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Anti-chikungunya activity of luteolin and apigenin rich fraction from *Cynodon dactylon*

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ABSTRACT

Objective: To obtain luteolin and apigenin rich fraction from the ethanolic extract of *Cynodon dactylon* (L.) (*C. dactylon*) Pers and evaluate the fraction's cytotoxicity and anti-Chikungunya potential using Vero cells. **Methods:** The ethanolic extract of *C. dactylon* was subjected to silica gel column chromatography to obtain anti-chikungunya virus (CHIKV) fraction. Reverse phase-HPLC and GC-MS studies were carried out to identify the major phytochemicals in the fraction using phytochemical standards. Cytotoxicity and the potential of the fraction against CHIKV were evaluated *in vitro* using Vero cells. Reduction in viral replication was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) after treating the viral infected Vero cells with the fraction. **Results:** Reverse Phase-HPLC and GC-MS studies confirmed the presence of flavonoids, luteolin and apigenin as major phytochemicals in the anti-CHIKV ethanolic fraction of *C. dactylon*. The fraction was found to exhibit potent viral inhibitory activity (about 98%) at the concentration of 50 μg/mL as observed by reduction in cytopathic effect, and the cytotoxic concentration of the fraction was found to be 250 μg/mL. RT-PCR analyses indicated that the reduction in viral mRNA synthesis in fraction treated infected cells was much higher than the viral infected control cells. **Conclusions:** Luteolin and apigenin rich ethanolic fraction from *C. dactylon* can be utilized as a potential therapeutic agent against CHIKV infection as the fraction does not show cytotoxicity while inhibiting the virus.

1. Introduction

Chikungunya is one of the major arboviral infections in tropical countries and it is transmitted mainly by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Chikungunya virus (CHIKV) belongs to the Semliki Forest antigenic complex of the genus *Alphavirus*, family *Togaviridae*[1] and caused a number of explosive and unpredictable epidemics in Africa, South Asia and Americas[2,3]. The re-emergence as a virulent pathogen interspersed by periods of disappearance that lasts several years to a few decades has become a major global health concern[4]. Increasing prevalence of CHIKV infection currently results in a wide range of atypical clinical manifestations involving neurological, cardiovascular, skin, ocular, and renal systems and persistent debilitating rheumatological symptoms that may last for years, invariably affecting all age groups of both genders[5,6]. Clinical symptoms of CHIKV infection are similar to that of other alphaviruses such as dengue virus, Sindbis

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virus, West Nile virus and Ross River virus, with arthralgia being the hallmark feature[7].

CHIKV infected patients are treated symptomatically, with administration of nonsteroidal anti-inflammatory drugs or corticosteroids to control arthralgia and myalgia[8] as there is no specific antiviral drug or vaccine available for treating this infection. The level of viremia correlates with the severity of disease with high viremia often seen in arboviral diseases including CHIKV. Hence, antivirals that can reduce the level of viremia or the viremic phase could possibly reduce the severity of the viral diseases. The drugs used to treat the disease on the basis of symptoms are not only expensive but also exhibit toxicity or adverse effects, and are not helpful in reducing the viral loads effectively[9,10]. A double-blind, randomized clinical trial was conducted to evaluate the efficacy of chloroquine in acute CHIKV viremia, but the study did not show any benefits in terms of the duration of viremia or the severity and duration of clinical symptoms[11]. Reports on synthetic broad-spectrum alphavirus inhibitors targeting cellular enzymes are available; however, these compounds are limited by their narrow therapeutic index or immunomodulatory effects that are considered unfavorable for the treatment of clinical infection[12-14]. Arbidol, a broad spectrum synthetic antiviral drug was reported to have effective CHIKV inhibitory activity by blocking the viral replication cycle at an early stage *ie*., during virus attachment or at viral entry; however, the origin of ARB resistant mutant strains limited its useness against the virus[9,15]. Presently, none of the antiviral drugs have been approved for treating CHIKV infection.

