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Alginate-based microcapsules loaded with Brazilian green propolis decrease reactive oxygen species production, reduce inflammatory cytokines, and mitigate intestinal inflammation

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ABSTRACT

Ulcerative colitis causes intestinal inflammation, with treatments often limited in efficacy and safety. New technologies enable the controlled release of bioactive compounds, and Brazilian green propolis could benefit in managing inflammation. This study proposed developing alginate-based microcapsules loaded with ethanolic extract of green propolis (EEGP-MC), evaluating their effects on inflammatory cytokines, reactive oxygen species, and experimental colitis. The results demonstrated that the EEGP-MC reached peak release of phenolic compounds in the intestinal phase (IP) at 4 h (76.9 %) and 6 h (75.0 %). Similarly, Artepillin C peaked at 22.3 ± 1.2 mg/g at 4 h and 22.5 ± 1.1 mg/g at 6 h in the IP. In THP-1 cell cultures, pretreatment with EEGP-MC (1000 μg/mL) and IP (300 and 1000 μg/mL) reduced TNF-α and IL-6 levels and ROS production. Additionally, oral administration of EEGP-MC at 300 mg/kg demonstrated superior protective activity in the colonic mucosa, reducing lesions by 86.1 % compared to 54.9 % with EEGP alone. Finally, the treatment with EEGP-MC suppressed TNF-α, IL-6, and IL-1β cytokines in the intestinal tissue. No toxicity was observed for the EEGP-MC. These findings highlight EEGP-MC as an innovative technology with promising applications for managing chronic inflammation in the food and pharmaceutical industries.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease that affects the mucosa of the large intestine (colon) and rectum, impacting millions of people worldwide [1]. It is primarily characterized by destroying intestinal epithelial structures caused by a localized inflammatory response [1,2]. The development of UC is associated with a combination of genetic predisposition, environmental factors, imbalances in gut microbiota, and alterations in immune responses [1,2]. The treatment of UC aims to improve patients' quality of life by reducing intestinal

Abbreviations: AAE, ascorbic acid equivalent; CUPRAC, cupric reducing antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EEGP, ethanolic extract of green propolis; EEGP-MC, sodium alginate-based microcapsules loaded with ethanolic extract of green propolis; ELISA, enzyme-linked immunosorbent assay; FRAP, ferric reducing antioxidant power; HPLC, high-performance liquid chromatography; IL-1β, interleukin-1 beta; IL-6, interleukin-6; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFR2, nuclear factor erythroid 2–related factor 2; TNF-α, tumor necrosis factor-alpha; TPC, total phenolic content; UC, Ulcerative colitis.

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inflammation and alleviating disease-related symptoms [3]. The main classes of available medications include anti-inflammatory agents (*e.g.*, 5-aminosalicylic acid), immunosuppressants (*e.g.*, corticosteroids), and biologics (*e.g.*, anti-cytokine agents) [1]. However, these treatments often have limitations in efficacy and are frequently associated with significant long-term side effects [4]. In this context, there is an urgent need to develop new therapeutic strategies for UC, with natural products representing a promising alternative for developing treatments to prevent and control inflammatory processes, such as in UC [5,6].

Brazilian green propolis is a natural product collected by *Apis mellifera* bees in the Southeast region and northern Paraná, Brazil [7]. This type of propolis offers numerous therapeutic benefits for human health, including anti-inflammatory activity, as evidenced by the suppression of edema and inflammatory cell influx in experimental models [8–10]. Moreover, inflammatory targets such as iNOS and NF-κB were inhibited by green propolis extract in cell culture studies [10]. Additionally, bioactive compounds have been isolated and identified in green propolis, with Artepillin C as one of the main contributors to its therapeutic effects [11]. However, despite its numerous pharmacological advantages, the clinical application of green propolis is often limited by its low solubility, poor bioavailability, and inherent instability, making it susceptible to degradation reactions that compromise its efficacy [12]. In this context, developing novel pharmaco-technical strategies emerges as a promising approach to enhance the performance and stability of active compounds, control their release, target specific biological sites, prolong their residence time at the action site, and increase their tissue penetration [13,14].

Microencapsulation is a versatile technology that protects solid, liquid, or gaseous materials within microparticles. This technique has been widely applied in various fields, including medicine, cosmetics, and functional foods, due to its ability to enhance stability, control release, and preserve the properties of encapsulated compounds [15,16]. Several technologies are used to microencapsulate bioactive compounds in small pouches (1 to 100 μm), such as freeze-drying, fluidized bed, coacervation, pan coating, and spray-drying. However, the vibrating nozzle ionotropic gelation technology is advantageous because it creates more uniform microcapsules and does not involve temperature, preserving the bioactive compounds from thermal degradation [17]. Spray-drying and freeze-drying have been used to microencapsulate propolis [18], and royal jelly has been microencapsulated using ionotropic gelation [19]. However, studies focusing on stabilising propolis using the vibrating nozzle technology have to be performed to unveil its feasibility to be scaled up.

These findings suggest that Brazilian green propolis can benefit from microencapsulation with sodium alginate using vibrating nozzle ionotropic gelation technology, which enhances the protection of Artepillin C and facilitates its targeted release at the desired site. In this study, we successfully developed sodium alginate-based microcapsules loaded with ethanolic extract of green propolis (EEGP-MC). We evaluated their antioxidant and anti-inflammatory properties using chemical models and cell cultures. Furthermore, we assessed their therapeutic effects in a murine model of intestinal inflammation and investigated their systemic toxicity using the *Galleria mellonella* larvae model.

2. Materials and methods

2.1. Chemical reagents

Lipopolysaccharide (LPS) from *Escherichia coli* (#L2630), phorbol 12-myristate 13-acetate (PMA) (#P1585), 2,2-diphenyl-1-picrylhydrazyl (DPPH, #1898-66-4), Folin-Ciocalteu's phenol reagent (#F9252), gallic acid (#149–91-7), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ, #93285), Artepillin C (>98 % purity, #72944–19-5), acetic acid (#64–19-7), ferric chloride hexahydrate (#10025–77-1), 2′,7′-dichlorofluorescein diacetate (DCFH-DA, #35845), copper(II) chloride ($CuCl_2$) (#10125–13-0), neocuproine (#121908), ammonium acetate, methanol (HPLC grade, #67–56-1), ascorbic acid (#A92902), dimethyl sulfoxide (DMSO, #D8418), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, #M5655) were acquired from Sigma-Aldrich-Merck (Darmstadt, Germany). Fetal bovine serum (FBS, #A5256801), RPMI 1640 medium (#11875093), L-glutamine (#25030–024), and penicillin-streptomycin (#15140122) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dextran sulfate sodium (DSS, #160110) was obtained from MP Biomedicals (Santa Ana, USA). Sodium alginate (#CHE3268/ Batch 315,678) and calcium chloride (#CHE1476) were obtained from Scientific Laboratory Supplies (Dublin, Ireland).

