Development and evaluation of topical formulations that contain hydroethanolic extract from *Schinus terebinthifolia* against HSV-1 infection

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The therapeutic drugs to treat *Herpes simplex* virus (HSV) infections have toxic side effects and there has been an emergence of drug-resistant strains. Therefore, the search for new treatments for HSV infections is mounting. In the present study, semi-solid formulations containing a crude hydroethanolic extract (CHE) from *Schinus terebinthifolia* were developed. Skin irritation, cutaneous permeation, and *in vivo* therapeutic efficacy of the formulations were investigated. Treatment with the ointment formulations did not result in any signs of skin irritation while the emulsions increased the thickness of the epidermis in Swiss mice. The cutaneous permeation test indicated that the CHE incorporated in the formulations permeated through the skin layers and was present in the epidermis and dermis even 3 h after topical application. *In vivo* antiviral activity in BALB/c mice treated with the CHE ointments was better than those treated with the CHE emulsions and did not significantly differ from an acyclovir-treated group. Taken together, this suggests that the incorporation of CHE in the ointment may be a potential candidate for the alternative topical treatment of herpetic lesions.

Keywords: HSV-1. *Schinus terebinthifolia*. Topical formulations. Skin irritation. Photoacoustic. *In vivo* efficacy.

INTRODUCTION

Infections caused by *Herpes simplex* virus type 1 (HSV-1) are common in the population and typically appear as oral lesions known as "cold sores". Recurrent episodes of herpetic lesions are usually painful and long lasting, which may cause discomfort or embarrassment to the individual (Arduino, Porter, 2008; Pierre, Bartlett,

Schlosser, 2009). The episodes of herpetic lesions are more severe in immunocompromised patients, involving the oral cavity and extending throughout the face (Fatahzadeh, Schwartz, 2007; Cunningham *et al.*, 2012).

Herpetic lesions are treated using oral or topical antivirals. The goal of antiviral therapy is to block viral replication, seeking to shorten the duration of symptoms and accelerate the healing of lesions (Cunningham *et al.*, 2012; Spruance *et al.*, 1977). Some topical therapies are available for cold sores, such as acyclovir, penciclovir, and docosanol. However, the emergence of strains that are resistant to conventional treatments has

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made it more difficult to treat lesions, particularly in immunocompromised patients (De Clercq, 2004).

Schinus terebinthifolia Raddi, popularly known as "aroeira," is used in folk medicine for its antimicrobial, antiinflammatory, antiulcerogenic, febrifuge, analgesic, and depurative effects and to treat illnesses of the urogenital system and mucosal lesions (Cavalher-Machado *et al.*, 2008; Lorenzi, Matos, 2008; Soares *et al.*, 2007; Guerra *et al.*, 2000). Antihistaminic (Nunes-Neto *et al.*, 2017) and antihypertensive (Glória *et al.*, 2017) activities have also been attributed to *S. terebinthifolia* in recent studies.

In previous studies the chemical composition of the crude hydroethanolic extract (CHE) from stem bark of *S. tereninthifolia* based on liquid chromatography-diode array detector-mass spectrometry (LC-DAD-MS) and MS/MS was established, which indicated that this extract is rich in condensed tannins (Nocchi *et al.*, 2017). Furthermore, this extract was shown to be active against HSV-1 *in vitro* and *in vivo* (Nocchi *et al.*, 2016; Nocchi *et al.*, 2017).

The present study evaluated the anti-HSV-1 activity of the CHE when incorporated into semi-solid formulations for topical use. In addition to developing formulations that contain the extract, skin irritation, cutaneous permeation, and *in vivo* therapeutic efficacy were evaluated.

MATERIAL AND METHODS

Plant material and extract preparation

Bark from the stems of *S. terebinthifolia* was collected from the Universidade Estadual de Maringá (UEM) campus, Maringá, Paraná, Brazil, in December 2011. The species was identified by Prof. Dr. Maria Auxiliadora Milaneze Gutierre, and a voucher specimen was deposited in the herbarium of UEM (HUEM no. 22057). The bark was air-dried and pulverized (Tigre ASN-5). Extraction was performed according to Nocchi *et al.* (2017), yielding 24.3% of dry residue. The chemical composition of this extract was determined based on liquid chromatography-diode array detector-mass spectrometry (LC-DAD-MS) and MS/MS (Nocchi *et al.*, 2017).

Topical formulations

Excipients that were used to develop the formulations were chosen in order to provide a film of the product with water resistance and adhesion in the region of the lesions caused by HSV-1. Raw materials for the formulations were commercially available. The formulations were produced by conventional techniques. An ointment and a non-ionic o/w emulsion (base formulation) were developed with the following compositions:

Base ointment (BO): lanolin (25.00%), paraffinum liquidum and polyethylene (Crodobase SQ; 20.00%), acetylated lanolin alcohol (Crodalan LA; 5.00%), petrolatum (30.00%), caprylic/capric triglyceride (2.00%), propylparaben (0.20%), butylated hydroxytoluene (BHT; 0.05%), paraffinum liquidum (17.75%).

Base emulsion (BE): ceteareth-20 (5.81%), glyceryl stearate (4.19%), cetyl alcohol (1.50%), decyl oleate (10.00%), caprylic/capric triglyceride (2.00%), PVP/eicosene copolymer (3.00%), propylparaben (0.10%), BHT (0.05%), stearic acid (4.00%), propylene glycol (10.00%), disodium ethylenediaminetetraacetic acid (0.10%), methylparaben (0.20%), imidazolidinyl urea (0.50%), water (58.55%).

