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Research Article

Screening of *In vitro* Antiviral Activity of Purified Terpenoid Extracts of Selected Seaweeds against Chikungunya Virus

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ABSTRACT

Currently, the search for novel phytochemicals with unique biological potentialities is a prerequisite for designing ideal drugs for the humankind. Seaweeds are bioresources with a broad spectrum of medicinal properties with minimal side effects. Kerala, the southern state of India, reported high incidence of chikungunya virus (CHIKV) infections in the last several years. No specific virucidal therapy or effective vaccines are available. This emphasizes the need to search for phytochemicals as drugs with less cost and more effective. Therefore, an attempt was made in screening purified terpenoid extracts of selected seaweeds as anti-CHIKV potential. In this study, the terpenoids composition from the red algae *Hypnea musciformis*, *Kappaphycus alvarezii*, and *Gracillaria dura* were identified and analyzed by thin-layer chromatography and gas chromatography-mass spectrum. The methanolic extract of seaweeds was purified by column chromatography, and each fraction was eluted by using petroleum ether and ethyl acetate as solvent combination. The analysis of the purified fraction of *H. musciformis* and *K. alvarezii* revealed the presence of several terpenoids and *G. dura* showed only four major terpenoids, respectively. Vero cell lines were employed in the antiviral assays, infected to CHIKV, and treated with varied doses of purified terpenoid extracts. In the antiviral activity, terpenoid extracts of *G. dura* showed remarkable and promising EC₅₀ inhibitory effect at 1.25 µg/mL. Further, the terpenoid extracts displayed efficient virucidal activity against CHIKV (inhibit around 90%) with 5 µg/mL dosage. As the last phase, terpenoid extracts added at time intervals of 0, 1, 2, and 3 post-infection periods still maintained a significant inhibitory potential against CHIKV viral replication. Thus, the overall study suggests that the terpenoid extracts of *G. dura* may be effectively used in the prevention and treatment of CHIKV infections. Clinical studies may be warranted for designing a promising new anti-CHIKV drug.

INTRODUCTION

The CHIKV, a re-emerging arbovirus, leads to crippling musculoskeletal inflammatory disorders in humans featured by high fever, polyarthralgia, myalgia, rash, and headache. CHIKV is carried via the *Aedes* mosquito vector and is capable of an out breaking epidemic, urban transmission cycle with intensified infection rates. Since 2004, CHIKV has spread to various countries, causing the disease on a global scale, and the potential for its epidemics still remains high. No proven vaccines

or virucidal therapies are available, even though it has caused millions of cases and significant economic loss in the affected countries.

Africa in 1950s, record the first CHIKV infection, subsequently, CHIKV epidemics noticed throughout the last half of the 20th century in Asia and sub-Saharan African countries. CHIKV phylogenetic sequence analyses revealed that it was originated over 500 years ago from Africa.^[1] The common lineage was diverted into two distinct lineages, referred to as West African and East/ Central/ South

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African.^[2] West African strains have been linked mainly with enzoonotic transmission and small focal outbreaks of human disease in countries located in western Africa. In contrast, strains from East/ Central/ South African branch have repeatedly spread to new zones to cause remarkable urban epidemics. The first emergence of an East/ Central/ South African strain outside of Africa was noted to have occurred between 70 and 150 years ago in Asia.^[3] The virus continued to circulate in this region, evolving independently of the East/ Central/ South African category into a distinct Asian genotype, which results in numerous CHIKV disease outbreaks in this region.

India was impacted by the major outbreak of chikungunya fever during 2006-2007. Kerala, the worst affected state with a contribution of 55.8%. However, no systematic information is available on infection status of CHIKV in the region. Hence, a post-epidemic survey was carried to estimate seroprevalence status [immunoglobulin G (IgG)] in the community using indirect immune-fluorescence test that is specific and sensitive for CHIKV infection. Mid-highlands region which harbor rubber plantations evidenced 68% of the population to be seropositive for CHIKV IgG. This may be due to high occupational risk of the male population engaged in plantation activities exposed to mosquito infective bites. The current study provides an insight into screening of efficacy of purified terpenoid extracts from selected seaweeds against CHIKV.

