UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

TUANY RAFAELI SCHMIDT

ESTUDO DO EFEITO DA FORMULAÇÃO MUCOADESIVA DO EXTRATO DE CURCUMA LONGA L. NA MUCOSITE ORAL QUIMIOINDUZIDA EM HAMSTER

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Cirurgião-Dentista.

Orientadora: Profª. Drª. Manoela Domingues Martins

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Porto Alegre, 08 de julho 2019.

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DEDICATÓRIA

À minha família, em especial minha mãe e meu irmão, que sempre estiveram presentes de alguma forma durante toda minha jornada acadêmica. Minha eterna gratidão pela educação, carinho, amor e proteção; por sempre acreditarem em mim e nos nossos sonhos.

AGRADECIMENTOS

A minha família, padrinhos/madrinhas, afilhados e amigos, por sempre acreditar em mim, por entender minhas ausências. Amo vocês!

A minha mãe, inspiração dos meus dias, por dar prioridade a educação e estudos dos seus filhos. Por todo carinho, atenção e amor incondicional. Essa conquista é nossa!

Ao meu irmão, meu anjo, viveu esse sonho comigo. Sempre participou dos meus estudos, me guiando pelo melhor caminho. Minha eterna gratidão ao amor e cuidado; levarei tuas palavras sempre comigo: “Não se esqueça, torço muito por você e sempre estarei ao seu lado”.

Aos pacientes que contribuíram tanto para minha formação, que Deus os abençoe com muita saúde e felicidades. Minha eterna gratidão pela confiança e carinho.

Aos meus colegas de faculdade, foram sete anos de convívio diário. Obrigada por tornarem minhas noites tão alegres e prazerosas; crescemos juntos e construímos lembranças que ficarão para sempre em meu coração.

Aos “manoeletes” e residentes da estomatologia, obrigada por tantos ensinamentos, paciência e carinho. Vendo vocês eu sonhava com o meu futuro, me identificava com tantos sentimentos. Muito obrigada por tornar a pesquisa e ambulatório ainda mais enriquecedores e especiais.

Aos profissionais do centro de pesquisas do HCPA e da Unidade de Experimentação Animal do HCPA, muito obrigada por tantos ensinamentos e paciência nos momentos experimentais.

Aos programas de bolsa, BIC UFRGS e PROBIC FAPERGS-UFRGS, nos quais atuei como bolsista de iniciação científica de 2015 a 2019, e ao Programa de Fomento à Pesquisa, pelo auxilio financeiro mensal e para participação em congressos nacionais.

Ao professor Vinicius, obrigada por me abrir as portas da patologia. Obrigada pela paciência, ensinamentos e oportunidades.

Ao professor Marco, obrigada por me apresentar o universo da estomatologia e o ambiente hospitalar. Gratidão pelos ensinamentos, paciência e conduta exemplar para com os pacientes e alunos.

A minha orientadora Manô, por acreditar em mim mesmo antes de eu acreditar. Obrigada por me acolher na iniciação cientifica, por me pegar na mão e me mostrar um mundo que eu achava tão distante. Foram cinco anos de muito aprendizado, carinho e companheirismo. Minha eterna gratidão a tantos ensinamentos, por ser meu exemplo de todos os dias, por ser minha mãe cientifica.

“Somos todos anjos de uma só asa, e só podemos voar quando nos abraçamos uns aos outros.”

Fernando Pessoa

RESUMO

A mucosite é uma complicação comum no tratamento citorredutor do câncer. A Curcuma longa L. tem sido proposta como candidata ao tratamento de várias doenças por possuir propriedades antioxidante, antitumoral e anti-inflamatória. O objetivo do presente estudo foi avaliar os efeitos da formulação mucoadesiva de Curcuma longa L. (FMC) na mucosite oral (MO) induzida por 5-fluoracil (5-FU) em hamster. Foram utilizados 72 hamsters sírios dourados separados aleatoriamente em 4 grupos: Grupo controle (apenas manipulação), Grupo Placebo (uso tópico de óleo neutro), Grupo Controle Positivo (Camomila- uso tópico de AdMuc) e Grupo Teste FMC (uso tópico da FMC). Para indução da mucosite foram realizadas injeção intraperitoneal de 5-FU nos dias 0 e 2 e escarificação da mucosa bucal nos dias 3 e 4. Os animais receberam duas aplicações diárias do produto de acordo com o grupo experimental. Nos dias 8, 10 e 14 dias foram eutanasiados 6 animais de cada grupo. A área das feridas foi calculada e cortes histológicos de 3µm foram corados pela HE para análise semi-quantitativa da reepitelização e grau de inflamação tecidual. Imunohistoquímica foi usada para análise de TGF-B1 e CD31. A principal diferença entre os grupos ocorreu aos 8 dias. O grupo tratado com FMC mostrou maior redução clínica das lesões, maior grau de reepitelização, menor processo inflamatório, menor angiogênese e marcação epitelial de TGF-B1 quando comparado aos grupos placebo e controle ($p < 0,05$). FMC e camomila foram semelhantes. Conclui-se que a Curcuma longa L. possui efeito terapêutico acelerando o reparo de lesões de mucosite quimioinduzida em hamster.

Palavras-chave: Cicatrização. Angiogênese. Curcumina. Mucosite oral.

ABSTRACT

Mucositis (OM) is a common complication in the cytoreductive treatment of cancer. Curcuma longa L. has been proposed as a candidate for the treatment of different diseases because of its anti-inflammatory, antioxidant and antitumor effects. The objective of the present study was to evaluate the clinical, histopathological and immunohistochemical effect of mucosal formulation of Curcuma longa L. (FMC) on oral mucositis induced by 5-fluorouracil (5-FU) in hamsters. Seventy-two golden Syrian hamsters were randomly separated in 4 groups: Control group (manipulation only), Placebo Group (topical use of neutral oil (no active substance), Positive Control Group (AdMuc® Topical Chamomile) and FMC Test Group (topical use of FMC.) In order to induce mucositis, it was performed intraperitoneal injection of 5-FU (days 0 and 2) and scarification of the buccal mucosa (days 3 and 4). The animals received two daily applications of the product according to the experimental group. Wound area was calculated and histological sections of 3µm were stained by HE for semi-quantitative analysis of re-epithelization and degree of tissue inflammation. For the Immunohistochemical staining, the slides were incubated at room temperature for 2h with anti-CD31 and for 18h with anti-TGF-B1. Data were compared using the Kruskall Wallis test and Tukey poshoc. The main difference between groups occurred at day 8. The FMC-treated group showed a greater clinical reduction of the lesions, a higher degree of re-epithelization and a lower inflammatory process, with a lower angiogenesis and a lower TGF-B1 epithelial marking when compared to the other groups ($p < 0.05$). It is concluded that Curcuma Longa L. has a therapeutic effect accelerating the repair of chemo-induced mucositis lesions in hamsters.

