Evaluation of antiviral activity of *Andrographis paniculata* and *Tinospora cordifolia* using *in silico* and *in vitro* assay against DENV-2

Anubrata Paul, Arpana Vibhuti and V Samuel Raj

DOI: https://doi.org/10.22271/phyto.2021.v10.i2f.13847

Abstract

**Background:** Dengue is one of the most widespread arthropod-borne viral infections without any effective treatment. The anti-DENV-2 mechanism of plants *Andrographis paniculata* (whole plant), *Tinospora cordifolia* (stem & leaves), their bioactive synthetic compounds depend on acute febrile treatment, is poorly understood for new anti-dengue therapy development.

**Objectives:** The current study was undertaken to evaluate *in silico* and *in vitro* study on crude extracts, bioactive fractions, bioactive synthetic compounds of *A. paniculata*, *T. cordifolia* against anti-DENV-2.

**Methods:** *In silico* study was evaluated by Lipinski’s rule of five, drug-likeness score and molecular docking against DENV-2 NS2B-NS3. After *in silico* study, the antiviral activity was performed under *in vitro* conditions with cytotoxicity, pre-incubation, post-incubation, and protective assay.

**Findings:** It was observed that *in silico* studies, the best docked compounds andrographolide (-11.58 kcal/mol), magnoflorine (-9.22 kcal/mol) and their combination (50:50); ethanolic extract of *A. paniculata*, aqueous-ethanolic (50:50) extract of *T. cordifolia* and their combination (50:50) extract, their bioactive fractions with possible phenolic glycosides, pyridinecarboxylic acid, flavone, phenols, phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid, diterpenoids, quinic acid, isopalmitic acid and sesquiterpenoids compound class category, showed 50% minimum effective and inhibitory concentration.

**Conclusions:** The crude extracts, bioactive fractions and bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* and combination (50:50) could be the potential anti-DENV-2 therapy in *in silico* and *in vitro* infection model.

**Keywords:** Dengue virus, *Andrographis paniculata*, *Tinospora cordifolia*, Molecular docking, PRNT assay

**Introduction**

Dengue virus (DENV), transmitted by female *Aedes aegypti* mosquito has affected over 700 million people globally and 40 million of Indian population mostly in tropical and subtropical countries of world [1]. It has caused adverse economic effects across world due to its morbidity and mortality every year in urban and rural areas [2]. Dengue has been first recorded in Chinese Medical Encyclopedia in 265-420 AD (Jin Dynasty) as “Water Poison” [3]. The first epidemic of dengue occurred in Calcutta in 1963 while the outbreak of DENV was first identified in Madras in 1780 [4]. In 1967, an outburst of dengue was reported in Delhi where more than 10,000 people were hospitalized and 423 were declared dead [5]. WHO has accepted the mosquitoes as “Public Enemy number One” because today dengue ranks as most important mosquito borne disease [6]. Acute febrile illness is the most common early stage symptoms of dengue [7]. Dengue fever (DF) and Dengue hemorrhagic fever (DHF), known as “Break borne fever” are acute febrile disease symptoms caused by arthropod-borne DENV 1-4 serotypes of the genus Flavivirus family “Flaviviridae” in human. The maximum number of DENV-2 cases, and most frequently identified, prevalent serotype infection outbreaks were reported globally, mainly in India to be a public cause of acute febrile fever [8]. The DENV genome, a positive-sense single-stranded RNA, and its size is 10.7kb. It encodes a single poly-protein precursor that contains of three structural proteins (Envelope, Capsid and Membrane) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [6]. The efficient profiling research indicates that based on the dengue life cycle, NS2B-NS3 protease of DENV 1-4 shares very similar peptide substrate structure activity relationships, measured as a crucial goal for anti-DENV medicine development [9].
According to WHO factsheet of primary health care, 90% of the inhabitants depend on traditional medication, a significant source for therapeutic manufacturing in African and Asian countries due to geographical and economic constraints [10]. The plant-based extracts, bioactive fractions and bioactive synthetic compounds are used in large amount because of relatively low cost, effectiveness, less side effects, easy availability and cultural acceptance in disease prevention. Various parts of plants such as leaf, stem, bark, and root were being used to prevent tropical and acute febrile illness disease like malaria, diarrhea, tuberculosis, pneumonia and asthma etc. for many centuries [11]. Therefore, the screening of herbal plants may help to treat dengue infection and also may help in regulating dengue infection and has been widely used as a part of Indian folk medicine and Ayurveda for several eras to prevent tropical and acute febrile illness disease [12, 13].

In the present study Andrographis paniculata, Tinospora cordifolia were evaluated for their antiviral activity using in silico and in vitro assay and have been widely used as a part of Indian folk medicine and Ayurveda for several eras to prevent tropical and acute febrile illness disease. This new approach involve screening of crude extract, bioactive fractions and bioactive synthetic compounds with antiviral potential. Phytochemical analysis is a simple and quick procedure to analysis of phytochemicals like alkaloids, flavonoids, glycosides, saponins, phenols, tannins, steroids etc. present in A. paniculata and T. cordifolia plant [14, 15]. Column chromatography, flash chromatography and thin layer chromatography (TLC) with different solvents should be used to isolate, identify and separation of the bioactive fractions from plant crude extracts by solvent extraction using different solvent systems [16, 17]. In vitro screening of crude extracts, bioactive synthetic compounds, bioactive fractions of A. paniculata and T. cordifolia was done using MTT based cytotoxicity assay and plaque reduction neutralization assay (PRNT) to evaluate antiviral activity [18]. In silico study is a new approach for a rapid identifying drug compounds to analysis of molecular properties, drug-likeness score and molecular docking to predict the best inhibitors using bioinformatics tools and software [9]. So, in this study the some acute febrile symptoms relied based plants like A. paniculata and T. cordifolia were chosen for the antiviral study against DENV-2.

