Royal jelly and propolis therapies reduce inflammation and stimulate healing of oral mucositis in rats

Mara Luana Batista Severo
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude

Stéfanie Thieme
Universidade Federal do Rio Grande do Sul Faculdade de Odontologia

Felipe Martins Silveira
Universidade Estadual de Campinas

Raquel Padilha Martins Tavares
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude

Amanda Katarinny Goes Gonzaga
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude

Silvana Maria Zucolotto
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude

Aurigena Antunes Araújo
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude

Marco Antonio Trevizani Martins
Universidade Federal do Rio Grande do Sul Faculdade de Odontologia

Manoela Domingues Martins Domingues Martins
Universidade Federal do Rio Grande do Sul Faculdade de Odontologia

Ericka Janine Dantas Silveira (ericka_janine@yahoo.com.br)
Universidade Federal do Rio Grande do Norte Centro de Ciências da Saúde: Universidade Federal do Rio Grande do Norte Centro de Ciencias da Saude https://orcid.org/0000-0003-2128-0147

Research Article

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Abstract

Purpose This study aimed to evaluate the effects and mechanisms of action of royal jelly (RJ) and propolis in an animal model of oral mucositis (OM).

Methods Seventy-two male Wistar rats were randomly allocated to four groups (n = 18): control (no treatment), PBMT (intraoral laser, 6 J/cm2), RJ, and propolis. On days 0 and 2, animals received injection of 5-fluorouracil (5-FU). The buccal mucosa was scratched (days 3 and 4) and the treatments were initiated on day 5. Six animals of each group were euthanized on days 8, 10, and 14. Phytochemical analysis (thin-layer chromatography-TLC), clinical, histopathological, immunohistochemical of pS6, pAKT and NF-κB and oxidative stress markers were also investigated.

Results TLC revealed the presence of large amounts of sucrose (Rf 0.34) in RJ and flavonoids in propolis. On day 8, lower clinical OM scores (and day 10) and morphological were observed in the PBMT, RJ and propolis groups ($p < 0.05$). On day 8, immunoexpression of pS6, pAKT and NF-κB was increased when compared to control. On day 14, GSH antioxidant levels were increased in the propolis group compared to control ($p < 0.05$).

Conclusions Our results showed that RJ and propolis, as well as PBMT, are effective in the treatment of OM. The RJ and propolis may explain their excellent wound healing activity and anti-inflammatory effects. Considering that not all patients who develop OM have access to PBMT, the present study demonstrated that topical application of RJ and propolis may be an important alternative for the treatment of OM.

Introduction

Oral mucositis (OM) is a common and severe complication of cancer treatment. It is estimated that more than 90% of patients undergoing radiotherapy for head and neck cancers have some degree of OM. This condition is therefore considered one of the most important acute side effects observed in patients with head and neck cancer submitted to radiotherapy and/or chemotherapy [1–4].

In general, OM clinically manifests as erythematous, erosive and/or ulcerative lesions of variable clinical severity. The ulcers are usually painful and therefore interfere directly with the quality of life of the patient. Additionally, OM increases the risk of secondary infections, which may lead to a delay, interruption or discontinuation of oncological treatment, with a consequent increase in the morbidity and mortality of cancer patients [3, 5].

The pathogenesis of OM comprises several stages: initiation, positive regulation and activation that result in the production of inflammatory mediators, signal amplification, ulceration, and healing. The initiation phase is characterized by tissue damage due to oxidative stress resulting from the production of reactive oxygen species (ROS), direct DNA damage, and activation of the innate immune response. This oxidative stress causes damage to cell membranes and triggers the activation of macrophages and

nuclear factor kappa B (NF-κB). Activated NF-κB induces gene expression and the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, and cyclooxygenase 2 (COX-2). The healing phase occurs after the harmful stimuli are eliminated and is characterized by increased epithelial cell proliferation and differentiation, associated with reorganization of the extracellular matrix [4, 6–10].

Some authors have linked the development of OM and aphthous ulcerations to activation via mammalian target of rapamycin (mTOR) [4, 11, 12]. The phosphoinositide 3-kinase (PI3K)-mTOR pathway is activated by AKT (serine/threonine kinase). This pathway is part of two complexes: mTORC1 (that leads to the phosphorylation of S6) and mTORC2 (that leads to the phosphorylation of AKT) [4, 12, 13]. The activation of PIK3-mTOR regulates the synthesis of proteins necessary for cell cycle progression and cell growth and survival, increasing the proliferation and migration of epithelial cells and thus accelerating wound healing [7, 13–16].

Studies have investigated the use of natural compounds in an attempt to identify alternatives for the prevention and treatment of OM since photobiomodulation therapy (PBMT), the gold standard for the prevention and treatment of OM, is not always available at cancer treatment centers [3]. Additionally, some studies have sought to understand the physiopathology of OM and the effects of treatment with these natural compounds on signaling pathways involved in the development of this condition [2, 5, 17–20]. Among the natural products available for the treatment of different conditions, royal jelly (RJ) and propolis, two important compounds produced by honey bees of the genus *Apis*, have been used worldwide as traditional nutrients and in pharmacology since ancient times [21–26].

Within this context, the present study aimed to investigate the wound healing and anti-inflammatory effects of RJ and propolis in an animal model of OM by clinical and histological analyses. In addition, the mechanisms of action were assessed based on the oxidative stress pattern and analysis of NF-κB and PI3K/mTOR/AKT.