MATERIALS AND METHODS

The marine algae *H. musciformis*, *G. dura*, and *K. alvarezii* were collected during March 2018, from the Mandapam coast (latitude 9°17'N, longitude 79°22'E), Gulf of Mannar. Identity was confirmed by referring algal flora and substantiated by matching with samples of CMFRI, Kochi. The thalli of *K. alvarezii*, *H. musciformis*, and *G. dura* were washed thoroughly until all the debris was removed. Then, macerated into pieces, shade dried, and powdered mechanically. Initially, 50 grams of each of the dried algal powder was subjected to Soxhlet extraction with 250 mL of methanol. The extraction was repeated thrice. The extract was then filtered and kept at room temperature for evaporation. Purification of each sample was done by using silica gel column chromatography (CC). Each fraction was eluted by using different combinations of petroleum ether:ethyl acetate solvents. The column eluted fractions were analyzed for the presence of terpenoids and subjected to thin layer chromatography (TLC), and further determined by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analysis

For GC-MS analysis, the sample was injected into an HP-5 column (30 m × 0.25 mm i.d. with 0.25 μm film thickness), Agilent Technologies 6890 N JEOL GC Mate II GC-MS model. Helium was used as carrier gas with a flow rate of 1 mL/min. A chromatogram was obtained,

and the mass spectrum of the unknown components was identified by comparing it with the spectrum of the known components available in the NIST library.

Cytotoxicity Assay

The cytotoxic effect of purified terpenoid extracts from selected seaweeds was analyzed on Vero cells with 90% confluence in 96 well plates. The cells were pre-treated with 1, 2, 4, 6, 8, 10, and 12 mg/mL of purified terpenoid extracts from selected seaweeds and incubated for 48 to 72 hours in Dulbecco's modified eagle medium (DMEM) and 5% of fetal bovine serum (FBS) at 37°C in CO₂ incubator. Further, 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was mixed after 72 hours post-terpenoid extracts treatment and incubated for 3 hours at 37°C. Gently, the medium was removed, followed by the addition of 100 μM dimethyl sulfoxide (DMSO), and cells were incubated at 37°C for 15 minutes to dissolve the formazan crystals as prescribed.^[4] The optical density (OD) was recorded at 550 nm using ELISA plate reader. The live active cell % was compared with the cell's control, and cellular cytotoxicity was calculated. Three independent experimental was carried to substantiate the results.

Antiviral Activity by Virus Plaque Reduction Protocol

Antiviral potentiality was validated using a virus plaque reduction protocol. Vero cell lines were cultured on 24 well plates under prescribed environment and subsequently infected with CHIKV [multiplicity of infection (MOI) = 0.1] with or without the varied doses of purified terpenoid extracts (0.625, 1.25, 2.5, 5, 10, and 20 μg/mL) at 37°C. After 1-hour of adsorption, the residual inoculum was changed by medium containing methylcellulose (1%) and the corresponding dose of each compound. Plaques were counted after 72 hours of incubation at 37°C at 5% CO₂. The inhibitory concentration 50% (EC₅₀) was measured as the terpenoid dose required to reduce virus plaques by 50%. All experimental were carried twice and each in triplicate. This was employed to evaluate the number of plaques forming virus units (PFU/mL) compared to mock CHIKV control.^[5]

Virucidal Profile

This part of the experiment was carried to assess efficacy of terpenoid extract that has a direct effect on the infectivity of CHIKV. Approximately 200 plaque-forming units of the viruses were incubated at 37°C in DMEM containing concentration of 5 μg/mL of terpenoid extract. As the control, the same amount of virus was incubated in DMEM containing 1% DMSO, and a similar condition was also made for ribavirin. The remaining virus titer obtained after each experiment was determined by plaque assay on Vero cells, as described above.^[5]

Time Interval of Drug Addition

The effects of treatment at different time periods were tested to analyze the period of the viral life cycle that was



affected by the terpenoid extract. Monolayers of Vero cells were cultured on 24 well plates as previously narrated and were infected with CHIKV at an MOI of 0.1 at time period of 0. Cells were treated with terpenoid extract of *G. dura* to a dose of 5 µg/ 0, 1, 2, 4, 6, 8, 12, and 16 hours. After 12 hours of incubation, the culture supernatants were collected, and the cells were topped with 3% carboxy methyl (CM) cellulose in culture medium. After 72 hours of incubation at 37°C, the cells were fixed with 20% formaldehyde for 2 hours, and stained with crystal violet for 5 minutes. Plaques formed after each treatment was counted, and titer value was calculated.^[5]

Statistical Analysis

The cytotoxicity (CC₅₀) as well as the effective minimal cytotoxic concentration and IC₅₀ of each terpenoid extract, were assessed from dose-response curve (Prism, Graph Pad). Results were compared using Student's t test. The statistical significance level was set at p-values < 0.05.

