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In vitro antiviral activity against Zika virus from a natural product of the Brazilian red seaweed *Bryothamnion triquetrum*

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Summary. – Zika virus (ZIKV) is an arthropod-borne flavivirus that reemerged in 2007 and, since then, has caused several outbreaks and spread to over 80 countries worldwide. Along with this, ZIKV infections have been associated with severe clinical outcomes, including neurological manifestations, especially in newborns, posing a major threat to human health. However, there are no licensed vaccines or specific antiviral agents available yet; thereby, there is an urgent need for the discovery of novel therapeutic strategies to fight this infection. In this context, seaweeds are proven sources of biologically relevant products, including antiviral ones, that remain poorly explored. Herein, we evaluated the antiviral potential of the dichloromethane extract of the red seaweed *Bryotamnion triquetrum* against ZIKV. MTT assay was carried out to evaluate the extract's toxicity in Vero cells, while standard plaque assays were performed for viral titer quantification in the antiviral assays. The *B. triquetrum* extract possessed great inhibitory activity on the ZIKV replication in Vero cells, with an EC_{50} of 1.38 µg/ml and a higher selectivity index than ribavirin (289.85 and 75.20, respectively), a licensed antiviral drug. The investigation of its mechanism of action revealed a moderate virucidal effect while it strongly impaired virus replication at both early and late steps of the virus replication cycle with moderate inhibition at the attachment stage. Finally, the *B. triquetrum* extract presented a remarkable synergistic effect with ribavirin at suboptimal concentrations, which also highlights the promising antiviral potential of this product as a drug candidate to combat ZIKV infection.

Keywords: Rhodophyta; Algae; arbovirus; antiviral; Zika

Introduction

Over the past century, humanity has been witnessing the (re)emergence of several arthropod-borne viruses (arboviruses), such as the Zika virus (ZIKV) (Gould *et al*., 2017). ZIKV is a positive-sense single-stranded RNA virus that belongs to the *Flaviviridae* family, the Flavivirus genus, and is primarily transmitted by *Aedes* mosquitoes (Vorou, 2016). This virus was first isolated in 1947 from a non-human primate in the Zika forest, Uganda, and remained confined in African and a few Asian countries for several decades (Dick, 1952; Karkhah *et al*., 2018). As of 2007, the first massive outbreak outside these regions occurred in the Yap islands, followed by another one in French Polynesia (2013–2014) (Song *et al*., 2017). In the same period, ZIKV was introduced in to the Americas

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Abbreviations: PFU = plaque forming unit; SI = selectivity index; ZIKV = Zika virus

(Massad *et al.*, 2017) and later, it was responsible for the major epidemic that started in Brazil in 2015 when the virus spread to many other American countries (Faria *et al.*, 2016; Zhang *et al.*, 2017).

ZIKV often causes self-limited and mild illness commonly characterized by fever, rash, myalgia, arthralgia, and conjunctivitis (Dias *et al.*, 2018). However, severe manifestations were associated with this disease, such as Guillain-Barré syndrome (Cao-Lormeau *et al.*, 2016) and Zika congenital syndrome in infants (Brasil *et al.*, 2016; Honein *et al.*, 2017). Along with this clinical presentation, ZIKV has currently spread to over 80 countries worldwide and has become a global public health threat (Gubler *et al.*, 2017; World Health Organization, 2019). Also, the economic and social burden of ZIKV infections is of great concern (Peiter *et al.*, 2020; Thompson *et al.*, 2020).

Accordingly, global health authorities should be concerned in the near future. As a result of climate changes, urbanization, and globalization, nearly 50% of the world population is expected to be at risk for arbovirus transmission in 2050 due to the increased distribution of *Aedes* mosquitoes (Kraemer *et al.*, 2019). Also, alternative routes of transmission of ZIKV (*e.g.,* sexual, vertical, and through blood products) pose a challenge to fight the spread of the virus (Gregory *et al.*, 2017). Despite the efforts of many countries, there are no approved vaccines or antivirals to tackle ZIKV infections yet, which makes the search for therapeutic strategies an urgent need (Baz and Boivin, 2019; Lunardelli *et al.*, 2021).

