

Antiviral Activity of Crude Hydroethanolic Extract from *Schinus terebinthifolia* against *Herpes simplex Virus Type 1*

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ABSTRACT

Herpes simplex virus infections persist throughout the lifetime of the host and affect more than 80% of the humans worldwide. The intensive use of available therapeutic drugs has led to undesirable effects, such as drug-resistant strains, prompting the search for new antiherpetic agents. Although diverse bioactivities have been identified in *Schinus terebinthifolia*, its antiviral activity has not attracted much attention. The present study evaluated the antiherpetic effects of a crude hydroethanolic extract from the stem bark of *S. terebinthifolia* against *Herpes simplex virus type 1* *in vitro* and *in vivo* as well as its genotoxicity in bone marrow in mammals and established the chemical composition of the crude hydroethanolic extract based on liquid chromatography-diode array detector-mass spectrometry and MS/MS. The crude hydroethanolic extract inhibited all of the tested *Herpes simplex virus type 1* strains *in vitro* and was effective in the attachment and penetration stages, and showed virucidal activity, which was confirmed by transmission electron microscopy. The micronucleus test showed that the crude hydroethanolic extract had no genotoxic effect at the concentrations tested. The crude hydroethanolic extract afforded protection against lesions that were caused by *Herpes simplex virus type 1* *in vivo*. Liquid chromatography-diode array detector-mass spectrometry and MS/MS identified 25 substances, which are condensed tannins mainly produced by a B-type linkage and prodelfinidin and procyanidin units.

Introduction

HSV-1 (*Herpes simplex virus type 1*) is commonly associated with oral or facial infection, and more than 80% of the human population worldwide is infected with this pathogen [1]. After primary

infection, HSV-1 establishes latency mainly in sensory ganglia. Sporadic reactivation of the latent virus leads to virus replication, followed by recrudescence infection. HSV-1 can also cause conjunctivitis, eczema herpeticum, and other diseases [2].

The treatment of HSV infection is currently based on the use of modified nucleosides or their prodrugs, which act mainly as inhib-

ABBREVIATIONS

ACV	acyclovir
ANVISA	Agência Nacional de Vigilância Sanitária
CHE	crude hydroethanolic extract
FIC	fractional inhibitory concentration
GAE	gallic acid equivalent
HRF	heterocyclic ring fission
MNPCE	micronucleated polychromatic erythrocyte
PDE	prodelphinidin
PCY	procyanidin
QM	quinone methide
RDA	retro-Diels-Alder
SD	standard deviation

itors of DNA polymerase [2], such as acyclovir. Nevertheless, the intensive use of these drugs has led to undesirable effects, such as drug-resistant strains, and this encourages the search for new antiviral drugs [3].

Schinus terebinthifolia Raddi, belonging to the Anacardiaceae family and popularly known as “Aroeira”, is used in folk medicine for its antimicrobial, anti-inflammatory, antiulcerogenic, febrifuge, analgesic, and depurative effects and to treat illnesses of the urogenital system [4–6].

According to de Lima et al. [7], the chemical analysis of an ethanolic extract from the bark of *S. terebinthifolia* revealed the presence of phenols, triterpenes, and anthraquinones, and this extract was active against *Staphylococcus aureus*. Jorge and Markmann [8] found that the leaves and bark of *S. terebinthifolia* were equally rich in tannins and essential oils, suggesting that its anti-inflammatory properties might enhance the healing effect of the tannins. Although diverse bioactivities have been identified in *S. terebinthifolia*, its antiviral activity has not yet attracted much attention.

The aim of the present study was evaluate the efficacy of CHE against HSV-1 *in vitro* and *in vivo* and establish its chemical composition based on liquid chromatography-diode array detector-mass spectrometry (LC-DAD-MS) and MS/MS.

Results and Discussion

The biological applications of *S. terebinthifolia* have been known for many years, and its properties have been described since the first edition of the Brazilian Pharmacopoeia that was published in 1926 [9].

Recently, the optimization of extraction solvent from the bark of *S. terebinthifolia* was developed to get a higher polyphenol concentration in the extracts, since they are related to its biological properties [10]. In this study, we found that the total phenolic content in CHE is 277.4 mg GAE · g⁻¹ (27.74%), which is consistent with that found by DiCiaula et al. [10] in which the total phenolic content corresponded to 29.39%.

Tannins are among the major compounds of *S. terebinthifolia* bark. The total tannin content in CHE found in this study was 124.1 mg GAE · g⁻¹. The biological activity of tannins is attributable to three properties: complexation with metal ions, an antioxidant effect, and the ability to complex with macromolecules, such as proteins and polysaccharides [11]. An antiherpetic effect has been reported for a few isolated tannins, which could be related to its ability to bind the protein coat of the virus or the membrane of the host cell [12–15].

Although proanthocyanidins have already been detected in the bark of *S. terebinthifolia* and are related to its anti-inflammatory, antibacterial, and antifungal properties [11, 15], the identification of tannin chemical structures has not been described. This is important because the degree of polymerization and composition of flavan-3-ol units have been reported to influence some biological properties [16–18].

