

# Inhibition of DNA Virus: Herpes-1 (HSV-1) in cellular culture replication, through an antioxidant treatment extracted from rosemary spice

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This work aimed to evaluate antiviral properties in antioxidants from spices. Phenolic compounds extracted from rosemary (*Rosmarinus officinalis*, L) by hot water, had their antioxidant activity determined by spectrophotometry using  $\beta$  carotene/linoleic acid system. The rosemary extract was evaluated by antiviral assay of Herpes Virus type-1 (HSV-1) replication in VERO cells, in the presence or absence of the spice. 10,000 TCID<sub>50</sub>/mL of the HSV-1 was kept for 3 h at 4° C, with 300 ppm of rosemary extract, and 100 ppm of butyl hydroxyl toluene (BHT). Then, these viruses were inoculated in VERO cells incubated at 37° C in CO<sub>2</sub>-5 %, for seven days. Daily, they were examined and the end point was based on 100% of CPE in virus control (without antioxidants). The HSV-1 replication inhibition percentage (IP) measured the antiviral action from antioxidants, showing viral reductions of the 82.0, 82.5%, in the presence of rosemary and rosemary + BHT, respectively. As an extension, cell test corresponded to the similar viral decrease (IP = 85.0 and 86.3%) in both aforementioned situations. Results lead to conclude that phenolic compounds from rosemary revealed an antiviral action on herpesvirus-1.

**Uniterms:** Natural antioxidants. Virus replication. Phenolic compounds. Herpesvirus. Rosemary.

Neste estudo foi avaliada a ação antiviral de antioxidantes de especiaria. Extrato aquoso de alecrim (*Rosmarinus officinalis*, L), que apresentou atividade antioxidante através de espectrofotometria usando o sistema  $\beta$  caroteno/ácido linoléico, foi avaliado em ensaios com vírus herpes-1 na replicação em células VERO. Nestes ensaios foram utilizados 10.000 TCID<sub>50</sub>/mL do vírus HSV-1, mantidos em contato com 300 ppm do extrato de alecrim e com 100 ppm de butil hidroxi tolueno (BHT), durante 3h a 4°C. Esses vírus, em seguida, foram inoculados em células VERO incubadas a 37 °C/5% de CO<sub>2</sub> por sete dias. Pelo efeito citopático (ECP) e o “end point” de ECP do controle de vírus (sem antioxidante), foi possível observar que houve reduções na replicação viral de 82 e 82,5% na presença do alecrim e do alecrim + BHT, respectivamente. Nessa situação, avaliou-se ainda a redução da adsorção viral às células, que apresentou índices similares de 85,0 e 86,3% de redução na capacidade da adsorção. Estas reduções no desempenho do HSV foram medidas pela fórmula de porcentagem de inibição da replicação viral (PI). Os resultados levam a concluir que os compostos fenólicos do alecrim apresentam ação antiviral sobre o HSV-1.

**Unitermos:** Antioxidantes naturais. Replicação viral. Compostos fenólicos. Vírus herpes. Alecrim.

## INTRODUCTION

Since the early studies of antiherpetic drugs, their

role has been observed on the decrease of viral multiplication, *in vitro*, as well as *in vivo*. The generally used synthetic antiviral acyclovir [9(4-hydroxy-3-hydroxy methylbutyl)guanine] is a nucleoside analog, and in spite of its competent action, there are problems with acyclovir-resistant strains of herpesvirus. Even with the

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regular dose recommended for parenteral or topical uses, collateral effects are mentioned, such as neurotoxicity and renal dysfunction. Drugs with activity against DNA viruses, present a mechanism of action dependent on viral thymidine kinase, which requires phosphorylation, cellular metabolism of the drug, and a certain grade of sensitivity to viral polymerase (Cotarelo *et al.*, 1999; Mibu *et al.*, 2007). Macrophages from mice infected with herpesvirus simplex type 2 (HSV-2) demonstrated extrinsic antiviral resistance. This was observed only with the adherent peritoneal macrophage population. Such antiviral effects required viable macrophages, as the cell lysates did not inhibit virus growth (Morahan, Morse, Mc George, 1980).

Therefore, studies have been performed in order to investigate antiviral properties from natural sources, visualizing not only their use alone, but as a complement to synthetic antivirals, through the synergistic action between both. Extracts from plants, algae and essential oils from seeds or fruits presenting compounds with antiviral effects have been the most studied items within this category. Flavonoid compounds such as quercetin revealed decrease or inhibition of the yield of *Herpes simplex* virus (HSV 1), and also of type 2 virus, thanks to a probable virucidal action, as the result was an inhibition of viral DNA replication (Nolkemper *et al.*, 2006).