Methods

Experimental Procedure

The study was approved by the Ethics Committee on Animal Use (CEUA, Approval number 2018 – 0298) of the University Hospital of Porto Alegre (HCPA). Seventy-two male Wistar rats (*Rattus norvegicus albinus*), 8 to 12 weeks old and weighing 275 ± 25 g, were used. The animals were housed as described by Curra et al. (2015) [27] and were divided randomly into four groups:

- Control group (n = 18): induction of OM, not treated and only handled daily;
- Photobiomodulation therapy (PBMT) (n = 18): induction of OM and intraoral laser application;
- Royal jelly (RJ) (n = 18): induction of OM and topical application of royal jelly;
- Propolis gel (n = 18): induction of OM and topical application of propolis gel.

All animals were submitted to the protocol of OM induction proposed by Sonis et al. (1990) and modified by Leitão et al. (2007) (moderate severity) [28, 29]. Briefly, 5-fluorouracil was applied intraperitoneally on days 0 (60mg/kg) and 2 (40mg/kg), in a volume of up to 5 mL/kg. On days 3 and 4, anesthesia was induced in the animals with isoflurane (4–5%) and maintained (1–2%) with vaporized isoflurane in 0.5 L/min of 100% oxygen. Both sides of the buccal mucosa were scratched twice with the tip of a sterile needle by the same operator. The treatments were initiated on day 5.

Parameters of Photobiomodulation Therapy

The animals of the PBMT group received one daily intraoral laser application with an aluminum gallium indium phosphide (InGaAlP) diode (MM Optics, São Carlos, SP, Brazil). The buccal mucosa of the animals was irradiated followed the protocol of Thieme et al. (2019)[20]. The following irradiation parameters were used: wavelength of 660 nm, spot size of 0.04cm^2, irradiance of 1W/cm^2, power of 100mW, and energy density of 6J/cm^2. Irradiation was performed for 14.4 seconds in direct contact with the mucosa, resulting in a total energy density of 1.44 J per point.

Specifications of the Natural Products

Royal jelly was used in the present study in fresh form (100%, 18 g). Propolis gel was made from propolis tincture (Florien Fitoativos®; Piracicaba, SP, Brazil) at a concentration of 10%, adding 100 g of carboxymethylcellulose as mucoadhesive agent. This mucoadhesive propolis formulation was manufacturer by a compounding pharmacy (Fitonfarma®, Porto Alegre, RS, Brazil).

Phytochemical Analysis of Royal Jelly and Propolis Gel by Thin-Layer Chromatography

Thin-layer chromatography (TLC) was performed using glass chromatoplates with 60 Fuv$_{254}$ silica gel as absorbent (0.25 mm, Macherey-Nagel®, Fisher Scientific, UK). The mobile phase was chosen according to the secondary metabolites of interest. Various tests using different solvent systems were carried out. Finally, a solvent system consisting of toluene: ethyl acetate (TLC) and formic acid (5:5:0.5, v/v/v) (co-TLC) was used for the analysis of 10% propolis gel, and 1-isopropanol: ethyl acetate: distilled water: formic acid (4:0.5:0.5:0.5, v/v/v/v) was used for the analysis of fresh RJ. The results were compared to standards by observing the chromatographic spot color and retention factor (*Rf*).

After elution of the mobile phase, the plates were developed using two regents: sulfuric vanillin as universal developer and 0.2% Natural Reagent (diphenylboryloxyethylamine) specific for flavonoids. The plates were observed under ultraviolet (UV) light at 365 nm. Sucrose was used as standard for fresh RJ because of the presence of sugars. Propolis gel was compared to the flavonoid standards luteolin, quercetin, and kaempferol (Sigma-Aldrich®).

Euthanasia

On day 8, 10 and 14, six animals of each group were euthanized with an overdose of the anesthetic isoflurane (severity: no recovery). Isoflurane was vaporized in 100% oxygen (oxygen flow of 0.5 L/min) at a concentration ≥ 5% and administered by inhalation (chamber, nose cone, or face mask). Before biopsy, the right buccal mucosa was photographed for clinical examination and then fixed in 10% buffered

formalin for histopathological and immunohistochemical analysis. The left buccal mucosa was stored in liquid nitrogen in a freezer at -80°C

Clinical and Histopathological Analyses

The clinical and histopathological analyses were carried out by a previously calibrated and blinded professional according to the parameters proposed by Lima et al. (2005)[30].

Immunohistochemical Method and Analysis

For immunohistochemistry, 3-µm histological sections were mounted on silanized slides, deparaffinized in xylene, rehydrated in alcohol, and immersed in 0.3% hydrogen peroxide in methanol for the blockade of endogenous peroxidase. The sections were submitted to antigen retrieval and then incubated with the primary antibodies: anti-phospho-S6 ribosomal protein (Ser240/244) (1:800, for 1h), anti-phospho-Akt (Ser473) (1:200, for 1h), and anti-NF-κB (p65/sc-109) (1:400, overnight). The amplification reaction was performed using the HiDef detection™ – HRP Polymer System (Cell Marque, Rocklin CA, USA). The reactions were developed using 0.03% of 3,3'-diaminobenzidine (DAB, DakoCytomation, USA) as chromogen. The sections were counterstained with Mayer's hematoxylin. Positive controls were included in all reactions according to manufacturer instructions. As negative control, the primary antibodies were replaced with 1% bovine serum albumin diluted in Tris-HCl, pH 7.4.

