

## In vitro antiviral activity of *Rhizophora mangle* L. aqueous bark extract and the butanolic fraction against Canine Distemper Virus and Bovine Herpes Virus type 1

### Actividad antiviral *in vitro* del extracto acuoso de corteza de *Rhizophora mangle* L. y la fracción butanólica contra el virus del Moquillo Canino y el virus del Herpes Bovino Tipo 1

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**ABSTRACT:** There is currently an increasing need for new substances or secondary metabolites from natural products with antiviral activity. The aim of this study was to determine the antiviral activity of *Rhizophora mangle* L. against DNA and RNA virus. With this aim, *R. mangle* aqueous bark extract (RMABE) and its butanolic fraction (BF-RMABE) were evaluated for the cytotoxicity and cytopathic effect inhibition assay. The selectivity index was also calculated. An *in vitro* antiviral activity was demonstrated for RMABE and BF-RMABE against the RNA prototype of Canine Distemper Virus (CDV) (CI<sub>50</sub> 83.30 and 86.10 µg/mL, respectively) and the DNA prototype of Bovine Herpes Virus type 1 (BHV-1) (CI<sub>50</sub> 87.50 and 90.63 µg/mL, respectively). The results showed the promising antiviral effect of the evaluated *R. mangle* extracts, suggesting the need for further tests to confirm its *in vivo* activity.

**Key words:** *in vitro* antiviral activity, CDV, VHB-1, polyphenols, *Rhizophora mangle*.

**RESUMEN:** Actualmente existe una creciente necesidad de nuevas sustancias o metabolitos secundarios a partir de productos naturales con actividad antiviral. El objetivo de este estudio fue determinar la actividad antiviral de *Rhizophora mangle* L. frente a virus prototipos de ADN y ARN. En este sentido, se evaluó el extracto acuoso de corteza de *R. mangle* (RMABE) y su fracción butanólica (BF-RMABE) mediante las pruebas de citotoxicidad e inhibición del efecto citopático. De la relación entre estas pruebas se calculó el índice de selectividad. Se demostró, además, actividad antiviral *in vitro* para RMABE y BF-RMABE frente al virus del moquillo canino (CDV) como prototipo de virus ARN (CI<sub>50</sub> 83,30 y 86,10 µg/mL, respectivamente) y el Herpes Virus Bovino tipo 1 (BHV-1) como prototipo de ADN (CI<sub>50</sub> 87,50 y 90,63 µg/mL, respectivamente). Los extractos de *R. mangle* evaluados, mostraron efecto antiviral, lo que sugiere la necesidad de pruebas adicionales para confirmar su actividad *in vivo*.

**Palabras clave:** actividad antiviral *in vitro*, CDV, BHV-1, polifenoles, *Rhizophora mangle*.

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## INTRODUCTION

Plants are a natural source of therapeutically recognized compounds with diverse biological activities offering treatment for several human and animal diseases. Viral infections represent an important research focus for the identification of new phytopharmaceuticals (1).

The discovery of antivirals is a relatively recent issue. At present, the antiviral therapy is mostly limited to human diseases with few applications in veterinary medicine. Currently the research carried out on antivirals against animal viruses is mostly aimed at providing new insights for the development of chemotherapeutics against phylogenetically correlated human viruses. There is in fact an increasing interest to the animals and their pathologies as possible models for human diseases. In the veterinary field, antivirals may represent an alternative when vaccination is not feasible in case of sudden epidemic outbreaks and they would offer advantages when in-contact animals need protection during emergency vaccinations. Although the primary focus has been on the synthetic products, the number of natural compounds with antiviral action for different DNA and RNA viruses that are being studied is increasing (2).

Tannins are secondary metabolites characterized by their polyphenol nature and they can be found in many plants as individual components or forming complex mixtures (3). There is a wide range of the pharmacological activity of the plants containing polyphenols as reported by Keerthi *et al.* (4). The wide biological activities of polyphenols have been related to their ability to bind macromolecules such as proteins and polysaccharides (5,6,7).