In this scenario, marine organisms are outstanding reservoirs of bioactive products with a variety of pharmacological activities, yet little explored so far (Carroll *et al.*, 2019; Malve, 2016). In the last decades, seaweeds have been targeted by different bioprospecting campaigns (Carroll *et al.*, 2019; Pereira and Costa-Lotufo, 2012). Excitingly, a recent chemometric study proved the high structural diversity between the secondary metabolites of seaweeds, particularly the green and red ones, which might be of pharmaceutical interest (Al Sharie *et al.*, 2020). Among the red seaweeds (Rhodophyta), *Bryothamnion triquetrum* possesses a broad spectrum of biological activities, such as anti-inflammatory, antinociceptive (Cavalcante-Silva *et al.*, 2012; Fontenelle *et al.*, 2018), vascular relaxant (Lima *et al.*, 2004), neuroprotective (Fallarero *et al.*, 2006, 2003), anticancer (Moo-Puc *et al.*, 2009), and antiparasitic (Moo-Puc *et al.*, 2008). Recently, our research group uncovered the antiviral activity against the Chikungunya virus of the dichloromethane extract of this seaweed collected at the Brazilian coast (Cirne-Santos *et al.*, 2019). This finding prompted us to investigate the *in vitro* antiviral properties of the dichloromethane extract of the Brazilian seaweed *B. triquetrum* towards ZIKV.

Materials and Methods

Algae and extraction. The algae *Bryothamnion triquetrum* (S. G. Gmelin) M. Howe was collected at Atol das Rocas reef, Rio Grande do Norte State (lat. 03°51'03", long. 33°40'29"). The seaweeds were separated from sediments, epiphytes, and other associated organisms, washed with seawater and air-dried (approximate temperature 28–30°C for 7–10 days) until the total evaporation of any water. *B. triquetrum* was extracted with dichloromethane. The extracts were evaporated under reduced pressure, yielding crude extracts of each species (15–20 mg), of which 2–5 mg were used in tests against the ZIKV. The crude extracts were dissolved in 100% dimethylsulfoxide (DMSO) and the working concentrations were obtained by dilution with cell culture medium.

Cell culture and virus. Vero cells (ATCC CCL-81) cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5% fetal bovine serum (FBS; LGC Biotecnologia, Brazil), 2.5 µg/ml amphotericin B (Cultilab, Brazil), and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. These conditions were kept unless otherwise stated. The African Zika virus MR766 strain was kindly provided by Dr. Davis Fernandes Ferreira, Universidade Federal do Rio de Janeiro, RJ, Brazil. ZIKV stocks were grown in Vero cells at a multiplicity of infection (MOI) of 0.1 and supernatants of infected cells were harvested, clarified by centrifugation at 1,500 rpm for 10 min, and stored at -80°C until use. Virus titer was determined by standard plaque-forming assay and defined as plaque-forming units per milliliter (PFU/ml).

Cytotoxicity assay. Cell viability of Vero cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) method (Mosmann, 1983). Confluent cell cultures in 96-well plates were exposed to different concentrations of the *B. triquetrum* extract at different concentrations (50, 100, 200, 400, 800, and 1000 µg/ml) for 72 h at 37°C and 5% CO_2 atmosphere. After this period, the medium containing the extract was removed and MTT solution (0.5 mg/ml) was added to cells which were further incubated for 2 h at 37°C and 5% CO_2. Finally, MTT solution was discarded, and ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, the absorbance was measured in a microplate reader at 545 nm. Since ribavirin was used as a control in the antiviral assays, this compound had its toxicity on Vero cells evaluated using the same conditions described for the extract concentrations at the following concentrations: 50, 100, 200, 400, 800, and 1000 µM. DMSO (1%) was used as the solvent control. The experiments were carried out in triplicate in three independent assays and the concentration required to reduce 50% of cell viability (CC_{50}) was calculated by linear regression of the dose-response curves using GraphPad Prism 5.