Polysaccharides are reported as a complex group of macromolecules possessing a wide range of therapeutically important biological properties, and they are known to affect the growth of animal viruses. These compounds have been extracted from different algae species. *Prunella vulgaris* (L), a perennial plant commonly found in China and Europe, and two others such as brown algae *Sargassum patens* and *Spirulina maxima* present the active principle sulphated polysaccharide, with virucidal capacity in herpesvirus replication in vitro. Beside these, saponins are other example of compounds extracted from both *Anagallis arvensis Primulaceae* and a planktonic blue-green alga, *Spirulina maxima*, which among their several uses, present antiviral action (Amoros, Fauconnier, Gierre, 1987; Corona *et al.*, 2002; Chiu *et al.*, 2004; Zhu *et al.*, 2004).

The phenolic compounds extracted from aromatic plants, seeds, stem bark, roots and spices that have been used as natural antiviral agents, are mentioned also in the literature. Eugenol (4-allyl-1-hydroxy-2-methoxybenzene), the most studied, is mainly found in oil of cloves and in essential oils of cinnamon, and basil. This compound presents inhibitory effects on lipid peroxidation and an effective antiviral replication of either RNA or DNA virus. This antiviral has a mechanism of action that disables the viral lipidic envelope (Benencia, Courreges, 1999; Cota-

relo *et al.*, 1999; De Logu *et al.*, 2000; Garcia *et al.*, 2003; Minami *et al.*, 2003).

More scientific investigations on this matter revealed that the essential oil from peppermint is able to exert a direct virucidal effect on herpesvirus (HSV), including an acyclovir resistant strain of HSV. It affected the virus before adsorption, but not after penetration into host cells. Considering the lipophilic nature of the oil, which enables it to penetrate the skin, it could be suitable for topical therapeutic use in herpes infection, mainly as adjuvant for synthetic drugs (Schumaker *et al.*, 2003; Saddi *et al.*, 2007).

The antiviral effects of mangiferin and isomangiferin were also evaluated through comparison with antiviral control drugs as acyclovir, idoxuridine, and cyclocytidine. The action mode of these natural antiviral agents is presumably due their capability of inhibiting virus replication within cells (Thompson, Dragar, 2004; Zheng, Lu, 1990).

Many of these antiviral agents can be used internally, as these plants and herb extracts were evaluated as non-toxic by appropriate tests, or they are flagrantly eatable, as confirmed by their prolonged use, as for instance, in the case of spices.

There are recent attempts to obtain a synergistic action between both, natural and synthetic antiviral agents. The advantages of such combinations are: a) dose decreasing of potentially toxic compounds, b) improvement of drug resistant viruses, and c) potentialization of the antiviral action (Hayashi *et al.*, 1997).

In our previous work with phenolic compounds from spices, active effects were verified on the inhibition of the RNA viruses, like Parainfluenza (PI3) isolated from natural hosts, such as humans (Mancini-Filho *et al.*, 2005). In this present study, the phenolic compounds fraction was evaluated from the spice rosemary (*Rosmarinus officinalis*, L), alone and in combination with synthetic antioxidants, aiming to verify their antiviral effects on DNA viruses, such as Herpes – type 1.

## MATERIAL AND METHODS

### Spice extraction

The rosemary spice (*Rosmarinus officinalis*, L.) was acquired at a local market, in the city of São Paulo, Brazil. The aqueous extract was obtained from a 4g sample of the spice, which was incubated in 40 mL of hot water (80°C) while shaking for 10 min, followed by centrifugation at 3,000g for 20 min. After residue removal, water was added to the supernatant to complete 40 mL. The amount of dry material in the extract was determined gravimetrically. The

determination of the total phenolic compounds was accomplished by spectrophotometry, using Folin – Denis' reagent and catechins as standard. The antioxidant activity was measured in the  $\beta$ -carotene and linoleic acid model system, as previously described by Mancini-Filho *et al.* (2005).

## Cells and virus

African Green Monkey Kidney cells (VERO), provided by the Instituto Adolfo Lutz, São Paulo, Brazil, were cultured in Leibovitz-15 ( $L_{15}$ ) medium and supplemented with 5% fetal calf serum (FCS) from CUTILAB®, Brazil, with the addition of antibiotics.

Herpes Virus type I (HSV-1) was obtained from Instituto de Medicina Tropical, São Paulo, Brazil; its titer (expressed by TCID<sub>50</sub>) was determined by cytopathic effect in cells culture.