It has been found that the natural products such as plant extracts and plant derived compounds (phytochemicals) can be used as potential sources for the development of effective and novel antiarboviral drugs as they do not cause adverse effects and many are less toxic or non toxic[10]. The antiviral mechanism of these agents is validated on the basis of their antioxidant and anti-inflammatory activities, scavenging capacities, immune-stimulatory properties inhibiting viral DNA and RNA synthesis, inhibition of viral entry *etc*[16]. Though reports on antiviral activities of plant extracts and/or phytochemicals against arboviruses such as dengue are available, studies on anti-CHIKV activities of phytochemicals are limited[10,17-19].

The medicinal plant *Cynodon dactylon* (L.) (*C. dactylon*) Pers. belongs to the family Poaceae, commonly named as Bermuda grass, and has been known to exhibit antimicrobial, antiviral, antioxidant, antidiabetic, hypolipidemic and hepatoprotective effects and much of the medicinal properties are attributed to glycosides, polysaccharides, flavonoids, terpenes, alkaloids, phenolics, amino acids, and phytosterols[20,21]. This plant has been used in the traditional medicine for treating various ailments such as calcus, snake bites, hypertenstion, epilepsy, syphilis, wound infection, piles, dropsy, and convulsions. The extracts of the plant were reported to show antiviral activity against white spot syndrome virus of shrimp both *in vitro* and *in vivo*[22] and porcine reproductive and respiratory syndrome virus[23]. However, reports on the therapeutic potential of the plant against human viruses are scarce and there is no report on anti-arboviral activity from the constituents of the plant so far. Hence, in this study, an attempt has been made to use luteolin and apigenin rich fraction from the ethanolic extract of *C. dactylon* for its protective efficacy against CHIKV *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture reagents and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Culture medium, dimethyl sulfoxide (DMSO), penicillin G, streptomycin, fungizone, diethyl pyrocarbonate, luteolin and apigenin (HPLC purified) were purchased from Sigma Aldrich (St. Louis, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Hi Media, India. HPLC grade solvents (Fisher Scientific, India) were used for the phytochemical extraction and HPLC analysis.

2.2. Plant material and isolation of active fractions having anti-CHIKV components

The whole plant of *C. dactylon* was collected during December, 2012 in the Western Ghats region around Madurai District of Tamilnadu, India. The plant sample was authenticated by Botanical Survey of India, Coimbatore and the voucher specimen (No.BSI/SRC/2/23/2013-14.TECH.471) was deposited in Botanical Survey of India, Coimbatore, Tamilnadu.

2.3. Preparation of solvent extract

The fresh plant *C. dactylon* was washed, air dried in shade at ambient temperature and the dried material was made to a fine powder (1 200 *g*; particle size ~0.25 mm) using a laboratory mill and defatted with 1 000 mL petroleum ether (60-80 ℃) for 3 to 4 h in a Soxhlet apparatus. The resulting residue was dried and extracted further with 95% ethanol. The ethanolic extract was filtered using Whatman No.1 filter paper and concentrated on a rotary evaporator at 40-50 ℃ under reduced pressure. The powder is dissolved in 0.2% DMSO and stored at -20 ℃ until further use.

2.4. Isolation of active fraction from C. dactylon

The ethanolic extract was chromatographed on silica gel column (3 cm×50 cm) eluted with ethanol/water (90:10, v/v) to obtain fractions

based on the absorbance at 210 nm using UV-Vis spectrophotometer. The fractions were reconstituted, concentrated and checked for *in vitro* anti-CHIKV activity. The fraction showing anti-CHIKV activity (F4) was further subjected to Reverse Phase-HPLC analysis (ABL1525, UK) with a Phenomenex Hypersil C18 reversed phase column (5 μm; 4.6 mm×250 mm) using ethanol-acetonitrile-acetic acid-phosphoric acid-H_2O (100:50:10:10:100, v/v) as a mobile phase at a flow rate of 1.0 mL/min. The injector was a Rheodyne model valve with 20 μL loop. The absorbance of eluent was detected at 200-400 nm using photodiode array detector. The phytochemicals in the active fraction such as luteolin and apigenin were detected by retention time (RT) with respective to their standards. To prepare standard stock solutions, 50 mg each of luteolin and apigenin weighed and transferred separately into a 50 mL volumetric flask, dissolved in 50 mL of ethanol and sonicated for 5 min to produce 1 mg/mL stock solution.