**Materials and Methods**

**In silico studies**

From the protein databank (PDB) (www.rcsb.org/pdb/), the crystal structure (PDB ID: 2FOM) of the DENV-2 NS2B-NS3 was acquired and for molecular visualization and energy minimization of DENV-2 NS2B-NS3, SPDB viewer (www.expasy.org/spdbv/) was used and for active site and binding pocket detection studies of DENV-2 NS2B-NS3 were done by using CASTP (Computed Atlas of Surface Topography of Proteins). Based on IC₅₀ value, toxicity, larvicidal activity and medicinal uses, structure of 82 bioactive compounds of A. paniculata and T. cordifolia was collected from Pubchem database, generated from the SMILES notation (Simplified Molecular Input Line Entry Specification) by using Open Babel Software. Geometry optimization and energy minimization were carried out using the chimera software after construct the structures [19]. The drug-likeness score and Lipinski’s rule of five of the bioactive compounds of A. paniculata and T. cordifolia depend on acute febrile symptom treatment were investigated by molinspiration and molsoft online server to evaluate pharmacological and biological properties with orally active toxic or nontoxic in human. Lipinski’s rule of five (Ro5) consists of HBA/ HBD value up to 10 and 5, respectively; MW less than 500, LogP value less than 5 and total polar surface area (TPSA) value less than 140 Å. Molecular Docking calculations were carried out with Auto Dock 4.0 software.

This docking procedure was applied for all bioactive compounds of A. paniculata and T. cordifolia against NS2B-NS3 protease receptor of DENV-2 (PDB: 2FOM). Gasteiger partial charges, non-polar hydrogen atoms, rotatable bonds, essential hydrogen atoms, Kollman charges and salvation parameters were selected and defined to implement for docking studies with 23 Å² affinity grid point maps and 0.375 Å² spacing using the auto grid program. Docking simulations were achieved using Lamarckian Genetic Algorithm (LGA) and derived from 10 different runs after a maximum of 250000 energy estimations, translational step of 0.2 Å [20].

**Experimental studies**

**Plant extraction, fractionation and characterization**

Dried and powder samples of A. paniculata (stem, leaves and roots) and T. cordifolia (stem, leaves) were extracted by Soxhlet hot extraction and cold maceration techniques with 250ml of methanol, ethanol, aqueous-methanol (50:50) and aqueous-ethanol (50:50). The collected plant samples were authenticated in CSIR-NISCAIR, New Delhi. A. paniculata’s Ref. No. NISCAIR/RHMD/Consult/2018/3222-23–l and T. cordifolia’s Ref. No.-NISCAIR/RHMD/Consult/2018/3222-23-2. The resulting solutions were filtered separately and the solvent was evaporated under reduced pressure, thereby the percentage of extraction was calculated. The phytochemical screening was done following the standard procedure as described. Evaluations of the major phytochemicals such as alkaloids, flavonoids, tannins, saponins, steroids, glycosides, phenols were conducted. Each dry methanolic extract of A. paniculata and ethanolic extract of T. cordifolia was subjected to flash chromatographic column over silica gel (200-400 mesh size), with a gradient of hexane-ethyl acetate and chloroform-methanol respectively, as the mobile phase, at increasing polarities. In total, more than 200 fractions were obtained and TLC, LC-MS were used for further analysis, thus enabling molecular mass characterization of the active fractions by LC-MS analysis based on profiles and predictive analysis. The MS analysis was performed using ESI in the negative mode. The MS analysis was carried out using Mass Spectrometer. The mass spectrometry parameters were: Retention time 0.268-0.451 min, 115 scans, and frag= 60.0 V, m/z range= 0- 400 [21]. The resolved compounds were then identified using online software i.e., MassBank of North America (MoNA) which is a public repository natural product library for sharing mass spectral data. The identification of bioactive compound class was based on mass and intensity obtained via records [22].

**In vitro studies**

Vero (African green monkey kidney) cells used in this study were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% inactivated FBS at 37°C, 5% CO₂ and 75% Humidity. During the time of virus propagation and antiviral assay the FBS concentration of the cell culture medium was reduced to 2%. The DENV-2 strain was isolated from ICGEB, New Delhi.
The DENV-2 strain was propagated in Vero cells and harvested after full cytopathic effect was observed. Viruses were further characterized by plaque assay and the obtained stock was aliquoted and stored at -80°C\[^{18, 23}\].

**Cell viability assay**

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)] assay was performed to evaluate the cytotoxicity of crude extracts, bioactive fractions, bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* in Vero cells using previously standardized protocols. Briefly, monolayers of Vero cells were grown in 96-well plate and were treated with different concentrations in 8 replicates together with negative control (media containing 0.1% Dimethyl sulfoxide (DMSO)). It was followed by incubation at 37°C with 5% CO\(_2\) for 48 hours before the MTT assay was performed. Two days post-treatment, 10µl of 5 mg/ml MTT solution was added to the cells and incubated for 4 hours at 37°C with 5% CO\(_2\) followed by the addition 150µl of DMSO, prior to absorbance detection at 495 nm wavelength using multiplate reader. Percentage survival of cells after treatment was determined through this assay using Graph Pad Prism 5\[^{[23]}\].

**Antiviral activity of crude extracts, bioactive fractions, bioactive synthetic compounds of plants**

*In vitro* screening of crude extracts, bioactive synthetic compounds, bioactive fractions of *A. paniculata* and *T. cordifolia* was done using plaque reduction neutralization assay (PRNT) to evaluate antiviral activity. In PRNT assay, Vero cells were infected with DENV-2 strains at a multiplicity of infection (MOI= 1) in the presence of extracts, bioactive fractions and synthetic compounds at non-cytotoxic concentrations and DENV-2 was removed after 1 hour 37°C incubation time. After that the infected cells were covered with 150µl of DMEM medium after adding 1% CMC and incubated at 37°C for 2 days. Finally, cell plaques and virus titers were determined by using crystal violet solution (pfu/100µl). After the *in vitro* screening assay, PRNT was executed which was defined the half-maximal inhibitory concentration (PRNT50=50% inhibition of the plaque count) for each concentration (µg/ml) of the extracts, their combinations and bioactive fractions against DENV-2, with reference to the virus control (100% infection or 0% inhibition)\[^{[18]}\].

