CLINICAL AND HISTOMORPHOMETRIC EVALUATION OF RATS ORAL MUCOSA SUBMITTED TO TOPICAL APPLICATION OF CHAMOMILE

AVALIAÇÃO CLÍNICA E HISTOMORFOMÉTRICA DA MUCOSA ORAL DE RATOS SUBMETIDA À APLICAÇÃO TÓPICA DE CAMOMILA

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Abstract

Introduction: Among the diversity of plant species in Brazil, *Matricaria recutita*, known as chamomile, is widely used as a medicinal herb for its action in digestive disorders,

antimicrobial, analgesic and anti-inflammatory properties. Experimental studies demonstrate the ability of this medicinal plant to increase tissue repair speed and thus promote faster re-epithelialization. **Objective:** Evaluate tissue pattern after topical application of different presentations of chamomile in the normal oral mucosa of rats. **Methodology:** Thirty-five male *Wistar* rats allocated into four groups: Control/baseline (G1), Chamomile fluid extract - 2x/day (G2), Chamomile Infusion 1 - 2x/day (G3), Chamomile Infusion 2 - 3x/day (G4). Euthanasia of animals took place on days 7 and 14 after the beginning of applications of medicinal plant on the buccal mucosa of the right cheek. Sections stained with Hematoxylin and Eosin were evaluated by descriptive morphology (degeneration of epithelial basal layer; presence of inflammatory infiltrate and collagen expression) and morphometry (distance from basal layer to epithelial surface; distance from epithelial crest to epithelial surface; width of crest epithelial thickness; intercrest distance; and stratum corneum thickness). The quantity and organization pattern of total collagen was evaluated using Sirius Red staining. **Key results and conclusions:** None of the experimental groups showed significant statistical differences ($p>0.05$) between them in morphological and morphometric aspects, in addition to being similar to G1, although the average value of G2 showed greater divergence in the percentage of collagen expression at day 7 and 14, compared to the other periods and groups investigated ($p=0.0569$). Topical use of chamomile, regardless of the dosage or formulation investigated, did not cause tissue damage in normal oral mucosa of rats, and can be considered a therapeutic agent well tolerated by tissues.

Keywords: Chamomile; Phytotherapy; Buccal Mucosa; Histology.

Resumo

Introdução: Dentre a diversidade de espécies vegetais no Brasil, a Matricaria recutita, conhecida como camomila, é amplamente utilizada como erva medicinal por sua ação em distúrbios digestivos, além de suas propriedades antimicrobianas, analgésicas e anti-inflamatórias. Estudos experimentais demonstram a capacidade de essa planta medicinal acelerar a reparação tecidual e, consequentemente, promover uma reepitelização mais rápida. **Objetivo:** Avaliar o padrão tecidual após aplicação tópica de diferentes formulações de camomila na mucosa oral normal de ratos. **Metodologia**: Trinta e cinco ratos Wistar machos foram alocados em 4 grupos: Controle/linha de base (G1), Extrato fluido de camomila - 2x/dia (G2), Infusão de Camomila 1 - 2x/dia (G3), Infusão de Camomila 2 - 3x/dia (G4). A eutanásia dos animais ocorreu nos dias 7 e 14, após o início das aplicações da planta medicinal na mucosa bucal da bochecha direita. Secções coradas com hematoxilina e eosina foram avaliadas por morfologia descritiva (degeneração da camada basal epitelial; presença de infiltrado inflamatório e expressão de colágeno) e morfometria (distância da camada basal à superfície epitelial; distância da crista epitelial à superfície epitelial; largura da espessura da crista epitelial; distância intercrestal; e espessura do estrato córneo). A quantidade e o padrão de organização do colágeno total foram avaliados utilizando coloração com Sirius Red. **Resultados e conclusões principais**: Nenhum dos grupos experimentais apresentou diferenças estatísticas significativas ($p>0.05$) entre si nos aspectos morfológicos e morfométricos, além de serem semelhantes ao G1, embora o valor médio do grupo G2 tenha apresentado maior divergência na porcentagem de expressão

de colágeno nos dias 7 e 14, em comparação com os demais períodos e grupos investigados (p=0,0569). O uso tópico de camomila, independentemente da dosagem ou formulação investigada, não causou danos teciduais na mucosa oral normal de ratos e pode ser considerado um agente terapêutico bem tolerado pelos tecidos.

Palavras-chave: Camomila; Fitoterapia; Mucosa bucal; Histologia.

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1. Introduction

Brazil has a popular pharmacopoeia with diverse medicinal plants, resulting from cultural miscegenation involving Africans, Europeans and indigenous peoples, with the introduction of exotic species by colonizers and enslaved persons[1]. According to the World Health Organization[2], the definition of a medicinal plant is "every plant that contains, in one or several of its organs, substances that can be used for therapeutic purposes or precursors of substances used for such purposes."

Among the diversity of plant species and biomes found in Brazil, the plant *Matricaria recutita* (*M. recutita*), popularly known as chamomile, is widely used throughout the world, mainly in the form of teas/infusion and decoction[3-6]. It contains bioactive constituents such as flavonoids, matricin, camazulene and terpenoids, which give this plant anti-inflammatory, antispasmodic and antibacterial properties[7,8]. Among the possible medicinal uses of *M. recutita*, is indicated to control stomach disorders in general, diarrhea, nausea, urinary tract inflammation and menstrual disorders, in addition to eye and mouth inflammation[9].

Some experimental studies demonstrate the ability of this medicinal plant to increase tissue repair speed and thus promote faster re-epithelialization of ulcers[6,10,11]. This activity explains the suggestion for using chamomile on wounds that are difficult to heal and on skin conditions in general[9].

Due to its antimicrobial and biostimulating actions in tissue repair, chamomile has been used in dentistry to control microorganisms present in dental biofilms[12] and as an adjuvant in the therapy of conditions such as xerostomia[13], mucositis[14], gingivitis[15], oral infections[16] and recurrent aphthous ulceration[17].

Despite the extensive investigation of chamomile over the years, most available studies have focused on its therapeutic effects in injured or inflamed tissues. Consequently, the histopathological effects of its topical application on normal oral tissues remain poorly characterized.

This represents an important knowledge gap because, regardless of the clinical indication, topical chamomile preparations inevitably come into contact with healthy oral mucosa during use. Understanding the tissue response under these physiological conditions is essential to establish the biological safety and tissue tolerance of different chamomile formulations. Therefore, this study aimed to evaluate tissue tolerance after the topical application of different formulations and concentrations of chamomile (*Matricaria recutita*) to the normal oral mucosa of rats.

