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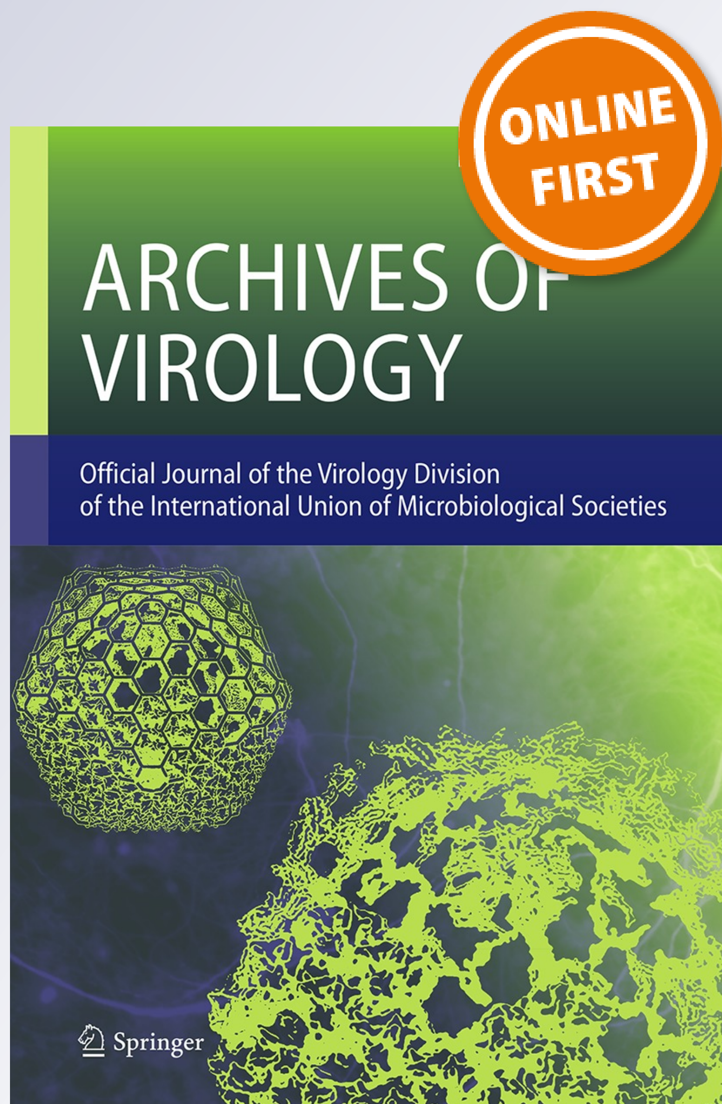

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ORIGINAL ARTICLE

Salidroside exhibits anti-dengue virus activity by upregulating host innate immune factors

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Abstract Dengue is an arboviral disease with no effective therapy available. Therefore, there is an urgent need to find a potent antiviral agent against dengue virus (DENV). In the present study, salidroside, a main bioactive compound of *Rhodiola rosea*, was evaluated for its antiviral potential against DENV serotype-2 infection and its effect on host innate immune factors. Antiviral effects of salidroside were examined in DENV-infected cells by western blotting, flow cytometry and real-time PCR. Its underlying mechanism involved in antiviral action was determined by evaluating expression of host innate immune factors including RIG-I, IRF-3, IRF-7, PKR, P-eIF2α and NF-κB. Salidroside potently inhibited DENV infection by decreasing DENV envelope protein expression more than tenfold. Salidroside exerts its antiviral activity by increasing expression of RNA helicases such as RIG-I, thereby initiating a downstream signaling cascade that induces upregulation of IRF-3 and IRF-7. It prevents viral protein synthesis by increasing the expression of PKR and P-eIF2α while decreasing NF-κB expression. It was also found to induce the expression of IFN-α. In addition, the number of NK cells and $CD8^+$ T cells were also found to be increased by salidroside treatment in human PBMCs, which are important in limiting DENV replication during early stages of infection. The findings presented here suggest that salidroside exhibits antiviral activity against DENV by inhibiting viral protein synthesis and boosting host immunity by increasing the expression of host innate immune factors and hence could be considered for the development of an effective therapeutic agent against DENV infection.

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Abbreviations

DENV	Dengue virus
IC_{50}	Half-maximal inhibitory concentration
RIG-I	Retinoic acid inducible gene
IRF-3	Interferon regulatory factor 3
PKR	RNA-activated protein kinase
P-eIF2α	Phosphorylated eukaryotic initiation factor 2α
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IFN-α	Interferon alpha
NK	Natural killer cells
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
DC-SIGN	Dendritic cell-specific ICAM3-grabbing non-integrin
hPBMCs	human peripheral blood mononuclear cells
FBS	Fetal bovine serum: MOI: multiplicity of infection
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide
NO	Nitric oxide
TNF-α	Tumor necrosis factor alpha

Introduction

Dengue is an acute systemic arboviral disease caused by dengue virus (DENV), which belongs to the family *Flaviviridae*. The disease symptoms range from mild flu-like syndrome known as dengue fever (DF) to a severe, fatal disease characterized by hemorrhage and shock, known as

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dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [12]. There are four serotypes of DENV (1, 2, 3 and 4). The DENV-2 serotype is reported to be more virulent and a major cause of DHF [3, 6]. Hence, our study focused on antiviral activity against DENV-2. DENV primarily infects human monocytes, macrophages and dendritic cells [11]. The *Aedes aegypti* mosquito vector, carrying DENV, when it bites a person, injects DENV into skin tissues, where it infects immature dendritic cells through dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN) receptor [19]. Infected dendritic cells then mature and migrate to regional lymph nodes, where they present viral antigens to $CD8^+$ T cells, initiating the cellular and humoral immune responses, [28].

Presently, there is no licensed anti-dengue drug available against DENV, as there are four closely related but antigenically distinct, serotypes of the virus that cause disease. Immunity generated due to infection by one serotype does not ensure protection of the patient from a subsequent exposure to any of the other three serotypes [23]. On the contrary, individuals that have acquired humoral immunity against one dengue serotype have more chances of developing DHF/DSS when subsequently infected with a heterologous serotype through antibody-dependent enhancement (ADE) [10]. A successful dengue vaccine must therefore generate long-lasting protective immunity against all four dengue serotypes simultaneously [5]. In recent years, the Sanofi Pasteur CYD tetravalent dengue vaccine has shown good immunological responses in clinical trials [9]; however, its long-term safety and efficacy have not been fully established. In contrast to the dengue vaccine approach, the therapeutic drug approach circumvents the immunopathological complications of dengue and directly targets the acute viral infection.

Natural products have been the main source of test materials in the development of antiviral drugs based on traditional medical practices [15]. Currently, not a single natural product has actually been approved as an antiviral drug against DENV, although a few are reported to have antiviral activity [16, 20, 30].

Salidroside (p-hydroxyphenethyl-β-D-glucoside, $C_{14}H_{20}O_7$), is a main bioactive compound of *Rhodiola rosea L.* (Crassulaceae). *Rhodiola rosea* is reported to have neuroprotective, anti-inflammatory and antiviral properties [4, 17]. Other *Rhodiola* species have also shown potential effects. For example, *Rhodiola imbricata* has immunomodulatory properties [21], and *Rhodiola kirilowii* has antiviral properties against hepatitis C virus of the family *Flaviviridae* [31]. *Rhodiola rosea* contains only 0.8-1 % salidroside in its phenyl ethanol derivatives fraction, which contain tyrosol as well. Salidroside has been shown to be an effective antiviral agent against coxsackievirus B3 infection [27]. It has many other pharmacological properties, including antidepressant, antioxidative, anti-fatigue, neuroprotective, and cardioprotective properties [8, 18, 29]. However, no information is available in the literature about the effects of salidroside against DENV. Therefore, the objective of the present study was to investigate the possible antiviral effects and underlying immunological mechanism of salidroside against DENV *in vitro*. Salidroside was tested for its antiviral activity in a human monocytic cell line (THP-1), a green monkey kidney epithelial cell line (Vero), and human peripheral blood mononuclear cells (hPBMCs).

We observed that salidroside inhibited DENV infection in THP-1 cells by enhancing host innate immune factors such as RIG-I, IRF-3, IRF-7, IFN-α, PKR and P-eIF2α. In addition, the numbers of NK cells and $CD8^+$ T cells were also found to be increased by salidroside treatment in human PBMCs, which are important in limiting DENV replication during early stages of infection. The present study showed that salidroside has potent anti-dengue-virus activity.