The antioxidant and radical scavenging activities of tannins are often linked to the interaction with metal ions, forming chelated complexes (8). The antiviral activity of the phenolic compounds against several DNA and/or RNA viruses has been well documented by Chattopadhyay *et al.* (9), Bhanuprakash *et al.*

(10), Elsebai *et al.* (11). Thus, Gamaleldin *et al.* (12), reported that plants, rich in chlorogenic acids (CGAs), caffeic acids and their derivatives, have been found to exert the antiviral effects against influenza virus neuraminidase. The crude and aqueous extract and the ethyl acetate fractions of *Trichilia catigua* (a Brazilian native plant) have been reported to exert the antiviral activity during the replication of the Herpes Simplex Virus (HSV-1), BHV-1 and Poliovirus (PV-1), which are responsible for important diseases in humans and animals (13).

The phenolic compound eugenin (ellagitannin), extracted from *Geum japonicum* and *Syzygium aromaticum*, clearly demonstrated the anti-HSV activity by decreasing the DNA polymerase activity (14).

In a systematic study of the antiviral activity of a very wide range of natural products, Van den Berghe *et al.* (15) concluded that polyphenols act principally by binding the virus and/or the protein of the host cell membrane being able to inhibit the absorption of several viruses. Further researches demonstrated the capability of polyphenols to inactivate proteins from the cell surface, to block the viral adsorption and to inhibit the viral enzymes such as reverse transcriptase and RNA polymerase and poly (ADP-ribose) (16).

Steinmann *et al.* (17) reported the antiviral activity of epigallocatechin-3-gallate (EGCG), the main constituent of *Camellia sinensis* (green tea), showing different mechanisms of action on diverse families of viruses, such as *Retroviridae*, *Orthomyxoviridae* and *Flaviviridae*, as well as important human pathogens such as the human immunodeficiency, influenza A and hepatitis C viruses. Furthermore, the molecule demonstrated to be able to interfere with the replication cycle of DNA viruses like hepatitis B virus, herpes simplex virus and adenovirus.

Another example of the antiviral effect of polyphenols is that of bakuchiola phenolic isoprenoid with novel enantiomer-selective anti-influenza A virus activity involving Nrf2

activation. Compounds like (+)-(S)-bakuchiol and its enantiomer, (-)-(R)-bakuchiol, inhibit influenza A viral infection and growth and reduce the expression of viral mRNAs and proteins in these cells. Furthermore, these compounds markedly reduced the mRNA expression of the host cell influenza A virus-induced immune response genes, interferon- $\beta$  and myxovirus-resistant protein 1. Interestingly, (+)-(S)-bakuchiol had a greater efficacy than (-)-(R)-bakuchiol, indicating that chirality influenced the anti-influenza virus activity (18).

*Rhizophora mangle* L, red mangrove, is recognized in the Cuban traditional medicine and in different Caribbean countries as well (19). Its aqueous bark extract showed to be rich in polyphenols (20). *R. mangle* revealed interesting pharmacological effects with therapeutic potentiality such as: antimicrobial (21), wound healing promoter (22), anti-inflammatory (23), in the treatment of aphthous ulcer (24), antioxidant (25), and antiulcerogenic (26). The chemical composition of *Rhizophora mangle* aqueous bark extract and its fractions were also studied in previous researches (26,27).

It is worth mentioning that polyphenols may modulate the production of IL-2 and interferon gamma (IFN-gamma). Thus following that logic, it was confirmed that the structural diversity of polyphenols present in *Rhizophora mangle* L has a capacity to stimulate the release of IL-2, indicating that such polyphenols play an important role in the biological regulations (27).

Orf, commonly referred as contagious ecthyma, is a highly contagious viral disease that causes important economic losses in sheep and goats, being one of the most skin distressing diseases affecting these species. The topical application of *Rhizophora mangle* aqueous bark extract on lambs experimentally infected with Orf virus shows a faster healing than in the treated group (28).

The current work researched the *in vitro* antiviral activity of *Rhizophora mangle*

aqueous bark extract (RMABE) and its butanolic fraction (BF-RMABE) against BHV-1 and CDV as prototypes of DNA and RNA viruses respectively.