Antiviral assay. Vero cell monolayers grown in 24-well plates were infected with ZIKV at an MOI of 1.0 for 1 h at 37°C and 5%

CO_2. After attachment, the virus inoculum was removed, cells were washed with phosphate buffer solution (PBS; pH 7.4) and DMEM containing 1.5% methylcellulose, 2% FBS, and various concentrations of *B. triquetrum* extract (0.65, 1.25, 2.5, 5.0, 10, and 20 μg/ml) were added. Cells were incubated at 37°C and 5% CO_2 and, after 48 to 72 hours post-infection (h.p.i), cells were fixed and stained with 20% formaldehyde and 1% crystal violet and plaques were counted. Currently, there is no licensed drug to treat ZIKV infection, so we chose ribavirin (a broad-spectrum antiviral agent) as a positive control because it was shown to potently inhibit the ZIKV replication in Vero cells previously (Kamiyama *et al.*, 2017). The same conditions used for the algae extract antiviral evaluation was employed for ribavirin (assayed at 0.65, 1.25, 2.5, 5.0, 10, and 20 μM). DMSO (1%) was used as the solvent control. The inhibition rate of the compounds was determined by comparison to the infected and untreated cells. All determinations were performed thrice in triplicate and the concentration of the compounds that reduce virus plaque formation by 50% (EC_{50}) was calculated by linear regression of dose-response curves using the GraphPad Prism 5.

Virucidal assay. A suspension of ZIKV containing 10^4 PFU was pre-treated for 2 h with different concentrations of the *B. triquetrum* extract (5.0, 10.0, or 20.0 μg/ml) and ribavirin (5.0, 10.0, or 20.0 μM) at 37°C. Untreated virus suspensions were also incubated under the same conditions. The virus suspension was then added to confluent Vero cells in 24-well plates and allowed to attach for 1 h at 37°C and 5% CO_2 atmosphere. After this time, virus inoculum was discarded, cells washed with PBS, and DMEM supplemented with 1.5% methylcellulose and 2% FBS was added and incubated for 48 to 72 h. Finally, cells were fixed and stained with 20% formaldehyde and 1% crystal violet, and viral plaques were counted. Three independent experiments were carried out in triplicate. The virucidal activity rate was determined by comparing the number of plaques formed by untreated and treated virus inoculum.

Attachment assay. Confluent monolayers of Vero cells cultured in 24-well plates were infected with ZIKV (MOI = 1.0) for 1 h at 4°C. Different concentrations of the *B. triquetrum* extract (2.5, 5.0, and 10 μg /ml) or ribavirin (2.5, 5.0, and 10 μM) were also added during the attachment period. Infected and untreated cells control was also performed. After attachment time, cells were washed with cold PBS to remove unabsorbed virus particles and further lysed by freezing and thawing cycles. The cell lysates were collected, and the virus titer was determined by standard plaque assays in Vero cells. The experiments were performed thrice and each one was measured in triplicate. The inhibition rate of the compounds was determined relative to the infected and untreated cells.

Time-of-drug-addition assay. A time-of-drug-addition assay was conducted to evaluate which stage of the viral life cycle was affected by the *B. triquetrum* extract and ribavirin. Confluent monolayers of Vero cells grown in 24-well plates were infected with ZIKV at an MOI of 1.0 at time zero. Then, the virus inoculum was discarded, cells were washed with PBS and DMEM was added in the absence or presence of 10 μg/ml of the extract or 10 μM of ribavirin, which were added at different time points, 0, 1, 2, 4, 6, 8, 12 and 16 h.p.i. After 16 h.p.i, cells were further incubated for 12 h at 37°C and 5% CO_2 atmosphere. Then, the medium was replaced by fresh DMEM supplemented with 2% FBS and 1.5% methylcellulose for 48 to 72 h. Finally, cells were fixed and stained with 20% formaldehyde and 1% crystal violet and viral plaques were counted. Untreated and DMSO-treated controls were also carried out. Three independent experiments were conducted in triplicate.

