Andrographolide inhibits chikungunya virus infection by up−regulating host innate immune pathways

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ABSTRACT

Objective: To investigate the therapeutic efficacy of andrographolide, a plant derived compound, against chikungunya virus (CHIKV) infection. **Methods:** Using flow cytometry and immunoblotting assay, *in vitro* viral protein expression was studied in THP-1 cells line. In Balb/c mouse neonates, viral RNA copy number was determined by real time PCR. **Results:** The results showed reduced CHIKV protein expression on andrographolide treatment in CHIKV-infected human peripheral blood mononuclear cells, Vero cells and THP-1 cell line. *In vivo*, andrographolide treatment to CHIKV-infected neonates reduced viral RNA copy number. Further, andrographolide also increased cytotoxic T lymphocytes both *in vitro* and *in vivo*. Andrographolide also activated host innate immune pathways, *viz.*, protein kinase R, phosphorylated eukaryotic initiation factor 2 α , retinoic acid inducible gene-I and interferon regulatory factor 3/7, thereby increasing IFN- α secretion. CHIKV-induced nuclear factor κ light chain enhancer of activated B cells and tumor necrosis factor- α was also reduced on andrographolide treatment. **Conclusion:** Andrographolide inhibits CHIKV by suppressing viral protein expression and up-regulating host innate immunity and hence could be an effective therapeutic agent against CHIKV infection.

1. Introduction

Many vector borne diseases are known worldwide with no drug or vaccine available for the treatment. Chikungunya is one of vector-borne diseases without treatment available. Chikungunya virus (CHIKV), a RNA virus, is the causative agent of this disease, which belongs to family Togaviridae and genus *Alphavirus*. It is an enveloped virus with spherical structure of 60–70 nm. Infected *Aedes* mosquito transmits CHIKV to human and other primate hosts leading to fever, headache, rashes, arthralgia and myalgia. Persistent inflammation in joints sustains the symptoms of arthralgia and myalgia for years[1]. The disease was first described in Tanzania in 1952 and now Chikungunya has spread all over the world including Asia, Africa, Europe and America[2]. The ubiquitous presence of vector pose great threat to human population worldwide[3]. Apart from symptomatic treatment, there is no drug or vaccine available for the treatment of chikungunya till date[4].

Various strategies are being employed for the management of chikungunya. Pentosan polysulfate, a glycan, has been reported to

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reduce alphavirus-induced inflammation and cartilage destruction[5]. Artificial miRNAs have also shown inhibitory effects on CHIKV replication[6]. Attenuated CHIKV candidate vaccines have been tested in interferon (IFN) compromised mice and found to provide both short and long term protection[7]. However, no drug or vaccine has been approved for the treatment of chikungunya. The present study highlights the management of chikungunya by a plant derived antiviral.

Andrographolide is a purified major bioactive compound of plant *Andrographis paniculata* (family Acanthaceae) and has known anti-inflammatory properties[8-13]. It is a labdane diterpenoid majorly present in the aerial parts of the plant. The compound has been found to mitigate inflammation in various diseases like rheumatoid arthritis[14], melanoma[15], diabetes[16], chronic rhino sinusitis[17], cardiovascular disease[18], and cerebral ischemia[19]. It has also been known to have antiviral effects against variety of viruses including H1N1, herpes simplex virus and Epstein Barr virus[20]. Wintachai *et al.* has also demonstrated anti-CHIKV effects of andrographolide in HepG2 cells[21]. However, *in vivo* effects of andrographolide treatment on CHIKV and the pathway involved in virus elimination were not studied earlier.

To combat CHIKV infection, multitudinous approaches are required to inhibit virus propagation and virus-induced inflammation and also to strengthen the host antiviral immune response to eliminate virus from the body. The present work demonstrated the anti-CHIKV effect of andrographolide both *in vitro* and *in vivo*. Andrographolide reduced CHIKV-induced cytotoxicity *in vitro* and CHIKV RNA copy number and thus mortality *in vivo*. Moreover, andrographolide treatment enhanced antiviral CD8 T cells and also activated host innate immune pathways, *viz.*, retinoic acid inducible gene-I (RIG-I) and protein kinase R (PKR). RIG-I activation induce IFN secretion, exerting antiviral effects. Further, PKR activation leads to phosphorylation of eukaryotic initiation factor 2 α (EIF2 α) thus attenuating viral protein synthesis. The present study therefore substantiated the antiviral and immunomodulatory effects of andrographolide and highly recommended further screening of andrographolide in other CHIKV animal models for an early development of anti-CHIKV drug.

