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Evaluation of *in vitro* antiviral activity of *Vitex Negundo* L., *Hyptis suaveolens* (L) poit., *Decalepis hamiltonii* Wight & Arn., to Chikungunya virus

Sangeetha Kothandan, Rajarajan Swaminathan

PG and Research Department of Microbiology and Biotechnology, Presidency College (Aut), Chennai, India

PEER REVIEW

Peer reviewer

PJ. Shanmugam, Professor, Department of Microbiology, Sri Manakula Vinyagar Medical College and Hospital, Puducherry, India.
Tel: 09486623094
E-mail: drjshanmugam@gmail.com

Comments

Owing to the increasing epidemics of chikungunya virus, the present study is of utmost importance to the research community especially in the field of drug development. Although it is a preliminary study it would add valuable insights for the development of drugs in near future.
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ABSTRACT

Objective: To screen the three Indian plants for the antiviral activity to chikungunya virus since chikungunya infections are treated symptomatically without specific drugs till date.

Methods: *In vitro* cytotoxicity assay of the lyophilised extracts was assessed in vero cells for the determination of maximum non toxic concentration and *in vitro* antiviral assay was evaluated by the inhibition of virus induced cytopathic effect.

Results: Aqueous and aqueous ethanolic extracts of *Hyptis suaveolens* exhibited partial inhibition to Asian strain of chikungunya virus.

Conclusion: Of all the three plants tested for antiviral activity to both the lineages of chikungunya virus, *Hyptis suaveolens* were found to be effective to Asian strain of chikungunya virus.

KEYWORDS

Chikungunya virus, Cytotoxicity assay, Antiviral assay, *Hyptis suaveolens***1. Introduction**

Chikungunya viral infections are characterized by fever and debilitating prolonged arthralgia syndrome primarily affecting the peripheral small joints[1]. Although acute phase of fever resolves within a few days, incapacitating pain associated with atrophy lasts for months causing serious socioeconomic impact on the affected communities.

Other atypical manifestations of chikungunya virus infections are myocarditis, sinus tachycardia, haemorrhagic manifestations including haematemeses and melaena, neurological disease. Also varied ocular manifestations

like non-granulomatous anterior uveitis, episcleritis, panuveitis, granulomatous anterior uveitis, optic neuritis, sixth nerve palsy, retrobulbar neuritis, retinitis with vitritis, neuroretinitis, keratitis, central retinal artery occlusion, choroiditis, exudative retinal detachment, secondary glaucoma, unilateral papillitis, bilateral papillitis, retrobulbar neuritis, perineuritis, neuroretinitis had been reported during recent epidemics[2].

After 1973 epidemics of chikungunya virus had disappeared and since then, no case was reported till end of 2005. Chikungunya infections has resurged since 2005 causing annual recurrence of infections in India. The

*Corresponding author: Dr. Rajarajan Swaminathan, Coordinator, Bioinformatics Infrastructure Facility Center of DBT, PG & Research Department of Microbiology and Biotechnology, Presidency College (Aut), Chepauk, Chennai-05, India.

E-mail: rajarajan7101@gmail.com

Tel: +919282321575

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current infection rate is 4% to 45% affecting thousands of people each year^[3]. About 18 countries throughout Asia including Malaysia, Singapore, Thailand, Indonesia had reported thousands of infections and Europe, North America documented imported cases of chikungunya fever^[4].

Till now no specific antiviral drugs have been developed to treat the infections and are treated symptomatically with antipyretics, anti-inflammatory agents such as paracetamol, acetaminophen, ibuprofen, steroid therapy, indomethacin, and aspirin. Chloroquine failed to show any signs of clinical or biological difference between treated and untreated patients in placebo studies. A few compounds namely glyceric acid, apigenin, harringtonine, narygenin, sylybin, arbidol was reported to inhibit either Asian or East Central South African (ECSA) strain of chikungunya virus^[5–8]. Except a study of the antiviral activity of the Vietnamese plant species *Trigonostemon howii*^[9] there is a lack of study on the antiviral activity of plant species to both the Asian and ECSA lineage of chikungunya virus. Hence in this study, antiviral activity of few Indian plants that had known to possess cytoprotective properties were assessed to both the Asian and ECSA strain of chikungunya virus.

