In vitro anti-Chikungunya efficacy, antioxidant and anti-inflammatory potential of *Murraya koenigii* ethanolic extract

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Abstract

Chikungunya virus is an arthropod-transmitted Alphavirus that causes severe arthralgia which may persist for weeks or years. Currently no licensed antivirals or vaccines are accessible against Chikungunya infection. The present study focused on in vitro anti-chikungunya efficacy and characterization of Murraya koenigii ethanolic extract. Fourier transform infrared spectroscopy and gas chromatography mass spectrometry determine bioactive compounds. The anti-chikungunya potential was determined and quantified via a foci forming unit assay. Additionally, the anti-inflammatory and antioxidant assays were also performed. The Fourier transform infrared spectrophotometry showed the presence of surface hydroxyl groups, phenols, alcohols, alkanes, aldehydes, aromatic compounds, alkyl and aryl halides and amines.

Gas chromatography mass spectrometry revealed 24 bioactive compounds. The maximum non-toxic dose of Murraya koenigii (7.8 µg/mL) was used for the antiviral assay. It lowered the virus titer from 8.10 (virus control) to 7.36 mean log_{10} FFU/ml with 82.25% of viral inhibition. Murraya koenigii also demonstrated remarkable DPPH scavenging (IC_{50} value 53.77 µg/mL) and protein denaturation (IC_{50} value 146.4 µg/mL) activities. The results revealed that Murraya koenigii ethanolic extract at MNTD of 7.8 µg/mL showed significant inhibition of Chikungunya virus fabrication at multiple stages of its replication cycle and may serve as a preventive or curative agent against the Chikungunya virus.

Keywords: *Murraya koenigii*, Antiviral, Chikungunya, FFU assay, Antioxidant, Anti-inflammatory.

Introduction

Chikungunya virus (CHIKV) belongs to the *Togaviridae* family of the Alphavirus genus which contains positive-sense RNA[36]. CHIKV is mostly transmitted to others through infected female *Aedes* mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus*, which contribute to the urban transmission cycle between humans and mosquitoes[21]. Chikungunya virus (CHIKV) was first identified on the Makonde plateau in Tanzania in 1952 and has subsequently emerged as a major global health concern. The virus first appeared in Sub-Saharan Africa and Asia and afterwards invaded Europe and the Americas. Chikungunya fever is identified by the acute onset of a high fever lasting 3-10 days, coupled with severe joint pain (polyarthralgia), muscular pain, headaches, nausea, vomiting and distinctive skin rashes.

The term "that which bends up" comes from the Kimakonde language, which reflects the crippling joints pain that is a hallmark of the infection[22]. 50% of Chikungunya patients may have persistent arthralgia for months to years after the initial infection, which is characterized by joint pain and stiffness, although this condition is typically not fatal[33]. In addition to directly infecting cells, viruses indirectly cause disease by stimulating cell proliferation, inhibiting apoptosis and activating inflammatory signaling pathways including cytokines[46].

The viral structure is composed of RNA genetic material enclosed in a protein capsid and encircled by an envelope containing glycoproteins E1 and E2, which facilitate cellular entry. The severity and persistence of symptoms, particularly chronic arthralgia, are associated with viral load during acute infection. This suggests that long-term complications may be prevented by reducing viral replication[45].

Currently, there are no targeted antiviral therapies for Chikungunya fever[17,40]. Patient care is predominantly focused on symptomatic alleviation using analgesics such as paracetamol and non-steroidal anti-inflammatory medications. Despite the development of an FDA-approved vaccination, it is not yet broadly accessible for general uses. The primary preventive strategy remains vector control targeting *Aedes* mosquito populations. The Coalition for Epidemic Preparedness Innovations (CEPI) and the World Health Organization acknowledged the urgent need for research and development of viable treatment options due to the pandemic potential of CHIKV[9]. The virus's capacity to induce massive outbreaks and prolonged damage in affected populations indicates the urgent need for innovative antiviral medications that might diminish viral load and prevent chronic effects.

A number of viral diseases can be treated with natural products from plants[15]. Plant extracts offer an improved

method to discover novel compounds exhibiting medicinal benefits that are safer and can be applied to make synthetic drugs with greater effectiveness. Eighty percent of people worldwide are believed to continue to resort to and employ plant medication-based treatments[34]. *Murraya koenigii* (Meethi neem) is a 6-meter tall aromatic deciduous tree[32]. It is a member of the *Rutaceae* family and generally referred to as "curry leaf" in English[35]. Its leaves are commonly used in small amounts to add flavor because a particular fragrance originates from the volatile compounds[39]. The leaves are known to act as a depressant, blood purifier and anti-inflammatory agent[2].