The formulations that contained the extract were produced by incorporating the CHE of *S. terebinthifolia* (2% and 5%, w/w) in the base formulations at room temperature.

Animals

All of the animal experiments were approved by the Animal Ethics Committee of UEM (permission no. 018/2013) and conducted in accordance with internationally accepted principles for laboratory animal care and use (EEC Directive of 1986; 86/609/EEC). The animals were maintained at 22.0 °C ± 2.0 °C under a 12 h/12 h light/dark cycle for at least 5 days before starting the experiments. Food and water were provided *ad libitum*.

Photoacoustic spectroscopy

Photoacoustic spectroscopy (PAS) was used to evaluate the cutaneous permeation of CHE in semi-solid formulations. The PAS measurements were performed using a monochromatic light from a 1000 W Xenon arc lamp with a stable power supply (Oriel Instruments, Stratford, CT, USA) and monochromator (Oriel Instruments, model 77250). The light beam was modulated with a mechanical chopper (Stanford Research Systems, Sunnyvale, CA, USA). The photoacoustic cell was constructed in-house from an aluminum block that

was machined to hold samples with maximum dimensions of ~5 mm diameter and 3 mm thickness, allowing light to enter through a highly transparent quartz window (6 mm diameter, 2 mm thickness). The microphone chamber was 15 mm away and connected to the sample holder chamber by a 1 mm diameter duct. The capacitive microphone had a 12 mm diameter (model 2639, Bruel & Kjaer, Norcross, GA, USA). The lock-in amplifier was obtained from EG & G Instruments (model 5110, North Branford, CT, USA). The photoacoustic spectra were obtained at a modulation frequency of 25 Hz and recorded between 200 and 800 nm. Data acquisition was performed with a computer, and the photoacoustic spectra were normalized to the carbon black signal as a function of the incident radiation wavelengths.

Determination of cutaneous permeation by photoacoustic spectroscopy

Male Wistar rats (8 weeks old, 250-350 g, n = 2-3 per group) were used to evaluate the skin permeation depth profile of the formulations by PAS. The amount of topically applied formulation was 2 or 5 mg/cm^2. The animals were divided into 25 groups according to topical treatment: (G1) no treatment (skin analysis), (G2) 2 mg/cm^2 BE for 40 min, (G3) 5 mg/cm^2 BE for 40 min, (G4) 5 mg/cm^2 BE for 90 min, (G5) 5 mg/cm^2 BE for 180 min, (G6) 2 mg/cm^2 emulsion that contained 2% extract (EE2) for 40 min, (G7) 5 mg/cm^2 emulsion that contained 2% extract (EE2) for 40 min, (G8) 5 mg/cm^2 emulsion that contained 2% extract (EE2) for 90 min, (G9) 5 mg/cm^2 emulsion that contained 2% extract (EE2) for 180 min, (G10) 2 mg/cm^2 emulsion that contained 5% extract (EE5) for 40 min, (G11) 5 mg/cm^2 emulsion that contained 5% extract (EE5) for 40 min, (G12) 5 mg/cm^2 emulsion that contained 5% extract (EE5) for 90 min, (G13) 5 mg/cm^2 emulsion that contained 5% extract (EE5) for 180 min, (G14) 2 mg/cm^2 BO for 40 min, (G15) 5 mg/cm^2 BO for 40 min, (G16) 5 mg/cm^2 BO for 90 min, (G17) 5 mg/cm^2 BO for 180 min, (G18) 2 mg/cm^2 ointment that contained 2% extract (OE2) for 40 min, (G19) 5 mg/cm^2 ointment that contained 2% extract (OE2) for 40 min, (G20) 5 mg/cm^2 ointment that contained 2% extract (OE2) for 90 min, (G21) 5 mg/cm^2 ointment that contained 2% extract (OE2) for 180 min, (G22) 2 mg/cm^2 ointment that contained 5% extract (OE5) for 40 min, (G23) 5 mg/cm^2 ointment that contained 5% extract (OE5) for 40 min, (G24) 5 mg/cm^2 ointment that contained 5% extract (OE5) for 90 min, and (G25) 5 mg/cm^2 ointment that contained 5% extract (OE5) for 180 min.

The animals were epilated in the cervical spine region. After 24 h, the formulations were applied topically on the clean surface of the skin in a defined area (1 cm × 1 cm). After the time of contact of the sample with the skin (40, 90, or 180 min), the animals were sacrificed, and the treated skin was removed. The photoacoustic measurements of the dissected skin (mean thickness of 663 µm) were performed approximately 15 min after sacrifice.

In vivo skin irritation assay

Healthy male Swiss mice (6 weeks old, 25-30 g, n = 5 per group) were used for the skin irritation test. The animals were divided into groups according to the following topical treatments: (G1) no treatment, (G2) aqueous solution that contained 2% extract, (G3) aqueous solution that contained 5% extract, (G4) BE, (G5) emulsion that contained 2% extract (EE2), (G6) emulsion that contained 5% extract (EE5), (G7) BO, (G8) ointment that contained 2% extract (OE2), and (G9) ointment that contained 5% extract (OE5).

The animals were epilated in the cervical region. After 24 h, the formulations were applied topically to the epilated area of each animal. The animals were treated once daily for 7 days and evaluated daily for signs of irritation (i.e., erythema, edema, and desquamation (Chorilli *et al.*, 2009). The skin was removed on day 8 for histological and morphometric analyses.

The skin samples were fixed in Bouin solution, dehydrated in ethyl alcohol solutions of increasing concentrations (70%, 80%, 90%, and 100%), diaphanized in xylol, and embedded in paraffin. Semi-serial histological sections (5 µm thickness) were stained with hematoxylin-eosin (HE).