2.2. Collection, extraction, and chemical analysis of Brazilian green propolis

Brazilian green propolis was purchased from Guaxupé (−21°16′20.1"S, 46°42′52.4"W), Minas Gerais, in March 2022 (SisGen #AFDE9B6). The extraction process was performed according to Tiveron et al. [20], with modifications. Green propolis (200 g) was extracted with 660 mL of 80 % ethanol (#64–17-5) in water (*v*/v) for 24 h using a magnetic stirrer at room temperature, protected from light. After extraction, the mixture was centrifuged to separate the solid and liquid phases. The liquid phase was subjected to rotary evaporation (IKA Rotary Evaporators RV 3 V®) to remove the solvent at 45 °C. The resulting ethanolic extract of green propolis (EEGP) was freeze-dried (Fig. S1) and stored at −20 °C. The final yield of the ethanolic extract was 82 g (41 % of the original propolis weight). The chemical profile of the EEGP was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.2.1. LC–ESI- QTOF -MS/MS analysis of the EEGP

LC-MS/MS analysis on the EEGP was performed using an Agilent 6545 ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-Q/TOF-MS). Briefly, EEPG samples were prepared for UHPLC-ESI-QTOF-MS profiling by weighing 10 mg of each and dissolving in a 1:1 *v*/v acetonitrile-water mixture. The solutions were then transferred to injection vials for analysis. The analysis was conducted on an Agilent 1290 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA) featuring an autosampler. Chromatographic separation was achieved using an Agilent ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm), maintained at a temperature of 30 °C. The mobile phases consisted of (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. The gradient elution was programmed as follows: 0 min: 5 % B; 2 min: 5 % B; 20 min: 100 % B; 25 min: 100 % B. The flow rate was set at 0.3 mL/min, with an injection volume of 2 μL. The HPLC system was coupled to an Agilent 6545 LC/Q-TOF MS mass spectrometer operated in negative ion modes (ESI-). The system utilized a dual Agilent Jet Stream ESI, with the drying gas set to nitrogen at a temperature of 325 °C and a flow rate of 8 L/min. The sheath gas was also nitrogen, heated to 360 °C, with a flow rate of 12 L/min. Nebulizer pressure was maintained at 35 psi. The capillary voltage was set at 3000 V, and the fragment voltage at 135 V. The acquisition mass range was from *m/z* 100 to 1700. The peaks were identified by comparing the results obtained to an existing 1 mg/mL Artepillin C standard, determining relative retention times, and confirming ion fragmentation. The standards and samples were [M-H]- 299.220 in extracted ion chromatograms and had the same retention time.

2.3. Preparation of EEGP-MC

The preparation of EEGP-MC was performed according to Keskin et al. [21], with modifications. Sodium alginate (1 %) was dissolved in water under constant stirring (180 rpm) on a hot plate at 100 °C. After dissolution and cooling, 2 mL of 10 % EEGP was added to 98 mL of the alginate solution. Alginate and propolis-alginate beads were formed

using vibrating nozzle ionotropic gelation technology (B-390 Encapsulator, Buchi) with the following parameters: frequency of 200 Hz, electrode voltage of 1000 V, and pressure of 300 mbar. The encapsulator dispensed the droplets of alginate and propolis-alginate into a 5 % $CaCl_2$ solution in distilled water. The beads were left in the solution for 15 min to ensure complete gelation. Finally, the beads were filtered and washed with distilled water, then dried at room temperature for 24 h.

2.3.1. Encapsulation efficiency

A 0.2 g sample of EEGP-MC was suspended in 5 mL sodium phosphate buffer (0.2 M, pH 7.5) and shaken continuously at 37 °C at 180 rpm for 3 h. The total phenolic content in the sodium citrate solution was determined using the Folin-Ciocalteu method. Encapsulation efficiency (EE %) refers to the percentage of phenolic compounds incorporated into the microcapsules and was calculated using the following equation:

$$EE\ (\%) = (A\alpha/Ae) \times 100$$

EE (%) is the encapsulation efficiency, Aα is the total phenolic content of the EEGP-MC, and Ae is the total phenolic content of the EEGP.

2.3.2. Moisture and water activity (aw)

EEGP-MC's water activity (aw) was determined using a water activity meter (AQUALAB 4TE, Aqualab, Decagon Devices, USA). The moisture content was measured using a moisture analyzer (Ohaus, MB27, USA).

2.3.3. Quantification of total phenolic content

The total phenolic content (TPC) was measured as the Folin-Ciocalteu reducing capacity, following the method of Singleton et al. [22] and Margraf et al. [23]. Briefly, 25 μL of EEGP, EEGP-MC, or a blank (water or ethanol) was added to 200 μL of ultrapure water and 25 μL of Folin-Ciocalteu reagent solution. The plates were homogenized, and after 5 min, 25 μL of 10 % sodium carbonate solution was added to the mixture, followed by further homogenization. The absorbance was measured at 725 nm after 60 min using a microplate reader (Microplate Spectrophotometer, BioTek - Eon®). The Folin-Ciocalteu reducing capacity was expressed in mg gallic acid equivalents per gram of sample (mg GAE/g), calculated from the regression line obtained from the standard curve.

2.3.4. Scanning electron microscopy

The EEGP-MC were photographed using scanning electron microscopy (SEM). Initially, the sample underwent lyophilization to eliminate moisture, followed by a gold coating. Subsequently, the morphological analysis was performed using the JEOL JSM-5600LV microscope and the JEOL software [24].

2.4. Release assay of EEGP-MC and evaluation of antioxidant activity

Briefly, 0.2 g of EEGP-MC was weighed and placed in separate 10 mL solutions of simulated gastric fluid (composition, pH = 1.5), which were subjected to agitation (180 rpm) on a hot plate at 37 °C for 4 h. The mixture was then centrifuged, and the precipitated microspheres were dispersed in 10 mL of simulated intestinal fluid (composition, pH = 7.2) for 6 h at 37 °C and 180 rpm. Every 2 h, an aliquot of 1 mL was individually collected from the solutions, and the polyphenols were quantified using the Folin-Ciocalteu method. In contrast, Artepillin C was quantified using high-performance liquid chromatography (HPLC) [25].

2.4.1. Chemical analysis by HPLC

To quantify the Artepillin C compound in each phase of particle digestion, a Shimadzu HPLC system equipped with a C18 column (5 μm; 4.6 × 250 mm) and a photodiode array detector (PDA) was used to achieve compound separation. The oven temperature was 37 °C, and the sample injection volume was 20 μL. The mobile phase (flow rate: 0.8 mL/min) consisted of (A) acetic acid/water (0.5 %/99.5 %; *v*/v) and (B) 100 % methanol, with the following gradient: 30 % solvent B (0 min); 40 % solvent B (15 min); 50 % solvent B (30 min); 60 % solvent B (45 min); 75 % solvent B (65 min); 90 % solvent B (95 min); and returning to 30 % solvent B (105 min) until 120 min. The identity of Artepillin C was confirmed by comparison with an authentic standard from Sigma-Aldrich. The same standard was used to create an analytical curve consisting of eight dilution points (2 μg/mL – 0.0062 μg/mL). Linearity was determined, and the limits of detection (LOD) and quantification (LOQ) were calculated according to Massarioli et al. [26].

