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Evaluation of Antiherpetic Activity and Genotoxic Effects of Tea Catechin Derivatives

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This study evaluated the antiherpetic activity and genotoxicity of catechin and some of its derivatives using the MTT colorimetric and comet assays, respectively. The results showed that all compounds have antiviral activity with selective indices varying from 1.3 to 13, depending on the tested HSV-1 strain. It was observed that the same concentration of the compounds that protects the Vero cells against the viral infection induces genotoxicity. It was also observed that the molecules containing three hydroxyl groups on the B ring caused less DNA damage and showed better antiviral effect than those with two hydroxyls on the same ring, but if there is an additional galloyl group, these results can be altered. The bioavailability and stereochemistry could be related to the antiviral and genotoxic effects detected.

KEYWORDS: Catechin derivatives; antiviral; HSV-1; MTT assay; genotoxicity; comet assay

INTRODUCTION

Teas are classified according to the procedures used to manufacture them from the leaves of *Camellia sinensis*. Tea catechins are the major polyphenolic components of fresh tea leaves as well as the soluble matter in green tea. The main catechins present in green tea are epicatechin, epigallocatechin, and epigallocatechin gallate. All of these catechins have the structure of flavan-3-ol and additional pyrogallol or galloyl groups that contribute to their biological activities (*1*). Tea catechins have been proven to have a variety of pharmacological actions. It is noteworthy that catechins act as antioxidants in vitro and in vivo because in general this activity may be closely related to preventive effects on various diseases including arteriosclerosis, liver injury, and carcinogenesis (*2*, *3*). In addition, some studies have demonstrated that catechin and derivatives possess significant bioactivities such as anticancer, anti-inflammatory, antiallergic, antimutagenic, and antiaging, among others (*4*−*6*). Tea catechins have also been studied concerning their antiviral activity against adenovirus (*7*, *8*), Epstein−Barr virus (*9*), HIV-1 (*1*), herpes simplex viruses (*8*, *10*−*12*), and influenza virus (*13*), among others. In the present study, catechin and some derivatives were tested for their cytotoxicity and antiherpetic activity. To complement this study, the potential cell DNA damage caused by two concentrations of these compounds at which they have previously showed antiherpetic activity was also evaluated by using the comet assay.

MATERIALS AND METHODS

Source of Catechin Derivatives. Catechin (**I**), gallocatechin (**II**), epicatechin (**III**), and epigallocatechin (**IV**) were obtained commercially from Extrasynthèse; epigallocatechin gallate (**V**) and acyclovir (**VII**) were obtained from Sigma; robinetinidol(4α-6)gallocatechin (**VI**) was kindly provided by Professor João Carlos Palazzo de Mello, Universidade Estadual de Maringá, PR, Brazil (*14*).

Preparation of Stock Solutions. The compounds (**I**−**VII**) (**Figure 1**) were dissolved in 1% (v/v) of dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) diluted in Minimum Essential Medium (MEM) (Sigma Chemical Co., St. Louis, MO), vortexed, and filtered through 0.22 μm filters (Millipore, Bedford, MA). All stock solutions were stored at 4 °C and protected from light until used.

Biological Activities. *Cell Culture and Virus.* The cell line used was Vero cells (ATCC:CCL 81, Rockville, MD) grown in MEM supplemented with 5% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), penicillin G (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (25 μg/mL) (Gibco BRL). The cell culture was maintained at 37 °C in a humidified 5% CO_2 atmosphere. Herpes simplex virus type 1 (HSV-1) was used (KOS and 29R/acyclovir resistant strains; Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Rennes, France). Stock viruses were prepared as described previously (*15*), and the infected cells' supernatant fluids were harvested, titered, and stored at −80 °C until used. HSV-1 titer was obtained by the limit-dilution method and expressed as 50% tissue culture infection dose per milliliter ($TCID_{50}$/mL) (*16*).