Materials and methods

Salidroside

Salidroside was purchased from Sigma Aldrich (Fluka, USA, cat. no. 4386). Stock solution (5 mg/ml) was prepared in 70 % ethanol, aliquoted, and stored at -20 °C until needed. At the time of the experiment, it was diluted to 1 mg/ml in sterile PBS and filtered through a syringe filter with a 0.2-μm pore size (Millipore, USA).

Cells

The THP-1 cell line, maintained in RPMI (Sigma, USA), was used as an infection model for DENV, and the C6/36 cell line, maintained in MEM (Sigma, USA), was used to propagate DENV. RPMI was supplemented with 10 % Fetal bovine serum (FBS) (Sigma, USA), 100 U penicillin (Sigma, USA), and 100 μg streptomycin (Sigma, USA) per ml at 37 °C in a 5 % CO_2 atmosphere in an incubator (Sanyo, Japan).

Development of virus infection model

The DENV serotype 2 New Guinea C strain was propagated in C6/36 cells supplemented with 2 % FBS in MEM at 28 °C in the absence of CO_2. It was harvested after cytopathic effects (CPE) were observed on day 14 postinfection. Supernatant was collected and stored at -80 °C as virus stock. Virus propagation in C6/36 cells was examined by immunoblotting of the envelope protein (cat. no. PA5-32246, Thermo Scientific, USA). Similarly, virus infection was determined in THP-1 cells.

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Infection of cells

Human PBMCs (hPBMCs) were obtained from heparinized blood by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Ten ml of blood was collected from healthy individuals, diluted in a 1:1 ratio with phosphate-buffered saline (PBS) and layered on Lymphoprep in a 15-ml tube. Tubes were centrifuged at 2,000 rpm for 30 min. The buffy layer along with white cells was collected in a 15-ml centrifuge tube and washed twice with PBS. Finally, mononuclear cells were suspended in incomplete RPMI. THP1 cells and hPBMCs were infected with DENV at multiplicity of infection (MOI) of 3, as productive viral infection was observed at this dose.

THP-1 cells (2×10^6 cells/ml) were infected with DENV in serum-free medium at 37 °C for 2 h. For optimal virus and cell contact, the culture plates were gently agitated, and unadsorbed virus was removed by washing the cells three times with incomplete medium. The mock- and DENV-infected cells were replenished with fresh medium supplemented with 2 % FBS. Infected cells were then treated with or without salidroside at a concentration of 166 µM and further incubated for 48 h. After incubation, cells were harvested, and the cell-free supernatant was stored at -80 °C until assayed for cytokine profiling. Similarly Vero cells (1×10^6 cells/ml) were infected with DENV at an MOI of 3 for 1 h in a 24-well culture plate (BD-Falcon, USA). Cell supernatant was harvested 5 days postinfection for virus titer determination.

Cell viability assay

Cytotoxicity of salidroside in THP-1 and Vero cells was determined by MTT assay. Heparin (cat. no. 58389 (084842) [9041-08-1], SRL, India) was used as a positive control [13] for antiviral activity against virus infection. THP-1 and Vero cells were treated with salidroside at concentrations of 33, 83, 166, 333 and 666 µM for 24 h, 48 h and 72 h. Similarly, THP-1 cells were treated with heparin at concentrations of 25,125, 250, 500 and 625 units/ml for 24 h, 48 h and 72 h. Both THP-1 and Vero cells were plated at a concentration of 1×10^6 cells/ml in a 96-well plate, treated with the above-mentioned doses of salidroside and incubated for the stated time period. The cytotoxicity of salidroside and heparin in THP-1 and Vero cells was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich, USA) dye. Ten µl of MTT stock was added to the cells, which were then further incubated for 4 h at 37 °C. MTT, a yellow tetrazole, was reduced to purple formazan crystals by NADPH-dependent oxidoreductase enzyme present in viable cells. The crystals were dissolved by addition of DMSO, and the optical density was measured at 570 nm using a spectrophotometer (Biotek Instruments, USA).

Determination of antiviral activity of salidroside

Mock- and virus-infected THP-1 cells were treated with or without salidroside at 166 µM and incubated for 48 h. A whole-cell lysate was prepared, and protein was estimated by the Bradford method. Forty µg of protein from each sample was separated by electrophoresis on a 10 % sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Nonspecific binding of antibodies was blocked using 3 % BSA in TBS buffer (0.1 M Tris–HCl, pH 7.4, 0.9 % NaCl), followed by washing with $TBST_{20}$ (0.1 % Tween-20 in TBS) and incubation with primary antibody, rabbit anti-dengue envelope protein. The PVDF membrane was then washed three times with $TBST_{20}$ and incubated with biotinylated goat anti-rabbit immunoglobulin (IgG) (cat. no. B8895, Sigma, USA) and streptavidin peroxidase (cat.no. S2438, Sigma, USA). The proteins were detected by chemiluminescence.

Intracellular staining of DENV

DENV-infected THP-1 cells and hPBMCs at a concentration of 2×10^6 cells/ml were treated with salidroside (166 µM) and incubated for 48 h. Heparin was used as a positive control. Cells were harvested and then washed twice with 0.01 M PBS, fixed, and permeabilised with Cytofix/ Cytoperm buffer (BD Biosciences, USA) for 20 min followed by incubation with anti-DENV FITC-conjugated monoclonal antibodies (cat. no. orb15512, Biorbyt, UK) at a 1:200 dilution for 60 min. Unbound antibodies were removed by washing with PBS, and the cells were suspended in 0.5 ml of PBS and analyzed using a flow cytometer (BD FACS Calibur) with Cell Quest Pro software.

RNA extraction and real-time PCR

The antiviral effect of salidroside on viral RNA copy number was determined quantitatively by real-time PCR. Virus-infected and salidroside (166 µM)-treated Vero cells were incubated for 5 days. Viral RNA was isolated using GenoSen's Viral RNA Extraction Mini Kit (Genome Diagnostics, India). Viral RNA stock was further used for setting up the real-time PCR reaction using the DENV-specific primer, (forward primer, 5′-AAACTGCAGATCTATGGCAGCAA TCCTGGC-3′; reverse primer, 5′-AAACTGCAGTCACTA TCCTTTCTTAAACCAGTTGAG-3′) and TaqMan probes (Genome Diagnostics, India) using a thermal cycler (Corbett

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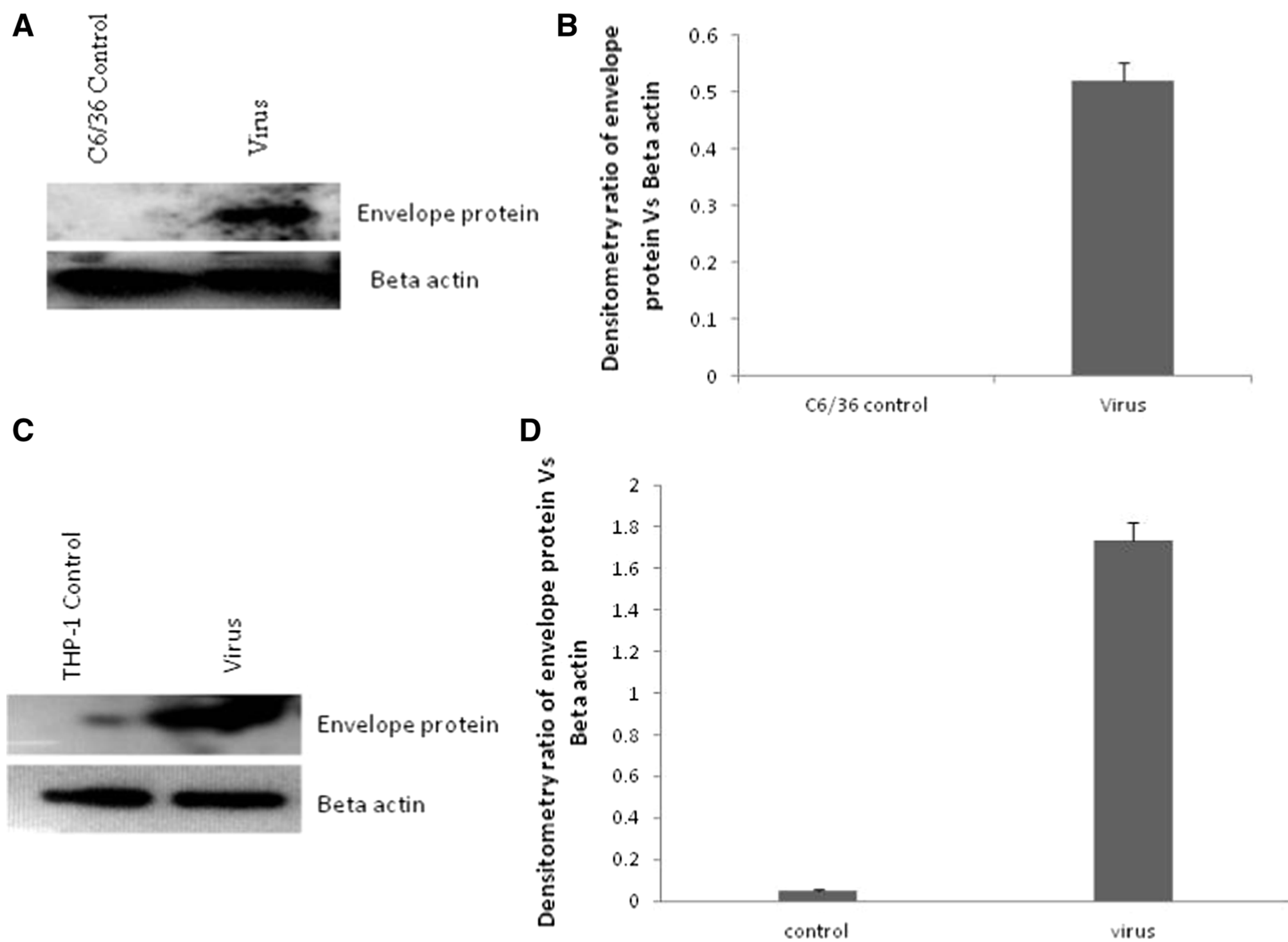