The occurrence of particular phytocompounds plays a pivotal role in pest control. *M. koenigii* is considered one of the greatest plants for controlling mosquitoes. It provides mosquito protection for a duration of six hours. The derivatives of *M. koenigii* are effective for vector control and have various other applications[25]. Its leaves are effective in treating diabetes, diarrhea, cancer, HIV, ulcers, obesity, inflammation and skin disorders. In addition, *M. koenigii* has been documented and proven to have cytotoxic, anti-trypanosomal, anticancer, anti-diabetic, anti-dermatophyte, antioxidant, hepatoprotective, antimicrobial, antihypertensive, mosquitocidal, antifungal, larvicidal, antiprotozoal and hypolipidemic properties[42].

Approximately 80% of the world's population uses herbal treatments to manage and to treat various illnesses[43]. The development of various drugs depends on these traditional remedies. Here, we explored *in vitro* antiviral, anti-inflammatory and antioxidant properties of an ethanolic extract of *M. koenigii* against CHIKV. Our findings confirm *M. koenigii*'s antiviral qualities as a potential candidate that shows promise for more research aimed at developing an anti-CHIKV herbal drug.

Material and Methods

Cells and virus: The Vero cell lines (CCL-81) were grown in Minimal Essential Medium (MEM; HiMedia) supplemented with 1X antimycotic-antibiotic solution (Sigma-Aldrich, St. Louis, MO, USA) and 10% Fetal Bovine Serum (FBS, Gibco™, New York, USA). The cells were maintained at 37°C in a 5% CO_2 humidified condition. The National Institute of Virology, Pune, India, provided the CHIKV strain and two passages were performed in Vero cells. A multiplicity of infection (MOI) of 0.01 was employed to infect Vero CCL-81 cells with the CHIKV. After 75% of the cells showed a cytopathic effect, the supernatants were aliquoted and stored at -80°C. The FFU antiviral assay quantified viral stock titers.

Preparation of plant extract and stock solution: Healthy and fresh leaves of *M. koenigii* were obtained from the Maharshi Dayanand University, Rohtak, India. The location of the collection site is 28.958146 at latitude and 76.54042 longitude. The specialist verified the plant sample. Dry the plant part at room temperature and grind it into powder form. The extract was prepared using the maceration process. Plant powder was macerated in ethanol (99.9% pure) with continuous stirring for 48 hours. Filtration was done using Whatmann filter paper no. 1 and a rotary evaporator helps to evaporate the solvent. The concentrated extract was stored at -4°C. The plant extract's stock solution (10 mg/ml) was prepared using DMSO (50%) and filtered through syringe filter of 0.22 µM pore size. Additional dilution of the stock solution was performed in basal media (MEM) as necessary. Store the filtered stock solution at -20°C until it is used.

Fourier Transform Infrared Spectroscopy (FTIR): It is the most effective technique to identify the categories of functional groups and chemical bonds present in compounds. The chemical bond is characterized by the wavelength of light-absorbed. The FTIR analysis was performed using dried powder (10 mg) of various solvent extracts from each plant material. The resolution of 4 cm^{-1} and scan range of 400-4000 cm^{-1} were used.

Gas Chromatography-Mass Spectrometry (GC-MS): This technique helps to identify the presence of organic volatile compounds in plant extracts. The Shimadzu GC-MS-QP2020 was utilized to perform GC-MS. Helium is the carrier gas, which flows at a rate of 1.0 ml/min. on the SH-Rxi-5Sil MS column, consisting of 30 m × 0.25 mm I.D. dimension and a film thickness of 0.25 µm. A splitless infusion was delivered with a volume of 1 µL. The oven temperature was initially set at 40°C for 2 minutes, then increased to 150°C at a rate of 10°C/min over 5 minutes and finally raised to 250°C at a rate of 20°C/min over the next 10 minutes.

The temperatures for the ion source and interface were 230°C and 280°C respectively. The mass spectrometer was set to scan from 35 to 500 m/z at a rate of 2000 amu/sec. with a solvent delay of 3 minutes and an electron impact mode set to 70 eV. The National Institute of Standards and Technology (NIST) Library's ionization spectra were used to compare the bioactive components, their retention indices and mass spectra.