Cytotoxicity Evaluation: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Method (17, with Minor Modifications). Vero cell culture (2.5×10^5 cells/mL) was prepared in 96-well tissue culture plates (Corning). After a 24 h incubation period at 37 °C in a

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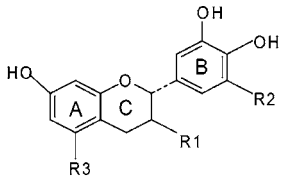
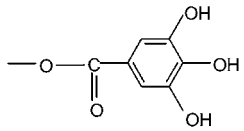
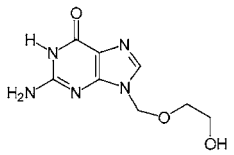




Galloyl group

	Substances	Configuration	R1	R2	R3
I	(-)-catechin	2S 3R	OH	H	OH
II	(-)-gallocatechin	2S 3R	OH	OH	OH
III	(-)-epicatechin	2R 3R	OH	H	OH
IV	(-)-epigallocatechin	2R 3R	OH	OH	OH
V	(-)-epigallocatechin gallate	2R 3R	galloyl group	OH	OH
VI	Robinetinidol(4α-6)gallocatechin	-	gallocatechin	OH	H



VII. Acyclovir

Figure 1. Chemical structures of tested compounds.

humidified 5% CO_2 atmosphere, the cell monolayer was confluent, and the medium was removed from each well and replenished with 200 μL of different concentrations of compounds per well (each concentration was diluted at 1:2, v/v) prepared in MEM with 5% (v/v) FBS. Only 200 μL of MEM was added to the cells as cell control. The plates were incubated at the same conditions cited above for 96 h. After incubation, the medium was removed by suction from all wells, and 50 μL of MTT solution prepared in MEM (1 mg/mL; Sigma Chemical Co., St. Louis, MO) was added to each well and the plates were incubated for an additional 4 h. The MTT solution was removed without disturbing the cells, and 100 μL of DMSO (Nuclear, São Paulo, Brazil) was added to each well to dissolve formazan crystals. After the plates had been gently shaken for 10 min, whereby crystals were completely dissolved, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, Elx 800) at 540 nm. The percentage of cytotoxicity was calculated as $[(A - B)/A \times 100]$, where A and B correspond to the absorbances of control and treated cells, respectively. The CC_{50} value was defined as the concentration of each compound that reduces the absorbance of treated cells by 50% when compared to cell controls.

Antiviral Evaluation: MTT Method (18, with Minor Modifications). The same method used to evaluate cell viability by MTT as described above was followed. Vero cell culture (2.5×10^5 cells/mL) was prepared in 96-well tissue culture plates (Corning). After 24 h of incubation, cell monolayer was confluent, and the medium was removed from each well and replenished with 100 μL of noncytotoxic concentrations of each catechin derivative and 100 μL of each of the HSV-1 virus strains (KOS and 29-R). Cell and viral controls were performed by adding 200 μL of MEM only and 200 μL of virus suspension (MOI = 0.5) diluted in MEM without FBS, respectively. The 50% antiviral effective concentration of each compound (EC_{50}) was calculated as $[(A - B)/(C - B) \times 100]$, where A, B, and C indicate the absorbances of tested compounds, viral, and cell controls, respectively. Acyclovir [9-(2-hydroxyethoxymethyl)guanosine, 10 μg/mL, Sigma Chemical Co.] was used as positive control for HSV-1 inhibition. The selective index (CC_{50}/EC_{50}) was calculated for each substance.