2.4.2. Antioxidant capacity

For the ferric reducing antioxidant power (FRAP), 20 μL of the gastric phase (GP) at 0, 2, and 4 h and the intestinal phase (IP) at 2, 4, and 6 h of the EEGP-MC were mixed with 180 μL of freshly prepared FRAP reagent. The samples were incubated for 30 min, after which the absorbance was measured at 593 nm using a microplate reader (Microplate Spectrophotometer, BioTek - Eon®, Agilent Technologies, Santa Clara, CA, USA). The antioxidant capacity was assessed using ascorbic acid as the standard. The results were expressed as mg of ascorbic acid equivalent (AAE) per gram (mg AAE/g) [27].

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the GP (0, 2, and 4 h) and IP (2, 4, and 6 h) of the EEGP-MC was assessed using a spectrophotometric assay. Aliquots of 40 μL of the samples were added to a well of a 96-well plate, along with 260 μL of a 0.10 mmol/L methanolic solution of DPPH. The mixture was reacted in the dark at 25 °C for 30 min, and the decrease in DPPH absorbance was measured at 517 nm using a microplate reader. The antioxidant capacity was assessed using ascorbic acid as the standard. The results were expressed as mg AAE/g [28].

For the cupric reducing antioxidant capacity (CUPRAC), aliquots of 50 μL of the GP (0, 2, and 4 h) and IP (2, 4, and 6 h) of the EEGP-MC were mixed with 500 μL of each solution: Copper(II) chloride ($CuCl_2$), neocuproine solution, and ammonium acetate (NH_4Ac), along with 500 μL of water. Absorbance was recorded at 450 nm after 30 min of incubation. The antioxidant capacity was assessed using ascorbic acid as the standard. The results were expressed as mg AAE/g [29].

2.5. Cell-based assays

2.5.1. THP-1 macrophage-like cell culture and differentiation

THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 150 U/mL penicillin, 200 U/mL streptomycin, and 0.05 mM 2-mercaptoethanol. The cells were maintained in an incubator with 5 % CO_2 at 37 °C. For differentiation into macrophages, the cells were seeded in 96-well plates at a density of 20,000 cells per well and treated with PMA (100 nM) for 3 days.

2.5.2. Viability assay

THP-1 cells were pretreated with EEGP-MC (30, 300, and 1000 μg/mL), and the IP of the EEGP-MC (30, 300, and 1000 μg/mL). After 24 h, the supernatant was removed, and RPMI medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 3 h, the supernatant was removed, and 200 μL of absolute ethanol was added. The absorbance was measured at 540 nm using a microplate reader [30].

2.5.3. Cytokine quantification assay

The experiment was conducted according to Mulvey et al. [31], with modifications. THP-1 cells were pretreated with EEGP-MC (300 and 1000 μg/mL), and the IP of the EEGP-MC (300 and 1000 μg/mL) for 30 min before stimulation with LPS at 100 ng/mL. After 6 h (for TNF-α - R&D Systems #DY410) and 24 h (for IL-6 - R&D Systems #DY406), the supernatant was collected, and cytokines were quantified using enzyme-linked immunosorbent assay (ELISA) with DuoSet ELISA Development

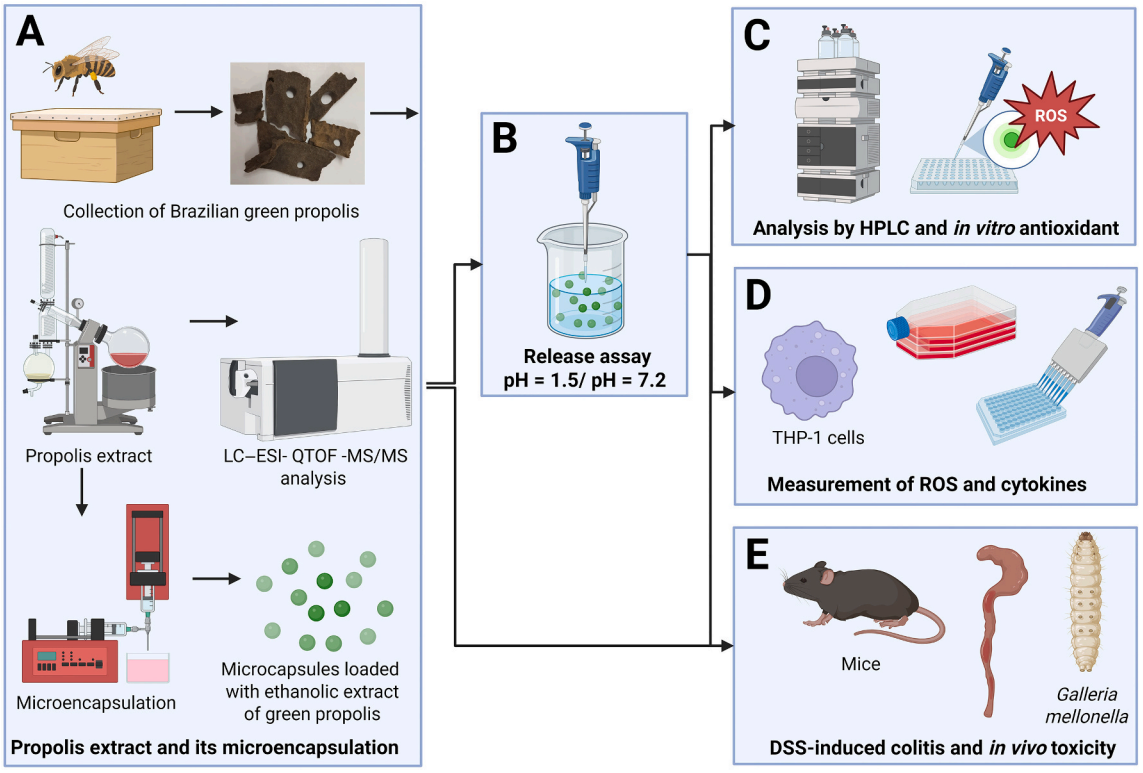

Fig. 1. Experimental design. (A) Collection, extraction (80 % ethanol in water (*v*/v) for 24 h using a magnetic stirrer at room temperature), and chemical analysis (LC–ESI-QTOF-MS/MS) of Brazilian green propolis, followed by the preparation of sodium alginate-based microcapsules loaded with ethanolic extract of green propolis (EEGP-MC). (B) Release assay of EEGP-MC at pH 1.5 (gastric phase) and 7.2 (intestinal phase). (C) Chemical analysis by HPLC and *in vitro* antioxidant assays (FRAP, DPPH, and CUPRAC). (D) Cell-based assays using THP-1 macrophage-like cells to measure ROS (DCFH-DA) and cytokines (TNF-α and IL-6). (E) DSS-induced colitis model with evaluation of body weight change (%), colon length (cm), histological analysis, and levels of TNF-α, IL-6, and IL-1β in the colon. *In vivo* evaluation of systemic toxicity in *Galleria mellonella*. Figure created using https://BioRender.com.

Systems kits according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader.

2.5.4. Measurement of reactive oxygen species (ROS)

THP-1 cells were pretreated with EEGP-MC (300 and 1000 μg/mL), and the IP of the EEGP-MC (300 and 1000 μg/mL), diluted in DCFH-DA (5 mmol/L), for 30 min before stimulation with LPS at 100 ng/mL. After 6 h, the supernatant was removed, and the cells were washed with PBS. Subsequently, 100 μL of HANKS solution was added, and fluorescence was detected using a microplate reader (BioTek Synergy H1 Multimode Reader®, Agilent Technologies, Santa Clara, CA) with emission at 538 nm and excitation at 485 nm [31–33].