Cytotoxic Activity: The cytotoxicity of *M. koenigii* ethanolic extract was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay[4,27]. After Vero CCL-81 (35×10^3 cells/well) grew into a monolayer in 96-well microplates, the medium was replaced with 10% fresh MEM and different concentrations of *M. koenigii* ethanolic extract (500-1.9 µg/mL) were added to their respective wells. Cells were cultured with the treatments for 72 hours at 37°C. Each well was treated with 15 µL of an MTT solution (prepared in PBS) and incubated for 4 hours. Then, 100 µl of the solubilization solution (DMSO) was introduced to each well to dissolve the formazan. A 96-well microplate reader was employed to assess the optical density (OD) of the wells at 570 nm. The percentage of treated cells compared to untreated control cells was used to measure the cell viability.

GraphPad Prism 10.5.0 (GraphPad Software Inc.) was utilized to generate the dose-response curve.

Antiviral Assay: For the antiviral assay, Vero cells were used to propagate the CHIKV (MOI of 0.01). Virus inoculum was added to a monolayer of Vero cells and incubated for 1 hour for proper adsorption following the removal of viral inoculum. Add MNTD of plant extract. Cells were cultured for 48 hours after infection. The morphological alterations of infected Vero cells were observed using an inverted microscope. The reduction of the viral cytopathic effect (CPE) was used to determine the antiviral effectiveness of the plant extract. The 96-well plate was subjected to freeze-thaw cycles at 80°C to collect the culture supernatants. The focus-forming units (FFU) assay quantified the infectious virus titer in culture supernatants obtained from the multiple treated wells. All the assays were conducted in triplicate.

Focus-forming unit (FFU) assay: The FFU assay was used to quantify viral particles[26,28]. A 96-well plate was seeded with approximately 35×10^3 cells per well and incubated for 24 hours. The cells were treated with tenfold serially diluted culture supernatants and incubated for about an hour. Add 2% MEM and carboxymethyl cellulose (1.8%) and keep for five days in a CO_2 incubator at 37°C. Washing was done with PBS comprising tween 20 detergent and a chilled solution of acetone and methanol (1:1) used for fixation. Add a blocking buffer solution (1% BSA in PBS) and incubate for 40 minutes at 37°C. After repeating the washing, add anti-CHIKV monoclonal antibody (MAb ClVE4/D9 clone) at a ratio of 1:300 for 40 minutes and then anti-mouse IgG HRP conjugate (Sigma-Aldrich, USA) at a dilution of 1:1000 and incubat for another 40 minutes. Each addition was followed by a washing process. The final step involved the addition of substrate (True Blue Peroxidase Substrate) for 15 minutes in the dark. The blue tinge appeared and removed the substrate prior to dry it. The virus titer was quantified through the number of foci.

Antioxidant assay: The extract's ability to scavenge free radicals is measured using the DPPH radical scavenging assay[7]. DPPH is a chemical that possesses a stable free radical. A 0.3 mM DPPH solution, a standard 1 mg/ml ascorbic acid solution and various concentrations of plant extract were also made in methanol (50-300 µg/mL) and prepared to a final volume of 3 ml. DPPH reagent (1 ml) was added carefully to each preparation. After 30 minutes of dark incubation at room temperature, absorbance was measured at 517 nm. Diluted DPPH reagent in methanol was taken as a blank. The radical scavenging potential was determined using the given formula:

Radical scavenging activity (%): $(Ab_{control} - Ab_{sample}) / Ab_{control} \times 100$

Anti-inflammatory assay: *In vitro* egg albumin denaturation method determines the anti-inflammatory activity of *M. koenigii* ethanolic extract[1,29]. The test sample consisted of a mixture of egg albumin (0.2 ml), PBS (2.8 ml) and various concentrations (31.25, 62.50, 125, 250, 500 and 1000 µg/mL) of *M. koenigii* ethanolic extracts (2 ml). The same procedure was followed for the reference drug diclofenac. Double distilled water is used as a control. The reaction mixtures were kept for 15 minutes at 37°C and then incubated for 5 minutes at 70°C. The absorbance was measured at 660 nm after cooling down the mixture. The 50% inhibition (IC_{50}) was calculated through the dose-response curve. The inhibition percentage was calculated using the formula:

Percentage Inhibition% = $(Ab_{control}-Ab_{sample})/Ab_{control} \times 100$

Statistical analysis: All analyses were done with GraphPad Prism 10 (GraphPad Inc., USA). Multiple comparisons were performed using One-way Analysis of Variance (ANOVA) and Tukey's post hoc test. Following the comparison of the two the Student's t-test, a *p*-value under 0.05 was significant. All experiments were performed in triplicate. All results are displayed as the mean ± SD.

