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Antiviral Activity of Marine Actinobacteria against Bovine Viral Diarrhea Virus, a Surrogate Model of the Hepatitis C Virus

Juliana Cristina Santiago Bastos[1], Cláudia Beatriz Afonso de Menezes[2], Fabiana Fantinatti-Garboggini[2], Marina Aiello Padilla[1], Clarice Weis Arns[1], Luciana Konecny Kohn[1]*

[1]Laboratory of Virology, Department of Genetics, Evolution and Bioagents, University of Campinas - UNICAMP, Cx. Postal 6109, CEP 13083-970, Campinas, SP, Brazil.

[2]Multidisciplinary Center for Biological and Agricultural Chemical Research, University of Campinas - UNICAMP, Av. Alexandre Cazelatto, 999, Betel, CEP 13148-218, Paulínia, SP, Brazil.

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***For Correspondence**

Konecny Kohn, Laboratory of Virology, Department of Genetics, Evolution and Bioagents, University of Campinas - UNICAMP, Cx. Postal 6109, CEP 13083-970, Campinas, SP, Brazil, Tel: +55-019-2139-2893

E-mail: lucianakohn@gmail.com

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ABSTRACT

The Hepatitis C virus (Flaviviridae family, *Hepacivirus* genus) represents a major public health problem worldwide and it is responsible for chronic infections in humans, which can develop to liver cirrhosis and hepatocellular carcinoma. As this virus does not replicate efficiently in cell culture and in animals, bovine viral diarrhea virus (BVDV) is used as a surrogate model for screening assays of antiviral activity, and mechanism of action assays. From marine invertebrates and their microorganisms isolated, we prepared extracts and fractions, and we isolated substances for assessment of their possible antiviral activity. Of the 71 tested, seven were considered promising presenting protection percentage of more than 80%. The best inhibition results were obtained from the extracts produced by the *Gordonia* bacteria samples with 99.9% inhibition and by Micrococcus with 99% inhibition. Furthermore, most of the extracts selected by the protection percentage showed selectivity index values considered promising, especially the extracts of the bacteria *Williansia* (SI=27) and *Brachybacterium* (SI=39). On the action mechanism, most of the promising extracts showed activity in the inhibition of intracellular replication steps, although it has been observed action of different extracts in several stages of viral replicative cycle. Thus, various extracts stood out and may lead to the development of drugs that ensure an alternative therapy for the treatment of hepatitis C.

INTRODUCTION

The bovine viral diarrhea virus (BVDV) is a member of Flaviviridae family, which is responsible for causing significant clinical diseases in both humans and animals [1]. These viruses comprise a single strand of positive sense RNA and membranous envelope [2].

The BVDV virus shares similarities as biology and genetic organization with the hepatitis C virus [3]. They share a high degree of homology in terms of genome organization and strategies of replication and protein expression [4].

Due to these similarities with HCV and it is easy to *in vitro* culture, BVDV is an effective surrogate virus model for identifying new drugs with potential antiviral activity against HCV and its mechanism of action [5-9].

The HCV infection is a major cause of human hepatitis worldwide and the World Health Organization estimates that virus chronically infected about 170 million people [10-11]. These patients usually do not show clinical symptoms until they evolve into serious cases [12]. This represents a major public health problem worldwide, because patients with chronic HCV infection present a high risk of developing liver cirrhosis and even hepatocellular carcinoma [13].

Given the lack of available vaccine and an effective treatment, it is necessary to find new compounds that can be used as antiviral and may represent an alternative to the existing treatment. The search for new drugs from natural resources has become a global trend [14]. In recent years, researches involving marine organisms have significantly contributed to the discovery of new potentially bioactive substances for therapeutic purposes [15]. These studies showed that marine invertebrates belonging to the Porifera, Mollusca, Cnidaria, Anthozoa, Echinodermata and Bryozoa families are rich sources of various bioactive metabolites [16,17]. However, evidence suggests that in many cases the active compounds isolated from them are from symbiotic microorganisms [16,18].