2.6. DSS-induced colitis

Male C57BL/6 mice (20–22 g) were obtained from the Central Vivarium of the University of São Paulo in Ribeirão Preto. The animals were randomly (https://www.graphpad.com/quickcalcs/randomize1/) divided into groups (5 per group) and housed in the Central Vivarium of the School of Medicine of Ribeirão Preto under controlled conditions (22–25 °C, 40–60 % humidity, 12 h light/dark cycle). They were provided with a standard pellet diet and water *ad libitum*. The study received ethical approval from the research ethics committee (Reference: 190/2018) and followed the ARRIVE guidelines. The mice received oral treatments (DietGel® Recovery, #72-06-5022, Fig. S2) with EEGP-MC (100 or 300 mg/kg), EEGP (300 mg/kg), PBS with 2 % DMSO (Control), or aqueous gel with empty microcapsules (Control) once daily for 12 days. Colitis was then induced by adding 2 % DSS (MP Biomedicals, Santa Ana, United States) to autoclaved drinking water for 6 days, with the water replaced every 2 days. On day 6, DSS was removed, and normal water was provided until euthanasia on day 8. Bodyweight and survival were monitored throughout the experiment. The clinical activity index of the disease was assessed using clinical scores based on fecal consistency, weight loss, and bleeding: Score 0: body weight decrease (%) ≤1, stool consistency = normal, rectal bleeding = normal; Score 1: body weight decrease (%) = 1–5; Score 2: body weight decrease (%) = 5–10, stool consistency = loose stools; Score 3: body weight decrease (%) = 10–20; Score 4: body weight decrease (%) ≥20, stool consistency = diarrhea, rectal bleeding = gross bleeding. After euthanasia, colons were collected to measure their length. Dissection was performed from the ileocecal junction to the rectum, with length measured from the cecocolonic junction to the rectum. A distal colon fragment was fixed in 4 % paraformaldehyde (#01P100501AH) for histological analysis and processed into sections. Colitis severity was assessed *via* hematoxylin (#H9627) and eosin (#E4009) (H&E) staining, evaluating eight components: inflammatory infiltrate, goblet cell loss, hyperplasia, crypt density, muscle thickness, submucosal infiltration, ulcerations, and crypt abscesses, each scored from 0 to 3. The total histological score ranged from 0 to 24. Finally, the remaining colon was frozen at −70 °C for quantifying inflammatory cytokines. Subsequently, 1× PBS containing a protease inhibitor (#11697498001) was added to the tissue, and processing was performed using a homogenizer (QUIAGEN TissueLyzer II). The quantification of the cytokines TNF-α, IL-6, and IL-1β (#DY401) was performed in the collected supernatant using an enzyme-linked immunosorbent assay (ELISA) with DuoSet ELISA Development Systems kits, following the manufacturer's instructions. The results were expressed as pg/mg of tissue [34].

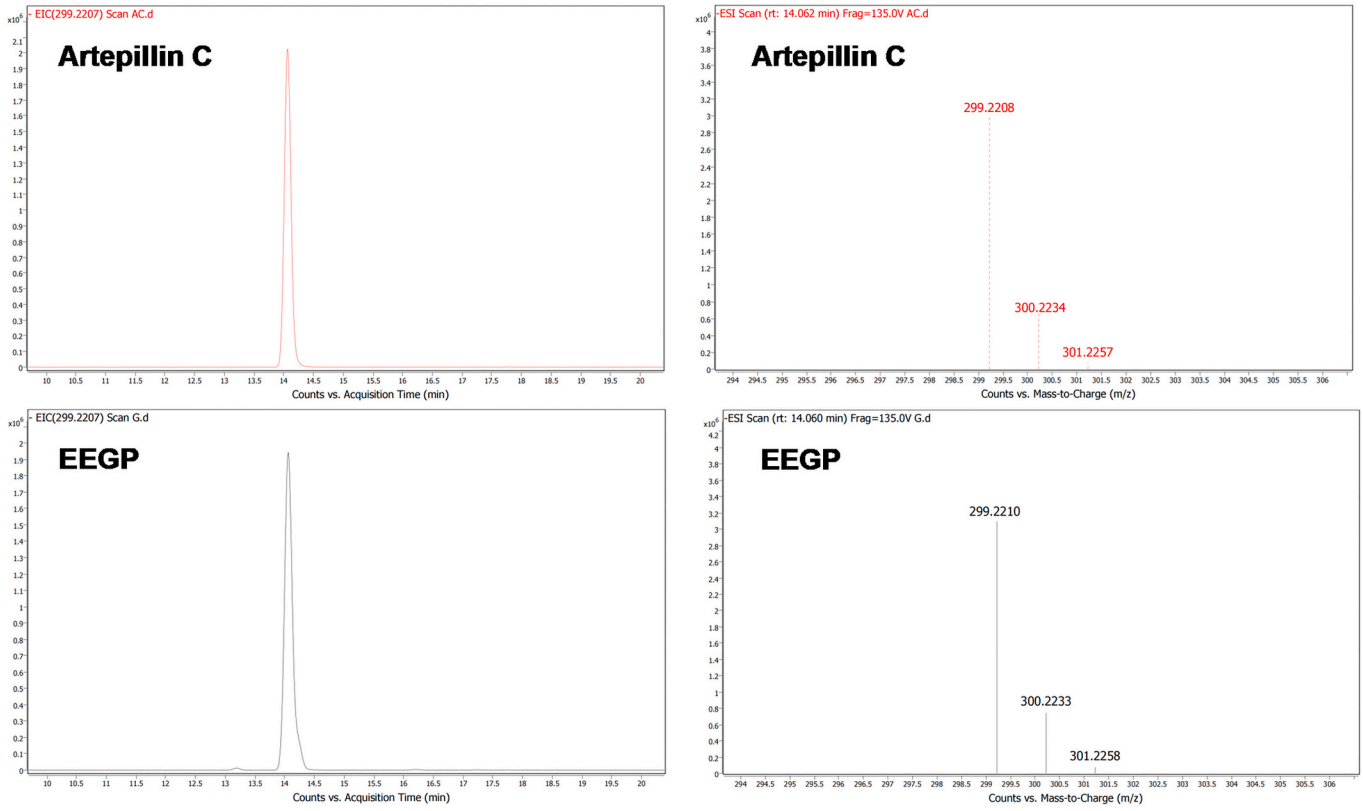

Fig. 2. HPLC-ESI (-)-Q-ToF Extracted ion chromatograms (EIC) of Artepillin C standards and comparisons with ethanolic extract of green propolis (EEGP).

