In-vitro screening of selected Indian medicinal plants against Chikungunya virus and their phytochemical analysis

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Abstract

The continuous spread, high morbidity, lack of approved treatment, and unsatisfactory effects of currently used synthetic drugs make the Chikungunya virus (CHIKV) a significant public health concern. In India, various plants are used traditionally for managing infectious diseases owing to their antiviral potential. However, the effectiveness of these plants in treating CHIKV infection has not yet been studied. Therefore, this study aims to assess the antiviral potential of four selected plant extracts against CHIKV and conduct a phytochemical analysis to correlate with the observed activities. The antiviral activity of the selected plants was estimated using a focus-forming unit (FFU) assay in pre-treatment, co-treatment, and post-treatment. Phytochemical analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS) to determine the potential phytochemicals responsible for the observed activity. Extract of *Glycyrrhiza glabra* reduced the virus titre from 7.324 to 6.884 and from 7.623 to 7.149 mean $\log_{10}$ FFU/ml under co- and post-treatment, respectively. *Ocimum tenuiflorum* and *Tinospora cordifolia* extracts showed activity under pre- and post-treatment (7.623 to 7.462, 7.623 to 7.376), while *Trigonella foenum-graecum* extract showed activity under post-treatment (7.623 to 7.463) only. GC-MS analysis identified many bioactive compounds like inositol, stigmasterol, β-sitosterol, γ-sitosterol, lupeol, phytol, and campesterol, etc., which are well correlated with their reported pharmacological effects and their antiviral potency against CHIKV. The present study demonstrates the effect of selected plant extracts against CHIKV. The screened plants hold promising potential for further exploration toward developing effective therapeutics for Chikungunya.

Keywords: Chikungunya Virus, Antiviral activity, FFU assay, Medicinal plant extracts, GC-MS analysis, ethanol extract

1 Introduction

Chikungunya virus (CHIKV) is a mosquito-borne RNA virus classified as a member of the *Alphavirus* genus and the *Togaviridae* family. Its genome is approximately 11.8 kb in length and is capable of functioning as mRNA [1]. The genome has two open reading frames: one encodes non-structural proteins, and the second encodes structural proteins of the virus [2]. After its first appearance in 1952, this viral infection spread globally [1, 3-5]. As of 2022, the European Centre for Disease Prevention and Control reported 383,357 cases and 76 deaths worldwide, with the majority of cases occurring in Brazil (265289 cases and 75 deaths) [6].

CHIKV infection primarily manifests as fever and joint pain, with some cases also experiencing headaches, muscle pain, joint swelling, and skin rashes [7]. These symptoms can persist for several weeks or even years, leading to severe disability in patients. Currently, there are no approved vaccines or specific antivirals for treating CHIKV, and patients are treated symptomatically only [8]. The high morbidity and severe social and economic impacts of this infection in affected countries have led the scientific community to explore effective therapies against CHIKV [9]. In recent years, the use of phytochemicals in drug development has gained popularity due to their potential and proven results in the past. Given these factors, we conducted the present study to evaluate the efficacy of selected plant extracts against CHIKV.

Modern scientific research has reported countless adverse effects related to synthetic antiviral drugs [10]. As a result, there has been a significant shift towards developing safe medications using medicinal plants and their phytochemicals. Ayurvedic plant-based products have been used since centuries to treat various chronic and infectious diseases. Studies have shown that plants such as *Andrographis paniculata, Zingiber officinale, Mangifera indica, Curcuma longa, Allium sativum, Azadirachta indica,* and *Camella sinensis* are effective against viral diseases, and their phytochemicals can prevent viral replication with little to no side effects or impact on the host physiology [10-12]. Additionally, these compounds may improve the host's immunological response to viral infections [13]. Therefore, the search for new antivirals from plants holds great potential.

Several scientific studies have reported the remarkable anti-CHIKV activity of some antiviral plant extracts such as *Plumeria alba, Ancistrocladus heyneanus, Bacopa monnieri, Cucurbita maxima, Andrographis paniculata, Zingiber officinale, Cynodon dactylon* [5, 14-17] as well as many phytochemicals like baicalein, fisetin, quercetagetin, silymarin, nobiletin, and resveratrol [18-20]. Therefore, it is important to screen plants that have been reported to

possess antiviral potential, particularly against other arboviruses like dengue. Additionally, it is imperative to evaluate those plants that have been traditionally used in Ayurveda to increase the body's resistance to infectious diseases but have not been scientifically evaluated. Thus, this study aims to investigate medicinal plants that are part of traditional folklore practices and have reports of having antiviral potential.