Results

According to our investigation, the ethanolic extract of *M. koenigii* exhibits potent antiviral activity against the Chikungunya virus by inhibiting virus replication.

Phytochemical Analysis

FTIR: The FT-IR spectra of *M. koenigii* ethanolic extract were recorded within the 400-4000 cm^{-1} range illustrated in figure 1 and table 1. The bands at 740.81 and 652.97 cm^{-1} assigned to C-H bending, showed aromatic compounds. The band at 1058.28 cm^{-1} attributed to C-N stretching in the amine group. The peak at 1266.68 cm^{-1} corresponds to C-F stretching, showing the presence of alkyl and aryl halides. The presence of peaks at 1916.35, 1744.48 and 1695.96 cm^{-1} are assigned to C-H bending. 2360.76 cm^{-1} assigned to carbon dioxide. Peaks at 2923.64 and 2855 cm^{-1} represent C-H stretching of alkanes and aldehyde groups. Peaks at 3740.06, 3675.07 and 3615.48 cm^{-1} are attributed to O-H stretching having phenol and alcohol groups. The surface hydroxyl group band observed at 3841.34 cm^{-1} indicates O-H and N-H stretching.

Gas chromatography-mass spectrometry (GC-MS) analysis: The GC-MS library was used to compare the evaluate bioactive chemicals of the ethanolic extract of *M. koenigii*. The chromatogram shows 24 compounds. The active compounds with their corresponding peak areas, retention times, molecular formulas and molecular weights are displayed in figure 2 and table 2. The two main components are hexadecanoic acid eicosyl ester and tetradecanoic acid hexadecyl ester, with 14.99% and 15.18% of the total area respectively. These are long-chain fatty acid esters found in natural waxes and fats. Certain identified compounds, such as hexadecanoic acid[5,30] and neophytadiene[6,8] exhibited antibacterial, anti-inflammatory

and antimicrobial effects. Heptasiloxane, hexadecamethyl, showed antioxidant, anticancer, antibacterial and antifungal activities. Beta-sitosterol acetate demonstrated an analgesic effect, increasing pain tolerance by 157%[44] and exhibiting antinociceptive action[3].

Table 1
FTIR spectrum analysis of *M. koenigii* ethanolic extract

S.N.	Wave number (cm^{-1})	Wave number range (cm^{-1})	Vibration/ stretching	Functional groups
1.	3841.34	4000-3800	O-H and N-H stretch	Surface hydroxyl groups
2.	3740.06	3800-3700	O-H stretch	Alcohols and Phenols
3.	3675.07	3500-3700	O-H stretch	Alcohols and Phenols
4.	3615.48	3500-3700	O-H stretch	Alcohols and Phenols
5.	2923.64	2990-2850	C-H stretch	Alkanes
6.	2855	2900-2800	C-H stretch	Aldehydes
7.	2360.76	-	O=C=O stretch	carbon dioxide
8.	1916.35	1650-2000	C-H bending	Aromatic compound
9.	1744.48	1650-2000	C-H bending	Aromatic compound
10.	1695.96	1650-2000	C-H bending	Aromatic compound
11.	1266.68	1000-1400	C-F stretch	Alkyl and Aryl Halides
12.	1058.28	1020-1250	C-N stretch	Amine
13.	740.81	650-900	C-H bending (out-of-plane)	Aromatic compound
14.	652.97	650-900	C-H bending (out-of-plane)	Aromatic compound

