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Original Article

Green propolis extract for the treatment of alkali-induced superficial corneal ulcers: local and systemic analyses

Extrato de própolis verde para o tratamento de úlceras superficiais da córnea induzidas por álcalis: análises locais e sistêmicas

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Abstract

Green propolis, produced by honeybees from the resin of certain plants, has garnered attention for its potential health benefits. Green propolis contains compounds like artepillin C, which exhibits antioxidant, anti-inflammatory, and antimicrobial properties. This study's objective was to examine the effects of green propolis extract containing 21.05% artepillin C, topically administered (5, 10 and 15 mg/mL) in rats with induced superficial corneal ulcers with alkali, applied four times daily, for 96 hours. Rats treated with the green propolis extract exhibited of ulcer reduction more significant reduction after 24 hours of treatment than untreated animals, suggesting a similar healing activity of the commercial drug. After 96 hours of treatment, the rats were euthanized, and the corneas were submitted for histopathological analysis; no statistical difference was detected between rats treated with green propolis and those not treated in terms of polymorphonuclear and neovessel counts in the corneal stroma. In the toxicogenetic analysis, by the micronucleus test, no significant differences were observed in the frequencies of chromosomal damage and the ratio of polychromatic erythrocytes to the total number of erythrocytes between treated and untreated animals, revealing the absence of genotoxicity and cytotoxicity, respectively. The biochemical results did not indicate a hepatotoxic or nephrotoxic effect of the formulations containing green propolis. Like this, the standardized green propolis extract induced corneal epithelialization without toxic effects, rendering it a promising option for treating superficial keratitis.

Keywords: alkali, artepillin C, superficial keratitis, natural product, reepithelization, toxicity.

Resumo

A própolis verde, produzida por abelhas a partir da resina de certas plantas, tem atraído atenção por seus potenciais benefícios à saúde. A própolis verde contém compostos como a artepillin C, que exibe propriedades antioxidantes, anti-inflamatórias e antimicrobianas. O objetivo deste estudo foi examinar os efeitos do extrato de própolis verde contendo 21,05% de artepillin C, administrado topicamente (5, 10 e 15 mg/mL) em ratos com úlceras de córnea superficiais induzidas com álcali, aplicado quatro vezes ao dia, por 96 horas. Os ratos tratados com o extrato de própolis verde exibiram redução de úlcera mais significativa após 24 horas de tratamento do que os animais não tratados, sugerindo uma atividade de cura semelhante ao medicamento comercial. Após 96 horas de tratamento, os ratos foram sacrificados e as córneas foram submetidas à análise histopatológica; nenhuma diferença estatística foi detectada entre os ratos tratados com própolis verde e aqueles não tratados em termos de contagens de polimorfonucleares e neovasos no estroma da córnea. Na análise toxicogenética, pelo teste do micronúcleo, não foram observadas diferenças significativas nas frequências de danos cromossômicos e na razão de eritrócitos policromáticos em relação ao número total de eritrócitos entre os animais tratados e não tratados, revelando a ausência de genotoxicidade e citotoxicidade, respectivamente. Os resultados bioquímicos não indicaram efeito hepatotóxico ou nefrotóxico das formulações contendo própolis verde. Assim, o extrato padronizado de própolis verde induziu epitelização corneana sem efeitos tóxicos, tornando-se uma opção promissora para o tratamento de ceratite superficial.

Palavras-chave: alcalino, artepillin C, ceratite superficial, produto natural, reepitelização, toxicidade.

1. Introduction

Superficial corneal ulcers are a common condition because of the constant exposure of the organ to the environment. Etiological factors include alkali burns (Bizrah et al., 2019), which are an ocular emergency because they can damage the surface and anterior segment of the eye (Labetoulle et al., 2019; Ahmmed et al., 2021). Alkaline products penetrate

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the cornea and hydroxyl ions cause damage by promoting the saponification of triglycerides and cell membrane lysis. Furthermore, pH changes result in the hydrolysis and denaturation of proteins and enzymes and the consequent loss of cell function and death (Martin et al., 2021).

Although several therapeutic modalities exist for alkali burns, none of them is completely effective. Thus, there is a need for new drugs need, for example natural products that can be used alone or in combination with already established drugs (Lee et al., 2021). Within this context, green propolis is an interesting option because of its documented medicinal effects such as anti-inflammatory (Franchin et al., 2018), antioxidant (Beserra et al., 2021), antimicrobial (Przybyłek and Karpiński, 2019), antitumor (Beserra et al., 2021), neuroprotective (Park et al., 2020), anti-inflammatory (Lan et al., 2016), and healing properties (Al-Waili, 2018; Stojko et al., 2021). These activities are attributed to the presence of flavonoids (quercetin and pinocembrin), terpenes (baccharin), and phenolic acids (artepillin C, p-cumaric acid, caffeic acid, and ferulic acid) (Martinotti and Ranzato, 2015; Moise and Bobiş, 2020; Tran et al. 2020). Artepillin C is considered a marker of green propolis (Beserra et al., 2021).