Fig. 1 Dengue envelope protein expression in virus-infected C6/36 and THP-1 cells. **a** DENV envelope protein expression in C6/36 cells used for virus propagation. **b** Density ratio of the DENV envelope protein band to the Vs beta actin band. **c** DENV envelope protein expression in THP-1 cells used as a virus infection model. **d** Density ratio of the DENV envelope protein band to the beta actin band. The results shown are representative data from three independent experiments

Research, Australia) with fluorescence measurement capability.

Determination of the effect of salidroside on innate immune factors by immunoblotting

DENV-infected or mock-infected THP-1 cells were treated with or without salidroside (166 µM). Cytoplasmic and nuclear extracts and whole-cell lysates were prepared. Samples containing 40 µg of proteins were separated on 10 % SDS gels and transferred to PVDF membranes. The membranes were incubated for 1 h with 3 % BSA in TBS buffer to block nonspecific binding, followed by washing with $TBST_{20}$ and incubation with the following primary antibodies: rabbit anti-NF-kB (cat. no. 3038, Bio-Vision, USA), which was used to probe the nuclear extract proteins, and rabbit anti-RIG-I (cat. no. PRS3953, Sigma, USA), rabbit anti-IRF-3, (cat. no. SAB3500280, Sigma, USA), rabbit anti-IRF-7 (cat. no. PRS3941, Sigma, USA), rabbit anti-PKR (cat. no. SAB3500326, Sigma, USA), rabbit anti-P-eIF2α (cat. no. PAI-14138, Thermo scientific), rabbit anti-eIF2α (cat. no. SAB4500729 Sigma, USA), and mouse anti-beta actin peroxidase (cat. no. A3854, Sigma, USA), which were used to probe whole-cell lysate proteins. Subsequently, the membranes were washed three times for 10 min with $TBST_{20}$ and incubated with the secondary antibody, biotinylated goat anti-rabbit IgG, and streptavidin peroxidase. The proteins were detected by chemiluminescence.

Cytokine detection by ELISA

Virus-infected THP-1 cells and hPBMCs were treated with salidroside (166 µM) and incubated for 24 h in IRPMI supplemented with 2 % FBS. Supernatant was collected and kept at -80 °C. IFN-α and IRF-3 expression levels were quantified by ELISA (Blue Gene ELISA kit and Kinesis DX, Los Angeles, respectively) following the manufacturer's instructions.

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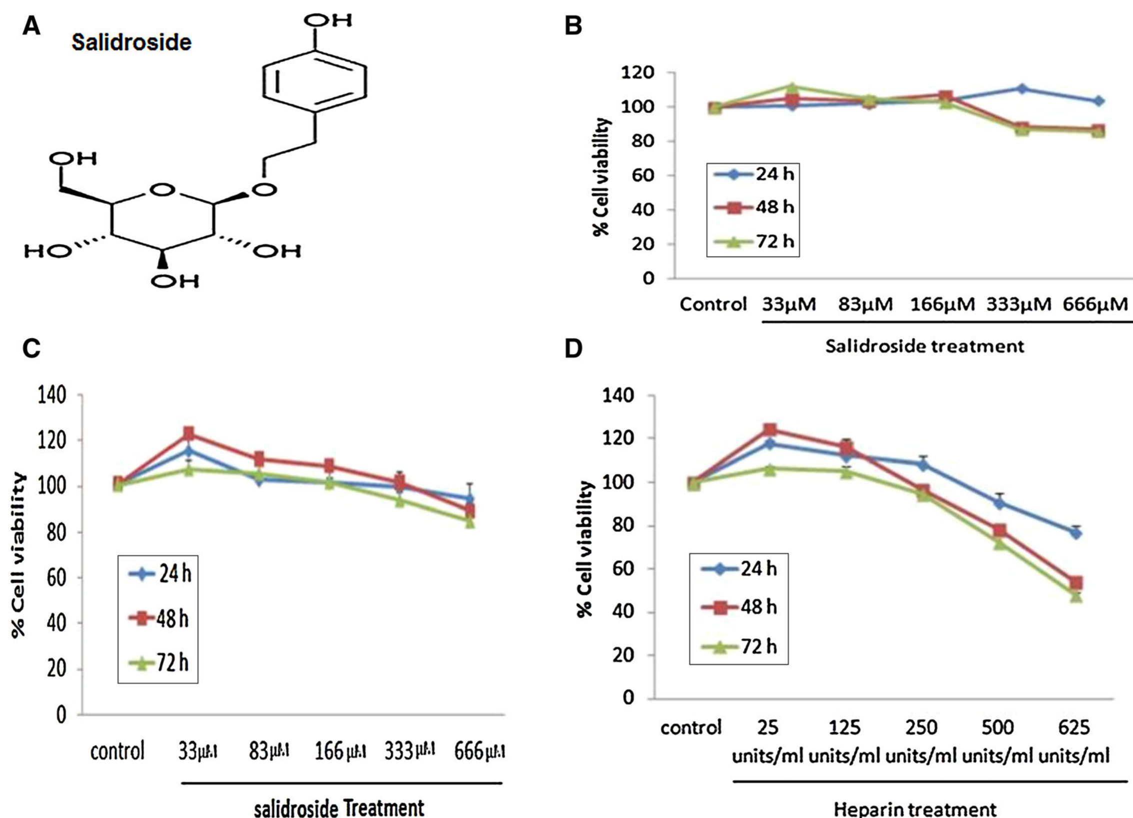

Fig. 2 Cytotoxicity of salidroside and heparin in THP-1 and Vero cells. **a** Structure of salidroside obtained from *Rhodiola rosea.* **b** Cytotoxicity of salidroside in THP-1 cells. **c** Cytotoxicity of salidroside in Vero cells. **d** Cytotoxicity of heparin used as a positive control for DENV infection in THP-1 cells. All experiments at the 24 h, 48 h and 72 h time points were conducted in triplicate

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from THP-1 cells using an RNeasy Protect Mini Kit (QIAGEN, Germany), following the manufacturer's instructions. Amplification of the target gene was done using a OneStep RT-PCR Kit (QIAGEN, Germany) with specific primers, following manufacturer's protocol. The primer pairs and their product sizes were as follows: beta actin: forward primer, 5′-GAGACCTTCAACACCCCAGCC-3′; reverse primer, 5′GGATCTTCATGAGGTAGTCAG-3′, 207 bp. RIG-I: forward primer, 5′TGTGGGCAATGTCATCAAAA-3′; reverse primer, 5′-GAAGCACTTGCTACCTCTTGC-3′, 67 bp. The RIG-I PCR products that were obtained were analyzed by electrophoresis on a 3 % agarose gel, and the beta actin PCR product was analyzed on a 2 % agarose gel.

Staining for cell-surface marker

Human PBMCs were mock infected or infected with DENV at an MOI of 3, treated with or without salidroside (166 µM), and cultured in RPMI-1640 supplemented with 2 % FBS in a 5 % CO_2 incubator for 48 h. Cells were harvested and incubated with anti-CD16-FITC/anti-CD56-PE and anti-CD8-PE monoclonal antibodies (BioLegend, USA) for 45 min in the dark. Subsequently they were washed twice with PBS and resuspended in 0.5 ml of PBS, and 10,000 cells were analyzed using a flow cytometer (BD FACSCalibur) with CellQuest Pro software.