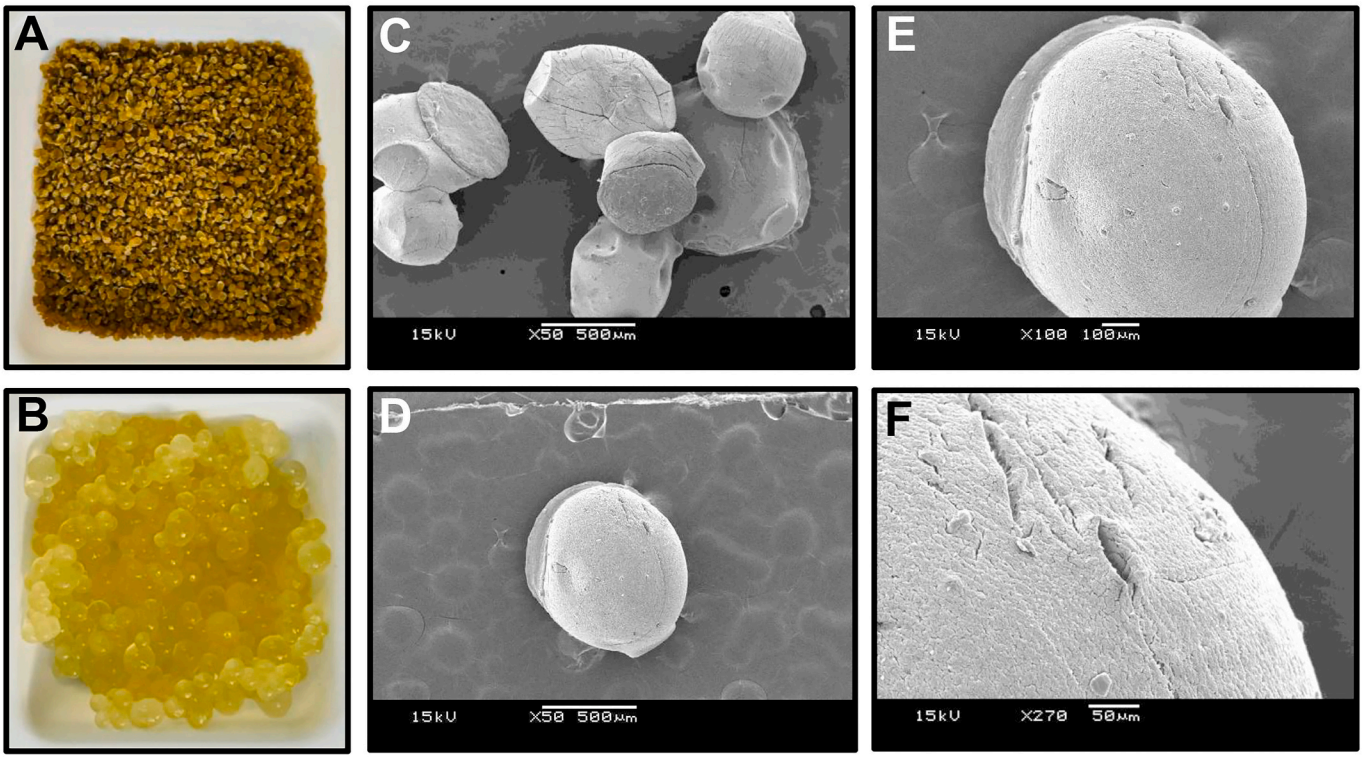

Fig. 3. (A) Dry microcapsules loaded with ethanolic extract of green propolis (EEGP-MC). (B) Hydrated EEGP-MC. (C-F) Scanning electron microscopy of dry EEGP-MC.

Table 1
Characterization of alginate-based microcapsules loaded with ethanolic extract of green propolis (EEGP-MC).

Sample	Analysis	Results
EEGP-MC	Encapsulation efficiency (%)	64.3 ± 4.8
	TPC (mg GAE/g)	32.25 ± 1.55
	Moisture (%)	23.4 ± 0.2
	Hygroscopicity (g/100 g capsule)	37.7 ± 0.6

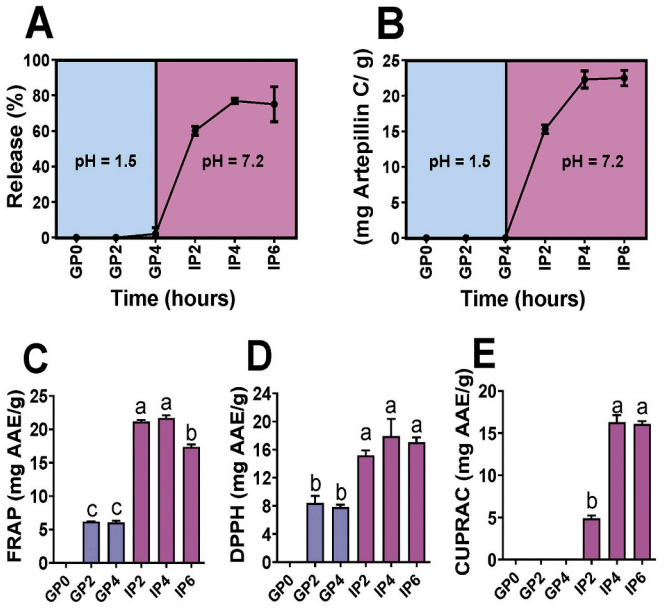

Fig. 4. Release dynamics of microcapsules loaded with ethanolic extract of green propolis (EEGP-MC) and antioxidant activity. (A) Quantification of total phenolic content in the gastric phase (GP) at 0 (GP0), 2 (GP2), and 4 h (GP4), and in the intestinal phase (IP) at 0 (IP0), 2 (IP2), 4 (IP4), and 6 h (IP6) of the EEGP-MC. (B) Artepillin C quantification by HPLC in the GP (0, 2, and 4 h) and the IP (0, 2, 4, and 6 h). (C) Antioxidant activity in the GP (0, 2, and 4 h) and the IP (0, 2, 4, and 6 h) using the ferric reducing antioxidant power (FRAP) assay. (D) Antioxidant capacity in the GP (0, 2, and 4 h) and the IP (0, 2, 4, and 6 h) using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. (E) Antioxidant activity in the GP (0, 2, and 4 h) and the IP (0, 2, 4, and 6 h) using the cupric reducing antioxidant capacity (CUPRAC) assay. Results are expressed as mg ascorbic acid equivalent (AAE) per g of extract. Data are presented as mean ± SEM, $n = 3$. Different letters indicate statistically significant differences within groups.

2.7. In vivo toxicity in Galleria mellonella

To assess the *in vivo* toxicity of EEGP, EEGP-MC, and the IP, we conducted a survival assay on *G. mellonella* larvae [35,36]. Larvae were reared at 28 °C, and those weighing between 200 and 250 mg were randomly selected ($n = 10$ per group). Each larva received 10 μL of EEGP (at doses of 30, 130, 300, 400, 500, and 1000 mg/kg), EEGP-MC (300 mg/kg), IP (300 mg/kg), or a vehicle control of 2 % DMSO (dimethyl sulfoxide) in PBS. The injections were administered into the hemocoel *via* the last left proleg. All groups were incubated at 28 °C, and survival was monitored at 24, 48, and 72 h. Larvae exhibiting high melanization and no movement upon touch were considered dead.