As mentioned above, corneal ulcers are a common condition. Considering the therapeutic properties of green propolis and the lack of studies on the use of this product in ophthalmology, the aim of the present study was to evaluate the effect of different concentrations of standardized green propolis extract on the healing of corneal superficial ulcers induced with alkali in rats, as well as its anti-inflammatory and angiogenic activity and possible deleterious systemic effects (genotoxic, hepatotoxic, and nephrotoxic).

2. Material and Methods

2.1. Preparation of the hydroalcoholic crude extract of green propolis and extraction of artepillin C

Green propolis obtained from *Baccharis dracunculifolia* was kindly provided by Apis Flora Comercial Ltda (Ribeirão Preto, SP, Brazil; lot: 65400918).

The natural product was extracted by maceration with ethanol and water (7:3) in the Laboratory of Chemistry of Natural Products, University of Franca (UNIFRAN). The crude hydroalcoholic extract was obtained after removal of the solvent under vacuum. Artepillin C was extracted by filtration of green propolis with ethyl acetate and the content of this compound was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). Analysis was performed by RP-HPLC with photodiode array detection (RP-HPLC-PDA) using a Shimpack VP-ODS column (250 mm x 4.6 mm I.D., 5 µm; Shimadzu, Kyoto, Japan) and a gradient system consisting of acetic acid-water (0.1: solvent A) and acetonitrile (solvent B) as mobile phase: 30% B (0-10 min), 30-55% B (10-11 min), 55% B (11-32 min), 55-30% B (32-33 min), and 30% B (33-42 min). The column temperature was set at 40ºC, the flow rate at 1.0 mL/min, the injected volume at 20.0 µL, and the detection wavelength at 290 nm (Figure 1). The concentration of artepillin C (Supplementary Material) obtained was 266.86 µg/mL (21.05%).

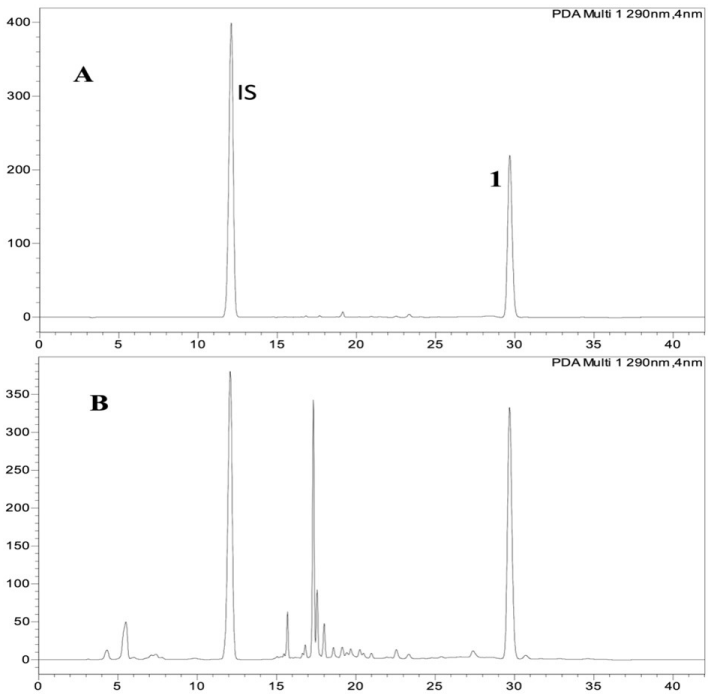

Figure 1. HPLC-PDA chromatograms showing (A) the analytical standard (Artepillin C) and coumarin (internal standard) peaks; and (B) acid-base extract and coumarin (internal standard), obtained in the established analytical conditions. The injected volume was 20 µL, flow rate was set at 1 mL·min^{-1}, column temperature was set at 40 °C, and detection was at 290 nm.

2.2. Preparation of the ophthalmic formulations

Topical formulations containing the standardized green propolis extract were prepared at the Natural Products Research Laboratory of UNIFRAN. The green propolis extract was solubilized in polyethylene glycol 400 (PEG400, Êxodo Científica, Sumaré, SP), polysorbate 80 (Tween 80®, Sigma-Aldrich, St. Louis, MO, USA), and distilled water at a ratio of 8:2:90 (v/v/v), respectively. The final concentrations of the solutions were 5, 10, and 15 mg/mL (Table 1). The extracts were solubilized every 2 days, autoclaved before use, and stored in amber containers at room temperature.

2.3. Selection of animals and induction of superficial corneal ulcers

The study followed the guidelines of the Association for Research in Vision and Ophthalmology - ARVO (National Institutes of Health Publication number 85-23) and was approved by the Animal Use Ethics Committee of UNIFRAN (No. 60611231118). Forty young, uncastrated male Wistar rats (*Rattus norvegicus*, albinus) were used. To exclude undesirable variables, the inclusion criteria were normal general clinical and ophthalmic parameters for the species. The rats were kept in the animal house of UNIFRAN in a ventilated and dry environment under controlled conditions of temperature (23±2ºC), humidity (50±10%), and light (12-hour light/dark cycle).