Statistical analysis

Data were analyzed using a commercially available statistics software package (SPSS for Windows, version 14.0, Chicago, USA). A one-way analysis of variance (ANOVA) test was performed. Results are presented as means ± standard error (S.E.M), p-values less than 0.05 were regarded as statistically significant.

Results

Virus infection model established in THP-1 cell line

DENV was propagated in the C6/36 cell line. The supernatant obtained 14 days postinfection was probed for dengue envelope protein. Expression of the envelope

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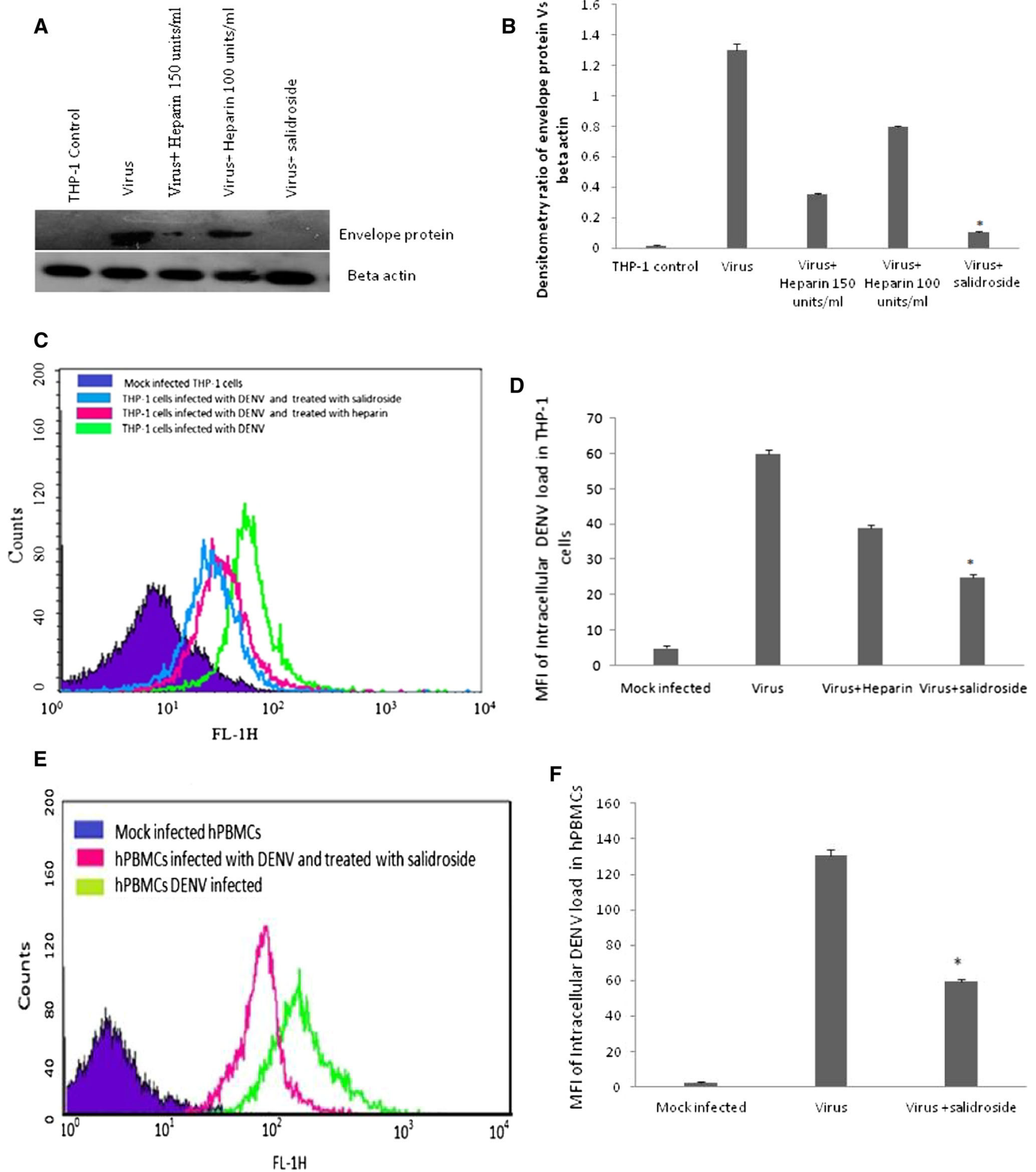

protein confirmed that the cells were infected (Fig. 1a, b). The cell supernatant was then used as virus stock to infect THP-1 cells at an MOI of 3. Expression of envelope protein in DENV-infected THP-1 cells confirmed the establishment of a virus infection in THP-1 cells (Fig. 1c, d).

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◀**Fig. 3** Effect of salidroside on dengue virus infection. **a** Immunoblot of envelope protein expression in salidroside-treated and DENV-infected THP-1cells. **b** Density ratio of the envelope protein band to the beta actin band in extracts of DENV-infected and salidroside-treated THP-1 cells. Two doses of heparin, 150 units/ml and 100 units per ml, were used as a positive control. Salidroside at a concentration of 166 µM was used in all of the experiments. *, $p < 0.01$ vs. virus. **c** Histogram showing the intracellular viral load in DENV-infected THP-1 cells treated with 166 µM salidroside. Heparin was used at 150 units/ml as a positive control. **d** Bar diagram showing a significant decrease in the intracellular viral load in THP-1 cells. *, $p < 0.05$ vs. virus-infected cells. **e** Histogram showing the intracellular viral load in DENV-infected hPBMCs treated with 166 µM salidroside. **f** Bar diagram showing a significant decrease in the intracellular viral load in hPBMCs. *, $p < 0.02$ vs. virus-infected cells. The results shown are representative data from three independent experiments

Cell viability assay

The cytotoxicity of salidroside (Fig. 2a) and heparin in THP-1 and Vero cells was tested by MTT assay. Treatment of cells with salidroside at concentrations of 33, 83 and 166 µM did not result in any cytotoxicity in THP-1 or Vero cells, even up to 72 h of incubation. Lower doses of salidroside up to 166 µM, in fact, resulted in cell proliferation. Cell viability at 333 µM and 666 µM decreased slightly to 88.8 % ± 0.21% and 87.1 % ± 0.08 % at 48 h of incubation in THP-1 cells, while in Vero cells, 666 µM salidroside exhibited toxicity of 88.9 % at 48 h (Fig. 2b, c). Lower doses of heparin (25 and 125 units/ml) did not causes toxicity in THP-1 cells up to 72 h of incubation. Low toxicity at 72 h of incubation was observed with a 250 units/ml dose of heparin. Higher doses (500 and 625 units/ml) were highly toxic both at 48 h and 72 h of incubation (Fig. 2c). Lower doses of salidroside and heparin, 33, 83 and 166 µM and 100 and 150 units/ml, respectively, were chosen to determine their antiviral activity.

Determination of antiviral activity of salidroside

The effect of salidroside on DENV infection was determined by evaluating DENV envelope protein expression by western blotting. DENV-infected and salidroside (166 µM)-treated THP-1 cells showed a marked decrease in envelope protein expression in comparison to DENV-infected cells (Fig. 3a). Salidroside at a dose of 166 µM showed more antiviral activity than at 83 µM (data not shown), so 166 µM was used in all further experiments to determine its antiviral activity. Salidroside at 166 µM showed even more antiviral activity than heparin at 150 units/ml. The density ratio of envelope protein in DENV-infected and salidroside-treated cells to beta actin decreased more than tenfold in comparison to virus-infected cells without salidroside treatment (Fig. 3b), indicating the antiviral activity of salidroside.

