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MODULAÇÃO DO PROCESSO CICATRICIAL DE FERIDAS CUTÂNEAS EM CAMUNDONGOS DIABÉTICOS POR EXTRATOS DE PRÓPOLIS VERMELHA ASSOCIADOS À MEMBRANA DE CELULOSE BACTERIANA

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Caxias do Sul

2018

Dados Internacionais de Catalogação na Publicação (CIP)
Universidade de Caxias do Sul
Sistema de Bibliotecas UCS - Processamento Técnico

P598m Picolotto, Aline

Modulação do processo cicatricial de feridas cutâneas em camundongos diabéticos por extratos de própolis vermelha associados à membrana de celulose bacteriana / Aline Picolotto. – 2018.

viii, 44 f. : il. ; 30 cm

Dissertação (Mestrado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Ciências da Saúde, 2018.

Orientação: Sidnei Moura e Silva.

Coorientação: Leandro Tasso, Jozi Godoy Figueiredo.

1. Diabetes. 2. Cicatrização de ferimentos. 3. Própole. 4. Materiais biomédicos. 5. Curativos. I. Silva, Sidnei Moura e, orient. II. Tasso, Leandro, coorient. III. Figueiredo, Jozi Godoy, coorient. IV. Título.

CDU 2. ed.: 616.379-008.64

Catalogação na fonte elaborada pela(o) bibliotecária(o)
Paula Fernanda Fedatto Leal - CRB 10/2291

UNIVERSIDADE DE CAXIAS DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

COORDENADOR DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

PROF. DR. ASDRUBAL FALAVIGNA

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Dissertação de Mestrado submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul, como parte dos requisitos necessários para a obtenção do título de Mestre em Ciências da Saúde, Linha de Pesquisa: Farmacologia e Biomarcadores.

Caxias do Sul, 06 de julho de 2018.

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Dedico...

Aos meus pais

Pelo amor incondicional e por toda renúncia e dedicação para fazer de minha educação uma prioridade em suas vidas, tornando possível a realização de mais esta etapa.

Agradecimentos

Ao meu orientador, Prof. Dr. Sidnei Moura e Silva por ter aceitado e confiado em minha proposta de trabalho, pelo apoio e compreensão nos momentos mais difíceis e por compartilhar seus conhecimentos.

Ao Prof. Dr. Leandro Tasso e Profª. Drª. Mariana Roesch Ely pelas orientações e colaboração fundamental para a conclusão deste trabalho.

À Dra. Jozi Godoy Figueiredo, pelas incansáveis orientações, dedicação, comprometimento e amizade.

Ao Dr. Hernane da Silva Barud e Dr. Guilherme Pacheco Pereira pela parceria e empenho fundamental para o andamento desta pesquisa.

Aos professores do Mestrado em Ciências da Saúde, pelos ensinamentos passados e pelo exemplo de profissionais.

Aos colegas, pela excelente convivência e pelas experiências compartilhadas.

Aos Laboratórios de Biotecnologia de Produtos Naturais e Sintéticos e de Fisiologia da Universidade de Caxias do Sul pela disponibilidade e auxílio da parte de seus técnicos e responsáveis.

Aos meus colegas de trabalho, que com muita dedicação “seguraram as pontas” na minha ausência e me incentivaram nesta caminhada.

Aos meus pais, pela dedicação que sempre tiveram por mim, pelo apoio financeiro, por entenderem minha ausência durante esta jornada e me incentivarem a não desistir nos momentos mais difíceis e desanimadores, enfim, por todo amor e cuidado.

Ao meu namorado Guilherme, pelo apoio incondicional, por estar presente nos momentos mais difíceis me incentivando a continuar, por ficar sentado comigo aos finais de semana estudando, e principalmente por ser meu porto seguro e meu amor.

A todos que contribuíram direta ou indiretamente para a concretização deste trabalho.

Muito Obrigada!

Sumário

Esta dissertação de Mestrado Acadêmico Stricto Sensu é apresentada no formato exigido pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul. A mesma é constituída da secção de “Introdução com referências bibliográficas”, a inclusão do artigo original submetido/publicado em periódico Qualis A na classificação da Coordenação de Aperfeiçoamento de Pessoal em Nível Superior (CAPES), e as “Considerações Finais e Perspectivas”.

1 INTRODUÇÃO

O diabetes mellitus é considerado uma das maiores epidemias do século XXI, tendo acometido mais de 400 milhões de pessoas no mundo, representando uma prevalência superior a 8% da população adulta. Estimativas indicam que em 2040 este número será superior a 600 milhões (1). Dados revelam que a doença causou aproximadamente 1,5 milhões de mortes em 2012, sendo a 8ª causa de morte em todo o mundo (2).

Os altos níveis de glicose no sangue em indivíduos diabéticos favorece o aparecimento de complicações, incluindo doenças cardiovasculares e neuropatia periférica com comprometimento dos membros inferiores podendo levar ao surgimento de feridas crônicas (1, 3). Assim, estas lesões afetam pelo menos 15% dos indivíduos diabéticos, as quais precedem 85% das amputações não traumáticas de membros inferiores relacionadas à doença (4).