2 Material and Methods

2.1 Reagents and chemicals

Cell growth media was Minimal Essential Medium (MEM) which was supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), antibiotic–antimycotic solution, sodium bicarbonate, and Phosphate Buffer Saline (PBS) (Hi-Media laboratories). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Hi-media laboratories), Maintenance media (MEM supplemented with antibiotic–antimycotic solution, sodium bicarbonate, and 2% heat-inactivated FBS), 1% Trypsin Phosphate Versene Glucose (TPVG), cell culture tested Dimethyl sulfoxide (DMSO).

2.2 Sample collection, extraction, and preparation

Plant samples (the best useful parts) were selected based on their antiviral activity and traditional uses in infectious diseases (Table 1) [15, 21-26]. Two plant samples, i.e., *Ocimum tenuiflorum* (Tulsi) leaves and *Tinospora cordifolia* (Giloy) stem, were collected from the Herbal Garden of Maharshi Dayanand University, Rohtak, and the samples of *Glycyrrhiza glabra* (Liquorice) roots and *Trigonella foenum-graecum* (Methi) seeds were purchased from the local market of Rohtak. All samples were authenticated by an ethnobotanist at, the Department of Botany, Maharshi Dayanand University, Rohtak. Voucher specimens (Pharm/PhCog/2022/237-240) were kept in the department for future reference. To prepare the extracts, plant samples were cleaned, dried, powdered, and then macerated (in a 1:10 ratio) in absolute alcohol (ethanol with a minimum of 99% purity) for 3 days (one cycle per day). The extracts were filtered through Whatman filter paper No. 1, dried in a rotary evaporator (IKA, RV10 auto) (temperature 40°C, rpm 49, vacuum 175), and then labeled appropriately. Stock solutions of the extracts were prepared in dimethyl sulfoxide (0.1%), then filtered through a syringe filter (pore size = 0.2 μM), and stored at −20°C until use.

2.3 Cell and virus culture

Vero cell-lines (ATCC No. CCL-81) were grown in the cell growth medium by incubating in a CO_2 incubator in a humid environment at 37 °C. The growth of the Vero cells was observed daily, and the media was changed after 3–4 days of incubation. The healthy cells were collected in 96-well tissue culture plates and incubated until confluent. CHIKV (Strain No. 061572, P-2, African genotype) was used and propagated in Vero cells supplemented with an infection medium (MEM supplemented with antibiotic–antimycotic solution, sodium bicarbonate and 2% FBS (heat-inactivated), 1% TPVG). Cells were harvested when the cytopathic effect (CPE) had been observed and kept at −80 °C until used.

2.4 Cytotoxicity assay of extracts

The stock solutions (concentrations: 1 mg/ml) of the extracts were prepared and serially diluted (two-fold) in cell growth media. The cytotoxicity of crude extracts was evaluated by cytotoxicity assay using the MTT method. Briefly, each well of the 96-well plate was seeded (35000 cells per well) with Vero cells and incubated at 37 °C in a 5% CO_2 incubator for 48 hours with different increasing dilutions of extracts (100µl per well, concentration range from 500µg/ml to 0.98µg/ml). A blank control (medium only) and a cell control (cells only) were also plated. After incubation, MTT solution (10µl) was added to each well and incubated at 37 °C for 3 hours in the dark. The MTT solution was removed, and 100µl of DMSO was added to each well. Absorbance was recorded at 570 nm with the help of a microplate reader (BioTek Synergy, USA). The cell viability and toxicity were determined by the following formulas:

$$\textbf{Cell viability (percentage)} = \left[\frac{(A_{sample} - A_{blank})}{(A_{control} - A_{blank})}\right] \times 100$$

$$\textbf{Cell toxicity (percentage)} = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \times 100$$

Here A_{sample} = absorbance of treated cells, $A_{control}$ = absorbance of untreated cells, A_{blank} = absorbance of medium without cells.

2.5 Determination of in vitro antiviral activity of extracts

The extracts were assayed using their respective maximum non-toxic dose (MNTD) (100µl) for anti-CHIKV activity in three treatment conditions (pre-, co-, and post-treatment) as given in the literature [17].

In pre-treatment, the cells were first incubated with plant extract for 24 hours at 37 °C, after which the culture supernatant was removed. They were then infected with 20µl of CHIKV inoculum and incubated at 37 °C for 1 hour in a 5% CO_2 incubator. Following incubation, the cells were washed twice with PBS to remove any unbound virus particles and incubated after the addition of 100µl of maintenance medium into each well. During co-treatment, the cells were infected with a mixture of virus (20µl) and plant extract (100µl) for 1 hour at 37 °C. In post-treatment, the cells were infected with the virus for 1 hour and treated with the extracts after 24 hours.