2. Methodology

The description of the experimental stages as well as characteristics of the animals and analysis carried out followed recommendations and parameters required by ARRIVE guideline [18].

Study design

After approval by the Ethics Committee on the Use of Animals of the Institute of Health Sciences, Federal University of Bahia (ICS/UFBA) under number 3562050618, thirty-five Wistar rats, were distributed into four experimental groups (Figure 1).

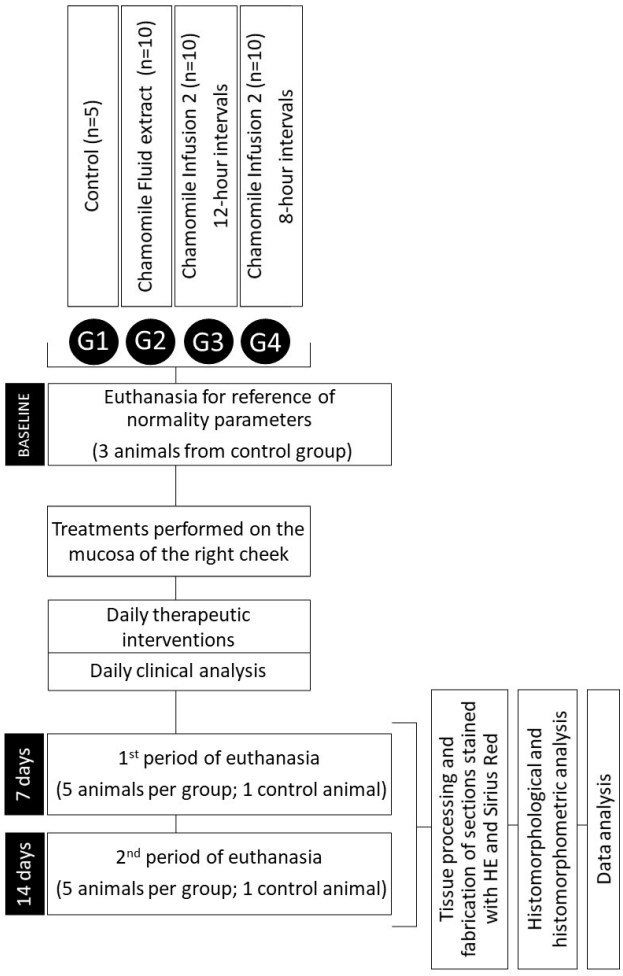


Figure 1 - Flowchart representing the stages of the study. Source: own authorship.

Control Group/baseline (G1): Negative control. Five animals were allocated to this group. Three served as a negative control, where no therapy was used, and two were submitted to the application of saline solution (0.9%) at 12-hour intervals, with the use of a flexible plastic swab with cotton in the extremities (Swabs, Johnson & Johnson, São Paulo, Brazil)

Chamomile Fluid Extract Group (G2): Experimental group. Ten animals were subjected to topical application of chamomile fluid extract (Ad-Muc®, Biolab, São Paulo, Brazil), with a plastic swab (Swabs, Johnson & Johnson, São Paulo, Brazil) at 12-hour intervals, as recommended by the drug manufacturer.

Chamomile Infusion 1 Group (G3): Experimental group. Ten animals submitted to topical application of chamomile infusion obtained by infusing 01 tea bag (Chás Real®, Paraná, Brazil) submerged in 250 mL of boiling water for 3 minutes, according to the manufacturer's instructions. Refrigerated at a temperature of 20-22°C, stored for a period of up to 24 hours, applied at intervals of 12 hours, with the aid of a dropper.

Chamomile Infusion 2 Group (G4): Experimental group. Ten animals submitted to topical application of chamomile infusion obtained as same protocol as in the Infusion 1 Group (G3), however, the application occurred three times a day, at 8-hour intervals.

Treatments described were performed on the mucosa of the right cheek of all animals included in this experimental research.

Sample size

Considering that initial primary objective of the study was represented by the number of inflammatory cells. The smallest difference between groups would correspond to 15 cells per field in the positive control, compared to 7 cells per field in the negative group. it was estimated that a sample of five animals per group was required. A standard deviation of 5, 5 alpha % and power of the study of 80% was considered. Tirty-five rats were allocated in the four experimental groups for the experiment.

Inclusion and Exclusion criteria

Inclusion criteria of animals involved: male, healthy *Rattus norvegicus albinus*, resulting from the ICS/UFBA vivarium, weighing an average of 240g, with eight to ten weeks old. Exclusion criteria involved: animals that showed any sign of systemic alterations, HE-stained histological sections that presented lack of biological material in good condition for evaluation.

During the experiment, no animal presented any clinical condition that would make it unfeasible to remain in the experiment. However, there were one sample loss of a G3 section, referring to a case of 7 days of sacrifice.

Randomization

Animals were randomly allocated into experimental groups by a simple randomization, which used 35 folded papers, where contained one of the four group options already described. The respective demarcations numbering and identification of animal groups were performed on the tails, with a permanent marker, as well as cages identification, to avoid possible confusion during the manipulation of the animals.

Blinding/Masking

All experimental steps that involved

handling animals were carried out with the researchers aware of the respective groups. Thus, daily clinical evaluation performed and the manipulation of each animal were not conducted blindly, to avoid confounding variables.

In terms of histological analysis, slide sections were masked to be evaluated by a previously calibrated and examiners (blinded).

Outcome measures

First outcome is related to clinical aspects of the mucosa and the second to the histological characterization of tissue. The description of analysis appears in the order of data collection.

Clinical evaluation: Macroscopic characteristics of the mucosa were analyzed daily with the aid of ambient light, wooden spatulas and rubber bands were used to open mouths of the animals. This analysis was performed through direct visual inspection of buccal mucosa and evaluation involved aspects of the surface of the buccal mucosa of the animals in terms of color, surface texture, changes in relief, shine/hydration. On the days of euthanasia, it was possible for visual inspection to be more accurate.