2. Materials and methods

Roots of *Decalepis hamiltonii* Wight & Arn, leaves of *Vitex negundo* L. and *Hyptis suaveolens* (L) poit were collected from Chennai, Tamilnadu and outer part of the root were removed gently and fresh leaves were rinsed with the sterile water. The leaves were spread evenly and dried in shade for 3 to 4 d and ground finely to obtain a fine powder. Fresh roots were then ground finely.

2.1. Preparation of aqueous, aqueous ethanolic and ethanolic extracts of plant parts

Aqueous, aqueous-ethanolic and ethanolic extract of each plant parts were prepared by soaking about 20 g of dried powder in 100 mL of water (100%) water: ethanol (50%: 50%) and ethanol (100 %) and stored at - 4 °C overnight. Then the extract was squeezed in gauze cloth and centrifuged at 5000 r/min for 15 min. The clarified extract was filtered using 0.22 µm Millipore filter and the filtered extracts were lyophilised at -70 °C at reduced pressure till the extracts are dried.

2.2. Stock preparation and dilution of the lyophilised extracts

Stock solution of 1 mg/1mL of the extracts was prepared by weighing 1 g of the extracts and dissolved either in

ethanol/water based on the solubility and made up to 1 mL in minimum essential medium (MEM) (Sigma Aldrich, India) by adjusting the pH to 7.0 with HCl or NaOH. The dissolved extract was filtered using 0.22 µm syringe filter (Sartorius). The stock solution was diluted from the concentration of 500 µg/mL to 3.9 µg/mL in log₂ dilution.

2.3. Estimation of maximal nontoxic concentration of the lyophilised extracts by in vitro cytotoxicity assay

Vero cell line (NCCS, Pune) was procured from NCCS, Pune and it have been regularly subcultured and maintained in MEM supplemented with 10% FBS (Himedia), L-Glutamine and antibiotics (penicillin, streptomycin, amphotericin B). Cells were trypsinised with 0.25% trypsin phosphate versene glucose (Himedia) and about 10 mL of 10% MEM was added to the flask to dislodge the cells. A total of 100 µL of dislodged cells were plated into the 96 well tissue culture micro titer plate and to that 100 µL of 10% MEM was added into each wells. The micro titre plate was then incubated at 37 °C for 12 h under 5% CO₂ atmosphere.

After the establishment of cell in 96 well tissue culture plate, 100 µL of the diluted aqueous, aqueous-ethanolic, ethanolic extract of varying concentrations (500 µg/mL to 3.9 µg/mL in log₂ dilution). A total of 100 µL of 2% MEM was added into the wells and incubated at 37 °C for 4 d. The highest concentration of the compound that showed no morphological variations or alterations as observed under inverted phase contrast microscope (Nikon) were considered as maximum nontoxic concentration of the drug. Cytotoxicity 50% was calculated from the concentration of the extract that reduced the cell viability by 50% to that of the control by plotting a graph.

2.4. Viral stocks and estimation of TCID₅₀

Asian and ECSA of chikungunya virus was procured from National Institute of Virology, Pune and the tissue culture infective dose (TCID₅₀) was estimated by Reed and Meunch Method, 1938 and the TCID₅₀ was calculated as

$$TCID_{50} = \log_{10} \text{ dilution factor} \frac{\{\% \text{ infection at a next dilution above } 50\% - 50\}}{\{\% \text{ infection at next dilution } 50\% - \% \text{ infection at next dilution below } 50\% \}}$$

2.5. Estimation of the antiviral assay to Asian and East Central South African lineage of chikungunya virus

Cells were infected with the virus at the multiplicity of infection of 1 of TCID₅₀ and incubated for 1 h at 37 °C. After adsorption of virus, maximum nontoxic concentration of

the drug was added to the adsorbed virus and incubated at 37 °C. Cell control and virus control with untreated drug were maintained. Each experiment was done in triplicates. The effective concentration of the extracts was estimated in comparison to the virus control by plotting a dose response curve. The selectivity index of each extract was estimated by the ratio of the cytotoxic concentration 50% of the extracts to that of the effective concentration 50% of the extracts.