2. Materials and methods

2.1. Virus

The East Central South African strain of CHIKV isolated from major epidemic in India in year 2006 (DRDE-06, GenBank Accession No. EF210157) was obtained from Defence Research and Development Establishment (DRDE), Gwalior, India. CHIKV was propagated in Vero cell line. Supernatant obtained after 3 d of infection was filtered through 0.22 μm filter and stored at -80 ℃ until use. CHIKV concentration was calculated by plaque assay and

0.2 multiplicity of infection (MOI) was used for infection.

2.2. Preparation of formulation

Andrographolide (Sigma, USA) was dissolved at a concentration of 10 mg/mL in cell culture grade dimethyl sulfoxide (DMSO) (Sigma, USA) and further diluted in 0.01 mol/L phosphate buffer saline (PBS).

2.3. Cells

THP-1 and BHK-21 cell line were obtained from National Center for Cell Sciences (NCCS), Pune, India. Vero cell line was the generous gift from Dr. Sudhanshu Vrati, National Institute of Immunology (NII), New Delhi, India.

Human peripheral blood mononuclear cells (PBMCs) were isolated from the healthy volunteers by density gradient centrifugation. Informed consent was obtained from each healthy volunteer. Blood was collected in heparinized tubes and was equally diluted in PBS. Blood was then layered on histopaque (Sigma, USA) and then subsequently centrifuged at $1\ 100 \times g$ for 30 min for density gradient separation. Human PBMCs were isolated as buffy layer in a separate tube and then washed and suspended in media.

THP-1 and human PBMCs were maintained in RPMI-1640 medium (Sigma, USA) and BHK-21 and Vero cell lines were maintained in MEM medium (Sigma, USA) both supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), 100 U/mL penicillin (Sigma, USA), and 100 μg/mL streptomycin (Sigma, USA). Cells were maintained at 37 ℃ in humidified 5% CO_2 incubator (Sanyo, Japan).

2.4. In vitro study

2.4.1. In vitro CHIKV infection and andrographolide treatment

Human PBMCs were infected with CHIKV at 0.1 and 0.2 MOI. Then, 0.2 MOI was found to have predominant infection and therefore used for further infection in Vero cells, human PBMCs and THP-1 cells. For infection, cells were suspended in media without FBS and rotated for 2 h at room temperature for virus binding. Cells were washed with media to remove unbound virus and then plated at a concentration of 1×10^6 cells/mL in media supplemented with 2% FBS. Following infection, andrographolide treatment was given to the cells and incubated for 72 h at 37 ℃ in 5% CO_2 atmosphere. After incubation, cells were harvested for lysate preparation or flow cytometry experiments and supernatants were collected and stored at -80 ℃ until assay.

2.4.2. Immunoblotting assay

Cells after incubation were harvested for cell lysate preparation as per the standard protocol[22]. Protein concentration was estimated

using Bradford's reagent (Sigma, USA) and 40 μg of protein was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel[23]. Protein was then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA)[24]. The membrane was incubated for 1 h in 3% BSA dissolved in TBS buffer (0.1 mol/L Tris–HCl, pH 7.4, 0.9% NaCl) to block non-specific binding. Followed by washing in TBST20 (0.1% Tween-20 in TBS), membrane was incubated with antibody against following proteins: CHIKV (Abcam, USA, catalogue number. ab130852), RIG-I (Sigma, USA, catalogue number PRS3953), PKR (Sigma, USA, catalogue number SAB3500326), interferon regulatory factor 3 (IRF3) (Sigma, USA, catalogue number SAB3500280), interferon regulatory factor 7 (IRF7) (Sigma, USA, catalogue number PRS3941), phosphorylated eukaryotic initiation factor 2 α (pEIF2 α) (Thermofisher Scientific, USA, catalogue number PA1-14138), and nuclear factor κ light chain enhancer of activated B cells (NF- κ B) (in nuclear extract) (Biovision, catalogue number 3038). After incubation, membrane was washed with TBST20 and then incubated with respective secondary antibodies including anti-mouse IgG 2b-HRP (AbD Serotec, UK), anti-rabbit IgG biotinylated (Sigma, USA, B8895), streptavidin-peroxidase polymer (Sigma, USA, S2438) and the protein was detected by chemiluminescence method (Sigma, USA).