The morphometric analysis was performed according to Endo *et al.* (2007) with minor modifications. The measurements of the epithelial thickness of the samples (Fig. 1) were performed using ImagePro Plus 4.5 software (Media Cybernetics) with a 40× objective. The images were captured with a BX-41 optical microscope (Olympus, Tokyo, Japan) with an Olympus Q Color 3 high-resolution camera. Two points (R1 and R2) were evaluated (Figure 1) with 100 measurements of R1 and 100 measurements of R2 for each animal, for a total of 1000 measurements per group.
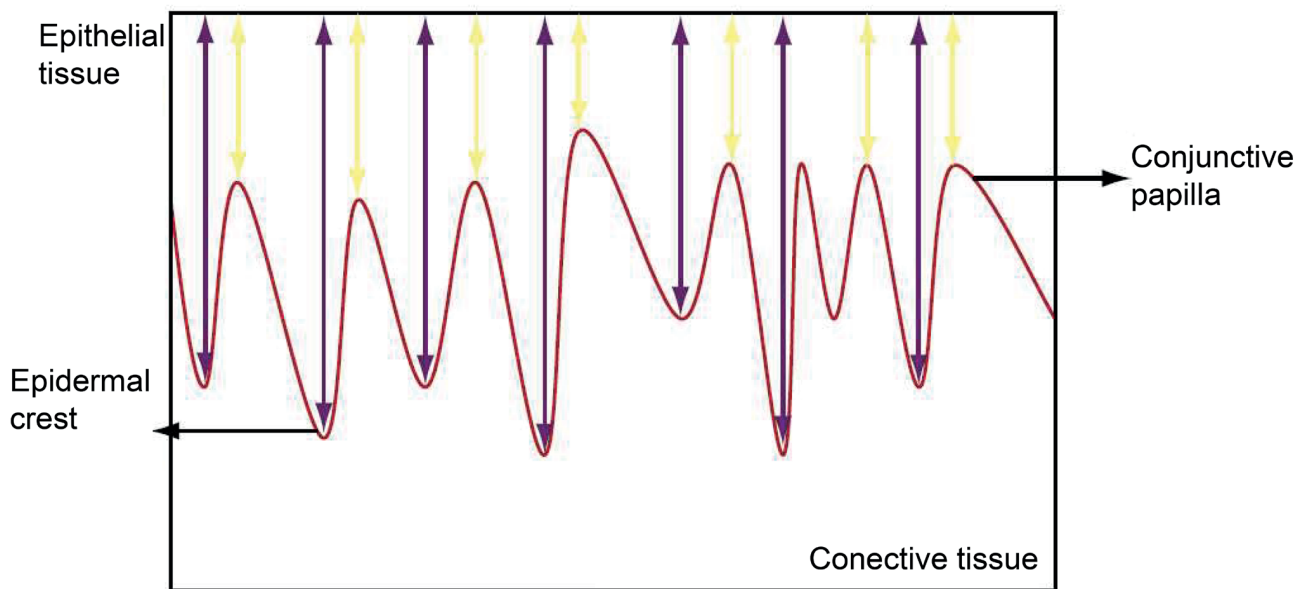
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FIGURE 1 - Representative scheme of the morphometric analysis performed on the histological sections of the skin of the Swiss mice. The purple-colored arrows represent the epidermal regions with epidermal ridge formation (R1) and the yellow arrows, epidermal regions with dermal papilla formation (R2). Adapted from Endo *et al.* (2009).

In vivo antiviral activity

This experiment was performed as described previously (Nocchi *et al.*, 2017; Chunasa *et al.*, 2008; Lipipun *et al.*, 2003; Kurokawa *et al.*, 1995). Male BALB/c mice (5 weeks old, 18-21 g, *n* = 10 per group) were used. The right midflank of each mouse was clipped and epilated. 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. The mice were randomly divided into groups: (G1) control group (infected and untreated), (G2) infected and treated with BE, (G3) infected and treated with emulsion that contained 2% extract (EE2), (G4) infected and treated with emulsion that contained 5% extract (EE5), (G5) infected and treated with BO, (G6) infected and treated with ointment that contained 2% extract (OE2), (G7) infected and treated with ointment that contained 5% extract (OE5), and (G8) infected and treated with acyclovir (Zovirax® cream). The treatments were applied topically with a sterile swab 1 h after HSV-1 inoculation and five times daily for 10 consecutive days. The appearance of skin lesions was continuously observed and scored daily according to Chunasa *et al.* (2008) and Lipipun *et al.* (2003) as follows: 0, no lesion;

1, vesicles in local region; 2, erosion and/or ulceration in local region; 3, mild zosteriform lesion; 4, moderate zosteriform lesion; 5, severe zosteriform lesion or death.

Statistical analysis

For skin irritation assay, the comparison between the treatments in the R1 and R2 regions of the epidermis, in relation to the thickness, was performed by one-way ANOVA followed by the Tukey post-test. For the *in vivo* antiviral activity assay, the comparison between the treatments was carried out by the Kruskal-Wallis nonparametric test. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The chemical composition of the CHE from *S. terebinthifolia* based on LC-DAD-MS and MS/MS, as well as its anti-HSV-1 activity *in vitro* and *in vivo*, was recently reported (Nocchi *et al.*, 2017).

In the present study this extract was incorporated into semi-solid formulations and skin irritation, cutaneous permeation, and therapeutic anti-HSV-1 efficacy *in vivo* were evaluated.