2.8. Statistical analysis

The results are expressed as mean ± SEM. Antioxidant and anti-inflammatory activities *in vitro* and *in vivo* were analyzed using one-way and two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The survival percentage of *G. mellonella* larvae was compared using the log-rank Mantel–Cox test. All data analyses were performed using GraphPad Prism version 10.2 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Preparation and release dynamics of EEGP-MC and their modulatory effects on inflammatory cytokines and ROS in THP-1 cells

The experimental design of this study is presented in Fig. 1. Brazilian propolis from *Apis mellifera* exhibits a complex chemical composition that varies depending on the location and time of year when it is collected [37]. Furthermore, due to this variability in chemical composition, analyses such as identifying markers are essential to ensure the quality and reproducibility of studies involving propolis [38–12]. The present study obtained EEGP using 80 % ethanol in water. The identification of the characteristic marker of Brazilian green propolis, Artepillin C, was confirmed by LC-MS/MS (Fig. 2). Brazilian green propolis has *Baccharis dracunculifolia* D.C. (Asteraceae) as its primary botanical source and features a complex profile of phenolic compounds, with Artepillin C standing out as a key marker of its chemical composition [39,40]. Moreover, it is the main compound in Brazilian green propolis and is involved in numerous biological activities, such as antioxidant, antitumor, antimicrobial, and anti-inflammatory effects [40]. In this context, in addition to the chemical evaluation, the pursuit of tools that facilitate the incorporation and creation of a protective barrier for bioactive compounds, such as Artepillin C, while preserving their biological activities, holds significant interest and importance for applications in the food and pharmaceutical industries [17].

In this study, EEGP-MC were obtained using the vibrating nozzle ionotropic gelation technology (Fig. 3). Table 1 shows that microcapsules loaded with the green propolis had a high encapsulation efficiency (64.3 ± 4.8 %), TPC (32.25 ± 1.55 mg GAE/g), moisture content (23.4 ± 0.2 %), and hygroscopicity (37.7 ± 0.6 g/100 g capsule). Additionally, the release dynamics of phenolic compounds and Artepillin C from the EEGP-MC were evaluated, along with their antioxidant activity, using the FRAP, DPPH, and CUPRAC assays (Fig. 4). During the IP, the peak release of phenolic compounds (Fig. 4A) was observed at 4 h (76.9 %) and 6 h (75.0 %). Similarly, the peak release of Artepillin C (Fig. 4B) during the IP was recorded at concentrations of 22.3 ± 1.2 mg/g (4 h) and 22.5 ± 1.1 mg/g (6 h). Regarding antioxidant activity assessed through chemical assays, the EEGP-MC exhibited significantly better performance during the IP than the GP ($p < 0.05$). The maximum effect was observed at 4 h: FRAP 21.7 ± 0.71 mg AAE/g (Fig. 4C), DPPH 17.9 ± 4.23 mg AAE/g (Fig. 4D), and CUPRAC 16.3 ± 1.30 mg AAE/g (Fig. 4E). The benefits of propolis encapsulation are well-documented in the literature, highlighting its ability to provide stability and solubility to bioactive compounds while also reducing its characteristic odor [41]. However, our studies are pioneering in using vibrating nozzle ionotropic gelation technology with propolis. Furthermore, the use of sodium alginate facilitated the microencapsulation of green propolis compounds, specifically Artepillin C. Sodium alginate is a natural anionic polymer that forms a gel matrix in the presence of Ca^{2+} ions, offering low toxicity, biodegradability, and biocompatibility [42]. In addition, it enables controlled bioactive release, as demonstrated by the release tests *in vitro*.

As presented in Fig. 5, the modulatory activity of the EEGP-MC and their IP on the release of ROS and pro-inflammatory cytokines (TNF-α and IL-6) was evaluated in activated THP-1 cells. Initially, to determine the toxic and non-toxic doses of the tested samples, the viability of THP-1 macrophages (MTT assay) was assessed after pre-treatment with EEGP-MC and the IP of the EEGP-MC for 24 h. According to the results, the EEGP-MC (30, 300, and 1000 μg/mL) (Fig. 5A) and the IP (30, 300, and 1000 μg/mL) (Fig. 5E) did not affect macrophage viability at any of the tested doses ($p > 0.05$). Subsequently, the samples' ability to modulate the release of inflammatory cytokines and ROS in THP-1 cells

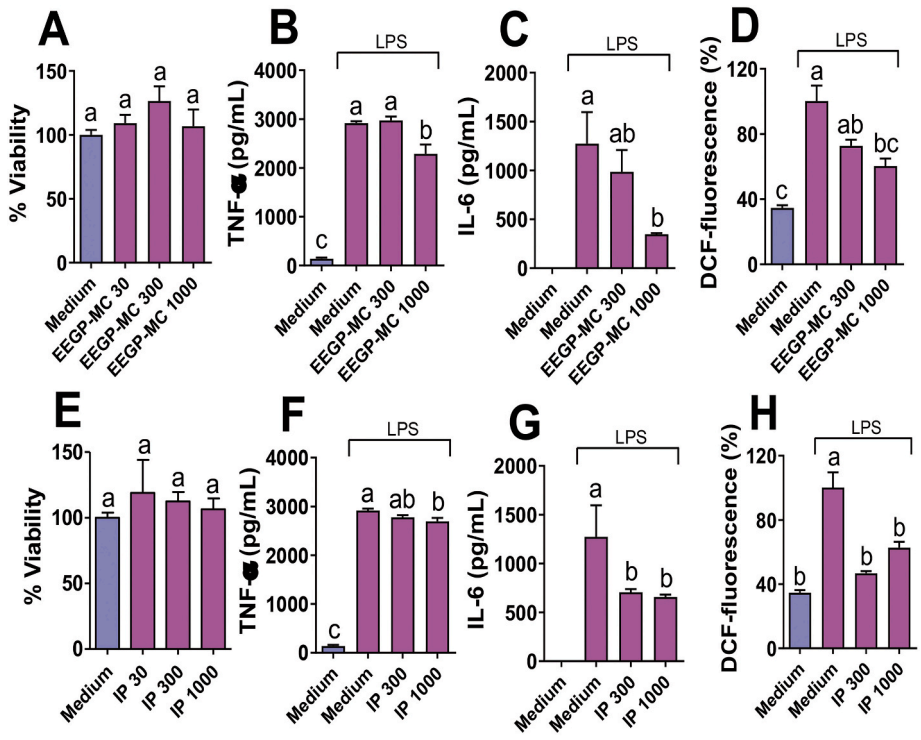

Fig. 5. Microcapsules loaded with ethanolic extract of green propolis (EEGP-MC) and their intestinal phase (IP) reduce reactive oxygen species (ROS) and pro-inflammatory cytokines in activated THP-1 macrophages. THP-1 macrophages were pretreated with EEGP-MC at 30, 300, and 1000 μg/mL, IP at 30, 300, and 1000 μg/mL, or medium (vehicle), 30 min before stimulation with LPS at 100 ng/mL. (A, E) Cell viability of THP-1 macrophages incubated for 24 h with the tested samples. (B, F) TNF-α levels in the supernatant of THP-1 macrophages stimulated with LPS for 6 h. (C, G) IL-6 levels in the supernatant of THP-1 macrophages stimulated with LPS for 24 h. (D, H) ROS levels in the supernatant of THP-1 macrophages stimulated with LPS for 6 h. Data are expressed as mean ± SEM ($p < 0.05$; one-way ANOVA followed by Tukey's test); $n = 4$–5. Different letters indicate statistically significant differences within groups.