**Pre-incubation assay**

Vero cells were seeded in 96-well plates (20,000 cells/ well), a day in advance. DENV-2 (MOI=1) were separately pre-incubated with serial dilutions of crude extracts, bioactive fractions, bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* at non-cytotoxic concentrations in 100µl volume, at 37°C for 2 hours and at 4°C overnight (12 hours). The pre-incubation mixture was diluted with an equal volume of medium (DMEM+2% Fetal bovine serum (FBS)) and used to infect Vero cells (3 wells for each concentration at 200µl/well) in the 96-well plate. After 2 hours of adsorption in the incubator (37°C, 5% CO\(_2\)), infected cells were overlapped with 150µl of methylcellulose-containing growth medium and treated thereafter as described for the standardized plaque assay. To measure any potential cytotoxicity, cells were exposed to respective samples in the absence of DENV-2 infection with mock-infected (negative control) include cell and infected DENV-2 without possible anti-dengue agents (positive control). This assay was designed to identify the ability to block DENV-2 from entering susceptible cells.

**2. Post-treatment assay**

Vero cells in 96-well plates (20,000 cells/well) were infected with DENV-2 (MOI = 1) without pre-incubating with the crude extracts, bioactive fractions, bioactive synthetic compounds of *A. paniculata* and *T. cordifolia*. After 2 hours of adsorption, the virus inoculum was removed, the monolayer rinsed with 1X Phosphate-buffered saline (PBS), and then fed with complete medium containing the respective samples (corresponding non-cytotoxic concentrations). After 2 hours of contact, the monolayer was removed and overlaid with growth medium containing methylcellulose and plaques were settled after 48 days. This assay was considered to evaluate the capacity of plant samples to inhibit DENV-2 within the infected cell.

**3. Protective assay**

Vero cells in 96-well plates (20,000 cells/well) were treated with the various non-cytotoxic concentrations of crude extracts, bioactive fractions, bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* for 12 hours. Post treatment, the cells were washed two times with 1X PBS and infected with DENV-2 (MOI=1) for 2 hours. After 2 hours of adsorption, the mix was expressed, the monolayer was washed with 1XPBS and overlapped with growth medium containing methylcellulose and plaques were recognized after 48 days.

**Results & Discussions**

Dengue is one of the most important global pathogen and may represent a global pandemic. Each year, dengue infections results in approximately 2500 deaths occurs that 40% of the world’s population live in areas at risk for dengue. Investigations about dengue have relevance because it is the fastest spreading vector-borne viral disease, and it is endemic in over 100 tropical and sub-tropical countries. Most of the studies directly or indirectly used natural product as sources for their antiviral study since the active substances for most therapies derived conventionally from natural sources. However, there is an urgent need to rationalize the system by actually isolating the bio active constituents which could be active against dengue virus. Hence the present study has compiled on evaluating as efficient antiviral drugs which could be very effective on DENV-2. So, *A. paniculata* and *T. cordifolia* were selected as possible sources of anti-DENV-2 therapy based on its ethno pharmacological characteristics on acute febrile illness relief and known antiviral activity.

**In silico studies**

The 3D structure of the refined protein is most significance in providing insight into the molecular functions which will help in the identification of binding sites and may lead to the designing of new drug compounds. Swiss-Pdb Viewer was used an empirical energy function (residues, bonds, angles, torsion, improper, non-bonded, electrostatic constraint, total E) for energy minimization in (Supplementary Table 1). Energy minimization (E= -6.994.08 KJ/mol) adjusts the structure of the molecule in order to lower the energy of the system. A binding site analysis was carried out by using Castp online server on the basis of docking of anti-dengue drugs with the receptor protein 2FOM was searched for its active site. The study discovered that the residues GLU54 and SER75 were the major determinant of binding pocket and shows the interaction of bioactive synthetic compounds of *A. paniculata*, *T. cordifolia* in the form of hydrogen bonds.
(Supplementary Figure 1). The molecular properties and drug-likeness score of bioactive synthetic compounds from \textit{A. paniculata}, \textit{T. cordifolia} compounds showed various properties according to Lipinski's rule of five (Ro5) and drug-likeness score which indicated that Deoxyandrographolide, neoandrographolide, 5-hydroxy-7,8,2',3'-tetramethoxyflavone, 5-hydroxy-7,8,2',5'-tetramethoxyflavone, 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8-dimethoxyflavone from \textit{A. paniculata}; magnoflorine, syringin, tinocordiside, choline, jatrorrhizine from \textit{T. cordifolia} were found to have good drug likeness property (Supplementary Table 2, 3). As per previous study andrographolide (-5.66 kcal/mol) and 14deoxy11oxoandrographolide (-7.37 kcal/mol) had the best binding interactions with dengue virus NS5 protein (PDB ID: 3P97) \cite{24}. But there was no research on \textit{in silico} docking study of bioactive compounds of \textit{A. paniculata} against DENV-2 NS2B-NS3 protein (PDB ID: 2FOM). The binding analysis (-11.58 kcal/mol) of andrographolide showed those ligands had significant inhibitory activity against the target and could be a valuable drug candidates against dengue (Table 1, Figure 1 a). Also in earlier study, tinosponone in \textit{T. cordifolia} was found to be potent inhibitor of NS2B-NS3 receptor in DENV-2 with binding affinity (-2.8 kcal/ mol) \cite{25}. But our present study revealed that magnoflorine (-9.22 kcal/mol) in \textit{T. cordifolia} could be a potential drug candidate against DENV-2 NS2B-NS3 (PDB ID: 2FOM) (Table 2, Figure 1 b).

\textit{In vitro} assay

\textbf{Figure 2: MTT based cytotoxicity assay}

\textbf{Fig 2a: MTT based cell cytotoxicity assay for the different extracts of \textit{A. paniculata} and \textit{T. cordifolia}}

\textbf{Figure 2b: MTT based cell cytotoxicity assay for the ethanolic extract of \textit{A. paniculata} and aqueous-ethanolic extract of \textit{T. cordifolia} and their combination}
Figure 4: Post-Treatment assay

Fig 2c: MTT based cell cytotoxicity assay for the bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* and combination study (50:50)

Fig 4a: Post-treatment assay of different extracts of *A. paniculata* with DENV-2