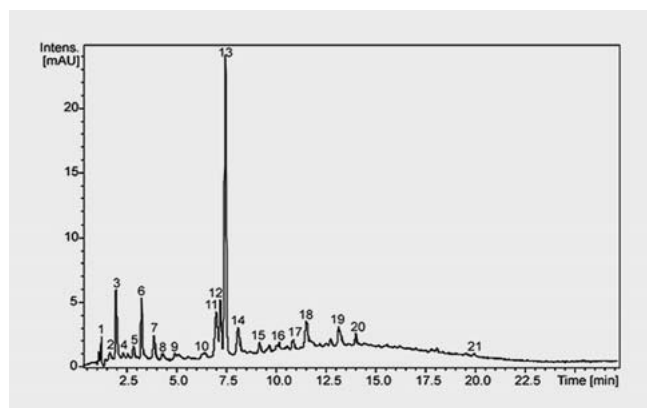
In the present study, 25 condensed tannins were identified in CHE (► **Table 1** and **Fig. 1**; **Figs. S1** and **S2**, Supporting Information) based on UV, MS, and MS/MS data compared with published data, [16, 19–21]. They were mainly produced by a B-type linkage and PDE and PCY units (► **Fig. 2**). The main compound that was identified in CHE was catechin (chromatographic peak 13, ► **Fig. 1**), and the other constituents produced a complex mixture that consisted of monomers (peaks 6 and 16), dimers (peaks 3, 6, 7, 8, 11, 12, 14, 17, 18, 20, and 21), and trimers (peaks 1–5, 9, 10, 14, 15, and 19) of flavan-3-ols (► **Table 1**). Therefore, for identification, we observed the accurate mass to find and confirm the molecular formulas, and observed protonated/desprotonated ions and fragmentation by HRF, RDA, and QM reactions [16, 19–22]. The loss of 168 and 152 *u* by the RDA reaction confirmed the presence of the units PDE and PCY, respectively, followed by the loss of a water molecule. For example, compound 11 (*m/z* 577 [M – H]⁻), similar to compound 12, produced intense fragment ions at *m/z* 425 [M – H-152]⁻ and 407 [M – H-152-H₂O]⁻, and compound 7 (*m/z* 595 [M + H]⁺) produced fragment ions at *m/z* 443 [M + H-152]⁺ and 425 [M + H-152-H₂O]⁺. The QM reactions produced the loss of flavan-3-ol units, confirming the top and terminal units, such as for compound 8 that produced fragment ions at *m/z* 307 and 289, which is compatible with PDE and PCY in the top and terminal positions (► **Fig. 3**).

Nocchi et al. [23] performed the bioguided fractionation of CHE and isolated catechin and galocatechin. Among them, only catechin exhibited anti-HSV-1 activity. Of the extract, fractions, and isolated compounds tested, the CHE showed the most promising results, so in the present study, the cytotoxicity and the antiviral activity of CHE were evaluated *in vitro* against different strains of HSV-1. CHE did not affect Vero cell viability at the highest concentration tested (CC₅₀ > 500 µg/mL) and had anti-HSV-1 activity against all of the tested strains (► **Table 2**). To elucidate the targets and its action mechanism, a set of experiments was performed to determine the stages of the viral replication cycle that CHE affects. The results showed that CHE exerted an inhibitory effect at multiple steps of the HSV-1 life cycle. Nocchi et al. [23], using a sulforhodamine B method, found that stem bark of *S. terebinthifolia* was active against a clinical strain of HSV-1, acting in the early stages of replication and also directly on the virus particle (► **Table 3** and **Fig. 4**). It is unclear, however, whether this ac-

► **Table 1** Identification of the constituents from hydroethanolic extract of stem bark of *S. terebinthifolia* by LC-DAD-MS/MS.