Determination of cutaneous permeation by photoacoustic spectroscopy

The photoacoustic spectra of the base formulations, BO and BE, and the extract in formulation, OE2, OE5, EE2, and EE5, were first obtained by PAS to verify the absorption profiles in the range of 200 to 800 nm (Figure 2) for further evaluation after application to the skin. The extract exhibited intense absorption from 200 to 600 nm, with Gaussian curves centered at 209, 239, 292, 357, 454, and 560 nm. The base formulations (BO and BE) did not absorb at wavelengths greater than 350 nm. Formulations that contained the extract (OE2, OE5, EE2, and EE5) exhibited absorption from 200 to 560 nm. The 450-560 nm region was thus selected, comprising regions where the base formulations were not absorbed.

For the evaluation of permeation profiles, the formulations were applied to the animals in different amounts (2 and 5 mg/cm^2). After different contact times, skin samples were removed and first excited on the epidermal face where the formulations were applied and then turned and excited on the dermal face to determine whether the substances propagated through the skin.

The results suggested the presence of the extract in the epidermis after 40 min of treatment, reflected by an increase in absorption in the region of 450 to 560 nm (spectral region where the epidermis and the base formulations were not absorbed) compared with the skin spectrum without application of any formulation (epidermis control). A higher amount of extract in the epidermis was observed for both the ointment and emulsion formulations containing 5% CHE. Increases in the amount of formulation that was applied also resulted in a higher concentration of the extract in the epidermis, reflected by an increase in optical absorption when 5 mg/cm^2 of the formulations was applied compared with 2 mg/cm^2 (Figure 3).

To evaluate permanence of the formulations on the epidermal surface over time, skin samples were removed 40, 90, and 180 min after the application of 5 mg/cm^2 of the formulations containing the CHE. The results indicated a decrease in the amount of the formulations in the epidermis as the contact time increased (Figure 4), indicating that the reduction was attributable to permeation to deeper layers over time.

The depth to be analyzed in the sample is known through the thermal diffusion length and is related to the light modulation frequency according to the following equation: $\mu_S = (D / \pi f)^{1/2}$, where f is the frequency, and D is the thermal diffusivity of the skin ($D_{human\ skin} = 4.0 \times 10^{-4} cm^2/s$ (Baesso, Snook, Andrews, 1994).

The thermal diffusion length at which the photoacoustic signal was generated was on the order of 23 μm, considering the thermal diffusivity of the skin and the 25 Hz modulation frequency. The greater absorption that was detected on the dermal face of the skin samples indicated that constituents of the formulations propagated through the skin and were present in the dermis (Tunin *et al.*, 2016).

Control skin samples that were excited on the dermal face exhibited absorption in the region of 200-300 nm. The absorption band was centered around 400 nm and associated with the presence of blood in the dermal region (Tunin *et al.*, 2016). The results showed the presence of the extract in the dermis when both ointment and emulsion formulations were applied at both 2 and 5 mg/cm^2, indicated by an increase in absorption in the 450 to 560 nm region compared with untreated skin (dermis control). As verified on the epidermal face, a greater amount of the extract in the dermis was observed 40 min after application of the formulations that contained 5% CHE and for the treatment with 5 mg/cm^2 of the formulation (Figure 5).

The results showed the presence of the extract in the dermis even 180 min after skin application of 5 mg/cm^2 of the formulations, both ointments and emulsions, and in a larger amounts when the formulations contained 5% CHE compared to 2% (Figure 6). After 180 min of topical treatment with OE2 and with EE2, a reduction in the amount of the extract that was present in the dermis was observed relative to the other time points. When OE5 and EE5 were applied, the amount of the extract that was present after 90 and 180 min was similar, which were both lower than 40 min of treatment.

Skin permeation depends on the skin composition, and the characteristics and concentration of both the active substance and the other components of the formulation, among other factors. The results indicated that the extract that was incorporated into both the ointment and emulsion permeated through different skin layers. The extract was present in the epidermis and dermis even 180 min after topical application. The amount of the extract that is present in the deep dermis depends on both the concentration of the CHE in the formulation and the amount of the formulation that is applied to the skin, in that larger amounts are present when higher concentrations are used and more is applied.
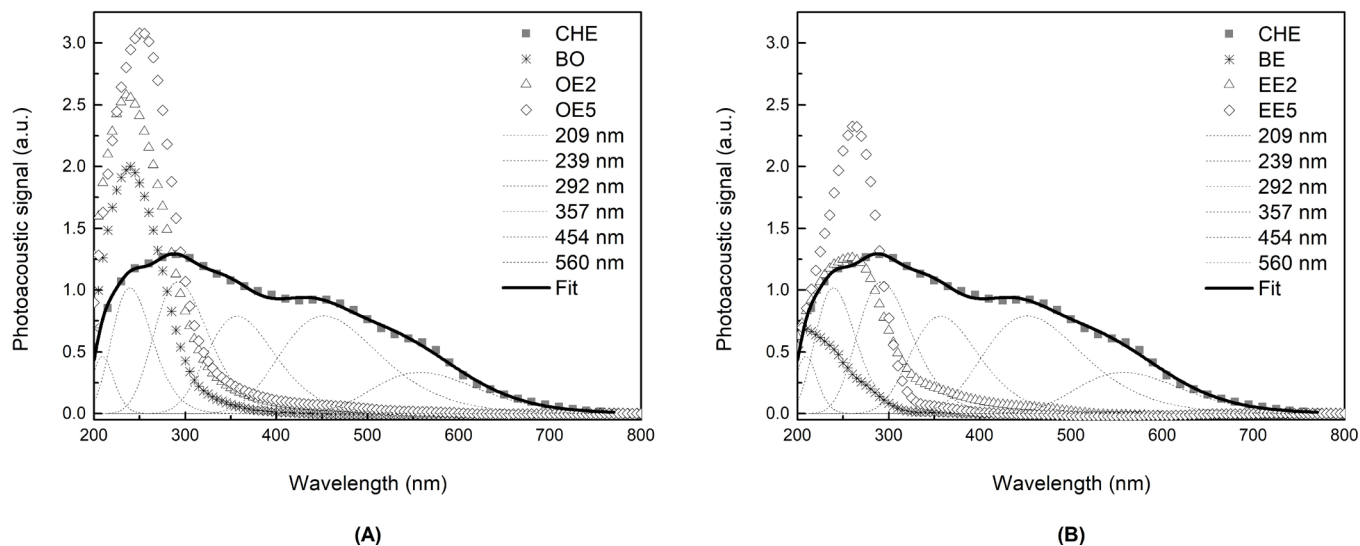
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(A) **(B)**