2.4.3. Reverse transcriptase polymerase chain reaction (RT–PCR)

Viral RNA was isolated from 140 μL of Vero and THP-1 cell supernatant/serum collected from Balb/c mouse neonates, using viral RNA isolation kit (Qiagen, Germany). Following the manufacturer's instructions, 50 μL of viral RNA was isolated and eluted from the column in sterile centrifuge tube and stored at -80 ℃ until use.

CHIKV RNA was amplified to cDNA by RT-PCR using One-step RT-PCR kit (Qiagen, Germany). Using primers for *E1* gene (CHIKV E1 forward primer 5'-ACG CAA TTG AGC GAA GCA C-3', CHIKV *E1* reverse primer 5'-CTG AAG ACA TTG GCC CCA C-3'), a 204-bp product was obtained and run on 1% agarose gel. The image of the product was analyzed on Gel Doc molecular imager (Biorad, USA).

2.4.4. Cell viability test

Cytotoxicity caused by CHIKV was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human PBMCs were infected with CHIKV and treated with andrographolide at different concentrations. The suitable dose of andrographolide was further tested in Vero cells for its antiviral effects.

CHIKV-infected human PBMCs and Vero cells were suspended at a concentration of 1×10^6 cells/mL respectively in RPMI-1640 medium and MEM medium supplemented with 2% FBS. Cells were plated in 96-well culture plate (BD Falcon, USA) and incubated for 24, 48 and 72 h. After incubation, 0.5 mg/mL MTT (Sigma, USA) was added to cells followed by incubation for 4 h. MTT get converted

into blue formazan crystals by the active mitochondria of the living cells. The crystals were dissolved in DMSO (200 μL/well) and optical density was measured at 570 nm using 96-well plate reader (Biotek Instruments, USA). Percent cytotoxicity in human PBMCs and percent proliferation in Vero cells were calculated in reference to healthy untreated cells.

2.4.5. Intracellular staining

THP-1 cells after 72 h of infection were harvested and washed twice with 0.01 mol/L PBS. Cells were fixed at 4 ℃ using 4% formaldehyde for 20 min and permeabilized using 0.01% Triton X-100 in PBS for 10 min. After washing, cells were incubated with anti-CHIKV antibody (Abcam, USA, catalogue number ab130852) for 60 min at 4 ℃. Cells were fluorescently labeled using anti-mouse IgG-PE antibody (BD Biosciences, USA). Followed by washing, cells were suspended in 0.5 mL PBS and 10 000 cells were acquired on Flow Cytometer (BD FACS caliber) using CellQuest Pro software.

2.4.6. In vitro extracellular staining

Surface markers were studied in human PBMCs obtained after 72 h post CHIKV infection. PBMCs were washed with PBS and fixed for 20 min using 4% formaldehyde and then incubated at 4 ℃ with anti-human CD8-PE (Bio-legend, USA) antibody for 45 min. Followed by washing, cells were suspended in 0.5 mL of PBS and 10 000 cells were acquired on Flow Cytometer (BD FACS caliber) using CellQuest Pro software.

2.4.7. Cytokine estimation by ELISA

The cell supernatant of PBMCs and THP-1 was collected for the tumor necrosis factor α (TNF- α) and IFN- α estimation. TNF- α (PeproTech, USA) and IFN- α (BOSTER Immunoleader, USA) were measured using human ELISA kit following manufacturer's instructions. Optical density was obtained from spectrophotometer (Biotek Instruments, USA) and concentration was estimated in pg/mL from the standard curve.

2.5. In vivo study

2.5.1. Grouping of animals

Balb/c mouse neonates were used as CHIKV animal model and ethical clearance was obtained from the Institutional Ethical committee. Animals were divided into three groups (n=18), namely, group 1: healthy control; group 2: CHIKV-infected; and group 3: CHIKV-infected and andrographolide-treated group (CHIKV+ A100).