## 50% tissue culture infectious doses (TCID<sub>50%</sub>)

VERO cells were used to prepare the viral micro-titration in 96-wells culture plates (Nunc Multidish® – USA). HSV-1 virus was ten-fold diluted ( $10^{-1}$  to  $10^{-10}$ ) in  $L_{15}$  medium and was inoculated in each plate well, with 25  $\mu$ L of each dilution in 5 wells, and incubated for 2 h at 4 °C, after which a further 25  $\mu$ L of  $L_{15}$  medium was added. The micro plate was incubated for seven days at 37 °C in CO<sub>2</sub> (5%), and examined daily for evidence of CPE. Titers of all cases were determined by Reed Muench's method.

## Antiviral assays

The antiviral activity of rosemary (*Rosmarinus officinalis*, L.) extract was assayed using VERO cells grown in 96-wells culture microplates (Nunc Multidish-USA). Cells were infected with 10,000 TCID<sub>50/mL</sub> of HSV-1 (titer TCID<sub>50</sub>  $10^{9.5}$ ), previously treated with 300 ppm from rosemary extract (v/v) for 3h at 4 °C. The cells inoculated were incubated for seven days at 37 °C in CO<sub>2</sub> (5%). The virus inhibition was measured by the decrease in percentage of the CPE levels (formula IP) (Benencia, Courreges, 1999).

## Attachment assay

This test described by De Logu *et al.* (2000) was performed as follows: VERO cells monolayer grown in 24 wells culture plate, were pre-chilled at 4 °C for 2 h. The medium was aspirate and the cell monolayer was infected with 1,000 TCID<sub>50/mL</sub> of HSV-1, in absence or presence of 300 ppm from rosemary extract. After incubation of infected cells at 4 °C for 3 h, the medium was drawn to remove

the non-adsorbed viruses. Then the monolayer was washed with PBS three times and overlaid with maintenance medium. The culture was maintained for seven days at 37 °C in CO<sub>2</sub> (5%). The percent of inhibitory effect of rosemary for HSV-1 attachment to the cells was calculated using the formula for calculation of virus inhibition percentage (Benencia, Courreges, 1999).

## Synergistic effect

With both, 300 ppm from natural (rosemary extract) and 100 ppm from synthetic antioxidant (butyl hydroxyl toluene - BHT), the synergistic effect was assayed by the mentioned antiviral assay using rosemary alone and in combination with synthetic antioxidant, at the same time of contact with HSV-1 diluted at  $10^{-2}$  TCID<sub>50/mL</sub> (V/V). Beyond this, the synergistic effect between natural and synthetic antioxidants was assayed by lipidic oxidation inhibition.

Formula for virus inhibition percentage calculation (Benencia, Courreges, 1999):

$$IP = \frac{1 - [(TCID_{50/mL} \text{ of HSV-1 treated with Rosemary})] \times 100\%}{TCID_{50/mL} \text{ titer of Control HSV-1}}$$

## RESULTS AND DISCUSSION

In the present work, the results were obtained by means of antiviral evaluations of either natural antioxidant agents from rosemary (*Rosmarinus officinalis*, L.) alone or in combination with a synthetic antioxidant (butyl hydroxy toluene - BHT). These data, concerning HSV-1 replication in cells, revealed similar levels of inhibition percentage (IP) of the viral replication (82.0% and 82.5%). These antiviral action is attributed to rosemary and rosemary combined with BHT, respectively (Tables I and II).

**TABLE I** – Viral inhibition action of natural antioxidant from rosemary (*Rosmarinus officinalis*, L.) 300ppm on HSV-1 replication in vero cells

Contact Virus X Antioxidants (hours)	HSV-1 TCID <sub>50/mL</sub>	Percent of Virus Inhibition
0	10,000	NONE
3	1,800	82.0

Formula to determine the virus inhibition percentage:

$$IP = \frac{1 - [(TCID_{50\%/mL} \text{ of HSV-1 treated with Rosemary})] \times 100\%}{TCID_{50\%/mL} \text{ titer of Control HSV-1}}$$

**TABLE II** –Viral inhibition of the natural antioxidant from rosemary (*Rosmarinus officinalis*, L.) 300 ppm and synthetic antioxidant butyl hydroxyl toluene (BHT) 100 ppm on HSV-1 replication in VERO cells

Contact Virus X Antioxidants (hours)	HSV-1 TCID <sub>50%/mL</sub>	Percent of Virus Inhibition
0	10,000	NONE
3	1,750	82.5

Formula to determine the virus inhibition percentage:

$$IP = \frac{1 - [(TCID_{50\%/mL} \text{ of HSV-1 treated with Rosemary + BHT})] \times 100\%}{TCID_{50\%/mL} \text{ titer of Control HSV-1}}$$

Regarding to the attachment assay, the observed results were of a percent inhibition (PI) of the 85.0% and 86.3% on HSV-1 replication in VERO cells obtained from both antioxidants: rosemary and rosemary plus BHT, respectively (Tables III and IV).