Genotoxicity Evaluation: Comet Assay (19). To determine the extent of cell DNA damage, alkaline microgel electrophoresis was performed with minor modifications. Vero cell culture (2.5×10^5 cells/mL) was prepared in 24-well tissue culture plates (Corning). After 24 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, cell monolayer was confluent, and the medium was removed from each well and replenished with 500 μL of two concentrations of each catechin derivative, both noncytotoxic and with antiherpetic activity. As a negative control, only MEM was added to the cells. As a positive control, hydrogen peroxide 30% solution (v/v) (at final concentrations of 100 and 200 μM, Nuclear, São Paulo, Brazil) was added. After 90 min of incubation, the medium was removed, cells were trypsinized, and an equal volume of 0.4% (w/v) Trypan blue dye aqueous solution was added to an aliquot of the cell suspension. Viable cells were counted under the phase contrast microscope. The percentage of viable cells was estimated by comparison with cell controls. After the cell viability confirmation, immediately after the treatment, cells were harvested and processed for the assay as follows. Briefly, 40 μL of the cell suspension was mixed with 60 μL of 0.5% (w/v) low melting point agarose (Gibco BRL) dissolved in phosphate buffer saline (PBS) Ca^{2+}- and Mg^{2+}-free at 37 °C. Then, 100 μL of this mixture was spread on a chilled microscope slide previously precoated with one layer (100 μL) of 1.5% (w/v) normal melting point agarose (Gibco BRL) in PBS Ca^{2+}- and Mg^{2+}-free at 37 °C. A glass coverslip was immediately placed on the top of the slide, and the agarose/cell mixture was allowed to cool by placing the slide at 4 °C for 15 min. Next, the coverslip was gently removed and the slide was immersed into an ice-cold lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% (v/v) DMSO, 1% (v/v) Triton X-100, pH 10] at 4 °C for at least 1 h. After treatment, the slides were placed in a submarine gel electrophoresis chamber containing 300 mM NaOH and 1 mM EDTA, pH 13, for 30 min, before being electrophoresed at 25 V (300 mA) for 30 min, at 4 °C. After electrophoresis, the slides were rinsed (three times, 5 min each) with neutralizing buffer (0.4 M Tris-HCl, pH 7.5), stained with 30 μL of ethidium bromide (20 μg/mL), and covered with coverslips. Cell analysis was performed by visual score (*20*). Briefly, fluorescent stained nucleoids were counted using an epifluorescence microscope (Olympus BX 40) with a 515−560 nm excitation filter and a 590 nm barrier filter at 400× magnification. Fifty comet cells per slide were scored at random (in the central area of each codified slide) and classified into five classes based on the shape of the comets: type 1, no tail; type 2, comets with small tails (tail length less than a fourth of head diameter); type 3, comets with medium tails (tail length between a fourth and full head diameter); type 4, comets with long tails (tail length greater than head diameter); type 5, comets with poorly defined or small head or without head. Numerical values of 0−4 were assigned to the comet types 1−5, respectively, and the product of the numerical values was calculated for each sample and used for the analysis. The total score for 50 comet cells could range, therefore, from 0 (all undamaged) to 200 (all maximally damaged).

Data Analysis. The CC_{50} and EC_{50} values were estimated from concentration−effect curves after linear regression analysis and represent mean ± standard error of the mean values of three independent experiments.

For the comet assay, all of the experiments were performed in triplicate, and two slides were prepared for each compound concentration, as well as for the positive and negative controls. The data presented are the mean ± standard error of the mean values. Statistical analyses were performed using GraphPad InStat, version 3.0 (GraphPad Software, San Diego, CA). Data were tested for normality using the Kolmogorov−Smirnov test. Trends were followed, when appropriate, by a one-tailed Dunnett's test, comparing each tested concentration against the negative control group, with a significance level of $p < 0.05$.

RESULTS

According to the results shown in **Figures 2** and **3**, it was possible to calculate the selective indices ($SI = CC_{50}/EC_{50}$) for the tested compounds (**Figure 4**), and $SI > 4$ indicates potential antiviral activity (*21*). All compounds demonstrated antiherpetic activity, at noncytotoxic concentrations. Epigallocatechin (**IV**) and robinetinidol(4α-6)gallocatechin (**VI**) demonstrated promising antiherpetic activity against both tested virus strains, and epicatechin (**III**) demonstrated potential antiherpetic activity just against the 29R acyclovir-resistant tested strain.