For analysis of immunostaining, the slides were photographed (Pannoramic MIDI, 1.15 SPI, 3D HISTECH®, Budapest, Hungary) selecting three different fields in the oral mucosal epithelium using the borders of the wound or the area with evidence of previous tissue damage as a reference. The slides were analyzed by a single examiner using the Image J program (National Institute of Mental Health, Bethesda, Maryland, USA) in a blind analysis. A modified semiquantitative immunoreactivity score (IRS) (REMMELE; STEGNER 1987) (Table 1) was calculated for pS6 (positive cytoplasmic staining) and$pAkt^{473}$ [31]. Immunoexpression of NF-κB (positive nuclear staining) was analyzed quantitatively by calculation of the labeling index (LI) as follows (SEVERO et al., 2018): LI (%) = (number of immunopositive cells)/(total number of counted cells) × 100 [32].

Table 1
Immunohistochemical classification based on immunoreactivity score (IRS)

Percentage of positive cells (PP)	Staining intensity (SI)	IRS = PPxSI
0 absence of positive cells	0 no staining	0–1 negative
1 <20% positive cells	1 weak staining	2–3 low positive
221 to 50% positive cells	2 moderate staining	4–8 moderate positive
351 to 80% positive cells	3 intense staining	9–12 strong positive
4 >80% positive cells		

Oxidative Stress Assays

Reduced glutathione

Reduced glutathione (GSH) exerts antioxidant activity by deactivating free radicals. The method described by Costa et al. (2006) was used to measure GSH levels in OM. Briefly, the buccal mucosa samples (n = 18 per group) were homogenized in 0.25 mL of 5% EDTA and 320 µL distilled water and 80 µL of 50% trichloroacetic acid (TCA) were added [33]. The samples were centrifuged at 3000 ×*g* for 15 min at 4^{o}C. The supernatant (400 µL) was combined with 800 µl of0.4 M Tris buffer (pH 8.9) and 20 µL of 0.01 M dithiobis-2-nitrobenzoic acid. Absorbance was read at 420 nm and the results are reported as GSH unit/mg tissue.

Superoxide dismutase

The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide. SOD exerts an important antioxidant activity in cells exposed to oxygen. The activity of SOD was estimated following the method of Beauchamp and Fridovich (1971)[34]. A homogenate of the sample was prepared (100 mg of buccal mucosa tissue in 1 mL 0.4 M phosphate buffer, pH 7.0). The preparation was centrifuged at 10,000 rpm for 15 min at 4^{o}C and the supernatant was used in the assay (30 µL). The homogenate was divided into four groups: 1) test: addition of 0.25 mL methionine, 0.03 mL riboflavin, 0.01 mL NBT, 0.01 mL; 2) control: addition of 0.25 mL methionine, 0.03 mL riboflavin, 0.01 mL phosphate buffer (pH 7.8), 0.01 mL;3) standard: addition of 0.25 mL methionine, 0.03 mL riboflavin, 0.01 mL NBT, 0.01 mL phosphate buffer; 4)blank: addition of 0.25 mL methionine, 0.03 mL riboflavin, 0.02 mL phosphate buffer (pH 7.8). The sites labeled test, standard and control were exposed to a 15-W fluorescent lamp for 10 min in a chamber covered with aluminum foil. Immediately after light exposure, the optical density of all reactions was read at 560 nm. The units of enzyme present in the samples were calculated using the following formula: SOD activity × 100/total protein (mL/100µL).

Malondialdehyde

Malondialdehyde (MDA) is an end product of membrane lipid peroxidation. In this study, MDA levels were quantified in the samples analyzed as described by Siddique et al. (2012) and Bradley et al. (1982)[35, 36]. The buccal mucosa samples were resuspended in Tris-HCl (1:5,w/v) and cut into small pieces with scissors for 15 seconds on an ice-cold plate. The resulting suspension was homogenized for 2 min in an automated Potter homogenizer and centrifuged at 2,500 × *g*for 10 min at 4^{o}C. The MDA content was determined in the supernatants by reading absorbance at 586 nm. The results were expressed as nanomole MDA per gram tissue.

Statistical Analysis

The clinical, histopathological, pS6 and pAkt^{473} immunoexpression, and oxidative stress data were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. The Mann-Whitney test was used for the analysis of transcription factor NF-κB. Statistical analysis was

performed with the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA), adopting a level of significance of 5% ($p < 0.05$).

Results

The thin-layer chromatography (TLC) analysis of RJ revealed a dark brown color spot (*Rf* 0.42), suggesting the presence of sugars due the characteristic color. RJ showed a spot with the same *Rf* and color of the standard sucrose (*Rf* 0.34) (Fig. 1A).

The chromatograms of propolis developed with sulfuric vanillin revealed a predominance of compounds with a terpene-like structure (red-brown, yellow-brown or dark green, blue and blue-purple bands), flavonoides (yellow and green bands) and saponins, (blue, blue-purple and sometimes red or yellow-brown bands) (Fig. 1B).

Co-TLC of propolis eluted and developed with 0.2% Natural Reagent A (specific for the detection of flavonoids) followed by UV detection at 365 nm showed the presence of yellow and green fluorescence, indicating the presence of aglycone flavonoids such as quercetin (yellow-orange fluorescence and *Rf* 0.41), luteolin (yellow fluorescence and *Rf* 0.40), and kaempferol (green color and*Rf* 0.58), using the color characteristics and *Rf* as reference (Fig. 1C and 1D).

All animals developed OM on day 5. Figure 2 shows the clinical features of OM in all groups over the experimental period (8, 10, and 14 days). In general, animals of the treated groups (PBMT, RJ and propolis) exhibited lower clinical OM scores on days 8 and 10 compared to the control group ($p < 0.05$) (Fig. 2A and 2B). No significant differences were observed between the groups studied on day 14 ($p > 0.05$).