Neste contexto, a partir do momento que surge uma lesão inicia-se o processo de cicatrização, no qual o organismo desencadeia mecanismos fisiológicos com o objetivo de reparação tecidual (5). Desta forma, o processo de cicatrização é uma sequência de eventos biológicos que envolvem processos moleculares e celulares, como recrutamento de leucócitos, inflamação, angiogênese, deposição de colágeno e reepitelização (6). A cicatrização se divide em três fases: (i) inflamatória, (ii) proliferativa e (iii) maturação. No entanto, estas se sobrepõem, possibilitando encontrar em uma mesma ferida diferentes estágios de cicatrização (6-8).

A primeira fase de cicatrização se inicia com a ruptura dos vasos sanguíneos e o extravasamento de sangue, seguida pela agregação plaquetária e cascata de coagulação. Estes eventos resultam na formação de moléculas insolúveis de fibrina e hemostasia. Posteriormente, os macrófagos e neutrófilos são recrutados (9, 10). Os neutrófilos iniciam o desbridamento do tecido lesado e fagocitose de agentes infecciosos através da liberação de componentes citotóxicos, especialmente a enzima mieloperoxidase (MPO) presente nos grânulos primários (azurófilos) e considerada um importante marcador da presença de neutrófilos em tecidos inflamados (11). Esse processo inflamatório pode durar semanas, meses ou até anos em se tratando de feridas crônicas (12).

A partir do momento que a atividade fagocitária dos macrófagos começa a reduzir, assim como os sinais clínicos da inflamação, inicia-se a segunda fase da cicatrização (12). Durante esta fase, conhecida como proliferativa, ocorre a formação do tecido de granulação com aspecto róseo e granular, caracterizado pela intensa proliferação de novos vasos sanguíneos e de fibroblastos. Esta se estende até a epitelização total da ferida, e em alguns casos, também ocorre a contração das lesões. A última fase do processo de cicatrização, denominada de maturação, consiste na reorganização das fibras de colágeno, iniciando-se com a formação do tecido cicatricial. Desta forma, esta fase se caracteriza pelas mudanças na forma, tamanho e resistência da cicatriz (9, 12).

Nas lesões crônicas, decorrentes do diabetes, ocorre um fenômeno de estagnação nos mecanismos da cicatrização que as tornam incapazes de completá-lo de maneira ordenada e linear. Isso acontece devido à doença vascular periférica que leva o diabético a uma situação de isquemia nos membros inferiores. Assim, quando uma infecção se instala, a liberação de oxigênio fica prejudicada traduzindo-se em uma cicatrização demasiadamente longa, com redução da angiogênese e formação de tecido viável, o que pode evoluir para uma amputação caso a lesão não seja tratada adequadamente (12-14).

O crescente aumento no número de pacientes portadores de lesões provenientes do diabetes conduz a uma busca constante por novos curativos e biomateriais, que possam ser eficazes e resolutivos no tratamento de feridas crônicas (15). Têm sido reportadas inúmeras opções de produtos que podem ser utilizados para o tratamento de feridas, além de uma diversidade de biomateriais, alguns inclusive já disponíveis comercialmente.

As membranas de celulose bacteriana sintetizadas a partir de bactérias do gênero *Gluconacetobacter* têm recebido destaque a partir da sua comprovada eficácia no tratamento de lesões, sendo empregadas como substitutas temporárias da pele humana em diferentes tipos de feridas (16, 17). Estas membranas têm apresentado adequada aderência às lesões e são resistentes a tensões semelhantes à pele humana. Quando aplicadas, mantém o meio adequadamente úmido por apresentarem grande quantidade de água em sua composição, aliviando a dor e promovendo a rápida regeneração tecidual. Entretanto, estas não

apresentam atividade anti-inflamatória e antimicrobiana para que possam ser utilizadas na fase inflamatória da cicatrização e em feridas infectadas (17).

Produtos e extratos naturais, como a própolis tem chamado a atenção pelas suas propriedades antibacterianas, antioxidantes e anti-inflamatórias (18). Estas características têm relação direta com sua composição química, a qual é baseada em compostos fenólicos, principalmente os flavonoides e ácidos fenólicos (19). A própolis é uma substância resinosa produzida pelas abelhas para isolar as colmeias. Sua composição química varia de acordo com as características fitogeográficas do ambiente da colmeia, bem como pela sazonalidade climática (20).

Dos diversos tipos de própolis existentes no Brasil, a própolis vermelha, classificada como o 13º subtipo, é comumente encontrada na região nordeste e merece destaque por apresentar uma composição química peculiar. Esta é abundante em compostos fenólicos, principalmente isoflavonoides (21, 22). Estudos *in vitro* demonstram que este composto possui atividade antimicrobiana e antioxidante, além de apresentar propriedades antitumorais e cicatrizantes (22-25). Devido a essas características, extratos, frações e produtos isolados de própolis têm sido estudados e utilizados para controle microbiológico (25, 26).

Tendo em vista as propriedades das membranas de celulose bacteriana, tem se buscado incrementar a capacidade de cicatrização das mesmas com a adição de derivados naturais, como o relatado recentemente na produção de biocurativos associados a própolis verde (17).