Peak	RT (min)	Compound	UV (nm)	FM	Negative mode (m/z)		Positive mode (m/z)	
					MS (*)	MS/MS	MS (*)	MS/MS
1	1.3	PDE-PDE-PDE (B-type)	280	C ₄₅ H ₃₈ O ₂₁	913.1853 (2.2 ppm)	–	915.1974 (0.1 ppm)	443, 425, 317, 305, 287, 263
2	1.7	PDE-PDE-PCY (B-type)	280	C ₄₅ H ₃₈ O ₂₀	897.1898 (1.6 ppm)	543, 467, 423, 407, 303, 289	899.2024 (0.6 ppm)	731, 595, 443, 425, 317, 305, 287
3	2.1	PDE-PDE (B-type)	280	C ₃₀ H ₂₆ O ₁₄	609.1248 (0.2 ppm)	423, 305	611.1369 (4.2 ppm)	425, 407, 299, 287, 275
		PDE-PCY-PCY (B-type)		C ₄₅ H ₃₈ O ₁₉	881.1922 (1.4 ppm)	577, 407, 289	883.2076 (0.4 ppm)	545, 533, 425, 409, 383, 365, 301, 287
4	2.4	PDE-PCY-PCY (B-type)	280	C ₄₅ H ₃₈ O ₁₉	881.1942 (0.8 ppm)	425, 407, 289	883.2049 (3.5 ppm)	441, 425, 409, 317, 305, 289, 287, 275
5	2.9	PCY-PCY-PCY (B-type)	280	C ₄₅ H ₃₈ O ₁₉	865.1971 (1.6 ppm)	577, 451, 407, 289	867.2097 (3.9 ppm)	697, 545, 409, 287
6	3.3	Gallocatechin st	280	C ₁₅ H ₁₄ O ₇	305.0667 (0 ppm)	219, 179, 165	307.0814 (0.5 ppm)	223, 195, 181, 163
		PDE-PCY (B-type)		C ₃₀ H ₂₆ O ₁₃	593.1285 (2.6 ppm)	423, 407, 303, 289	595.1425 (3.6 ppm)	443, 425, 305, 287, 275, 179
7	4.0	PDE-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₃	593.1279 (3.6 ppm)	423, 407, 305, 289	595.1417 (4.9 ppm)	443, 425, 409, 317, 305, 287, 275, 179
8	4.4	PCY-PDE (B-type)	280	C ₃₀ H ₂₆ O ₁₃	593.1272 (4.8 ppm)	423, 407, 305, 289	595.1437 (1.5 ppm)	425, 409, 307, 289, 275, 179
9	4.9	PDE-PDE-PCY (B-type)	280	C ₄₅ H ₃₈ O ₂₀	897.1884 (1.2 ppm)	407, 303, 289	899.2007 (2.5 ppm)	609, 595, 443, 423, 317, 305, 291, 287, 275
10	6.4	PDE-PCY-PCY (B-type)	280	C ₄₅ H ₃₈ O ₁₉	881.1873 (2.2 ppm)	407, 289	883.2061 (2.2 ppm)	595, 579, 425, 407, 305, 291, 287, 275
11	7.1	PCY-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₂	577.1316 (6.1 ppm)	425, 407, 339, 289	579.1486 (1.8 ppm)	427, 409, 291, 289, 275, 247
12	7.3	PCY-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₂	577.1312 (6.8 ppm)	407, 339, 289	579.1487 (1.7 ppm)	427, 409, 289, 275, 247
13	7.4	Catechin st	280	C ₁₅ H ₁₄ O ₆	289.0702 (5.4 ppm)	221, 203, 187, 175, 161, 151	291.0860 (1.0 ppm)	207, 189, 179, 165, 147
14	8.2	PCY-PCY-PCY (B-type)	280	C ₄₅ H ₃₈ O ₁₈	865.1952 (3.8 ppm)	407, 289	867.2139 (1.0 ppm)	409, 289, 275
		PDE-PCY-GAL		C ₃₇ H ₃₀ O ₁₇	745.1374 (4.9 ppm)	593, 575, 437, 407, 339, 289	747.1566 (1.4 ppm)	425, 299, 287, 275
15	9.2	PCY-PCY-PCY (B-type)	280	C ₄₅ H ₃₈ O ₁₈	865.1951 (3.9 ppm)	407, 289	867.2133 (0.2 ppm)	545, 527, 409, 289, 275
16	10.2	Epicatechin st	280	C ₁₅ H ₁₄ O ₆	289.0721 (1.3 ppm)	–	291.0866 (1.0 ppm)	–
17	10.8	PDE-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₃	593.1281 (3.4 ppm)	407, 289	595.1428 (3.1 ppm)	409, 317, 305, 291, 287, 275, 179
18	11.6	PCY-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₂	577.1336 (2.6 ppm)	407, 289	579.1490 (1.2 ppm)	409, 291, 289, 275, 257
19	13.2	PCY-PCY-GAL (B-type)	280	C ₃₇ H ₃₀ O ₁₆	729.1443 (2.5 ppm)	577, 559, 451, 433, 407, 289	731.1584 (3.1 ppm)	411, 291, 289
		PCY-PCY-PCY (B-type)		C ₄₅ H ₃₈ O ₁₈	865.1995 (1.1 ppm)	407, 289	867.2132 (0.5 ppm)	409, 291, 289, 275
20	14.0	PCY-PCY (B-type)	280	C ₃₀ H ₂₆ O ₁₂	577.1350 (0.3 ppm)	407, 289	579.1475 (3.8 ppm)	409, 291, 289, 271
21	20.3	PCY-PCY (A-type)	280	C ₃₀ H ₂₄ O ₁₂	575.1180 (2.6 ppm)	423	577.1334 (1.0 ppm)	425, 407

* Error in ppm; st: confirmed by authentic standard; PDE: prodelfinidin; PCY: procyanidin; GAL: galloyl



► **Fig. 1** Chromatographic profile at 280 nm of the hydroethanolic extract from *S. terebinthifolia*.

tivity is related to an interaction between CHE and virion envelope structures masking viral receptors, which are necessary for adsorption in host cells, or whether it is related to interference with cell structures that are related to the viral entry into the cell. In the present study, using the plaque reduction assay, we found that CHE did not inhibit the HSV-1 infectivity when the cells were pre-treated for 1 and 24 h before infection. However, CHE inhibited HSV-1 infectivity during the attachment and penetration stages (► **Table 3** and **Fig. 5**), thus blocking viral entry. This result was confirmed by the Western blot analysis of glycoproteins (gB, gC, and gD) from HSV-1 (► **Fig. 6**), in which the expression of the proteins decreased when CHE was added to cells during the adsorption and penetration steps, and when the viral particles were pre-treated before cell infection (virucidal assay).

Transmission electron microscopy revealed that the morphology of untreated HSV-1 was mostly conserved (► **Fig. 7 a, b**) and the treatment with 10 µg/mL CHE significantly changed the structure of HSV-1. Non-enveloped viruses (► **Fig. 7 c, d**) and an unstructured envelope (► **Fig. 7 c**) were systematically found in the preparations. These findings indicate that the inhibitory effects mainly depended on the ability of CHE to interact with the viral particles, rather than cellular components, thus preventing virus attachment to the cell surface and penetration into the cell. These findings, as well as those reported by Hong et al. [24] for the Hp 1036 and Hp 1239 peptides, indicate that CHE inactivates HSV-1 before its adsorption to the cell surface and effectively inactivates the virus that already bound to the cells. However, the possibility of alternative mechanisms of action cannot be ruled out considering that CHE is constituted of a complex mixture of compounds, which may act against different molecular targets, inhibiting viral attachment and penetration. In addition, the particles that were formed after a viral replication cycle were also exposed to the extract when released into the extracellular medium, thus interrupting the adsorption and penetration of the virus to other adjacent cells. This was confirmed by the plaque reduction assay and Western blot when CHE was added at the post-entry step of HSV-1 replication. In the plaque reduction assay, CHE inhibited the infectivity of HSV-1 strains at the post-entry step by approximately 40% (► **Fig. 8**). In the Western blot assay (► **Fig. 6**), CHE did not reduce

the expression of the proteins at a dose of 1 µg/mL. However, when 10 µg/mL CHE were added at the post-entry step, a significant reduction of the expression of the tested proteins was observed.