Regarding the histopathological findings (Fig. 3), accelerated healing of OM was observed on day 8 in the PBMT, RJ and propolis groups compared to control, with the difference in scores being statistically significant($p < 0.05$) (Fig. 3A and 3B). The main findings in these groups were reepithelization, mild or absent hyperemia, a mild to moderate chronic inflammatory infiltrate, and the absence of ulceration and abscesses (score 0 or 1). Particularly interesting findings were the increased number of new fibroblasts amidst the developing extracellular matrix and the presence of immature skeletal muscle cells (Fig. 3A). No significant differences were observed between the groups studied on day 10 or 14 ($p > 0.05$).

On day 8, the PBMT, RJ and propolis groups exhibited an increase in the immunoexpression of proteins pS6 and pAKT ($p < 0.05$) when compared to the control group (Fig. 4A, 4B and 4C). However, on day 10, only the expression of pS6 appeared to be increased in the RJ group compared to control ($p < 0.05$) (Fig. 4B). No significant differences in the immunoexpression of these proteins were observed between groups on day 14 ($p > 0.05$) (Fig. 4B and 4C).

Immunoexpression of NF-κB was increased in the treated groups compared to control throughout the experimental period ($p < 0.05$) (Fig. 4A and 4D). Comparison between the treated groups (PBMT, RJ and

propolis) showed no significant difference in these markers ($p > 0.05$), regardless of the day of analysis (Fig. 4).

Figure 5 illustrates the results of the oxidative stress assays (GSH, SOD and MDA). As can be seen, only on day 14 did the propolis group produce higher GSH levels when compared to the control group ($p < 0.05$) (Fig. 5A). No significant difference in SOD or MDA levels was observed when the control group was compared to the PBMT, RJ and propolis group on the days analyzed ($p > 0.05$) (Fig. 5B and 5C).

Discussion

Oral mucositis is a common and clinically significant side effect in patients undergoing antineoplastic treatments [3, 37]. These treatments frequently result in severe damage to the oral mucosa, with the patients developing ulcerative lesions that cause great discomfort and intense pain. If OM is not prevented or treated adequately, the discomfort generated can reduce the patient's ability to tolerate treatment [4, 20]. Within this context, considering that not all patients who develop OM have access to PBMT, the present study demonstrated that topical application of natural compounds derived from bees may be an important alternative for the treatment of OM. Our results showed that both RJ and propolis promoted clinical improvement of OM, increasing reepithelization and reducing inflammation.

One of the compounds studied was RJ, which has shown high healing, antibacterial, anti-inflammatory, vasodilating, disinfectant, and antioxidant activities, among others [24, 26, 38]. Fresh RJ is composed chemically of proteins, sugars, lipids, vitamins, and free amino acids [39]. Sugars make RJ an important nutritional source, while the other components confer pharmacological properties such as healing, anti-inflammatory, antimicrobial and antioxidant activity [40, 41]. The present result showed that the chromatographic band that could be visualized in the mobile phase used was dark colored, suggesting the presence of sugars, which prevented the visualization of other bands that could suggest more compounds.

Like RJ, propolis has extraordinary biological properties. Propolis is mainly composed of phenolic compounds (flavonoids, aromatic acids, benzopyrans), diterpenes, triterpenes, and essential oils (spathulenol, farnesol, benzyl benzoate, andprenylated acetophenones) [42, 43]. In the study of Wagner et al. (1984), TLC using 0.2% Natural Reagent and UV detection at 365 nm revealed bands of yellow, orange and green fluorescence indicating the presence of flavonoids [42]. Similarly, in the present study aglycones, which are flavonoids not bound to sugar molecules, were detected in propolis, including quercetin (yellow-orange fluorescence), luteolin (yellow fluorescence), and kaempferol (green fluorescence)[2, 38].

The phytochemical properties of RJ and propolis apparently had an impact on the clinical response and histochemical assays in the present study, promoting anti-inflammatory and healing effects in OM. We observed lower clinical and histopathological OM scores already on day 8, three days after the beginning of treatment, in the RJ and propolis groups compared to the control group. Similarly, Curra et al. (2015), Vitale et al. (2017), Thieme et al. (2019) (PBMT), Damyanliev et al. (1982) (10% propolis), Noronha et al.

(2017) (10% mucoadhesive propolis gel), Suemaru et al. (2008) (RJ) and Watanabe et al. (2013) (RJ) also reported a significant decrease in lesion size and the absence or reduction of ulcers and local inflammation [1, 20, 21, 23, 25, 27, 44]. We therefore consider that the use of fresh RJ and 10% propolis was as effective as PBMT by accelerating healing and reducing inflammation in cases of chemically induced OM.

We also evaluated the mechanisms of action of RJ and propolis. The results showed that the acceleration of healing by the two compounds is associated with activation of the PIK3-mTOR pathway. Studies report that activation of this pathway regulates the synthesis of different proteins involved in cell cycle progression and cell growth and survival, increasing the proliferation and migration of epithelial cells and thus accelerating wound healing [13–16].This study demonstrated increased immunoexpression of pS6 and pAKT on day 8, a critical period for tissue repair and wound closure, in animals treated with PBMT, RJ and propolis compared to the control group. Immunoexpression of pS6 continued to be high until day 10. In experimental studies on rats, Squarize et al. (2010) and Castilho et al. (2013) found that activation of mTOR drastically increases the proliferation and migration of epithelial cells and wound healing [13, 14]. Curra et al. (2015) observed clinical improvement of OM (day 10) and higher NF-κB activation levels (days 10 and 15) during the development and healing of OM in animals submitted to PBMT when compared to the control group [27]. The present results suggest that the accelerated clinical and microscopic improvement of OM in the PBMT, RJ and propolis groups was due to the activation of the PIK3-mTOR pathway in the early stages of tissue repair and to the high expression of NF-κB in the final stages. High levels of NF-κB were observed throughout the experimental period until day 14, stimulating proliferation, apoptosis, morphogenesis and cell differentiation until the end of the healing process [12].