For the induction of corneal ulcers, the animals were anesthetized with ketamine hydrochloride (50 mg/kg; Agener União Saúde Animal, Embu-Guaçu, SP) and xylazine hydrochloride (5 mg/kg; Konig, Mairinque, SP), intraperitoneally in a single application (Stokes et al., 2009). Additionally, ocular anesthesia was performed with a drop of oxybuprocaine hydrochloride (4 mg/mL; Cristália Produtos Químicos e Farmacêuticos Ltda, Cotia, SP) to desensitize the cornea and to prevent closure of the eyelid.

Corneal ulcers were induced using a Whatman 41 filter paper disk (GE Healthcare Life Science, São Paulo, SP), which was cut with a surgical trephine (punch, Brasmed Empresa Brasileira de Cirurgia Veterinária Ltda, Paulínia, SP) measuring 3.0-mm in diameter and soaked in 1 M alkaline sodium hydroxide solution (NaOH; Labsynth, Diadema, SP) for 15 seconds (Ormerod et al., 1989). The disk was placed in the center of the left cornea for 60 seconds and, after its removal, the ocular surface was washed with 0.9% saline (Eurofarma Laboratório S.A., Ribeirão Preto, SP) (Park et al., 2022). The injury was confirmed by instillation of 1% fluorescein (Pharmédice, Belo Horizonte, MG) and examination under an indirect ophthalmoscope equipped with a cobalt blue filter (Eyetec, São Carlos, SP).

Table 1. Composition of the formulations containing green propolis at concentrations of 5, 10, and 15 mg/mL and solvent control prepared for the treatment of superficial corneal ulcers.

Formulation	Composition
Green propolis 5 mg/mL	25 µL green propolis extract
	398 µL PEG400
	99.5 µL Tween 80®
	4.477.5 µL distilled water
Green propolis 10 mg/mL	50 µl green propolis extract
	396 µL PEG400
	99 µL Tween 80®
	4.455 µL distilled water
Green propolis 15 mg/mL	75 µL green propolis extract
	394 µL PEG400
	98.5 µL Tween 80®
	4.432.5 µL distilled water
Solvent control	400 µL PEG400
	100 µL Tween 80®
	4.500 µL distilled water

2.4. Experimental design

Rats were randomly divided into eight groups of five animals each: GP5, animals treated with formulations containing 5 mg/mL standardized green propolis extract; GP10, animals treated with formulations containing 10 mg/mL standardized green propolis extract; GP15, animals treated with formulations containing 15 mg/mL standardized green propolis extract; SC (solvent control), animals treated with a formulation containing green propolis solvents; PC (positive control), animals receiving commercial eyedrops containing 1 mg/mL sodium hyaluronate (Lunah 1 mg/mL; Cristália Produtos Químicos e Farmacêuticos Ltda, São Paulo, SP); UC (untreated ulcer control), animals treated with 0.9% saline; PCT (positive control for the genotoxicity tests), animals not submitted to corneal ulcer induction but receiving the potentially carcinogenic alkylating agent methyl methanesulfonate (MMS, 40 mg/kg/dose; Sigma-Aldrich, St. Louis, MO, USA), intraperitoneally in a single dose (OECD, 2016); NC (negative control for the biochemical and genotoxicity tests), animals not submitted to corneal ulcer induction or ophthalmic treatment.

The formulations (one drop) were instilled every 6 hours (Bollag et al., 2020) for 4 consecutive days (96 hours). Concomitantly, the analgesic tramadol hydrochloride (5 mg/kg; Cristália Produtos Químicos e Farmacêuticos Ltda, São Paulo, SP) was administered subcutaneously every 12 hours to all rats, except for those of the PCT and NC groups (Stokes et al., 2009).

2.5. Clinical analysis

Local intercurrent symptoms that suggested any ocular discomfort after the ophthalmic instillations were evaluated daily by direct inspection.

Before the daily ophthalmic treatments, fluorescein was instilled and the left corneas were then photographed (iPhone 11, Apple Inc. Cupertino, CA, USA) at 24, 48, 72 and 96 hours of treatment (T24, T48, T72 and T96, respectively) under cobalt blue light, at a standardized distance of 15 cm, in order to measure the percentage of the healed area compared to T0 (before treatment). The photographic images were analyzed using the ImageJ® software (ImageJ/Fiji® 1.50b) for manual delimitation of the borders of the ulcers and subsequent automatic calculation of the injured

areas. The results were compared statistically to those of the untreated group.