Keywords: Wound healing. Angiogenesis. Curcumin. Oral mucositis.

SUMÁRIO

1 ANTECEDENTES E JUSTIFICATIVA

A mucosite oral (OM) é uma inflamação da mucosa decorrente da toxicidade de tratamentos antineoplásicos, seja radioterapia ou quimioterapia. Ambas as terapias são inespecíficas, afetando tanto células malignas quanto células normais (SONIS, 2011). Clinicamente caracteriza-se por lesões eritematosas, erosivas e/ou ulceradas, que geram de pequenos desconfortos a dores intensas. Sua incidência é variável de acordo com os protocolos utilizados. Curra *et al*. (2018), através de uma revisão de literatura, concluiu que protocolos para o transplante de células-tronco hematopoiéticas implicam em alto risco de desenvolver mucosite oral, assim como altas doses dos quimioterápicos 5-fluoracil (5-FU) e citarabina, agentes alquilantes e derivados da platina. Esta complicação geralmente inicia em 5 a 10 dias após a infusão do quimioterápico e tem duração de 7 a 14 dias (CIDON, 2018).

De acordo com a classificação da Organização Mundial da Saúde (OMS) a mucosite varia de grau 0 a 4, levando em consideração critérios objetivos e subjetivos, como descrito a seguir: 0- ausência de alterações na mucosa; 1- inflamação e eritema; 2- eritema e ulceração (o paciente consegue engolir sólidos); 3- ulceração (o paciente consegue apenas ingerir líquidos) e 4-não é possível se alimentar por via oral. Conforme o grau, a mucosite pode diminuir a qualidade de vida e sobrevida do paciente através do aumento do risco de infecções, prescrição de opióides e tempo de internação hospitalar levando muitas vezes a necessidade de modificação ou até mesmo a interrupção do tratamento antineoplásico (CINAUSERO *et al*., 2017; CURRA *et al*., 2018; ELTING *et al*., 2003; PEREIRA *et al*., 2018; SONIS, 2013).

Tendo isso em vista, o controle da mucosite é extremamente importante. Os tratamentos propostos visam, principalmente, aliviar a sintomatologia dolorosa, intensidade das lesões e controlar possíveis quadros infecciosos e/ou hemorrágicos. Na literatura encontramos diversos tipos de tratamento para mucosite, dentre eles: laser de baixa intensidade; fatores de crescimento; enxaguatórios contendo analgésicos, anti-inflamatórios ou antimicrobianos; crioterapia e antissépticos (HE *et al*., 2018; MOSLEMI *et al*., 2016; VILLA; SONIS, 2016). No entanto, existem poucas terapias eficazes para prevenir e/ou tratar a mucosite oral recomendadas pela *Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology* (LALLA *et al*., 2014).

Agentes fitoterápicos vêm sendo estudados como novas modalidades terapêuticas para o tratamento de diversas doenças, incluído a mucosite (BAHARVAND; JAFARI; MORTAZAVI, 2017; CINAUSERO *et al*., 2017), devido seu fácil acesso, ausência de efeitos colaterais e baixo custo. Uma revisão de literatura aponta diversos fitoterápicos utilizados no manejo da mucosite oral; dentre eles, a camomila mostrou bons resultados reduzindo a intensidade da mucosite devido sua ação anti-inflamatória, antibacteriana, antifúngica e cicatrizante (AGHAMOHAMMADI; HOSSEINIMEHR, 2016). Estudos do nosso grupo apontam a camomila com um importante fitoterápico no tratamento de úlceras devido sua ação cicatrizante e anti-inflamatória (CURRA *et al*., 2013; MARTINS *et al*., 2009). O reparo acelerado pode ser explicado pelo fato da camomila diminuir os níveis de citocinas pró-inflamatórias (IL-1β e TNF-α) fazendo da camomila um importante fitoterápico no controle da mucosite oral (CURRA *et al*., 2013). Braga *et al.* (2015), em um ensaio clinico randomizado de fase II com uma amostra de 40 pacientes, mostrou a eficácia do uso de um enxaguatório bucal contendo extrato de camomila a 1% em relação a redução da incidência, intensidade e duração da mucosite comparado ao grupo controle, em pacientes adultos submetidos ao transplante de células-tronco hematopoiéticas.

Mais recentemente, a Curcuma (diferuloylmethane), muito utilizada na medicina popular, vem sendo estudada devido suas propriedades farmacológicas sobre o reparo tecidual. A curcuma é um polifenol extraído da raiz *Curcuma longa* L. (popularmente conhecido como açafrão-da-terra, açafrão-da-índia, cúrcuma, tumérico ou gengibre amarelo) (GUPTA *et al*., 2013; GOEL; KUNNUMAKKARA; AGGARWAL, 2008). A curcumina corresponde ao princípio ativo da planta, composta por flavonoides e compostos voláteis como tumerona, atlantona e zingiberona. Este agente fitoterápico possui ação anti-inflamatória, antioxidante, analgésico, imunoestimulante, antiviral, antibacteriano além de propriedades antitumorais (LÜER *et al*.,2011; MANTZOROU *et al*., 2018; SALEHI *et al*., 2019). Além disso, a curcumina é biologicamente segura. Os curcuminoides da *Curcuma longa* L. apresentam um bom perfil de segurança tanto em humanos (12g/dia) (LAO et al., 2006) quanto em animais (100mg/kg/dia), não manifestando toxicidade mesmo em altas doses (TEITEN *et al*., 2010).

Diversos estudos sugerem que a curcumina acelera o reparo através da diminuição do estresse oxidativo, modulação da resposta inflamatória, e redução da liberação de interleucinas através da inibição do NF-KB (HE *et al*., 2015; PAGANO *et*

al., 2018). No entanto, a curumina apresenta baixa biodisponibilidade, o que limita seu uso (KARKI *et al*., 2017). Devido a isso, desenvolvemos um sistema mucoadesivo capaz de solubilizar uma grande quantidade de curcuminóides. Além disso, o componente mucoadesivo (poloxamer 407) aumenta a interação da curcumina com a mucina que recobre a mucosa, promovendo maior contato do princípio ativo com o tecido (DOS SANTOS FILHO *et al*., 2015).

No entanto, mais estudos são necessários para elucidar os mecanismos de ação, dose de segurança e forma de administração da *Curcuma longa* L. no tratamento da mucosite oral. Dessa forma, o objetivo do presente estudo foi avaliar os efeitos da formulação mucoadesiva contendo curcuminoides (MFCs) de *Curcuma longa* L. em mucosite oral induzida por 5 - fluorouracil em hamsters, através da avaliação clínica, histopatológica e imunohistoquímica.