Furthermore, the ability of propolis to reduce the redox potential in tissues is widely reported in the literature. This antioxidant activity is related to the action of flavonoids. Flavonoids act as scavengers of free radicals that are produced in tissues and can cause extensive damage to macromolecules such as DNA. In addition, flavonoids can catalyze the final products of lipid peroxidation resulting from cell membrane damage [2, 43, 45]. In the present study, increased GSH levels were observed in animals of the propolis group in the final stages of healing. However, this finding did not negatively influence tissue repair because, although late, it suggests that GSH acted by deactivating ROS in the wound, which favored the healing process [41]. The presence of flavonoids explains the reduction of tissue oxidative stress in the propolis group.

In conclusion, this is the first study to investigate the PI3K-Akt-mTOR pathway in the healing process of chemically induced OM treated with RJ and propolis. The study reported promising results for the treatment of OM with RJ and propolis. These products exhibited biological properties, including high healing and anti-inflammatory activity demonstrated by the activation of NF-κB in more advanced stages of healing and of the PIK3-mTOR cell proliferation pathway. The findings were similar to the clinical, histopathological and molecular effects found in the group submitted to photobiomodulation.

Declarations

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Author contribution Mara Luana Batista Severo, Amanda Katarinny Goes Gonzaga, Marco Antonio Trevizani Martins, Manoela Domingues Martins and Éricka Janine Dantas da Silveira designed the research. Mara Luana Batista Severo, Stéfanie Thieme, Felipe Martins Silveira Marco Antonio Trevizani Martins, Manoela Domingues Martins, Raquel Padilha Martins Tavares, Silvana Maria Zucolotto and Aurigena Antunes de Araújo performed the experiments. Mara Luana Batista Severo, Stéfanie Thieme, Felipe Martins Silveira, Marco Antonio Trevizani Martins, Manoela Domingues Martins and Éricka Janine Dantas da Silveira analyzed the data. All authors have read wrote, reviewed and approved the final submitted manuscript.

Data Availability The data and materials as well as software application support their published claims and comply with field standards.

Code availability N/A.

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Consent to participate N/A.

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Conflict of interest The authors declare that they have not conflict of interest.

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Tables

Table 1 Immunohistochemical classification based on immunoreactivity score (IRS)

Percentage of positive cells (PP)	Staining intensity (SI)	IRS=PPxSI
0 absence of positive cells	0 no staining	0-1 negative
1<20% positive cells	1 weak staining	2-3 low positive
221 to 50% positive cells	2 moderate staining	4-8 moderate positive
351 to 80% positive cells	3 intense staining	9-12 strong positive
4> 80% positive cells		

Figures

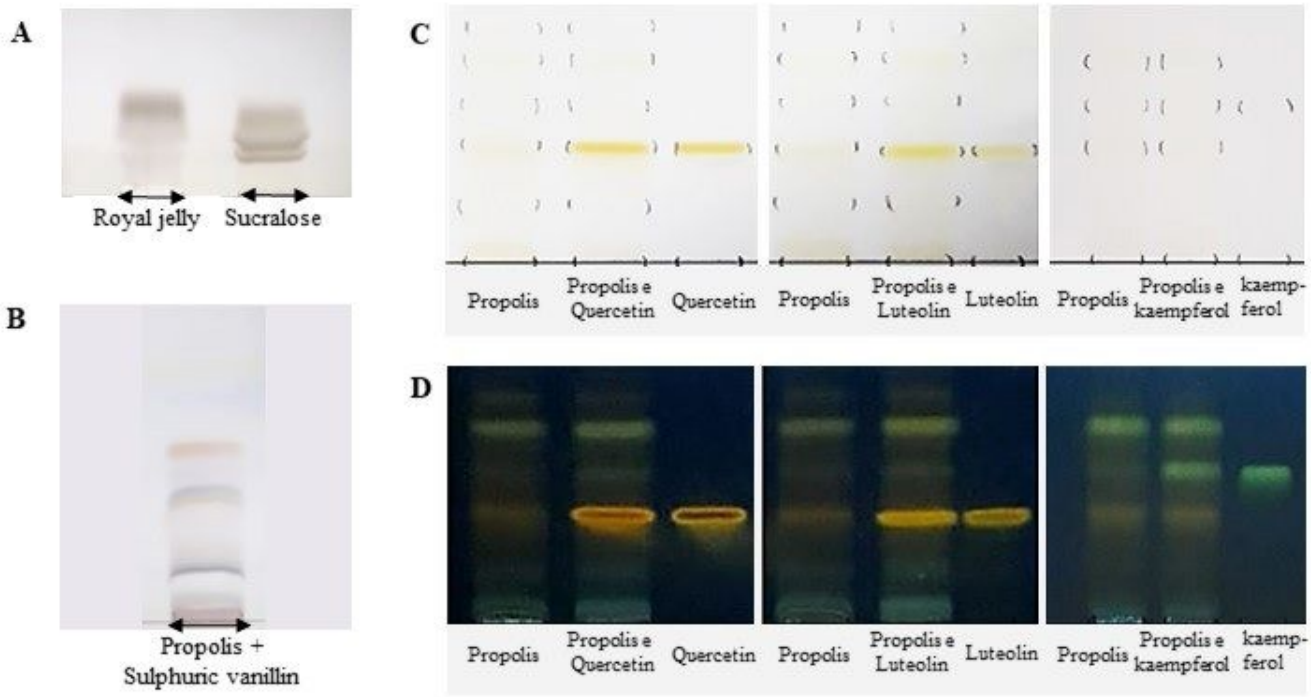

Figure 1

Phytochemical analysis of royal jelly and propolis. (A) Thin-layer chromatogram (TLC) of fresh royal jelly and sucrose. Dark brown bands (Rf 0.42) suggesting the presence of sugars. (B) TLC of propolis developed with sulfuric vanillin showing bands of varied colors, indicating the presence of terpenes, saponins, and essential oils. (C) and (D) Co-TLC of propolis tincture after elution and development with 0.2% Natural Reagent A. Visible inspection and UV detection at 365 nm, respectively. The yellow color of the bands indicates the presence of flavonoids in the quercetin, luteolin and kaempferol standards.