MATERIALS AND METHODS

Bacterial strains

Strains used in this study are preserved in the Brazilian Collection of Industrial and Environmental Microorganisms (CBMAI, CPQBA, UNICAMP, Campinas, SP, Brazil). The isolates were recovered from nine samples of marine macro organisms, including the sponges *Amphimedon viridis* (AV); *Axinella corrugata* (AC); *Dragmacidon reticulata* (DR); *Geodia corticostylifera* (GC); *Mycale laxissima* (ML) and *Mycale angulosa* (MA); the *ascidians Didemnum ligulum* (DL) and *Didemnum* sp. (DSP), and the algae *Sargassum* sp. (AS). The macro organisms were collected in January 2007, in beach areas named Praia Guaecá (23149S; 45125W), Ilha Toque-Toque (23151S; 45131W) and Ilhota da Prainha (23151S; 45124W), in São Sebastião, São Paulo, Brazil, at depths between 5 and 10 m. Bacterial strains were isolated from triturated samples after it were sterilized with 0.001 g/L mercury chloride in 5% ethanol and then washed twice with sterilized seawater. Aliquots of 100 µl (10^{-2}, 10^{-4} and 10^{-6}) were inoculated onto Petri dishes containing one of the bacterial specific media described below. Four culture media supplemented with cycloheximide (50 µg/ml) were used for bacteria isolation: GPY, TSA, M1 (soluble starch 10 g/L, yeast extract 4 g/L, peptone 2 g/L, agar 15 g/L) and Marine Agar (Difco™, USA). All media were prepared with artificial sea water (ASW): KBr 0.1 g/L, NaCl 23.48 g/L, $MgCl_2 \times 6H_2O$ 10.61 g/L, $CaCl_2 \times 2H_2O$ 1.47 g/L, KCl 0.66 g/L, $SrCl_2 \times 6H_2O$ 0.04 g/L, Na_2SO_4 3.92 g/L, $NaHCO_3$ 0.19 g/L, H_3BO_3 0.03 g/L. Agar plates were inoculated and incubated at 25°C for 1-4 weeks. Isolation of colonies was conducted from the 2nd to the 30th day of plating. Pure cultures were obtained after serial transfers to the same culture medium used to plate the macroorganism samples. The maintenance of the strains was performed in glycerol 20% at -80°C.

Crude Extracts

Extracts derived from the metabolism of the actinobacteria strain were obtained by the liquid-liquid separation method. A pre-inoculum of the isolates was cultured in 7 ml of Nutrient Broth (Oxoid) and was incubated at 30°C for 48 h at 100 rpm. After culture growth, the total volume was transferred to an Erlenmeyer flask containing 50 ml of the Nutrient Broth (Oxoid) and was kept at the same temperature and rotation for 24 h. Again, 50 ml of bacterial growth were transferred to a glass jar containing 500 ml of the same medium and were kept in the same conditions mentioned for three weeks. After this period, 500 ml of ethyl acetate were added and the mixture was triturated in a blender with high rotation. Then, the mixture was stirred at 100 rpm for 24 h. Initially, the mixture was filtered on a Buchner funnel containing a pad of celite and then the organic phase was recovered in an Erlenmeyer flask using a separating funnel. In the aqueous phase, the cell debris and the culture medium were retained, and in the organic phase, the possible biologically active metabolites were recovered.

The organic phase was then filtered through cotton and was transferred to a round bottom flask. Extracts contained in the organic phase were concentrated in a rotaevaporador (BuchiRotavapor R-215) in vacuum at 37°C until complete solvent drying. Then the extracts were suspended in methyl alcohol, filtered with cotton, and the volume transferred to new tubes glass using Pasteur pipette. These tubes were led to Savant vacuum centrifuge; model 210A Speedvac® Plus SC for evaporation. After complete solvent drying, the extracts were dissolved in 10% DMSO in medium AMH and were kept at 4°C until use in the antiviral activity assays.