Fig 4b: Post-treatment assay of different extracts of *T. cordifolia* with DENV-2
Table 1: Docking of *A. paniculata* compounds against DENV-2 NS3-NS2B

| Compounds                             | Est. Free Energy of Binding (kcal/mol) | Est. Inhibition Const, Ki |
|---------------------------------------|----------------------------------------|--------------------------|
| Andrographolide                       | -11.58                                 | 3.25 nM                  |
| Deoxyandrographolide                  | -9.52                                  | 105.52 nM                |
| Isoandrographolide                    | -10.95                                 | 9.33 nM                  |
| Neandrographolide                     | -10.80                                 | 12.09 nM                 |
| 5-hydroxy-7,8,2',3'-tetramethoxyflavone | -10.94                               | 9.60 nM                  |
| 5-hydroxy-7,8,2',5'-tetramethoxyflavone | -10.52                               | 19.56 nM                 |
| 5-hydroxy-7,8-dimethoxyflavone        | -10.35                                 | 25.98 nM                 |
| 5-hydroxy-7,8-dimethoxyflavone        | -10.39                                 | 24.33 nM                 |
Table 1: Docking results of T. cordifolia compounds against DENV-2 NS3-NS2B

| Compounds              | Estimated Free Energy of Binding (kcal/mol) | Estimated Inhibition Constant, Ki |
|------------------------|--------------------------------------------|-----------------------------------|
| Magnoflorine           | -9.22                                      | 173.74 nM                         |
| Syringin               | -4.22                                      | 802.85 nM                         |
| Tinocordifolin         | -8.44                                      | 645.53 nM                         |
| Tinocordiside          | -2.41                                      | 17.07 mM                          |
| Choline                | -4.55                                      | 466.62 uM                         |
| Jatrorrhizine          | -3.38                                      | 3.32 mM                            |
| Dimethyl nonanedioate   | -2.05                                      | 31.60 mM                           |
| N-methyl-2-pyroline    | -3.66                                      | 2.09 mM                            |

In vitro studies

For the crude extraction of A. paniculata and T. cordifolia, there was no research on combination extraction techniques like Soxhlet hot extraction and cold maceration technique. Maximum scientists were using Soxhlet hot extraction techniques for crude extraction of herbal plants [26]. In our present research, we were using (50:50) combination extraction technique of Soxhlet hot and cold maceration extraction. Our results show the maximum percent yield in ethanolic extract of A. paniculata (whole plant) (81.66%) and also in aqueous-ethanolic extract of T. cordifolia (stem & leaves) (45.12%) (Supplementary Table 4).

The phytochemical analysis of methanol, ethanol, aqueous-methanol (50:50) and aqueous-ethanol (50:50) extract of A. paniculata and T. cordifolia indicated the presence of bioactive compounds or phytochemicals that are existing in extracts like tannins, alkaloids, saponins, flavonoids, steroids, phenols, glycosides (Supplementary Table 5). Upadhyay et al. (2013) reported that the total phenolic content were present in ethanol extract and methanolic extract of T. cordifolia (stem) with 66.28±0.82 mg TA/g and 51.86±0.77 mg TA/g respectively [27]. Based on the study of Sivakumar et al. (2011), the high amount of alkaloids, phenolics and flavonoids was proven to the methanolic extract and in different fractions of methanolic, ethanolic, aqueous, chloroform extract of T. cordifolia (stem) [28]. In our study, the total content of phenolics, flavonoids and alkaloids of different extracts of A. paniculata and T. cordifolia ranged from 1.66±0.66 to 286.89±0.75 (mg/g), 0.30±0.05 to 252±1.17 (mg/g) and 0.39 ± 0.15 to 65.46±0.14 (mg/g). The ethanolic extracts of A. paniculata showed maximum phenolic content, the methanolic extract of A. paniculata showed the high amount of flavonoid, alkaloids content and aqueous-ethanolic extract of T. cordifolia presented sufficient amount of alkaloids content (Supplementary Table 6).

Gurupriya et al. (2018) reported that the total content of phenols, flavonoids and alkaloids of ethanolic extract of A. paniculata (stem) were 269.04±0.83 mg/gm, 237.02±0.59 mg/gm and 30.012 mg/gm respectively [29]. Also we revealed that the bioactive fraction of ethanolic extract of A. paniculata showed 180.42±0.15 amount of total flavonoids content. Also the bioactive fraction of ethanolic extract of A. paniculata showed 180.42±0.15 of flavonoids content with maximum amount. Furthermore 125.34±0.12 amount of alkaloids was present in high amount in aqueous-ethanolic extract of T. cordifolia (Supplementary Table 7). Owing to their antiviral and anti-inflammatory properties, phenols, flavonoids and alkaloids could have strong potential for being used in the search of new drugs for dengue.

Cell cytotoxicity of bioactive synthetic compounds from A. paniculata, T. cordifolia and generic medicines was determined using MTT assay in Vero cells and percent cell survival cells, non-cytotoxic were evaluated. In our present study it was observed that the maximum nontoxic concentration of different crude extracts of plants, their extract combination extract (50:50) was 250μg/ml, 400μg/ml respectively (Figure 2a, 2b). The similar results were reported in which the methanolic extract of (leaves) A. paniculata for 50μg/ml [30]. Also bioactive synthetic compounds of plants was exposed the maximum nontoxic concentrations were 10μg/ml (Figure 2c).

Further, three types of bioassays like pre-incubation, post-treatment and protective assay were developed to identify the concentration of best docked bioactive synthetic compounds of A. paniculata, T. cordifolia depend on acute febrile symptoms treatment for potential DENV-2 inhibitory activity. For all experiments viruses were prepared as mentioned in the material and method sections and were checked using PRNT to determine no. of plaques formed at MOI=1 and the virus used for the study was 3x10^6 pfu/100μl in concentration. In previous report revealed that those three types of assay were used for potential CHKV inhibitory activity [18].