## MATERIALS AND METHODS

### *Rhizophora mangle* aqueous bark extract (RMABE) and its butanolic fraction (BF-RMABE)

Fresh bark in distilled water (1:7.5 w/v) was boiled for 20 min. The plant material was separated by filtration and the supernatant (RMABE) was freeze-dried and preserved at 4-8°C.

BF-RMABE fractionation was carried out with n-butanol as follow: 50 mL of distilled water and 18 g NaCl were added to 1g of RMABE. The solution was stirred, heated (78°C) for 30 minutes and centrifuged at 3000 rpm for 10 minutes. The precipitate was then extracted with n-butanol using Büchi B 811 Extraction System (upper heating 4, lower heating 16, 2:00 hours) and dried by rotary evaporation (55°C).

### Cells and viruses

VERO cells (culture collection ATCC® number CCL-81™) and MDBK cells (culture collection ATCC® number CCL-22) were used for the *in vitro* growth of canine distemper virus (CDV) and bovine herpes virus- 1 (BHV-1) respectively. Cells were cultured at 37 °C in a 5 % CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium (Gibco, Invitrogen Corporation) supplemented with 10 % fetal calf serum (FCS), 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate and 7.5 % sodium bicarbonate. All the cells, medium components and virus strains were previously tested for the absence of *Mycoplasma* spp. and *Bovine Viral Diarrhea virus* (BVDV) by PCR assays. Bussell-CDV strain and BoHV-1 have been propagated in cells using a 2 % FCS medium and they were subsequently titrated. The viral titer was expressed as the 50 % tissue culture infectious dose (TCID<sub>50</sub>/mL).

### Cytotoxicity assay

The cell toxicity of RMABE, BF-RMABE, ribavirin (RBV) and HPMPC (S-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine was evaluated on the growing cell lines VERO and MDBK using a colorimetric assay based on the mitochondria metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Missouri). The toxicity of the compounds and DMSO used to dissolve them was measured as previously described by Scagliarini *et al.* (29). The 50 % cytotoxic concentration (CC<sub>50</sub>) was defined as the compound concentration able to reduce the number of viable cells to 50 % of cell control. The CC<sub>50</sub> values were expressed as the mean  $\pm$  the standard deviation of at least three independent experiments.

### Antiviral assays

The intracellular activity of the plant extracts and positive controls 1- $\beta$ -D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin, RBV) and (S)-1-(3-Hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC, Cidofovir, Vistide) against animal viruses was evaluated by the cytopathic effect (CPE) reduction assay (CPE-reduction assay) on confluent cells, using the protocol previously described by Scagliarini *et al.* (29).

### Statistical analysis

The selectivity index (SI) was obtained by calculating the ratio between the cytotoxicity and the antiviral activity values.

The 50 % inhibitory concentration (IC<sub>50</sub>), defined as the compound concentration required to reduce viral CPE by 50 % of the virus control, was calculated as the mean of three independent experiments  $\pm$  the standard deviation.

## RESULTS AND DISCUSSION

The cytotoxicity assays allowed determining the cytotoxic concentration (CC<sub>50</sub>) of RMABE and BF-RMABE in Vero

and MDBK cells (Table 1). The cytotoxicity assessment is clearly an important part of a potential antiviral agent evaluation because a useful compound should be selective for virus-specific processes with no or few effects on cellular metabolism and not showing toxicity against the host (30,31). Additionally, this step is critical because the virus uses the cell machinery to replicate, and it must be ensured that the virus has the ideal conditions for growth (32).

The present results showed relatively low CC<sub>50</sub> values for RMABE and BF-RMABE in Vero and MDBK cells compared to the control compounds HPMPC and RBV. These results are in accordance with previous results obtained with *Sesbania virgata* extracts showing CC<sub>50</sub> values between (3.91 and 250  $\mu$ g/mL) and (15.62 y 125  $\mu$ g/mL) for MDBK and Vero cells respectively. The polar nature of the active ingredient(s) in the antiviral activity of this plant was suggested (33).

Ruibal *et al.* (34) found high cytotoxicity for different polyphenolic fractions obtained from *Pinus caribaea* Morelet var. *caribaea* in MT4 cells. Fractions containing condensed tannins showed CC<sub>50</sub> = 100  $\mu$ g/mL; while low molecular weight phenolics were highly toxic to the cells. In this sense, it was previously demonstrated that the presence of hydroxyl groups is associated to the increment of intracellular reactive oxygen species (ROS), with consequences in the damage of the cells, although the relationship among compounds with similar structures and cytotoxicity is still unknown for particular types of cells (35).