Antiviral Activity Assay

Virus and cell lines

The Madin–Darby bovine kidney (MDBK) cell was propagated in monolayer cultures using minimal essential medium (MEM) with Earle's salts, supplemented with 10% equine serum. Cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures using 0.05% trypsin with 0.02% EDTA. The viral strain used was the BVDV Singer, a cytopathic BVDV-1 strain.

Cell cytotoxic effect

An aliquot of 100 µl of suspension per well was seeded in 96-well culture plates at a density of 1×10^5 cells/ml. The microtiter plates containing cells were pre-incubated for 24 h at 37°C to allow stabilizations prior to addition (100 µl) of samples at 11 concentrations (500 to 0.48 µg/ml). The maximum nontoxic concentration (MNTC) was determined microscopically by the observation of morphological changes of cells at 24, 48 and 72 h of incubation, and after 72 h the results were obtained with MTT assay.

MTT assay

The MTT assay is a sensitive *in vitro* assay for the measurement of cell proliferation or a reduction in cell viability. Cells were cultured in 96-well tissue culture plates. The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium

bromide) was added to the wells and the cells were incubated. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. Dimethylsulfoxide was then added to the wells, solubilizing the crystals so the absorbance could be read using a spectrophotometer on absorbance of 540 nm [19,20]. The data was analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell proliferation. The tetrazolium reduction rate is proportional to the cell proliferation rate and indirectly indicates the reduction in cell viability caused by the virus action.

Titration of viruses

The cells were seeded in 96-well culture plates at a density of 1×10^5 cells/ml and then incubated at 37°C in a humidified atmosphere containing CO_2 for 24 h. Serial dilutions of virus stocks were prepared and cells were infected with the dilution of virus. After an additional incubation (1–2 days), the cytopathic effect was recorded. The 50% tissue-culture infective dose (TCID50) per ml was calculated as described previously by Reed and Münch [21].

Antiviral activity

Determination of antiviral activity was based on cytopathic effect inhibition. All experiments were performed in quadruplicate. Briefly, for evaluation of inhibition, the cells were seeded in 96-well culture plates. After 24 h of incubation, the medium was replaced with 100 µl of MEM(E) containing the extracts at 50 µg/ml, and 50 µl of 100 TCID50/50 µl of viruses were added in quadruplicate and incubated for 3 days. Controls consisted of untreated infected (virus at 100 TCID50/50 µl), treated no infected (extract control), and untreated no infected. The cytopathic effect was observed after 72 h and the extracts with antiviral activity were determinate. For quantification of this activity, we performed the MTT assay. This assay allows the quantification of cell viability and indirectly allows quantification of cell extract protection against the virus.

The protection percentage was calculated using the following formula (29): (Absorbance of treated – absorbance of the control virus)/(Absorbance of the cellular control - absorbance of the control virus) × 100 [22].

The antiviral activity test was evaluated initially with a single dose of MNTC against 100 TCID50/ml of virus. The extracts with activity greater than 80% were considered promising.

To confirm activity, a concentration response curve with different concentrations of extract in the presence of 100 TCID50/ml of virus was determined by MTT assay, to determine antiviral concentration at 50% (EC50).

The EC50 was calculated from concentration-effect curves after linear regression analysis. The results were obtained from triplicate assays with at least five extract concentrations. The percentage of cytotoxicity was calculated as [(A – B)/A] × 100, where A and B are the OD540 of untreated and of treated cells, respectively. The percentages of protection were calculated as [(A − B) × 100/(C − B)], where A, B and C indicate the absorbance of the extracts, virus and cell controls, respectively. Each obtained EC50 value was defined as the effective concentration that reduced the absorbance of infected cells to 50% when compared with cell and virus controls. The 50% inhibition (IC50) for each compound was obtained from dose-effect-curves (not shown) generated by plaque assay after linear regression analysis. The EC50 and IC50 are the average of three assays with five concentrations within the compounds inhibitory range. The therapeutic index (i.e., selective index) was defined as EC50/IC50.