was evaluated. The results showed that the EEGP-MC at 1000 μg/mL (Fig. 5B) reduced TNF-α levels in the macrophage supernatant by 21.5 %, while the IP at 1000 μg/mL (Fig. 5F) reduced TNF-α levels by 7.6 %, compared to the medium control group (LPS-activated) ($p < 0.05$). Regarding IL-6, the EEGP-MC at 1000 μg/mL reduced its levels by 72.7 % (Fig. 5C), whereas the IP reduced IL-6 by 44.6 % and 48.4 % at concentrations of 300 and 1000 μg/mL, respectively (Fig. 5G) ($p < 0.05$). Finally, in the production of ROS in LPS-activated THP-1 macrophages, pre-treatment with EEGP-MC at 1000 μg/mL (Fig. 5D) resulted in a 39.9 % reduction. The IP reduced ROS production by 53.4 % (300 μg/mL) and 37.5 % (1000 μg/mL) (Fig. 5H) compared to the medium control group (LPS-activated) ($p < 0.05$). The effects of green propolis on the modulation of inflammatory cytokines in cell cultures are extensively documented in the literature [9]. Wu et al. [43] demonstrated, in MG6 microglia culture, that green propolis could inhibit the production of IL-1β, TNF-α, and IL-6. In another study, the ethanolic extract of Brazilian green propolis inhibited a wide range of inflammatory cytokines in J774A.1 macrophage culture, including TNF-α and IL-6 [44]. Thus, the results obtained in THP-1 cell culture indicate the potential modulatory effect of the bioactives released in the IP of EEGP-MC on the inflammatory process. This highlights the need to further investigate its anti-inflammatory activity using an animal model of inflammatory diseases, such as experimental colitis.

3.2. *EEGP-MC improve DSS-induced colitis*

The activity of the EEGP-MC in DSS-induced colitis in mice highlights the essential role of animal models in elucidating the pathophysiological aspects of intestinal inflammation and in developing new drugs [45]. Among the commonly used experimental models, DSS-induced colitis stands out for its characteristics, including increased expression of inflammatory cytokines (*e.g.*, TNF-α, IL-6, and IL-17), inflammatory infiltrates in intestinal tissue, and gut microbiota dysbiosis [46]. Moreover, this model offers rapid execution and high reproducibility, making it a valuable tool for preclinical studies [46]. In the present study, oral administration of EEGP-MC at 300 mg/kg (once daily for 8 days) effectively prevented pathological aspects of experimental colitis, including body weight loss (Fig. 6B) and colon shortening (Fig. 6C), compared to the control group (Gel) ($p < 0.05$). Additionally, when compared to the group of animals receiving only EEGP at 300 mg/kg, the EEGP-MC at the same dosage showed superior activity in protecting the colonic mucosa (Fig. 6D-E), with an 86.1 % reduction in lesions, compared to a 54.9 % reduction with EEGP alone ($p < 0.05$). The results of Mariano et al. [47] support our findings on the protective effect of Brazilian green propolis extract at 300 mg/kg in experimental colitis. However, our data demonstrated that microencapsulation of EEGP enhances its protective effects in colitis. For instance, Keskin et al. [21] showed that alginate-based microencapsulation of Turkish propolis promoted controlled intestinal release of phenolic compounds. In this study, we demonstrated *in vitro* that the EEGP-MC promote intestinal release, suggesting that the observed effects in experimental colitis are directly linked to gastric protection and the targeted release of phenolic compounds, such as Artepillin C, in the intestine. In addition, the synergistic effects of the phenolic compounds may contribute significantly to the protective effect observed in experimental colitis.

The mechanisms through which the EEGP-MC at 300 mg/kg modulate acute colitis were investigated. It was observed that the treatment significantly reduced the levels of inflammatory cytokines, such as TNF-α (Fig. 6F), IL-6 (Fig. 6G), and IL-1β (Fig. 6H), in the colon of DSS-induced acute colitis mice ($p < 0.05$). However, the results obtained with the EEGP-MC at 300 mg/kg did not differ significantly from those of the group receiving only EEGP ($p > 0.05$). In animal experimental models, previous studies with Brazilian green propolis have demonstrated its potential in modulating the immune system, cytokine

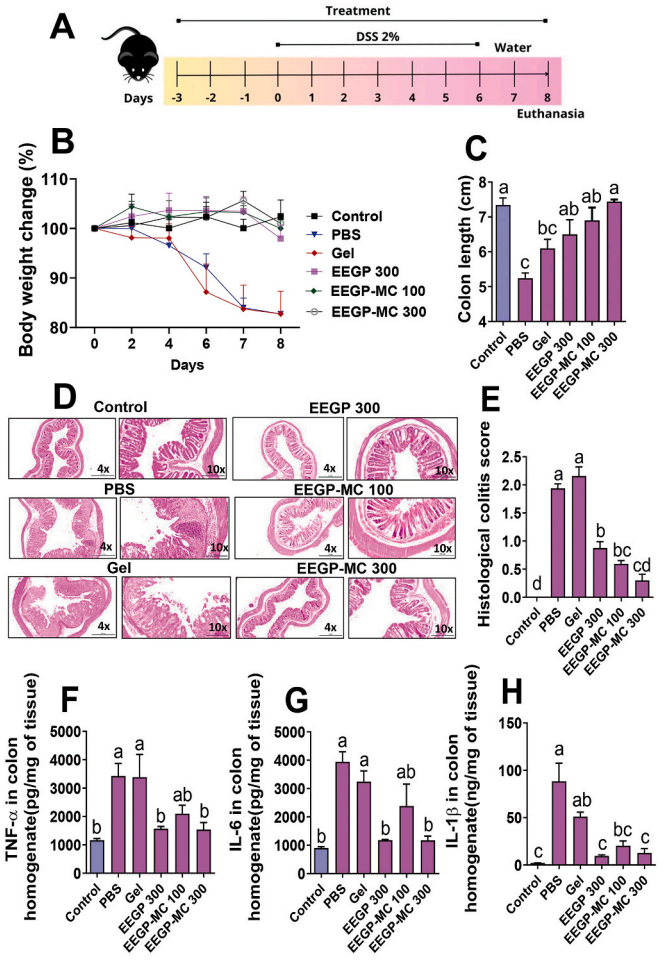

Fig. 6. Microcapsules loaded with ethanolic extract of green propolis (EEGP-MC) reduce intestinal inflammation in mice with DSS-induced colitis. Mice were treated orally (1×/day) with EEGP at 300 mg/kg, EEGP-MC at 100 and 300 mg/kg, PBS with 2 % DMSO (control), or aqueous gel (control), and challenged with 2 % DSS intraperitoneally. (A) Flowchart of DSS 2 % administration and treatment. (B) Daily assessment of body weight change (%) in mice over 8 days after DSS challenge, $n = 5$. (C) Colon length (cm) of mice after 8 days of DSS challenge, n = 5. (D, E) Histological analysis of the colon after 8 days of DSS challenge, n = 4–5. (F, G, H) Levels of TNF-α, IL-6, and IL-1β in colon homogenates after 8 days of DSS challenge, n = 5. Data are expressed as mean ± SEM ($p < 0.05$; ANOVA followed by Tukey's test), n = 5 (total number of 30 C57BL/6 mice). Different letters indicate statistically significant differences within groups.