Determination of intracellular viral load

The intracellular viral load in DENV-infected and salidroside-treated THP-1 cells and hPBMCs was determined by FACS analysis. The mean fluorescent intensity of FITC-labelled DENV-2-specific antibody decreased significantly from 60 ± 1.26 to 25 ± 0.84 (Fig. 3c, d) in salidroside-treated THP-1 cells in comparison to virus-infected cells. The decrease in viral load due to salidroside was even more

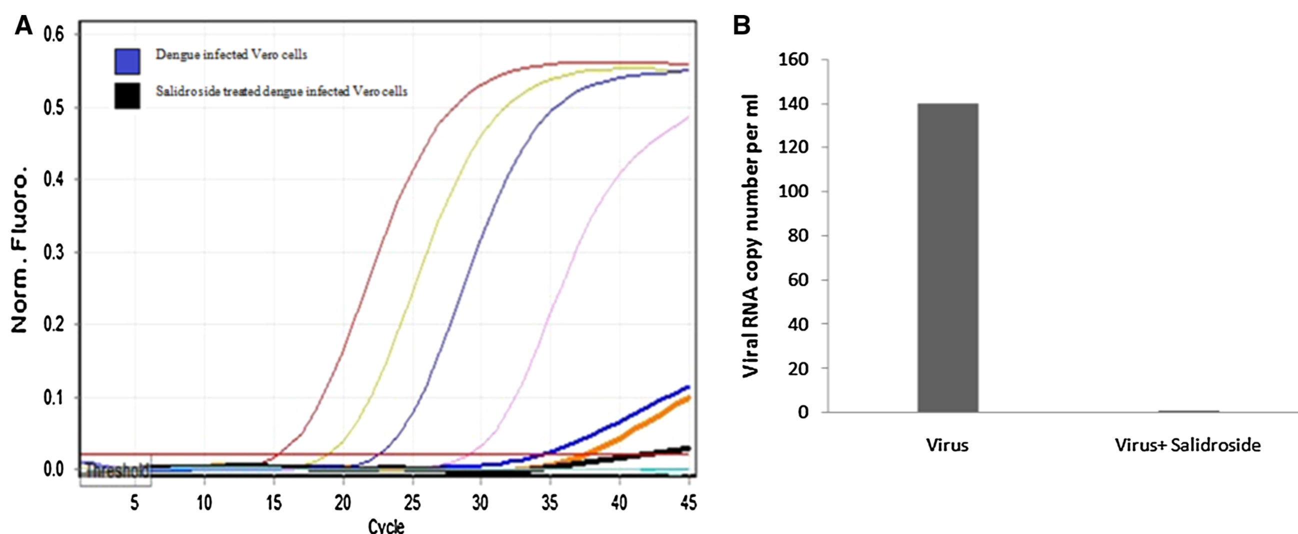

Fig. 4 **a** Determination of viral RNA copy number in DENV-infected Vero cells treated with salidroside, measured by real-time PCR. **b** Bar diagram showing the decrease in viral RNA copy number

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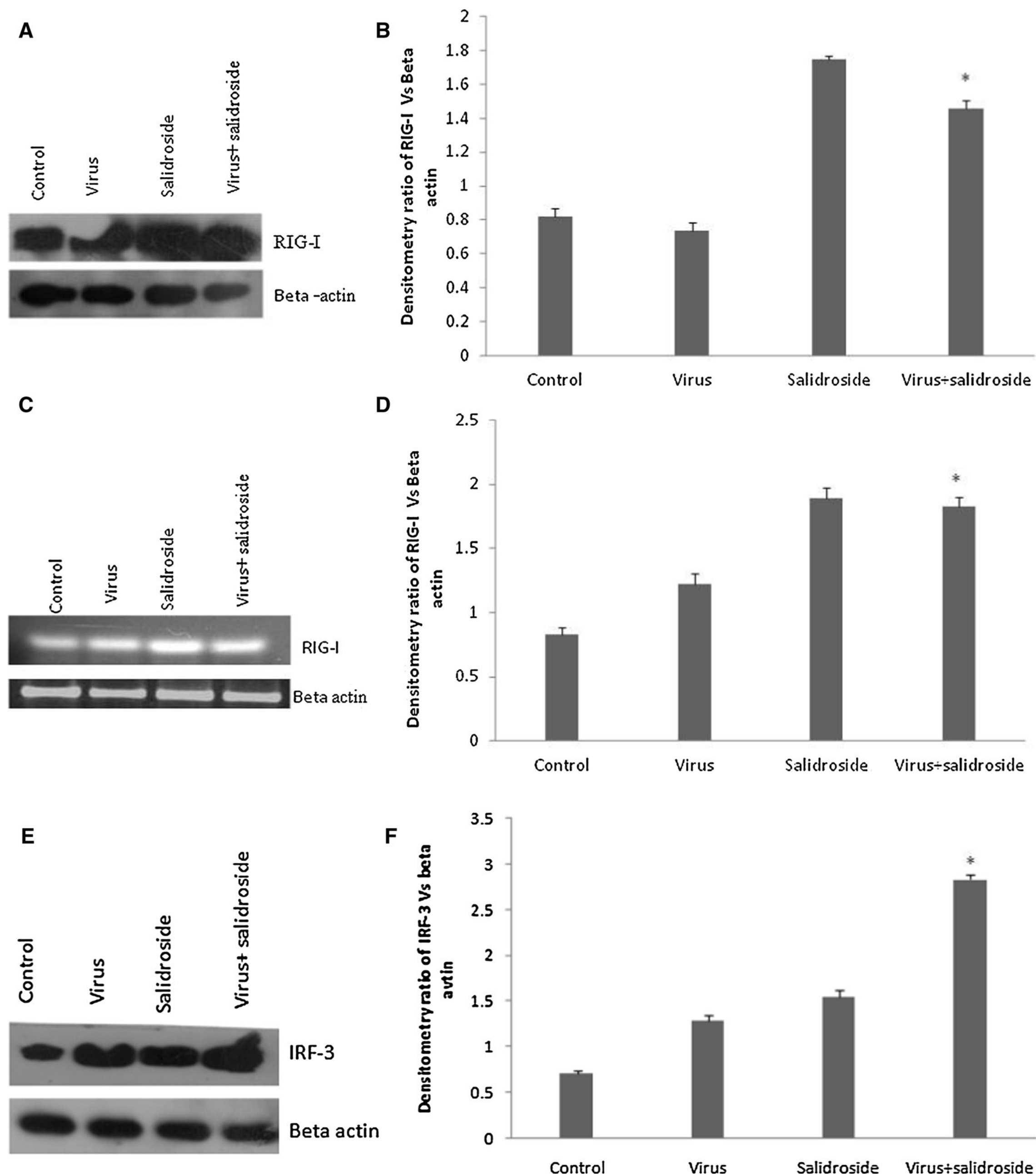

Fig. 5 Effect of salidroside on host innate immune factors. **a** Immunoblot of RIG-I expression in DENV-infected THP-1 cells after salidroside treatment. **b** Bar diagram showing the density ratio of RIG-I versus beta actin in DENV-infected THP-1 cell extracts after salidroside treatment. *, $p < 0.02$ vs. virus-infected cells. **c** Expression of RIG-I mRNA in salidroside-treated cells. **d** Bar diagram showing expression of RIG-I mRNA versus beta actin in DENV-infected THP-1 cells after salidroside treatment, measured by RT-PCR. *, $p < 0.05$ vs. virus infected cells. **e** Immunoblot of IRF-3 expression in DENV-infected THP-1 cells after salidroside treatment. **f** Bar diagram showing the density ratio of the IRF-3 band to the beta actin band in DENV-infected THP-1 cells after salidroside treatment. *, $p < 0.05$ vs. virus-infected cells. **g** Level of IRF-3 in cell supernatants of DENV-infected and salidroside-treated cells, measured by ELISA. **h** Immunoblot of IRF-7 expression in DENV-infected THP-1 cells after salidroside treatment. **i** Bar diagram showing the density ratio of IRF-7 to beta actin in DENV-infected THP-1 cells on salidroside treatment. *, $p < 0.02$ vs. virus-infected cells. **j** Cytokine analysis of IFN-α in THP-1 cells. *, $p < 0.05$ vs. virus-infected cells. The results shown are representative data from three independent experiments

than that observed in the heparin positive control (39 ± 0.92). A similar trend was observed in hPBMCs, with the viral load decreasing significantly from 131 ± 3.75 to 60 ± 1.37 in salidroside-treated hPBMCs (Fig. 3e, f), thus confirming the antiviral activity of salidroside against DENV.