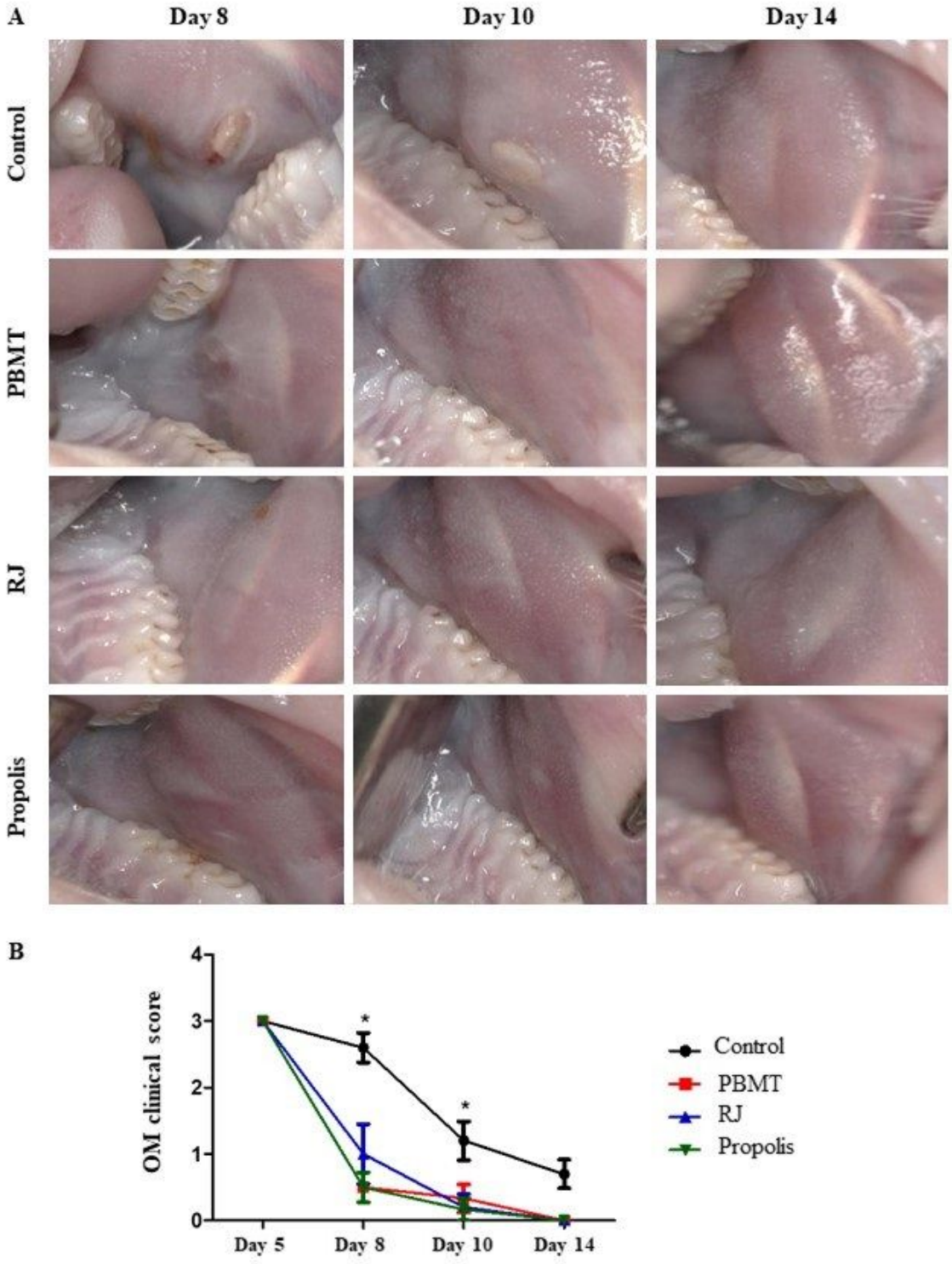

Figure 2

Clinical analysis of chemically induced oral mucositis (OM) in Wistar rats. (A) Clinical features of OM in all groups on days 8, 10 and 14. (B) Graph showing lower clinical OM scores on days 8 and 10 in the treated groups (PBMIT, RJ, and propolis) compared to control ($p<0.05$). The asterisk indicates a significant difference ($p<0.05$).