Evaluation of the synergistic effect with ribavirin. To evaluate the effect of the *B. triquetrum* extract compounds in combination with ribavirin, Vero cells were cultured in 24-well plates until confluency and then infected with ZIKV at an MOI of 1.0 for 1 h at 37°C and 5% CO_2. After the attachment period, virus inoculum was removed, cells were washed with PBS, and DMEM with 2% FBS was added in the absence or presence of different concentrations of the *B. triquetrum* extract (0, 0.5, and 10 μg/ml) or ribavirin (0, 0.5, and 10 μM). The combined treatment regimen was defined by taking the concentration of the extract and ribavirin required to significantly block the infection (inhibition rate > 90%) and the suboptimal ones that have shown inhibition rates lower than 20%. At 18 h.p.i, supernatants were collected, and the virus titers were determined by standard plaque assays. The assays were performed in triplicates in three independent experiments. The inhibition rate of the compounds was determined relative to the infected and untreated cells. Drug interactions were evaluated using the Bliss independence model available within the Combenefit v. 2.021 software (Di Veroli *et al.*, 2016).

Statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni test using GraphPad Prism version 5 program. For the synergism analysis using the Bliss model, a one-sample T-test was employed to analyze the statistical significance, using the Combenefit software. A p-value of <0.05 was considered statistically significant.

Results

Cytotoxicity and antiviral evaluation of the B. triquetrum extract against ZIKV replication in Vero cells

We first evaluated the toxicity of the *B. triquetrum* extract on Vero cells by the MTT method. The extract showed low cytotoxicity with a CC_{50} of 400 ± 13.5 μg/ml (Table 1), which is higher than the marketed antiviral drug ribavirin (297 μM or 72.53 μg/ml). The viability of Vero cells after treatment with 1% DMSO was 97.73 ± 2.44%, indicating that the solvent used did not significantly affect the cells and would not influence the results of further antiviral activity assays.

Table 1. Cytotoxicity, anti-ZIKV profile, and selectivity index for the *B. triquetrum* extract and ribavirin

Product	CC_{50} [a]	EC_{50} [b]	SI[c]
B. triquetrum extract	400 ± 13.5 µg/ml	1.38 ± 0.28 µg/ml	289.85
Ribavirin	297 ± 4.25 µM	3.95 ± 0.95 µM	75.2

Data represented as mean ± standard deviation from three independent experiments. [a]Concentration that reduced Vero cell viability by 50% when compared to untreated cells. [b]Concentration that reduced the ZIKV plaque formation by 50% when compared to untreated infected cells. [c]Selectivity index was defined as the ratio between CC_{50} and EC_{50}.

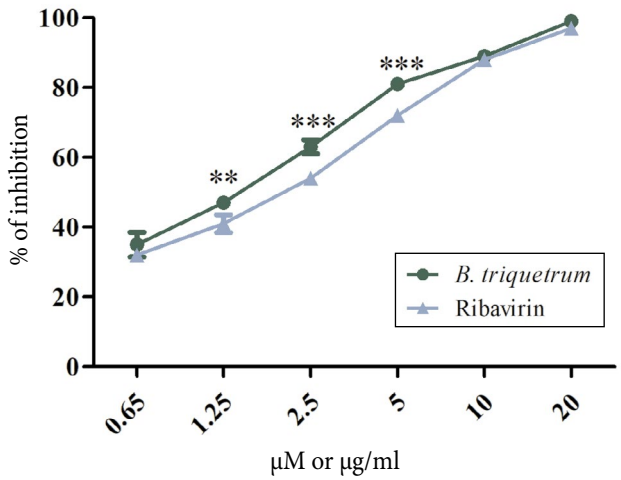

Fig. 1

Inhibition of ZIKV replication by *B. triquetrum* extract and ribavirin

Vero cells were infected with ZIKV and treated with different concentrations of the extract (0.65, 1.25, 2.5, 5, 10 and 20 µg/ml) or ribavirin (0.65, 1.25, 2.5, 5, 10 and 20 µM). The results are derived from three experiments measured in triplicates and data are presented as mean ± standard error (**p <0.01; ***p <0.001).