3. Results

Maximum nontoxic concentration of the aqueous extract was higher in the range of 500 µg/mL to 250 µg/mL followed by aqueous–ethanolic and ethanolic extracts of the plants tested in Vero cells (Table 1). Ethanolic extract of *Vitex negundo* was found to be highly toxic above the concentration of 15.62 µg/mL. Antiviral activities to both the Asian and ECSA strains was evaluated from the maximum nontoxic concentration of the extracts (Figure 1 and Figure 2). Ethanolic extract of *Vitex negundo* and aqueous ethanolic extract of *Hyptis suaveolens* inhibited the Asian strain of chikungunya virus (Table 2; Figure 3 and Figure 4). Ribavirin inhibited the Asian strain at 31.25 µg/mL and the East central South African strain at 250 µg/mL.

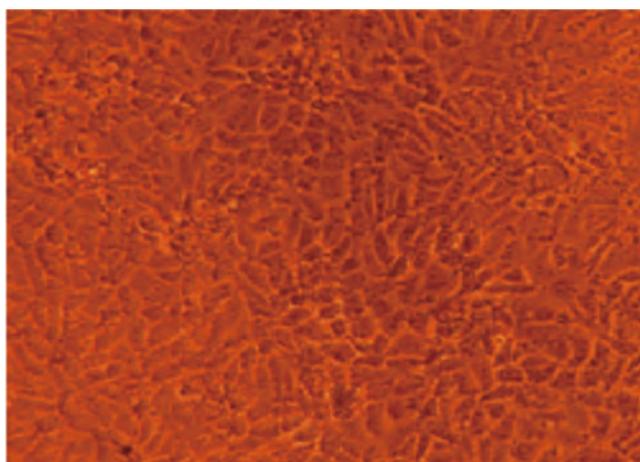


Figure 1. Control Vero cells (72 h).

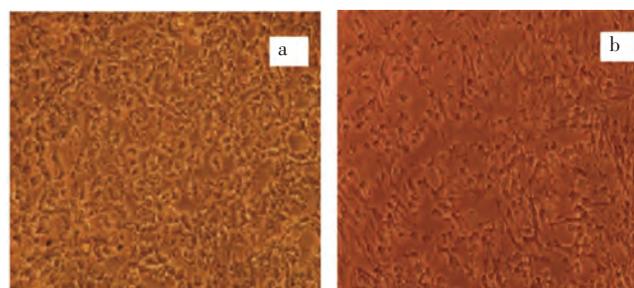


Figure 2. a: Cytopathic effect of Asian strain of Chikungunya virus in vero cells; b: Cytopathic effect of ECSA strain of Chikungunya virus in vero cells after 48 h.

Table 2

Antiviral activity of aqueous, aqueous ethanol and ethanolic lyophilised extracts of plants.

Plant	Extract	CC ₅₀ (µg/mL)	Asian strain		African strain	
			EC ₅₀ (µg/mL)	SI	EC ₅₀ (µg/mL)	SIs
<i>Vitex negundo</i>	Aqueous	500.00	NE	–	NE	–
	Aqueous–ethanolic	250.00	NE	–	NE	–
	ethanolic	31.25	15.62	2	NE	–
<i>Hyptis suaveolens</i>	Aqueous	500.00	NE	–	NE	–
	Aqueous–ethanolic	250.00	15.62	16	NE	–
<i>Decalepis hamiltonii</i>	ethanolic	250.00	NE	–	NE	–
	Aqueous	250.00	NE	–	NE	–
	Aqueous–ethanolic	125.00	NE	–	NE	–
	ethanolic	62.50	NE	–	NE	–
Ribavirin	–	500.00	31.25	16	250.00	2

