endemic regions by reducing early infection rates, lowering viral load, and reducing onward transmission[33,34]. In addition, *S. arvensis* L. ethanol extract was found to effectively inhibit viral attachment to host cells and subsequent post-attachment entry and fusion processes. These findings highlight *S. arvensis* L. ethanol extract's ability to interfere with the early stages of DENV infection, which are critical for establishing successful viral replication and pathogenesis.

Additionally, we hypothesized that the direct antiviral activity of *S. arvensis* L. might be attributable to the combined and synergistic actions of diverse phytochemicals in the crude extract, rather than a single bioactive compound. These compounds may interact with structural components and key viral proteins of DENV. Among the polyphenolic constituents of *S. arvensis* L., quercetin stands out as a major flavonoid with well-documented antiviral properties. Numerous studies have reported on the inhibitory potential of quercetin on DENV infection by blocking interactions between the viral E protein and host cell receptors and by interfering with receptor-induced conformational changes necessary for membrane fusion[35]. Furthermore, molecular docking analyses have revealed that quercetin can bind to the active residues of the NS2B-NS3 protease complex, a key viral enzyme involved in polyprotein processing, thereby potentially inhibiting DENV-1 and DENV-4 replication[36]. Similarly, terpenoids have been extensively investigated for their antiviral activities across a broad spectrum of viruses, including DENV. Limonene, a terpenoid present in *Sonchus* species and known for its anti-inflammatory properties, has been shown to reduce DENV replication when used as a pretreatment[37].

While the direct antiviral effects of *S. arvensis* L. extract are promising, it is essential to investigate its impacts on the host immune response. The innate immune system forms the first line of defense against viral infections, restricting early virus replication through antiviral and inflammatory pathways. However, the imbalance of these responses could lead to disease severity. Moreover, DENV harbors a number of virulence proteins that are able to evade the host's innate immune response, especially the type-I IFN responses that play a key role in establishing innate antiviral defense[38]. The NS4B, along with NS2A, NS2B, and NS3, plays a significant role in immune evasion by inhibiting IFN- α / β signaling[39,40]. Furthermore, NS5 also contributes to immune evasion by targeting signal transducer and activator of transcription 2 and inhibiting its phosphorylation, which is essential for IFN- α

signaling[41]. In this study, post-treatment with 30 µg/mL of *S. arvensis* L. ethanol extract did not alter *IFN-β* gene expression in DENV-1- or DENV-2-infected cells compared to virus controls, suggesting that its antiviral activity may be independent of IFN-β induction. Future investigations could focus on exploring alternative mechanisms underlying its antiviral effects, such as modulation of oxidative stress or interactions with other host immune signaling pathways, to better understand and harness its therapeutic potential against dengue.

Instead, our results showed that 30 µg/mL of *S. arvensis* L. ethanol extract likely exerts its antiviral properties by modulating the inflammatory response. Specifically, the extract reduces DENV-1 and DENV-2-induced activation of the key transcription factor NF-κB, leading to remarkable suppression of the associated pro-inflammatory cytokines TNF-α and IL-6. These findings suggest that the extract may mitigate DENV-induced excessive inflammation while maintaining a balanced immune response. The NF-κB pathway plays a central role in regulating multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammation[6,7]. DENV exploits this pathway to facilitate its replication, prevent apoptosis, and mediate the host immune response[8]. NF-κB activation is also intricately linked to the cytokine storm observed during DENV infection, driving the production of key pro-inflammatory cytokines that contribute to severe immunopathology, including hepatic injury, cardiac damage, pulmonary failure, and neurological complications[8,42,43]. Therefore, targeting NF-κB activation presents a promising approach to modulate the inflammatory response and reduce the severity of dengue pathogenesis. These findings are supported by earlier studies, which revealed that inhibition of NF-κB leads to reduced viral replication. For instance, NF-κB suppression using gefitinib resulted in decreased DENV replication and cytokine production[44]. Hence, we postulated that the inhibition of the virus by *S. arvensis* L. ethanol extract may be mediated *via* suppression of NF-κB signaling that influences the host inflammatory response. Furthermore, the multifaceted mechanisms of *S. arvensis* L. extract establish its potential as a therapeutic candidate for inhibiting viral replication while modulating detrimental inflammation.

In conclusion, *S. arvensis* L. extracts demonstrated potent *in vitro* anti-DENV activity against DENV-1 and DENV-2 (Figure 6). Among the tested extracts, the ethanol extract at 30 µg/mL showed notable inhibitory effects, which may involve direct virucidal activity, prophylactic protection, and interference with viral binding and internalization into host cells. Additionally, the observed anti-DENV effects of 30 µg/mL of *S. arvensis* L. ethanol extract may be mediated by inhibition of the NF-κB signaling pathway and the resulting reduction of pro-inflammatory cytokines (TNF-α and IL-6), rather than the induction of the IFN-β-mediated innate immune response. However, as the active compounds responsible for these

effects have not yet been identified, further studies are required to isolate and characterize the bioactive constituents, assess their toxicity and pharmacological properties, and clarify their precise mechanisms of action.

Conflict of interest statement

All authors have no competing interests to disclose.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation was performed by NNZ, KLC, NZ, and DKW. Data collection and analysis were conducted by NNZ and KLC. NNZ and KLC drafted the original manuscript. KLC, as the corresponding author, supervised the overall organization of the work. NZ, DKW, and MS revised and refined the manuscript. All authors approved the final version of the manuscript.

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