2.5.2. Andrographolide treatment

Infection was established by infecting 20 μL CHIKV (1×10^6 pfu/mL) subcutaneous to 1-day-old suckling neonates on day 0 and after 3–4 h, intraperitoneal andrographolide treatment (100 μL per injection) (100 mg/kg body weight in 1% DMSO) was given for 3 alternate

days (day 0, 2 and 4). Andrographolide treatment at 100 mg/kg body weight dose was standardized for the mouse neonates considering the minimal dose and solvent cytotoxicity. In healthy animals, 1% DMSO was given for 6 alternate days.

Treatment regime of 6 d was given as highest mortality in the virus-infected group occurred after 6 d and therefore experiments were terminated on day 6. Viral RNA was collected from the blood for quantitative PCR analysis and splenocytes were collected for surface marker study.

2.5.3. Quantitative RT–PCR

The viral RNA was isolated from the serum collected from blood of CHIKV-infected and andrographolide-treated Balb/c mouse neonates and quantified using SS III Platinum one step quantitative RT-PCR kit (Invitrogen, USA) following manufacturer's instructions in Mx3005P system (Stratagene, La Jolla, USA). The primers targeting *E1* gene with forward sequence 5'-ACG CAA TTG AGC GAA GCA C-3' and reverse sequence 5'-CTG AAG ACA TTG GCC CCA C-3' was used to quantify CHIKV RNA following the method described by Agarwal *et al*[25]. Thermal profile included 30 min of reverse transcription at 50 ℃. After initial denaturation of 5 min at 95 ℃, 40 cycles of amplification were run at 95 ℃ for 30 s, 55 ℃ for 1 min and 72 ℃ for 30 s. Melting curve analysis was done using Mx3005P melting curve software.

2.5.4. In vivo extracellular staining

Surface markers were studied in splenocytes of Balb/c mouse neonates' obtained on day 6 after *in vivo* CHIKV infection and andrographolide treatment. Following the extracellular staining method mentioned above, harvested splenocytes were incubated with anti-mouse CD 25-PE (Ebioscience, USA) and anti-mouse CD8-PE (Ebioscience, USA) antibody for 45 min at 4 ℃. Followed by washing 10 000 cells were acquired on Flow Cytometer (BD FACS caliber) using CellQuest Pro software.

2.6. Statistical analysis

The results are expressed as mean±SEM and all the statistical comparisons were carried out using Students' independent *t*-test and one-way analysis of variance (ANOVA) wherever applicable for repeated measurements. All analyses were performed using GraphPad Prism 5 software. Significance level was set at $P \leq 0.05$.

3. Results

3.1. Confirmation of THP–1 as suitable model for CHIKV infection

To confirm THP-1 as suitable model for CHIKV infection, THP-1 and Vero cells were infected with CHIKV and viral RNA was amplified by RT-PCR. The electrophoresis of RT-PCR products showed increase in viral RNA in THP-1 cells, thus confirming THP-1 as suitable model for *in vitro* study. Viral RNA from Vero cells was taken as positive control.

3.2. In vitro antiviral effects of andrographolide on CHIKV infection

3.2.1. In human PBMCs

Inhibitory effects of andrographolide on virus-induced cytotoxicity were assessed by MTT assay. Andrographolide (at concentration of 0.5, 1, 5 and 10 μg/mL) against CHIKV in human PBMCs at 24, 48 and 72 h post infection (p.i.) were studied. Andrographolide at concentrations of 5 and 10 μg/mL showed increased cytotoxicity both with and without CHIKV infection. Andrographolide at the concentration of 1 μg/mL showed less toxicity without CHIKV as compared to with CHIKV at 24, 48 and 72 h p.i. However, andrographolide at the concentration of 0.5 μg/mL showed the least cytotoxicity both with and without CHIKV infection at 72 h of infection, though the changes were not significant. Therefore, andrographolide at the concentration of 0.5 μg/mL showed antiviral effects in human PBMCs and hence this dose was used for further *in vitro* experiments (Figure 1A).