CC₅₀: cytotoxic concentration 50% of the extracts; EC₅₀: effective concentration 50% of the extracts; SI: selectivity index; NE: not effective

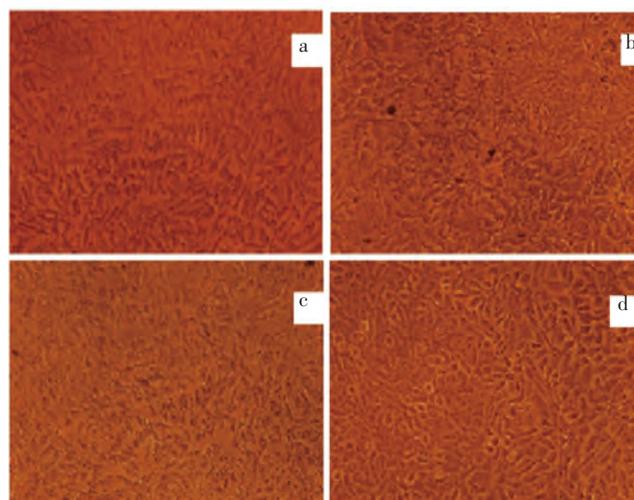


Figure 3. Antiviral activity of plants to Asian strain of Chikungunya virus. Inhibition of Asian strain of Chikungunya virus by a: Aqueous–ethanolic extract of *Hyptis suaveolens* at 15.62 µg/mL; b: Ethanolic extract of *Vitex negundo* at 15.62 µg/mL; c: Aqueous–ethanolic extract of *Decalepis hamiltonii* at 250 µg/mL; d: Ribavirin at 31.25 µg/mL.

Table 1

Maximum nontoxic concentration of the drug with the physico–chemical characteristics.

Plant	Extract	Color of the extract	Solubility	pH of the extracts after dissolving in the MEM	Maximum nontoxic concentration of the drugs
<i>Vitex negundo</i>	Aqueous	Light brownish fine crystals	Water	7.0	500.00
	Aqueous–ethanolic	Light brownish fine crystals	Water	7.0	250.00
	Ethanolic	Green pasty	Ethanol	7.5	15.62
<i>Hyptis suaveolens</i>	Aqueous	Dark brownish crystals	Water	7.0	500.00
	Aqueous–ethanolic	Dark brownish crystals	Water	7.5	250.00
	Ethanolic	Greenish crystals	Ethanol	6.5	250.00
<i>Decalepis hamiltonii</i>	Aqueous	Red amorphous powder	Water	7.0	250.00
	Aqueous–ethanolic	Red amorphous powder	Water	7.0	125.00
	Ethanolic	Red powder	Ethanol	7.5	62.50

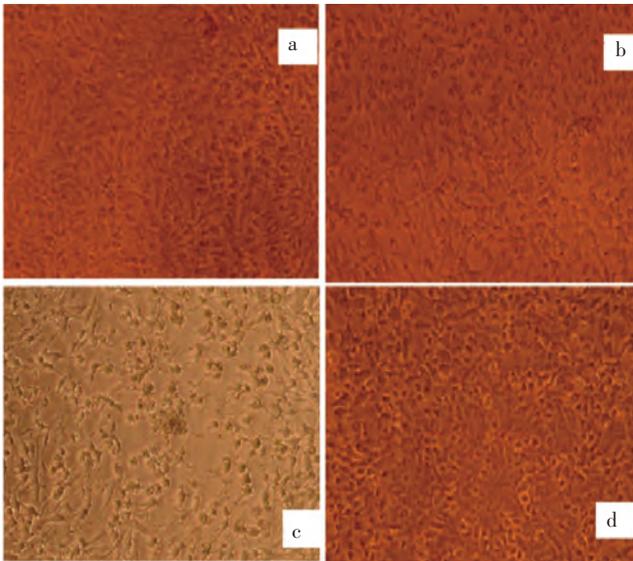


Figure 4. Antiviral activity of plants to the African strain of Chikungunya virus. a: Partial inhibition of aqueous ethanolic extract of *Hyptis suaveolens* at 15.62 µg/mL; b: Ineffectiveness of ethanolic extract of *Vitex negundo* at 15.62 µg/mL; c: Ineffectiveness of aqueous ethanolic extract of *Decalepis hamiltonii* at 250 µg/mL; d: Inhibition of African strain by Ribavirin at 250 µg/mL.