FIGURE 2 - Photoacoustic spectra of CHE from *S. terebinthifolia* and formulations developed. **A**: base ointment (BO), ointments containing 2% (OE2) and 5% (OE5) of CHE; **B**: base emulsion (BE), emulsions containing 2% (EE2) and 5% (EE5) of CHE.

(A) **(B)**
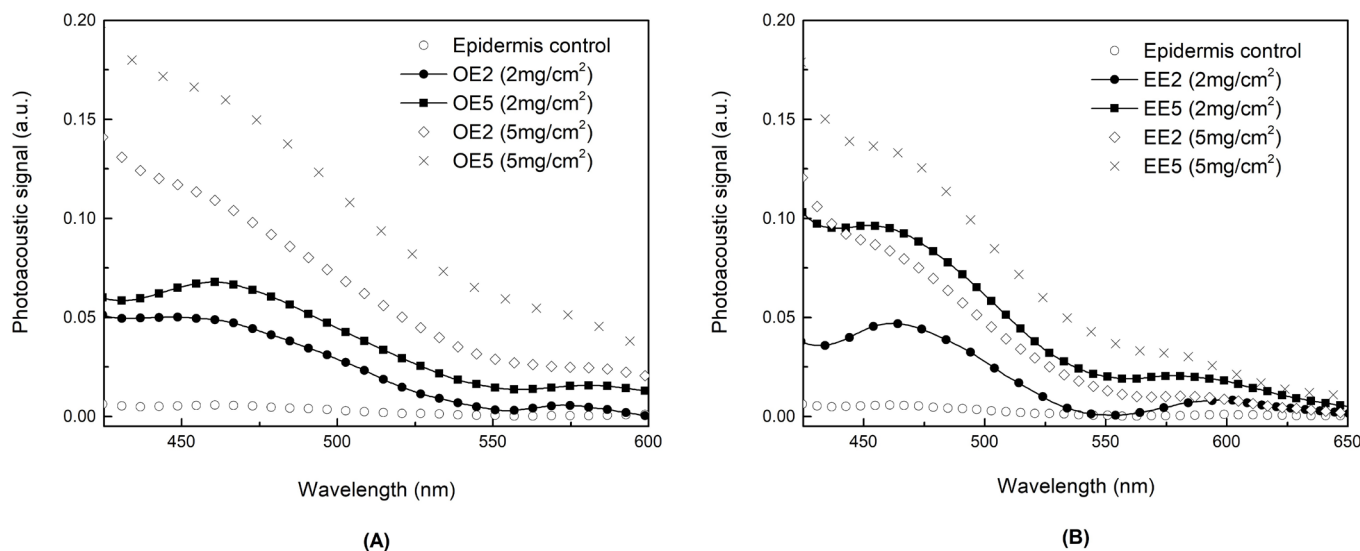
FIGURE 3 - Photoacoustic spectra of the epidermal face of skin samples obtained after 40 min of topical treatment with either 2 mg/cm^2 or 5 mg/cm^2 of the formulations containing CHE or without treatment (epidermis control). The light modulation frequency was 25 Hz and the thermal diffusion length was 23 μm. **A**: ointments containing 2% (OE2) and 5% (OE5) of CHE. **B**: emulsions containing 2% (EE2) and 5% (EE5) of CHE.
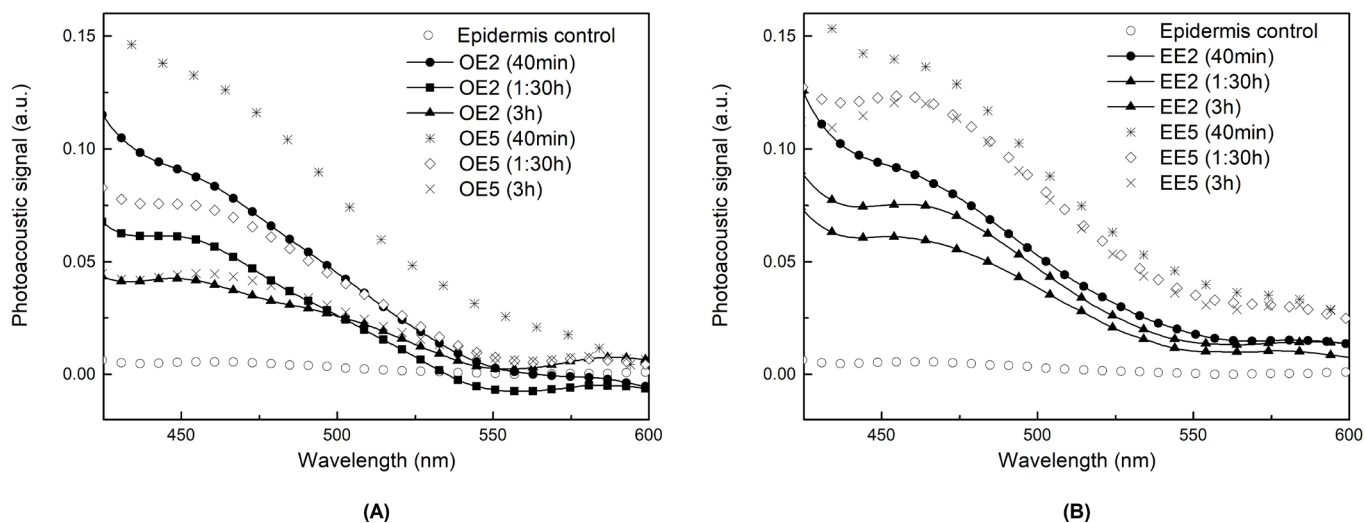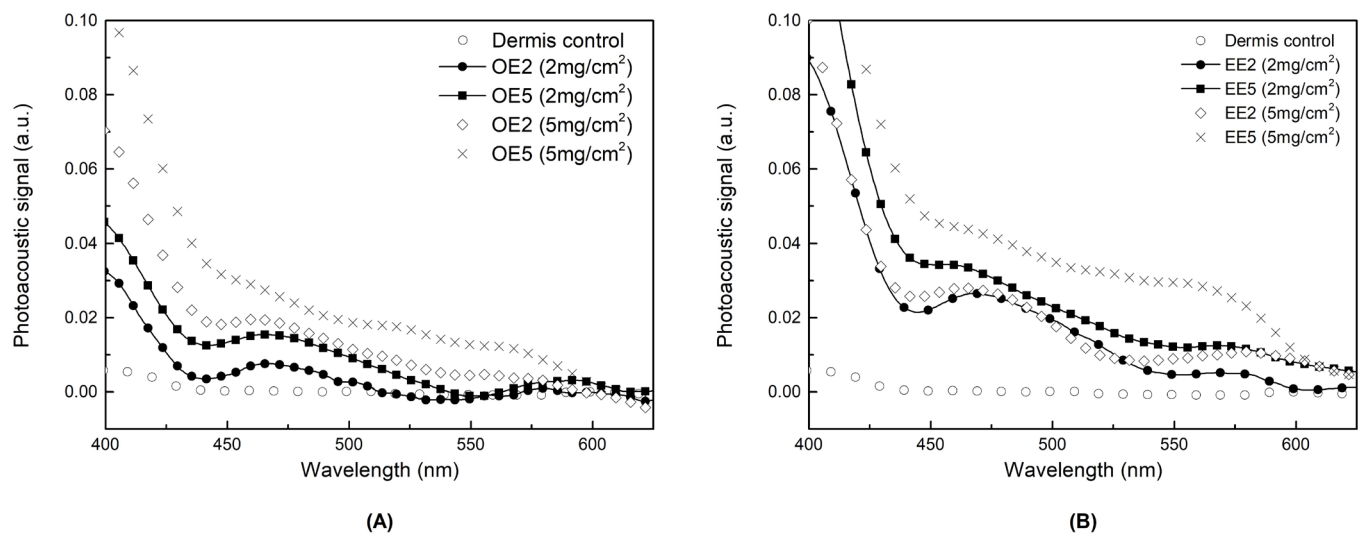
FIGURE 4 - Photoacoustic spectra of the epidermal face of skin samples obtained after 40 min, 90 min and 180 min of topical treatment with 5 mg/cm^2 of the formulations containing CHE and without treatment (epidermis control). The light modulation frequency was 25 Hz and the thermal diffusion length was 23 μm. **A**: ointments containing 2% (OE2) and 5% (OE5) of CHE. **B**: emulsions containing 2% (EE2) and 5% (EE5) of CHE.