Morphological and morphometric evaluation: Histological analysis of HE-stained sections were performed at the Oral Biochemistry Laboratory (LBO) of ISC/UFBA, by a previously calibrated and blinded examiner. Presence of degeneration of the epithelial basal layer, inflammatory infiltrate and collagens expression were evaluated in a semi-quantitative manner, coded with scores ranging from intense (+++), moderate (++), mild (+) and absent (0), as described by Medrado et al.[19]. The analysis took place in triplicate, with a minimum interval of one week between them by a single blinded evaluation by a previously calibrated examiner as performed by Dantas et al.[20].

Morphological analysis involved staining the sections with Picrosirius Red solution to the manufacturer protocol. Analysis followed metrics described by Dantas et al.[20]. were evaluated and classified as either normal or enlarged: the pattern of deposition of total collagen fibers, the thickness of fibers (thick or delicate), the pattern of elongation (fragmented or not fragmented), and the depth of deposition of the fibers at the site (normal or enlarged).

Also, parameters such as if thick or delicate, if fragmented or not fragmented, and the depth of deposition of the fibers were evaluated. The analysis previously described by Dantas et al.[20] were made in triplicate, with one week interval between them, analyzing the slides, running entirely under light microscope.

The Motic BA410 microscope (Quimis, Sao Paulo, Brazil) and Motic Images Plus 2.0 software (Motic Asia, Hong Kong, China) was used for the morphometric analysis. As described by Fortuna et al.[21] It was performed by capturing images of three distinct areas of each of the edges (right and left edges) and the center of the wound contained in tissue sections stained in HE, in a 10x objective lens, through the camera coupled to the microscope. In cases where there was no ulcer present, the number 0 (zero) was assigned as a measure. Linear measurements, performed in µm, included: the distance from the basal layer to the epithelial surface and the thickness of the stratum corneum. With the same sections, distance from basal layer to epithelial surface (DBLES), distance from the crest apex to the epithelial surface (DAECES), width of

epithelial crest (WEC), inter-crest distance (ID), and thickness of the stratum corneum (TSC) which refers to the stratum corneum, were also evaluated in triplicate by the same blind examiner.

In relation to the total area of the tissue, quantitative analysis of the percentage of total collagen was performed using a free software, ImageJ (National Institutes of Health, Bethesda, MD, USA), with the standardization of the image area of 720,000 µm2 (800 x 900 µm), previously described by Fortuna et al.[21]. Two blind examiners, previously calibrated performed this analysis also from the capture of three distinct areas of tissue sections of the two edges and center of the ulcer, stained in Picrosirius Red, with the 10x objective lens.

Statistical methods

All analysis were performed using the R software (Foundation for Statistical Computing, Vienna, Austria), with a significance level of 5%. A database was created in Microsoft Excel 2016 for the descriptive analysis in order to identify the general and specific characteristics of the sample studied, including quantitative analysis of the collagen percentage.

Data on morphological variables were presented with absolute and relative frequencies and mode.

Inter-rater reproducibility analysis were performed for the variables percentage of collagen area and linear morphometric measurement. For this, paired t test and intraclass correlation analysis were used. The interpretation of the intraclass correlation coefficient (ICC) was performed according to Fleiss, Levin, Paik[22], who consider coefficients below 0.40 as poor, from 0.40 to below 0.75 as satisfactory, and equal to or above 0.75 as excellent. The average found by the two evaluators for these variables was then calculated.

Descriptive and exploratory analysis of the data were then carried out. Exploratory analysis were performed to verify whether the data meeted the assumption of an analysis of variance (ANOVA). To this end, a graphical evaluation of the studentized residuals was performed, identifying discrepant values and analyzing the homogeneity of variance. The normality of the errors was analyzed using the box plot, quantile-quantile graph and the Shapiro-Wilk test. The relationship between means and variances was also verified, evaluating the λ value, by the Box-Cox method. These previous analysis indicated that the data do not meet the assumptions of an ANOVA, and then generalized linear models were adjusted.

Linear models were used considering group and time effects, as well as the interaction between them, to analyze the percentage of collagen area. For morphometric measurements, the effects of group, time and TSC, as well as the double and triple interactions between them, were considered in the model. For the clinical evaluation of the ulcer, a mixed generalized linear model for repeated measures over time was used. The associations between the group and the presence of ulcer were analyzed using Fisher's exact test.

Experimental animals

Animals included in this experiment were healthy, male, *Rattus norvegicus albinus*, weighing an average of 240g, with eight to ten weeks old. Specimens were housed in specific plastic cages, in groups of five, under good lighting (12/12 hour light/dark cycle) and temperature conditions (±26°C). They were fed a commercial diet (Ração Nuvilab, Quimtia, Colombo-PR,

Brazil) and received water ad libitum throughout the experiment.

They underwent adaptation in the vivarium environment for a period of seven days. During the remaining days of the experiment, animals were subjected to minimal stress, which only occurred during manipulation moments for application of the investigated therapies.

Experimental procedures

Animal euthanasia: The animals were euthanized by intraperitoneal administration of 90mg/kg of Ketamine Hydrochloride 10% (Cetamin®, Synthec, São Paulo, Brazil) associated with 10mg/kg of Xylazine Hydrochloride 2% (Xilazin®, Synthec, São Paulo, Brazil), sufficient dose to produce the absence of the corneal reflex and confirmation of death. The established periods of death were 7 and 14 days after the beginning of topical application in mucosa of the right cheek (5 animals/day).

Histological processing: After the death was confirmed, the right cheek of the animals was removed with the aid of a #15 scalpel blade (Descarpack, Santa Catarina, Brazil) and goldman fox scissors (Golgran, São Paulo, Brazil). Fragment was fixed in buffered formalin at 4%, with pH 7.4 and later sent for routine histological processing to obtain slides stained with Hematoxylin and Eosin (HE) and Picrosirius Red at the Histology and Embryology Laboratory of the Faculdade Adventista da Bahia (FADBA), in Cachoeira-BA, Brazil.

3. Results

The clinical evaluation carried out through visual inspection immediately after death, did not show the presence of alterations in the normal aspects of the surface of the buccal mucosa of the animals in terms of color, surface texture, changes in relief, shine/hydration.

Although all sections obtained were analyzed, the statistical results presented refer to the analyzes in HE of groups G2, G3 and G4 (Figure 2),

In the HE-stained histological sections of the control group (G1), a normal corneal layer was observed, with a presence of epithelial ridges in the entire extension of the epithelium and a mononuclear inflammatory infiltrate residing in the lamina propria in the animals of all sacrifice periods (Figure 2). Regardless of the period investigated, a pattern was observed in the expression of total collagen. Regarding the degeneration of the basal layer, it was absent in all analyzed sections. In the sections stained with Sirius Red, it was noted the presence of thick collagen fibers, in dense bundles, arranged in all directions (Figure 3).