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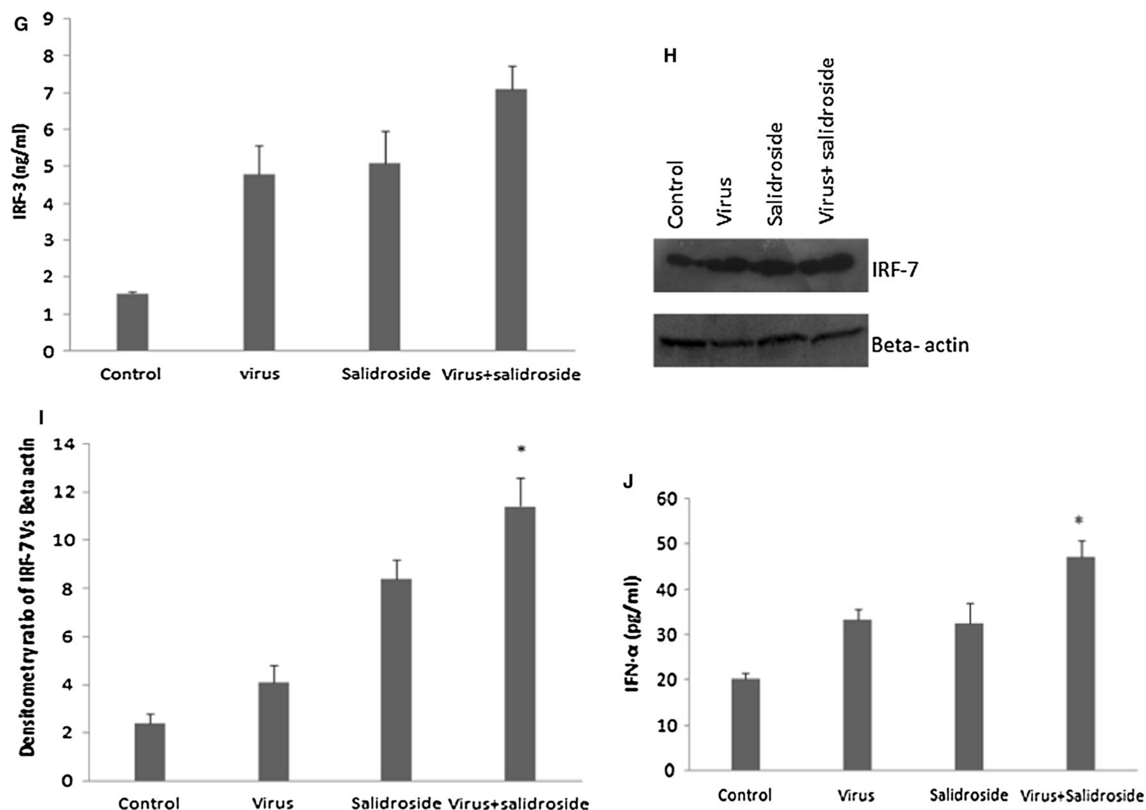

Fig. 5 continued

Real-time PCR analysis in DENV-infected Vero cells

Quantitative determination of the viral RNA copy number was performed in DENV-infected Vero cells treated with or without salidroside. The viral RNA copy number was 140 copies/ml in DENV-infected Vero cells, whereas it was drastically reduced to only 1 copy/ml in salidroside-treated Vero cells, as determined by real-time PCR (Fig. 4a, b) confirming the excellent antiviral activity of salidroside against dengue virus.

Effect of salidroside on innate immune factors in DENV-infected cells

In order to identify the underlying mechanism of the antiviral activity of salidroside, expression of various host innate immune factors on DENV infection and salidroside treatment were evaluated. Salidroside was found to increase the expression of RIG-I thus increasing recognition of DENV. IRF-3 and IRF-7 protein expression also increased after salidroside treatment, resulting in antiviral activity. Salidroside inhibits viral protein synthesis by increasing the expression of PKR and P-eIF2α and boosts host immunity by increasing the expression of $CD8^+$ T cells and NK cells.

Effect of salidroside on the expression of RIG-I, IRF-3, IRF-7, and IFN-α in DENV-infected THP-1 cells

Expression of the intracellular RIG-I helicase protein as well as its mRNA during virus infection and subsequent salidroside treatment was measured by western blotting and RT-PCR. RIG-I expression in DENV-infected and salidroside-treated cells was found to increase twofold when compared to virus-infected cells (Fig. 5a–d). Cells treated with salidroside alone also showed significantly increased expression of RIG-I, both at the mRNA and protein level. Salidroside treatment also increased interferon regulatory factor-3 (IRF-3) gene expression by approximately twofold in DENV-infected cells (Fig. 5e, f). Production of IRF-3 was also found by ELISA to be increased after salidroside treatment in DENV-infected cells from 4.8 ± 0.79 ng/ml in virus infected cells to 7.1 ± 0.62 ng/ml in salidroside-treated and DENV-infected cells (Fig. 5g). Similarly, the IRF-7 level in DENV-infected cells was also found to be significantly increased by more than 2.5-fold after salidroside treatment (Fig. 5h, i).

Changes in the expression of IFN-α cytokines during DENV infection and salidroside treatment were

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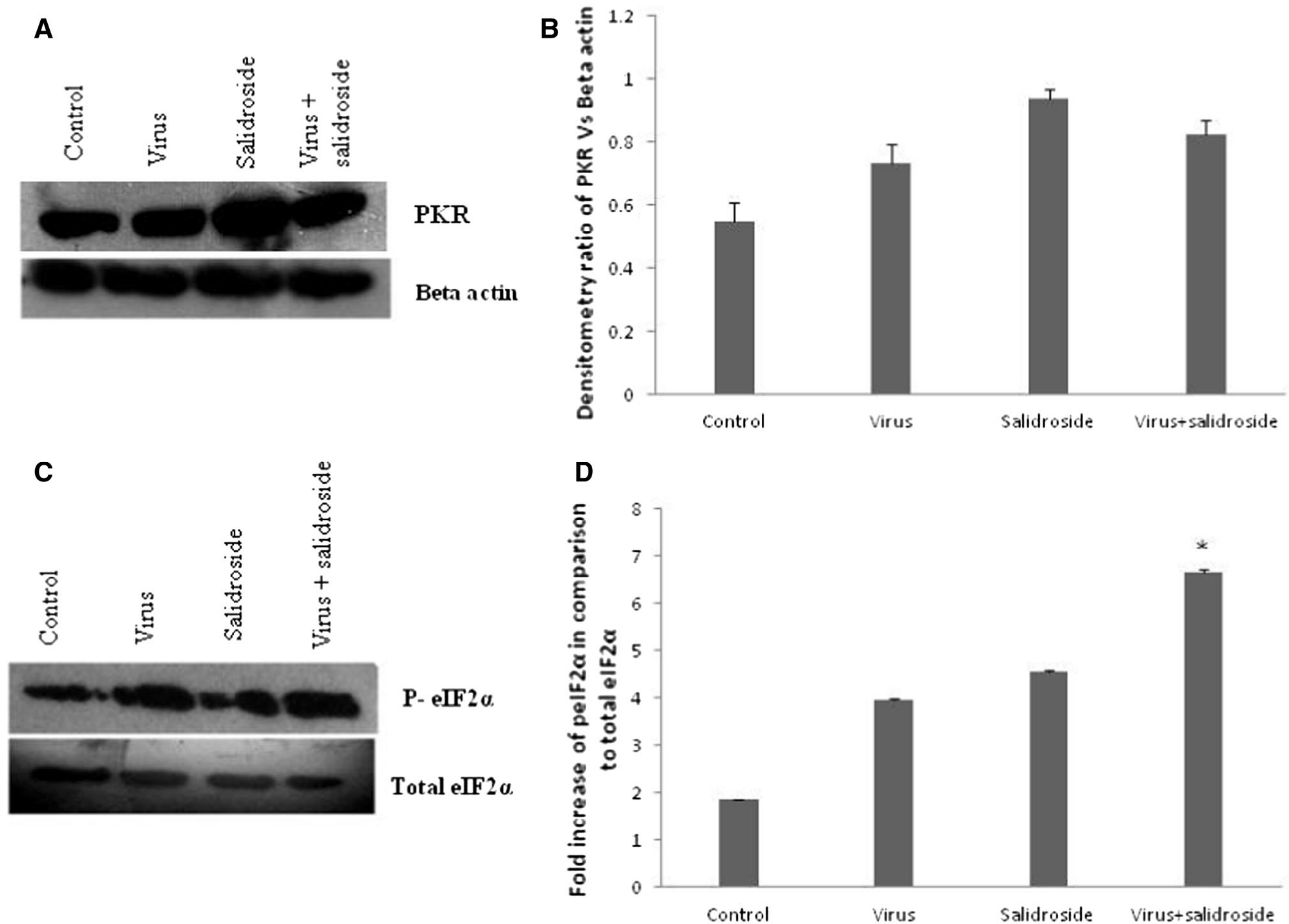

Fig. 6 Effect of salidroside on PKR and P-eIF2α expression. **a** Immunoblot of PKR in DENV-infected and salidroside-treated cells. **b** Bar diagram showing the density ratio of PKR in DENV-infected and salidroside-treated THP-1 cells. **c** Immunoblot of P-eIF2α and total eIF2α in DENV-infected and salidroside-treated cells. **d** Bar diagram showing the fold increase in P- eIF2α vs. total eIF2α in DENV-infected and salidroside-treated THP-1 cells. The results shown are representative data from three independent experiments. *, $p < 0.01$ vs. virus-infected cells

investigated by ELISA. The expression of IFN-α was found to be almost twofold higher in DENV-infected THP-1-cells treated with salidroside than in the control, increasing significantly from 33.3 ± 2.4 pg/ml to 47.3 ± 3.8 pg/ml (Fig. 5j).