Some scientists reported the inhibitory concentration of methanolic extract of A. paniculata (leaves) in Vero E6 cell line with DENV-1 at the concentration of 50μg/ml, but the aqueous, aqueous-ethanolic and ethanolic extract of A. paniculata (leaves) didn’t show antiviral activity against DENV-1 [30, 31]. Also it discovered in earlier study that the anti-dengue activity of ethanolic extract of the whole plants of A. paniculata (leaves) were found to be 25μg/ml in Vero cells at 50% minimum concentrations using pre-incubation, post-treatment and protective assay [23]. Further we have observed that the ethanolic extract of A. paniculata (whole plant) showed the best antiviral activity result with 46.67% inhibition at 3.905μg/ml using pre-incubation assay and 49.44% effective inhibition at minimum concentration of 7.81μg/ml using post-treatment assay with DENV-2-induced cytopathic effect on Vero cells. No protective effect of ethanolic extract of A. paniculata (whole plant) observed against DENV-2 (Figure 3a, 4a). Also Ramalingam et al. (2018) reported that ethanolic extract of A. paniculata showed a total 75% of inhibition against DENV using pre-treatment assay [32]. Till now none of the researchers have reported the antiviral screening of aqueous-ethanolic (50:50) and aqueous-methanolic (50:50) extracts of A. paniculata against DENV-2. Our present study revealed that aqueous-ethanolic (50:50) and aqueous-methanolic (50:50) extracts of A. paniculata showed 32.75% and 42.77% of plaque inhibition at lower concentration of 62.5μg/ml using pre-incubation assay and 48.33% and 46.11% of plaque inhibition at lower concentration of 125μg/ml using post-treatment assay against DENV-2 (Figure 3a, 4a).

Another research described that as an ayurvedic medicine T. cordifolia (giloy) can be given to dengue patients because it raises the platelet and also Vitamin C (ascorbic acid) with 500 mg dose raises interferon to protect from dengue like viral diseases with strong immune system [13]. Till now none of the researchers have reported the antiviral screening of methanolic, ethanolic, aqueous-methanolic (50:50) and aqueous-ethanolic (50:50) extracts of T. cordifolia (leaves, stem) against dengue. In our study revealed that the aqueous-ethanolic (50:50) extract of T. cordifolia proved to be effective dose against DENV-2, at minimum concentration of 1.95μg/ml with 49.44% inhibition using pre-incubation assay and 48.89% inhibition using post-treatment assay (Figure 3b, 4b).
Till now none of the researchers have reported the antiviral screening of combination formulation of ethanolic extract of \textit{A. paniculata} and aqueous-ethanolic (50:50) extract of \textit{T. cordifolia} against dengue. We have observed in our research that the combination of ethanolic extract of \textit{A. paniculata} and aqueous-ethanolic (50:50) extract of \textit{T. cordifolia} exhibited 0.975µg/ml with 47.22% inhibition using pre-incubation assay and 48.89% inhibition using post-treatment assay. Protective assay of this combination didn’t unveil any defensive effect against DENV-2 infection (Figure 3c, 4c). No researchers worked on bioactive fractions isolated from ethanolic extract of \textit{A. paniculata} and aqueous-ethanolic extract of \textit{T. cordifolia} against dengue. Our present study discovered that the fraction from ethanolic extract of \textit{A. paniculata} 48.34% at 100µg/ml; 47.33% at 200µg/ml and the fraction from aqueous-ethanolic extract of \textit{T. cordifolia} 49.44% at 50 µg/ml; 48.89% at 100 µg/ml showed the effective inhibition against DENV-2 (Figure 3d, 4d). Also there were no report on anti-DENV-2 activity of bioactive fractions from ethanolic extract of \textit{A. paniculata} and aqueous-ethanolic extract of \textit{T. cordifolia} using flash chromatography technique with ethyl acetate: hexane and chloroform: methanol solvent system and LC-MS technique to predict bioactive compound classes. In our study, the LC-MS data were useful to identify and characterize the LC-ESI MS data obtained in bioactive fraction of ethanolic extract of \textit{A. paniculata} peak and aqueous-ethanolic (50:50) extract of \textit{T. cordifolia} at retention time 0.268-0.451 min had predicted molecular mass range with m/z values which will be phytochemical class of phenolic glycosides, pyridinecarboxylic acid, flavone, phenols, phenylpropanoids, flavonoids, quinic acid, isopalmitylic acid and sesquiterpenoids (Supplementary Table 8, 9).

In previous research revealed that andrographolide from \textit{A. paniculata} had significant anti-DENV-2 activity with 50% effective concentrations for DENV-2 of 21.304µM and 22.739µM for HepG2 and HeLa respectively using post-infection assay \cite{20}. In our study we evaluated the antiviral activity of andrographolide compound against DENV-2 in Vero cells with 50% and 47.78% inhibition at 5µg/ml minimum concentration using pre-incubation and post-treatment assay, respectively (Figure 3e, 4e). There is no research reported on any other compounds or combination formulation from \textit{A. paniculata}, so in my present research we determined the antiviral activity of magnoflorine (bioactive synthetic compound from \textit{T. cordifolia}) against DENV-2 with 48.33% for both pre-incubation and post-treatment assay at 5µg/ml and 10µg/ml minimum concentration respectively and also the combination (50:50) of andrographolide+magnoflorine exhibited the anti-DENV-2 activity with 45.56% inhibition at 2.5µg/ml and 10µg/ml minimum concentration by the pre-incubation and post-treatment assay, respectively (Figure 3e, 4e).

In our published \textit{in silico} research paper in 2015 explained that (+)-catechin hydrate showed the best docking result against dengue receptor protein, but no researcher published any \textit{in vitro} research paper on (+)-catechin hydrate compound against dengue \cite{20}. In our \textit{in vitro} screening study showed that (+)-catechin hydrate have the potential anti-DENV-2 activity at 2.5µg/ml with 46.67% and 10µg/ml with 50% inhibition using pre-incubation and post-treatment assay, respectively (Figure 3e, 4e).

**In silico studies**

### Supplementary Table 1: Energy of refined protein after minimization

| Protein Receptor | Residues | Bonds KJ/mol | Angles KJ/mol | Torsion KJ/mol | Improper KJ/mol | Non-Bonded KJ/mol | Electrostatic constraint KJ/mol | Total E KJ/mol |
|-----------------|----------|--------------|---------------|----------------|-----------------|-------------------|-------------------------------|----------------|
| Refined 2FOM    | HTT A 43 to OXT B 167 | 103.56 | 551.74 | 935.75 | 185.42 | -5949.36 | -2821.22 | -6994.08 |