FIGURE 5 - Photoacoustic spectra of the dermal face of skin samples obtained after 40 min of topical treatment with 2 mg/cm^2 and 5 mg/cm^2 of the formulations containing CHE and without treatment (dermis control). The light modulation frequency was 25 Hz and the thermal diffusion length was 23 μm. **A**: ointments containing 2% (OE2) and 5% (OE5) of CHE. **B**: emulsions containing 2% (EE2) and 5% (EE5) of CHE.
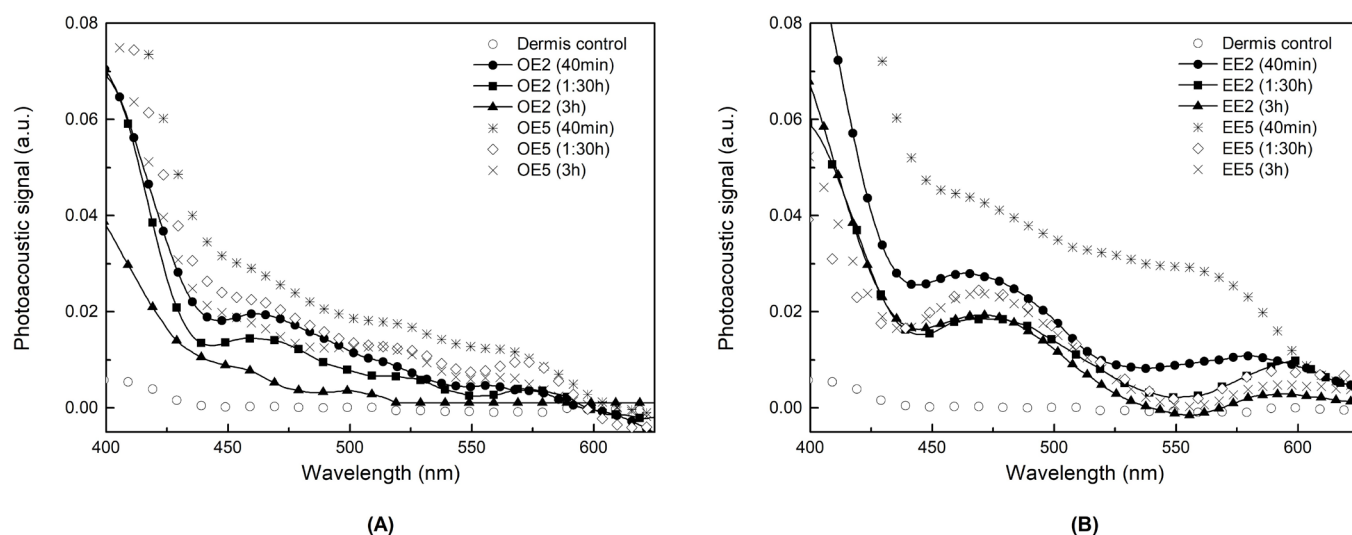
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(A)