3.2.2. In Vero cells

Antiviral effects of andrographolide against CHIKV-induced cell cytotoxicity were also confirmed in Vero cells at 24, 48 and 72 h post CHIKV infection. Vero cell line, being IFN α/β-deficient, do not interfere in virus propagation and drug solely inhibit virus propagation. Andrographolide (0.5 μg/mL) without CHIKV infection showed significant increase in proliferation at 24, 48 and 72 h as compared to control cells ($P \leq 0.05$), signifying its non-cytotoxic and proliferative effects. However, Vero cells after 72 h of CHIKV infection showed the least proliferation because of virus-induced cytotoxicity. Whereas, andrographolide treatment (at 0.5 μg/mL) to CHIKV-infected cells significantly increased the cell proliferation as compared to CHIKV-infected cells at 24, 48 and 72 h p.i ($P \leq 0.05$). Thus andrographolide at the concentration of 0.5 μg/mL 72 h post CHIKV infection exhibited anti-CHIKV effects in Vero cells by promoting cell proliferation and reducing virus induced cytotoxicity (Figure 1B).

3.2.3. In THP–1 cells

Anti-CHIKV effects of andrographolide were also confirmed in human monocytic cell line (THP-1) by CHIKV protein expression study. Through immunoblotting, it was found that andrographolide treatment reduced CHIKV protein expression in CHIKV-infected cells significantly as compared to that in CHIKV-infected cells without treatment ($P \leq 0.05$) (Figure 1C). Intracellular viral protein expression was studied by flow cytometer and results showed inhibitory effects of andrographolide on viral protein expression.

CHIKV infection in THP-1 cells increased the mean fluorescent intensity to (628±55) as compared to control cells (58±0). However, andrographolide treatment (at 0.5 µg/mL) significantly reduced mean fluorescent intensity to (161±5) ($P \leqslant 0.05$) (Figure 1D).
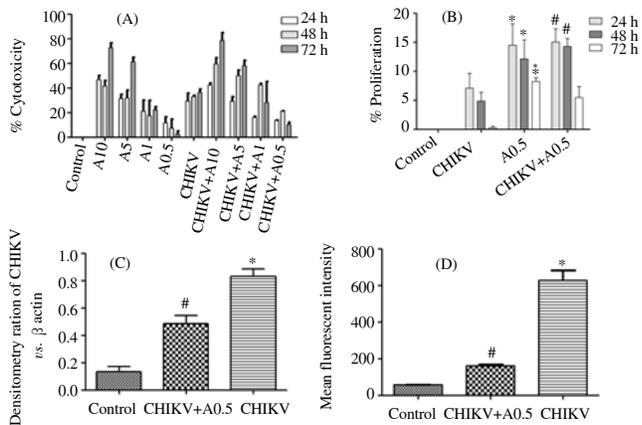

Figure 1. *In–vitro* antiviral effects of andrographolide on CHKIV infection.
Figure 1A represents percent cytotoxicity in human PBMCs (*n*=4) at 24, 48 and 72 h post CHIKV infection at various doses of andrographolide, *i.e.*, 10, 5, 1, and 0.5 µg/mL (A10, A5, A1 and A0.5). CHIKV represents CHIKV infection alone, while CHIKV+A10, CHIKV+A5, CHIKV+A1 and CHIKV+A0.5 represents CHIKV infection in combination with andrographolide 10, 5, 1 and 0.5 µg/mL, respectively. Figure 1B represents percent proliferation in Vero cells 24, 48 and 72 h post CHIKV infection (*n*=3). Figure 1C represents densitometry ratio of immunoblot performed in THP-1 cell line (*n*=3) 72 h post CHIKV infection. Figure 1D represents flow cytometry data performed using THP-1 cells (*n*=3) 72 h post CHIKV infection. In Figures 1A, 1B, 1C and 1D, CHIKV represents CHIKV-infected cells; A0.5 represents andrographolide treatment at 0.5 µg/mL; CHIKV+A0.5 represents the CHIKV-infected cells treated with andrographolide at 0.5 µg/mL; and control represents the healthy cells. *P<0.05 *vs* control; #P<0.05 *vs* CHIKV-infected cells.