Considerando as propriedades já comprovadas das membranas de celulose bacteriana e as características peculiares da própolis vermelha, entende-se que avaliar a efetividade da associação dos mesmos se torna fundamental na busca por biomateriais que auxiliem na cicatrização de feridas crônicas.

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3 ARTIGO

Bacterial cellulose membrane associated with red propolis as phytomodulator: improved healing effects in experimental models of diabetes mellitus

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ABSTRACT

Ethnopharmacological Relevance: Red propolis has been highlighted due to its popular use as an antimicrobial, and it has anti-inflammatory and healing properties, which is associated with its chemical composition. Here, we combine a bacterial membrane with red propolis to treat diabetic wounds.

Aim: This work aims to evaluate a biocurative from bacterial cellulose joint with red propolis in mice in a diabetic wound healing model.

Materials and Methods: Biocuratives from bacterial cellulose membrane and different extracts of red propolis were produced. The qualification and quantification of the presence of propolis chemical compounds in the membrane were made using high resolution mass spectrometry (HRMS). The activity tests of the biocuratives were performed on Swiss, male, diabetic (induced by estroptozotocin) mice. The animals were submitted to a surgical procedure and a single lesion was produced in the dorsal region, which was treated with the biocuratives. Macroscopic assessments were performed at 2, 7 and 14 postoperative days, and biopsies were collected on days 0, 7 and 14 for histological analysis, myeloperoxidase enzyme activity (MPO) and cytokines (TNF-α, IL-1β, and TGF-β).

Results: In the membranes, ten compounds were identified while five were quantified. The ethyl acetate extract showed more red propolis markers, and the most prevalent was Formononetin with 4423.00–2907.00 $\mu g.g^{-1}$. In *in vivo* experiments, macroscopic analyses demonstrated that the two groups treated with red propolis (GMEBT and GMEAE) showed significantly greater healing compared to the control groups (GS and GMS). In histology, an increase in leukocyte recruitment was observed, confirmed by the activity of the enzyme myeloperoxidase (MPO) in the GMEBT and GMEAE groups. The levels of TNF-α were significantly higher in wounds stimulated with red propolis as well as in TGF-β (GMEBT and GMEAE) on day 7. This was different from the IL-1β levels that were higher in the control groups (GS and GMS).

Conclusions: In summary, the biocuratives produced in this work were able to accelerate the wound healing process in a diabetic mouse model. In this way, the traditional knowledge of red propolis activity helped to create a biotechnological product, which can be used for diabetic wound healing.

Keywords: Red propolis, Diabetes mellitus, Wounds healing, Flavonoids

1 Introduction

Since the beginning of human history, natural products with specific features have been used as essential components in traditional medicine (Fang et al., 2005). Today, it is estimated that around 80% of the world population depends on plant-derived products for primary health care (Kettner et al., 2005; WHO, 2013). The traditional knowledge of natural products has been highlighted in modern pharmaceuticals (Rates, 2001; Newman and Cragg, 2016;).

A complex mixture resulting from chemical compounds collected by bees from plants in the region associated with salivary secretions, wax, and pollen is known as propolis. This resinous product used for hive protection has been classified in accordance with the region where it is collected, its chemical composition, and colour (Ghisalberti, 1979; Brasil, 2001). The red propolis found in the Northeast region of Brazil has been widely used in traditional medicine due to its antimicrobial, anti-inflammatory, healing, antioxidant, and antitumor activities (Ghisalberti, 1979; Marcucci et al., 2001; Pereira et al., 2002; Alencar et al., 2007; Frozza et al., 2013; Freires et al., 2016). Regarding the chemical composition, the biologically active compounds are mainly flavonoids and may also contain cinnamic acid derivatives, esters, and some terpenes, which are derived from the plant *Dalbergia ecastophyllum (L) Taub* (Marcucci et al., 2001; Salatino et al., 2005; Daugsch et al., 2008).

Diabetes mellitus is a progressive metabolic disorder mainly characterized by persistent hyperglycemia (IDF, 2015). This disease is among the largest epidemics of the 21st century, having affected more than 400 million people worldwide. The estimates indicate that in 2040 this number will exceed 600 million (IDF, 2015; WHO, 2016). Among the main complications of diabetes is peripheral neuropathy with impairment of the lower limbs, leading to the appearance of chronic wounds (Reiber and Ledoux, 2003; IDF, 2015).

Wound healing involves a sequence of biological processes, which can be divided into the following three phases: (i) inflammatory, (ii) proliferative, and (iii) maturation (Fonder et al., 2008). In chronic lesions caused by diabetes, there is a phenomenon of cicatrisation mechanism stagnation (Hansen et al., 2003; Neidrauer et al., 2010; Soldevilla Agreda and Torra i Bou, 2012). The treatment of diabetic

wounds represents an important socioeconomic impact, as well as the quality of life of the patients. In this sense, new drugs, technologies, and biomaterials are being used for the treatment of these lesions (Serafini et al., 2014). Natural products have been highlighted for their potential therapeutic uses and have been used in new types of dressings. Ethnopharmacological knowledge has helped in the search for new active natural products to cure diabetic wound healing. Kumar et al. (2007) has identified a large number of Indian plants that can be used for this purpose.