For all treatment conditions, 0.01 MOI of the virus from the virus stock with the known titre of FFU/ml, was used for infection, and plates were incubated for 48 hours after infection, freeze-thawed, the supernatant collected, and the virus titre was estimated using the focus-forming unit (FFU) assay method. Triplicates of each experiment were run during the whole experiment. Positive control (cells + virus) and negative control (cells only) wells were also plated.

2.6 Gas Chromatography-Mass Spectroscopy (GC-MS) analysis of the extracts

All the extracts were subjected to GC-MS analysis using the instrument Shimadzu GC-2010 Ultra, in which the initial column temperature was set to 70 °C (hold time: 5 minutes) and programmed to rise to 310 °C at 5°C per minute, which was held for 10 minutes. Sample injection volume was 1µl, helium as carrier gas in split mode, column flow 1.0 ml/min, total flow 14 ml/min. After separation in the column, the components were first subjected to electron ionization and then to mass spectrometric analysis. The phytochemicals were identified by matching the spectra in the data libraries (WILEY 8 and NIST 11), and phytochemicals with > 70% similarity index were considered [27].

2.7 Statistical Data Analysis

The cell viability was plotted against the concentration of the extract to calculate the 50% cytotoxic concentrations (CC_{50} values) for all the extracts. The data was expressed as mean ± standard error, and the data of virus-control cells and extract-treated cells was compared online with an independent t-test using GraphPad. $P < 0.05$ was considered significant.

3 Results

3.1 Extraction yield

The extractive values of the plant samples were calculated based on the %w/w amount of the extract yield obtained after complete drying of the extract (Table 1). The yield of *Glycyrrhiza glabra* extract (8.5% w/w) was found comparable to previous studies 7.1% w/w [28] and 9.73% w/w [29], while the yield of *Trigonella foenum-graecum* (10.5% w/w) was found lower than an earlier research report (14.77% w/w) [30]. *Ocimum tenuiflorum* extract yield (2.5% w/w) was lower than observed (7.65% w/w) in another study [24]. Our result of 5.6% w/w yield of *Tinospora cordifolia* was almost similar to the result (5.8% w/w) of Gupta *et al*. [31]. The variations observed could be due to several factors like plant sample, solvent system, extraction method etc.

Table 1. Description of plant extracts and their cytotoxicity observed on Vero cells

Extract Name	Plant Name	Family	Common Name	Part used	MNTD (µg/ml)	Extract Yield (w/w)	CC_{50} (µg/ml)	Ethnobotanical uses
GG	*Glycyrrhiza glabra* L.	Fabaceae	Liquorice, Mulethi	Root	15.62	8.55%	35.16	Antitussive, anti-asthmatic, antimicrobial, antiviral[19], anti-inflammatory, immunostimulatory[20]
TG	*Trigonella foenum-graecum* L.	Fabaceae	Methi, Fenugreek	Seed	7.81	10.5%	28.26	Hypotensive, antioxidant, anti-arthritic, antiviral, anticancer, anti-inflammatory, galactagogue, laxative, anticholesterolemic, antimicrobial[21,22]
OS	*Ocimum tenuiflorum* L.	Lamiaceae	Tulsi, Holy basil	Leaf	15.62	2.5%	70.40	Antibacterial, antioxidant, antiviral, anti-inflammatory[23]
TC	*Tinospora cordifolia* (Willd.)	Menispermaceae	Giloy	Stem	31.25	5.6%	119.25	Fever, jaundice, anticancer anti-arthritic, antiviral, , anti-inflammatory, chronic diarrhoea, cancer, dysentery[24,25]

3.2 Cytotoxicity evaluation of extracts

Among four plant extracts, Tinospora extract showed the least cell toxicity, while Trigonella extract showed the highest toxicity. The highest concentrations of plant extracts that showed non-toxicity (cell viability ≥ 90%) to Vero cells were considered their maximum non-toxic dose (MNTD) values and are given in Table 1. The effects of varying concentrations of the extracts on cell viability, with MNTD and CC_{50} values, are given in Appendix 1.

3.3 Antiviral effects of extracts on CHIKV

The results of the antiviral assay are presented as the mean $\log_{10}$ focus-forming unit/ml ± standard error (Figure 1). Various treatment conditions, viz., pre-treatment, co-treatment, and post-treatment, represent their prophylactic (prevention), virucidal, and therapeutic utility, respectively.