3.3. In vitro effect of andrographolide treatment on CD8 T cells infected with CHIKV

CHIKV infection to human PBMCs did not affect the number of CD8 T cells as compared to control cell [(15±1)% *vs.* (15±5)%]. Andrographolide treatment to CHIKV-infected cells increased the CD8 T cell number to (18±7)%, whereas, CD8 T cells on andrographolide treatment alone (at 0.5 µg/mL) was (14±5)%. Though, the changes were not significant.

3.4. Andrographolide mediated activation of innate immune pathway

3.4.1. PKR
PKR expression decreased significantly on CHIKV infection ($P \leqslant$ 0.05) (Table 1), whereas andrographolide treatment to CHIKV-infected cells increased PKR expression, hence activating host antiviral pathway to eliminate CHIKV.

3.4.2. pEIF–2 α
Andrographolide treatment to CHIKV-infected cells significantly increased the pEIF-2 α expression ($P \leqslant 0.05$) which is required for CHIKV propagation, thus attenuating the viral replication by inhibiting cellular protein synthesis (Table 1).

3.4.3. RIG–I
Andrographolide treatment to CHIKV-infected cells up-regulated RIG-I protein expression significantly as compared to CHIKV-infected cells ($P \leqslant 0.05$) (Table 1), thereby reducing CHIKV propagation in the host.

3.4.4. IRF3 and IRF7
CHIKV inhibited activation of IRF3 and IRF7 protein expression. However, andrographolide treatment significantly activated IRF3 and IRF7 protein expression in CHIKV-infected and uninfected cells ($P \leqslant 0.05$) (Table 1).

3.4.5. NF– κ B
CHIKV infection initiated the inflammatory pathway inside the host which led to NF- κ B activation and its nuclear translocation; whereas, andrographolide with and without CHIKV infection inhibited NF- κ B translocation significantly ($P \leqslant 0.05$), ultimately inhibiting CHIKV-induced inflammation (Table 1).

3.5. Effect of andrographolide on cytokine levels

3.5.1. IFN– α secretion
Chikungunya induced activation of IFN- α secretion in human PBMCs and THP-1 showed activation of virus clearance machinery of host. However, andrographolide treatment to CHIKV-infected cells further activated the host innate immune system by increased IFN- α production.

Table 1
Effect of andrographolide on host innate immune pathway in CHIKV-infected THP-1 cells.

Groups	PKR	pEIF-2 α	RIG-I	IRF-3	IRF-7	NF- κ B
Control	1.6±0.1	0.5±0.2	1.0±0.1	0.4±0.0	0.2±0.0	1.0±0.0
A0.5	1.3±0.3	0.7±0.1	0.8±0.0	1.0±0.0*	0.4±0.0*	0.3±0.0*
CHIKV	0.4±0.1*	0.6±0.1	0.6±0.0**	0.9±0.0	0.1±0.0	1.3±0.0
CHIKV+A0.5	0.9±0.1	1.0±0.1*	0.9±0.1#	1.5±0.1#	0.3±0.0	0.6±0.2#

The data shown represents the densitometry ratio of different innate immune pathway proteins expressed on CHIKV infection in THP-1 cells. The values are expressed as mean±SEM. PKR, pEIF-2 α , RIG-I, IRF-3, IRF-7 and NF- κ B proteins expression were estimated by immunoblotting (*n*=3) using THP-1 lysate 72 h post CHIKV infection. CHIKV represents CHIKV-infected cells; A0.5 represents andrographolide treatment at 0.5 µg/mL; CHIKV+A0.5 represents the CHIKV-infected cells treated with andrographolideat at 0.5 µg/mL; and control represents the healthy cells. *P≤0.05 *vs* control; **P≤0.01 *vs* control, #P≤0.05 *vs* CHIKV-infected cells.

CHIKV infection in human PBMCs increased the IFN-α secretion compared to control cells. However, in THP-1 cells there was a slight decrease in IFN-α on CHIKV infection as compared to control cells. Further, treatment of andrographolide to PBMCs and THP-1 increased IFN-α secretion. Moreover, combination of andrographolide and CHIKV further increased the IFN-α in PBMCs and THP-1 (Table 2).

Table 2

In vitro modulation of andrographolide treatment on IFN-α and TNF-α secretion in CHIKV-infected cells (pg/mL).

