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

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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 *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 activity 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 IC_{50} 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 A^0 affinity grid point maps and 0.375 A^0 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 A^0 [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, atincreasing 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^{O}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^{O}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^{O}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^{O}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^{O}C incubation time. After that the infected cells were covered with 150µl of DMEM medium after adding 1% CMC and incubated at 37^{O}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 ($PRNT_{50}$=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^{O}C for 2 hours and at 4^{O}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^{O}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 trampled 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 *A. paniculata*, *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-dimethoxyflavavone, 5-hydroxy-7,8-dimethoxyflavone from *A. paniculata*; magnoflorine, syringin, tinocordiside, choline, jatrorrhizine from *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) [24]. But there was no research on *in silico* docking study of bioactive compounds of *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 *T. cordifolia* was found to be potent inhibitor of NS2B-NS3 receptor in DENV-2 with binding affinity (-2.8 kcal/ mol) [25]. But our present study revealed that magnoflorine (-9.22 kcal/mol) in *T. cordifolia* could be a potential drug candidate against DENV-2 NS2B-NS3 (PDB ID: 2FOM) (Table 2, Figure 1 b).

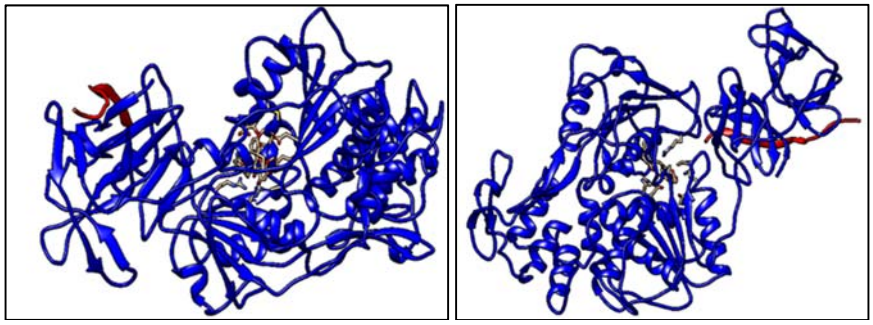


Fig 1: 3D image of a) Andrographolide b) Magnoflorine after Molecular docking

***In vitro* assay**

Figure 2: MTT based cytotoxicity assay

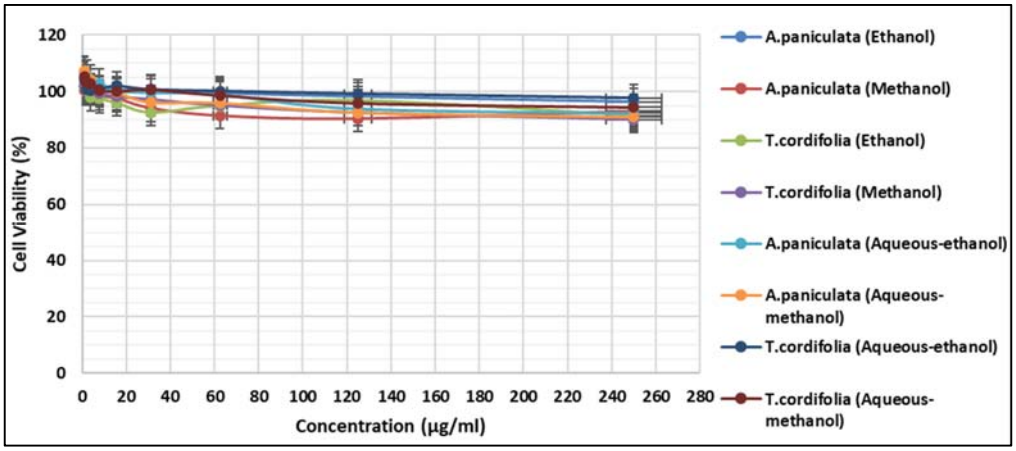


Fig 2a: MTT based cell cytotoxicity assay for the different extracts of *A. paniculata* and *T. cordifolia*