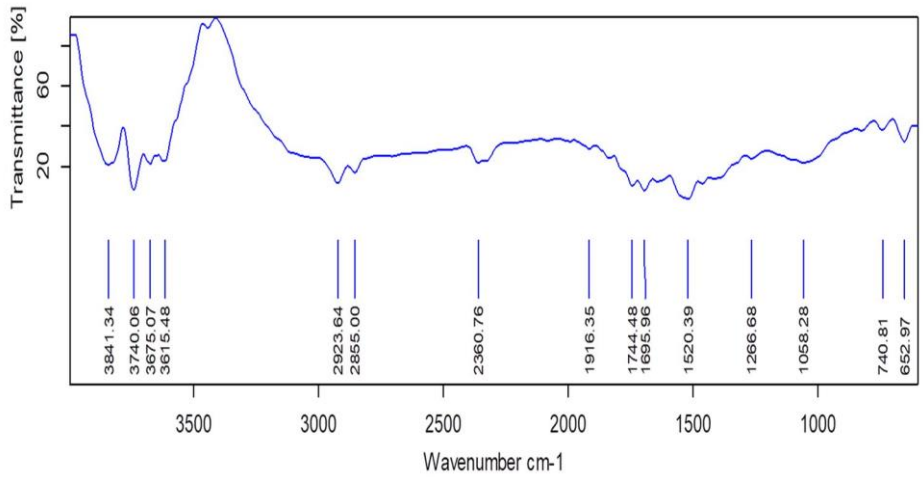

Figure 1: FTIR spectrum of *M. koenigii* ethanolic extract

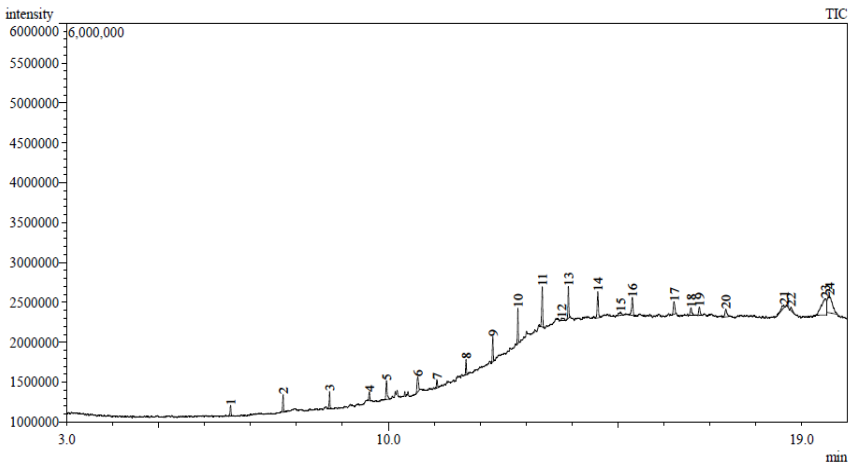

Figure 2: The GC-MS Chromatogram of *M. koenigii* ethanolic extract showing 24 peaks

Table 2
Bioactive compounds found in *M. koenigii* ethanolic extract through GC-MS

Peak #	Retention Time	Area%	Compounds	Class	Molecular formula	Molecular weight (g/mol)
1	6.566	1.73	Cyclohexasiloxane, dodecamethyl-*	Cyclic siloxane	$C_{12}H_{36}O_6Si_6$	444.92
2	7.709	2.60	Cycloheptasiloxane, tetradecamethyl-*	Organosilicon	$C_{14}H_{42}O_7Si_7$	519.07
3	8.720	2.39	Cyclooctasiloxane, hexadecamethyl-*	Cyclic siloxanes	$C_{16}H_{48}O_8Si_8$	593.2
4	9.588	1.34	Cyclononasiloxane, octadecamethyl-*	Cyclic siloxane	$C_{18}H_{54}O_9Si_9$	667.4
5	9.962	4.47	Neophytadiene	Sesquiterpenoids	$C_{20}H_{38}$	278.5
6	10.645	4.21	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	Phthalate	$C_{22}H_{34}O_4$	362.5
7	11.060	1.49	Cyclooctasiloxane, hexadecamethyl-*	Cyclic siloxanes	$C_{16}H_{48}O_8Si_8$	593.2
8	11.693	2.31	Cyclononasiloxane, octadecamethyl-*	Cyclic siloxanes	$C_{18}H_{54}O_9Si_9$	667.4
9	12.275	3.77	Cyclononasiloxane, octadecamethyl-*	Cyclic siloxanes	$C_{18}H_{54}O_9Si_9$	667.4
10	12.821	5.29	Cyclononasiloxane, octadecamethyl-*	Cyclic siloxanes	$C_{18}H_{54}O_9Si_9$	667.4
11	13.352	7.24	Tetracosamethyl-cyclododecasiloxane*	Cyclic siloxanes	$C_{24}H_{72}O_{12}Si_{12}$	889.8
12	13.773	1.49	Pregn-4-ene-3,20-dione, 17,21-dihydroxy-, bis(O-methyloxime)	Steroid hormones	$C_{23}H_{36}N_2O_4$	404.5
13	13.923	5.62	Cyclononasiloxane, octadecamethyl-*	Cyclic siloxanes	$C_{18}H_{54}O_9Si_9$	667.4
14	14.562	5.70	Tetracosamethyl-cyclododecasiloxane*	Cyclic siloxanes	$C_{24}H_{72}O_{12}Si_{12}$	889.8
15	15.054	1.46	Stearic acid hydrazide	Fatty acid derivative	$C_{18}H_{38}N_2O$	298.5
16	15.313	4.79	Tetracosamethyl-cyclododecasiloxane*	Cyclic siloxanes	$C_{24}H_{72}O_{12}Si_{12}$	889.8
17	16.223	3.41	Tetracosamethyl-cyclododecasiloxane*	Cyclic siloxanes	$C_{24}H_{72}O_{12}Si_{12}$	889.8
18	16.593	2.38	Cholesta-4,6-dien-3-ol, (3.beta.)	Steroids	$C_{27}H_{44}O$	384.6
19	16.774	2.11	beta-Sitosterol acetate	Phytosterol	$C_{31}H_{52}O_2$	456.7
20	17.352	2.92	Tetracosamethyl-cyclododecasiloxane*	Cyclic siloxanes	$C_{24}H_{72}O_{12}Si_{12}$	889.8
21	18.613	2.11	Stearic acid hydrazide	Fatty acid derivative	$C_{18}H_{38}N_2O$	298.5
22	18.769	0.99	Heptasiloxane, hexadecamethyl-	Organosilicon	$C_{16}H_{48}O_6Si_7$	533.1
23	19.514	14.99	Hexadecanoic acid, eicosyl ester	Fatty acid ester	$C_{36}H_{72}O_2$	537
24	19.605	15.18	Tetradecanoic acid, hexadecyl ester	Fatty acid ester	$C_{30}H_{60}O_2$	452.8