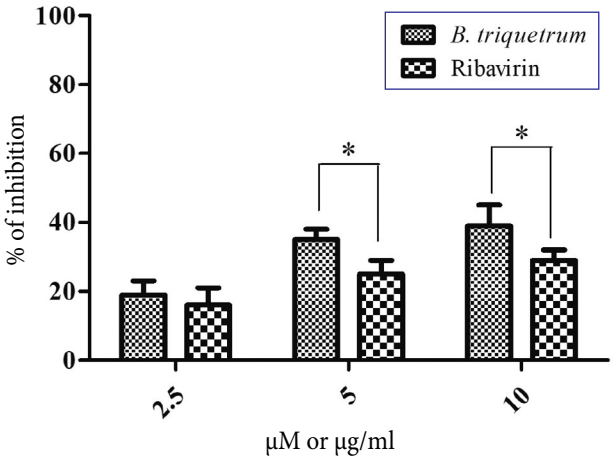

Fig. 2

Virucidal activity of the *B. triquetrum* extract (at 2.5, 5.0, and 10 µg/ml) or ribavirin (at 2.5, 5.0, and 10 µM) against ZIKV

ZIKV suspensions (10^4 PFU) were pre-treated for 2 h at 37°C with different concentrations of the extract or ribavirin and then, virus titers were quantified using standard plaque assays in Vero cells. Data are presented as mean values and error bars indicate the standard deviation obtained from three independent experiments (*p <0.05).

Consequently, we investigated the anti-ZIKV activity of this extract. This product was able to almost completely block the virus cytopathic effects at 20 g/ml. The algae product inhibited around 40% of virus plaques at the lowest concentration assayed similarly to the control ribavirin at 20 µM (Fig. 1). We further determined the potency of this product, yielding an EC_{50} of 1.38 µg/ml which is comparable to the one observed for ribavirin (EC_{50} = 95 µM or 0.96 µg/ml; Table 1). The *B. triquetrum* extract showed a significantly higher selectivity index (SI, Table 1) than ribavirin, an already licensed drug, indicating an interesting safety and efficacy profile as an antiviral drug candidate.

Evaluation of the virucidal action of the B. triquetrum extract on the ZIKV particles

To begin the characterization of the mechanism of antiviral action of the *B. triquetrum* extract, we carried out a virucidal activity assay to investigate the direct effects of this product on the ZIKV particles. Overall, the extract did not present a significant virucidal effect since only ~40% plaque formation inhibition rate was reached after treating only ZIKV particles at the highest concentration tested (10 µg/ml) (Fig. 2). As well, ribavirin did not exhibit a virucidal profile at the tested concentrations.

Time-course evaluation of the antiviral activity of B. triquetrum extract against ZIKV

We further investigated at which step of the virus replication cycle the *B. triquetrum* extract could act by the time-of-drug-addition assay (Fig. 3). The extract showed a potent antiviral activity and decreased virus titer about 5 Log_{10} PFU/ml when added at 0, 1 and 2 h.p.i, indicating that this product acts at early phases of the replication cycle of ZIKV similarly to ribavirin. Unlike ribavirin, a strong activity is still observed when the extract is added at 6 h.p.i and 12 h.p.i with a titer reduction of ~2 Log_{10} PFU/ml, at least, suggesting that this extract might exert its antiviral action by blocking early and late steps of the virus replication.

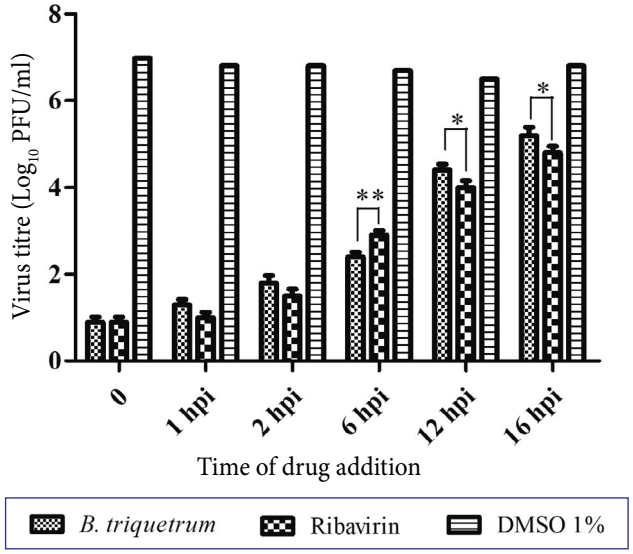

Fig. 3

Antiviral activity of the *B. triquetrum* extract (10 µg /ml) or ribavirin (10 µM) added at different time points of ZIKV infection