2.6. Histopathological analysis

At the end of the ophthalmic treatments, the rats were euthanized with 120 mg/kg sodium pentobarbital (Cristália Produtos Químicos e Farmacêuticos Ltda, São Paulo, SP), intraperitoneally in a single application (Stokes et al., 2009). Next, the left ocular bulbs were removed by the conventional subconjunctival enucleation technique and cut for processing and staining of the corneas with hematoxylin-eosin following a routine histological technique. The prepared slides were placed in a bright-field microscope (Leica Microsystems® DMLB, Wetzlar, Germany) and digitized with a camera (Tucsen GT-5 using a 1/2-inch 5MP CMOS sensor; China) attached to the microscope for polymorphonuclear cell (400x) and neovessel (100x) counts in three distinct areas in the corneal stroma, avoiding those close to the limbus. The mean of these measurements was compared statistically to the untreated group.

2.7. Toxicogenetic analysis

Bone marrow samples were collected to evaluate the genotoxic potential of the ophthalmic treatments by the micronucleus test (Macgregor et al., 1987; OECD, 2016), including the PCT and NC groups. For this purpose, the frequency of micronucleated polychromatic erythrocytes (PCEMNs) was obtained by the analysis of 2,000 polychromatic erythrocytes (PCEs) per rat. The same slides were used for cytotoxicity assessment, in which 500 erythrocytes/animal were analyzed and the ratio of PCE/PCE+NCE (normochromatic erythrocytes) was calculated.

2.8. Biochemical analysis

To evaluate the possible hepatotoxic and nephrotoxic effects of the ophthalmic treatments, blood samples were collected from all groups, except for PCT, for the measurement, respectively, of serum liver enzymes [aspartate aminotransferase (AST, U/L) and gamma-glutamyltransferase (GGT, U/L) and urea and creatinine (mg/dL). The analyses were performed in a Mindray® automatic analyzer (Mindray Medical Brazil Ltda., São Paulo, SP) using enzymatic colorimetric kits (Dialab, Vienna, Austria). The results were compared statistically to those obtained for the NC group. The reference values used to interpret the biochemical parameters analyzed in this study were obtained from the literature and include: AST (54–300 U/L), GGT (0–20 U/L), urea (40–70 mg/dL), and creatinine (0.5–1.2 mg/dL) (Chatterjee and Dey, 2003).

2.9. Statistical analysis

The results were analyzed statistically by simple analysis of variance (ANOVA) for completely randomized experiments, with the calculation of F statistics and their respective p-values. In cases in which $p \leq 0.05$, treatment means were compared by the Tukey test and the minimum significant difference was calculated for $\alpha = 0.05$ using the GraphPad Prism 8.0® program.

3. Results

3.1. Clinical findings

There was no evidence of discomfort after the instillations, suggesting that the tested formulations did not cause irritation or local damage and are harmless to the ocular surface.

Regarding the percentage of corneal ulcer reduction compared to T0 (Table 2), significantly greater reductions ($p \leq 0.05$) were observed in the treated groups compared to the untreated group (UC) at T24 (Figure 2). However, there was no difference between the treated groups at this time point nor at T48, T72 or T96. These results suggest a similar healing activity of green propolis and the commercial drug. Among the groups treated with the standardized green propolis extract, GP5 exhibited complete corneal healing within a short period of time (T48), followed by GP10 (T72) and GP15 (T96).

3.2. Histopathological findings

There was no significant difference in polymorphonuclear cell or neovessel count in the corneal stroma between groups (Table 3 and Figure 3).

Table 2. Percentage of corneal ulcer reduction in rats submitted to different ophthalmic treatments with standardized green propolis extract and their respective controls after 24, 48, 72 and 96 hours (T24, T48, T72 and T96, respectively) compared to T0 (before treatment).

Group	T24	T48	T72	T96
GP5	75.12%	100%	100%	100%
GP10	82.53%	98.78%	100%	100%
GP15	84.64%	96.70%	99.57%	100%
SC	79.06%	100%	100%	100%
PC	96.93%	100%	100%	100%
UC	23.51%[a]	96.06%	100%	100%

GP5: group treated with the formulation containing 5 mg/mL of green propolis; GP10: group treated with the formulation containing 10 mg/mL of green propolis; GP15: group treated with the formulation containing 15 mg/mL of green propolis; SC: group treated with solvent; PC: positive control group treated with commercial eyedrops containing 1 mg/mL sodium hyaluronate; UC: untreated ulcer control group. [a]Significantly different from the other groups at T24 ($p \leq 0.05$).