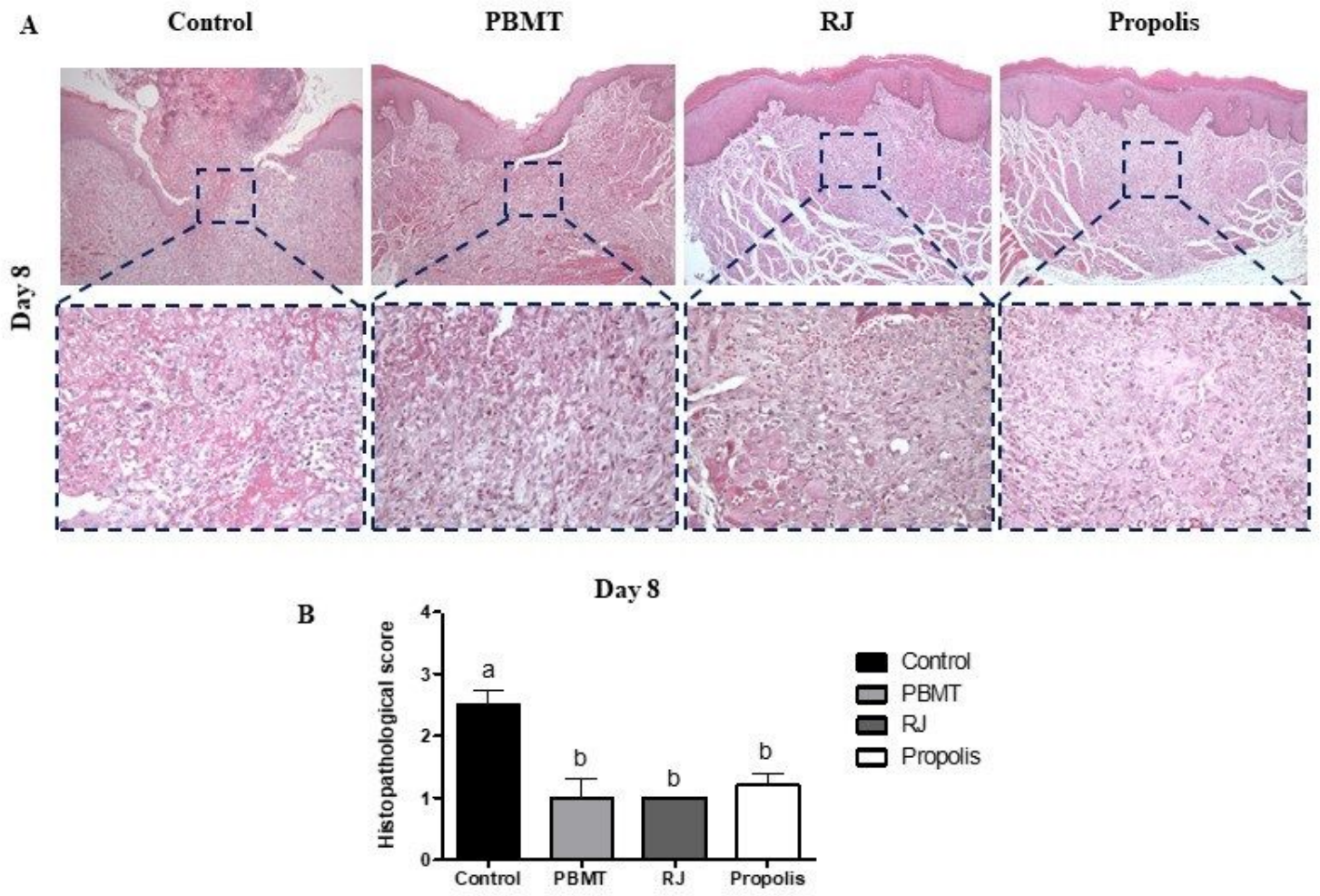

Figure 3

Histopathological analysis of oral mucositis in all groups on day 8. (A) Photomicrographs showing ulceration and a neutrophilic inflammatory infiltrate in the control groups. However, complete reepithelization and a mild/moderate chronic inflammatory infiltrate were observed in the other groups (HE, original magnification: ×100 and×400). (B) The bar graph indicates accelerated repair of oral mucositis in the PBMT, RJ and propolis groups compared to control ($p<0.05$). Different lowercase letters (a and b) above the bars (intergroup analysis) indicate a significant difference ($p<0.05$).