2 ARTIGO CIENTÍFICO

MUCOADHESIVE FORMULATION CONTAINING CURCUMA LONGA L. REDUCES ORAL MUCOSITIS INDUCED BY 5-FLUOROURACIL IN HAMSTERS

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Este trabalho de conclusão de curso foi publicado na revista Phytotherapy Research 2019; 33: 881–890.

Fator de impacto 2018- 3.766

Qualis Capes- A2- Odontologia

Received: 12 September 2018 | Revised: 5 December 2018 | Accepted: 13 December 2018
DOI: 10.1002/ptr.6279

RESEARCH ARTICLE

WILEY

Mucoadhesive formulation containing *Curcuma longa* L. reduces oral mucositis induced by 5-fluorouracil in hamsters

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Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil(CAPES), Grant/Award Number: finance code 001; Postgraduate Research Group, Hospital de Clínicas de Porto Alegre, Grant/Award Number: GPPG/FIPE:14-0613

We explored the effects of a mucoadhesive formulation containing curcuminoid (MFC) from *Curcuma longa* L. extract on oral mucositis (OM) induced by 5-fluorouracil (5-FU) in hamsters. Seventy-two golden Syrian hamsters were randomly allocated into four groups: control, placebo, chamomilla, and MFC. Animals received an intraperitoneal injection of 5-FU at Days 0 and 2. On Days 3 and 4, the buccal mucosa was scratched. Therapy was initiated on Day 5. Animals received two applications of the substances per day according to the experimental group. Six animals were euthanized on Days 8, 10, and 14. Clinical analysis were performed using photography and histopathological sections of 3 μm were stained by hematoxylin–eosin for semiquantitative analysis of re-epithelization and inflammation. Immunohistochemistry was used for angiogenesis (CD31) and transforming growth factor beta 1 (TGF-β1) analysis. On Day 5, all groups exhibited OM. Clinical and histopathological findings revealed that on Day 8, both MFC and chamomilla groups exhibited better wound healing. In addition, the MFC group demonstrated lower angiogenesis and TGF-β1 levels on Day 8 compared with placebo and control groups. Collectively, these findings suggest that MFC has a therapeutic effect on OM, accelerating wound healing through re-epithelization and anti-inflammatory action as modulation of angiogenesis and TGF-β1 expression.

KEYWORDS

angiogenesis, chemotherapy, curcumin, oral mucositis, TGF-β1, wound healing

1 | INTRODUCTION

Oral mucositis (OM) is considered one of the most important acute side effects observed in patients undergoing chemotherapy, head and neck radiotherapy, and targeted agents. It is clinically characterized by erythematous, erosive, and/or ulcerative lesions. The ulcers can be extremely painful, interfere in oral functions (eat, speak, swallow), increase the risk of infection, and cause schedule delays, interruptions, or discontinuations of treatment (Cinausero et al., 2017). In parallel, additional hospital costs have been reported (Elting, Cooksley, Chambers, & Garden, 2007). Therefore, the control of this condition is important for patients' quality of life and prognosis (Pereira et al., 2018).

There are few effective therapies to prevent and/or treat OM recommended by the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (Lalla et al., 2014). Among them, cryotherapy, recombinant human keratinocyte

growth factor-1 (KGF-1/palifermin), low-level laser therapy, and benzydamine mouthwash were reported. In addition, other anti-inflammatory agents have also been studied in the management of OM (Barbosa et al., 2018). Herbal medicines have been considered as new modalities to be studied for the management of inflammatory conditions, including mucositis (Baharvand, Jafari, & Mortazavi, 2017; Cinausero et al., 2017), because they are safe, easily accessible, and inexpensive. Among several phytotherapeutic agents, our study group previously showed that chamomilla is effective in the treatment of OM in a hamster model reducing the tissue levels of interleukin 1 beta and tumor necrosis factor alpha (TNF-α; Curra et al., 2013).

Curcuma longa L. is a rhizomatous perennial herb that belongs to the family *Zingiberaceae* and has been widely used in food and drinks worldwide as a spice and yellow colorant. Curcuminoids are a diphenolic compound of *C. longa* that has been studied as a phytotherapeutic agent due to diverse pharmacologic activities including anti-inflammatory, antioxidant, and antitumor properties (Lüer, Troller, Jetter, Spaniol, & Aebi, 2011; Mantzorou, Pavlidou, Vasios, Tsagalioti, & Giaginis, 2018). Several studies have suggested that curcumin accelerates wound healing, decreases oxidative stress, modulates inflammatory response, and reduces the release of different interleukins through inhibition of nuclear factor κappa-B (He et al., 2015; Pagano, Romano, Izzo, & Borrelli, 2018). The rationale that OM pathogenesis involves the activation of nuclear factor κappa-B initiating an inflammatory response and an increase in subepithelial vascularity (Cinausero et al., 2017) suggests that curcumin may represent a potential therapeutic agent against mucositis (ClinicalTrials. gov, 2016; Dos Santos Filho et al., 2018). Nevertheless, curcumin presents low oral bioavailability and poor water solubility, which has been considered a limiting factor for its therapeutic use (Karki, Kulkarni, Swamy, & Sheeba, 2017). To overcome this limitation, we developed a mucoadhesive system capable of solubilizing a high amount of curcuminoids. In addition, the mucoadhesive constituent (poloxamer 407) probably improved the interaction of the curcumin with the mucin that covers the mucosa, promoting a prolonged and localized contact between the pharmaceutically active agent and the absorptive tissue (Dos Santos Filho et al., 2015).

The objective of the present study was to evaluate the effects of a mucoadhesive formulation containing curcuminoids (MFCs) from *C. longa* on OM induced by 5-fluorouracil (5-FU) in hamsters.

2 | MATERIAL AND METHODS

This is a prospective, randomized, controlled, and blinded animal model study. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and received approval from the Ethics Committee on Animal Use of the Porto Alegre University Hospital (HCPA, Brazil, n.14–0613).

2.1 | Preparation of MFC

The extract of curcuminoids from *C. longa* was obtained from Gamma Comércio Importação & Exportação LTDA (São Paulo, Brazil). The curcuminoids content (curcumin, desmetoxicurcumin, and bidesmetoxicurcumin) in this extract was 96.4% (*w*/w). Transcutol HP® (diethylene glycol monoethyl ether) was kindly donated by Gattefossé (Lyon, France). Poloxamer 407 and Soluplus® were obtained from Basf (Ludwigshafen, Germany). Propylene glycol and polyethylene glycol 400 were sourced from Labsynt (São Paulo, Brazil).