2.5. Identification of active compounds by GC-MS

Ethanolic fraction of *C. dactylon* was analysed by GC-MS (JEOL GCMATE-][ ) equipped with VF-5 MS capillary column (30 m× 0.25 mm *i.d.*, 0.25 μm); helium was used as a carrier gas at a flow rate of 1 mL/min. The samples were injected with a split ratio of 1:10. Injector and detector temperatures were 80 ℃ (for 2 min) and 275 ℃, respectively. Mass spectra were recorded over 40-400 amu range in multiple ion detection mode with 70 eV of ionization energy and ion source temperature of 240 ℃. Mass spectra were acquired in positive ion mode. Identification of components of *C. dactylon* was performed by matching the obtained mass spectra data with NIST MS search version on Wiley-8 and FAME library.

2.6. Cell propagation and scoring of viral titers

The Vero cells were obtained from National Centre for Cell Science, Pune, India and sub-cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 100 μg/mL penicillin G, 100 μg/mL streptomycin and 100 μg/mL fungizone. The cultures were maintained at 37 ℃ in a humidified 5% CO_2 atmosphere. CHIKV culture was obtained from National Institute of Virology, India, activated with 10 μg/mL trypsin for 60 min at 37 ℃ and propagated in Vero cells cultured in a 96-well microplate. After 72 h, the viral titers were scored for cytopathic effect (CPE) and the results were expressed as 50% tissue culture infectious dose ($TCID_{50}$) per mL.

2.7. Cytotoxicity studies

Cytotoxicity of the ethanolic fraction of *C. dactylon* was determined by MTT reduction assay[24]. Briefly, monolayer of Vero cells (at a density of 2×10^4 cells/well) seeded in 96-well microplates was treated with different concentrations of ethanolic fraction ranging from 5 to 500 μg/mL (5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 μg/mL) for 72 h. Both solvent (0.2% DMSO) and cell controls were included in the study. Then 20 μL of MTT solution (1 mg/mL) was added to each well. The plates were incubated at 37 ℃ for 3 h followed by the addition of 0.2% DMSO to each well. The absorbance of each well was measured at 620 nm using 96-well microplate reader (Thermo Multikan EX, USA). The cytotoxic concentration (CC_{50}) was determined for the fraction.

2.8. Antiviral activity studies

Antiviral activity was assessed by the ability of the ethanolic fraction to inhibit the CPE of CHIKV on the treated Vero cell monolayers cultivated in 96-well microplates. Briefly, the cells (2×10^4 cells/well) were seeded onto 96-well microplates and grown to confluence. Then, the culture medium was removed in all wells followed by the addition of 200 μL of the activated CHIKV inoculum ($TCID_{50}$ = 2.81×10^{-5}/mL; 1.94×10^5 PFU/mL) to infect cells for 2 h at 37 ℃ in 5% CO_2 atmosphere. Subsequently, the infected cells were treated with the non-toxic concentrations ranging from 5 to 200 μg/mL (5, 10, 25, 50, 75, 100, 150 and 200 μg/mL) of fraction in fresh medium with 2% of fetal calf serum. After 72 h incubation period, the viability of viral infected cells was studied by MTT assay as described earlier along with cell and virus controls.

2.9. RNA isolation and RT-PCR analysis

The viral infected and noninfected cells were washed with 1 PBS thrice, treated with trypsin and centrifuged at 10 000 rpm for 5 min to remove dead cells and debris. Total RNA was extracted by using QIA amp Viral RNA Mini Kit (Qiagen, USA) according to manufacturer's instructions and the extracted RNA was dissolved in 1% DEPC treated water. The amount of RNA was determined by measuring absorbance at 260 nm. cDNA was synthesized and amplified by using *CHIKV E2* gene specific primers: F 5'TATCCTGACCACCCAACGCTCC-3' (Genome position: 9403-9424) and R 5' ACATGCACATCCCACCTGCC-3' (Genome position: 9693-9712)[25] from total RNA in an RNase free condition. PCR-amplified products were resolved on 1.5% agarose gels containing ethidium bromide (0.2 μg/mL) and visualized under UV light. The housekeeping gene β-actin served as reference control.