Several biomaterials have been tested for the treatment of wounds and bacterial cellulose membranes have been highlighted. These have been used with or without additional chemical compounds or natural extracts, such as green própolis (Barud et al., 2013). Thus, from traditional knowledge of propolis use with biomaterials, a biotechnological product for the treatment of wounds has been generated (de Almeida et al., 2013). This study aims to evaluate the effect of bacterial cellulose membrane associated with red propolis on the healing of cutaneous wounds in diabetic mice. For this purpose, membranes were prepared with the addition of different extracts of the natural product, which were tested for chemical composition and activity on wounds in a mouse model.

2 Materials and methods

2.1 Chemicals

Methanol, ethanol (HPLC grade), streptozotocin, daidzein, liquiritigenin, isoliquiritigenin, formononetin and biochanin standards were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Acetonitrile (ACN, HPLC grade), was purchased from Tedia (Fairfield, OH, USA). Tramadol hydrochloride was purchased from Laboratory Teuto Brasileiro S.A (Goiás, Brazil). Xylazine hydrochloride and ketamine hydrochloride were purchased from Sespo Industry and Commerce Private Limited Company (São Paulo, Brazil). Hexane, ethyl acetate, acetonitrile, butanol, acetic acid, ethanol, hematoxylin, eosin, formaldehyde, paraffin, sodium dibasic phosphate heptahydrate, citric acid monohydrate, magnesium sulphate heptahydrate, and sodium hydroxide were supplied by Merck (São Paulo, Brazil). The 0.5% alcoholic chlorhexidine antiseptic solution was purchased from Pharmaceutical Industry

Rioquímica Ltda (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q system (Millipore®). All chemicals were used without further purification.

2.2 Propolis extraction and fractionation

The red propolis samples were collected in the Alagoas state (9º 34 '12 "S, 36º 33' 0" O) in the Northeastern region of Brazil. The samples were protected from light and refrigerated at -20 °C until extract preparation. The extract was prepared in accordance with Frozza et al. (2013). Briefly, the sample was ground using porcelain grade and pistil, with the addition ofliquid nitrogen. After the complete evaporation of nitrogen, the solid was extracted with 70% ethanol (1 g in 10 mL). This hydroalcoholic extract (HE) was filtered and concentrated in a rotary evaporator at 37 °C. Then, the HE extract was fractionated by liquid-liquid extraction. Fractionation followed the method indicated by a previous study (Rufatto et al., 2018). Briefly, ultrapure water (10g in 200 mL) was added to the dry HE, which was extracted with hexane (3 × 200 mL). The organic phases were jointed – extract He, and evaporated in a rotary evaporator at 37 °C. In the same way, the hydroalcoholic phase was extracted with ethyl acetate (Ea) and butanol (Bu) (Scheme 1). All extracts were stored in the dark and under refrigeration.

Scheme 1:

2.3 Membrane preparation

The bacterial cellulose membranes were cultivated and supplied by the Laboratory of Biopolymers and Biomaterials, University of Araraquara (SP, Brazil). These were obtained from *Komagataeibacter rhaeticus* as previously reported (Dos Santos et al., 2014; Machado et al., 2016). Posteriorly, around 80% of their net weight was removed by a manual compression process. Then, 10 mL of each extract of red propolis (1% m/v, diluted in 40% ethanol) were added individually to the membranes until the contents were completely absorbed. For the control membrane, only the 40% ethanol solution was used. All the membranes were pre-sterilized in glass containers using an autoclave system. All membrane preparation processes were in a laminar flow hood using sterilized gloves. The final concentration of red propolis in the membranes was 1.1 $mg.cm^{-2}$.

2.4 Chemical analysis

For compound extraction from embedded membranes with different propolis extracts, about 0.07 g of the extract was individually diluted in 10 mL of ethanol. The suspension was vortexed for 10 min, posteriorly filtered through a 0.45 µm pore synthetic fibre polyamide, and the solvent was evaporated in a rotary evaporator at 37 °C.

For quantification, the residual mass was determined and dissolved in 0.5 mL of ACN (HPLC grade) and ultrapure H_2O (1:1) with 0.1% formic acid. The compounds were quantified on days 0, 2, 7 and 14 after membrane preparation. The solutions were separated by a UFLC Shimadzu model 20AD (Kyoto, JP), which was equipped with a binary pump system (LC-20AD), automatic injector (SIL-20A) and controller (CBM-20A). An isocratic method was performed using an NST C18 column (250 × 4.6mm × 5µm) with ultrapure water and 0.1% acetic acid in pump A and acetonitrile in pump B at a total flow of 0.5 $mL.min^{-1}$ (1:1) during a 30 min run (total time). A calibration curve of flavonoids (daidzein, liquiritigenin, isoliquiritigenin, formononetin, and biochanin) ranging from 18.75–600.00 $\mu g.L^{-1}$ was determined. The quantification was performed using the external standardization method, correlating the compound peak area with the linear response (Küçükboyaci et al., 2013).

For qualitative chemical composition, the ethanol solution in the first step was diluted in ACN:H_2O (1:1) and 0.1% formic acid and infused directly into the ESI source by a syringe pump (Harvard Apparatus) at a flow rate of 150 $\mu L.min^{-1}$.