The extract of *Glycyrrhiza glabra* showed anti-CHIKV activity under all treatment conditions and reduced the virus titre from 7.674 to 7.507, 7.324 to 6.884, and 7.623 to 7.149 mean log10 FFU/ml under pre-, co-, and post-treatment, respectively. It reduced virus titre more under post-treatment than pre-treatment, which shows its utility in treatment better than prevention. *Trigonella foenum-graecum* reduced the viral titre under both co- and post-treatment. Although co-treatment resulted in a greater decrease in viral titre (7.324 to 7.024) than post-treatment (7.623 to 7.463), only post-treatment showed a significant reduction (P = 0.044), which shows its utility in treatment only, not for prevention. *Ocimum tenuiflorum* showed activity under pre-, and post-treatment, and significantly reduced the virus titre from 7.674 to 7.496 and 7.623 to 7.462 mean $\log_{10}$ FFU/ml. However, co-treatment did not reduce virus titre significantly (7.324 to 7.291). *Tinospora cordifolia* reduced the CHIKV titre under pre- and post-treatment conditions and showed mild anti-CHIKV activity. However, post-treatment reduced virus titre by twice as much compared to pre- and co-treatment. Hence, it is better for treatment than prevention.

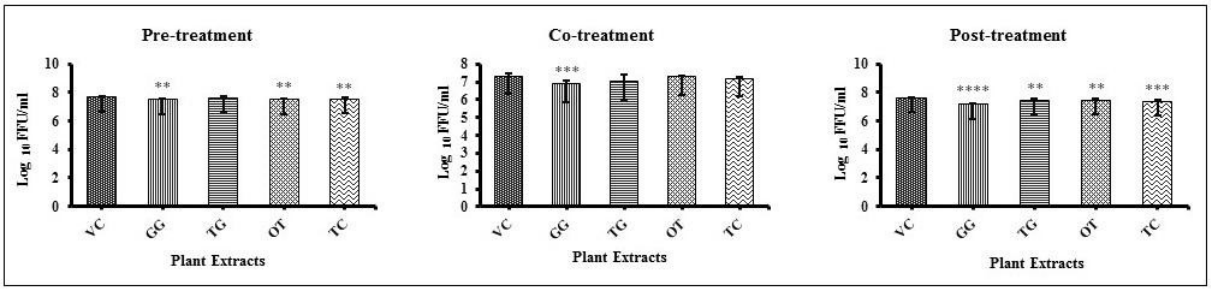

Figure 1. Antiviral effects of plant extracts at their respective MNTD against CHIKV under different treatment conditions and compared with virus control (VC). **P < 0.05, ***P < 0.03, ****P < 0.003

3.4 GC-MS analysis of the extracts

The GC-MS analysis identified a total of 61 phytochemicals (Figures 2) in all the extracts, which are given in Tables 2 to 5, along with their retention times and similarity matches with the library. The major phytochemicals present were sterols (stigmaterol, β-sitosterol, γ-sitosterol, campesterol, and stigmasta-5,22-dien-3-ol), diterpenoids (phytol), triterpenoids (squalene and lupeol), polysaccharides (inositol), and alkanes (hexadecane and tetradecane). Several fatty acids and their esters were also identified, such as hexadecanoic acid, (E)-9-octadecenoic acid, ethyl ester; ethyl (9z,12z)-9,12-octadecadienoate; 9, 12-octadecadienoic acid, ethyl ester; 9,12,15-octadecatrienoic acid. Other medicinally important phytochemicals present were 15-heptadecenal and some phthalic acid ester derivatives [32].