2.10. Statistical analysis

All *in vitro* experiments were carried out in eight well replicates and the experiments were repeated thrice. The results were statistically analyzed by SPSS software 17.0 package and the values were

presented as Mean ± SD. The level of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation of anti-CHIKV active ethanolic fraction

The yield of defatted ethanolic extract from *C. dactylon* was about 48 g which upon re-suspension in distilled water followed by filtration and concentration using vacuum evaporator yielded 15.26 g. The concentrated extract was analysed for the presence of phytochemicals such as alkaloids, flavonoids, saponins, tannins, sterols, glycosides and carbohydrates, and the biochemical analysis showed the presence of alkaloids, flavonoids and glycosides (data not shown). The concentrated powder (5 g dissolved in DMSO) was subjected to silica gel chromatography for obtaining five different fractions (fractions F1 to F5; Figure 1). The fractions were reconstituted, lyophilized, dissolved in DMSO and evaluated for anti-CHIKV activity at the concentration of 50 μg/mL using Vero cell lines (data not shown). Only a single fraction (F4) showed anti-CHIKV activity and this fraction was further evaluated for its cytotoxicity and antiviral studies.

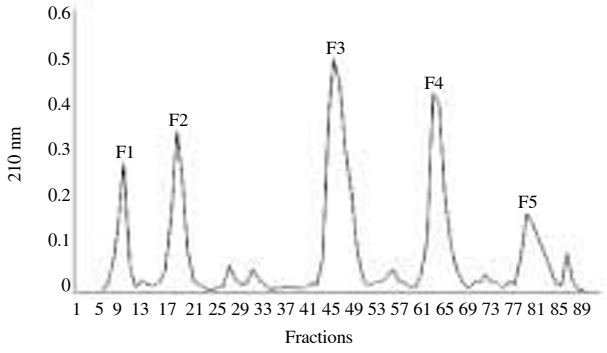

Figure 1. Separation of fractions from ethanolic extract of *C. dactylon* by silica gel chromatography.

3.2. HPLC and GC MS studies on identification of phytochemicals in ethanolic fraction

The partitioned ethanolic fraction (F4) of *C. dactylon* that exhibited antiviral activity was analyzed by RP-HPLC after separation of various active components by increasing the polarity of the solvents (Figure 2). Though the HPLC chromatogram of anti-CHIKV active fraction was found to contain several well resolved peaks with distinct RT, two prominent peaks corresponding to the RT of about 20 min and 24 min were observed at 270 nm (Figure 2a). The phytochemicals standards for flavonoids such as luteolin and apigenin of *C. dactylon* were procured and resolved by HPLC using the same conditions adopted for the fraction sample (Figure 2b and 2c). The results showed that the RT of the luteolin and apigenin standards matched with that of the RT of components in the fraction indicating the presence of the two predominant phytochemicals in the anti-CHIKV active fraction. GC-MS spectrum revealed the presence of three prominent peaks having the molecular weight of 207, 267 and 281 of which the peaks corresponds to 267 and 281 matched with the molecular weight of apigenin (MW 270.24) and luteolin (MW 286.2) (Figure 3). Hence, the presence of these phytochemicals as major constituents in the anti-CHIKV active fraction was confirmed. Other peaks having the molecular weight ~207 and ~295 were identified to be hexamethyl cyclotrisiloxane (MW 222) and phytol (MW 296) respectively from the ethanolic fraction (F4) of *C. dactylon*.