It was also verified that the compounds epicatechin (**III**), epigallocatechin (**IV**), and robinetinidol(4α-6)gallocatechin (**VI**) showed substantial effects against HSV-1 (29-R strain) at 107.14, 70.42, and 72.19 μM, respectively. In relation to HSV-1 (KOS strain), the compounds **IV** and **VI** showed significant effects at 173.56 and 69.85 μM, respectively.

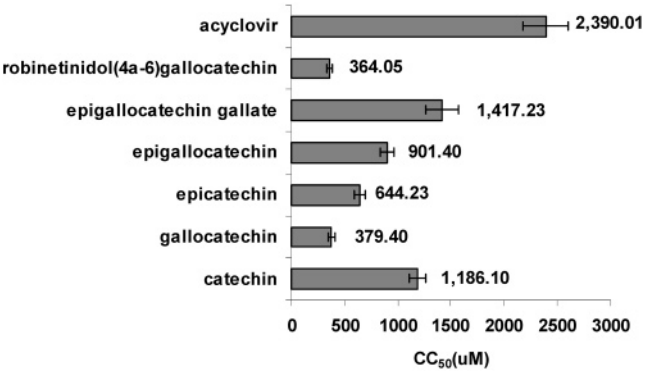


Figure 2. Cytotoxicity of catechin derivatives and acyclovir by using MTT assay on Vero cells. CC_{50} = cytotoxic concentration at 50% (values represent mean ± standard error of the mean values of three different experiments).

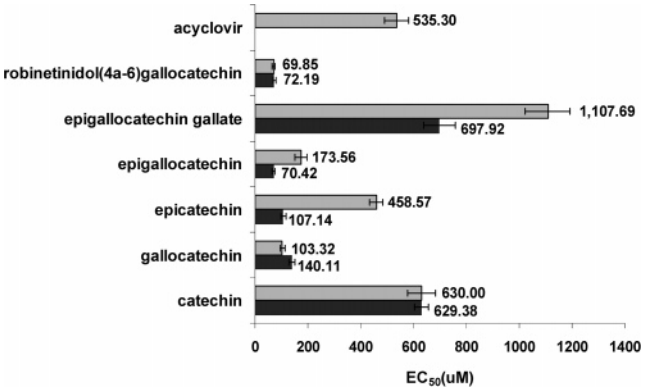


Figure 3. Antiherpetic activity [HSV-1 KOS (gray bars) and 29-R (black bars) strains] of catechin derivatives and acyclovir by using MTT assay. EC_{50} = effective concentration required to inhibit 50% of viral replication (values represent mean ± standard error of the mean values of three independent experiments). Titer of HSV-1 KOS strain: $5 \times 10^{6.625}$ $TCID_{50}$/mL. Titer of HSV-1 29-R strain: $5 \times 10^{6.5}$ $TCID_{50}$/mL.

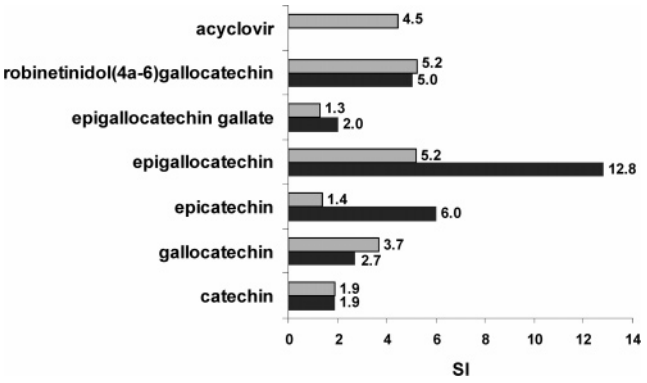


Figure 4. Selective indices (SI) of catechin derivatives and acyclovir for HSV-1 [KOS (gray bars) and 29-R (black bars) strains] on Vero cells. SI = CC_{50}/EC_{50}.