The mucoadhesive ingredient (poloxamer 407, 15%, *w*/v) was dispersed in an organic phase comprising a mixture of polyethylene glycol 400 (PEG 400) and propylene glycol, and kept under constant magnetic stirring at 65°C. Next, the curcuminoids were added to this mixture to a final concentration of 20 mg/mL. An aqueous fraction was also prepared by adding Transcutol HP®, Soluplus®, and two antioxidants (sodium metabisulphite and sodium bisulphite) to purified water under constant stirring. The aqueous fraction was then heated to 65°C and poured into the organic phase. The resulting mixture was kept under stirring for an additional 30 min. A blank formulation was prepared as described above but replacing the curcumin for equal mass of PEG 400. Citric acid solution (0.1 M) was used to adjust the pH of the formulations (as 6.5). These formulations were stored in amber flasks at room temperature until time to use.

2.2 | Experimental procedure

Seventy-two male golden Syrian hamsters (mean weight *SD*: 150 g; mean age: 8 weeks old) were used. Four animals were kept in each plastic box under standard conditions of temperature (22 ± 2°C), relative humidity of 40–60%, air exhaust system and light/dark cycles (12 hr/12 hr), with solid chow and water ad libitum.

The animals were randomly divided into four groups (n = 18):

- Control group: No treatment, only daily handling.
- Placebo group: Topical treatment with mucoadhesive formulation without active substance.
- Chamomilla group: Topical treatment with fluid extract of *Chamomilla recutita* (L.) Rauschert chamomilla, Ad-Muc®. This extract was chosen to serve as a phytotherapeutic positive control based on previous positive results (Curra et al., 2013; Pavesi et al., 2011).
- *C. longa* group: Topical treatment with MFCs from *C. longa*.

For the induction of OM, each animal received an intraperitoneal injection of 5-FU (60 mg/kg) at Day 0 followed by a second injection (40 mg/kg) at Day 2, according to the protocol proposed by Sonis, Tracey, Shklar, Jenson, and Florine (1990) and modified by Leitão et al. (2007). At Days 3 and 4, the animals were anesthetized with isoflurane diluted in oxygen and the buccal mucosa was scratched twice using the tip of a sterile needle by the same operator. The treatments according to each experimental group were initiated on Day 5. Each animal received two applications of the substances per day (morning and evening) using a 0.5-g measuring spoon to standardize the amount used. Animals of the control group were handled twice per day under identical conditions, but no substance was applied.

Six animals from each group were euthanized on Days 8, 10, and 14 using overdose of isoflurane (inhalant anestehetic). The buccal mucosa was photographed and removed. Posteriorly, the buccal mucosa was fixed in 10% buffered formalin solution and embedded

in paraffin for histopathological analysis. All analyses were performed using all 72 samples obtained.

2.3 | Macroscopic findings

Clinical analysis was performed by three calibrated and blinded evaluators. Macroscopic appearance such as erythema, bleeding, epithelial ulcers, and abscesses were evaluated through scores from 0 to 3 according to the method described by Lima et al. (2005), as follows: Score 0—normal buccal mucosa, with absence of or discreet erythema and hyperemia, with no areas of bleeding, ulceration, or abscesses; Score 1—moderate erythema and hyperemia, with no areas of bleeding, ulceration, or abscesses; Score 2—severe erythema and hyperemia, presence of areas of bleeding, small ulcers or eschars, but no abscesses; and Score 3—severe erythema and hyperemia, presence of areas of bleeding, extensive ulcers, and abscesses.

3 | HISTOPATHOLOGICAL ANALYSIS

For histopathological analysis, serial sections of 5 μm in thickness were obtained and stained with hematoxylin–eosin. A descriptive analysis of each group/evaluation time was performed, followed by a semiquantitative evaluation by means of scores. Three pathologists blind to the experimental groups analyzed the sections by a consensual final score. The degree of re-epithelialization was determined by a grading system (0 to 4), as described elsewhere (Sinha & Gallagher, 2003): Grade 0—re-epithelialization at the end of the wound; Grade 1—re-epithelialization covering less than half the wound; Grade 2—re-epithelialization covering more than half of the wound; Grade 3—re-epithelialization covering all of the wound with irregular thickness; Grade 4—re-epithelialization covering the entire wound and of normal thickness.

Inflammation was also evaluated through scores that assessed the resolution phases of the inflammatory process, as described by Camacho-Alonso and López-Jornet (2007): Grade 1—acute inflammation (pyogenic membrane); Grade 2—predominance of diffuse acute inflammation; Grade 3—predominance of chronic inflammatory process; Grade 4—resolution and healing (reduction or disappearance of chronic inflammation).

3.1 | Immunohistochemistry

For immunohistochemical staining, specimens were cut into 3-μm thick sections, deparaffinized in xylene, and hydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide in two 15-min baths. Antigen retrieval was performed for 18 hr in a citrate buffer solution heated to 90°C in a water bath. Slides were incubated at room temperature for 2 hr with rabbit anti-CD31 (polyclonal, 1:50, Abcam, Boston, MA, USA) and for 18 hr with anti-TGF-β1 (sc-146,1:100, Santa Cruz, Santa Cruz, CA,USA). The detection system used was the polyvalent HRP plus kit (Spring Bioscience, Pleasanton, CA, USA). Sections were then incubated with 3, 3′-diaminobenzidine tetrahydrochloride (DAB; Sigma- Aldrich Corp., St. Louis, MO, USA) and counterstained with Mayer's hematoxylin. Cases of mouse intestine served as appropriate positive controls, and the negative control was provided by suppressing the primary antibody.

3.2 | TGF-β1 analysis

Slides stained with TGF-β1 were analyzed semiquantitatively by three blinded and previously calibrated observers. Only cytoplasmic expression in epithelial cells was considered positive. Each case was classified according to Gonçalves et al. (2017), where the immunoreactive score was calculated by multiplying the percentage of positive cells (stained 0–2) by staining intensity (stained 0–3). The percentage of positive cell was scored as follows: 0, no cells stained; 1, <25% of cells stained; and 2, ≥25% of cells stained, and staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

3.3 | Microvessel density

Vessel counts at the wound area were determined by image analysis of CD31-immunostained sections. One investigator performed microvessel density (MVD) assessment. Four high-power fields (×400 magnification) of the lesion were captured: two at the wound edges and two at the deepest part of the lesion. Vessel counts were performed using the manual tagging feature in the ImageProPlus software package (NIH, Bethesda, MD, USA). Vessels in each section were defined by the circular or ovoid image of the brown endothelial walls and luminal space. Capillaries, arterioles, and venules were counted. Sections from different treatment groups were indistinguishable from one another, which allowed counting to be performed in a blinded manner.