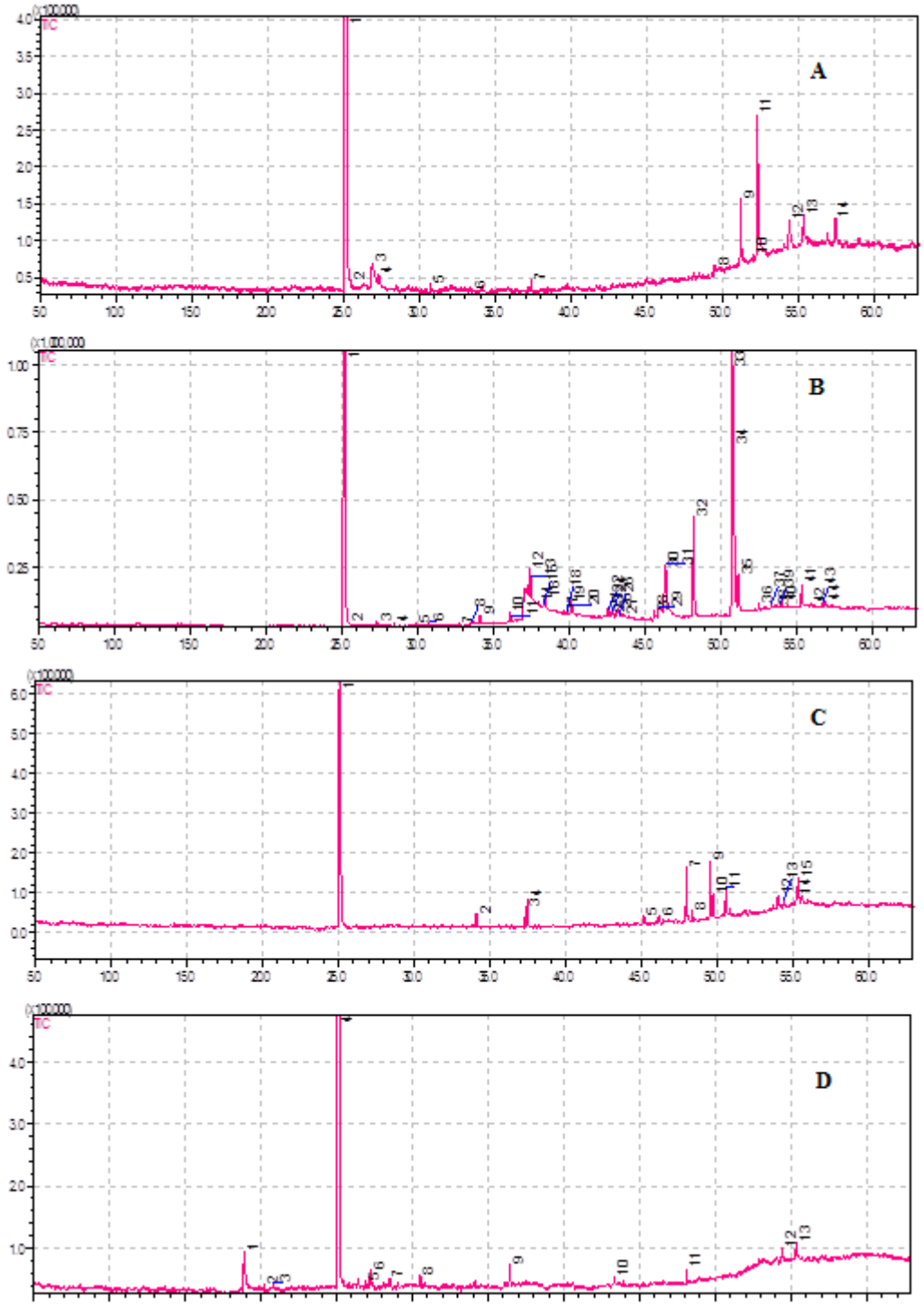

Figure 2. GCMS chromatogram of (A) *Glycyrrhiza glabra* (B) *Trigonella foenum-graecum* (C) *Ocimum tenuiflorum* (D) *Tinospora cordifolia*

Table 2. Phytochemicals identified in the ethanol extract of *Glycyrrhiza glabra* root

Sr. No	Retention Time (minutes)	Peak Area	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Similarity Index (%)	
						Wiley8	NIST11
1.	25.199	60808985	1,2-Benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	222	97	96
2.	25.417	77414	Hexadecane	$C_{16}H_{34}$	226	80	80
3.	26.874	184664	Mome inositol	$C_7H_{14}O_6$	194	89	83
4.	30.726	32418	Phthalic acid, ethyl 2-methylbutyl ester	$C_{15}H_{20}O_4$	264	79	79
5.	37.365	49354	2-Isopropenyl-5-methyl-6-hepten-1-ol	$C_{11}H_{20}O$	168	82	83
6.	54.401	186598	Stigmasterol	$C_{29}H_{48}O$	412	75	75
7.	55.355	157398	γ-Sitosterol	$C_{29}H_{50}O$	414	88	88

Table 3. Phytochemicals identified in the ethanol extract of *Trigonella foenum-graecum* seeds