The potential stage in the viral infection cycle

Cells and viruses were incubated with active extracts at different stages during the viral infection cycle in order to determine the mode of antiviral action. Cells were pretreated with samples before viral infection; viruses were incubated with samples before infecting the cells; and the cells were infected with the virus and incubated before the samples addition. Samples were used at the maximum no cytotoxic concentration. Subsequent assays were performed to differentiate the active extracts in the pretreatment between adsorption and penetration.

Values were expressed as titer (TCID50/µl) and inhibition percentage (IP) as described in [23]. The inhibition percentage was calculated by the formula: (IP) = (1 – T/C) × 100, where T is the antilog of the extract-treated viral titers and C is the antilog of the control (without extract) viral titers. IP was considered positive at greater than or equal to 98%.

Virucidal action

In order to evaluate possible extracellular viral inactivation by extracts, equal volumes (100 µl) of the 10-fold serially diluted virus suspension and MNTC of the extracts were mixed and were incubated for 1 h at 37°C. Each mixture was then added to the cell monolayer, and infectious titers were compared with controls.

Statistical analysis

The results were expressed as mean ± S.E.M. The selectivity index (SI) was determined by the ratio of CC50 to EC50. The statistically different effects of tested extracts on the inhibition of virus replication were compared with the control group using the Student's t-test with $p \leq 0.05$ for significant result.

Bacteria identification by 16S rRNA gene sequencing analysis

The genomic DNA was obtained by the extraction method described by Pospiech and Neumann [24]; 16S ribosomal RNA gene was amplified by PCR using the primers p27f and p1401r [25] for universal Bacteria Domain. Fifty microliter reaction mixtures contained 0.4 µM of each primer, 0.2 mM of dNTP (GE Health Care) mix, 2 U Taq DNA polymerase (Invitrogen®), and approximately 100 ng genomic DNA. The Polymerase Chain Reaction (PCR) amplifications were done using an initial denaturation step of 2 min at 94ºC, followed by 10 cycles of 1 min at 94ºC, 30 s at 62 and 57ºC (0.5°C decrease at each cycle), and 2 min at 72ºC for extension, followed by another 20 cycles of 1 min at 94ºC, 2 min at 57ºC and 2 min at 72ºC, in an Eppendorf thermal cycler. The PCR DNA product was purified on GFX™ PCR DNA and a Gel Band Purification kit (GE HealthCare) was used for automated sequencing in the MegaBace DNA AnalysisSystem 1000 using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE HealthCare), according to the manufacturer's recommendations. The sequencing was carried out using the 10f (5´ GAG TTT GAT CCT GGC TCA G3´); 765f (5´ATT AGA TAC CCT GGT AG3´); 782r (5´ACC AGG GTATCT AAT CCT GT3´) and 1100r (5´AGG GTT GGG GTG GTT G 3´) primers. 16S ribosomal RNA gene partial sequences obtained from isolates were assembled in a contig using the phred/Phrap/CONSED program [26,27].

Identification was achieved by comparing the contiguous 16S ribosomal RNA gene sequences obtained with 16S ribosomal RNA sequence data from reference and type strains available in the database GenBank and RDP (Ribosomal Database Project, Wisconsin, USA) using the BLASTn and RDP sequence match routines, respectively. The sequences were aligned using the CLUSTAL X program [28] and analyzed with MEGA software [29]. Evolutionary distances were derived from sequence-pair dissimilarities calculated as implemented in MEGA, using Kimura's DNA substitution model [30]. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm [31], with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software. The sequences obtained were deposited in GenBank.

RESULTS

Antiviral activity

Antiviral activity was assessed initially using a standard titration assay, wherein the actinobacteria extracts, at nontoxic concentrations, were added to the cell monolayer simultaneously to the virus, in order to select those with some antiviral activity against the BVDV virus. Among the 71 actinobacteria extracts tested, seven had potential activity against the BVDV virus, which corresponds to approximately 9.8%.