* Indicates contaminated compounds.

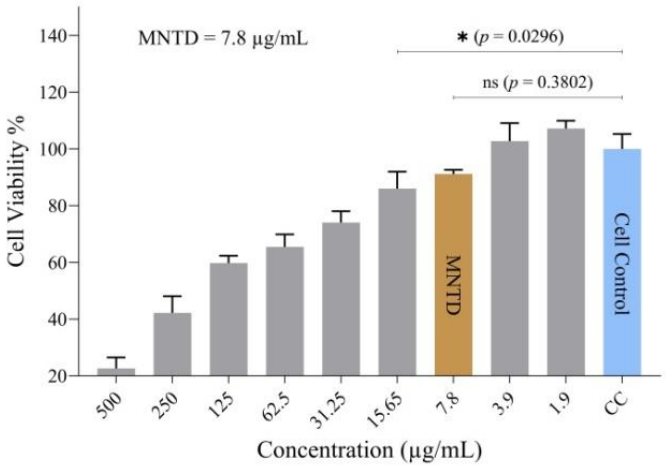

Figure 3: Cell viability Percentage by MTT assay at different concentrations of *M. koenigii* ethanolic extract

***In vitro* antiviral screening**

Cell Cytotoxicity Assays: The MTT assay was used to determine the cytotoxic effects of *M. koenigii* ethanolic extract on Vero cell viability. Cell viability percentages are depicted in figure 3. The maximum non-toxic dosage of extract that permitted over 90% cell viability was employed to evaluate antiviral efficacy. At dosages of 7.8 µg/mL or less, cell viability was over 90%, with an estimated IC_{50} of 147.6 µg/mL. Later studies used MNTD at 7.8 µg/mL, indicating non-toxicity to cells. All tests were done in triplicate.

Morphological screening of treated Vero cells: The antiviral potential of *M. koenigii* extract in infected Vero cells was evaluated using microscopic morphological screening after 48 hours to determine the cytopathic effect (CPE) of CHIKV. The uninfected control cells were characterized by fibroblastic, elongated, spindle-shaped cells with evident intercellular boundaries. In contrast, virus-infected cells with severe CPE resulted in multinucleated syncytia, cell rounding and substrate detachment, leading to monolayer degradation. Extract treated infected cells help in reducing the ability of virus replication, providing substantial cellular protection, maintaining a nearly normal fibroblastic shape and inhibiting syncytia development. The extract successfully arrested the Chikungunya-induced cellular damage.

Effect of *M. koenigii* ethanol extract on CHIKV replication: The MNTD (7.8 µg/ml) of the extract was used against infected Vero cells to examine the inhibition of virus replication. The results revealed that the extract at maximum non-toxic concentrations (7.8 µg/mL) exhibited anti-CHIKV potential, compared to the virus control as shown in figure 4. Following the antiviral test, the FFU assay was conducted after 48 hours to determine the virus titer. In the FFU assay, the 4 hour post-infection treatment with extract at 7.8 µg/ml concentration resulted in foci reduction from 8.10 (virus control) to 7.36 mean log10 FFU/ml (82.25% reduction in FFU titer; p=0.005).