In both cases, high resolution mass spectrometry (HRMS) was used with ESI(+)-MS and tandem ESI(+)-MS-MS for compound identification using a hybrid high-resolution and high accuracy (5 $\mu L.L^{-1}$) microTof (Q-TOF) mass spectrometer (Bruker® Scientific, Billirica, USA). The equipment conditions were as follows: capillary and cone voltages were set to + 3500 V and + 40 V, respectively, with a de-solvation temperature of 100 °C. For ESI(+)-MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. Diagnostic ions in different fractions were identified by the comparison of their ESI(+)-MS/MS dissociation patterns with compounds identified in previous studies or standards. For data acquisition and processing, QTOF-control data analysis software (Bruker® Scientific) was used. The data was collected in an m/z range of 70–1100 at a speed of two scans per second, providing a resolution of 50,000 (FWHM) at *m/z* 200. No

199 important ions were observed below *m/z* 100 or above *m/z* 800; therefore, ESI(+)-MS
200 data is shown in the *m/z* 100−800 range.
201
202 *2.5 Animals*
203 Male, Swiss lineage mice *(Mus musculus)* weighing approximately 30g were
204 used. The animals were housed in polypropylene boxes suitable for rodents, filled
205 with pine needles in a ventilated environment with air exhaustion, controlled
206 temperature (22 ± 2 ºC) and humidity (55 ± 15%), and a light-dark cycle of 12 h. They
207 had access to water (filtered in drinking fountains) and were deprived of food
208 (industrial to mouse brand Nuvilab CR-1) 4 h before induction of diabetes and the
209 surgical procedure. During the other periods, they received water and food *ad libitum*.
210 All procedures were in accordance with the Ethical Principles of Animal
211 Experimentation adopted by the Brazilian College of Animal Experimentation and
212 norms of the Ethics Committee on Animal Use of the University of Caxias do Sul. The
213 approval protocol of the project is 021/2016.
214
215 *2.5.1 Experimental model of diabetes mellitus*
216 The protocol for the induction of diabetes agreed with previous studies
217 (Hozzein et al., 2015; Wang et al., 2016), applying minor modifications. Initially, the
218 animals were weighed and their glycemic indexes recorded with the aid of a Bayer®
219 brand glucose meter (Contour TS). Food fasting was then started for a period of 4 h
220 with *ad libitum* water supply. The induction of diabetes was performed through the
221 single injection of streptozotocin (STZ) at a dose of 100 $mg.kg^{-1}$(Wang et al., 2017).
222 The STZ was dissolved in citrate buffer (pH 4.5) and injected intraperitoneally. After a
223 period of 7 days, the mice were fasted for 4 h and blood glucose was again recorded.
224 Animals that presented fasting glycemia (>220 $mg.dL^{-1}$) were considered diabetic
225 and included randomly in the study groups.
226 *2.5.2 Experimental surgical procedure - skin lesion*
227 This model involves the complete removal of the epidermis, dermis, and
228 subcutaneous tissue to a significant extent (Davidson, 1998). After confirmation of
229 diabetes induction, the animals were anesthetized with 10% ketamine (83 $mg.kg^{-1}$)
230 and 2% xylazine (17 $mg.kg^{-1}$) intraperitoneally following the anesthetic protocol
231 adapted from the Laboratory of Animal Experimentation of the University of Caxias

do Sul (Viana, 2014; Lapchik et al., 2017). After the anesthetic procedure, the animals were positioned in the ventral decubitus position and submitted to trichotomy with the aid of a razor blade in the dorsal region, followed by antisepsis with 0.5% alcoholic chlorhexidine solution.

Sterilized operative fields were placed on the animal and then a single lesion was produced (dorsal region, midline) with the aid of a 10 mm diameter surgical *Punch* with cutting blade at the edge. The skin and the subcutaneous tissue were removed with the aid of a thin-pointed straight iris scissors, exposing the dorsal muscular fascia. The area hemostasis was performed by digital compression with sterile gauze for approximately 1 min when necessary.

After the lesion induction, the animals were randomly divided into the following four groups (n=18/group): GS, saline control group (treated with 0.9% physiological solution directly applied to the lesion); GMS, saline membrane control group (treated with bacterial cellulose membrane associated with 40% ethanol); GMEBT, membrane group butanol extract 1% (treated with bacterial cellulose membrane associated with red propolis, 1% butanol extract); GMEAE, group membrane extract ethyl acetate 1% (treated with bacterial cellulose membrane associated with red propolis, 1% ethyl acetate extract). The membranes were cut with a *Punch* (12 mm in diameter) and placed on the lesions with the aid of anatomical forceps. Dressings were not occluded with gauze or other secondary coverage. All components of the surgical apparatus used were previously sterilized.

The animals were kept in a warm place under supervision until complete anesthetic recovery and were housed in individual boxes to avoid contact with the operative wounds.

In order to reduce pain in the postoperative period, the drug tramadol was used as the analgesic in the dose of 6 $mg.kg^{-1}$ for 12 h over 3 days by a subcutaneous route (Viana, 2014; Lapchik et al., 2017).