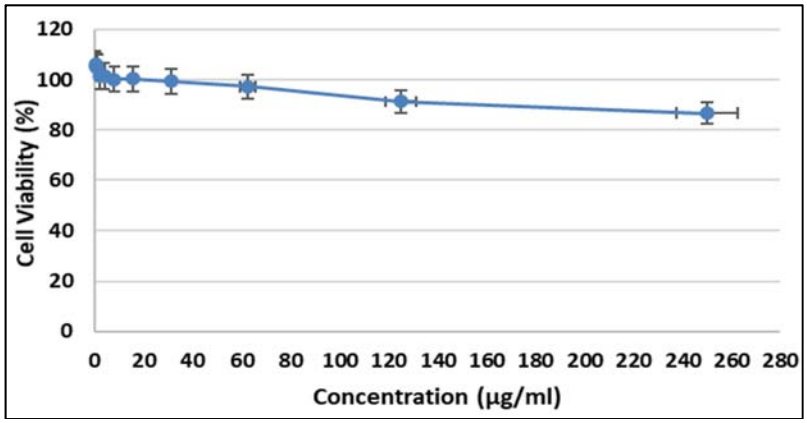


Figure 2b: MTT based cell cytotoxicity assay for the ethanolic extract of *A. paniculata* and aqueous-ethanolic extract of *T. cordifolia* and their combination

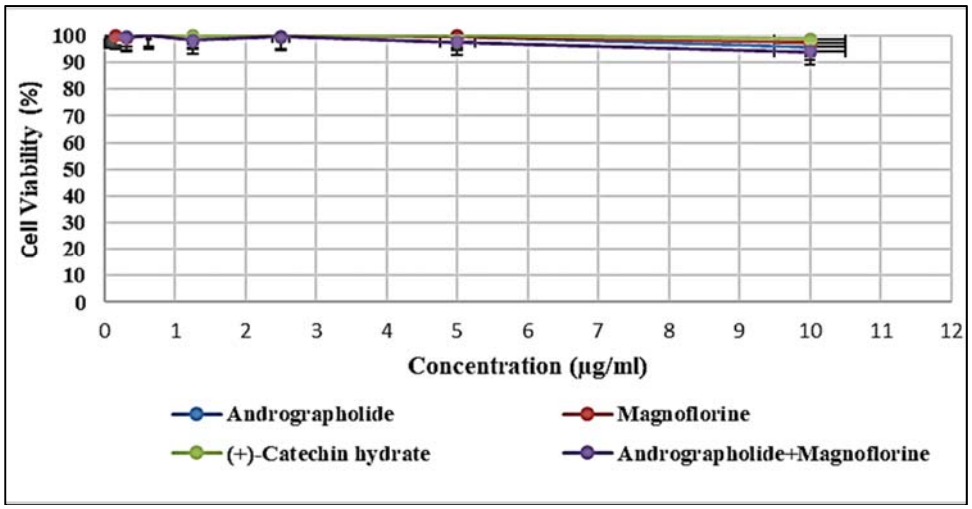


Fig 2c: MTT based cell cytotoxicity assay for the bioactive synthetic compounds of *A. paniculata* and *T. cordifolia and* combination study (50:50)

Figure 4: Post-Treatment assay

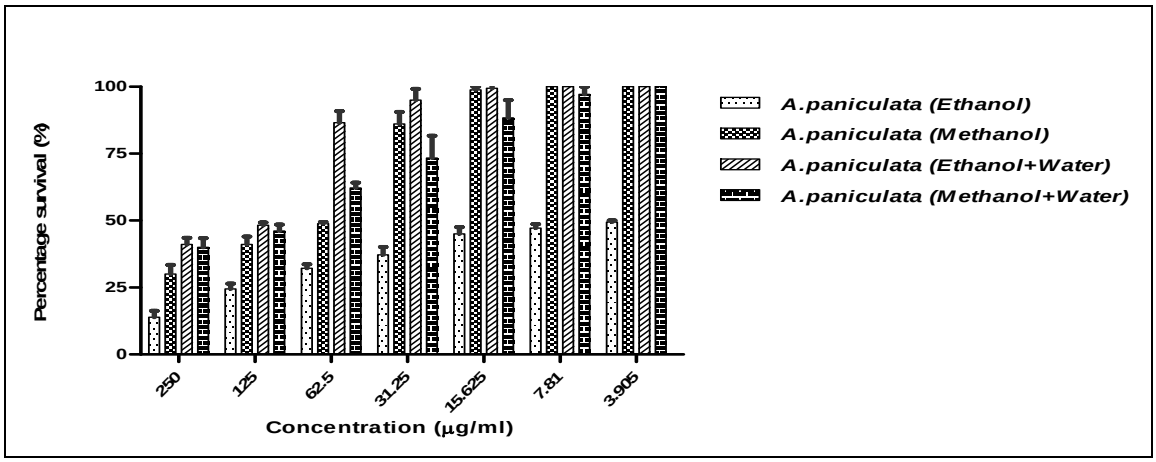


Fig 4a: Post-treatment assay of different extracts of *A. paniculata* with DENV-2