The susceptibility of the AR-29 strain (acyclovir-resistant strain) (► **Tables 2** and **3**) indicated that CHE has significant anti-HSV-1 activity with a mode of action that is different from ACV. This is highly important since there are huge difficulties for immunocompromised patient treatment in which the development of ACV-resistant strains is high. CHE also exhibited a synergistic antiviral effect with ACV *in vitro*, with an FIC value of 0.47. This synergism could be explained by the fact that the samples acted at different steps of HSV-1 replication. These results make CHE even more promising as a candidate for the treatment of HSV-1.

Some studies have evaluated the genotoxic/mutagenic effects of *S. terebinthifolia* [25–27], but not in mammalian cells. The present study performed a micronucleus test using bone marrow from rodents to evaluate the possible genotoxic effects of CHE. This test is recommended by the ANVISA [28] when herbal medicines for topical use are indicated for continuous or prolonged use in humans, as is the case of immunocompromised patients, particularly those with a coinfection with HIV/HSV. Micronuclei consist of cytoplasmic chromatin that appears as a result of chromosome breakage by clastogenic induction or from changes in mitosis (aneuploidy). The presence of micronuclei can be used as an indicator of the effects of DNA-damaging agents. [29, 30]. Our results showed that CHE exerted no genotoxic effects at the concentrations tested (► **Table 4**). No significant differences in the frequency of MNPCEs were observed compared with the negative control.

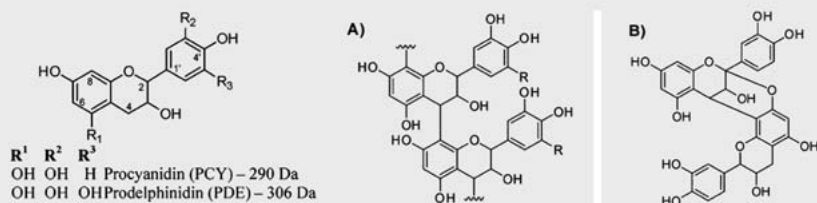
In the *in vivo* antiviral activity assay, CHE also reduced the lesions caused by HSV-1 (► **Fig. 9**). Compared with the untreated control group, herpetic lesions in the CHE-treated animals took longer to appear and lesions were softer and resolved more quickly. The animals treated with the highest dose of CHE had fewer lesions that were softer than the treatment with the lower dose, and, thus, suggest its effects are dose-dependent. Additionally, no significant difference was found between the groups that were treated with CHE and ACV.

The development of an alternative topical treatment for HSV-1-induced lesions is important for public health and has important economic and social relevance. For the low-income population, medicinal plants are an important social and cultural component and sometimes are the only alternative for the treatment of health problems. Although much work still needs to be done to develop medications that contain CHE as a topical antiherpes therapy, it is worthy to establish the safety and efficacy of the preparations from the stem bark of *S. terebinthifolia* used in traditional medicine for the treatment of HSV-1 infections.

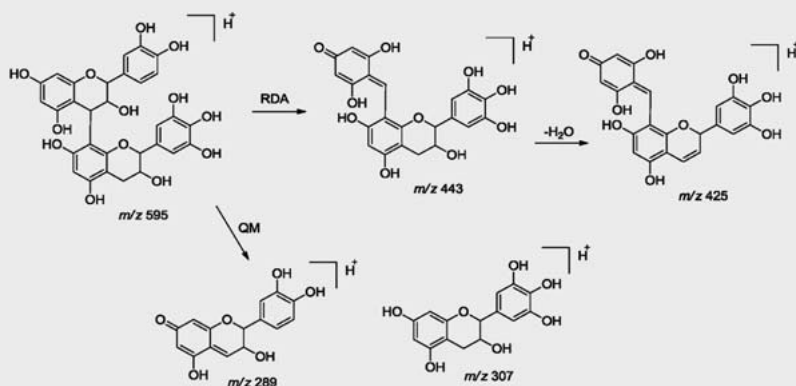
Materials and Methods

Plant material and extract preparation

Bark from the stems of *S. terebinthifolia* was collected at the Universidade Estadual de Maringá (UEM) campus, Maringá, Paraná, Brazil, in December 2011. The species was identified by Prof. Dr.



► **Fig. 2** Monomers of condensed tannins from *S. terebinthifolia*; typical linear type B (A) and type A (B).



► **Fig. 3** Some characteristic fragmentation pathways for B-type proanthocyanidins. RDA: retro-Diels-Alder, QM: quinone methide.

► **Table 2** Effects of CHE and ACV on cell growth and replication of HSV-1.

Sample	Strain	CC ₅₀ (µg/mL) ^a	EC ₅₀ (µg/mL) ^b (95% C. I.) ^c	SI ^d
CHE	HSV-1 KOS	> 500	10.20 (9.40–11.56)	> 49.0
	HSV-1 clinical strain	> 500	14.20 (10.10–18.36)	> 34.9
	HSV-1 AR-29	> 500	13.90 (11.35–15.51)	> 35.9
ACV	HSV-1 KOS	> 1000	0.35 (0.24–0.46)	> 2857.1
	HSV-1 clinical strain	> 1000	2.10 (1.71–2.71)	> 476.1
	HSV-1 AR-29	> 1000	–	–

CHE: crude hydroethanolic extract from *S. terebinthifolia*. ACV: acyclovir.
^a CC₅₀: 50% cytotoxic concentration for Vero cells in µg/mL, determined by the SRB assay. ^b EC₅₀: concentration that inhibited 50% of plaque formation, determined by plaque reduction assay. ^c 95% C. I.: 95% confidence interval. ^d SI: Selectivity index determined by the ratio of CC₅₀ to EC₅₀ (CC₅₀/EC₅₀). The values represent the mean of three independent experiments.