Groups	IFN-α secretion		TNF-α secretion
	THP-1 cells	Human PBMCs	
Control	297±7	5±4	8±8
A0.5	356±9	9±7	0±0
CHIKV	306±22	135±46	225±97
CHIKV+A0.5	410±21*#	146±50	225±97

Values are expressed as mean±SEM. IFN-α secretion was estimated by ELISA in THP-1 cells and human PBMCs (n=3) 72 h post CHIKV infection. TNF-α was estimated by ELISA in human PBMC supernatant 72 h post CHIKV infection (n=3). CHIKV represents CHIKV-infected cells; A0.5 represents andrographolide treatment at 0.5 μg/mL; CHIKV+A0.5 represents the CHIKV-infected cells treated with andrographolide at 0.5 μg/mL; and control represents the healthy cells. *P<0.05 *vs* control; #P<0.05 *vs* CHIKV-infected cells.

3.5.2. TNF–α secretion

CHIKV infection in human PBMCs increased TNF-α production compared to control cells. However, andrographolide treatment alone suppressed the TNF-α secretion and in combination with CHIKV infection, though the changes were not significant (Table 2).

3.6. In vivo anti–CHIKV effects of andrographolide in CHIKV–infected Balb/c mouse neonates

3.6.1. Andrographolide increased weight gain and percent survival in mouse neonates

Balb/c mouse neonates infected with CHIKV undergo weight loss due to arthralgia and myalgia. Andrographolide treatment at various concentrations was tested in neonates and andrographolide at the dose of 100 mg/kg body weight was found effective and prevented the weight loss compared to that in the CHIKV-infected neonates without treatment (Table 3). CHIKV infection along with weight loss also led to mortality in neonates. Andrographolide treatment to CHIKV-infected neonates did not allow CHIKV to cause mortality in neonates and therefore maintained the survival of neonates to 100% as compared to CHIKV-infected neonates with survival rate of 83% till the 5th day of infection (Table 3).

3.6.2. Andrographolide reduced CHIKV RNA in mouse neonates

The serum collected from CHIKV-infected Balb/c mouse neonates on treatment with andrographolide at the dose of 100 mg/kg body weight showed a significant ($P \leqslant 0.05$) decrease in CHIKV RNA as compared to CHIKV-infected neonates ($\log_{10}$ RNA copy number 4.39 *vs* 6.80). This was confirmed by one step quantitativePCR analysis with a positive control RNA from CHIKV positive patient serum (with $\log_{10}$ RNA copy number of 5.38). Inhibition of CHIKV replication in blood of Balb/c neonates indicated the inhibitory effects of andrographolide on viral replication.

3.6.3. Andrographolide increased CD25 and CD8 T cells in mouse neonates

Andrographolide treatment to CHIKV-infected Balb/c mouse neonates induced the number of CD25 positive cells significantly ($P \leqslant 0.05$) and CD8 T cells as compared to that in the CHIKV-infected neonates. In healthy control neonates, the number of CD25 and CD8 was (46±8)% and (10±2)%, respectively.

4. Discussion

Chikungunya is a debilitating disease leading to fever, rashes, arthralgia and myalgia at early stage of infection, whereas immense pain in joints and muscles can last for months and years after the infection. The inability of immune system to suppress virus replication and inflammatory response in joints leads to CHIKV-induced pathogenesis. Presently, there is no drug or vaccine available for chikungunya treatment which can inhibit CHIKV replication and virus-induced inflammation and can induce host innate immune response for CHIKV elimination.

Andrographolide is a potent anti-inflammatory[26] and antiviral agent in variety of pathogenic conditions[20]. It has also demonstrated its immunomodulatory effects[27-29]. The present study showed the anti-CHIKV effects of andrographolide both *in vitro* and *in vivo*. In

Table 3

In–vivo effect of andrographolide in CHIKV infected mouse neonates.