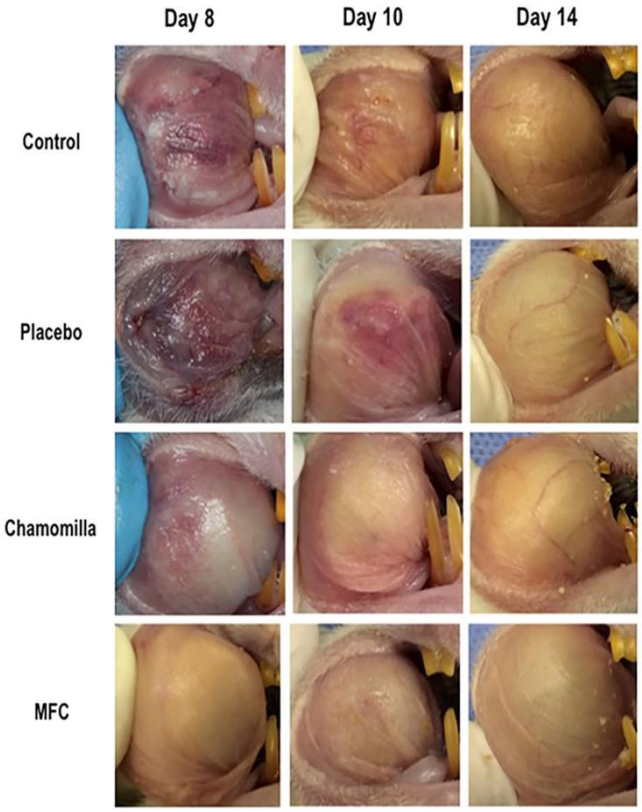
3.4 | Statistical analysis

Clinical and histopathological analyses were performed using the two-way analysis of variance, followed by Tukey's multiple comparison tests. MVD and TGF-β1 analyses were conducted by one-way analysis of variance followed by Tukey's multiple comparison tests using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The level of significance was 5% ($p < 0.05$).

4 | RESULTS

4.1 | MFC promotes clinical reduction of 5-FU-induced OM

All animals exhibited OM on Day 5. Figure 1 illustrates the clinical analysis of OM in all groups during the experimental period (8, 10, and 14 days). There were differences between groups on Day 8. Mild OM was observed in groups treated with chamomilla compared with control ($p < 0.05$) and placebo ($p < 0.01$). MFC revealed less severe OM compared with control ($p < 0.01$) and placebo ($p < 0.001$). No differences were observed among groups on Days 10 and 14.



Group	Day 8	Day 10	Day 14
Control	1.83 ± 0.75^{Aa}	0.83 ± 0.98^{ABa}	0.16 ± 0.99^{Ba}
Placebo	2.16 ± 1.16^{Aa}	0.83 ± 1.16^{ABa}	0.00 ± 0.00^{Ba}
Chamomilla	0.50 ± 1.22^{Ab}	0.33 ± 0.81^{Aa}	0.00 ± 0.00^{Aa}
MFC	0.00 ± 0.00^{Ab}	0.16 ± 0.40^{Aa}	0.00 ± 0.00^{Aa}

FIGURE 1 Macroscopic appearance: (a) Clinical aspects of hamster buccal mucosa on Days 8, 10, and 14 in control, placebo, chamomilla, and mucoadhesive formulation containing curcuminoid (MFC) groups. Note that on Day 8, the control and placebo groups exhibited extensive ulceration, whereas the chamomilla and MFC groups showed complete wound healing. (b) Table demonstrating the mucositis score according to the experimental time and group. Observe that on Day 8, control and placebo groups presented higher scores compared with chamomilla and MFC groups [Colour figure can be viewed at wileyonlinelibrary.com]

MFC accelerates reepithelization and resolution of inflammatory process in 5-FU-induced OM associated to decrease of angiogenesis and TGF-β1 level.

Histopathological analysis of epithelial and inflammatory components showed important results, particularly at Day 8, which represent the height of OM severity in the hamster model. The analysis of epithelium illustrated that, at Day 8, groups treated with chamomilla and MFC exhibited accelerated re-epithelialization in comparison with the control ($p < 0.001$) and placebo ($p < 0.001$) groups. Both phythotherapeutic agents exhibited neoformed epithelial tissue covering all of the wound thickness. Some animals from control and placebo groups still had areas of ulceration with re-epithelialization covering more than half of the wound. At Days 10 and 14, all groups showed re-epithelialization covering all of the wound thickness (Figure 2). The only statistically significant difference observed at day 10 was between placebo and MFC ($p < 0.05$).

Figure 2 also demonstrates the inflammation response grades in all groups. At Day 8, control and placebo groups showed similar scores with predominance of diffuse acute inflammation. Chamomilla and