Maria Auxiliadora Milaneze Gutierre and a voucher was deposited in the herbarium of UEM (HUEM no. 22057).

The bark was air-dried and pulverized (Tigre ASN-5). The extraction was performed according to Nocchi et al. [23], yielding 24.3% of dry residue.

Total phenolic content

As similarly proposed by Herald et al. [31], 50 µL of deionized water, sample, or standard, and Folin-Ciocalteu reagent (1 : 1 v/v, deionized water) were added in each well. After 6 min, 100 µL of Na₂CO₃ at 75 g/L were added to the mixture in the wells, mixed again, and after 90 min (dark), it was measured (at 765 nm by spectrophotometric microplate reader). The analyses were performed in triplicate. The total phenolic content is expressed as mg (milligrams) of GAE · g⁻¹ (per gram) of CHE.

Total tannin content

The CHE was solubilized in water (4 mg/mL), placed in contact with skin powder (20 mg, Hide Powder- protease substrate purchased from Sigma-Aldrich), and stirred for 60 min. After centrifugation, the supernatant was used to follow the phenolic content described above. The concentration of total tannins was calculated as the difference of the concentration of total phenolic and no tanning phenols. Total tannin content is expressed as mg of GAE · g⁻¹ of CHE.

► **Table 3** Mode of antiviral activity of CHE.

Mechanism of action	EC ₅₀ (µg/mL) ^a (95% C.I.) ^b		
	Clinical strain	KOS strain	AR-29 strain
Pretreatment (1 h)	–	–	–
Pretreatment (24 h)	–	–	–
Attachment	0.24 (0.17–0.33)	0.22 (0.20–0.24)	0.25 (0.13–0.37)
Simultaneous	0.26 (0.24–0.28)	0.21 (0.05–0.38)	0.20 (0.05–0.39)
Post-entry	–	–	–
Virucidal	0.21 (0.19–0.25)	0.17 (0.14–0.21)	0.18 (0.01–0.34)

CHE: crude hydroethanolic extract from *S. terebinthifolia*. ^aEC₅₀: concentration that inhibited 50% of plaque formation, determined by plaque reduction assay. ^b95% C.I.: 95% confidence interval. The values represent the mean of three independent experiments.

Identification of constituents by liquid chromatography-diode array detector-mass spectrometry

A Shimadzu Prominence UFLC Shimadzu device coupled to a diode array detector (DAD) and mass spectrometer MicrOTOF-Q III (Bruker Daltonics) was used. The column was a Kinetex C18 column (2.6 µm, 150 × 2.1 mm, Phenomenex). The injection volume, flow rate, and oven temperature were 1 µL, 0.3 mL/min, and 50 °C, respectively. The mobile phase consisted of acetic acid 1% (v/v) in both water (solvent A) and acetonitrile (solvent B), using a gradient elution profile (0–2 min: 3% B; 2–25 min: 3–25% B; 25–40 min: 25–80% B; 40–43 min: 80% B). The analyses were performed in negative and positive ion modes. Nitrogen was applied as a nebulizer gas (4 Bar) and dry gas (9 L/min). The capillary voltage was 2.5 kV. The extract was solubilized (2 mg/mL), filtered and injected.

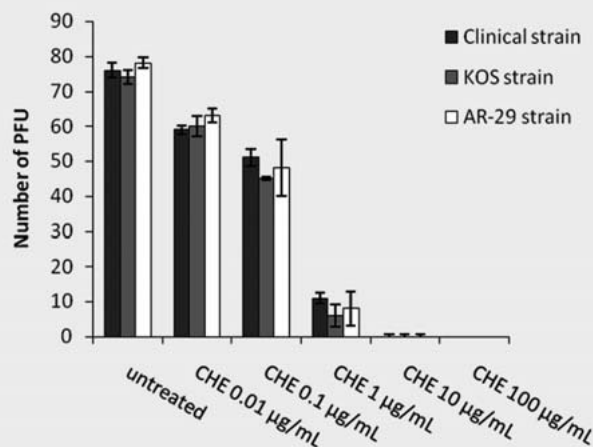
Cells and viruses

Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated FBS (Gibco) and 50 µg/mL gentamicin in an incubator at 37 °C with 5% CO₂ and 95% relative humidity.

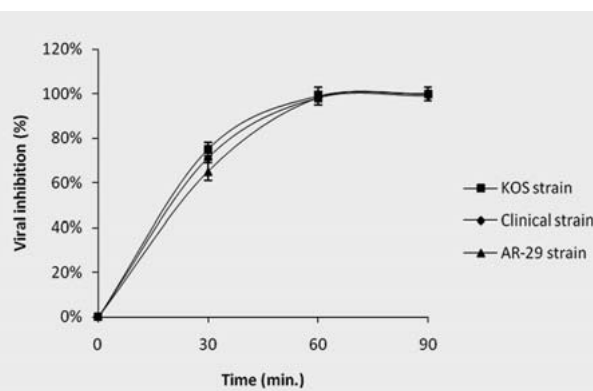
Three strains of HSV-1 were used in the experiments: HSV-1 KOS strain and AR-29 strain (which is resistant to ACV) were kindly provided by Prof. Dr. Marcelo Alves Pinto, Oswaldo Cruz Institute/Oswaldo Cruz Foundation – IOC/FIOCRUZ, Rio de Janeiro, Brazil, and a clinical strain (that is more resistant to ACV than KOS strain, but more sensible to ACV than AR-29 strain) was kindly provided by the Virology Department of the State University of Londrina, Paraná, Brazil. HSV-1 stocks were propagated in Vero cells and titrated. Afterward, the virus was stored at –20 °C until use.