RESULTS AND DISCUSSION

GC-MS Analysis

The crude methanolic extracts of *H. musciformis*, *K. alvarezii*, and *G. dura* were purified by using silica gel G (230–400 mesh size) column chromatography. Each fraction was eluted using petroleum ether:ethyl acetate solvent combinations. Elution was started with 100% petroleum ether (PE) and gradually increased the polarity up to 100% Ethyl Acetate (EA). The column eluted fractions were analyzed for the presence of terpenoids and subjected to TLC using 6 mL toluene:1 mL ethyl acetate as solvent combination and further to GC-MS analysis. The fraction eluted using 95:5 solvent combinations of *H. musciformis*, 50:50 solvent combinations of *K. alvarezii*, and 90:10 of *G. dura* showed significant amount of terpenoids.

The 95:5 PE:EA eluted fractions of *H. musciformis* showed a single band on TLC with R_f value 0.89. The isolated band was subjected to GC-MS revealed eight terpenoid compounds, such as, eicosane, heneicosane, 2-pentadecnone, hexadecanoic acid methyl ester, n-hexadecanoic acid, hexadecanoic acid ethyl ester, heptadecanoic acid methyl ester, 11-octadecanoic acid methyl ester. 50:50 solvent combinations of *K. alvarezii* showed two bands of terpenoids and its GC-MS analysis showed the presence of hexadecane, eicosane, heptadecane, octadecane, heneicosane, tricosane, hexadecanoic acid, methyl ester, and beta amyrin. The 90:10 (PE:EA) purified fraction of *G. dura* also showed single prominent band on TLC (R_f values 0.47) and revealed the presence of terpenoid fractions, such as, hexadecanoic acid methyl ester, n-hexadecanoic acid, 11-octadecanoic acid, and phytol.

Evaluation of the Cytotoxicity

The CC₅₀ of the purified terpenoid extracts from the red algae *G. dura*, *K. alvarezii*, and *H. musciformis* were

evaluated by MTT reagent. The results in Table 1 reveal the significant CC₅₀ value showed by *G. dura* as compared to the other seaweeds, such as, *K. alvarezii* and *H. musciformis*, i.e., 6 mg/mL. Subsequently, terpenoid extract of the seaweeds were evaluated further for their virucidal potentialities.

CC₅₀ is the 50% cytotoxic concentration, the dose that reduces cell viability by 50% compared to control.

EC₅₀ is the dose that reduced CHIKV replication by 50%, compared to infected control.

Selectivity index the ratio between CC₅₀ and EC₅₀, and represents the safety for *in vitro* assays.

Anti-CHIKV Profile and Selectivity Index

For this analysis, different dosages of the terpenoid extracts (0.625 to 20 µg/mL) from *G. dura*, *K. alvarezii*, and *H. musciformis* were used. Purified terpenoid extracts from *G. dura* showed a concentration-dependent % inhibition, i.e., 90% inhibition at 5 µg/mL concentration, 96% inhibition at 10 µg/mL, and 98.45% inhibition at 20 µg/mL extract. The EC₅₀ value of the purified terpenoid extract from *G. dura* was 1.25 µg/mL. The obtained data suggest that terpenoid extracts from *G. dura* were able to inhibit the replication of CHIKV by inhibiting plaque formation at various concentrations steadily (Fig. 1). Based on the % inhibition results, the selectivity index (SI) was evaluated that reflects the degree of reliability of the drug for future plausible usage and that reflects the relationship between CC₅₀ and EC₅₀, corroborating the perspectives that the extract with the lowest EC₅₀ also had a higher index of selectivity. Purified terpenoid extracts of *G. dura* presented a remarkable index of 4,800 (Table 1).