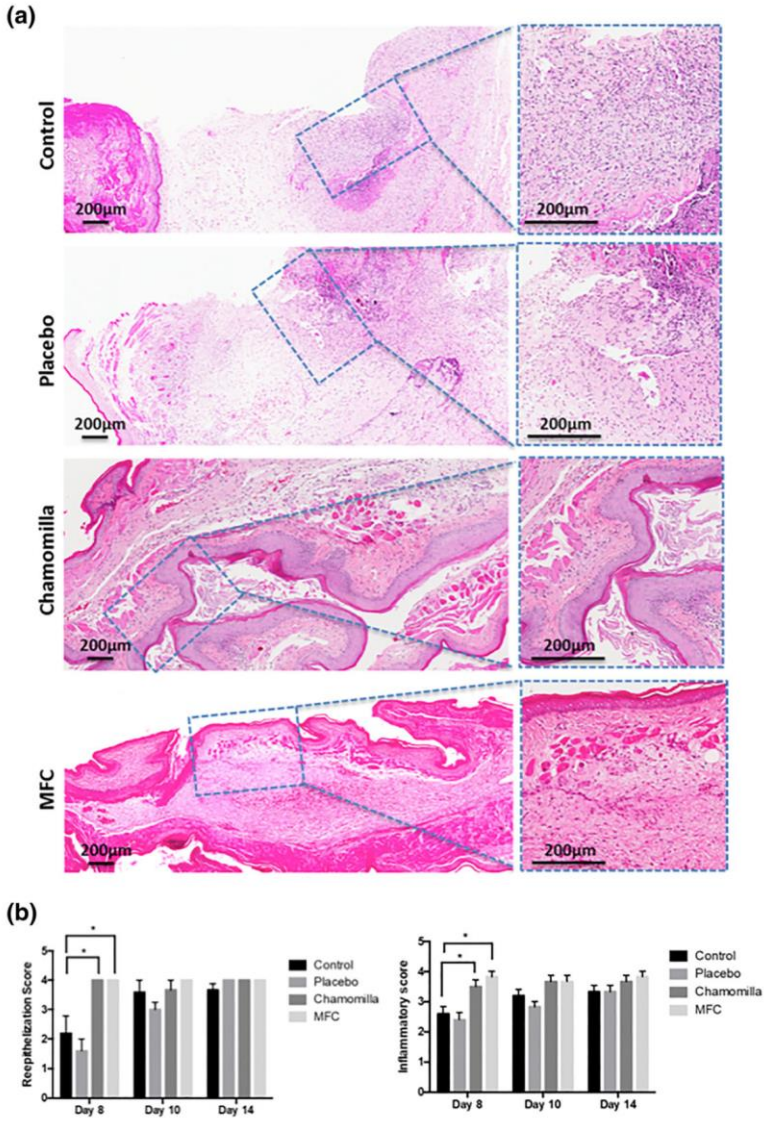


FIGURE 2 Microscopic appearance: (a) Histological aspects of all groups at Day 8. Observe that groups treated with Chamomilla and mucoadhesive formulation containing curcuminoids (MFC) exhibited accelerated re-epithelialization covering all of the wound thickness comparing with control and placebo groups. They also revealed a significant reduction and chronification of the inflammatory process compared to control and placebo groups; these latter groups presented areas of ulceration and predominance of diffuse acute inflammation. (haemotoxylin and eosin, ×40 and ×100). (b) Graphs illustrating the re-epithelization and inflammatory scores. On Day 8, chamomilla and MFC exhibited significant re-epithelization and resolution of the inflammatory process compared with the control group. [Colour figure can be viewed at wileyonlinelibrary.com]

MFC revealed a significant reduction and chronification of inflammatory process comparing with control ($p < 0.05$; $p < 0.001$) and placebo groups ($p < 0.01$; $p < 0.001$). At Day 10, chamomilla and MFC still demonstrated a lower inflammatory process compared with the placebo group ($p < 0.05$). At Day 14, all groups presented resolution and healing (reduction or disappearance of chronic inflammation).

The analysis of MVD demonstrated that at Day 8, animals treated with MFC (10.4 ± 3.9) presented significant less angiogenesis (MVD assessment) than the control (22.2. ±10.6; $p < 0.001$) and placebo (19.1 ± 3.6; $p < 0.05$). The mean of vessels in MFC was similar to chamomilla (11.9 ± 5.2; $p > 0.05$; Figure 3). At Days 10 and 14, the number of vessels in all groups was similar. In parallel, TGF-β1 analysis at Day 8 showed that the MFC group exhibited significantly lower labeling of this cytokine compared with placebo ($p = 0.019$) and control groups ($p = 0.019$). At Days 10 and 14, no difference was found among groups (Figure 4).

5 | DISCUSSION

OM is a common short-term side complication associated with antineoplastic treatment. Several therapeutic approaches have been employed to date to prevent and treat OM lesions (Lalla et al., 2014). However, efforts remain focused on the continuing search for new therapies for mucositis (Baharvand et al., 2017; Dos Santos Filho et al., 2015). Herbal products have attracted significant attention of researchers in this field (Koohi-Hosseinabadi et al., 2015; Koohi-Hosseinabadi et al., 2017; Mardani et al., 2016; Showraki et al., 2016). Therefore, we decided to evaluate the effect of MFC on OM induced by 5-FU in hamsters. Soleimani, Sahebkar, and Hosseinzadech (2018), through a literature review, concluded that the use of curcuma is safe, even at high doses; however, further studies are needed regarding the use of various formulations of curcumin, especially in humans. Our results showed that treatment with MFC accelerates clinical healing of OM lesions, leading to improvement on re-epithelialization and decrease of the inflammatory process. In parallel, curcuminoids were related to reduction in angiogenesis and TGF-β1 level on Day 8, which was interpreted as evidence of a premature advanced healing stage achieved by this group in comparison with the control and placebo groups.

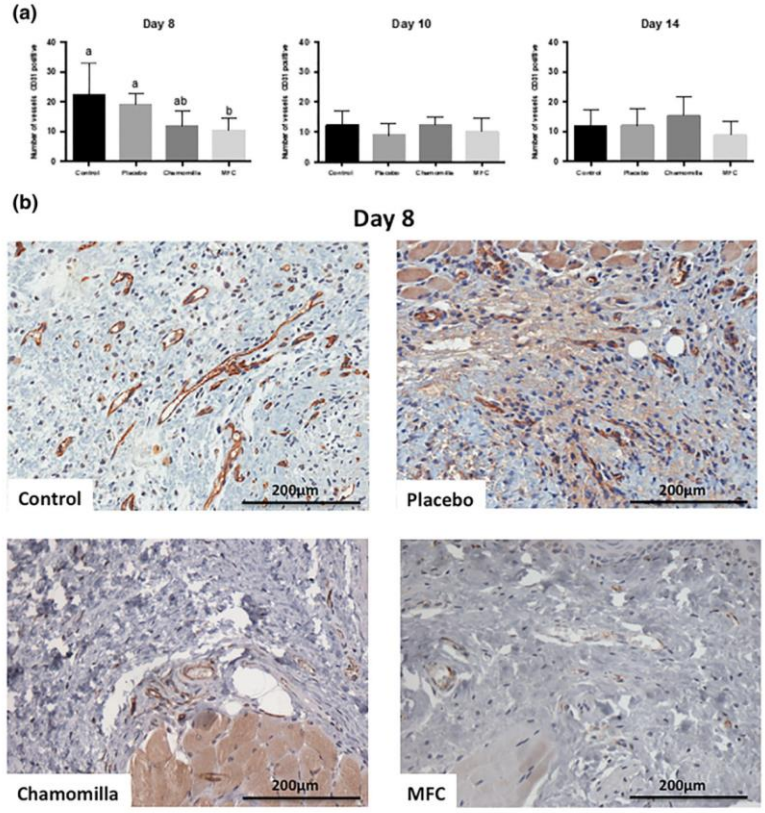


FIGURE 3 CD31 Analysis: (a) Quantification of blood vessels CD31 positive in different groups according to each experimental day. On Day 8, a reduction of vascularization was observed in chamomilla and mucoadhesive formulation containing curcuminoid (MFC) groups. (b) Illustration of immunohistochemical labeling of CD31 in all groups on Day 8. Note the reduction of angiogenesis in the chamomilla and MFC groups (original magnification: ×400) [Colour figure can be viewed at wileyonlinelibrary.com]

(a)

	Day 8	Day 10	Day 14
Control	8.0 ± 1.7^{A}	3.66 ± 2.5^{A}	1.33 ± 0.5^{A}
Placebo	8.0 ± 1.7^{A}	5.33 ± 1.1^{A}	3.33 ± 2.5^{A}
Chamomilla	4.66 ± 1.5^{BC}	5.33 ± 1.1^{A}	1.33 ± 0.5^{A}
MFC	1.0 ± 0.0^{C}	3.33 ± 1.1^{A}	1.66 ± 1.1^{A}

(b)