Monolayers of Vero cells were infected with ZIKV at an MOI of 1.0 at time zero. Either the extract or the ribavirin was added at the indicated times and cells were incubated at 37°C. At 28 h.p.i, the medium was replaced by DMEM with 2% FBS and 1.5% methylcellulose for 48 to 72 h and virus titer was quantified by plaque-forming assay. Data are derived from three independent experiments, performed in triplicate, and are expressed as the mean ± standard error (*p <0.05; **p <0.01).

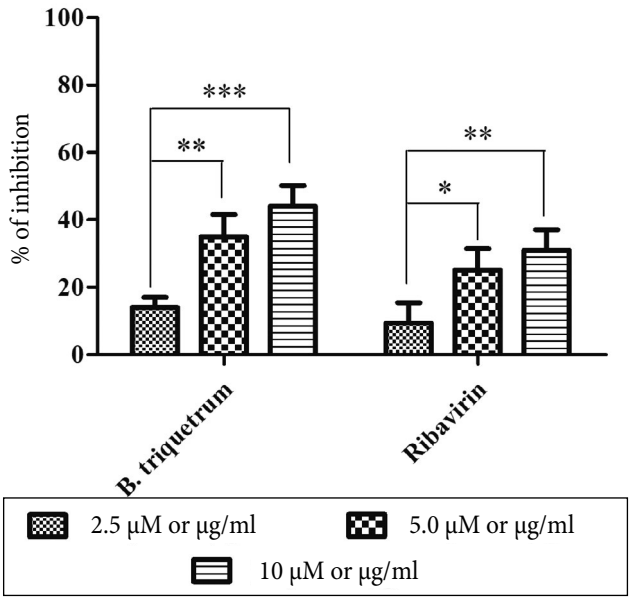

Fig. 4

Inhibitory activity of the *B. triquetrum* extract (2.5, 5.0, and 10 µg /ml) or ribavirin (2.5, 5.0, and 10 µM) on the attachment of ZIKV

Vero cells were infected with ZIKV at an MOI of 1.0 for 1 h at 4°C in the presence or absence of different concentrations of the extract or ribavirin. After this time, virus inoculum was removed, cells were washed with cold PBS to remove the unabsorbed virus, and further disrupted by freezing and thawing. Lysates were collected and the virus was tittered by the plaque assays. Data are shown as mean and error bars indicate the standard deviation from three independent experiments each measured in triplicate. No statistical significance was observed.

Attachment assay

Since the *B. triquetrum* extract acts at the early steps of the ZIKV life cycle, we investigated whether this product could inhibit virus attachment. The extract had moderate effects on virus attachment since it was able to inhibit around 40% of plaque formation at the highest concentration tested (Fig. 4). Likewise, ribavirin showed modest effects on ZIKV attachment, even at the highest concentrations studied and no significant differences were observed when compared to the extract's effects.

Evaluation of the antiviral activity of the B. triquetrum extract in combination with ribavirin against ZIKV

Considering ribavirin is a broad-spectrum antiviral agent approved to treat viral infections, we investigated the antiviral effects of the combination of this drug with the *B. triquetrum* extract against ZIKV replication. After treating infected cells with 10 µg/ml of extract or 10 µM of ribavirin, we observed a high inhibition rate of ZIKV plaques in Vero cells, but this effect was drastically reduced when the infected cells were treated with only 0.5 µg/ml of the extract or 0.5 µM of ribavirin. Interestingly, the combination of both ribavirin and the extract at 0.5 µg/ml and 0.5 µM, respectively, resulted in significant antiviral activity, showing an inhibition rate of ~100% of plaque formation (Fig. 5).