(B)

FIGURE 6 - Photoacoustic spectra of the dermal face of skin samples obtained after 40 min, 90 min, and 180 min of topical treatment with 5 mg/cm^2 of the formulations containing CHE and without treatment (dermis control). The light modulation frequency was 25 Hz and the thermal diffusion length was 23 μm. **A**: ointments containing 2% (OE2) and 5% (OE5) of CHE. **B**: emulsions containing 2% (EE2) and 5% (EE5) of CHE.

In vivo skin irritation assay

To assess the issue of skin irritation, the animals were evaluated macroscopically for signs of irritation, such as erythema, edema, and desquamation. The groups that were treated with the emulsions (BE [G4], EE2 [G5], and EE5 [G6]) presented skin desquamation from day 3 of treatment, with an increase in desquamation (i.e., small crusts) on day 4 and the absence of desquamation and general improvement on day 7. The other groups exhibited no visible changes.

The morphology of the skin was then observed. Stratified keratinized epidermis was evaluated by light microscopy to detect the occurrence of acanthosis. All the samples showed normal skin structure. The epidermis was organized into regions with and without ridges. Below the epidermis was loose connective tissue and dense connective tissue that formed the dermis. Under the dermis, acidophilic collagen fibers with a dense irregular arrangement were observed. The presence of hair follicles and typical sebaceous glands was also observed. Aggregates of inflammatory cells were not evident in the dermis. However, skin samples treated with the emulsion formulations, G4, G5, and G6, exhibited a significant increase in the thickness of the epidermis ($p < 0.05$) compared with the untreated control (G1; Table I, Figure 7), confirming the macroscopic observations. The other groups (G2, G3, G7, G8, and G9) did not exhibit any microscopic signs of cutaneous irritation. The measurements of the epidermis in these groups were not significantly different from G1.

TABLE I - Skin irritation test. Thickness of the epidermis of Swiss mice after treatment.

Group	Topical treatment	Regions of the epidermis (mean ± SD)	
		R_1 (µm)	R_2 (µm)
G1	Not treated (control)	19.16 ± 2.31[a]	8.23 ± 1.78[c]
G2	Aqueous solution containing 2% of extract	19.29 ± 2.18[a]	8.28 ± 1.84[c]
G3	Aqueous solution containing 5% of extract	18.92 ± 2.22[a]	8.72 ± 2.70[c]
G4	Base emulsion (BE)	41.18 ± 6.91[b]	19.25 ± 6.63[d]
G5	Emulsion containing 2% of extract (EE2)	40.92 ± 6.71[b]	18.83 ± 6.57[d]
G6	Emulsion containing 5% of extract (EE5)	41.51 ± 5.85[b]	18.67 ± 6.39[d]
G7	Base ointment (BO)	18.81 ± 3.06[a]	8.15 ± 2.57[c]
G8	Ointment containing 2% of extract (OE2)	18.83 ± 3.14[a]	8.17 ± 2.70[c]
G9	Ointment containing 5% of extract (OE5)	19.19 ± 2.50[a]	8.97 ± 2.25[c]

Different letters in the columns indicate a significant difference between treatments (one-way ANOVA followed by Tukey test, p<0.05).
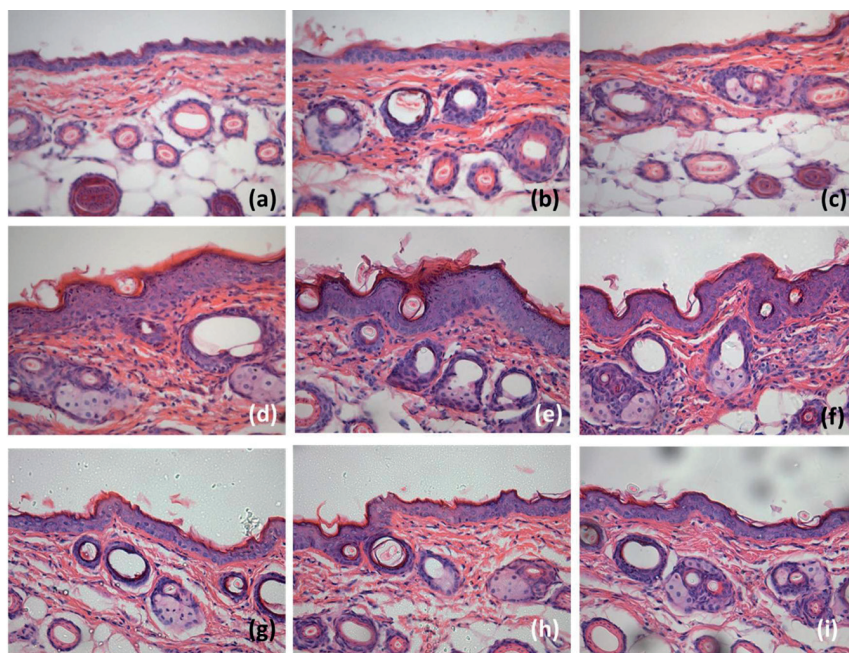SD: standard deviation