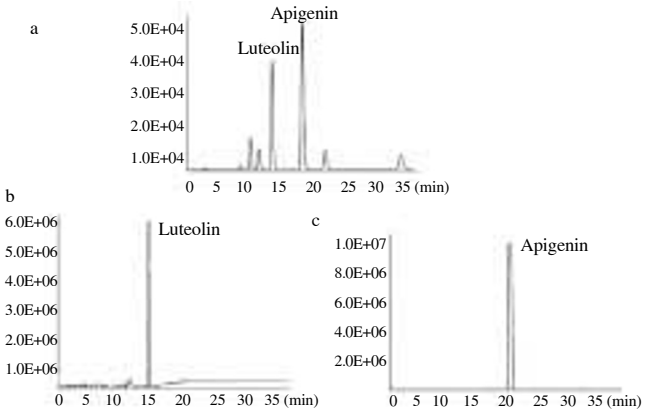

Figure 2. RP-HPLC resolution of (a) luteolin and apigenin in the anti-CHIKV ethanolic fraction of *C. dactylon*; (b) luteolin and (c) apigenin standards.

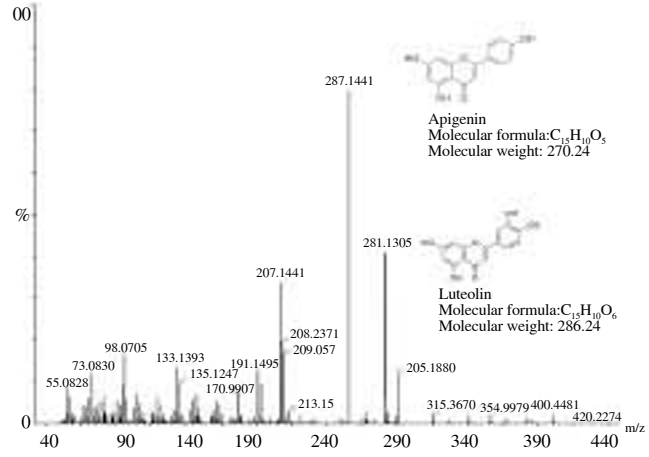

Figure 3. GC-MS analysis of anti-CHIKV active ethanolic fraction of *C. dactylon*.

3.3. Cytotoxicity studies

The studies on cytotoxicity of ethanolic fraction at the concentration of 5-500 μg/mL in Vero cell line showed that the fraction treated cell lines developed confluence upto 250 μg/mL concentration

at 48 h as comparable to that of controls (untreated and DMSO treated cells). However, cells treated with >250 μg/mL of ethanolic fraction exhibited toxicity as observed by microscopic analysis, *ie.*, changes in morphology of cells, loss of monolayer, granulation and vacuolization in the cytoplasm, and cell damage when compared to untreated as well as cells treated with <250 μg/mL concentration (Figure 4). Hence, the ethanolic fraction up to 250 μg/mL (CC_{50}) could be used to test the antiviral activity without affecting much of the cell viability (Figure 5).

Figure 4. Cytotoxicity studies on Vero cells treated with various concentration of anti-CHIKV fraction.
(A) Control; (B) 25 μg/mL; (C) 50 μg/mL; (D) 75 μg/mL; (E) 100 μg/mL; (F) 150 μg/mL; (G) 200 μg/mL and (H) 250 μg/mL.

Figure 5. Antiviral activity of ethanolic fraction of *C. dactylon* on Vero cells.
(A) Virus control showing CPE; (B) Cells treated with 10 μg/mL of fraction; (C) 25 μg/mL of fraction and (D) 50 μg/mL of fraction.

3.4. Antiviral activity by MTT assay and RT-PCR

Antiviral activity of the ethanolic fraction against CHIKV was determined by CPE reduction and MTT assay after treating the viral infected Vero cells with the non toxic concentration (5 to 200 μg/mL) for 72 h (Figure 6). Results revealed that the untreated infected control cells showed maximum CPE; among the concentrations tested, the fraction at the concentration of 10, 25 and 50 μg/mL exhibited significant antiviral activity *ie*. 68%, 88% and 98% respectively as observed by the reduction or nil CPE whereas the fraction at the concentration of 5 μg/mL showed minimum inhibitory effect (36%) as observed by the low degree of CPE (Figure 5 and 6).