As for the Fluid Extract group (G2), when evaluating the characteristics of the epithelium with 7 days and 14 days of sacrifice, they showed no significant changes in most of the samples (Figure 2). Regarding the degeneration of the basal layer, as in the Control group (G1), this parameter was also absent in all investigated periods (100%) (Table 1). As for the presence of mononuclear inflammatory infiltrate, it was discreet in the entire sample (100%), in both sacrifice periods. Finally, regarding collagen expression, in most samples, it was presented in a discrete way (100% in 7 days and 80% in 14 days) (Table 1). As for the analysis by staining with Sirius Red, the collagen fibers were thick, arranged in all directions, with dense, homogeneous bundles along the entire length (Figure 3).

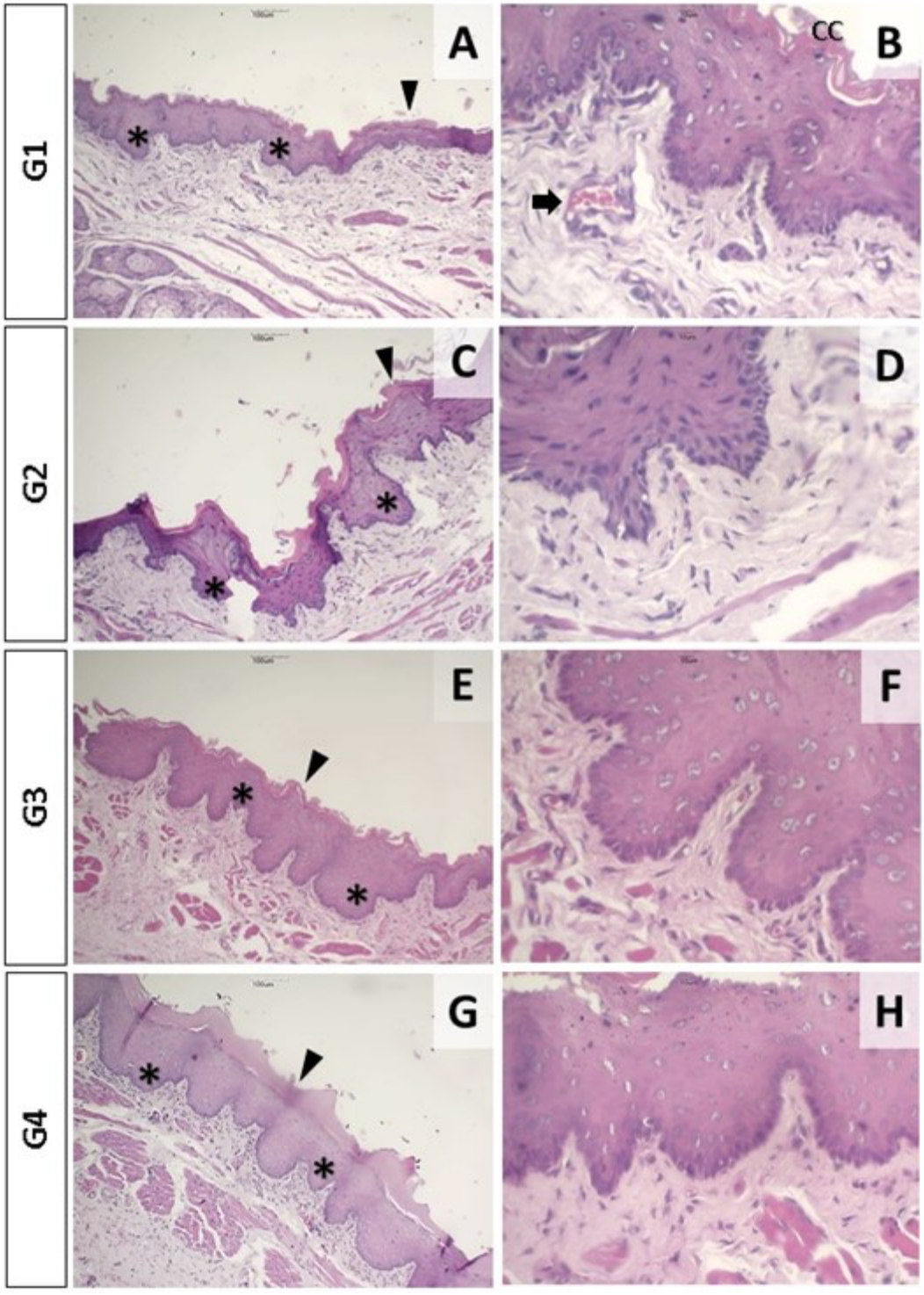


Figure 2 – Representative photomicrographs of HE-stained histological sections of buccal mucosa seen in the 10x (A, C, E and G) and 40x (B, D, F and H) objective, illustrating the morphology of the experimental groups. Thickness of the orthokeratinized stratified squamous epithelium can be seen, with the presence of epithelial ridges (asterisk), either voluminous or discrete. It is also observed varying degrees of stratum corneum thickness (arrowhead), which characterize the histomorphological pattern of the rat's buccal

mucosa. Arrow: blood vessels. Source: LBO/UFBA, 2022.