FIGURE 7 - Histological sections of Swiss mouse skin stained with Hematoxylin-Eosin (HE) (objective 40X). (a) control without treatment - G1; (b) treated with 2% CHE aqueous solution - G2; (c) treated with 5% CHE aqueous solution - G3; (d) base emulsion - G4; (e) emulsion containing 2% of CHE - G5; (f) emulsion containing 5% of CHE - G6; (g) base ointment – G7; (h) ointment containing 2% of CHE – G8; (i) ointment containing 5% of CHE – G9.

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In vivo antiviral activity

In the *in vivo* antiviral activity assay, the formulations that contained the CHE from *S. terebinthifolia* reduced lesions that were caused by HSV-1 (Figure 8).

The groups that were treated only with the BE or BO did not differ significantly from the untreated (control) group. On the other hand, the animals that were treated with the ointments or emulsions that contained the extract effectively reduced herpetic lesions, with a significant difference from the untreated group. In these groups herpetic lesions took longer to appear, and the lesions were softer and resolved more quickly.

Significant differences were also observed between the ointment and emulsion. The groups that were treated with the ointments containing the extract were more effective for the resolution of herpetic lesions compared to treatment with the emulsion containing the extract. Importantly, the ointment treatments did not significantly differ from the group that was treated with acyclovir.

The development of alternative topical treatments for HSV-1 infection is important for public health and has considerable economic and social relevance. The present study found that emulsions and ointments that contained the crude hydroethanolic extract of *S. terebinthifolia* permeated through the skin and were effective in treating HSV-1-induced lesions. Considering that the ointments were less irritating and had similar efficacy to acyclovir, these formulations may be promising for expanding the therapeutic arsenal for the treatment of HSV-1 infections.
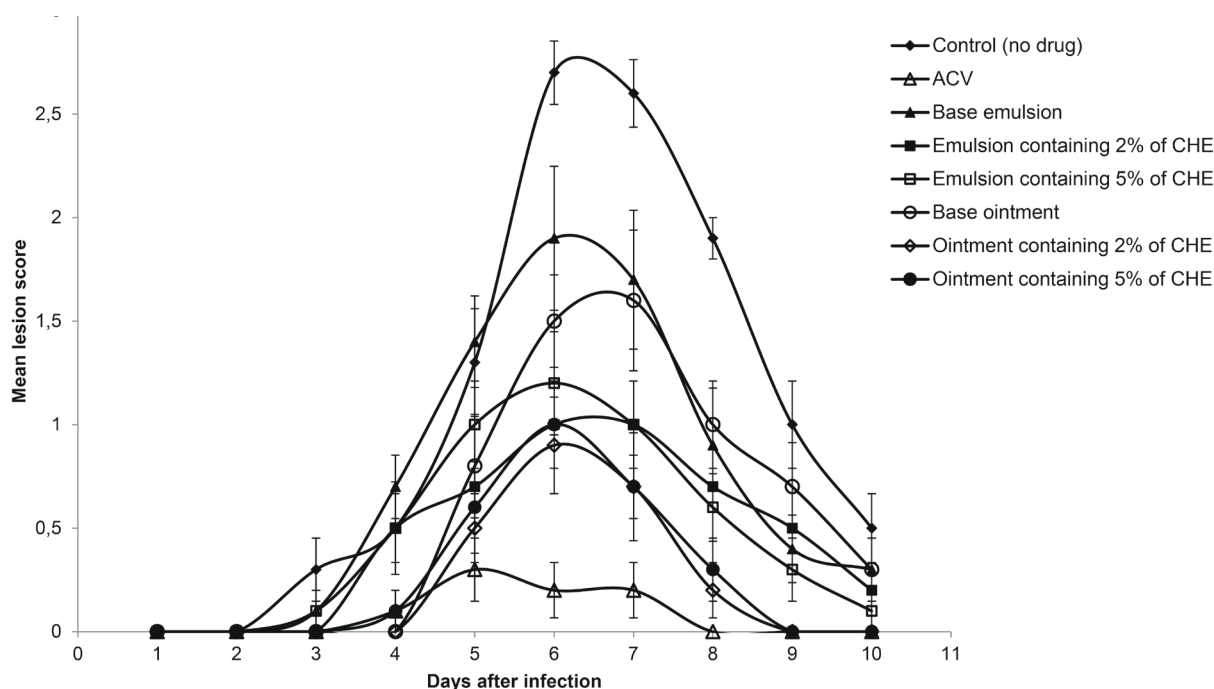

FIGURE 8 - Evaluation of the therapeutic efficacy of CHE incorporated into formulations (emulsion and ointment) on cutaneous lesions in HSV-1-infected BALB/c mice (1×10^6 PFU, KOS strain). Score lesions: (0) no lesion; (1) vesicles in local region; (2) erosion and/or ulceration in the local region; (3) mild zosteriform lesion; (4) moderate zosteriform lesion; (5) severe zosteriform lesion or death. The bars indicate the mean standard error.

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