Sr. No.	Retention Time (minutes)	Peak Area	Compound Name	Molecular Formula	Molecular weight (g/mol)	Similarity Index (%)	
						Wiley8	NIST11
1.	25.240	95628625	1,2-Benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	222	97	96
2.	25.425	31251	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	198	80	80
3.	28.431	23367	Phthalic acid, 5-methylhex-2-yl ethyl ester	$C_{17}H_{24}O_4$	292	--	80
4.	29.918	25054	E-15-Heptadecenal	$C_{16}H_{30}O$	252	92	92
5.	30.728	43246	Phthalic acid, ethyl pentyl ester	$C_{15}H_{20}O_4$	264	--	85
6.	32.733	20568	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	86	--
7.	33.539	95594	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	89	88
8.	34.114	112258	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	93	92
9.	36.079	98313	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	92	93
10.	36.206	73607	8,11,14-docosatrienoic acid, methyl ester	$C_{23}H_{40}O_2$	348	84	84
11.	37.348	299583	Ethyl (9z,12z)-9,12-octadecadienoate	$C_{20}H_{36}O_2$	308	93	91
12.	37.471	261687	7-Tetradecenal, (Z)-	$C_{14}H_{26}O$	210	--	86
13.	37.976	53019	Ethyl Nonadecanoate	$C_{21}H_{42}O_2$	326	79	--
14.	38.321	54473	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292	80	79
15.	38.447	151360	8,11,14-Eicosatrienoic acid, (z,z,z)-	$C_{20}H_{34}O_2$	306	87	87
16.	39.598	33368	2,6-Bis[2-(dimethylamino) ethoxy] pyridine	$C_{13}H_{23}N_3O_2$	253	--	79
17.	40.130	141543	1-Hydroxy-2,2,6,6-tetramethyl-3-(1-piperidinylmethyl)-4-piperidone	$C_{15}H_{28}N_2O_2$	268	71	71
18.	40.247	110119	1,7-Dioxaspiro[5.5]undec-2-ene	$C_9H_{14}O_2$	154	71	71
19.	42.517	106024	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C_{12}H_{23}NO_2$	213	--	87
20.	42.612	109542	Hexadecanal, 2-methyl-	$C_{17}H_{34}O$	254	81	81
21.	42.788	42405	9,12-octadecadienoyl chloride, (z,z)-	$C_{18}H_{31}ClO$	298	--	79
22.	42.861	77014	Hexadecanoic acid, 2 [(trimethylsilyl)oxy]-1,3-propanediyl ester	$C_{38}H_{76}O_5Si$	640	70	69
23.	45.611	58039	Cyclopropane, 1,1-dichloro-2,2,3,3-tetramethyl-	$C_7H_{12}Cl_2$	166	71	71
24.	46.293	621061	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{38}O_4$	354	91	91
25.	46.358	1211413	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl	$C_{39}H_{72}O_5$	620	81	--
26.	48.195	1673542	Oxalic acid, 3,5-difluorophenyl nonyl ester	$C_{17}H_{22}F_2O_4$	328	--	77
27.	50.754	7055338	8,11,14-docosatrienoic acid, methyl ester	$C_{23}H_{40}O_2$	348	79	79
28.	50.872	2981327	Methyl (Z)-5,11,14,17-eicosatetraenoate	$C_{21}H_{34}O_2$	318	--	83
29.	52.542	103086	2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-	$C_{31}H_{52}O_3$	472	85	82
30.	55.355	429792	γ-Sitosterol	$C_{29}H_{50}O$	414	93	93
31.	55.971	43056	13,15-Octacosadiyne	$C_{28}H_{50}$	386	--	58
32.	56.744	84930	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	$C_{30}H_{50}O$	426	75	75
33.	56.940	50251	Lupeol	$C_{30}H_{50}O$	426	--	72

Table 4. Phytochemicals identified in the ethanol extract of *Ocimum tenuiflorum* leaves

Sr. No.	Retention Time (minutes)	Peak Area	Compound Name	Molecular Formula	Molecular weight (g/mol)	Similarity Index (%)	
						WILEY8	NIST11
1.	18.876	284252	Phenol, 2-methoxy-4-(2-propenyl)-	$C_{10}H_{12}O_2$	164	92	88
2.	20.180	29012	Tetradecane	$C_{14}H_{30}$	198	85	84
3.	20.795	28903	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,	$C_{15}H_{24}$	204	91	--
4.	25.155	29005023	1,2-Benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	222	97	96
5.	26.905	81010	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-.alpha.-methylene-2-oxo-, methyl ester	$C_{16}H_{20}O_4$	276	84	84
6.	27.219	72120	(-)-5 Oxatricyclo [8.2.0.0(4,6)] dodecane, ,12-trimethyl- 9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-	$C_{15}H_{24}O$	220	85	--
7.	28.494	87470	3-Methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1-pentyn-3-ol	$C_{15}H_{24}O$	220	83	--
8.	30.520	44819	4AH-Cycloprop[E]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-, [1ar(1a.alpha.,4.beta.,4a.beta.,7.alpha.,	$C_{15}H_{26}O$	222	78	77
9.	36.415	83885	Phytol	$C_{20}H_{40}O$	296	95	96
10.	48.137	74252	Squalene	$C_{30}H_{50}$	410	92	92
11.	54.399	90673	Stigmasterol	$C_{29}H_{48}O$	412	84	84
12.	55.362	101692	γ-Sitosterol	$C_{29}H_{50}O$	414	85	85

Table 5. Phytochemicals identified in the ethanol extract of *Tinospora cordifolia* stem