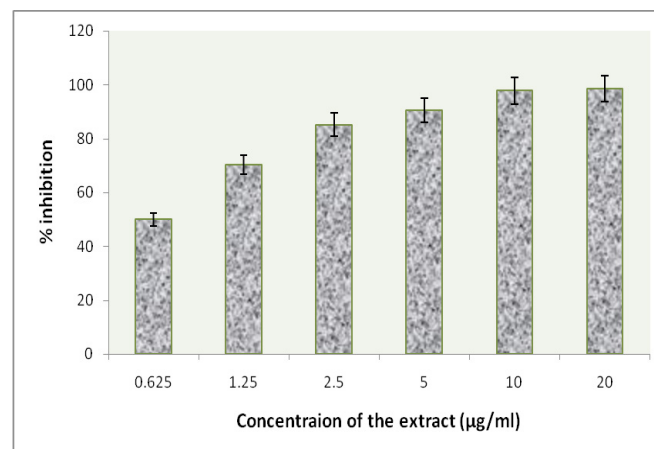


Fig. 1: Effect of the terpenoids of *G. dura* on CHIKV replication at different concentrations 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL

Table 1: Cytotoxicity, anti-CHIKV profile, and selectivity index of terpenoid extracts from the seaweeds

Terpenoids	CC ₅₀ (mg/mL)	EC ₅₀ (µg/mL)	SI
<i>G. dura</i>	6	1.25	4,800
<i>H. musciformis</i>	8	-	-
<i>K. alvarezii</i>	10	-	-
Ribavirin 297	0.25	2.5	100

Esteves *et al.*^[5] demonstrated that the CC₅₀ value of green algal extract of *Caulerpa racemosa* was 589.3 µg/mL. CHIKV is an arbovirus transferred to humans by *Aedes* mosquitoes. The virus penetrates the skin and enters the bloodstream. After the initial replication within dermal fibroblasts, it spreads via the bloodstream to the liver, muscles, joints, spleen, lymph nodes, and brain. The infection is featured by severe joint pain of abrupt onset, intense fever, and rash. Non-specific clinical symptoms are cervical adenopathy, asthenia, chills, nausea, vomiting, and diarrhea.^[6] Many studies have been carried out in search of novel drugs that can react to viral replication, decreasing the severity of the cases, and rejuvenating the health of the infected people.

For the last several years, strategies using secondary metabolites of marine weeds or from other biosources and their derivatives become popular mainly due to their poor toxicity and their potent activity against different viral strains as previously described.^[7-9] The present study revealed that the terpenoids of *G. dura* has less toxicity with a CC₅₀ value of 6 mg/mL, demonstrating that this molecule can be an ideal promising drug for trial. Similarly, they exhibit remarkable virucidal activity, i.e., the EC₅₀ value of CHIKV was 1.25 µg/mL and reflecting a SI of 4,800.

Evaluation of the Virucidal Potential

This part of the experiment was carried by evaluating the plaque formation and also the viral titer quantification. As shown in Fig. 1, terpenoids of *G. dura* were capable of arresting the CHIKV replication in 97.89% at the dosage of 5 µg/mL. However, the positive reference drug ribavirin yielded a lower value only, i.e., 95% of CHIKV virucidal activity.

Generally, the antiviral potential of drugs is evaluated by determining their potency in regulating epidemic outbreaks, i.e., by monitoring the drug as a preventive measure. The present data showed a concentration-dependent antiviral activity of inhibition

up to 100% of CHIKV activity at the dosage of 5 µg/mL used (Fig. 2).

Time of Addition Assay

Further, the impact of the terpenoids at different time intervals of addition on Vero cell lines infected by CHIKV was evaluated. The terpenoids were added at T = 0, 1, 2, and 3 hours (simultaneous) had a statistically remarkable inhibitory effect, i.e., sound inhibition rate on the viral particle and was comparable with the drug ribavirin (a sharp decline of its activity after 8 hours, meanwhile terpenoids displayed a decline accentuated even after 12 hours, as shown in the Fig. 3).