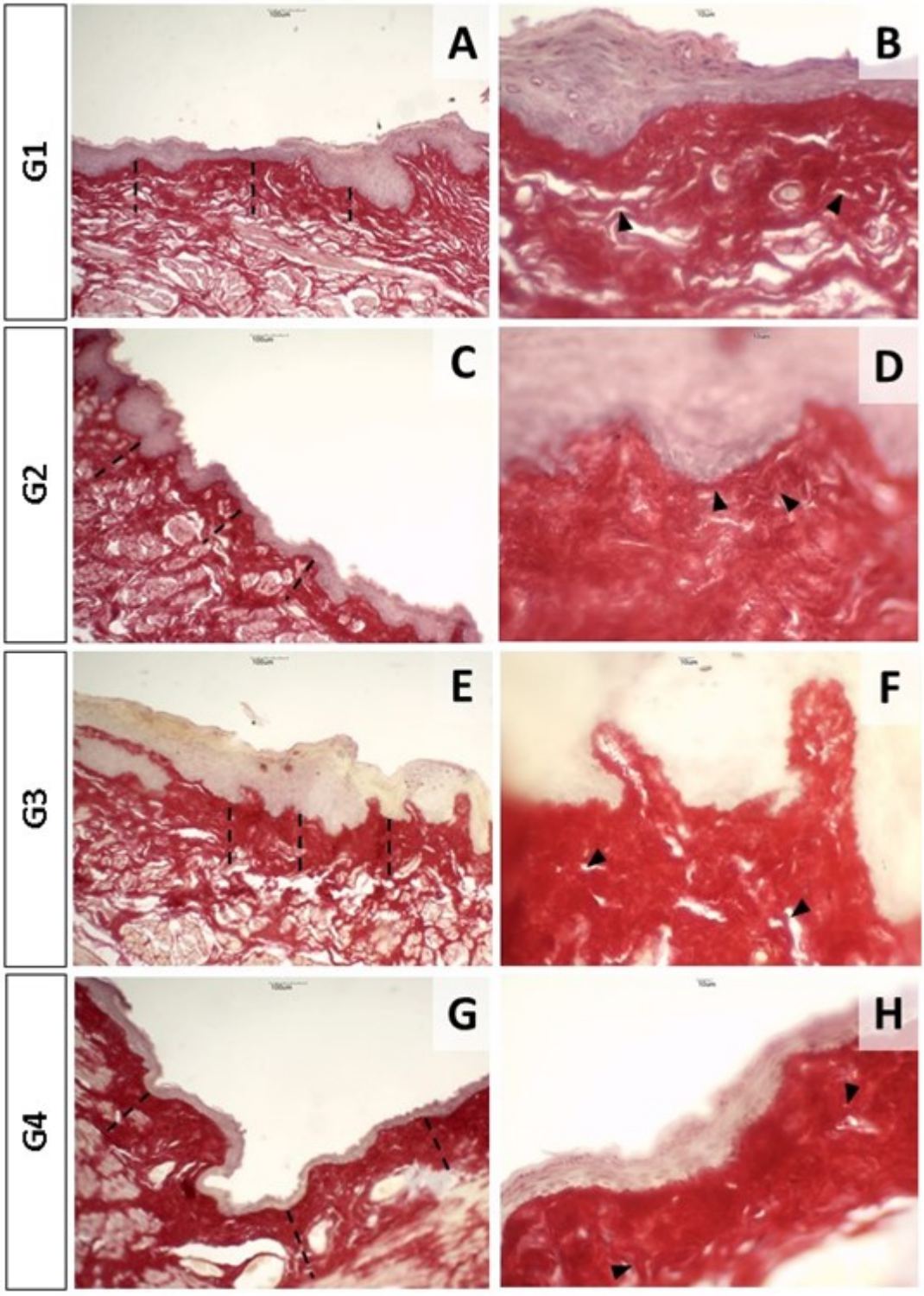


Figure 3 – Representative photomicrographs of histological sections of buccal mucosa stained by Sirius Red with 10x (A, C, E, G) and 40x (B, D, F, H) objective to illustrate the morphological pattern of collagen fibers in the groups, where it is noted the depth patterning of collagen deposition (broken lines) in the lamina propria, with dense fibers (arrowhead) dispersed in different organizational arrangements. Source: LBO/UFBA, 2022.

In the G3 group (Chamomile Infusion 1), the epithelial characteristics also showed no changes in their pattern in 7 or 14 days (Figure 2). Similar to what was observed in the G2 group, the degeneration of the basal layer was also absent in the entire sample (100%). The mononuclear inflammatory infiltrate variable was predominantly mild at both times of sacrifice (100% at 7 and 14 days). Collagen expression was totally (100%) discrete for both 7 and 14 days (Table 1). By staining with Sirius Red, the collagen fibers were also thick, arranged in all directions, with dense bundles, but with a fragmented aspect in part of the sample (Figure 3).

Table 1 – Distribution of frequencies of the results of the morphological analyzes according to the group and time of sacrifice (LBO/UFBA, 2022).

Variable	Result	G2		G3		G4	
		7 days	14 days	7 days	14 days	7 days	14 days
		n=5	n=5	n=4	n=5	n=5	n=5
Degeneration of the epithelial basal layer	Absent	5 (100,0%)	5 (100,0%)	4(100,0%)	5 (100,0%)	5(100,0%)	5 (100,0%)
	Discreet	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)
	Moderate	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)
Inflammatory infiltrate	Absent	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	1 (20,0%)	1 (20,0%)
	Discreet	5 (100,0%)	5 (100,0%)	4(100,0%)	5 (100,0%)	4 (80,0%)	4 (80,0%)
	Moderate	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)
Collagen expression	Absent	0 (0,0%)	1 (20,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)
	Discreet	5 (100,0%)	4 (80,0%)	4(100,0%)	5 (100,0%)	1 (20,0%)	2 (40,0%)
	Moderate	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	4 (80,0%)	3 (60,0%)

Source: own authorship

Finally, in the G4 group (Chamomile Infusion 2), the epithelial pattern did not change in any of the two evaluated periods (Figure 2). The degeneration of

the basal layer, as in the other groups, was also absent in the entire sample (100%). A slight inflammatory infiltrate was observed in most sections at both times (80%). Regarding collagen expression, this was moderately noted in most sections of the period (80% at 7 days and 60% at 14 days) (Table 1). As for the pattern of collagen fibers, by the coloring of Sirius Red, they were present in a thick form under dense bundles, arranged in all directions (Figure 3).

The results of the morphometric variables are shown in Table 2. For this analysis, the parameters of G1 (baseline) were considered, which included the DBLES (57.61 µm); DAECES (111.06 µm); the WEC (60.93 µm); the ID (39.67 µm) and the TSC (18.56 µm). Despite the measurement of DBLES in G2, at 7 days, it showed a higher value (95.23 µm) in contrast to the lowest value described in G4, at 14 days (59.51 µm), which was the group that came closest to the baseline mean, this difference was not statistically significant (p = 0.1757), as well as DAECES, which at 14 days of G2 presented the highest value (165.85 µm) compared to the lowest value found in G4 at 14 days, which was also the closest to the baseline (103.28 µm), but with p = 0.2040.

Regarding WEC, the G2 group, within 7 days of sacrifice, had the highest mean value (79.07 µm) in contrast to the lowest value (53.19 µm) found at 7 days of G4. The group on the 14th day of G3 (56.23 µm), which presented a mean closer to the baseline. No statistically significant difference (p = 0.4862) between groups.