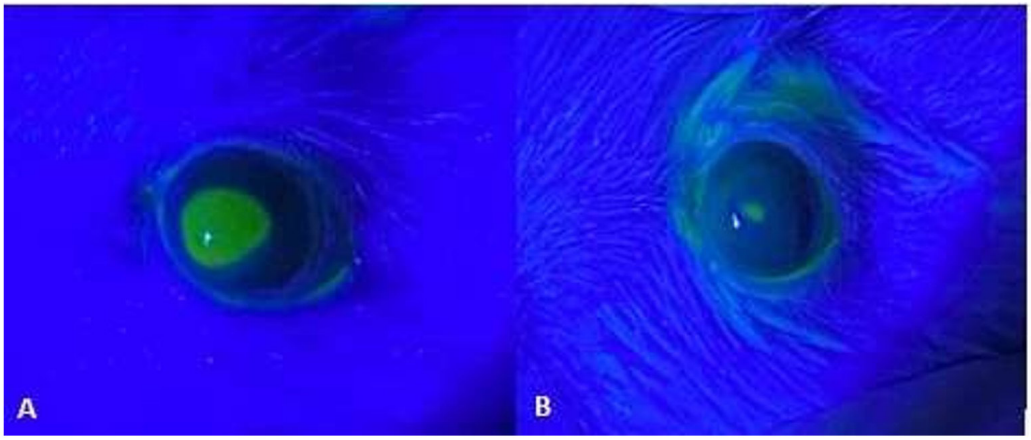

Figure 2. Photographic images of the left eyes of rats after 24 hours of treatment, showing a corneal ulcer stained with 1% sodium fluorescein obtained with an indirect ophthalmoscope equipped with a cobalt blue filter. (A) untreated animal; (B) animal treated with standardized green propolis at 5 mg/mL.

Table 3. Mean number and standard deviation of polymorphonuclear cells (PMNC) and neovessels in the corneal stroma after 96 hours of treatment with the ophthalmic formulations containing standardized green propolis and their respective controls.

Group	PMNC	Neovessels
GP5	6.25 ± 4.42	2.75 ± 4.27
GP10	6.00 ± 4.30	9.80 ± 11.39
GP15	10.60 ± 7.06	2.60 ± 7.00
SC	3.40 ± 2.30	1.00 ± 0.71
PC	3.80 ± 4.50	0.60 ± 0.89
UC	11.6 ± 7.80	0.25 ± 0.45

GP5: group treated with 5 mg/mL of green propolis; GP10: group treated with 10 mg/mL of green propolis; GP15: group treated with 15 mg/mL of green propolis; SC: group treated with solvent; PC: positive control group treated with commercial eyedrops containing 1 mg/mL sodium hyaluronate; UC: untreated ulcer control group. There was no significant difference between groups.

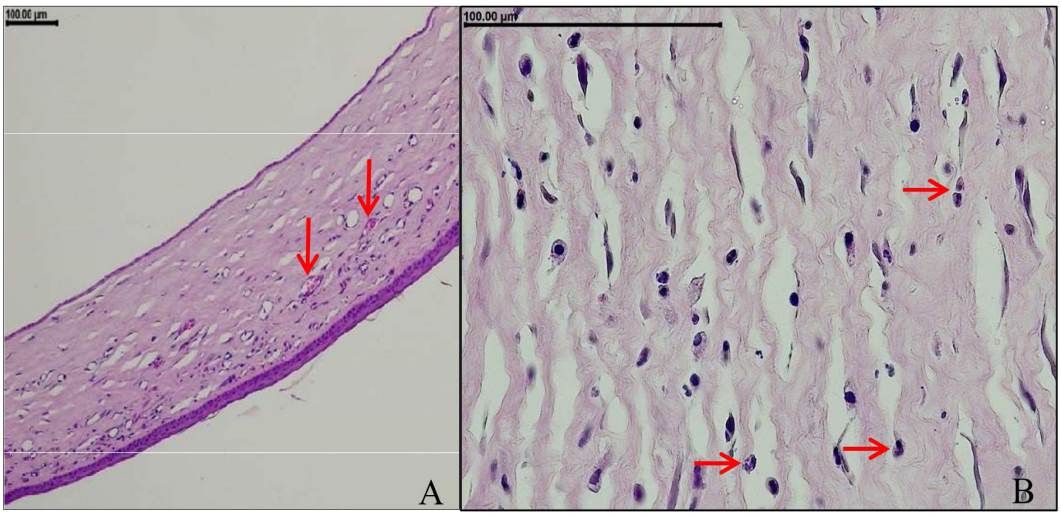

Figure 3. Digital photomicrographs of the left corneas of Wistar rats after 96 hours of treatment of alkali-induced superficial ulcers. (A) Presence of neovessels in the corneal stroma (arrows) of an animal treated with the standardized green propolis extract at 10 mg/mL; (B) presence of polymorphonuclear cells in the corneal stroma (arrows) of an animal treated with the standardized green propolis extract at 15 mg/mL.

3.3. Toxicogenetic findings

The results of the genotoxic assessments are presented in Table 4. There were no significant differences between animals treated with the different doses of green propolis and the NC group, revealing the absence of genotoxic and cytotoxic effects.

3.4. Biochemical findings

No significant differences were observed between groups compared to the NC group, suggesting the absence of hepatotoxicity (Table 5) and nephrotoxicity (Table 6) of the ophthalmic treatments administered.