### Supplementary Table 2: Calculation of Molecular Properties of \textit{A. paniculata}

| Ligand                          | miLogP | TPSA | MW   | nONnOHNH | vviolations | Drug-likeness score |
|---------------------------------|--------|------|------|----------|-------------|---------------------|
| Andrographolide                 | 1.05   | 86.99| 550.45| 5         | 3           | 0                   | -0.62             |
| Deoxyandrographolide            | 1.77   | 66.76| 334.40| 4         | 2           | 0                   | 0.53              |
| Isoandrographolide              | 1.14   | 76.00| 350.45| 5         | 2           | 0                   | -0.47             |
| Neandrographolide               | 1.17   | 125.69| 480.60| 8         | 4           | 0                   | 0.17              |
| 5-hydroxy-7,8,2',5'-tetramethoxyflavone | 3.09   | 87.38| 358.35| 7         | 1           | 0                   | 0.50              |
| 5-hydroxy-7,8,2',5'-tetramethoxyflavone | 3.31   | 87.38| 358.35| 7         | 1           | 0                   | 0.34              |
| 5-hydroxy-7,8-dimethoxyflavone  | 3.23   | 85.45| 364.23| 7         | 1           | 0                   | 0.42              |
| 5-hydroxy-7,8-dimethoxyflavone  | 3.27   | 68.91| 298.29| 5         | 1           | 0                   | 0.38              |
| 14-deoxy-11-oxoandrographolide  | 0.62   | 83.83| 348.44| 5         | 2           | 0                   | -0.36             |
| 3,14-dideoxyandrographolide     | 2.87   | 46.53| 318.46| 3         | 1           | 0                   | -0.31             |
| 3-oxo-14-deoxyandrographolide   | 3.45   | 49.31| 367.51| 3         | 1           | 0                   | -0.35             |
| 7-hydroxy-14-deoxyandrographolide | 3.23   | 50.34| 354.58| 3         | 1           | 0                   | -0.36             |

### Supplementary Table 3: Calculation of Molecular Properties of \textit{T. cordifolia}

| Ligand                      | miLogP | TPSA | MW   | nONnOHNH | Drug-likeness score |
|-----------------------------|--------|------|------|----------|---------------------|
| Magnoflorine                | -1.26  | 58.92| 342.42| 5         | 2                   | 0                   | 0.80               |
| Syringin                    | -0.66  | 138.08| 372.37| 9         | 5                   | 0                   | 0.12               |
| Tinocordifolin              | 2.17   | 49.83| 250.34| 3         | 1                   | 0                   | -0.84              |
| Tinocordiside               | 1.24   | 116.45| 396.48| 7         | 4                   | 0                   | 0.47               |
| Choline                     | -4.24  | 20.23| 104.17| 2         | 1                   | 0                   | 0.02               |
| Jatrorhizine                | -0.35  | 51.81| 338.38| 5         | 1                   | 0                   | 1.00               |
| Dimethyl nonanedioate        | 2.49   | 52.61| 216.28| 4         | 0                   | 0                   | -1.33              |
| N-Methyl-2-Pyrolidine       | 0.04   | 20.31| 97.12 | 2         | 0                   | 0                   | -1.00              |
**Supplementary Figure 1:** Active site of DENV-2 NS3-NS2B (2FOM)

**In vitro studies**

**Supplementary Table 4:** Percentage yield of different crude extracts of *A. paniculata* and *T. cordifolia*.

| Plants   | Solvents (250 ml) | Weight of whole plant (g) | Yield of extract | Percent (%) |
|----------|-------------------|---------------------------|------------------|-------------|
|          | Maceration         | Hot extraction            |                  |             |
| *A. paniculata* |                   |                           |                  |             |
| Methanol | 25                | 25                        | 65.33            |             |
| Ethanol  | 25                | 25                        | 81.66            |             |
| Aqueous-methanol | 25              | 25                        | 25.30            |             |
| Aqueous-ethanol  | 25              | 25                        | 37.38            |             |
| *T. cordifolia* |                   |                           |                  |             |
| Methanol | 25                | 25                        | 24.90            |             |
| Ethanol  | 25                | 25                        | 30.22            |             |
| Aqueous-methanol | 25              | 25                        | 36.78            |             |
| Aqueous-ethanol  | 25              | 25                        | 45.12            |             |

**Supplementary Table 5:** Preliminary phytochemical analysis of *A. paniculata* and *T. cordifolia*.

| Plants   | Solvents | Alkaloids | Flavonoids | Glycosides | Saponins | Phenols | Tannins | Steroids |
|----------|----------|-----------|------------|------------|----------|---------|---------|----------|
| *A. paniculata* | Methanol | +         | +          | -          | +        | +       | +       | +        |
|           | Ethanol  | +         | +          | +          | +        | +       | +       | +        |
|           | Aqueous-methanol | +     | +          | +          | +        | +       | +       | +        |
|           | Aqueous-ethanol  | +     | +          | +          | +        | +       | +       | +        |
| *T. cordifolia* | Methanol | +         | +          | -          | +        | -       | -       | -        |
|           | Ethanol  | +         | +          | +          | +        | +       | -       | -        |
|           | Aqueous-methanol | +     | +          | +          | +        | -       | -       | -        |
|           | Aqueous-ethanol  | +     | +          | +          | +        | +       | +       | -        |

+ Presence of compound, - Absence of compound

**Supplementary Table 6:** Quantitative phytochemical analysis of *A. paniculata* and *T. cordifolia* extracts.

| Plants   | Extraction | Total phenolics (mg/g) | Total flavonoids (mg/g) | Total alkaloids (mg/g) |
|----------|------------|------------------------|-------------------------|------------------------|
| *A. paniculata* | Methanol   | 262.67±0.84            | 252±0.17                | 65.46±0.14             |
|           | Ethanol    | 286.89±0.75            | 233±0.13                | 45.32±0.12             |
|           | Aqueous-methanol | 245.67±0.36 | 227±0.16                | 32±0.57                |
|           | Aqueous-ethanol  | 274.22±0.07  | 209±0.05                | 37.12±0.72             |
| *T. cordifolia* | Methanol   | 2.44±0.38              | 0.48±0.07               | 1.77±0.03              |
|           | Ethanol    | 4.88±0.07              | 0.67±0.06               | 0.39±0.15              |
|           | Aqueous-methanol | 1.66±0.06  | 0.30±0.05               | 1.25±0.04              |
|           | Aqueous-ethanol  | 8.11±0.57              | 0.82±0.03               | 4.62±0.08              |

**Supplementary Table 7:** Quantitative phytochemical analysis of bioactive fractions of *A. paniculata* and *T. cordifolia*.