Effect of salidroside on the expression of PKR, P-eIF2α, and total eIF2α in DENV-infected and salidroside treated THP-1 cells

In this study, treatment of DENV-infected cells with salidroside enhanced the expression of PKR (Fig. 6a). Subsequently, the expression of P-eIF2α, which is a PKR substrate, was also found to be significantly increased by approximately twofold in comparison to total eIF2α cells infected with DENV and treated with salidroside (Fig. 6c). The expression of these proteins was also increased in cells treated with salidroside alone when compared to the control. Thus, salidroside elicits a host response against DENV infection via the PKR pathway.

Effect of salidroside on the expression of NF-κB in DENV-infected and salidroside-treated THP-1 cells

Salidroside was found to decrease the expression of NF-κB in DENV-infected and salidroside-treated cells by more than twofold (Fig. 7a, b). Therefore, salidroside renders its antiviral effect against DENV by reducing inflammation.

Effect of salidroside on the expression of $CD8^+$ T cell and NK cell surface markers in DENV-infected hPBMCs

The role of salidroside in modulating $CD8^+$ T cells and NK cell expression was determined in DENV-infected

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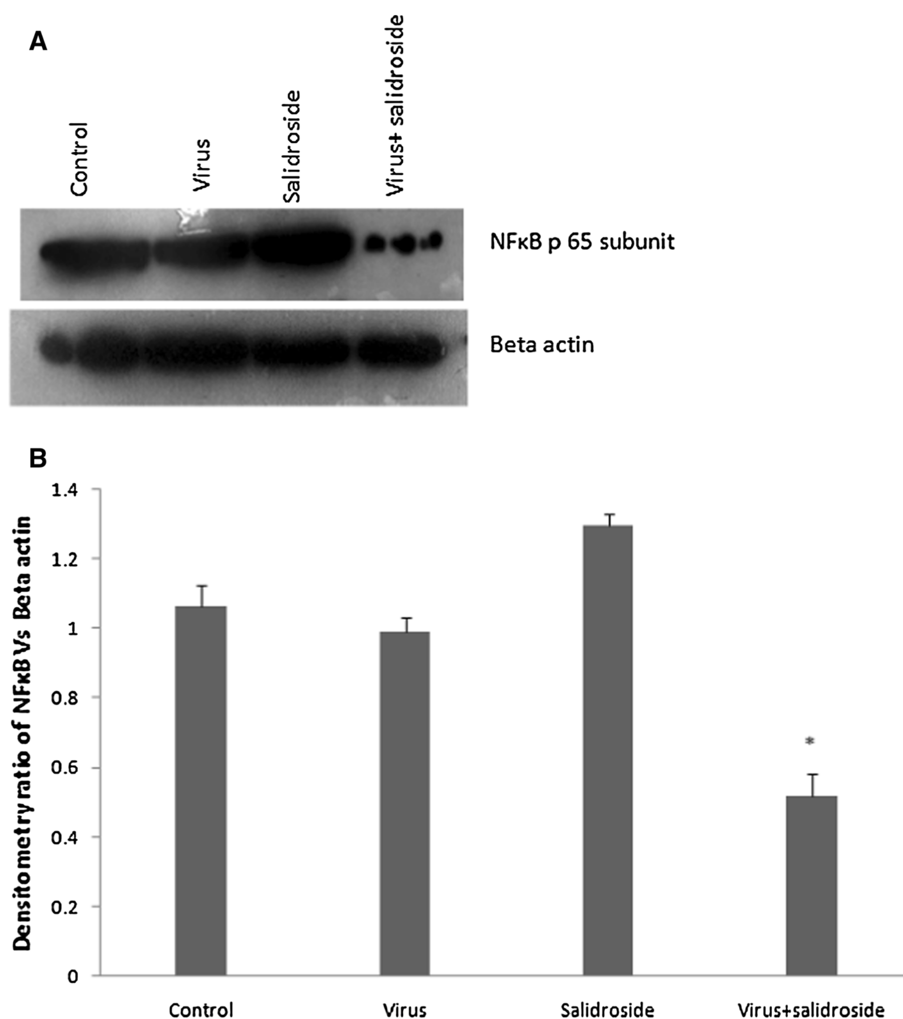

Fig. 7 Effect of salidroside on NFκB expression. **a** Immunoblot of NFκB in nuclear lysates of DENV-infected and salidroside-treated THP-1 cells. **b** Bar diagram showing the density ratio of NFκB to beta actin in DENV-infected and salidroside-treated THP-1 cells. *, $p < 0.01$ vs. virus-infected cells. The results shown are representative data from three independent experiments

hPBMCs. DENV-infected cells treated with salidroside showed an increase in the expression of $CD8^+$ T cells from 8.8 % (14.5 ± 3.3 %) cells in the mock-infected population to 14.3 % (18.6 ± 2.4 %) in the DENV-infected and salidroside-treated population (Fig. 8a; Table 1). The number of $CD16^+/56^+$ NK cells also increased from 38.73 % (28.4 ± 5.1 %) in mock-infected cells to 42.9 % (32 ± 5.4 %) in DENV-infected and salidroside-treated cells (Fig. 8b; Table 1).

Discussion

Dengue is a systemic viral infection that can become life-threatening to individuals with humoral immunity to a particular dengue serotype when infected with a heterologous serotype, due to ADE. DENV2 infection results in higher viremia in the human host in comparison to the other serotypes. Presently, there is no approved anti-dengue drug available, so, there is an urgent need to develop one. In the present study, we identified salidroside, a main bioactive component of *Rhodiola sp.* that exhibits anti-dengue activity. The antiviral properties of salidroside were investigated in *in vitro* experiments. When DENV- infected hPBMCs, THP-1 and Vero cells were treated with salidroside, virus titers decreased significantly. This observation indicated that salidroside had excellent antiviral activity *in vitro* and it itself boosted host immunity against dengue virus infection, as salidroside has antiviral properties irrespective of the cell type, be it immune cells (THP-1 and hPBMCs) or non-immune cells (Vero). In order to investigate the mechanism by which salidroside is able to block propagation of the virus in the host cell, we studied its immunomodulatory effects on host innate immune factors.

Mammalian cells are equipped with a sophisticated immune surveillance system for the detection of viral pathogens. These molecular sensors are classically termed ?"pattern recognition receptors"? (PRRs) and include ?RIG-I-like receptors? (RLRs) [7]. RLRs are RNA helicases that respond to ssRNA or dsRNA. A number of ssRNA viruses such as para- and orthomyxoviruses,

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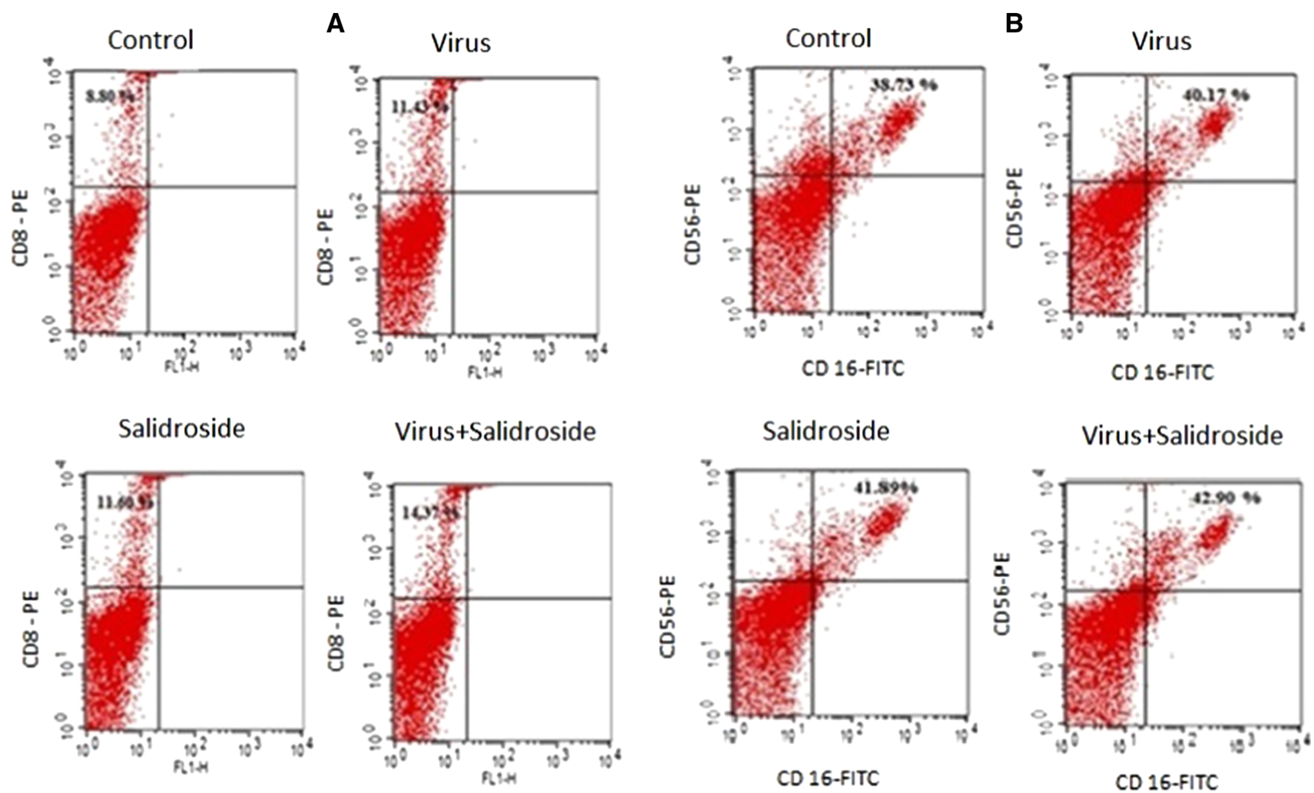