Anti-inflammatory Activity: The egg albumin denaturation method reveals the anti-inflammatory potential of *M. koenigii* ethanolic extract. The extract demonstrated dose-dependent inhibition (100-500 µg/ml) ranging from 16.29% to 64% and IC_{50} as 146.4 µg/mL. In contrast, diclofenac sodium (a reference medication) exhibited inhibition between 41.91% and 84.61% with an IC_{50} of 425.7 µg/mL (Figure 5). The results indicated that the plant extract exhibited moderate anti-inflammatory potential compared to diclofenac, suggesting that it could be therapeutically utilized as a natural anti-inflammatory agent.

Anti-oxidant Activity: The stable DPPH free radical has been widely utilized to estimate antioxidant free radical scavenging. The DPPH-scavenging capabilities of the ethanolic extract of *M. koenigii* are presented in figure 6. The extract exhibited moderate antioxidant activity with radical scavenging percentages ranging from 6% to 65% at various doses (100-500 µg/mL) and ascorbic acid (a standard) showed percentages ranging from 26% to 98%. The extract showed an IC_{50} of 53.77 µg/mL compared to the standard, with an IC_{50} of 81.12 µg/mL. These results indicate that *M. koenigii* ethanolic extract comparatively shows more moderate free radical scavenging capacity than the standard.

Discussion

At present, no antiviral medications have been developed against Chikungunya. The increasing CHIKV epidemics demand the development of efficient medicines to fight this devastating disease. Plant-derived antivirals are promising source for the development of new medication[19]. Antiviral effects have been found in a wide variety of traditionally used medical plants, herbs and other natural substances against numerous viruses including influenza[10,11], dengue[16,18], Chikungunya[23,38,41] and COVID-19[20,37] via *in vitro* methods. Hence, plant-derived anti-chikungunya pharmaceuticals may serve as an alternate treatment for mosquito-borne illnesses. In the present study, the *M. koenigii* ethanolic extract demonstrated a substantial reduction in CHIKV replication, indicating its potential as

an effective treatment against CHIKV infection. The phytochemical screening using FTIR analysis revealed that *M. koenigii* possesses a diverse array of functional groups including phenols, alcohols, alkanes, aldehydes, aromatic compounds, alkyl and aryl halides and amines. The GC-MS analysis has found 24 bioactive compounds, with hexadecanoic acid eicosyl ester and tetradecanoic acid hexadecyl ester being the predominant compounds, having 14.99% and 15.18% of the total area respectively. Long-chain fatty acid esters, along with neophytadiene and beta-sitosterol acetate, have been found to exhibit antibacterial, anti-inflammatory and analgesic properties which may also enhance antiviral activity.

The cytotoxicity of plant extracts was calculated prior to examine potential viral inhibitory methods. The MNTD of *M. koenigii* ethanolic extract was found to be 7.8 µg/mL. In the antiviral assay, the morphological screening of CHIKV-infected Vero cells treated with the extract revealed clear protective effects against viral cytopathic changes.

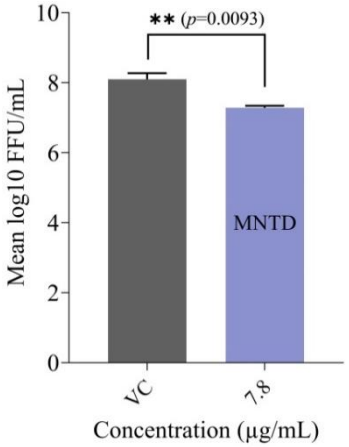

Figure 4: Focus forming unit assay CHIKV titre of *M. koenigii* ethanolic extract (7.8 µg/ml) and virus control

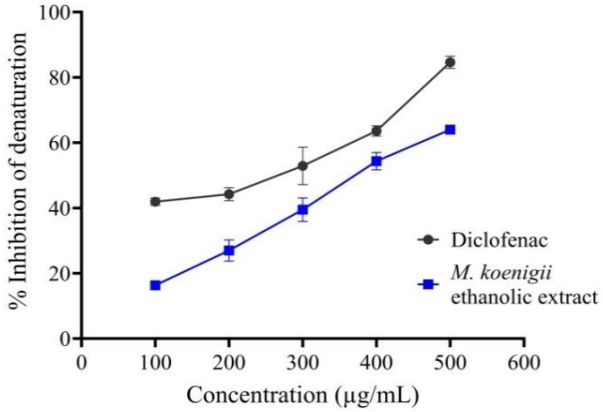

Figure 5: Dose-dependent percentage % inhibition of denaturation of *M. koenigii* ethanolic extract and diclofenac