| Plants   | Extraction    | Fractions | Total phenolics (mg/g) | Total flavonoids (mg/g) | Total alkaloids (mg/g) |
|----------|---------------|-----------|------------------------|-------------------------|------------------------|
| *A. paniculata* | Ethanolic  | Fraction 1 | 95.98±0.22             | 105.89±0.59             | 90.98±0.56             |
|           |               | Fraction 2 | 54.17±0.16             | 180.42±0.15             | 120.13±0.83             |
|           |               | Fraction 3 | 44±0.17                | 52±0.17                 | 60.54±0.15             |
| *T. cordifolia* | Aqueous-ethanolic | Fraction 1 | 110±0.38                | 85.21±0.26               | 125.34±0.12             |
|           |               | Fraction 2 | 71±0.66                | 92.88±0.10               | 108.76±0.21             |
|           |               | Fraction 3 | 81±0.69                | 114.31±0.17              | 91.13±0.39             |
Antiviral activity against dengue virus using *T. cordifolia* to accept identifying the anti-DENV-2 therapy using in silico and analysis to determine the effective concentration and its immune activity against DENV-2. Therefore, it needs to be subjected to further purification and analysis to determine the effective concentration and its immune activity against DENV-2.

### Suppmentary Table 8: LC-MS analysis of bioactive fraction of ethanolic extract of *A. paniculata*

| Range of (M - H)\(^+\) m/z | (M - H)\(^+\) m/z of peaks | Possible compound classes |
|---------------------------|-----------------------------|--------------------------|
| 100-150                   | 101.0, 119.9, 130.9, 146.9  | Flavone, phenolic acid, phenylpropanoids, pyridinecarboxylic acids, phenols, diterpenoids |
| 150-200                   | 170.8                        | Phenylpropanoids, phenols, diterpenoids |
| 200-250                   | 209.7                        | Phenols, flavone, Phenolic glycosides, Phenylpropanoids |
| 250-300                   | 255.2                        | Phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid |
| 300-350                   | 325.0                        | Phenylpropanoids, flavonoids |
| 350-400                   | 376.9                        | Quinic acids, flavonoids, diterpenoids |

Extracted ion chromatogram of peaks with Retention time 0.268-0.451 min

### Suppmentary Table 9: LC-MS analysis of bioactive fraction of aqueous-ethanolic extract of *T. cordifolia*

| Range of (M - H)\(^+\) m/z | (M - H)\(^+\) m/z of peaks | Possible compound classes |
|---------------------------|-----------------------------|--------------------------|
| 100-150                   | 101.0, 113.0, 120.0, 131.0, 147.2 | Flavones, Flavonoids, phenolic acid, phenols, sesquiterpenoids |
| 150-200                   | 172.9, 182.8                | Phenylpropanoids, sesquiterpenoids, phenols |
| 200-250                   | 209.9                       | Phenols, flavone, Phenolic glycosides, Phenylpropanoids |
| 250-300                   | 255.1                       | Phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid |

Extracted ion chromatogram of peaks with Retention time 0.268-0.451 min

### Conclusion

The explosive dengue fever with acute febrile illness epidemic of 2019 in India the need for appropriate DENV-2 antivirals. In our study, we have tried to explore the different perspectives for dengue management and treatment and the effectiveness of crude extracts, bioactive fractions and bioactive synthetic compounds from *A. paniculata* and *T. cordifolia*. So, in our research, we have accelerated to identifying the anti-DENV-2 therapy using in silico and in vitro study. In earlier, very few studies have been carried out to accept in silico and in vitro study on *A. paniculata* and *T. cordifolia* as anti-DENV-2 therapy.

According to the present survey, andrographolide, magnoflorine and their combination of *A. paniculata* and *T. cordifolia* with the best docking results have potential antiviral activity against dengue virus using in silico and in vitro assay respectively. Also it is revealed that the *A. paniculata* and *T. cordifolia* are the potential therapy against dengue as it holds a significant antiviral activity. The crude extracts, combination formulation (50:50) and presence of different bioactive compound class in bioactive fraction from ethanolic extract of *A. paniculata*, aqueous-ethanolic extract of *T. cordifolia* have the effective and inhibitory antiviral activity against DENV-2 and could inhibit DENV-2 entry into the host cell. Therefore, it needs to be subjected to further purification and analysis to determine the effective concentration and its immune activity against DENV-2. Additionally, the evaluation studies in in vivo systems and the lead compounds clinical testing are essential for establishing the potential compounds effectiveness.

### Acknowledgement

We thank to Dr. Sujatha Sunil and her Vector Borne Diseases Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India and Centre for Drug Design Discovery & Development (C4D) and SRM University, Delhi-NCR, Sonepat to carry out the research.