Table 4. Mean and standard deviation of the frequency of micronucleated polychromatic erythrocytes (PCEMNs) and ratio of polychromatic erythrocytes to total erythrocytes (PCE/PCE+NCE) in bone marrow of Wistar rats submitted to superficial left corneal ulcerations and treated with formulations containing standardized extract of green propolis and their respective controls.

Group	PCEMNs[a]	PCE/PCE+NCE[b]
NC	10.00 ± 3.3	0.64 ± 0.02
UC	6.60 ± 0.8	0.64 ± 0.01
SC	6.66 ± 1.5	0.63 ± 0.01
GP5	11.00 ± 1.4	0.59 ± 0.06
GP10	10.66 ± 3.0	0.56 ± 0.05
GP15	8.75 ± 1.5	0.59 ± 0.05
PC	7.00 ± 1.0	0.58 ± 0.03
PCT	87.00 ± 12.8[c]	0.53 ± 0.03

NC: negative control group (no ulcer and no treatment); UC: untreated ulcer control group; SC: group treated with solvent; GP5: group treated with 5 mg/mL of the standardized green propolis extract; GP10: group treated with 10 mg/mL of the standardized green propolis extract; GP15: group treated with 15 mg/mL of the standardized green propolis extract; PC: positive control group treated with commercial eyedrops containing 1 mg/mL sodium hyaluronate; PCT: positive control group of the toxicogenetic tests (no ulcer and treated with 40 mg/kg body weight of methyl methanesulfonate, intraperitoneally). [a]A total of 20.000 polychromatic erythrocytes were analyzed per treatment group; [b]A total of 5.000 erythrocytes were analyzed per treatment group; [c]Significantly different from the NC group ($p<0.005$).

Table 5. Mean and standard deviation of serum liver enzymes [aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT)] in Wistar rats submitted to superficial left corneal ulcerations and treated with standardized green propolis and their respective controls.

Group	AST (U/L)	GGT (U/L)
GP5	118.2 ± 26.3	10.0 ± 5.3
GP10	105.8 ± 6.6	9.6 ± 3.8
GP15	108.0 ± 24.6	14.4 ± 4.6
SC	111.4 ± 37.8	10.2 ± 4.4
PC	205.6 ± 23.6	19.8 ± 0.8
UC	205.4 ± 71.3	13.4 ± 4.2
NC	181.8 ± 47.5	13.6 ± 5.6

GP5: group treated with 5 mg/mL of green propolis; GP10: group treated with 10 mg/mL of green propolis; GP15: group treated with 15 mg/mL of green propolis; SC: solvent control group; PC: positive control group treated with commercial eyedrops containing 1 mg/mL sodium hyaluronate; UC: untreated ulcer control group; NC: negative control group for the biochemical and toxicogenetic tests. There was no significant difference between the experimental groups and the NC group.

Table 6. Mean and standard deviation of serum urea and creatinine in Wistar rats submitted to superficial left corneal ulcerations and treated with standardized green propolis and their respective controls.

Group	Urea (mg/dL)	Creatinine (mg/dL)
GP5	72.6 ± 1.9	0.8 ± 0.1
GP10	69.0 ± 4.3	0.7 ± 0.1
GP15	76.4 ± 8.6	0.7 ± 0.0
SC	75.2 ± 10.8	0.7 ± 0.1
PC	74.0 ± 10.4	0.8 ± 0.1
UC	68.0 ± 11.0	0.7 ± 0.1
NC	69.2 ± 7.3	0.8 ± 0.1

GP5: group treated with 5 mg/mL of green propolis; GP10: group treated with 10 mg/mL of green propolis; GP15: group treated with 15 mg/mL of green propolis; SC: solvent control group; PC: postive control group treated with commercial eyedrops containing 1 mg/mL sodium hyaluronate; UC: untreated ulcer control group; NC: negative control group for the biochemical and toxicogenetic tests. There was no significant difference between the experimental groups and the NC group.

4. Discussion

The orbital and corneal anatomy of rodents resembles that of humans. These species are therefore considered a reliable model for experiments in the field of ophthalmology (Kern, 1997) and are also widely used in genotoxicity and cytotoxicity safety tests (OECD, 2016). Only male animals were used in an attempt to minimize the influence of hormonal variations, which may occur in females, even over short experimental periods (OECD, 2016). The use of rodents is also in accordance with European guidelines. In addition to the easy obtainment, maintenance and handling of these animals, the guidelines also permit to increase the number of tested animals, an important factor for evaluating the biological and harmful effects of new products (Perches et al., 2012).

The induction of superficial corneal ulcers with alkali soaked in filter paper disks was based on the models of Ormerod et al. (1989) and Dias et al. (2017) to ensure uniformity of the lesions. Furthermore, according to Ormerod et al. (1989), NaOH concentrations higher than 1 M can induce profound stromal loss, with difficulty in healing and recurrent erosion that would require surgical treatment. These authors also suggested to avoid the limbus region because of the difficulty in healing due to damage to stem cells.