The extracts considered potentially active were obtained from actinobacteria isolated from different macro organisms. **Table 1** summarizes the information regarding these extracts, including their origin, microorganism identification and the protection percentage presented in the initial tests. Among them, the best activities against the BVDV virus were obtained with the extracts B204 (99.9%) and B232 (99.0%), extract from the Gordonia and Micrococcus bacteria, respectively.

Table 1. Identification and protection percentage presented by potentially active extracts

Extract	Source[a]	Culture conditions[b]	Identification (Genbank Number)[c]	Acronym in the culture collection[d]	Protection percentage (%)[e]
B232	Didemnun sp.(DSP)	GPY	*Micrococcus* sp.	-	99.0
B137	Amphimedon viridis (AV)	TSA	*Williansia* sp.	-	91.0
B204	Didemnun sp.(DSP)	M1	*Gordonia* sp. (JN615417)	CBMAI 1069	99.9
B255	Axinella corrugate (AC)	M1	*Janibacter* sp. (JN615420)	CBMAI 1072	93.0
B373	Geodia corticostylifera (GC)	M1	*Brachybacterium* sp. (JN615436)	CBMAI 1088	86.0
B149	Mycale angulosa (MA)	TSA	*Micrococcus* sp.	-	86.0
B177	Sargassum sp.(AS)	TSA	*Nocardioides* sp. (JN615452)	CBMAI 1107	81.0

[a]Source=macro organism from which the actinobacteria was isolated; [b]Culture conditions=medium used in the isolation and production of the extract; [c]Molecular identification based on sequence analysis of 16S ribosomal RNA gene and number of the sequence in the GenBank database; [d]Acronym in the Brazilian Collection of Industrial and Environmental Microorganisms - CBMAI; [e]Protection percentage of values refer to the results found with the concentration of 50 mg/ml of the extract against 100 TCID50/50 ml of quantified virus by the MTT colorimetric assay. Values in bold correspond to the active extracts (PP>98%)

Values regarding CC50 (extract concentration capable of reducing 50% cell growth), IC50 (extract concentration able to inhibit 50% viral replication) and resulting values of the ratio between them, corresponding to the selectivity index (SI), a value that determines the compound activity without causing damage to cell viability, were evaluated in this study **(Table 2).** Among the extracts evaluated, most of them showed selectivity index higher than four show, and therefore, are considered potentially active. The extracts B137 and B373 showed SI values considerably high, SI=27 and SI=39, respectively, when compared to the other's extracts values. The extracts B232 and B177 did not show the minimum value of selectivity index, resulting in SI=2.9 and SI=2.2, respectively **(Table 2).**

Table 2. Values of CC50, IC50 and SI of active and promising extracts

Extract	MNTC[a] (µg/ml)	CC_{50}[b] (µg/ml)	IC_{50}[c] (µg/ml)	SI[d]
B232	125	144	49	2.9
B137	500	918	34	27
B204	62.5	95	12	8
B255	125	78.25	14.6	5.35
B373	50	175	4.5	39
B149	125	187	30	6.2
B177	62.5	104	47	2.2

[a]MNCT: Maximum Nontoxic Concentration; [b]CC_{50}: extract concentration able to reduce 50% cell growth; [c]IC_{50}: extract concentration able to inhibit 50 % viral replication; [d]SI: selectivity index.

Activity of the extract in the viral replication cycle

The action mechanism tests indicate in what viral cycle stage the extract shows activity. Based on this, the extracts activity assays were conducted in three stages of the viral cycle either acting directly on the virus particle during the adsorption and/or penetration, or in the intracellular stages of replicative cycle. The results revealed different action mechanisms for the various extracts evaluated. The extracts B204, B616 and B137 acted by preventing adsorption, penetration and production of new viral particles. However, the extracts B232, B255 and B177 were able to act during the virus replicative cycle, preventing this to occur normally and preventing this to generate the same amount of new viral particles. Therefore, by interfering in the various replicative cycle stages, the active substances were able to prevent the infection progression. Finally, the extract B173 acted directly on the viral particle, inactivating it.