### Reference

1. Lt Col MS Mustafa, Col V Rasotgi, Col S Jain, et al. Discovery of fifth serotype of dengue virus (DENV-5): a new public health dilemma in dengue control. Med J Armed Forces India. 2015;70:67-70.
2. Sophie Yacoub, Juthathip Mongkolsapaya, and Gavin Screaton et al. Recent advances in understanding dengue. F1000 Research 2016;5:1-10.
3. Srinivasa Rao Mutheneni, Andrew P Morse, Cyril Caminade et al. Dengue burden in India: recent trends and importance of climatic parameters. Taylor & Francis Emerg Microbes Infect 2017;6(8):e70.
4. Ramakrishnan SP, Geljand HM, Bose PN, Sehgal PN, Mukherjee RN, et al. The epidemic of acute haemorrhagic fever, Calcutta, 1963; epidemiological inquiry. Indian J Med Res 1964;52:633-650.
5. Kumar Vikrama, Naggala BN, Veena Pande, Aruna Srivastava, et al. An epidemiological study of dengue in Delhi, India. Acta Tropica 2016;153:21-27.
6. Samir Bhatt, Peter W. Gething, Oliver J. Brady et al. The global distribution and burden of dengue. Nature 2013;496:504-7.
7. Zhang Wang, Peng Che, Chen Zhou, et al. NS1-based tests with diagnostic utility for confirming dengue infection: A meta-analysis. International Journal of Infectious Diseases 2014;26:5766.
8. Congcong Guo, Zixing Zhou, Zihao Wen, Yumei Liu et al. Global epidemiology of dengue outbreaks in 1990–2015: A systematic review and meta-analysis. Frontiers in Cellular and Infection Microbiology 2017;7:1-11.
9. Muhammad Tahir ul Qamar, Saleha Kiran, Usman Ali Ashraf, et al. Discovery of novel Dengue NS2B/NS3 protease inhibitors using pharmacophore modeling and molecular docking based virtual screening of the ZINC database. Int. J. Pharmacol 2016;12(6):621-632.
10. WHO 2013. WHO Traditional Medicine Strategy 2014-2023. http://www.who.int/medicines/publications/traditional/tr_m_strategy14_23/en/.
11. Azadeh GHIAEE, Farzaneh NAGHIBI, Somayeh ESMAEILI, et al. Herbal remedies connected to malaria like fever in Iranian ancient medicinal books- Brief review article. Iranian J Parasitol. 2014; 9(4): 553-559.
12. Abayomi Sofowora, Eyitope Ogunbodede, Adedeji Onayade et al. The role and place of medicinal plants in the strategies for disease. Afr J Tradit Complement Altern Med 2013;10(5):210-229.
13. Shasank Sekhar Swain, Debasmita Dudey, et al. Anti-dengue medicinal plants: A mini review. Research and Reviews: Journal of Pharmacognosy and Phytochemistry 2013;1(2): 5-9.
14. Wen-Wan Chao, Bi-Fong Lin. Review isolation and identification of bioactive compounds in *Andrographis paniculata*. Chinese Medicine 2010;5(17):1-15.
15. Sasidharan S, Chen Y, Saravanan D, *et al.* Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 2011;8(1):1-10.
16. Aarti Sharma, Sarita Kumar, Pushplata Tripathi. Evaluation of the larvicidal efficacy of five indigenous weeds against an Indian strain of dengue vector, *Aedes aegypti* L. (Diptera: Culicidae). Journal of Parasitology Research 2016, 1-8.
17. Hend Keskes, Sahla Belhadj, Lohana Jlail, *et al.* LC-MS–MS and GC-MS analyses of biologically active extracts and fractions from Tunisian *Juniperus phoenice* leaves. Pharmaceutical Biology 2017;55(1):88-95.
18. Jaspree Jain, Sujatha Sunil, *et al.* Standardization of *in vitro* assays to evaluate the activity of polyherbal siddha formulations against chikungunya virus infection. Virus Dis 2018;29(1):32-39.
19. Mohd Adnan Kausar, Ali A, Qiblawi S, *et al.* Molecular docking based design of Dengue NS5 methyltransferase inhibitors. Bioinformation 2019;15(6):394-401.
20. Anubrata Paul, Arpna Vibhuti, Samuel Raj. Molecular docking NS4B of DENV 1-4 with known bioactive phyto-chemicals. Bioinformation 2016;12(3):140-148.
21. Laode Rijai HK. Chemical Profile by LC-MS/MS and Some Bioactivities from Leafs of Kolowe (*Chydenanthus excelsus*): A Wild and Rare Plant from Indonesia. J. Pharm. Sci. & Res 2017;9(2):111-118.
22. Horai H, Arita M, Kanaya S, *et al.* MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom 2010;45(7):703-14.
23. Jaspree Jain, Ankit Kumar, *et al.* Antiviral activity of ethanolic extract of Nilavembu Kudineer against dengue and chikungunya virus through *in vitro* evaluation. Journal of Ayurveda and Integrative Medicine 2018, 1-7.
24. Nithya P, Chitra Jeiyaram, Meenakshi Sundaram K, *et al.* Anti-dengue viral compounds from *Andrographis paniculata* by *in silico* approach. World Journal of Alternative Medicine 2014;1(2):10-16.
25. Jesvin Bency B, Mary Helen PA. *In silico* identification of dengue inhibitors in Giloy (*Tinospora cordifolia*) and Papaya. Journal of Emerging Technologies and Innovative Research 2018;5(12):506-511.
26. Ruchi Sood, Ruchi Sood, Rajendra Raut, Poormina Tyagi, *et al.* Cissampelos pareira Linn: Natural source of potent antiviral activity against all four dengue virus serotypes. Plos one Neglected Tropical Diseases 2015;9(12):1-20.
27. Upadhayay AK, Kumar K, Kumar A, *et al.* *Tinospora cordifolia* (Willd.) Hook. f. and Thoms. (*Guduchi*) - Validation of the Ayurvedic pharmacology through experimental and clinical studies. Int J Ayurveda Res 2010;1(2):112-121.
28. Sivakumar V, Dhana Rajan MS. Standardization & characterization of *Tinospora cordifolia* (Willd.) Miers ex Hook. f. & Thoms. plant stem extract in different solvent fractions. Asian Journal of Biochemical and Pharmaceutical Research 2011;4(1):105-112.
29. Gurupriya S, Cathrine L, Pratheema P, *et al.* Preliminary phytochemical screening and GC-MS analysis of ethanolic stem extract of *Andrographis paniculata*. International Journal of Recent Scientific Research 2018;9(1):25300-25303.
30. Leon IC Tang, Ling AP, Koh RY, *et al.* Screening of anti-dengue activity in methanolic extracts of medicinal plants. BMC Complementary and Alternative Medicine 2012;12(3):1-10.
31. Anna Pick Kiong LING, Bee Fong KHOO Ching Hua SEAH *et al.* Inhibitory activities of methol extracts of *Andrographis paniculata* and *Ocimum sanctum* against dengue-1 virus. International Conference on Biological, Environment and Food Engineering 2014, 47-52.
32. Ramalingam S, Karuppannan S, Padmanaban P, *et al.* Anti-dengue activity of *Andrographis paniculata* extracts and quantification of dengue viral inhibition by SYBR green reverse transcription polymerase chain reaction. AYU 2018;39:87-91.
33. Madia Mehboob, Faisal Nooroz, Shumaila Noreen. Natural and Herbal Remedies for Dengue Prevention. Pakistan Journal of Clinical and Biomedical Research 2014;2(2):44-47.
34. Patcharee Panraksa, Ramphan S, Khongwichit S. Activity of andrographolide against dengue virus. Antiviral Research 2017;139:69-78.