Cytotoxicity assay

Cell viability was assessed by the sulforhodamine B assay as described by Camargo-Filho et al. [32].



► **Fig. 4** Virucidal activity of crude hydroethanolic extract (CHE) from *S. terebinthifolia* on different strains of HSV-1. The values represent the mean (± SD) of three independent experiments.



► **Fig. 5** Penetration assay. CHE from *S. terebinthifolia* was added after attachment of HSV-1 to cells at 4 °C for 1 h. Penetration was initiated by a temperature shift to 37 °C and stopped after 30, 60, and 90 min by washing the cells with pH 3.0 citrate buffer. The values represent the mean (± SD) of three independent experiments.

Determination of *in vitro* antiviral activity by the plaque reduction assay

This assay followed the procedures described by Cheng et al. [33] with minor modifications. Vero cells were seeded in 24-well microplates at 2.5×10^5 cells/well and infected with 60–80 PFU HSV-1/well. After 1 h, the infected cells were overlaid with different concentrations of CHE in medium containing carboxymethylcellulose (CMC). After 72 h, the cells were fixed with 10% formaldehyde, and the overlay medium was removed. Cells were stained with 0.5% crystal violet in 20% ethanol, and the plaques were counted under a magnifying glass. The CC₅₀ (cytotoxic concentration for 50% of the cells) and EC₅₀ (effective concentration that inhibited 50% of plaque formation) were estimated by non-linear regression of concentration-response curves. ACV (Sigma-

Aldrich, $\geq 99\%$ of purity) was used as a positive control. The selectivity index was calculated as the ratio of CC_{50} to EC_{50} .

Mode of antiviral action

In order to determine the mode of antiviral action of CHE, the plaque reduction assay was used. Cells and viruses were incubated with CHE at different stages during the viral replication cycle: *Pre-treatment* – cells were treated with CHE for 1 or 24 h prior to viral infection; *Attachment* – cells were treated with CHE concomitantly with viral adsorption at 4 °C; *Simultaneous treatment* – cells were treated with CHE concomitantly with viral infection at 37 °C; *Post-entry* – cells were infected with HSV-1 for 1 h and then were treated with CHE; *Virucidal* – the viral suspension was treated with CHE at 37 °C for 1 h and then this virus suspension was used to infect the cells.

Penetration assay

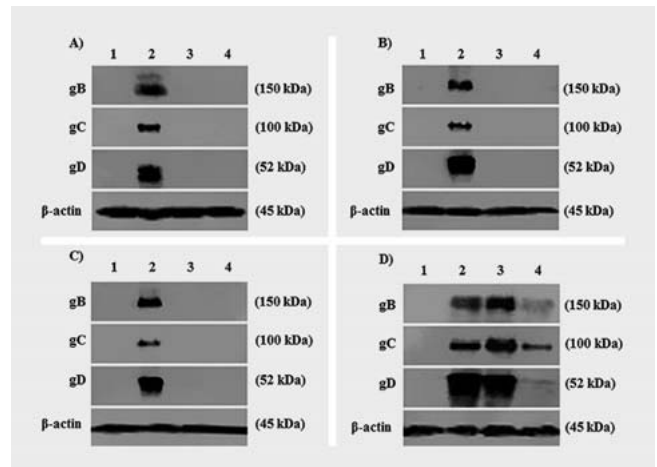
This assay was performed according to Gescher et al. [12] with modifications. Vero cells were prechilled at 4 °C for 1 h and infected with 60–80 PFU HSV-1 at 4 °C for 1 h to allow the attachment of HSV-1. The unbound viruses were removed, and the cells were treated with 10 $\mu\text{g}/\text{mL}$ CHE. The control group contained no CHE. The infected cells were incubated at 37 °C to maximize virus penetration. After 0, 30, 60, and 90 min, the infected cells were treated with citrate buffer (pH 3.0) for 1 min to inactivate the non-penetrated virus. The cells were washed with PBS and covered with medium. The virus control omitted CHE treatment, and the citrate buffer control omitted the citrate buffer. After 72 h of incubation, the cells were fixed and stained as described for the plaque reduction assay.

Evaluation of combination of CHE plus ACV

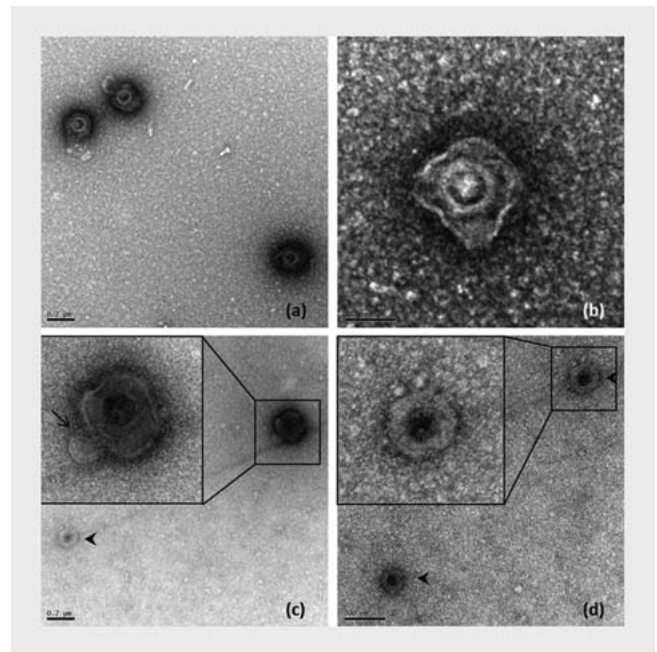
The effects of CHE combined with ACV were evaluated by the plaque reduction assay according to Chou [34] with minor modifications. Each sample alone or combined was tested at an equipotency ratio based on its corresponding EC_{50} value. The degree of interaction between CHE and ACV was calculated as the FIC and interpreted according to the combined FIC index ($FIC_{\text{CHE}} + FIC_{\text{ACV}}$) as synergism (≤ 0.5), no interaction (0.5–4), or antagonism (> 4) [35].