The determination of the mode of action is a strategy to substantiate whether the compound has a promising role and also to reveal its unique specificities. The search for phytochemicals that act at various stages of replication of the virus, in addition to its poor toxicity may be ideal for determining its possibility to adopt for clinical trial. The observed the poor cytotoxicity and sound inhibition of the CHIKV replication and the inhibitory role of the terpenoids added at various time intervals after viral infection suggests the above said direction. The noted data on addition of the molecule in the time intervals 0, 1, 2, and 3 hours post-infection stage still maintained an inhibitory potential of viral replication to nearly 100% for CHIKV. Parsania *et al.*^[10] screened antiviral activities of four plant extracts against acyclovir-resistant herpes simplex virus type-1. The antiviral effects of *Mentha pulegium* extract on HSV-1 were more remarkable than the virucidal potentiality. Visintini *et al.*^[11] established the effective *Bidens gaudichaudiana* as the most promising species in terms of *in vitro* antiviral activity among the herbal extracts of *Asteraceae* species. Kohn *et al.*^[12] analyzed *in vitro* antiviral activities of plants from Brazilian cerrado against the avian metapneumovirus (aMPV). Montanha *et al.*^[13] screened virucidal activities of Brazilian herbal extracts. Lelesius *et al.*^[14] documented *in vitro* antiviral activity of fifteen plant extracts against avian infectious bronchitis virus. Ahmad *et al.*^[15] explored

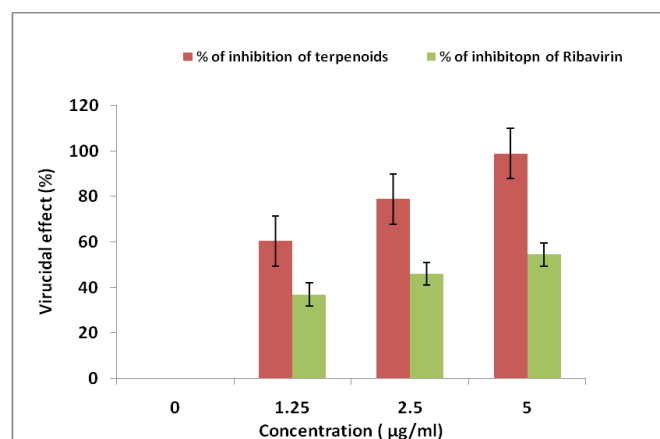


Fig. 2: Effect of terpenoid extract from *G. dura* on the infectivity of CHIKV; Vero cells were infected with CHIKV at an MOI of 0.1; the results were evaluated by plaque assay; error bars indicate that the standard deviation

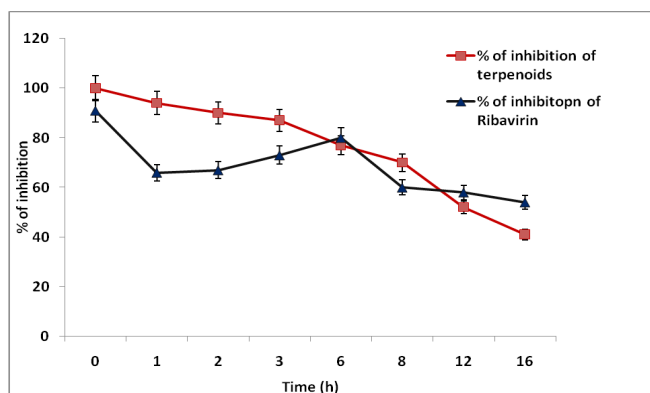


Fig. 3: Time course chart showing different treatment times (hr) at the concentration of 5 µg/mL with the action of the drugs in infection



in vitro cytotoxic and antiviral activities of different medicinal plants against infectious bursal disease (IBD) virus of Central Europe. Tello *et al.*^[16] proved cytotoxic, virucidal, and antiviral activity of South American plant species and algal extracts. Rosmalena *et al.*^[17] experimented with the antiviral effect of Indonesian medicinal plant extracts by *in vitro* and *in silico* condition against dengue virus. Hudson and Vimalanathan^[18] analyzed the phytochemicals of *Echinacea purpurea* under light-dependent and light-independent antiviral activities. Todorov *et al.*^[19] confirmed the antiviral activity of *Nepeta nuda*. Generally, the CHIKV replication cycle is approximately 6 hours.^[20] However, many reported reviews demonstrating the drug candidates that function at various periods of the replication cycle, but with an uncertainty of therapeutic efficacy. Further, the present study also demonstrated the efficacy of terpenoid via its antiviral activity, including the time scale potentiality.

The present results showed that the terpenoid extracted from *G. dura* displayed activity against CHIKV. With regard to the possible mode of action of terpenoid revealed sound virucidal pattern. The data tempted for searching the marine resources for antiviral drugs with activity against various viral strains that can remarkably decline the morbidity and mortality of infection. Virucidal activity of purified terpenoid extracts may be useful in developing a preventive strategy to effectively reduce the emergence of new pandemic issues.

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