**TABLE III** – Inhibitory action of natural antioxidant from rosemary (*Rosmarinus officinalis*, L.) 300 ppm on attachment cells for HSV-1

Contact Virus X Antioxidants (hours)	HSV-1 TCID <sub>50%/mL</sub>	Percent of Virus Inhibition
0	1,000	NONE
3	150	85.0

Formula to determine the virus inhibition percentage:

$$IP = \frac{1 - [(TCID_{50\%/mL} \text{ of HSV-1 in presence of Rosemary})] \times 100\%}{TCID_{50\%/mL} \text{ titer of Control HSV-1}}$$

**TABLE IV** – Inhibitory action of natural antioxidant from rosemary (*Rosmarinus officinalis*, L.) 300 ppm and synthetic antioxidant of butyl hydroxyl toluene (BHT) 100 ppm on attachment cells for HSV-1

Contact Virus X Antioxidants (hours)	HSV-1 TCID <sub>50%/mL</sub>	Percent of Virus Inhibition
0	10,000	NONE
3	137	86.3

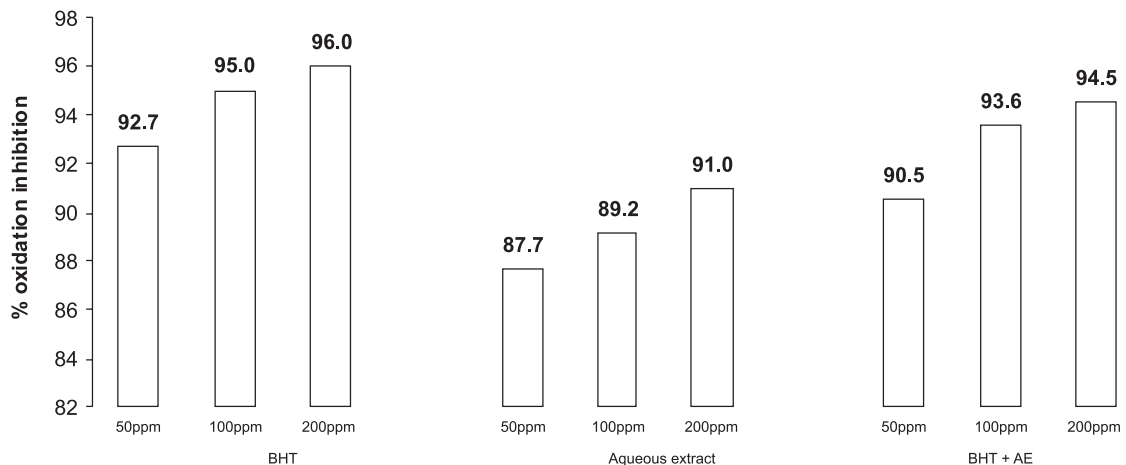
Formula to determine the virus inhibition percentage:

$$IP = \frac{1 - [(TCID_{50\%/mL} \text{ of HSV-1 in presence of Rosemary + BHT})] \times 100\%}{TCID_{50\%/mL} \text{ titer of Control HSV-1}}$$

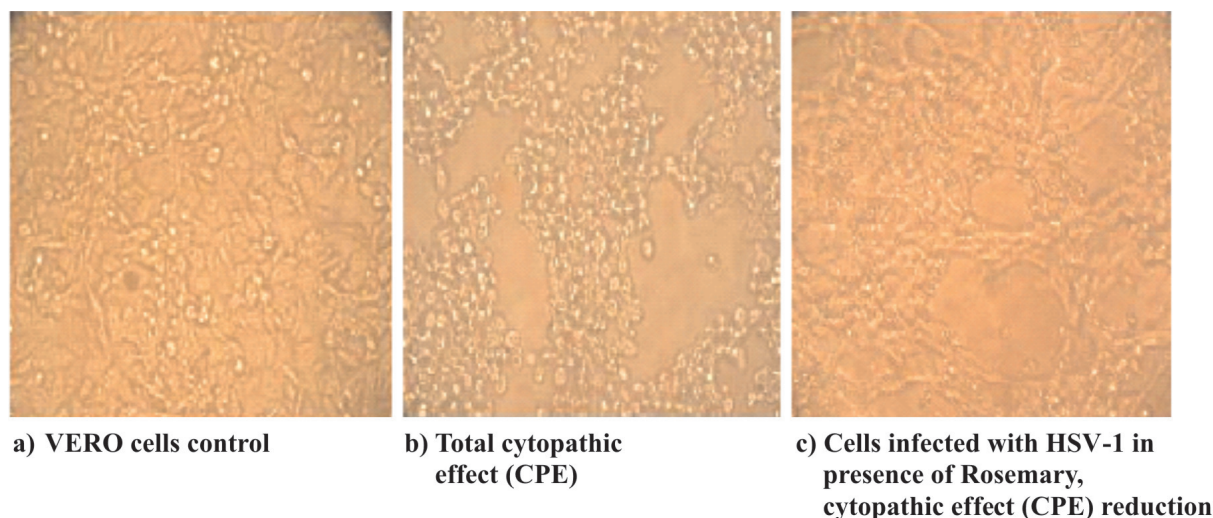
Nolkemper *et al.* (2006) also reported the antiviral action on both types of HSV, HSV-1 and HSV-2, in experiments using spices that presented significant reduction of these virus replications in RC – 37 cells.