production, and inflammation. Studies have highlighted the ability of green propolis extract to suppress lymphocyte differentiation [48] and influence the gut microbiome [9]. Furthermore, Artepillin C, the key bioactive compound of green propolis, has shown *in vitro* capability to suppress the production of inflammatory cytokines, such as TNF-α and IL-1β, as well as to inhibit the expression of the transcription factor NF-κB [49]. Therefore, our results demonstrated that microencapsulation effectively preserved green propolis's *in vitro* anti-inflammatory activities. These effects observed with EEGP-MC could provide significant clinical benefits in managing colitis inflammation by inhibiting inflammatory mediators and controlling cellular influx and edema. However, clinical studies need to be conducted to confirm the therapeutic efficacy of this product. Finally, this prototype demonstrates considerable potential for applications in the pharmaceutical and food industries, particularly for incorporation into nutraceuticals and therapeutic medications.

To better understand the toxicological profile of microcapsules, we

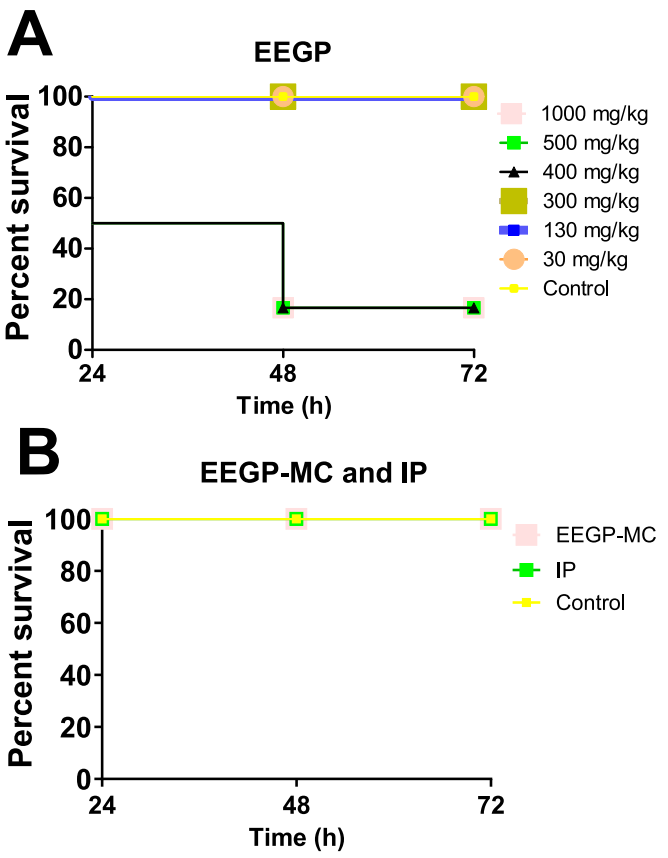

Fig. 7. Systemic toxicity of ethanolic extract of green propolis (EEGP), alginate-based microcapsules loaded with EEGP (EEGP-MC), and their intestinal phase (IP) in *G. mellonella*. (A) *G. mellonella* larvae were treated with EEGP at 30, 130, 300, 400, 500, and 1000 mg/kg. (B) *G. mellonella* larvae were treated with EEGP-MC at 300 mg/kg (mg extract/kg larvae), the IP at 300 mg/kg (mg/kg larvae), or PBS (control). The larvae were monitored at 24, 48, and 72 h after sample administration. Data are expressed as mean ± SEM ($p < 0.05$, log-rank test); $n = 10$.

investigated the systemic toxicity of EEGP (Fig. 7A) and EEGP-MC (Fig. 7B), as well as their IP (Fig. 7B), using the alternative model of *G. mellonella*. This model has proven to be an excellent method for screening the toxicological profile of natural products [50]. In the *G. mellonella* model, larval death is identified by melanization and/or lack of movement upon touch [50]. In this study, we observed that EEGP at concentrations of 30, 130, and 300 mg/kg did not induce systemic toxicity in larvae over a 72-h period. However, larval mortality was observed at 400, 500, and 1000 mg/kg doses. Regarding the EEGP-MC at 300 mg/kg and their IP at 300 mg/kg, no toxicity was detected in the *G. mellonella* model. These findings demonstrate that at the concentration of 300 mg/kg used for EEGP and its microencapsulation, no systemic toxicity was observed in *G. mellonella*, supporting the therapeutic effects evidenced in DSS-induced colitis. In addition, the data demonstrated that this microencapsulation prototype emerges as a promising tool to enhance the solubility, bioavailability, stability, and safety properties of EEGP.

4. Conclusion

The EEGP-MC exhibited stability in the GP and promoted the sustained release of phenolic compounds and Artepillin C in the IP, as demonstrated by their *in vitro* antioxidant and anti-inflammatory activities. In the DSS-induced colitis model in mice, the EEGP-MC improved inflammatory aspects of the colon and reduced the production of inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Lastly, no

systemic toxicity was observed for the EEGP-MC or their IP. The results of this study demonstrate the ability of alginate-based microcapsules to protect bioactive compounds and enhance the biological effects of EEGP. This innovative technology demonstrates the potential of EEGP-MC for use in the food industry to develop new products and in the pharmaceutical industry, with possibilities for incorporation into medications to manage chronic inflammation, such as ulcerative colitis. However, additional studies on stability and bioavailability are necessary to ensure its therapeutic efficacy and the preservation of the bioactive compounds in Brazilian green propolis.

CRediT authorship contribution statement

Marcelo Franchin: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana Sofia Martelli Chaib Saliba:** Writing – original draft, Methodology, Investigation. **Anderson dos Santos Ramos:** Writing – original draft, Methodology, Investigation. **Fernanda Papa Spada:** Writing – original draft, Methodology, Investigation. **Daniela Coelho dos Santos:** Writing – original draft, Methodology, Investigation. **Maísa de Oliveira Leandro:** Writing – original draft, Methodology, Investigation. **Eduardo Da Campo Junqueira Gonçalves:** Methodology, Investigation. **Bruno Bueno-Silva:** Writing – original draft, Methodology, Investigation. **Yandong Xu:** Writing – original draft, Methodology, Investigation. **Kai Wang:** Writing – original draft, Methodology, Investigation. **Jason Bennett:** Methodology, Investigation. **Thiago Mattar Cunha:** Writing – original draft, Methodology, Investigation. **Severino Matias de Alencar:** Writing – original draft, Methodology, Investigation, Funding acquisition. **Daniel Granato:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics approval and consent to participate

Approval was obtained from the Animal Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (CEUA FMRP/USP) (Ethical Approval: Reference number 190/2018).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author used ChatGPT to improve readability and language. After using this tool/service, the author reviewed and edited the content as needed and took full responsibility for the publication's content.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.143357.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Some data may be restricted due to privacy or ethical considerations.

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