2.6 Macroscopic Analysis

The lesions were analysed macroscopically on postoperative days 2, 7 and 14. These were photographed with a Nikon model D5300 digital camera mounted on a tripod at 34 cm from the surgical wound. Afterwards, they were measured with the aid of a 150 mm digital pachymeter, Leetools. The area of the lesion was calculated using the following equation: $A=\pi Rr$, where "A" represents the area, "R" is the largest

radius, and "r" is the minor radius of the wound (Prata et al., 1988). The percentage of wound contractionwas calculated using the following equation:

$$\text{Percentage of Wound Contraction } (\%) = 100 \times \frac{(A_0 - A_t)}{A_0}$$

where, A_0 represents the initial area and A_t is the final wound area in the time interval (Balakrishnan et al., 2006).

Different aspects of the cicatricial process were evaluated, including edema, hyperemia, exudation, crusts, and epithelial tissue. The following classification system was used: (0) absent, (1) mild, (2) moderate, and (3) intense for the parameters related to the inflammatory phase (edema, hyperemia, and exudation). On the other hand, the variable crusts and epithelization were classified as present or absent (Melo et al., 2011; de Figueiredo et al., 2014).

2.7 Histological Analysis

The collection occurred on days 0, 7 and 14 after anesthesia (according to the anesthetic procedure described above), including the entire extension of the lesion with a margin of approximately 0.5 cm of whole skin besides the scar. Immediately after removal, all the samples obtained were fixed in 10% formaldehyde for the purpose of preserving the morphological structures for further processing and analysis (Monday and Uzoma, 2013).

After resection of the lesions, the animals were submitted to euthanasia by cervical dislocation.

2.7.1 Preparation of histological slides

After removal of the formaldehyde, the pieces were dehydrated in ethanol, diaphanized in xylol, and included in paraffin blocks. Using a microtome, sections 5 µm thick were arranged on microscopic slides and stained with Hematoxylin-Eosin (HE) for histological study by light microscopy (Monday and Uzoma, 2013).

2.8 Analysis of myeloperoxidase enzyme activity (MPO)

Tissue samples from skin lesions from days 0, 7 and 14 post surgery were used to determine the activity of the myeloperoxidase enzyme (MPO) used as a marker for the presence of neutrophils in inflammed tissues. The procedures used for

MPO analysis were performed as previously described in the literature (Souza et al., 2001; Ramos et al., 2016).

2.9 Cytokine measurements

For the determination of cytokines, biopsies were homogenized individually in phosphate buffered saline (PBS) pH 7.4 and processed from days 0, 7 and 14 post surgery (Safieh-Garabedian et al., 2002). The levels of TNF-α, IL-1β and TGF-β in biopsy lysates were determined using a sandwich enzyme linked immunosorbent assay (ELISA). Briefly, microtiter plates (96 wells) were coated with 100 µL of anti-TNF-α, IL-1β and TGF-β primary antibodies (0.8 or 4 $\mu g.mL^{-1}$, kits from R&D Systems) and incubated overnight at 4 °C. The coating solution was removed, and the plates were washed by filling the wells with 290 µL of diluted wash buffer (R&D Systems).

Then, the remaining protein-binding sites were blocked in the coated wells by adding 290 µL of 1% bovine serum albumin in PBS pH 7.4. The plates were washed again. Aliquots of samples (100 µL) were added in duplicate to wells and incubated at 4 °C for 2 h. The plates were washed, and 100 µL of anti-TNF-α, anti-IL-1β or TGF-β secondary antibodies (R&D Systems) were added to the wells. After further incubation at room temperature for 2 h, the plates were washed and incubated with 100 µL of HRP-conjugated streptavidin. The plates were washed, and 100 µL of substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added in the absence of light. The enzyme reaction was stopped by adding 50 µL of H_2SO_4 (2 N), and the absorbance was measured at 450 nm.

The results are expressed as $pg.mL^{-1}$and reported as the mean ± the standard error of the mean (S.E.M). All analyses were performed in triplicate with samples obtained from three independent experiments, with results reproduced without significant differences.

2.10 Statistical analysis

Data is expressed as the mean ± S.E.M (standard error of the mean) or median, according to the variables evaluated.

Percentage contraction rates (%) are used to express the reduction of the lesion. For the evaluation of parameters related to inflammation, repeated measures

of analysis of variance (ANOVA) was used, followed by the Bonferroni post-test when the data distribution was normal, otherwise the Kruskal-Wallis test was used, followed by the Dunns post-test. The level of significance was set at $p<0.05$.

Statistical analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, USA).

3 Results

3.1 Chemical composition

As important as the biological evaluation is the confirmation of propolis compounds in the membrane. Qualitative and quantitative evaluations were performed using HRMS with direct infusion into the ESI source or using the UFLC system, respectively. As previously indicated, the membranes were prepared individually by the addition of He, Ea, and Bu extracts.

For qualification, around 0.7 g of the membrane was extracted with 10 mL of 40% ethanol. Then, the filtered solution was diluted (1:1) with acetonitrile/ultrapure water (1:1) with the addition of 0.1% formic acid, which was directly injected into the ESI source. As a result, propolis markers such as Liquiritigenin/Isoliquiritigenin (*m/z* 257.0808), Formononetin (*m/z* 269.0808), Medicarpine (*m/z* 271.0946), Biochanin A (*m/z* 285.0764) and Retusapurpurin B (*m/z* 523.1739) were observed (Table 1). Figure 1 shows a HRMS mass spectrum for the membrane embedded with Ea extract, which was the richest in the propolis flavonoid derivatives.