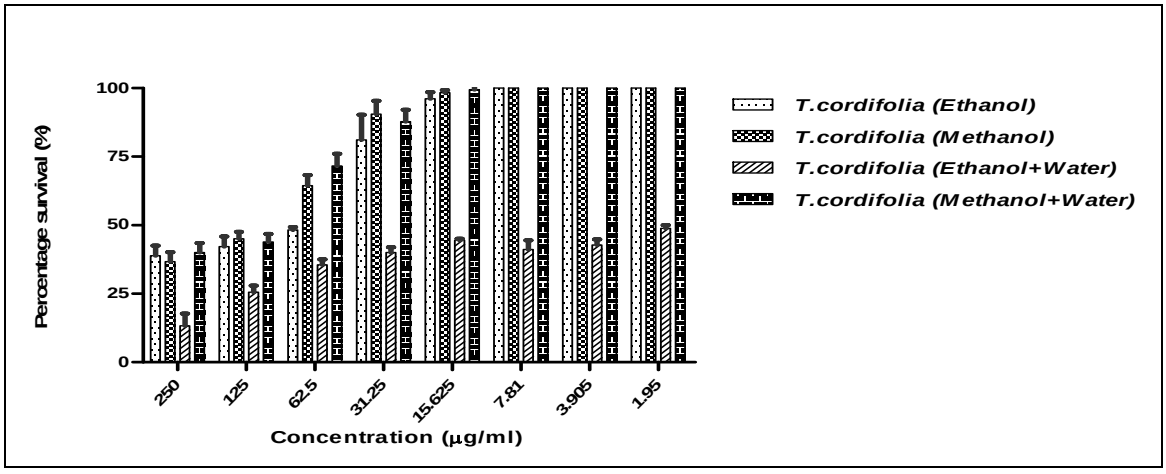


Fig 4b: Post-treatment assay of different extracts of *T. cordifolia* with DENV-2

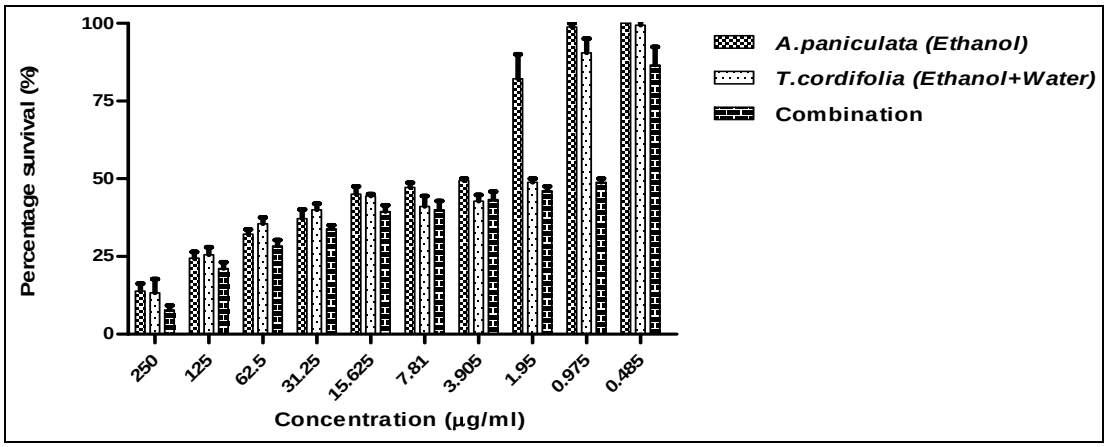


Fig 4c: Post-treatment assay of ethanolic extract of *A. paniculata* and aqueous-ethanolic extract of *T. cordifolia* and their combination with DENV-2