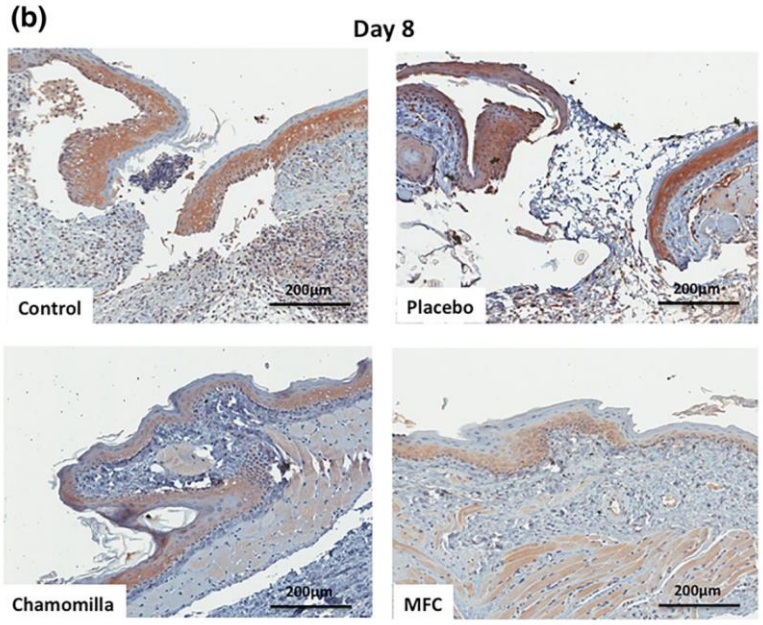


FIGURE 4 Transforming growth factor beta 1 (TGF-β1) analysis: (a) TGF-β1 analysis according to the experimental time and group. On Day 8, control and placebo groups presented higher scores compared with chamomilla and mucoadhesive formulation containing curcuminoid (MFC) groups. (b) Immunohistochemical labeling of TGF-β1 on Day 8 showed that the MFC group exhibited significantly lower labeling of this cytokine in epithelial tissue compared with placebo and control group (original magnification: ×200) [Colour figure can be viewed at wileyonlinelibrary.com]

C. longa. has been studied in wound healing due to its antioxidant properties and has shown many pharmacological activities such as anti-inflammatorial and antimicrobial (Akbik, Ghadiri, Chrzanowski, & Rohanizadeh, 2014; Dos Santos Filho et al., 2015; Lüer et al., 2011). All of these characteristics are very important for the treatment of oral ulcerative lesions such as mucositis. A limiting factor is its extremely low aqueous solubility, which hampers its use as a therapeutic agent (Karki et al., 2017; Naksuriya, Okonogi, Schiffelers, & Hennink, 2014). In an attempt to overcome this limitation and obtain better results with *C. longa*, our study group developed a mucoadhesive formulation that promoted a high level of curcumin solubilization with great potential to prolong the contact between the pharmaceutical dosage form and absorptive tissue (Karki et al., 2017; Worthington et al., 2011). Our study group has already demonstrated the effectiveness of this strategy with other agents in intestinal mucositis (Dos Santos Filho et al., 2015). Herein, we assessed for the first time the effect of this formulation containing curcuminoids from *C. longa*. as the active ingredients in OM. The results demonstrated that MFC is effective as an anti-inflammatory agent. The analysis of our clinical and histopathological findings revealed that, at Day 8, which represented the height of OM in this animal model, MFC promoted an acceleration of wound healing as compared with in the control and placebo groups. Other studies have demonstrated that curcumin in nonmucoadhesive formulation is effective in the treatment of mucositis induced by chemotherapy and radiotherapy, leading to a reduction in the incidence and severity of lesions in animals (Rezvani & Ross, 2009; van't Land et al., 2004). We believe that the mucoadhesive system is important for enhancing the beneficial effects of curcuminoids in the tissue, which may result in a greater acceleration of wound repair or more pronounced effects.

The clinical benefits of both chamomilla and MFC occurred in association with important histopathological modifications, which included the increase of epithelial proliferation/migration and a decrease of inflammation in the wound area. The analysis of the histopathological findings revealed that on Day 8, the chamomilla and MFC groups exhibited accelerated re-epithelialization along with a lower degree of inflammation compared with control and placebo groups. These results demonstrated that the positive therapeutic effects of MFC and chamomilla in OM are due to important cellular modulations at the initial phase of repair. Our study group already demonstrated the clinical and histopathological beneficial effects of chamomilla in OM; therefore, we used this extract as the positive control (Curra et al., 2013). This choice was also based on important similar characteristics of chamomilla and curcuminoids; notably, both are natural compounds and are applied topically twice a day. Moreover, AdMuc is approved by the Brazilian National Sanitary Surveillance Agency to treat mouth sores and is commercialized for this purpose, supporting the effectiveness of this compound. In the present study, MFC achieved similar or superior results than the positive control group, indicating a promising effect of this substance in OM. Our results corroborate with a previous study that demonstrated that curcuminoids induced intestinal epithelial mucosa proliferation in 5-fluoracil-induced duodenal mucositis (Dos Santos Filho et al., 2015). The increase in Ki-67 labeling in intestinal mucosa treated with MFC was associated with protective and healing effects. In accordance with this result, we presume that the faster re-epithelialization of oral mucosa in animals treated with MFC in the present study was associated with an accelerated turnover of epithelial cells.

Another important characteristic of curcumin is its anti-inflammatory properties. Here, we observed that MFC and chamomile groups presented lower inflammatory infiltrate and significant improvement of inflammation on Day 8 compared with control and placebo groups. Several studies have provided evidence that curcumin indeed reduces inflammation (Akbik et al., 2014; Mazieiro, Frizon, Barbalho, & Goulart, 2018; Naksuriya et al., 2014). Joe, Vijaykumar, and Lokesh (2004) thoroughly reviewed the numerous mechanisms by which curcumin modulates inflammation. Most notably, curcumin inhibited the production of TNF-α and interleukin-1, two main cytokines released from monocytes and macrophages that play important roles in the regulation of inflammatory responses. Aggarwal and Harikumar (2009) showed that curcumin can suppress inflammation through multiple pathways and may act in the prevention and treatment of various proinflammatory chronic diseases such as allergy, asthma, bronchitis, rheumatoid arthritis and other arthritic diseases, and psoriasis, among others. Mazieiro et al. (2018) reviewed the potential of curcumin in patients with inflammatory bowel diseases, concluding that this agent mediates the anti-inflammatory effects via the downregulation of inflammatory transcription factors, protein kinases, cytokines, and enzymes that promote inflammation. Besides its anti-inflammatory activity, it has the ability to scavenge free radicals, which are the major cause of inflammation during wound healing activity (Mohanty, Das, & Sahoo, 2012). It has been described that the activation of NF-kB as well as the increased production of tumor TNF-α, interleukin-1 and other cytokines are involved in OM pathogenesis. Therefore, treatments such as MFC can modulate the inflammatory process based on their capacity of inhibition of NF-kB and other important molecules.