From the Viral Inhibition Index values (VII) and Inhibition Percentage (IP) calculated in these tests, the extracts were classified as active (VII ≥ 1.5 and IP ≥ 98%) or promising (VII between 1.0 and 1.5; IP between 90 to 97%). The extracts B204, B616, B137, B232 and B373 were considered promising, because they had inhibition percentage between 90 and 97.

Table 3. Results of preliminary screening tests of action mechanism

Extract	[a]VII	[b]IP%	[c]Stage of viral cycle
B204	1.5	97%	Pretreatment: Adsorption
B616	1.0	90%	
B137	1.5	97%	Pretreatment: Penetration
B232	1.0	90%	Intracellular replication stages
B255	0.75	83%	
B177	0.75	83 %	
B373	1.0	90 %	Viral inactivation

[a]VII=Viral inhibition index; [b]IP=Inhibition percentage; [c]Stage in which the extract promoted greater activity against the virus, by reducing viral titer: pretreatment (adsorption or penetration) intracellular stages of the replicative cycle or viral inactivation

DISCUSSION

Diseases caused by viral pathogens, both in humans and in animals, are a major economic and social impact and highlight the need to develop new drugs, due to the virus increased resistance to available treatments as well as the emergence and/or identification of new viruses.

The marine environment exploitation is a promising strategy in the search for active compounds against infectious diseases [32]. In addition to their peculiar structures, marine natural products have an extraordinary diversity of molecular targets with remarkable selectivity, as ion channels, enzymes, microtubules, DNA, lysosomes, calmodulin, proteasomes and oxidative stress induction and immune system modulation. This greatly increases the pharmacological and therapeutic potential of these molecules [33].

The discovery of new drugs, and the adaptation of those existing, plays a key role against viral drug resistance. Hundreds of compounds have been isolated from marine organisms and evaluated as to their pharmacological properties. Some of them are already available commercially, such as acyclovir, vidarabine, cytarabine and zidovudine. Many compounds are in preclinical and clinical testing (avarol and cyanovirin-N). However, few were obtained from marine bacteria environment, including EPS-1 and EPS-2, isolated from Bacillus licheniformis and Geobacillus thermodenitrificans, respectively, and Macrolactin A, isolated from deep sea bacteria. Nevertheless, there are several isolated compounds from algae and marine sponges with antiviral activity against AIDS, HSV, herpesvirus [32]. In this study, through antiviral activity tests against BVDV, seven isolated actinobacteria extracts from marine organisms were considered potentially promising, corresponding to 9.8% of the total analyzed extracts. These extracts were considered potentially promising because they showed cell protection to over 80% against the virus at a 50 µg/ml concentration **(Table 1).** However, this activity can be increased in the tests by using the maximum non-toxic concentration of each extract. Furthermore, in this study we tested actinobacteria crude extracts and the active substances isolation can enhance the activity against BVDV. Thus, these extracts showed a great potential for identifying active substances against the viruses.

Recent researches have led to the isolation of new actinobacteria from different marine environments. They produce different types of new secondary metabolites, many of which have biological activities and have the potential to new therapeutic agent development. Marine actinobacteria are, therefore, an abundant source, yet still little explored for secondary metabolites discovery of therapeutic interest [34]. We considered potentially active, according to the protection percentage, presented in colorimetric assay, the extracts produced from colonies of Micrococcus (B232–99% and B149–86%), Williansia (B137–91%); Gordonia (B204–99.9%); Janibacter (B255 75:25 – 93%); Brachybacterium (B373 – 86%) and Nocardioides (B177 – 81%). Of these, the best activities were obtained from Micrococcus (99%) and Gordonia (99.9%) samples. These microorganisms are rarely analyzed for their biological activities.