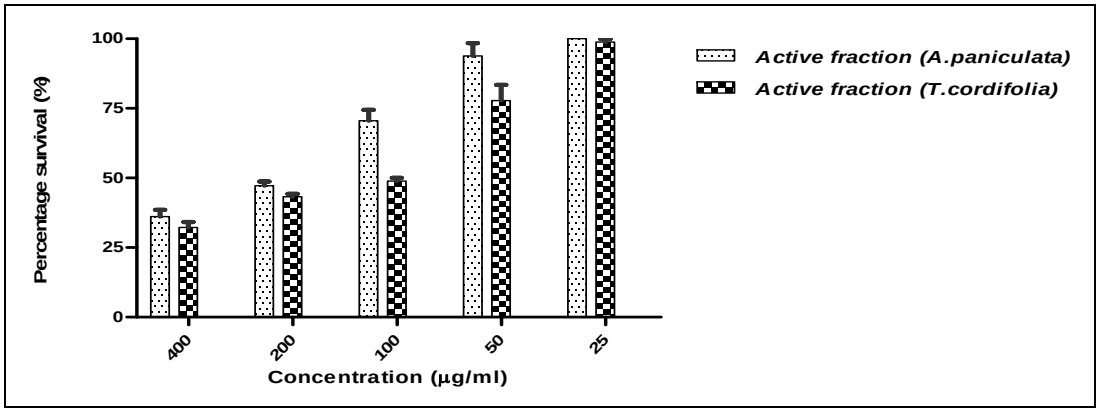


Fig 4d: Post-treatment assay of active fraction of *A. paniculata* and *T. cordifolia* with DENV-2

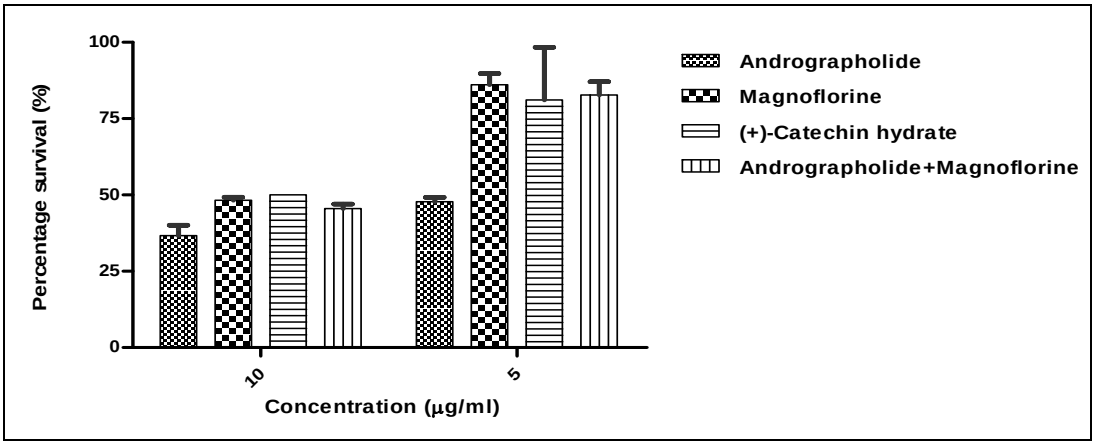


Fig 4e: Post-treatment assay of bioactive synthetic compounds of *A. paniculata* and *T. cordifolia* and combination study with DENV-2

Table 1: Docking of *A. paniculata* compounds against DENV-2 NS3-NS2B

Compounds	Est. Free Energy of Binding (kcal/mol)	Est. Inhibition Const, K_i
Andrographolide	-11.58	3.25 nM
Deoxyandrographolide	-9.52	105.52 nM
Isoandrographolide	-10.95	9.33 nM
Neoandrographolide	-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-dimethoxyflavavone	-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 uM
Tinocordifolin	-8.44	645.53 nM
Tinocordiside	-2.41	17.07 mM
Choline	-4.55	466.02 uM
Jatrorrhizine	-3.38	3.32 mM
Dimethyl nonanedioate	-2.05	31.60 mM
N-methyl-2-pyrrolidine	-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 ethanolic 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±0.17 (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±0.12 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 $3x10^8$ 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 [33]. 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 *A. paniculata* and aqueous-ethanolic (50:50) extract of *T. cordifolia* against dengue. We have observed in our research that the combination of ethanolic extract of *A. paniculata* and aqueous-ethanolic (50:50) extract of *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 *A. paniculata* and aqueous-ethanolic extract of *T. cordifolia* against dengue. Our present study discovered that the fraction from ethanolic extract of *A. paniculata* 48.34% at 100*µ*g/ml; 47.33% at 200*µ*g/ml and the fraction from aqueous-ethanolic extract of *T. cordifolia* 49.44% at 50 *µ*g/ml; 48.89% at 100 *µ*g/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 *A. paniculata* and aqueous-ethanolic extract of *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 *A. paniculata* peak and aqueous-ethanolic (50:50) extract of *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, phenolic acid, alkaloids, isopalmitic acid, diterpenoids, quinic acid, isopalmitic acid and sesquiterpenoids (Supplementary Table 8, 9).