A concurrent assessment of cytotoxicity is critically important for comet assay data interpretation (*19*). In this study, noncytotoxic concentrations of tested compounds, previously evaluated by MTT assay, were used and the cell viability was confirmed by Trypan blue dye exclusion method. It was observed that all tested compounds, including positive controls, showed >90% of cell viability (data not shown).

It was also observed (**Figure 5**) that all tested compounds, excepting acyclovir, showed genotoxic effects, at the tested concentrations, in which they had previously demonstrated

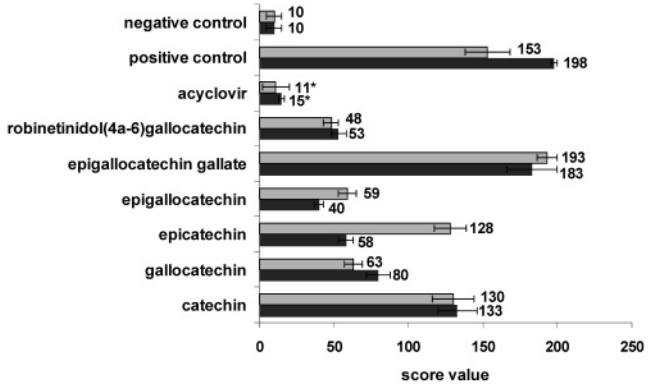


Figure 5. Genotoxicity evaluation of two antiherpetic concentrations [C1 (gray bars) and C2 (black bars)] of catechin derivatives and acyclovir on Vero cells by using comet assay. Score values represent mean ± standard error of the mean values of three independent experiments. C1, EC_{50} values obtained with HSV-1 KOS strain; C2, EC_{50} values obtained with HSV-1 29-R strain. Positive control (H_2O_2): C1 = 100 μM and C2 = 200 μM. The differences between negative controls and treatments were tested for statistical significance by a one-tailed Dunnett's test ($p < 0.05$). *, no statistical significance compared to negative control.

antiherpetic activity. Data showed that catechin (**I**), at 630 and 629.38 μM, and epigallocatechin gallate (**V**), at 1107.69 and 697.92 μM, presented higher levels of DNA damage and that epigallocatechin (**IV**), at 173.56 and 70.42 μM, and robinetinidol(4α-6)gallocatechin (**VI**), at 69.85 and 72.19 μM, showed low genotoxic effects in this study. These tested concentrations are related to the antiherpetic responses previously obtained.

DISCUSSION

Assessment of cytotoxicity is clearly an important part of the evaluation of a potential antiviral agent because a useful compound should be selective for virus-specific processes with no or few effects on cellular metabolism and not show toxicity against the host. Cytotoxicity evaluation in vitro is usually performed by using cell viability assays, such as the uptake of a dye by nonviable cells or by alterations in the mitochondrial function; these endpoints have been established for many years, in many cell types. The MTT assay is probably the most commonly used colorimetric indicator of cell viability, and it has been used to evaluate cytotoxicity in a quantitative way in contrast with cell morphology evaluation by inverted light microscopy, which is qualitative and more subjective (*22*, *23*).

The reduction of MTT in the cell assesses the functional intactness of mitochondria on the basis of the enzymatic reduction of this tetrazolium salt by the mitochondrial dehydrogenases in viable cells. MTT was first applied to quantify cellular proliferation, and it is now widely used for screening antitumoral (*24*−*28*) and antiviral (*18*, *22*, *29*−*33*) activities of a large number of synthetic and natural products. This assay has several advantages: it is easy to perform, the evaluations are objective, it can be automated using a personal computer, and the cytotoxicity measurement can be made in parallel with antiviral activity evaluation.