Western blot

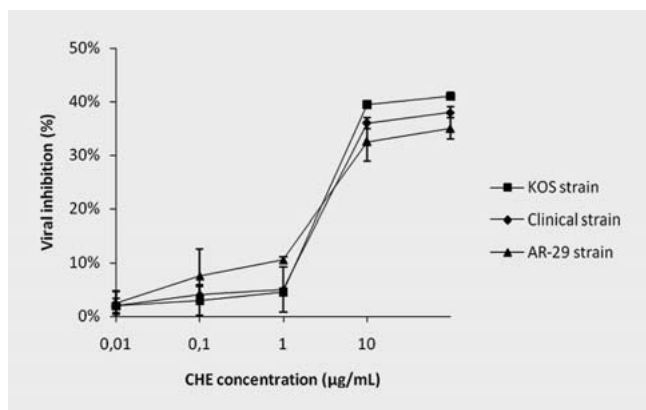
Western blot was performed as described by Cardozo et al. [36] with modifications. The density of the Vero cells was adjusted to 2.5×10^5 cell/mL and this suspension was added (2 mL/well) into a six-well flat-bottomed plate (TPP) without or with HSV-1 (at a multiplicity of infection [MOI] of 0.1). CHE (10 $\mu\text{g}/\text{mL}$) was added to the cells at different stages of viral replication (during adsorption at 4 °C, during early stages of replication, directly on virus particles, and at the post-entry step). The plates were then incubated in a 5% CO_2 humidified atmosphere at 37 °C for 48 h. The cells were harvested and lysed with lysis buffer (0.125 M Tris-HCl [pH 7.4], 30% glycerol, 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride, 2% sodium dodecyl sulfate [SDS], and 5% β -mercaptoethanol). The cell lysates were clarified by centrifugation and boiled, and equivalent amounts of proteins (15 μg) were separated on 10% SDS-PAGE. The proteins were transferred to polyvinylidene di-



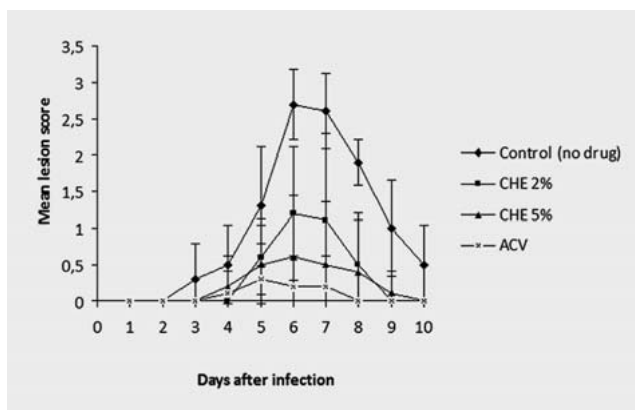
► **Fig. 6** Effect of CHE from *S. terebinthifolia* on HSV-1 (KOS strain) protein expression. Treated and untreated cells were analyzed by SDS-PAGE/Western blotting using specific antibodies for viral (gB, gC, or gD) and cellular proteins (β -actin). A Cells were treated during adsorption at 4 °C. B Cells were treated during the early stages of replication (adsorption and penetration simultaneously). C Virus were treated before infected cells. D Cells were treated after infection. Column 1: uninfected and untreated cells (cellular control). Column 2: untreated infected cells (virus control). Column 3: infected cells treated with CHE (1 $\mu\text{g}/\text{mL}$). Column 4: infected cells treated with CHE (10 $\mu\text{g}/\text{mL}$).



► **Fig. 7** Transmission electron microscopy micrographs of HSV-1 (KOS strain) treated with CHE from *S. terebinthifolia* (concentration of 10 $\mu\text{g}/\text{mL}$) at 37 °C for 1 h before being negatively stained with 2% phosphotungstic acid. Viral particles without treatment (a, b); viral particles after treatment with CHE (c, d) 200 nm, (b) 100 nm. The arrow shows unstructured envelope (c) and the arrowhead shows non-enveloped virus (c and d).



► **Fig. 8** Post-entry assay. The cells were infected with HSV-1 for 1 h at 37 °C. Unbound viruses were removed and the cells were overlaid with fresh medium and incubated more 1 h at 37 °C to ensure the penetration of bound viruses into the cells. Then, the cells were treated with crude hydroethanolic extract (CHE) from *S. terebinthifolia* at different concentrations. The values represent the mean (\pm SD) of three independent experiments.



► **Fig. 9** Effect of crude hydroethanolic extract (CHE) from *S. terebinthifolia* on skin lesions caused by HSV-1 in BALB/c mice. BALB/c mice were infected by the cutaneous route with 10^6 PFU HSV-1 and topically treated with 2% CHE, 5% CHE, or ACV (Zovirax cream 50 mg/g) 1 h after infection and 5 times daily for 10 days. Statistical analysis (Kruskal-Wallis test – comparison between groups) demonstrated significant differences between the control and test groups. The groups treated with 2% CHE, 5% CHE, and ACV showed no significant difference between them ($p < 0.05$). Each point represents the mean value of 10 animals per group (\pm SD).