The cells treated with various concentrations of the fraction were subjected to RT-PCR and the amplified gene products (E2) were resolved in 1.5% agarose gel. The presence of bands in the cells treated with fractions at the concentrations of 5 μg/mL and 10 μg/mL indicated that the concentration was too low to arrest the viral replication completely, whereas the concentrations 25 μg/mL remarkably exhibited antiviral activity as shown by the absence of bands in the gel (Figure 7a; i-v). The expression of house keeping gene β-actin (reference control) was observed in all wells including the controls and fraction treated infected cells (Figure 7b; i-vi).

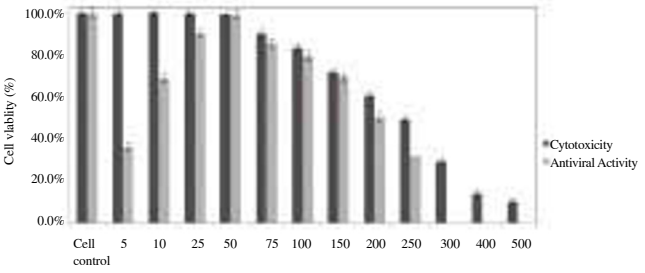

Figure 6. Cytotoxicity and antiviral effect of the anti-CHIKV active ethanolic fraction from *C. dactylon*.

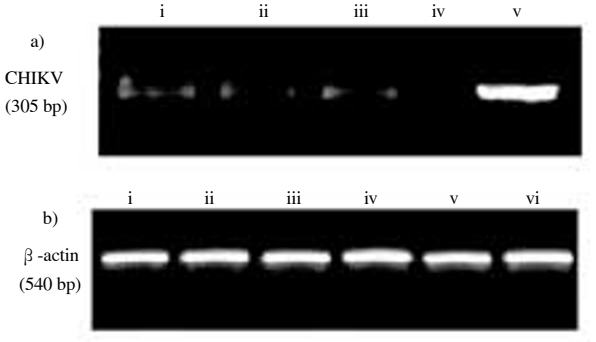

Figure 7. Studies on anti-CHIKV activity using ethanolic fraction from *C. dactylon* by RT-PCR.
(a) Expression of E2 viral gene (305 bp) from infected cells treated with fraction at the concentration of (i) 5 μg/mL, (ii) 10 μg/mL, (iii) 25 μg/mL, (iv) viral uninfected cells (negative control) and (v) expression of E2 gene (virus control);
(b) Expression of β-actin (540 bp) in viral infected cell lines treated with fraction at the concentration of (i) 5 μg/mL, (ii) 10 μg/mL, (iii) 25 μg/mL, (iv) 50 μg/mL, (v) negative control and (vi) virus control.

4. Discussion

Synthetic anti-CHIKV drugs such as chloroquine, ribavirin and arbidol did not show any significant therapeutic effects in clinical cases[14]. Hence, there is a necessity to explore the possibility of searching antiviral phytoconstituents with high efficiency, low toxicity and high viral inhibititory activity. In the present study, an attempt was made to study the anti-CHIKV activity of fractions from ethanolic extract of *C. dactylon* as the plant is known to exhibit several medicinal properties including antiviral activity and these properties are attributed to various phytochemicals[20-23].

Though the crude ethanolic extract of *C. dactylon* contained alkaloids, flavonoids and glycosides, the anti-CHIKV activity using Vero cell lines was exhibited by the flavonoid rich ethanolic fraction (F4) separated by silica gel chromatography. Further analysis by