Respecting to the evaluation of the lipidic oxidation inhibition regarding both, the natural and the synthetic antioxidants, a synergistic effect was observed between them (Figure 1).

No cytotoxic effect was observed in VERO cells in contact with 300 – 100 ppm of both, the natural and synthetic antioxidants. The normal cells with antioxidants (used as control) and the cytopathic effects from HSV-1 in VERO cells previously in contact with antioxidant, or



**FIGURE 1** – Percents of the oxidation inhibition from BHD and aqueous extracts (AE) of rosemary (*Rosmarinus officinalis* L.) and its synergic activity.



**FIGURE 2** – The HSV-1 cytopathic effect and its inhibition by the antioxidant rosemary.

not, are shown in Figure 2 (a, b, c). Garcia *et al.* (2003) reported that a range of 100–150 ppm of essential oils from aromatic plant extracts (San Luis – Argentina) demonstrated virucidal activity against HSV-1. This activity was obtained by direct contact between the virus and the extract, for 3h before its inoculation in cell culture.

An inhibition level of 99.9% on the HSV-1 yield was observed, when the extract from *Cordia salicifoliana* was added in Hela cell monolayers before the virus inoculation, during experiments performed by Palamara *et al.* (1995).

In both of these situations, the HSV-1 was inhibited either by viral inactivation obtained through direct contact between virus and the extract, or by the virus inhibition of the cell receptor contact.

An antiviral without interference on cellular metabolism, the glutathione (GSH), was reported by Palamara *et al.* (1995). This is because an antiviral, such as the DL-Galactan, extracted from the red seaweed *Gymnogongrus torulosus*, possesses an action mode of interference on the binding of the surface glycoprotein of the viral envelope to the cell receptor (Pujol *et al.*, 2002).

These glycoprotein potencies for antiviral activity are suggested to depend on the molecules' binding affinity for fetuin (Ooi *et al.*, 2008).

By afore mentioned test, all viral titrations of the HSV-1 were performed in VERO cells, observing their cytopathic effect (CPE) for seven days. The results of these experiments showed a range of 72 – 96 h for total CPE of virus controls. Mean titers of HSV-1 were  $10^{9.5}$  TCID<sub>50/μL</sub>. In order to evaluate antiviral activity from both, natural and synthetic antioxidants, antiviral tests with HSV-1 virus diluted at  $10^{-3}$  –  $10^{-4}$  were employed.

## CONCLUSIONS

Based on these data demonstrated in the results of this study, it is possible to argue that aqueous extract from rosemary spice (*Rosmarinus officinalis*, L) possesses antioxidant activity with antiviral action. This finding was confirmed by the inhibitor of lipidic oxidation test, as well as by the HSV-1 replication in VERO cell inhibition assays. Concentrations of 100 ppm and 300 ppm for rosemary and butyl hydroxy toluene (BHT), respectively, were considered appropriate for these tests, taking in account the lack of toxicity in this condition.

These experiments with HSV-1 replication in VERO cells, in the presence or absence of both, natural and synthetic antioxidants, have verified the virus inactivation and virus replication inhibition, in the range of 85 to 86 percent.

Therefore, considering the kind of antiviral actions of either natural extract or synthetic antioxidants, they could be suggested as virucidal agents, and also inhibitors of the viruses replication.

Although a synergistic effect has been verified by lipidic inhibition between these natural and synthetic antioxidants, it was not verified respecting the reinforcement of antiviral action from rosemary spice against the HSV-1, in this study.

The authors of this present investigation intend to reinforce and collaborate with experiments on herpesvirus therapy using extract from natural sources as adjuvants for synthetic antiviral agents.

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