To all these extracts we determined the value regarding MNTC **(Table 2).** The MNTC values are the maximum concentrations in which the extracts can be used without causing damage to the cell viability. This stage is crucial in the assessment of an extracts biological activity, since the virus uses the cell to replicate, therefore, it should be ensured that they find the proper condition to their multiplication [14]. Therefore, the promising compounds regarding the antiviral activity should have activity on the viability and/or viral replication, without interfering with the cell physiology. Among the crude extracts tested in this work, the B137 had the highest MNTC value (500 µg/ml); therefore, this extract had no toxicity even when used at high concentrations.

Values of CC50, IC50 and the Selectivity Index were calculated for each extract, and the SI value equal or higher than 4 was considered viable for antiviral activity. The extracts B137, B204, B255, B373 and B149 showed SI higher than 4 **(Table 2).**

We also carried out preliminary tests for the action mechanism of the various extracts against BVDV. These tests indicate in which stage the extract shows activity against the virus either acting directly on the viral particle, during adsorption, penetration or in intracellular stages of the replicative cycle.

At this stage, we calculated the inhibition percentages presented by each extract in the maximum activity stage. The extracts B204 and B137 showed IP equal to 97%; B616, B232 and B373 showed IP equal to 90% and the others showed IP equal to 83%. Considering that in the other stages the extracts also showed some activity, the protection percentage of initial value differs to this value, because this calculation takes into account only the stage where there was the highest activity (the highest IP), and calculating the protection percentage, the entire activity was considered simultaneously.

Three extracts were active in the pretreatment tests, two of which, the extracts B204 and B616, acted during viral adsorption and the extract B137 acted during penetration. By preventing BVDV adsorption or penetration, the extract is able to prevent any virus replicative cycle, since these are initial stages, without which the viral particle is not capable of replicating itself within the cell and producing new viral particles. This ensures treatment effectiveness, since to infect new cells viral particles are continuously released after infection. The active substances are, therefore, capable of intervening in the infection progression.

The extracts B232, B255 and B177 were able to act during the intracellular stages of the BVDV replicative cycle. They may, therefore have interfered in the nucleocapsid coating, in the RNA translation, or in the various stages involved in the new viral particles formation. By working in different intracellular stages of the replicative cycle, the active substances are able to prevent the infection progression.

The extract B373 acted directly on the viral particle, thus preventing the adsorption, penetration and production of new viral particles. If these extract active substances are able to remain bioavailability in the bloodstream for an adequate period, they are also capable of preventing the infection progression, as they may prevent the released viral particles, and these are no longer able to adsorb, penetrate the cell and replicate.

In this research phase, the extracts were tested on their maximum nontoxic concentrations, and from the titers found, we determined the viral inhibition index and the inhibition percentage. The inhibition percentage showed different values of the protection percentage presented in the initial tests. This was due to the use of different concentrations in the two tests as well as the use of different methodologies to ensure the objectives of each stage of this research.

These extracts, therefore, represent an important source for drugs development, besides the activities described above, they showed a great ability to inhibit the BVDV action and, from that, we can infer based on previous researches that they are also capable of inhibiting HCV activity, which may be demonstrated by specific tests for this virus.

Further studies are needed to reach the active substances in each of these extracts and to identify in detail their performance. The other extracts considered promising should also go through substances fractionation and isolation procedures so this activity potentiating can be checked. All of them showed their potential to develop an alternative therapy for treating an infection so important in the world.

CONCLUSION

Of the isolated microorganisms from marine organisms, we produced extracts containing compounds capable of inhibiting the BVDV action, model for HCV. Of 71 tested extracts, seven were selected. Most of them had selectivity index higher than four,

which determines their viability to continue the experiments. These extracts were capable of operating in several stages involved in viral replication, and 42.8% were active in the pretreatment tests; 42.8% were active in post-treatment tests and 14.2% were active by viral particle inactivation. Thus, in several ways, they have proved capable of inhibiting the infection progression.

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