RP-HPLC and GC-MS revealed the presence of flavonoids such as luteolin and apigenin as predominant phytoconstituents in the ethanolic fraction. Hence, the anti-CHIKV activity of *C. dactylon* could be mainly due to the luteolin and apigenin flavonoids in the ethanolic fraction. Flavonoids are widely distributed in edible plants that have been used in traditional medicine, and are likely to have minimal toxicity. Antiviral activities of flavonoids such as quercetin, fisetin, rutin, glabranine, baicalein and genistein are reported to display antiviral properties against some viruses including dengue, herpes simplex virus (HSV)-1, HSV-2, human cytomegalo virus and some types of human adenoviruses[20,26-29]. They exhibit antiviral activity by either preventing the entry of viruses into cells or inhibiting the intracellular replication of virus. Flavonoids with a 5,7-dihydroxyflavone structure such as apigenin, chrysin, silybin and naringenin are reported to inhibit replication of rhinoviruses, picornoviruses, CHIKV, HIV and Enterovirus-71[13,30,31]. However, there is no report on luteolin or the flavonoids from extracts of *C. dactylon* having anti-CHIKV activity.

The cytotoxicity studies showed that the ethanolic fraction up to 250 μg/mL concentration could be used to test the antiviral activity without affecting much of the cell viability. It was reported that the ethanol extract of *C. dactylon* exhibited replicatory inhibitory effect against porcine reproductive and respiratory syndrome virus propagated in MARC-145 cells at the concentration of 780 μg/mL without exhibiting cytotoxicity[23]. It was also reported that the ethanolic extract of *C. dactylon* was found to be safe at the oral dose level up to 400 mg/kg using mice and the concentration of extract between 100 to 400 mg/kg effectively prevented arthritis through reducing inflammation by suppression of inflammatory cells as well as secretion of proinflammatory mediators[32]. As arboviruses including CHIKV infection share rhematological manifestations *ie*., severe inflammation in joints, the ethanolic fraction of *C. dactylon* can be effectively used in reducing the inflammation in joints and thereby reducing the severity of the disease. Also, it was reported recently that luteolin and luteolin glycosides showed anti-inflammatory effect in lipopolysaccharide-stimulated macrophages and the phytochemicals were less toxic potential alternative to current anti-inflammatory drugs with promising use in pharmaceutical and food supplement industries[33]. Hence, the antiviral preparation of the present study is important not only in terms of exhibiting efficiency in antiviral activities with the presence of two predominant flavonoid phytochemicals but also reducing the CHIKV associated rheumatological manifestations through suppressing the severity of inflammation.

It was shown that natural compounds with a 5,7-dihydroxyflavone structure such as apigenin could inhibit CHIKV replication with IC_{50} value in the range of 22.5 μM to 126.6 μM[13]. The study reported the antiviral mechanism of the flavonoids having the dihydroxyflavone structure and their target site of action against CHIKV was replication rather than entry; it was shown that these flavonoids inhibited CHIKV replication once they gain entry into the host cells but were ineffective in preventing the entry of virus into the host cell. It was suggested that six different flavonoids including apigenin and luteolin were reported to inhibit the production of nucleoprotein by highly pathogenic A avian influenza virus *in vitro* using human lung epithelial (A549) cells without exhibiting toxicity[34]. Apigenin-7-O-glucoside and luteolin derived from two different plants such as *Melissa officinalis* and *Aspalathus linearis* were found to inhibit Rota virus infection using *in vitro* study employing MA104 cells at IC50 concentrations of 150 μM and 116 μM respectively[35]. The advantage of the present study is to provide a preparation having both apigenin and luteolin flavonoids exhibiting potential anti-CHIKV activity; and to our knowledge, this is the first report on the use of single plant based preparation of fraction rich in two anti-CHIKV flavonoids. Besides, the fraction of the current study has potential antiviral effect at minimal concentrations *ie*. 25 to 50 μg/mL and it did not exhibit cytotoxicity even upto the concentration of 250 μg/mL. However, earlier reports on antiviral activities of the plant suggested the use of extracts at higher IC_{50} than the one reported in this study. Besides anti-CHIKV activity, the fraction is envisaged to have the prospects of reducing the severity of CHIKV associated symptoms as the flavonoids of the fraction have anti-inflammatory and anti-arthritic activities. The application of the fraction can be potentially extended against other arboviruses such as dengue, Japanese encephalitis etc and treating the viral diseases through scale up as well as clinical and economical validation.

Conflict of interest statement

We declare that we have no conflict of interest.

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