In this study, it was observed that the compounds with free hydroxyls in their molecules showed toxicity to Vero cells. Robinetinidol(4α-6)gallocatechin (**VI**), which contains 10 free hydroxyls, was more cytotoxic than the other tested compounds (all of them with fewer hydroxyl groups); acyclovir, which has no free hydroxyls, showed the lowest cytotoxicity.

The presence of functional groups can contribute to the cytotoxicity of the tested compounds. It was observed that the

galloyl group on R1 of epigallocatechin gallate (**V**) decreased the cytotoxicity even though it contains the same hydroxyl groups on the B ring as epigallocatechin (**IV**). Compounds with galloyl groups in their molecules have also demonstrated low cytotoxicity in other studies (*34*−*37*).

In addition, the presence of one hydroxyl group on R2 of the B ring [for example, gallocatechin (**II**)] and/or a configuration modification [for example, epicatechin (**III**) or epigallocatechin (**IV**)] induced a cytotoxicity increase, at the tested conditions.

Nevertheless, compounds containing hydroxyl groups can interact with iron, copper, and other metals usually present in culture medium and fetal bovine serum (*36*, *38*), intensifying MTT reduction and increasing the blue coloration (*39*). Some compounds can react with iron, generating blue-black complexes (*40*), so the cytotoxicity detected in this study by the MTT assay could be overestimated.

The comet assay is a potent tool for genotoxicity evaluation, and it has been used to detect different kinds of DNA damage, including double- and single-strand breaks or alkali-labile sites. Fifty percent of the damage induced by natural light is efficiently repaired in normal cells within 15 min, and complete repair occurs within 1−2 h (*19*), justifying the treatment period of 90 min used in this study. The DNA damage detected for two tested concentrations of each catechin derivative could be explained by two hypotheses. First, it is possible that the damage is due to hydroxyl radical formation. Thus, it is envisaged that H_2O_2 (used here as positive control and also present by natural Fenton reaction), which easily crosses biological membranes, can penetrate the nucleus and react with iron and/or copper to form hydroxyl radicals. Because of the high reactivity of this radical and its resultant inability to diffuse significant distances within the cell, this mechanism is feasible only if the hydroxyl is generated very close to the DNA. A second possibility is that the metal ions might be released within the cell as a result of oxidative stress and then bind to the DNA. As the oxidative stress increases intracellular free calcium, it may cause rises in intracellular free iron and/or copper that could bind to DNA and make it a target for oxidative damage. An explanation of the ability of oxidative stress to cause DNA damage is that it triggers a series of metabolic events within the cell that lead to the activation of nucleases, which cleave the DNA backbone. Recently, there have been many debates concerning the hypothesis that oxidative stress can cause rises in intracellular free calcium that might break DNA by activating calcium-dependent endonucleases in a mechanism similar to apoptosis (*41*−*45*).

In this study, it was observed that the tested concentrations of the compounds containing three hydroxyl groups on the B ring (compounds **II**, **IV**, and **VI**) caused less DNA damage than those with two hydroxyls on the same ring (compounds **I** and **III**). Epigallocatechin gallate (**V**), the most genotoxic tested compound, also contains three hydroxyl groups on the B ring, but it can be distinguished from the other tested compounds because it presents an additional galloyl group on the R1 position. Therefore, this group could be responsible for the damage detected.

In addition, it was reported that epigallocatechin gallate can induce apoptosis mediated by activation of caspases 3 and 8 (*41*, *46*), and different groups of researchers have detected DNA damage induced by catechin and compounds containing galloyl group in their molecules (*34*, *41*, *47*, *48*). The bioavailability can be considered to be responsible for the lack of antioxidant effect of catechin, at the tested concentrations, which did not promote DNA repair and resulted in DNA oxidative damage (*47*, *48*).

It was also observed that the stereochemistry could be related to the antiherpetic response and also to the genotoxic effects. In this study, epicatechin (**III**) and epigallocatechin (**IV**) showed better antiviral response and lower genotoxicity than their corresponding epimers, catechin (**I**) and gallocatechin (**II**).