Groups	Weight gain (g)	Percent survival (%)	Surface markers (% gated population of cells)	
			CD25	CD8
Healthy control group	3±1	100	46±8	10±2
CHIKV-infected group	2±0	83	53±6	18±4
CHIKV + A100	3±0	100	87±2*#	29±9

The data shown represents the effect of andrographolide treatment on CHIKV infection in mouse neonates and expressed in terms of weight gain, percent survival, viral RNA copy number, and surface marker expressed like CD25 and CD8. CHIKV+A100 represents the CHIKV-infected cells treated with andrographolide at 100 μg/mL Viral RNA copy number was estimated in blood serum (n=18; as viral RNA was isolated from the blood pooled from six mouse neonates in each group), whereas surface markers (n=3) were studied in splenocytes on day 6 of experiment. For weight gain and survival studies number of animals taken were 18. Surface marker, weight gain and RNA copy number values are expressed as mean±SEM. *$P \leqslant 0.05$ *vs.* control and #$P \leqslant 0.05$ *vs.* CHIKV-infected group.

vitro andrographolide treatment to CHIKV-infected human PBMCs, THP-1 and Vero cells reduced virus-induced cytotoxicity and viral protein expression. In Bzxalb/c mouse neonates, andrographolide treatment reduced CHIVK RNA copy number bolstering its anti-CHIKV effects.

Andrographolide apart from decreasing CHIKV protein expression, exerts antiviral effects by activating host innate antiviral pathway and by subsiding the CHIKV-induced inflammation. PKR and RIG-I are host proteins involved in viral RNA detection and thereby activate host innate immune response. RIG-I can bind double stranded RNA as well as single stranded RNA with 5′ triphosphate[30], whereas PKR can bind double stranded RNA only[31]. CHIKV-inhibited PKR protein expression led to inhibition of phosphorylation of EIF-2 α protein, thus preventing downstream translation of proteins involved in mitigation of stress[32]. However, andrographolide treatment to CHIKV-infected cells activated the PKR protein leading to increased phosphorylation of EIF-2 α , thus resulting in inhibition of viral protein synthesis[33].

The function of RIG-I like receptors is regulated by IRF3 and IRF7 transcription factors[34]. RIG-I inhibition on CHIKV infection inhibits the downstream IRF3 and IRF7 transcription factors which in turn restricts the type I IFN production. However, andrographolide treatment to CHIKV-infected cells activated IRF3, IRF7 and thus IFN- α production. IFN production exhibit antiviral response and promote recognition of virus-infected cells by cytotoxic T cells[35-37]. Further, the increased number of CD8 T cells on andrographolide treatment explains its antiviral effects on virus-infected cells.

Inflammation is the cause of chikungunya associated ailments[38,39]. CHIKV exerts inflammatory effects by stimulating pro-inflammatory cytokine production by activation of transcription factor NF- κ B[40,41,42]. However, andrographolide is a known anti-inflammatory agent[9,12,17] and thus its treatment to CHIKV-infected cells reduced TNF- α and NF- κ B expression, thereby reducing virus-induced inflammation.

In vivo, CHIKV infection to Balb/c mouse neonates causes inflammation in joints and muscles, making them incapable to feed themselves with mother's milk. The inability to move and feed finally results in weight loss and ultimately death. The resultant weight loss in neonates causes stress to the mother which induced neonatal cannibalism by mother[43], further increasing the virus-induced neonatal mortality. Andrographolide treatment to CHIKV-infected neonates prevented weight loss and neonatal mortality by restricting the viral RNA replication and by increasing activated antiviral immune cells.

The present study therefore confirmed the antiviral effects of andrographolide against CHIKV by inhibiting virus propagation and virus-induced inflammation and also by activating the host innate immune pathway. Further screening of andrographolide in other CHIKV animal models can result in early development of a therapeutic drug against CHIKV infection. Anti-CHIKV as well as therapeutic effects of andrographolide in transmission of virus from infected mother to neonates can be further tested in adult mouse models suitable for CHIKV infection[44]. Andrographolide is also known for feticidal and ovicidal activity[45]. Inhibitory effects of andrographolide in CHIKV transmission from mosquito to either its host or to its progeny can be studied by feeding them with andrographolide inoculated blood meal[46]. Andrographolide, being anti-inflammatory and anti-rheumatic arthritis agent[14,47,48], can further help to ameliorate virus-induced arthritis.

Conflict of interest statement

Authors report no conflict of interest.

Acknowledgments

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