As a complementary analysis, we performed immunohistochemical labeling of CD31 that is an important marker of vascular components. Our results revealed that in parallel to reduction of inflammatory process, lower vascularization was observed in OM treated with MFC and chamomilla. Campos, Borges-Branco, and Groth (2007), through a literature review on wound healing, showed that the wound-healing process is divided into three main phases: inflammatory, proliferation, and remodeling/maturation. The inflammatory response begins with vasodilation, increased vascular permeability, chemotaxis of neutrophils, macrophages, and lymphocytes. During the proliferative phase, migration of endothelial cells to form new capillaries and fibroplasia are essential for correct healing. The final stage, termed maturation, is associated with reduction of vascular components and contraction of the wound (Pakyari, Farrokhi, Maharlooei, & Ghahary, 2013, review). Considering collectively the clinical and histopathological data, along with the MVD results, we can infer that OM treated with MFC and chamomilla exhibited faster wound healing than did both the placebo and control groups, accelerating two of the most substantial healing phases: inflammation and proliferation. Both protocols decreased acute inflammation and induced the re-epithelization and maturation of connective tissue. Similarly, Mohanty et al. (2012) tested curcumin-loaded oleic acid based polymeric bandage in rat skin wound healing and observed that comparatively the curcumin group presented faster healing response than control and void preparations. The curcumin group exhibited better organization of granulation tissue, which was responsible for a faster progression from the inflammatory to the proliferative phase. In skin, curcumin acts by reducing the inflammatory response and the damaged skin can more readily enter the later stages of healing such as proliferation and remodeling (Akbik et al., 2014). However, it is important to take precautions when comparing our results with observations of wound healing associated with curcumin in the literature because most studies were performed using different tissue models, such as skin, which has a different repair process than the oral mucosa healing.

Another point addressed by our study was the immunolabeling of TGF-β1 in the epithelial lining. It is well-known that the TGF-β1 family has key regulatory functions in a diverse spectrum of biological processes. In wound healing, it acts in inflammation, epithelial migration, stimulation of angiogenesis, fibroblast proliferation, collagen synthesis, and the deposition and remodeling of the new extracellular matrix (Kenneth, Praveen, & Anie, 2013; Pakyari et al., 2013). It is important to highlight, however, that this growth factor has a dual role in the inflammation process of wound healing. It exerts proinflammatory effects during the early stages and later contributes to the resolution of inflammation (Kenneth et al., 2013). Here, we focused on TGF-β1 immunolabeling in epithelial tissue because it has been described as a factor capable of regulating keratinocyte growth, migration, and differentiation (Gailit, Welch, & Clark, 1994; Suzuki, Pinto, & Senoo, 2017). In vivo, TGF-β1 knockout mice exhibit hyperproliferation of the epidermis, whereas overexpression of TGF-β1 in transgenic mice result in an inhibition of skin development (Gailit et al., 1994; Kenneth et al., 2013). Considering

that we analyzed OM on Days 8, 10, and 14 and that MFC group was already fully regenerated, it was expected that TGF-β1 would present lower expression. Previous studies have demonstrated that TGF-β1 expression appears to decrease as keratinocytes become differentiated (Pakyari et al., 2013). This process seems to be taking place in the context of the MFC group, in which we observed a more organized and differentiated epithelial tissue along with a lower expression of TGF-β1. Overexpression of TGF-β1 in keratinocytes has been previously associated with chronic inflammation in the wound, which leads to delayed healing (Pakyari et al., 2013), corroborating with the results observed in control and placebo groups. Our results demonstrate that OM treated with MFC exhibited lower labeling of this cytokine in epithelial tissue compared with all the other groups.

6 | CONCLUSION

Overall, we provided evidence that MFC is a phytotherapeutic agent that may contribute for OM treatment. The accelerated wound healing after topical MFC administration was associated with faster re-epithelization and modulation of the inflammatory process, angiogenesis, and TGF-β1. Expanding upon the evidence depicted herein, further studies are required to identify other factors related to the positive effects of curcumin in the healing process and OM pathogenesis in order to provide support for its clinical use.

ACKNOWLEDGEMENTS

The authors are grateful to Marta Justina Giotti Cioato and Flavia Rejane Giusti for technical support. This work was supported in part by the Postgraduate Research Group, Hospital de Clínicas de Porto Alegre (GPPG/FIPE:14-0613), Brazilian National Council for Scientific and Technological Development (CNPq student scholarship) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES)-finance code 001. Manoela Domingues Martins, Marize Campos Valadares and Aline Carvalho Batista are research fellows funded by the Brazilian National Council for Scientific and Technological Development (CNPq).

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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How to cite this article: Schmidt TR, Curra M, Wagner VP, et al. Mucoadhesive formulation containing *Curcuma longa* L. reduces oral mucositis induced by 5-fluorouracil in hamsters. *Phytotherapy Research*. 2019;33:881–890. https://doi.org/10.1002/ptr.6279

3 CONSIDERAÇÕES FINAIS

O presente estudo fornece evidências de que a formulação mucoadesiva de *Curcuma longa* L. é um agente fitoterápico que pode contribuir para o tratamento da mucosite oral. Atuando no reparo tecidual, através do aumento da reepitelização, modulação do processo inflamatório, diminuição da angiogênese e níveis de TGF-β1. Recentemente, um estudo clínico de fase I, avaliou a dose terapêutica e segura de um bochecho contendo extrato de curcuminóides e *Bidens Pilosa* L. (FITOPROT) de forma preventiva e terapêutica para mucosite oral, revelando sua segurança e eficácia para demais estudos clínicos (DOS SANTOS FILHO *et al*., 2018). Contudo, mais estudos são necessários para identificar outros fatores relacionados aos efeitos da curcumina no processo de cicatrização e patogênese da mucosite oral, a fim de sustentar o seu uso clínico.

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ANEXO - CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA

ANEXO 1

UFG

Ministério da Educação
Universidade Federal de Goiás
Faculdade de Farmácia

Goiânia, 28 de outubro de 2014.

Ao Comitê de Ética em Pesquisa CEP
Hospital de Clínicas de Porto Alegre - HCPA / UFRGS

Prezados Membros da Comissão Avaliadora,

Escrevo para esclarecer sobre alterações recentes no projeto intitulado "**ESTUDO DO EFEITO DA FORMULAÇÃO MUCOADESIVA DO EXTRATO DE *BIDENS PILOSA* L. E *CURCUMA LONGA* L. NA MUCOSITE QUIMIOINDUZIDA EM HAMSTER**". Sou responsável por fornecer a preparação farmacêutica utilizada no presente estudo, entretanto, devido a problemas relacionados à estabilidade da formulação contendo a referida associação, o estudo será conduzido com a formulação mucoadesiva contendo apenas o extrato de *Curcuma longa* L.

Coloco-me à disposição para maiores esclarecimentos.
Saudações universitárias,

Prof. Dr. Ricardo Neves Marreto
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