At 14 days, the same G2 was also the one that presented an ID value (57.32 µm) compared to the 14 days of G4, with a lower mean value (40.34 µm), again with a mean closer to that found at baseline, with p = 0.7009. Finally, for the TSC parameter, the average values in the sample ranged from 40.55µm and 17.61µm corresponding to the 7-day groups of G2 and 14 days of G3, respectively, with the average described for G4, the one most approached the baseline group. However, in terms of statistical evaluation, this difference was also not significant (p = 0.1386).

It was observed that, despite the divergence in the measurements of the variants in the investigated groups over time, this difference was not statistically significant (p>0.05).

A baseline percentage of 48.1 (G1) was considered for the quantitative pattern of total collagen expression. It is noted that the average value of G2 showed greater divergence in the percentage of collagen expression (41.5 at 7 days and 37.3 at 14 days) compared to the other periods and groups investigated, which determined a p-value close to the threshold (p=0.0569), but still without statistical significance.

Table 2 – Mean and standard deviation (SD) of morphometric measurements (µm) as a function of the group and time of sacrifice (LBO/UFBA, 2022).

Variable	Estatisticcs	G2		G3		G4	
		7 days	14 days	7 days	14 days	7 days	14 days
		n=5	n=5	n=4	n=5	n=5	n=5
DBLES	Mean (SD)	95,23 (27,38)	76,84 (20,93)	82,79 (24,75)	87,87 (26,75)	69,52 (23,50)	59,51 (39,75)
[1]p-values		p(groups)=0,1757; p(time)=0,4631; p(interaction)=0,6609					
DAECES	Mean (SD)	163,23 (28,73)	165,85 (33,75)	126,22 (483,26)	156,56 (55,75)	133,38 (92,32)	103,28 (51,54)
[1]p-values		p(groups)=0,2040; p(time)=0,9639; p(interaction)=0,5112					
WEC	Mean (SD)	79,07 (13,84)	67,73 (12,85)	73,20 (13,01)	56,23 (16,43)	53,19 (31,61)	65,68 (23,82)
[1]p-values		p(groups)=0,4862; p(time)=0,1437; p(interaction)=0,5783					
ID	Mean (SD)	49,16 (20,38)	57,32 (10,93)	45,37 (21,01)	49,55 (11,09)	46,51 (29,24)	40,34 (10,82)
[1]p-values		p(groups)=0,7009; p(time)=0,7208; p(interaction)=0,1740					
TSC	Mean (SD)	40,55 (27,37)	24,30 (64,71)	20,67 (7,62)	23,54 (10,08)	22,04 (12,04)	17,61 (10,49)
[1]p-values		p(groups)=0,1386; p(time)=0,2796; p(interaction)=0,3598					

[1]For purposes of statistical analysis, the control group was not considered. DBLES: Distance from basal layer to epithelial surface; DAECES: Distance from the crest apex to the epithelial surface; WEC: width of epithelial crest; ID: Inter-crest distance; TSC: Thickness of the stratum corneum. DBLES, DAECES and TSC two-way ANOVA analysis; WEC, ID analysis with generalized linear models. Source: own authorship.

For the analysis of the quantitative pattern of total collagen, a total of 30 histological sections stained with Red Sirius were obtained, with groups G1, G2, G3 and G4 composed of: 04, 10, 09 and 07 cases, respectively, depending on the difficulty analysis of the biological material of 01 case referring to the day 0 day G1; 01 case of 7 days of the G3; 01 case of 7 days and 02 cases of 14 days of the G4.

As in the results of the sections in HE, the analyzes referring to the control/baseline group (G1) were not included in the statistical analysis, although analyzed.

It can be seen in Table 3 that there was no significant difference between groups and time regarding the percentage of collagen area in relation to the total tissue area ($p>0.05$).

Table 3 – Mean and standard deviation (SD) of the percentage of collagen area in relation to the total tissue area as a function of the group and time of sacrifice (LBO/UFBA, 2022).

Group	7 days	14 days
	Mean (SD)	Mean (SD)
G2	41,5 (8,1)	37,3 (11,2)
G3	42,1 (5,0)	47,1 (8,9)
G4	50,0 (4,4)	49,1 (5,3)
p-value	p(group)=0,0569; p(time)=0,9173; p(interaction)=0,4928	

[1]For purposes of statistical analysis, the control group was not considered. Two-way ANOVA analysis. Source: own authorship

4. Discussion

Medicinal plants are not exempt from causing adverse reactions to the body, which may result from their own intrinsic components, inadequate dosage, or presence of contaminants during herbal preparations[23,24].

Due to their popularity, ease of access and low cost, these substances are widely used for therapeutic purposes, especially in the form of mouthwash and orabase in dentistry[14,15,17], in addition to the difficulty of limiting the use of these agents only in injured areas, makes this investigation of the topical action of *M. recutita* on normal tissue is also necessary. In this study, the histological characteristics proposed for analysis are the same found when a tissue is subjected to damage in the most diverse ways and causes tissue changes.

Results obtained in the present study, of the Control/baseline group (G1), were reference parameters for the other experimental groups. The analyzes of this group corroborate the descriptive study of Della Cella et al.[25], which describes buccal mucosa of Wistar rats, as formed by squamous lining epithelial tissue stratified orthokeratinized, with thick slender and presence of basal, spinous, granular and corneal layers, and also, crests at the epithelial-connective interface. In conjunctive tissue, authors highlight a significant concentration of collagen fibers and a considerable contingent of fibroblasts, in addition to blood vessels of small caliber and discrete inflammatory infiltrate resident.

The characteristics of tissue structure of the rat mucosa are similar to the mucosa of humans, both in terms of the lining epithelium and the lamina propria, except for the presence of the stratum corneum[25]. When evaluating the TSC parameter, it was not observed the thickening of the stratum corneum produced in response to a possible stress, through the increased production of keratin[26], both in the baseline and in the experimental groups. DBLES also did not show a pattern of significant thickening, which is histologically characterized as acanthosis, which is defined as an increase in the stratum spinosum due to the greater number of epithelial cells[26]. Since these parameters did not show significant discrepancies between the experimental groups, these findings show that there was no expressive response to a possible trauma. This could have occurred due to the friction of the cotton swab on the fabric during the application of the substance in the region, or even due to the stimulus caused by the substances present in the chamomile

formulations.