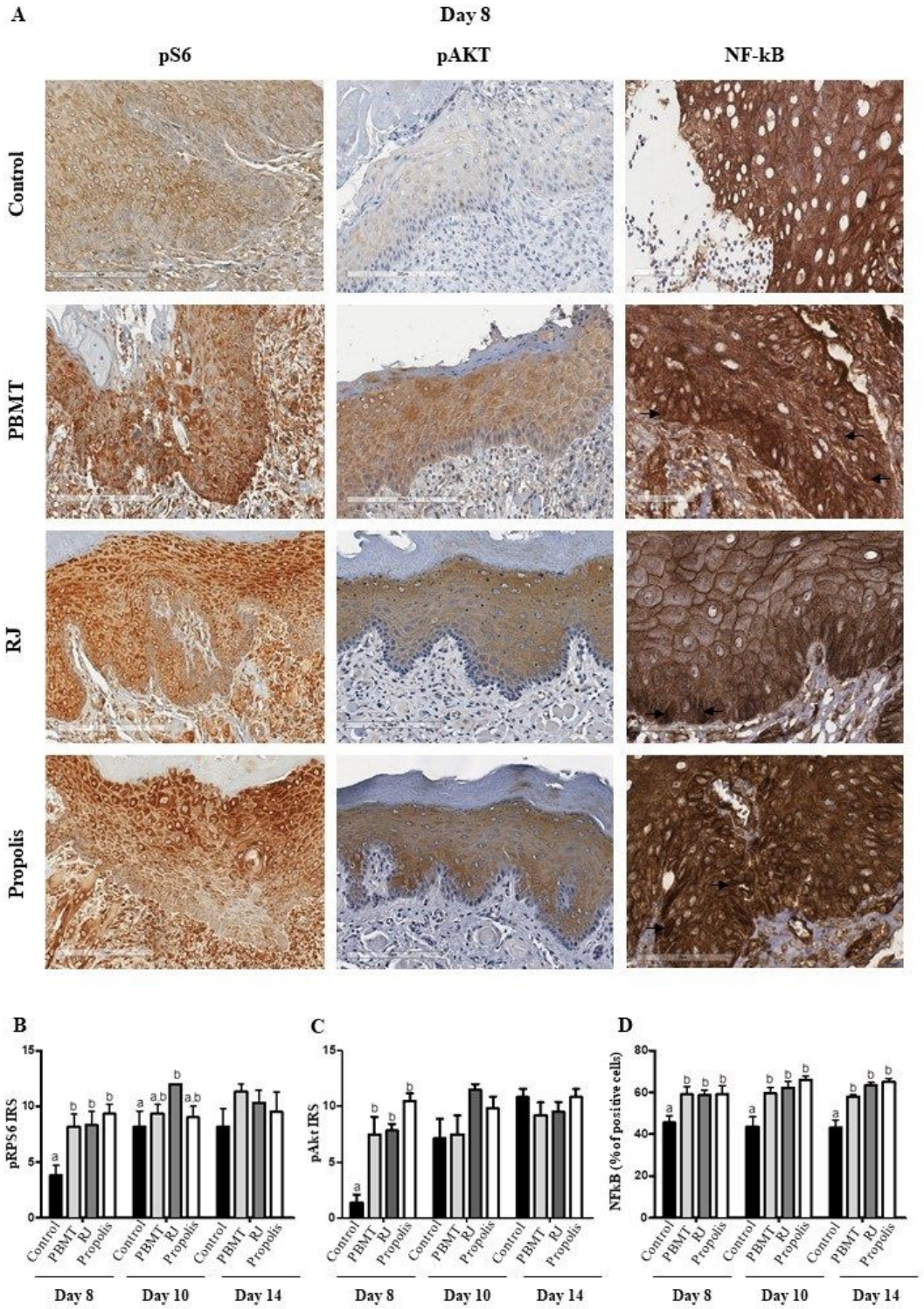

Figure 4

Analysis of the immunoexpression of proteins pS6 and pAKT and nuclear transcription factor NF-κB. (A) Photomicrograph of day 8 showing higher expression of pS6, pAKT and NF-κB (nuclear staining at the tip of the arrows) in the PBMT, RJ and propolis groups compared to control ($p<0.05$) (scale bars: 200μm, 200μm and 50μm, respectively). (B) Increased immunostaining of pS6 in all groups on day 8 and only in the propolis group on day10 ($p<0.05$). No significant difference between groups was observed on day 14

(p>0.05). (C) Increased immunostaining of pAKT in the PBMT, RJ and propolis groups on day 8 (p<0.05). There was no significant difference between groups on day 10 or 14 (p>0.05). (D) Increased immunostaining of NF-κB in the PBMT, RJ and propolis groups on day 8, 10 and 14 compared to control (p<0.05). Different lowercase letters (a and b) above the bars (intergroup analysis) indicate a significant difference (p<0.05).

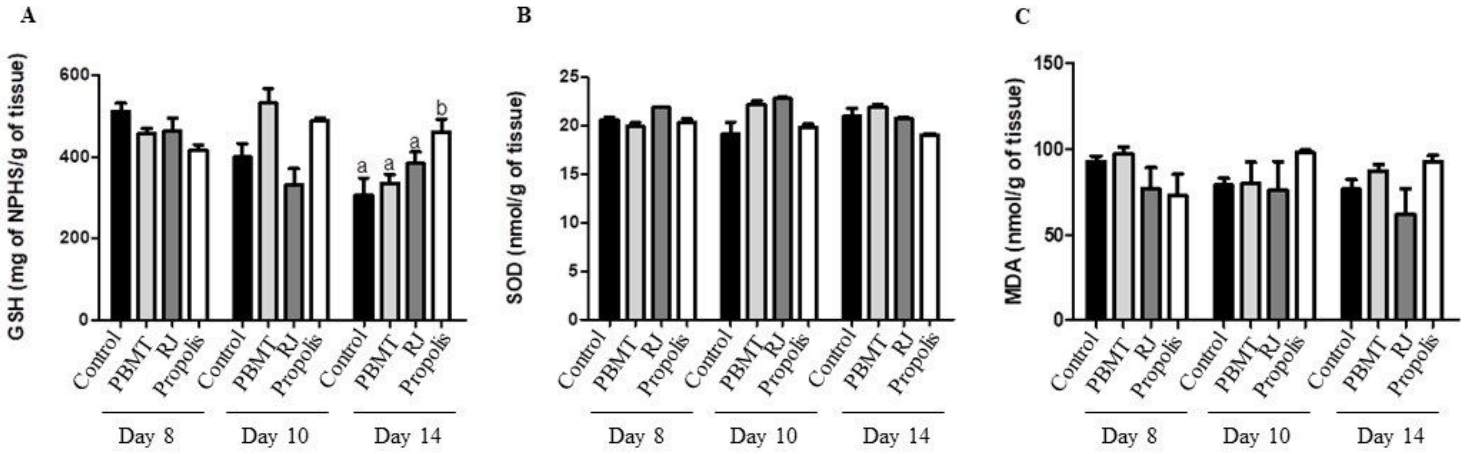

Figure 5

Analysis of oxidative stress in chemically induced oral mucositis in Wistar rats. (A) On day 14, the propolis group produced higher levels of GSH compared to the control group (p<0.05). Different lowercase letters (a and b) above the bars (intergroup analysis) indicate a significant difference (p<0.05). (B) and (C) There was no significant difference in SOD or MDA levels between groups on any of the days analyzed (p>0.05).