► **Table 4** Frequency of micronucleus in 2000 polychromatic erythrocytes (MNPCE) in bone marrow of mice after 24 h oral treatment with CHE at different doses.

Treatment	MNPCE (mean \pm S. D.)
CHE 2 g · kg ⁻¹	3.4 \pm 0.6 ^a
CHE 5 g · kg ⁻¹	3.5 \pm 0.6 ^a
C (+): cyclophosphamide 40 mg · kg ⁻¹	10.2 \pm 1.7 ^b
C (-): distilled water	2.2 \pm 0.7

CHE: crude hydroethanolic extract from *S. terebinthifolia*. C(+): positive control; C(-): negative control; MNPCE: micronucleated polychromatic erythrocytes; S. D.: standard deviation. ^aNo significant difference when compared to the negative control and significant difference when compared to the positive control (ANOVA or Kruskal-Wallis test; $p < 0.05$). ^bSignificant difference when compared to the negative control (ANOVA test; $p < 0.05$). The values represent the mean (\pm SD) of each group ($n = 10$ animals per group).

fluoride (PVDF) membranes (Millipore) and blocked with 5% albumin in blotting buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated for 90 min with the following primary antibodies: mouse monoclonal antibody against gB, gC, gD, and β -actin (Santa Cruz Biotechnology). After washing, the membranes were incubated with respective goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology) secondary antibodies for 1 h. The immunoblots were developed and detected using ECL Plus Western Blotting Detection Reagent (GE Life Science) and read using the STORM 860 Capture System (GE Healthcare).

Transmission electron microscopy

This assay was performed as described by Hong et al. [24] with minor modifications. The virus particle stock (HSV-1 KOS strain) cultivated in the media was either treated or untreated with CHE (10 μ g/mL) and incubated for 1 h at 37 °C. The samples were dripped onto 200-mesh formvar/carbon-coated copper grids. Each sample was negatively stained by placing a drop of 2% phosphotungstic acid (pH 7.2) on the grid. The sample grid was exposed to UV light (20 cm) for 10 min and air-dried. Finally, various areas of the grid were examined with a JEOL JEM-1400 transmission electron microscope that operated at 80 kV, and photographs were obtained.

Animals

All of the animal studies were approved by the Animal Ethics Committee of UEM under the number 018/2013 (March 13, 2013) and conducted in accordance with internationally accepted principles for laboratory animal care and use (EEC Directive of 1986; 86/609/EEC). The animals were obtained from the Central Animal House of UEM. The animals were housed under a 12-h/12-h light/dark cycle for at least 5 days before starting the experiments, with food and water available *ad libitum*. The temperature in the room was kept at 22 ± 2 °C.

Micronucleus assay

Female and male *Mus musculus* (Swiss) mice (8–12 weeks old; 25–40 g; $n = 10$ per group) were orally treated by gavage with 2.0 and 5.0 g/kg CHE and sacrificed 24 h after administration. A positive control group (40 mg/kg cyclophosphamide [Genuxal, Baxter Oncology]) and negative control group (distilled water) were also included. After the treatment period, the femurs were exposed

and sectioned, and the bone marrow was gently flushed out using FBS [37, 38].

After centrifugation at $3000 \times g$ for 5 min, the bone marrow cells were smeared on glass slides, coded for blind analysis, and air-dried. The smears were stained with May-Grunwald-Giemsa to detect MNPCEs. Three slides were prepared for each animal, and 2000 PCEs were counted to determine the frequency of MNPCEs. The slides were analyzed using an Olympus BH-2 microscope.

In vivo antiviral activity

This experiment was performed as described by Kurokawa et al. [39], Lipipun et al. [40], and Chunasa et al. [41] Female BALB/c mice (5 weeks old; 18–21 g; $n = 10$ per group) were used. The right midflank of each mouse was clipped and depilated. Three days later, the skin was scratched in a grid-like pattern using a sterile 27-gauge needle, and 20 μL of HSV-1 (KOS strain; 1×10^6 PFU) was applied to the scarified area. CHE was suspended in sterile water before administration. The mice were randomly divided into four groups: (i) control group – infected but received no treatment, (ii) group infected and treated with 2% CHE, (iii) group infected and treated with 5% CHE, and (iv) group infected and treated with ACV (Zovirax cream 50 mg/g, GlaxoSmithKline). CHE or ACV was administered topically with a sterile swab 1 h after HSV-1 inoculation and 5 times daily for 10 successive days. The appearance of skin lesions was continuously observed and scored daily as follows: 0, no lesion; 1, vesicles in local region; 2, erosion and/or ulceration in the local region; 3, mild zosteriform lesion; 4, moderate zosteriform lesion; 5, severe zosteriform lesion or death.

Statistical analysis

For the micronucleus test, homogeneous data were analyzed using one-way analysis of variance (ANOVA), and intergroup differences were analyzed using Dunnett's test. Heterogeneous data were analyzed using the Kruskal-Wallis test. For the *in vivo* antiviral activity assay, the Kruskal-Wallis nonparametric test for comparisons between groups was used. All of the statistical tests were performed at a $p < 0.05$ level of significance, and the results are expressed as mean \pm SD.

Supporting information

Chromatographic profiles and total ion chromatograms of the CHE of *S. terebinthifolia*, as well as the EC_{50} values of the CHE for different strains are available in Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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