Therefore, a *Phyllanthus orbicularis* extract was evaluated using MTT assay and cytotoxicity was determined for MDBK, FPH, HeLa and HEp-2 cells, showing CC<sub>50</sub> values of 262.05 $\pm$ 12.7, 669 $\pm$ 36, 212.62 $\pm$ 8.37 and 144.7 $\pm$ 3.7  $\mu$ g/mL, respectively (36).

However, compounds containing hydroxyl groups can interact with iron, copper and other metals usually present in culture media and fetal bovine sera, intensifying MTT reduction and increasing blue coloration. Some

compounds can react with iron, generating blue-black complexes that may influence the cytotoxicity estimation through MTT assay (30).

The results obtained in the *in vitro* antiviral assay are shown in Table 1.

Thus, current results showed antiviral activity of RMABE and BF-RMABE against BHV-1 and CDV in MDBK and Vero cells, respectively. Nevertheless, these preliminary results did not show differences between both types of viruses and IC<sub>50</sub> values were found between 83.30 and 90.63 µg/mL. To screen the antiviral activity of plant extracts, it is important to choose animal viruses containing the main differentiating features of viral morphology, like viruses with no envelope, those with an envelope, as well RNA and DNA viruses (32). In the current work, a prototype DNA virus (BHV-1) and an RNA virus, both enveloped, were used. This caused severe animal diseases. Particularly, CDV which is a Morbillivirus, is identified as an attractive target in the development of antiviral molecules, because human and animal tissues lack of a known homologue of the RNA-dependent RNA polymerase (33).

The current experiment also suggested that the evaluated extracts could affect viruses of the same genus or families, as well viruses with same genetic material. In fact, the topical application of *Rhizophora mangle* aqueous

bark extracts RMABE and BF-RMABE in the treatment of contagious ecthyma in lambs experimentally infected with Orf virus (*Poxviridae* family), shows efficacy at the initial state of the infection (28).

Chattopadhyay *et al.* (9) well documented the antiviral activity of the plant phenolic compounds against several DNA and/or RNA viruses. Cellular surface proteins are inactivated by polyphenols; they also interfere on viral adsorption, inhibit reverse-transcriptase and viral RNA polymerase, as well as DNA and RNA replication (16). Besides, studies related to structures and the antiviral biological activity of plants revealed that the sites and numbers of hydroxyl groups of phenols are responsible for their antiviral activity (9,15).

*Camellia sinensis* (green tea), catechins, epigallocatechin gallate (EGCG) have received the most attention and they have been researched deeply in its antibacterial, antiviral, and antifungal activities. EGCG was found to prevent influenza virus infections by binding the viral hemagglutinin, thereby preventing the attachment of viral particles to the target receptor cells. The antiviral action mechanisms of EGCG vary depending on its target virus (38).

A valuable review about the antimicrobial effect of polyphenols including the antiviral effect, by Daglia (39), mentioned that the

**TABLE 1.** *Rhizophora mangle* aqueous bark extract (RMABE) and its butanolic fraction (BF-RMABE), cytotoxicity and the *in vitro* antiviral evaluation activity. / *Extracto acuoso de corteza de Rhizophora mangle (RMABE) y su fracción butanólica (BF-RMABE), citotoxicidad y actividad de evaluación antiviral in vitro.*

Samples	MDBK Cells			Vero Cells		
	CC <sub>50</sub> (µg/mL)	BHV-1		CC <sub>50</sub> (µg/mL)	CDV	
		IC <sub>50</sub> (µg/mL)	SI		IC <sub>50</sub> (µg/mL)	SI
RMABE	92.20±15.78	87.50±6.83	1.05	112.66±21.5 3	77.50±4.79	1.45
BF-RMABE	120.42±19.7	90.63±9.98	1.33	106.71±17.8 6	86.10±12.7	1.24

CC<sub>50</sub>:50 % cytotoxic concentration; IC<sub>50</sub>:50 % inhibitory concentration; SI: selectivity index.