Despite the possibility of trauma caused using the flexible rod during the topical application of the fluid extract, the work by Myose et al.[27] tested the friction of instruments or artifacts in the rat mucosa by establishing an in vivo model of intraepithelial lesions to evaluate the effects on the inflammatory response of macrophages. To this end, the authors used as a methodology, the use of a flexible rod with cotton ends, another covered with gauze, a tufted brush and a sponge cut into 10 mm cubes, 30 times with an applied load of 50 g. Tissue damage was assessed when observed clinically, immediately after friction and microscopically. In their results, the authors observed that among the artifacts used, only the sponge brush was not capable of causing intraepithelial injury, which proves that constant loads of some materials can result in injury. Macroscopic damage such as erythema was not observed, however, the slight increase in macrophages in the subepithelial tissues shows that macrophages reacted to the mechanical injury. In addition to what the authors presented, the clinical and histological findings of the present study showed that the use of flexible swabs with cotton ends applied twice a day for 7 or 14 days, was not capable of causing macro-or microscopic damage to the tissue and that could be confused with tissue changes caused by the application of the substance.

Regarding the degeneration of the basal layer, represented by the loss of the integrity of the basal layer[28], the fact that it was absent in all experimental groups indicates a positive factor for chamomile, as it demonstrates that its topical application, regardless of the form of delivery, was not able to cause damage to the evaluated tissues during the 14 days of the experiment.

As for the presence of mononuclear inflammatory infiltrate, characteristic observations of the inflammatory process[26,29], which ranged from absent to discreet in the experimental groups at both times of sacrifice, a similarity with the Control/baseline group (G1) can be seen. It should be noted that, in addition to the extracellular matrix, the connective tissue is also composed of its own resident cells, such as macrophages, mast cells and leukocytes, capable of initially acting in the face of aggression[30]. This characteristic explains the presence of the discrete inflammatory infiltrate observed in the sections evaluated. Since the inflammatory response is due to damage, it can be suggested that there was no tissue damage in any of the forms of presentation and dosage of chamomile. This absence of adverse effects and good tissue tolerance are evident in several studies[14,15,17,31,32], which aimed to observe the anti-inflammatory effect of chamomile.

In the study by Oliveira et al.[33], the authors evaluated tumor necrosis factor alpha (TNF-α) and apoptosis in induced ulcers in the left cheek mucosa of rats divided into 5 groups: animals treated with saline solution (negative control group/rats normoglycemic and positive control group/diabetic rats); animals treated with chamomile extract (normoglycemic and diabetic rats) and; diabetic animals treated with triamcinolone. After ten days of the experiment, the histological analysis showed that the negative control group and the chamomile group with diabetic rats presented a score of 1 in the score, represented by the absence of ulcer, fibrosis and chronic inflammatory infiltrate; the positive control group scored 2, as it presented epithelial

ulceration; the triamcinolone group had a score of 4, characterized by the presence of ulcer and intense acute inflammatory process and; the chamomile group of normoglycemic animals showed a completely remodeled epithelium without inflammation, which represented a score of 0. The authors attributed the favorable results of chamomile to the anti-inflammatory capacity derived from its bioactive compounds, capable of inhibiting cyclooxygenase-2 and synthesis of inflammatory mediators. Furthermore, they report that the α-bisabolol present in chamomile is related to the formation of granulation tissue during the wound repair process. It is important to emphasize that, in this study, there is no information on normal tissue, which was not subjected to damage.

Salum et al.[34], in their experimental study, characterized the oral mucosa of different animal models commonly used in research. When evaluating the buccal mucosa of a rat (*Ratus norvegicus*), they describe that the epithelial thickness found ranged from 40 to 80 µm, of which 7 to 20 µm of keratin thickness. Such results are slightly different from those described in this study, since the thickness of the epithelial layer (DBLES) ranged approximately between 59 and 95 µm and the thickness of the stratum corneum from 17 to 40 µm. These differences in the data presented by the authors with the present study may be due to the difference in the sample number, since in the aforementioned study, only one animal was used for analysis. The difficulty in obtaining comparative studies or studies that include aspects of tissue normality in these animals justifies the relevance of this work and should be a stimulating factor for other publications containing this information.

In the present study, although the morphometric results of such linear measurements (DBLES and TSC) in the experimental groups were different from the Control/baseline, with a tendency to higher averages, when compared to each other (G2, G3 and G4) no differences were observed significant. Thus, it is suggested that the discrete thickening of the epithelial layer and the superficial keratinization, as well as the presence of the described epithelial ridges, are characteristic of the investigated species. However, further studies are suggested, with a larger sample size, to confirm these results.

Salum et al.[35], carried out a histological evaluation of the mucosa of the cheek pouch of sixty-six hamsters after application of topical thiocyanate (a daily dose of 55 mg/kg of gel per body weight), an ion that stimulates the salivary peroxidase enzyme system, with the aim of verifying whether such a substance would intensify epithelial proliferative activity and would be able to interfere with keratin deposition. The authors measured the thickness of the epithelium and the stratum corneum in the control group and found averages of 34.21 and 6.40 µm, respectively, in the experimental group, the averages obtained were 34.57 and 5.95 µm for the same investigated parameters. The thickness of the epithelium showed no statistically significant difference between the groups studied (p=0.677). However, the thickness of the stratum corneum showed a statistical difference between the two groups evaluated, with the mean of the experimental group being lower than that of the control group (p=0.035), according to the authors, the increase in the frequency of clear cells and the decrease in the height of the stratum

corneum promoted by ion administration, may be associated with increased keratin hydrophilicity. Despite the differences described, the authors concluded that thiocyanate was not able to promote the intensification of epithelial proliferative activity and the increase of keratin, as expected, therefore, without important macroscopic and histological alterations. Despite the description of the epithelial aspects in this study, it was not possible to compare such parameters with the present investigation due to the different substances used and species involved.

No previous studies were found in the consulted literature that brought quantitative results on the pattern of epithelial ridges in rats. The absence of epithelial ridges was described by Pinto[36] as a histological feature of the cheek pouch in hamsters. Thus, the results of the low averages for DAECES, WEC and ID found in the present study in all experimental groups, including the Control/baseline group (G1), only demonstrate the presence of a less imbricated interface between the analyzed tissues.