Moreover, the Bliss model was applied to evaluate quantitatively the combination effects observed (Fig. 5). The antiviral effects of the combination of ribavirin (0.5 µM) and the *B. triquetrum* extract (0.5 µg/ml) were synergistic. This combination showed an additional effect of 52% compared with the one predicted by the model used. The combination of ribavirin and the extract at 0.5 µM and 10 µg/ml, respectively, resulted in a slight synergism (8% additional effect) while no other drug interactions than additive effects were observed for other combinations tested.

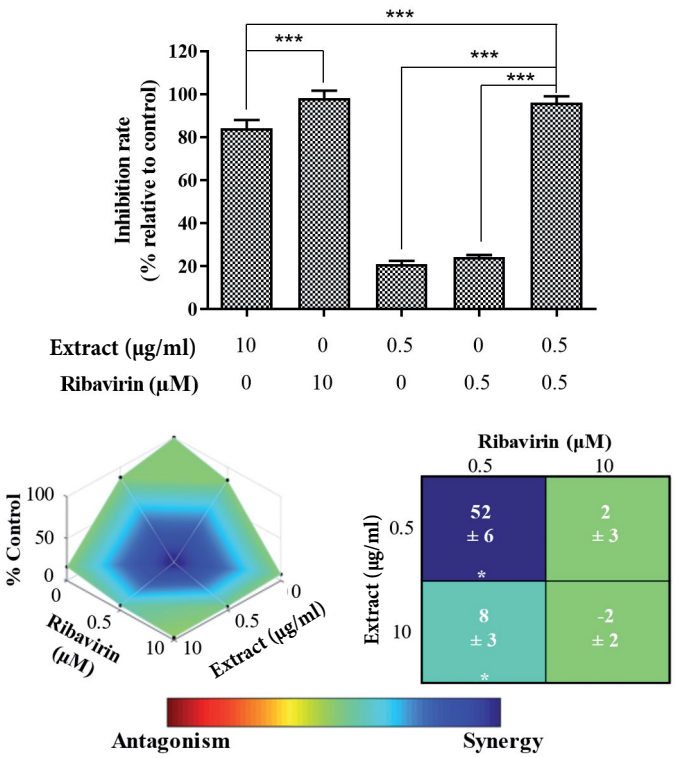

Fig. 5

Antiviral evaluation of the *B. triquetrum* extract combined with ribavirin against ZIKV replication

Inhibition rates of the different combinations according to standard plaque assays **(a)**, and synergy surface and matrix generated using the Bliss independence model within Combenefit software **(b)**. Vero cells were infected with ZIKV at an MOI of 1.0, incubated for 1 h, and subsequently treated with different concentrations of the products at different concentrations for 18 h. At 18 h.p.i, supernatants were harvested, and virus titer was determined by standard plaque assays. Data are derived from three independent assays measured in triplicate and expressed as the mean ± standard deviation (*p <0.05; ***p <0.001).

Discussion

To the best of our knowledge, only our group has reported the antiviral activity of seaweed products against the ZIKV (Pagarete *et al.*, 2021). Among them, another red seaweed, *Osmundaria obtusiloba*, has been evaluated against this virus, though another type of solvent was employed (ethanolic extract). The ethanolic extract exhibited similar results to the *B. triquetrum* extract, like a CC_{50} of 525 µg/ml and EC_{50} of 1.82 µg/ml (Cirne-Santos *et al.*, 2018). Additionally, other kinds of seaweeds have been investigated and similar extracts were assayed, but the dichloromethane extract of the brown algae *Dictyota menstrualis* inhibited only 42% of virus replication at 20 µg/ml (Cirne-Santos *et al.*, 2019). However, the dichloromethane extract of the brown algae *Canistrocarpus cervicornis* has also yielded a potent anti-ZIKV product (Cirne-Santos *et al.*, 2020) but the *B. triquetrum* extract still shows greater potency and SI.