Sr. No.	Retention Time (minutes)	Peak Area	Compound Name	Molecular Formula	Molecular weight (g/mol)	Similarity Index (%)	
						WILEY8	NIST11
1.	25.135	20680389	1,2-Benzenedicarboxylic Acid, Diethyl Ester	$C_{12}H_{14}O_4$	222	97	96
2.	34.109	108994	Hexadecanoic Acid, Ethyl Ester	$C_{18}H_{36}O_2$	284	93	92
3.	37.346	176381	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308	92	94
4.	37.468	237308	(E)-9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	310	90	92
5.	45.173	88201	Longicyclene DB5-1324	$C_{15}H_{24}$	204	78	78
6.	46.152	73289	5-(2,2-Dimethyl propylidene) hexahydro-1(2h)-pentalenone	$C_{20}H_{38}O_2$	192	75	75
7.	54.025	179795	Campesterol	$C_{28}H_{48}O$	400	83	83
8.	54.415	179795	Stigmasta-5,22-dien-3-ol	$C_{29}H_{48}O$	412	80	80
9.	55.348	322038	β-Sitosterol	$C_{29}H_{50}O$	414	89	89

4 Discussion

The lack of approved treatments for the CHIKV has prompted research into traditional plant-based remedies, which have been used for centuries to treat various infectious diseases. Studies have shown that plant extracts have potent antiviral properties against numerous viruses [5, 20], some of which are even more effective than their synthetic analogs [33]. In this context, this study aimed to evaluate the potential of plant extracts for treating CHIKV infection. Results indicated that the ethanol extracts of *Glycyrrhiza glabra*, *Ocimum tenuiflorum, Tinospora cordifolia*, and *Trigonella foenum-graecum* exhibited significant antiviral activity against CHIKV, making them promising candidates for further investigation.

Previous studies have reported the antiviral activity of *Glycyrrhiza glabra* root extract against numerous viruses, viz., human respiratory syncytial virus (HRSV), enterovirus 71, and hepatitis C virus (HCV) [34]. The probable mechanisms were proposed to inhibit viral attachment and post-entry steps of the viral life cycle against HRSV and HCV, respectively

[35-36]. However, the results emerged from the present study support both mechanisms for their anti-CHIK activity. The reduction in viral titre under co-treatment shows that it could prevent virus attachment to the cell by binding to the virus or may neutralize the virus (virucidal activity) outside the cell, while post-treatment results indicate its impact on post-entry steps like viral replication and/or assembly and release from the cell. Moreover, *glycyrrhiza*-derived antivirals also act primarily at post-entry steps [35]. Further research is required to elucidate the exact mechanism of the extract against CHIKV.

GC-MS analysis detected 7 phytochemicals in the extract of *Glycyrrhiza glabra* roots, and the identified antiviral phytochemicals such as inositol [37], stigmasterol [38], γ-sitosterol [39-40] and phthalic acid ester derivatives [32] may validate its antiviral activity against CHIKV. Stigmasterol might contribute to its anti-CHIV activity as it has significant antiviral potential against the dengue virus [38], which produces almost similar symptoms, transmitted by the same vector and commonly co-circulates and simultaneously co-infects with CHIKV [41-42]. Further, phthalic acid, ethyl 2-methylbutyl ester may be more potential phytochemical as phthalic acid ester derivatives showed potent antiviral activity against CHIKV [32]. Traditionally, *Glycyrrhiza glabra* roots have been reported to treat cough, inflammation, constipation, asthma, flatulence, arthritis, epilepsy, fever, and many more illnesses [21]. Therefore, this plant might also relieve CHIKV symptoms like fever, joint pain, etc., in addition to its antiviral action. Furthermore, this plant has also been reported to stimulate the immune system of the body in response to viral infections [13].

Trigonella foenum-graecum showed anti-CHIKV activity in post-treatment but lost activity in pre-treatment, which may be due to the metabolism of the active phytochemicals in the extract by the Vero cells. Though its antiviral potential was not prominent, it may reduce joint pain in CHIKV-infected patients due to its significant anti-arthritic and anti- inflammatory properties [22-23]. GC-MS analysis detected many bioactive phytochemicals. The presence of lupeol and phthalic acid ester derivatives is the scientific validation of its use against CHIKV because these have potent anti-CHIKV activity [32,43].