In normal tissue, the collagen deposition pattern results from the balance between fiber synthesis and degradation, which in turn matches the extracellular matrix formation process[37]. According to Pinto[36], the lamina propria of the oral mucosa of hamsters has a significant concentration of collagen fibers, which differs from the discreet pattern found in the tissue sections analyzed in the present investigation; however, this finding suggests the absence of tissue alteration, since all periods and groups investigated presented this aspect. With this, it is suggested once again that the chamomile applied in a systematic way did not interfere in the normality aspect of the tissue.

Experimental studies in animal models, with topical use of other medicinal plants, are described in the literature, such as the one by Abu-Al-Basal[38], who used diabetic mice to evaluate the effectiveness of the aqueous extract intraperitoneally and the topical use of essential oil of *Rosmarinus officinalis*, in skin healing. The authors evaluated the healing area by calculus through a digitized image and performed histological analysis on the sections stained with HE and Masson's Trichrome. According to the authors, significant differences were found between the groups that received the therapies compared to the control group, in different aspects of the healing process of diabetic wounds, such as wound contraction, re-epithelialization, granulation tissue regeneration, angiogenesis. and collagen deposition. In the present investigation, as there was no wound area to be measured, only visual inspection of the mucosa was performed in search of possible surface changes in animals submitted to topical application, with no changes in clinical aspects being observed. Sections stained with HE were also used for tissue morphological and morphological analysis and Sirius Red staining for collagen analysis.

The sections stained with Sirius Red also allowed the quantitative analysis of the percentage of collagen expression, which minimizes the subjectivity of the descriptive analysis through the numerical comparison between the groups, and confirms that the topical application of the phytotherapic was not able to generate biosynthesis stimuli or tissue collagen degradation, which could be perceived by the change in the expression of collagen fibers at the times studied[39].

Cavalcanti et al.[40] also in an experimental model, investigated the action of the use of a topical pharmaceutical formulation, developed including essential oil extracted from the leaves of *Croton zehntneri*, in the cutaneous healing of mice. In addition to the macroscopic semiquantitative analysis of edema and counting the number of capillaries and leukocytes in sections stained with HE, sections stained with Masson's Trichrome were used for quantitative analysis of fibroblasts and collagen fibers present in the photomicrograph obtained. Differently from the morphometric method used in the present investigation, the quantification of collagen content was performed according to the authors with the counting of all fibers, regardless of thickness, in five randomized fields, of six samples in each group. According to the authors, on the 15th day of investigation, groups treated with 20% *Croton zehntneri* essential oil, as well as with 20% trans-anethole, had a significant increase in the number of collagen fibers compared to controls and animals. treated with dexamethasone. This result differs from the present study, since no quantitative differences were observed in the experimental groups, with the methodology used.

The morphometric method used to count the percentage of collagen is similar to the one used in the study by Fortuna et al.[41], where forty Wistar rats were submitted to cutaneous surgery to characterize the vascular density and the fibroplasia process after the tissue repair stages, through the quantitative average per pixel, it was found that there was greater expression of collagen fibers in the group that received photobiomodulatory action of the laser with 4 J/cm^2 (3949 pixels in 14 days and 3828 pixels in 21 days) when compared to the control group, with animals irradiated with laser off (1876 pixels at 14 days and 21 days at 2885 pixels), with statistical significance of p=0.008 and p=0.016, respectively, which was related to the primary effects resulting from laser stimulation, such as the increase in the number of fibroblasts, myofibroblasts and collagen expression. However, once again, it is important to emphasize the absence of tissue injury prior to the analysis of the present investigation, which makes it difficult to compare results.

The analysis of collagen deposition pattern after tissue damage has long been investigated in the literature, especially in studies dealing with laser photobiomodulation in repair tissues[42,43]. It is important to understand that the inclusion of this work in this discussion aims to clarify that there are external agents capable of interfering with collagen production and deposition. Furthermore, the inclusion of these authors is justified by the difficulty in identifying publications that exhibit tissue patterns after application of herbal medicines.

In turn, Oliveira et al.[33] also evaluated the percentage of collagen deposition by the Sirius Vermelho stain. The authors identified that the negative control group, the positive control group and the chamomile group of normoglycemic rats showed an increase in collagen from the 5th to the 10th day of the experiment, compared to diabetic animals in the chamomile and triamcinolone-treated groups, which may be related to the fact that hyperglycemia modifies the biological profile of collagen deposition, which ends up interfering with wound healing.

It is worth remembering that, unlike these studies, the present study

evaluated the collagen expression of tissue that had not undergone previous damage, with this, the results show that there was no stimulus of collagen production through the topical action of chamomile, indicating good tolerance of the tissues in the presence of this herbal medicine. The fact that the p-value for groups was close to the threshold (p=0.0569) can be justified by the sample size, but there was still no statistical difference in the two periods and in all groups.

Although some authors report in their studies the genotoxic action of bioactive components of chamomile, such as flavonoids that have antiproliferative potential[44,45], the anti-genotoxic capacity of this herbal medicine is also described in the literature by other research groups, in experimental models with mice, bulbs of *Allium cepa*, *Drosophila melanogaster*[9,46,47]. Such contrast between authors highlights the need for research on the effects of chamomile on normal tissues. Thus, it is suggested that further studies should be carried out with larger samples, other forms of dosage and vehicles for the application of herbal medicines, as well as other forms of tissue analysis. Even so, the results found in the present study reaffirm the hypothesis that topical application of chamomile was not capable of causing tissue damage in the sample studied, through a histomorphometric analysis.

This study has some limitations that should be considered when interpreting the findings. To reduce the number of animals used, in accordance with the principles of animal research and following a methodology similar to that described by Pavesi et al., a smaller number of rats was allocated to the control/baseline group. Consequently, the morphological evaluation of this group was limited to a descriptive assessment, restricting more robust comparative analyses. In addition, it was not possible to perform specific analytical tests to determine the exact concentration of *Matricaria recutita* or to characterize the bioactive constituents present in the commercial formulations used. Therefore, potential variations in the composition of these formulations could not be assessed and may have influenced the observed biological responses.

5. Conclusion

In this study, the clinical, morphological and morphometric parameters evaluated did not show statistical differences between the experimental groups. Thus, the topical use of chamomile did not promote changes in the histological pattern of the normal oral mucosa of rats. Thus, chamomile can be considered a therapeutic agent well tolerated by tissues, in addition to being an economically viable alternative for the management of inflammatory lesions in the oral cavity.

6. Declaration of conflict of interests

The authors declare that they have no conflicts of interest that could influence the development of the study.

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