In previous research revealed that andrographolide from *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 [34]. In our study we evaluated the antiviral activity of andrographolide compound against DENV-2 in Vero cells with 50% and 47.78% inhibition at 5*µ*g/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 *A. paniculata,* so in my present research we determined the antiviral activity of magnoflorine (bioactive synthetic compound from *T. cordifolia*) against DENV-2 with 48.33% for both pre-incubation and post-treatment assay at 5*µ*g/ml and 10*µ*g/ml minimum concentration respectively and also the combination (50:50) of andrographolide+magnoflorine exhibited the anti-DENV-2 activity with 45.56% inhibition at 2.5*µ*g/ml and 10*µ*g/ml minimum concentration by the pre-incubation and post-treatment assay, respectively (Figure 3e, 4e).

In our published *in silico* research paper in 2015 explained that (+)-catechin hydrate showed the best docking result against dengue receptor protein, but no researcher published any *in vitro* research paper on (+)-catechin hydrate compound against dengue [20]. In our *in vitro* screening study showed that (+)-catechin hydrate have the potential anti-DENV-2 activity at 2.5*µ*g/ml with 46.67% and 10*µ*g/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

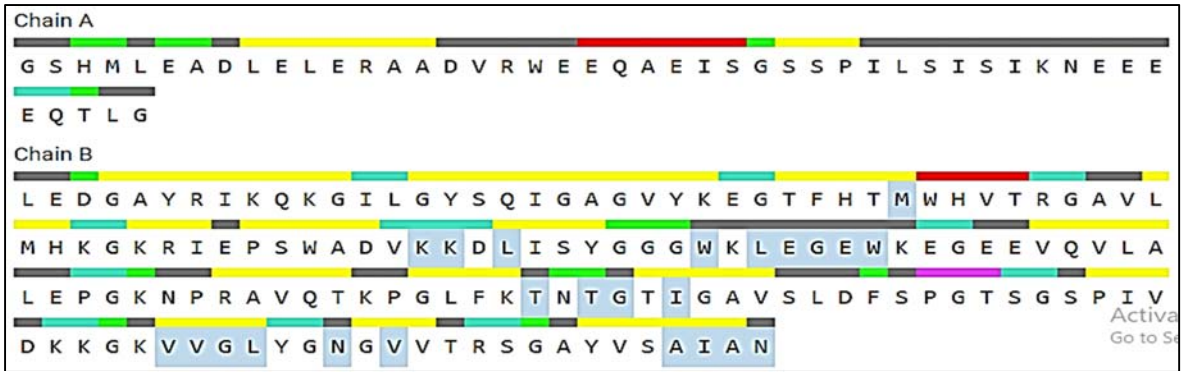
Protein Receptor	Residues	Bonds KJ/mol	Angles KJ/mol	Torsion KJ/mol	Improper KJ/mol	Non-Bonded KJ/mol	Electrostatic constraint KJ/mol	Total E KJ/mol
Refined 2FOM	HTT A 43 to OXT B 167	103.56	551.74	935.75	185.42	-5949.36	-2821.22	-6994.08

Supplementary Table 2: Calculation of Molecular Properties of *A. paniculata*

Ligand	miLogP	TPSA	MW	nON	nOHNH	nviolations	Drug-likeness score
Andrographolide	1.05	86.99	350.45	5	3	0	-0.62
Deoxyandrographolide	1.77	66.76	334.46	4	2	0	0.53
Isoandrographolide	1.14	76.00	350.46	5	2	0	-0.47
Neoandrographolide	1.17	125.69	480.60	8	4	0	0.17
5-hydroxy-7,8,2',3'-tetramethoxyflavone	3.09	87.38	358.35	7	1	0	0.50
5-hydroxy-7,8,2',5'-tetramethoxyflavone	3.31	87.38	358.38	7	1	0	0.34
5-hydroxy-7,8-dimethoxyflavavone	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 *T. cordifolia*

Ligand	miLogP	TPSA	MW	nON	nOHNH	nviolations	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
Jatrorrhizine	-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-Pyrrolidine	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
		Maceration	Hot extraction	Percent (%)
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±0.66	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	95.61±0.37	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

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

Range of $(M - H)^{-1}$ m/z	$(M - H)^{-1}$ m/z of peaks	Possible compound classes
100-150	101.0, 119.9, 130.9, 146.9	Flavone, phenolic acid, phenylpropanoids, pyridinecarboxylic acids, phenols, ditepenoids
150-200	170.8	Phenylpropanoids, phenols, ditepenoids
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

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

Range of $(M - H)^{-1}$ m/z	$(M - H)^{-1}$ 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.

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