Regarding green propolis derived from *Baccharis dracunculifolia*, data specifying its composition are scarce, especially in studies that investigate the effects on wound healing, a fact that limits comparisons between studies that use the same natural product (Martinotti and Ranzato, 2015; Oryan et al., 2018). In the present study, standardized green propolis extract containing 21.05% artepillin C was used to formulate the ophthalmic formulations. This compound is considered to be the main biological active ingredient of green propolis and has numerous proven medicinal properties (Przybyłek and Karpiński, 2019; Beserra et al., 2021).

Within this context, there are different methods of extracting compounds from natural products using minimal organic solvent. To identify the constituents of green propolis, in the present study, maceration with ethanol-water was essential for the identification of the pure compounds and rich extracts, as described by Yubin et al. (2014) and Torres et al. (2018), especially phenolic compounds such as artepillin C (Bankova et al., 2021).

The recommended dosage for ophthalmic instillations (one drop every 6 hours) was based on the description of Bollag et al. (2020) that frequent applications result in higher local tissue concentrations. Since this is a pioneering study on the use of standardized green propolis extract in superficial keratitis, the concentrations of the formulations were established at 5, 10 and 15 mg/mL and the choice of sodium hyaluronate as the commercial reference control was based on the viscoelastic properties of the compound, which ensure its permanence on the ocular surface for a longer period of time, favoring corneal healing by stimulating the proliferation, migration, and adhesion of epithelial cells (Gomes et al., 2004; Seino et al., 2020).

After the instillations, none of the tested products caused clinical symptoms that would suggest ocular discomfort secondary to irritation and/or local damage such as blepharospasm, epiphora, secretion, photophobia, and itching. The clinical findings associated with the histopathological findings were essential for demonstrating that the formulations were harmless to the ocular surface up to the last time point of analysis.

The topically treated groups exhibited a greater percent reduction in the ulcerated area than the untreated group at T24. This finding agrees with the literature regarding the average corneal healing time after treatment with a commercial sodium hyaluronate-based product, thus suggesting the promising potential of green propolis for the treatment of superficial keratitis. Martin et al. (2013) also reported a decrease in corneal ulcers induced with silver nitrate sticks in rats after 24, 48 and 72 hours of topical treatment with 1% Brazilian propolis (*Scaptotrigona* sp.). Martinotti and Ranzato (2015) attributed the healing effect of green propolis to flavonoid compounds that inhibit lipid peroxidation, reducing cellular necrosis. Rapid epithelialization of alkali-induced corneal ulcers is essential since delayed healing increases the risk of infection and inflammation and decreases stromal remodeling, resulting in the loss of corneal transparency and consequent sequelae that affect visual acuity (Ueno et al., 2005).

According to Stojko et al. (2021), in the skin, propolis accelerates wound healing by modulating the production of type I and III collagen, stimulating glycosaminoglycans and the accumulation of laminin, vibronectin, heparan sulfate, and heparin. In addition, green propolis increases hyaluronic acid, which favors wound hydration and thus contributes to healing (Olczyk et al., 2013).

Green propolis has low solubility in water, a fact that prevents tear substitutes or saline solution from being the exclusive solvents for the ophthalmic formulations tested. Thus, polyethylene glycol and Tween 80® were used, which possess surfactant and hydrophilic properties that are well established in the ophthalmological literature (Dias et al., 2017; Srinivasan and Manoj, 2021; Hou et al., 2022). The results obtained for the SC group regarding the percent reduction in the ulcerated area can probably be explained by these characteristics of the solvents that reduce superficial ocular tension, increasing contact with the tear film and consequent improvement in corneal lubrication, thereby favoring cell displacement adjacent to the wound border (Perches et al., 2012). Similar results have been reported by Zhang et al. (2018) who compared sodium hyaluronate and carboxymethylcellulose with polyethyleneglycol for the healing of mechanically induced corneal ulcers after 36 hours of treatment. Dias et al. (2017) found Tween 80® combined with copaiba oil to contribute to the healing of alkali-induced superficial corneal ulcers in rats.

The presence of an inflammatory infiltrate as a result of neovascularization that occurs within the first days after injury, triggered by the release of proinflammatory cytokines and chemotaxins in response to the insult, tends to favor the removal of cellular debris at the site and contributes to the defense against microorganisms. In addition, the inflammatory infiltrate induces the release of growth factors that stimulate fibroblasts, cells responsible for the production of collagen, which is essential for corneal wound healing (Martin et al., 2013). In the present study,