Fig. 8 Effect of salidroside on expression of **a** CD8$^+$ T cells and **b** CD16$^+$/56$^+$ natural killer cells in hPBMCs. The results shown are representative data from three independent experiments

Table 1 Expression of cell-surface markers of CD8$^+$ T cells and CD16$^+$/56$^+$ NK cells in DENV-infected and salidroside-treated hPBMCs

Sample (mean ± S.E.)	Population of % gated CD8$^+$ T cells	Population of % gated CD16$^+$/56$^+$ NK cells
Control	14.5 ± 3.3	28.4 ± 5.1
Virus	16.3 ± 3.3	29.7 ± 5.2
Salidroside	17.1 ± 3.1	30.5 ± 5.6
Virus + salidroside	18.6 ± 2.4	32 ± 5.4

rotaviruses, filoviruses and flaviviruses, are recognized by RIG-I. Its signaling involves binding of the C-terminal CARD domain of IPS-1, inducing the production of type I interferons. Salidroside increases RIG-I expression in DENV-infected or uninfected THP-1 cells. This increase in RIG-I expression suggests the antiviral potential of salidroside, as it increases host immunity by helping in recognition of DENV. As RIG-I is not induced during ADE, this drug could potentially be used for secondary infections. Upon recognition of pathogen-associated molecular patterns (PAMPs), PRRs initiate downstream signaling, resulting in the expression of antiviral molecules and many cytokines, including type I interferons (IFN-α/β). IFNs then induce an antiviral state in both infected and uninfected cells, further eliciting adaptive immune responses.

Innate antiviral mechanisms mediated by the IFN system are potentially the most important pathways of host cell defense, limiting viral replication during the critical early phase of DENV infection and prior to full recruitment of antigen-specific defenses [25]. In the present study, we observed that salidroside treatment increases the expression of IRF-3 and IRF-7 genes, thus helping to limit DENV infection in its initial stage.

During the initial phase of an immune response, many cytokines and chemokines are synthesized by cells of the innate immune system. The early cytokines have a multitude of functions, including antiviral activity and further activation of immune responses. Type-1 interferon downregulates viral promoters. Cytokines also downregulate expression of viral receptors and thus reduce viral entry. The type1 interferon level was found to be increased

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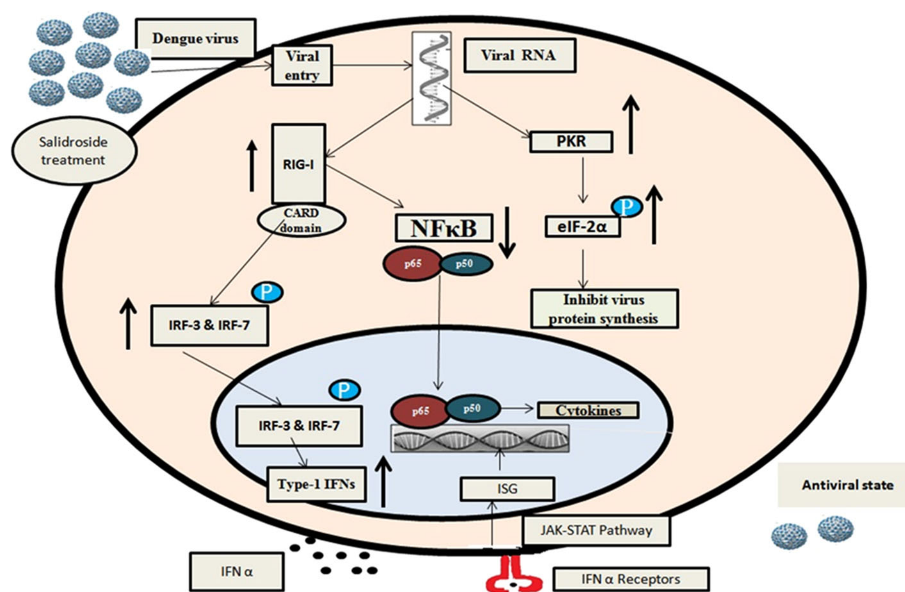

Fig. 9 Summary of the effect of salidroside on host innate immune factors in DENV-infected cells

significantly after salidroside treatment in DENV-infected cells, and this strengthens our hypothesis.

Activation of interferons and pathogen-induced stress in host cells have been known to increase the expression of the dsRNA–activated protein kinase PKR [24], which phosphorylates translation initiation factor eIF2α, causing inhibition of translation of both viral and cellular transcripts [22]. Salidroside treatment in DENV-infected cells increasing the expression of PKR, thereby increased the phosphorylation of eIF2α, which inhibits viral protein synthesis.

The NF-κB pathway provides an attractive target to viral pathogens. Activation of NF-κB occurs immediately, within minutes after exposure to a relevant inducer, and results in a strong transcriptional stimulation of several early viral and cellular genes [14]. During DENV infection, NF-κB is activated [2], leading to the activation of several proinflammatory mediators such as nitric oxide (NO) and TNF-α, which are involved in hemorrhage development by causing endothelial cell death. We observed that salidroside significantly decreases expression of NF-κB, and this feature may be attributed to its anti-inflammatory activity [8]. Thus, decreasing NF-κB expression subsequently limits DENV and its harmful effects.

NK cells are activated during early stages of DENV replication. Type 1 interferons and NK cells help in reducing viral replication during the early stages of DENV infection and thus limit subsequent pathogenesis [1]. NK cells may also produce cytokines that reduce inflammation and tissue injury. NK cells, upon activation, eliminate virus-infected cells by producing chemokines and anti-viral cytokines, mainly IFNγ and MIP1-β, by recognizing and eliminating infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) or by direct recognition through their activating receptors [26]. Salidroside was found to increase the expression of NK cells and $CD8^{+}$ T cells. NK cells are found in very low number, from 0.08 to 0.43×10^{6} cells/ml. Salidroside increases the number of NK cells by 1.11-fold, and although this increase is not statistically significant, it is still important for inhibiting DENV infection in its initial stage.

In summary (Fig. 9), our data show that salidroside is an excellent antiviral drug that decreases the intracellular DENV load not only in immune cells (THP-1 and hPBMCs) but also non-immune cells (Vero). Salidroside treatment helps to boost host innate immunity by increasing host innate immune factors such as RIG-I that specifically recognize viral RNA and alert the host immune system. The main mechanism behind the antiviral activity of salidroside is the subsequent activation of type 1 interferons *via* activation of IRF-3 gene. Furthermore, an increase in PKR activity and phosphorylation of eIF-2α leads to a decrease in viral replication and protein synthesis. Salidroside exhibits its anti-inflammatory effect by decreasing NF-κB production and preventing inflammation. In addition, salidroside was also found to increase the proportion of NK and CD-8 T cells in hPBMCs, which further helps the host to recognize the virus and helps in its clearance in its initial stage of infection. Our results thus give scientific support to the possibility of developing salidroside as an antiviral compound against DENV, which could be considered for the development of an effective therapeutic drug against DENV infection.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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