Besides ribavirin, several repurposing campaigns have identified novel opportunities for the treatment of ZIKV infections (Song *et al.*, 2020). Interestingly, the *B. triquetrum* extract explored in this study possesses a very promising potential as a drug candidate because it exhibits a stronger or comparable antiviral activity and three to six-fold higher SI than marketed drugs with anti-ZIKV activity, such as novobiocin (EC_{50} = 9.32 µg/ml; SI = 91.26) (Yuan *et al.*, 2017), suramin (EC_{50} = 51.63 g/ml; SI = 47.74) (Albulescu *et al.*, 2017), and nitazoxanide (EC_{50} = 0.45 g/ml; SI = 52.58) (Li *et al.*, 2017).

Aside from ZIKV, algae-derived products have been investigated targeting other flaviviruses, mainly the Dengue virus. For instance, fractions or isolated polysaccharides have been obtained from different red seaweeds, such as *Gymnogongrus torulosus*, *Gymnogongrus griffithsiae,* and *Cryptonemia crenulata,* and showed potent activity against DENV 1 to 4 (Pujol *et al.*, 2012, 2002; Talarico *et al.*, 2005). However, since a less polar solvent was employed to obtain the *B. triquetrum* extract, this type of compound is not likely found in the extract evaluated herein and, thus, its activity is triggered by other metabolites.

Furthermore, different assays were performed to understand the mechanism of anti-ZIKV action of the *B. triquetrum* extract. This extract showed moderate effects on ZIKV particle stability which can contribute in parts to its antiviral activity. Also, time-of-addition and attachment assays revealed that this extract impaired drastically ZIKV replication when added at early steps of replication and had a moderate effect on virus attachment in Vero cells. Interestingly, Koishi and coworkers (2012) uncovered the inhibitory activities of dichloromethane-methanol extracts of different seaweeds (*e.g.*, *C. cervicornis*, *Padina gymnospora*, *Palisada perforata*, and *Caulerpa racemosa*) against the replication of four DENV serotypes. No significant virucidal effect was observed against DENV 4 for the extracts, except for a partial effect of the *C. cervicornis*, whereas all the extracts were also able to inhibit early steps of DENV 4 replication, probably by inhibiting binding and internalization processes (Koishi *et al.*, 2012). More interestingly, our findings showed that the *B. triquetrum* extract also acts at late stages of ZIKV replication, pointing to multiple mechanisms of action of this product, which are yet to be investigated in-depth.

Drug combinations have become useful therapeutic strategies in the treatment of some viral infections like HCV and HIV. The combination therapy allows the use of lower concentrations of the drugs, reaching similar or greater effects with lower side effects, and could avoid the emergence of resistant strains by tackling the virus

by different mechanisms of action (Chaudhuri *et al.*, 2018; Ianevski *et al.*, 2020). In this regard, we combined ribavirin, a currently marketed antiviral drug, with the *B. triquetrum* extract at suboptimal concentrations (ribavirin 0.5 μM + *B. triquetrum* extract 0.5 μg/ml), which resulted in remarkable synergistic effects with extra effects of 52% according to the Bliss model. This combination could also form a basis for future therapeutic strategies to treat ZIKV infections. Excitingly, we have previously demonstrated that this extract also possesses inhibitory activity on the Chikungunya virus replication (EC_{50} = 3.30 μg/ml; SI = 121.20) (Cirne-Santos *et al.*, 2019), pointing to it as a potential broad-spectrum antiviral agent which is highly desirable to treat infections caused by a variety of reemerging and emerging viruses (Andersen *et al.*, 2020).

In conclusion, the dichloromethane extract of the red seaweed *B. triquetrum* was proved to exhibit potent antiviral activity against ZIKV replication in Vero cells with a high selectivity index in comparison to the licensed drug ribavirin. The antiviral activity of this extract occurs through multiple mechanisms of action, including the blockage of early and late stages of the virus lifecycle, and this product also synergizes with ribavirin, which results in a more powerful inhibitory effect. Collectively, our data highlight the *B. triquetrum* extract as a promising antiviral candidate to fight Zika fever and deserves further investigation.

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