the recruitment and permanence of these cells at T96 did not compromise tissue repair in the groups treated topically with the green propolis formulations, as confirmed by evaluation of the percentage of ulcer reduction. On the other hand, Martin et al. (2013) observed a significant reduction in neutrophils in rats with 1-mm corneal ulcers induced with silver nitrate sticks after 24 and 48 hours of treatment with a microemulsion consisting of 1% of propolis dry matter (produced by *Scaptotrigona* sp. bees) solubilized in polyethyleneglycol-6-caprylate/caprate, polyglyceryl-6-dioleate, caprylate/caprate glycerides, benzalcone chloride, and deionized water. Oztürk et al. (2000) also reported a decrease in polymorphonuclear cells on the first and seventh days of treatment with 1% of an ethanolic extract of propolis diluted in phosphate-buffered saline in rats with corneal ulcers induced with 1 M NaOH, suggesting an anti-inflammatory effect of the natural product. According to Franchin et al. (2018) and Beserra et al. (2021), the anti-inflammatory effect of green propolis can be attributed, at least in part, to the action of flavonoids, especially artepillin C. The latter decreases inflammatory signaling pathways by reducing the synthesis of cytokines such as TNF-α, IL-1β, IL-6, IL-8, IFN-γ, and NF-kB, in addition to reducing proinflammatory molecules such as nitric oxide, histamine, leukotrienes, prostaglandins, prostacyclins, and thromboxanes.

After one week of topical treatment (168 hours) of rabbits with corneal ulcers induced with a silver nitrate stick with a non-standardized 1% aqueous propolis extract, Hepşen et al. (1999) observed a reduction in new vessels. The authors attributed the finding to the inhibitory effect of the natural product on the activity of cyclooxygenase and lipoxygenase.

Despite the widespread use of green propolis in folk medicine and the belief that natural products, because they have been used for thousands of years for medicinal purposes, do not have side effects or health risks and do not contain toxic substances, pre-clinical toxicity studies are essential during the assessment of future medications in order to identify possible deleterious effects on non-target organs (Perches et al., 2012), as done in the present study. There are several *in vivo* tests that detect structural or numerical drug-induced chromosome damage in dividing cells, which can cause mutations in somatic cells. The micronucleus test was chosen to evaluate the toxicogenic potential of green propolis since it is a rapid test that is easy to perform and interpret, has wide applicability, is less subjective than other mutagenicity tests, and provides reliable results after acute treatments (OECD, 2016). Furthermore, the micronucleus test is accepted by international agencies and government institutions for the registration of new pharmaceutical and chemical products (Kang et al., 2013; OECD, 2016). In the present study, bone marrow samples were chosen because of the rapid proliferation of bone marrow cells and their predisposition for genetic changes (Agostini, 1993), while micronucleated erythrocytes in peripheral blood are quickly and effectively destroyed in the spleen (Hayashi, 2016). The ophthalmic formulations containing different concentrations of standardized green propolis did not show signs of toxigenicity; thus, the formulation will possibly not cause mutations in somatic cells or predispose exposed individuals to neoplasms or genetic diseases (Kirsch-Volders et al., 2011). Similar results have been reported by Senedese et al. (2011) who topically applied green propolis gel to skin burns in rats.

Although Farkouh et al. (2016) found high availability of lipophilic substances when applied in the form of eyedrops, rapidly increasing plasma concentrations and even passing through the hepatic circulation, the experimental groups of the current study treated with standardized green propolis did not exhibit liver damage secondary to the ophthalmic treatments when compared to the NC group, regardless of the concentration tested. These results corroborate the observations of Bertolami (2005), Botelho et al. (2010) and Larrey et al. (2017) that toxicity occurs when the serum levels of liver biomarkers, including AST and GGT, exceed three times the limits of normality, with clinical symptoms being rare. Furthermore, Hajiaghaalipour et al. (2013) reported that topical drugs generally have low systemic toxicity.

Analysis of the formulations containing standardized green propolis also indicated the absence of nephrotoxicity. According to Waki et al. (2010), the occurrence of renal toxicity requires significantly elevated serum urea and creatinine levels, with the animals consequently exhibiting some clinical symptoms. Similar results regarding hepato- and nephrotoxicity have been reported by Batista et al. (2012) who applied propolis-based ointments to skin wounds in rats, and by Zhu et al. (2018) who orally treated elderly people with propolis capsules containing 11 mg artepillin C for 24 months in order to prevent cognitive loss.

5. Conclusions

Based on the methodology used, the results obtained show that instillation of the ophthalmic formulations containing standardized green propolis extract at concentrations of 5, 10 and 15 mg/mL every 6 hours for 4 consecutive days were well tolerated by the ocular surface. Despite the absence of evident anti-inflammatory and angiogenic effects, the formulations were similar to the commercial reference product in terms of corneal ulcer healing, representing a less expensive and promising option that can be used alone or in combination with already established medications for the treatment of chemical superficial keratitis. Furthermore, the formulations did not exhibit hepatotoxic, nephrotoxic, or toxicogenetic effects, reinforcing their use for the development of new ophthalmic products. Despite the promising results, future experiments are needed to investigate lower concentrations, different solvents, and the association of green propolis with nanoparticles to further accelerate corneal wound healing.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. 1H(CDCl3, 400 MHz) NMR spectrum of artepillin C

Figure S2. 13C NMR spectrum (CDCl3, 100 MHz) of artepillin C

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