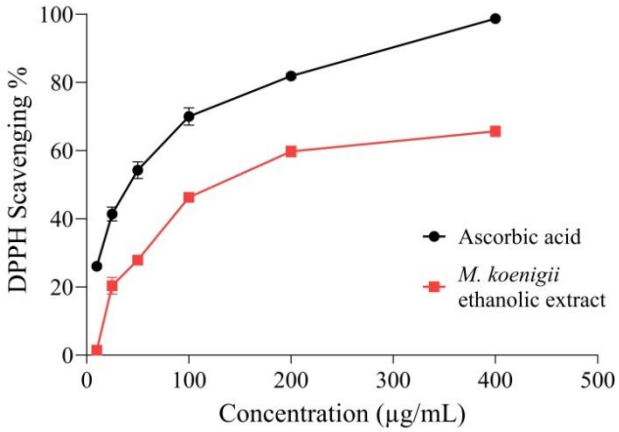

Figure 6: The DPPH scavenging activities of *M. koenigii* ethanolic extract

The treated cells maintained their normal fibroblastic morphology and prevented the formation of multinucleated syncytia, which is a characteristic of CHIKV infection. The antiviral activity was quantitatively confirmed using the FFU assay which evaluates functional infectious virus particles in infected Vero cells. It demonstrates an 82.25% inhibition in virus titer (from 8.10 to 7.36 mean $\log_{10}$ FFU/ml) upon treatment with 7.8 µg/mL of the extract. This significant reduction in viral replication suggests that the bioactive compounds in *M. koenigii* ethanolic extract may interfere with multiple steps of the viral life cycle including viral entry, replication and assembly. The mechanism of action may involve the interaction of phenolic compounds and fatty acid esters with viral envelope proteins or cellular receptors, inhibiting viral attachment and penetration.

The potential of plant extracts as anti-inflammatory drugs was assessed using a simple and viable protein denaturation bioassay. The diclofenac sodium was taken as a standard drug. The extract was found to be effective in preventing heat-induced denaturation of albumin. The extract shows promising anti-inflammatory properties, having IC_{50} of 146.4 µg/mL compared to diclofenac with IC_{50} of 425.7 µg/mL. This plant becomes significant potential candidate against inflammation leading to persistent arthralgia. The DPPH assay is an appropriate method for measuring the radical scavenging capability of a plant extract. The free radical scavenging activity increased with extract concentration. The ability of the extract to inhibit protein denaturation demonstrates its potential to regulate inflammatory processes, which could help reduce virus replication. The extract's moderate antioxidant activity (IC_{50} of 53.77 µg/ml) demonstrates its ability to neutralize reactive oxygen species during viral infection, preventing oxidative stress-induced cell damage.

Multiple studies have demonstrated that *M. koenigii* is capable of a broad range of biological processes including hepatoprotective, antipyretic, antioxidant, anti-inflammatory, neuroprotective, antibacterial, anti-diabetic, wound-healing, chemoprotective, antiviral, antifungal and anticancer properties. The leaf extract of *M. koenigii* exhibits significant antioxidant activities[13,24]. The DPPH assay of ethanolic extract of *M. koenigii* showed high scavenging activity (80%) compared to the quercetin (control)[31]. Several studies have confirmed the anti-inflammatory potential of *M. koenigii* through various approaches[12,14].

This study signifies that an ethanolic extract from *M. koenigii* exhibits various therapeutic effects including antiviral, anti-inflammatory and antioxidant properties and identifies it as a potential candidate for the development of a therapeutic drug against CHIKV. The presence of a wide range of phytocompounds suggests a synergistic approach to decrease the symptoms related to viral-borne diseases. Moreover, the conventional application of *M. koenigii* in traditional medicine and its recognized safety profile support the prospects for clinical translation of our findings. It may treat acute inflammatory diseases due to its anti-inflammatory properties. It may also be beneficial for CHIKV-infected patients in reducing arthritis. Further research is required to understand the mechanism of the plant extracts *in vivo*.

Conclusion

It was concluded that *M. koenigii* exhibited significant anti-CHIKV activity and showed potential as an herbal candidate against vector-borne illnesses. However, further animal model studies, molecular mechanisms of action and clinical trials are required to support it.

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