In contrast to the obtained cytotoxicity results, the presence of one hydroxyl group on R2 of the B ring [for example, gallocatechin (**II**)] and/or a configuration modification [for example, epigallocatechin (**IV**)] induced genotoxicity decrease, at the tested conditions.

Acyclovir, which was used as a positive control for antiviral assays, showed no significant genotoxicity, at the tested concentrations, when compared to negative controls, as expected for a drug currently in use (*49*).

The HSV-1 replication in the tested system without treatment (viral control) was sufficient to destroy Vero cell monolayers. The acyclovir, which is a reference drug for the treatment of herpes viruses infection, was effective in completely inhibiting the viral replication of HSV-1, KOS strain. In relation to the replication of HSV-1, 29-R strain, as expected, this drug was not effective because this strain is acyclovir-resistant.

In relation to the detected antiherpetic activity, it is proposed that the relationship between viral infection and reactive oxygen species (ROS) is probably modified during evolution, and the cell considers the entry of a virus in the cytoplasm as a signal for ROS production that should induce apoptosis in the infected cell before it becomes a source of massive infection for its neighbors. Another important consequence of the suggested mechanism should include a possibility of apoptosis induction in adjacent cells, which are in direct contact with the infected cells that are especially prone to infection. Hence, an apoptotic zone would be formed around the cell, which is a potential source of viral infection. This explains the phenomenon of bystanders: the cells not infected with the virus, but adjacent to infected cells, being eliminated together with the former (*50*). This could explain the antiherpetic activity and genotoxicity detected for the tested compounds (**Figures 3** and **5**). The genotoxicity could be probably induced by ROS released by infected cells and/or by catechin and its derivatives that were not antioxidants, at the tested concentrations, in which they demonstrated antiherpetic activity (*41*, *47*).

In this study, it was observed that the tested compounds containing three hydroxyl groups on the B ring and without galloyl radical on the R1 position (compounds **II**, **IV**, and **VI**) had the better selective indices, at the tested concentrations. Although showing high cytotoxicity, they had antiherpetic activity at low concentrations. These compounds were the same that caused less DNA damage, and this fact could reinforce the hypothesis that the antiherpetic activity of the compounds is related to their capacity to control the oxidative stress caused by the infection (*50*, *51*).

It was also observed that the tested concentrations of the compounds containing three hydroxyl groups on the B ring (compounds **II**, **IV**, and **VI**) show better antiherpetic response than those with two hydroxyls on the same ring (compounds **I** and **III**). Epigallocatechin gallate (**V**) also contains three hydroxyl groups on the B ring, but it can be distinguished from the other tested compounds because it presents an additional galloyl group on the R1 position.

Similarly to the obtained cytotoxicity results, the presence of one hydroxyl group on R2 of the B ring [for example,

gallocatechin (**II**)] and/or a configuration modification [for example, epigallocatechin (**IV**)] induced an increase of the antiviral response against HSV-1, at the tested conditions.

Considering the results obtained here, it could be concluded that free hydroxyls can contribute to the cytotoxicity of catechin and derivatives, but its number is an insufficient parameter to correlate the compounds' structures and their antiherpetic activity and genotoxicity; the molecules' configurations need to be considered, too.

All of the tested compounds showed anti-HSV-1 activity at noncytotoxic concentrations; and epigallocatechin (**IV**) and robinetinidol(4α-6)gallocatechin (**VI**) demonstrated promising antiviral activity and selectivity indices even higher than those of acyclovir, at the tested conditions.

However, genotoxic levels for the tested concentrations of the compounds studied here were also observed, probably due to the free radical formation, at the tested conditions, which could be responsible for the oxidative damage. This must be investigated, and the antioxidant potential of these substances requires further investigation.

The screening of bioactive substances, synthetic or from natural sources, and the determination of their structure−activity relationships, allied to technological advances, are required to develop new drugs that are more effective and safer than those already available.

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