activity can be attributable to both direct actions against bacteria, virus and fungi, as well as to the suppression of microbial virulence factors. *Rhizophora mangle* L shows ability to stimulate the *in vitro* release of IL-2, and such results could indicate that the polyphenols present in the plant play an important role as immune modulator as well (27). Gallina *et al.* (40) report that proanthocyanidin A2 extracted from *Heasculus hyppocastanum* exerted *in vitro* antiviral activity against CDV with a higher SI compared to Ribavirin. The *in vitro* antiviral activity of chestnut and quebracho wood extracts against avian reovirus and metapneumovirus has also been demonstrated by Lupini *et al.* (41).

Thus, Ikuno *et al.* (34) evaluated *Sesbania virgata* leaf extracts against BHV-1 and HVS-1 in MDBK and Vero cells. The results showed that all the fractions evaluated had a promising activity in MDBK cells against BHV-1. On the contrary, just two fractions (the most polar) showed moderated active in Vero cells against both BHV-1 and SHV-1 viruses. Different cell substrates were used for each virus. In this sense, the obtained results are not conclusive.

Although the massive use of antivirals in animals may be controversial over possible resistance, there are several animal diseases caused by DNA viruses like Orf and BHV-1 among others, which induce skin or mucosal erosive lesions needed to be treated with wound healing compounds. In such cases, RMABE and BF-RMABE, according to their healing properties (22), would be valuable therapeutic options contributing to animal welfare during infections. This is in agreement with the efficacy observed for these compounds in lambs experimental infected with contagious ecthyma (28). These viruses are quite stable reducing the probability of the emergence of resistant strains.

BHV-1 produces clinical manifestations characterized by pustular vulvo vaginitis and erosive skin lesions that may be treated with the evaluated compounds taking advantage of

their antiviral effect combined with the wound healing properties (22). In fact, some other herpesviruses also induce these kinds of erosive lesions that could be resolved by the potential similar mechanisms of antiviral effect.

Another factor influencing the antiviral activity is the virus strain. Thus, Savi *et al.* (30) reported different antiherpetic activities for epicatechin, epigallocatechin and epigallocatechin gallate against KOS and 29R/acyclovir resistant strains. Epicatechin showed the major activity.

Date and Destache (42) published data from various *in vitro* and *in vivo* studies carried out on epigallocatechin gallate, theaflavins (black tea polyphenols), resveratrol, genistein, and curcumin, highlighting their potential to prevent sexually transmitted infections caused by HIV (human immunodeficiency virus), HSV (herpes simplex virus) and HPV (human papilloma virus), confirming the potential of natural products as antiviral agents.

Moreover, the time and the moment for the plant extract sample application determine the intracellular or extracellular action. If the sample is not evaluated in an assay that is suitable for determining its mechanism of action, negative false results can be obtained and its antiviral potential could be limited. The present study evaluated RMABE and BF-RMABE against BHV-1 and CDV through an intracellular mechanism, inhibiting viral replication. This is an important issue because the severity of viral diseases and the ability of viruses to survive intracellularly pose a great challenge that is further aggravated by the non-availability of specific antiviral agents against veterinary pathogens (32). Other factors such as treatment time and infective viral doses could also influence the antiviral activity.

Besides, an intrinsic constituent of the antiviral testing is the determination of a selectivity index (SI) towards the supporting host cell. The SI refers to the ratio of the maximum drug concentration causing either 50 % or 90 % growth inhibition of normal cells

(CC<sub>50</sub>, CC<sub>90</sub>) and the minimum drug concentration at which 50 % or 90 % of the virus is inhibited (IC<sub>50</sub>, IC<sub>90</sub>) (43).

The present results showed low SI values for RMABE and BF-RMABE compared to the chemical compounds tested for the antiviral activity against CDV (43). Ribavirin in Vero cells is active against CDV with a time-dependent antiviral activity. However, Ribavirin is approved for use in human, with an SI (1.2) in part justified due to its cytostatic activity (29). The antiviral and toxic effects are not necessarily caused by the same mix components in crude extracts; that is why SI is not as valued as when it is calculated for the pure or synthetic compounds.

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