4. Discussion

Owing to the potential pharmacological properties of the plants *viz.*, antiinflammatory, antipyretic, antiarthritic, cytoprotective activities, the leaves of *Vitex negundo*, *Hyptis suaveolens* and roots of *Decalepis hamiltonii* were selected for the antiviral evaluation to chikungunya virus^[11–16]. Aqueous, aqueous–ethanolic and ethanolic extracts were prepared and used in this study to differentiate the activity exclusively on the basis of polar, nonpolar and combination of polar and nonpolar compound. Amongst the 15 extracts evaluated in this study, ethanolic and aqueous ethanolic extract was found to be effective to Asian strain exhibiting 50% inhibition at the concentration of 15.62 µg/mL depicting the involvement of non polar compounds predominantly in antiviral activity.

Selectivity index of *Hyptis suaveolens* was higher and found to be better than *Vitex negundo*. Although *Vitex Negundo* could inhibit the growth of Asian strains of chikungunya virus the selectivity index was very low showing narrow range of activity. As a drug to be used for therapeutic purpose, the maximum cytotoxic free value should be at least ten times higher than the concentration of the effective values hence the dependability for further phytochemical screening and evaluation of *Vitex negundo* to chikungunya infection will not be promising.

Selectivity index of an aqueous ethanolic extract of

Hyptis suaveolens to the Asian strain of chikungunya virus was significant in comparison to the standard drug ribavirin. Although ECSA strains were in circulation in recent epidemics of chikungunya virus however the same extract did not show promising activity for the ECSA strain of chikungunya virus. This could be attributed to the high virulence and replicative features of ECSA strain than the Asian strain of chikungunya virus^[17].

As chikungunya virus is able to induce cytopathogenicity by induction of apoptosis in the host cells, cytoprotective properties of *Decalepis hamiltonii* and *Hyptis sueveolens* were exploited to observe the virus induced cytopathogenicity but to our irony the plants failed to demonstrate any significant activity except aqueous ethanolic extract of *Hyptis suaveolens* to Asian strain. The activity of *Hyptis suaveolens* could be due to the presence of pentacyclic triterpenoids as the major constituent in the leaves reported in earlier studies.

Ethanolic extract of *Vitex negundo* at the toxic free concentration inhibited the Asian Strain but did not exhibit any potential activity for ECSA strain. To the best of our knowledge, this is the first study that has been evaluated for the antichikungunya activity of *Vitex negundo*, *Decalepis hamiltonii* and *Hyptis sueveolens* to both Asian and ECSA lineage.

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Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Chikungunya infections are reported since 2006 affecting millions of people with debilitating arthritis especially in Asian and African continent. Treatment of chikungunya infections are purely symptomatic and the antiviral drug is still in the development process for chikungunya virus.

Research frontiers

Lymphilized extracts of three Indian medicinal plants were screened for antiviral activity to chikungunya virus and selectivity index has been estimated to determine the therapeutic range.

Related reports

Aqueous extract of *Hyptis suaveolens* were found to be inhibitory to Asian strain in comparison to the African strain. Except a few studies on compounds and Vietnamese plants, anti-chikungunya activity on medicinal plants to both the lineages is lacking.

Innovations & breakthroughs

The plants screened for the antiviral activity in the present study has so far not been studied by other researchers for chikungunya. Hence the study is an attempt for the search for the plants showing antiviral activities.

Applications

The results obtained shows that *Hyptis suaveolens* could be further proposed for the isolation of compounds for the development of novel drugs.

Peer review

Owing to the increasing epidemics of chikungunya virus, the present study is of utmost importance to the research community especially in the field of drug development. Although it is a preliminary study it would add valuable insights for the development of drugs in near future.

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