Ocimum tenuiflorum reduced the virus titre under pre-treatment and post-treatment. Hence, this extract can inhibit virus entry and/or post-entry steps by interacting with virus or cell proteins involved in these processes [17]. Raghavendhar *et al.* also reported that the whole plant (aqueous extract) can inhibit virus attachment, entry, and replication [14]. The hydro-methanolic extract also showed potent antiviral activity against the H9N1 virus by the same antiviral mechanisms [44]. However, methanol extract exerted mild antiviral activity against

the dengue virus [45]. GC-MS analysis identified potential antiviral phytochemicals like phytol, stigmasterol, and γ-sitosterol, which could validate its use against CHIKV [39-40]. Furthermore, the anti-inflammatory potential of the leaves (ethanol extract) may contribute to this plant's usefulness in CHIKV infection [24]. *Tinospora cordifolia* stems showed mild anti-CHIKV activity under pre-treatment and post-treatment. Moreover, the aqueous extract of this stem reduced the production of inflammatory mediators and stimulated the immune system in response to viral infection, demonstrating its utility in CHIKV [26]. GC-MS analysis identified antiviral sterols (campesterol, β-sitosterol) which further supports its use in CHIKV infection [40,46].

To the best of our knowledge, this is the first report evaluating the anti-CHIKV activity of ethanol extracts of the selected plants. The results suggest that all the extracts have antiviral activity against CHIKV with varying degrees and could promote an effective antiviral environment through different mechanisms. However, further investigation is required to understand the sensitivity of extracts to the different virus strains. Although GC-MS does not provide an ultimate characterization of the phytochemicals, it is sufficient to present an idea about the phytochemicals present and thus can be correlated with the observed activity. It is suggested that further studies on characterization followed by isolation of responsible phytochemicals and testing for anti-CHIKV activity could be a future course of action. Furthermore, developing techniques to prepare low-cost formulations using active extracts that preserve their anti-CHIKV activity without batch-to-batch variations and further *in- vivo* investigations of those formulations are recommended.

5 Conclusion

The present study demonstrates the promising antiviral activity of *Glycyrrhiza glabra* and *Ocimum tenuiflorum* against CHIKV. However, further investigations focused on *in-vivo* studies on isolated phytochemicals are suggested for effective drug development against CHIKV.

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Data availability

Data is available in supplementary files

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Appendix 1

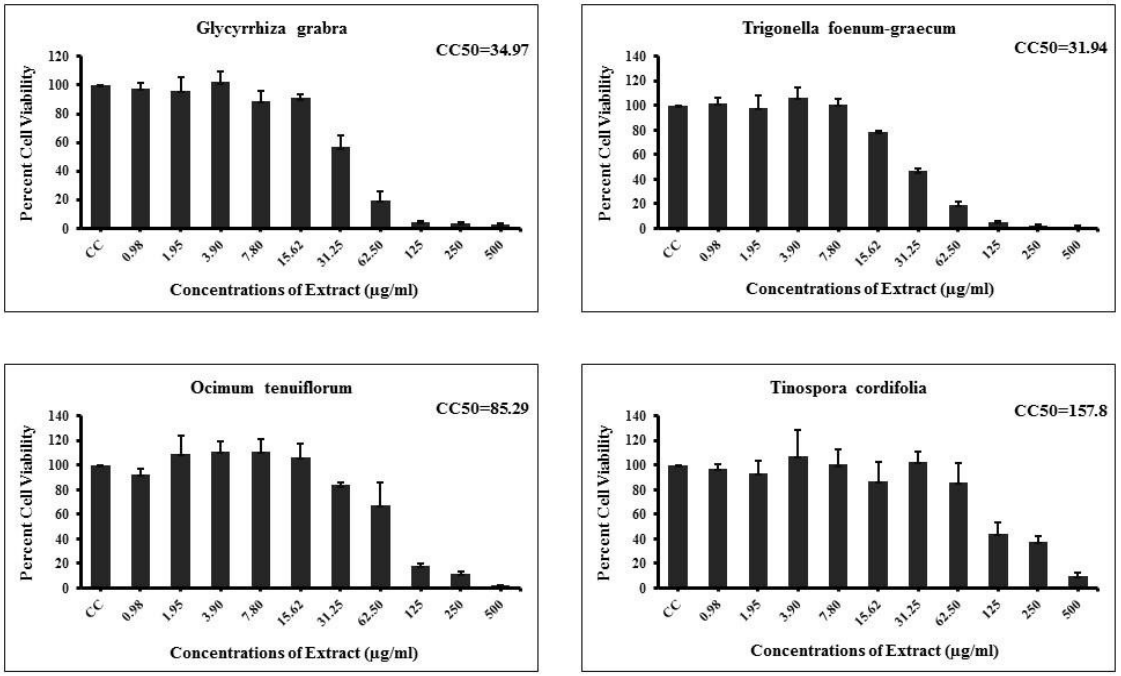

Figure: A. The effects of varying concentrations of different extracts on cell viability. The experiments were performed in triplicates, and the results are expressed as mean ± standard error and compared with cell control (CC).