Table 1.

Figure 1.

Meanwhile, for quantification, a method using UFLC-ESI(+)-QTOF was adapted (da Silva Frozza et al., 2016), using the available standards. Table 2 shows the analytical responses obtained for each standard in a concentration range of 18.75–600.00 $\mu g.L^{-1}$.

Table 2.

For this process, similarly a determinate amount of membrane embedded with different propolis extracts (He, Ea, Bu) was extracted with ethanol solution (40%) and diluted again in ultra-pure water: acetonitrile (1:1) with the addition of 0.1% formic acid. The compounds were quantified using the optimized method with UFLC-ESI-QTOF on days 0, 2, 7 and 14 after membrane processing (Table 3). These results were useful for the choice of Bu and Ea membranes for animal testing.

Table 3.

3.2 Macroscopic evaluation of clinical aspects

The clinical variables analysed macroscopically on postoperative days 2, 7 and 14 in the experimental groups (GS, GMS, GMEBT, and GMEAE) showed progressive closure of the lesions throughout the experiment (Figure 2). No necrosis or clinical signs of infection were observed in the GMS, GMEBT and GMEAE groups monitored during the experiment. In the GS, only one of the animals had an abscess formation with the presence of serous secretion on the 7^{th} day of evaluation.

Figure 2.

Crust formation occurred in all experimental groups days 2 and 7. At 14 days only the GS and GMS groups remained with crusts. The presence of epithelialization was evidenced only at 14 days and was present in more than 90% of the animals in all experimental groups.

All experimental groups presented edema at days 2 and 7 and at day 14 edema was evident only in the GS group (Figure 3A). It is noteworthy that 2 days after follow-up there was a significant reduction in the edema of the GMEBT group when compared to the GS control group of approximately 34%.

Regarding the hyperemia aspect, there was a statistically significant difference between the GMEBT and GMEAE groups at 7 days of follow-up when compared to the GS control groups, with a reduction of hyperemia by more than 50% (Figure 3B). On the second and seventh days after surgery, the cellulose membrane showed a resected appearance (GMS, GMEBT and GMEAE), while the GS group presented excessive exudation (Figure 3C).

Figure 3.

3.2.1 Morphometry

The analysis of the area of contraction of the lesions was performed in three different moments after the surgical procedure (2, 7 and 14). It is observed that 7 days after the beginning of the treatments the groups presented a similar reduction in the lesions (Figure 4). In contrast, on the 14th postoperative day the groups treated with bacterial cellulose membrane associated with red propolis (GMEBT and GMEAE) exhibited a significantly greater reduction compared to GS with a percentage of contraction of the lesions of 97.2% and 96.1%, respectively, while the GS was 80.8% (Figure 5).

Figure 4.

Figure 5.

3.3 Microscopic analysis

Histological analyses stained with H/E from different samples are represented in Figure 6. Mild inflammatory response with edema was observed immediately after lesion inductionin all groups (Figure 6A-6D). A moderate inflammatory process can be visualized at day 7 in all samples (Figure 6E-6L). Edema is seen after a week of postoperative procedure distributed between the connective tissue as highlighted with asterisk in all groups studied. Leukocyte recruitment seems to be more expressive in bacterial cellulose membrane associated to red propolis at seven days of follow up in groups GMEBT and GMEAE (Figure 6I-6L). Two weeks after postoperative day (Day 14), groups treated with bacterial cellulose membrane associated with red propolis GMEBT (Figure 6Q and 6R) and GMEAE (Figure 6S and 6T) exhibited accelerated wound healing activity compared to GS (Figure 6M and 6N) and GMS (Figure 6O and 6P).

Figure 6.

3.4 Myeloperoxidase activity levels

Leukocyte recruitment was stimulated in the animals treated with bacterial cellulose membrane associated to the red propolis at 7 days follow-up, evidenced by

MPO activity that presented an increase of over 100% in the GMEBT and GMEAE groups when compared to the GS control group (Figure 7). At 14 days, MPO decreased similar to the control groups.

3.5 Quantification of TNF- α, IL-1 β and TGF-β

Pro-inflammatory cytokine levels (TNF-α and IL-1β) were measured on wounds at 0, 7 and 14 days post-procedure (Figure 8A and 8B). TNF-α levels were significantly higher in red própolis stimulated wounds (GMEBT and GMEAE) at day 7, unlike the higher IL-1β levels in the control (GS and GMS) groups.

The same way that pro-inflammatory cytokines, TGF-β (anti-inflammatory) expression was assessed and peaked 7 days after injury, being higher in the groups treated with bacterial cellulose membrane associated with red propolis (GMEBT and GMEAE), representing a statistically significant difference when compared group to the GS control group. (Figure 8C).

Figure 7.

Figure 8.

4 Discussion

In the last decades, the prevalence of diabetes mellitus is increasing exponentially, becoming an important problem in public health due to its complications, including chronic wounds (Sartorelli and Franco, 2003; WHO, 2016;). Evidence refers to the constant need to improve the understanding of the pathophysiology of these lesions in order to develop new techniques and therapeutic approaches for wound care (Velnar et al., 2009).

The use of bacterial cellulose membrane for wound healing has been studied by several researchers, presenting promising results (Czaja et al., 2006; Czaja et al., 2007; Celes et al., 2016; Brassolatti et al., 2018). This has already been studied including associated with natural products like green propolis (Barud et al., 2013). Meanwhile, the red propolis is known to be rich in flavonoids (Frozza et al., 2013). This resinous product has been used in popular medicine mainly due to its antimicrobial and anti-inflammatory characteristics (Bankova and Marcucci, 2000;

462 Oldoni et al., 2011). In this way, this work proposed the use of a bacterial cellulose-
463 based bio-curative, associated with extracts of red propolis, to improve the healing of
464 wounds in diabetic mice.

465 The presence of chemical markers of propolis in the membrane was
466 performed through HRMS. This technique identifies chemical compounds according
467 to a set of information such as: exact mass (m/z), isotope ratio; and fragmentation.
468 Thus, HRMS has been used to determine flavanoids (Vessecchi et al., 2011),
469 terpenoids (Yang et al., 2007) and alkaloids (Nicola et al., 2013).

470 For direct infusion in the mass spectrometry system, red propolis markers
471 were identified, among them ten in the membrane embedded with Ea extract, six with
472 Bu, and three in He (Table 1). This result is directly related to the polarity of the
473 compounds and the solvents used. These markers have been described as
474 responsible for a number of biological activities, such as anti-inflammatory (Kole et
475 al., 2011), cytotoxic (Frozza et al., 2013), antibacterial and antifungal (Alencar et al.,
476 2007; Gaur et al., 2016; Bueno-Silva et al., 2017) among others. In ethanol extract
477 70% Frozza et al. (2013) found eight compounds, among them Liquiritigenin,
478 Biochanin-A and Formonometin.

479 In our study, five compounds were quantified, with Formononetin being more
480 prevalent, appearing in all the membranes tested. The extract Ea where this
481 compound ranged from 4423.71–2907.77 $\mu g.g^{-1}$. This result agrees with other
482 studies that point to Formononetin as one of the main components and most
483 important marker of Brazilian red própolis (López et al., 2014; Bueno-Silva et al.,
484 2016). Researchers have identified of 270.00; 1520.00 and 1666.00$\mu g.g^{-1}$ for
485 Biochanin A, Formononetin, and Liquiritigenin respectively in ethanolic extract
486 (Frozza et al., 2017).

487 In a study conducted by Rufatto et al. (2018) extracts and fractions of red
488 propolis were tested for antibacterial activity. In this bioguided process, fractions
489 containing Formononetin and Biochanin A were the most active against
490 *Staphylococcus aureus* and *Bacillus subtilis*. Gram-positive bacteria such as
491 *Staphylococcus aureus* are the most commonly found in wounds (Gjodsbol et al.,
492 2006; Shanmugam et al., 2013).

493 Other isoflavones were also found in samples of red propolis, such as
494 Daidzein, Biochanin A (Alencar et al., 2007; Awale et al., 2008; Bueno-Silva et al.,

2016), Isoliquiritigenin (Oldoni et al., 2011; Frozza et al., 2013) and Liquiritigenin (Frozza et al., 2013).

In vivo results demonstrated that the topical application of the bacterial cellulose membrane associated with extracts of red propolis accelerates the healing of diabetic wounds. This is demonstrated by the increase in the rate of contraction of the lesions, reduction of clinical signs of inflammation, as well as complete epithelization of the wounds without presence of crusts at 14 days. Similar results have been reported in the literature with topical application of propolis alcoholic extract from Saudi Arabia (Hozzein et al., 2015). However, Barud et al. (2013) reported that bacterial cellulose membranes associated with green propolis were not able to increase the degree of cicatrization evaluated macroscopically in the model of a non-diabetic mouse.

The microscopic analyses of tissues revealed an initial mild inflammatory response with edema after lesion induction. This inflammatory process evolved greatly until the end of the first postoperative week, decreasing after two weeks. Through histological analysis, an expressive amount of leukocyte recruitment could be observed in tissues with bacterial cellulose membrane associated to red propolis (GMEBT and GMEAE) one week follow-up, which were also evidenced by increased levels of MPO and TNF-α activity. Two weeks after the postoperative day, however, the same groups submitted to red propolis extracts association with membrane exhibited accelerated wound healing activity compared to GS and GMS. These results are in accordance with the morphometry that exhibited a significantly greater reduction in the area of contraction compared to control groups two weeks postoperative.

It is known that cellular and molecular interactions occur throughout the cicatricial process, usually in an orderly and synchronized manner with the purpose of repairing the damaged tissue (Pereira and Bartolo, 2016). In diabetic patients, different factors interfere with the metabolism of these processes, leading to the chronicity of the wounds, mainly due to the persistent inflammation (Deveci et al., 2005; Dinh et al., 2012). In this inflammatory phase, there is initially an increase in vascular permeability and infiltration of neutrophils, which are responsible for the debrided tissue damage, mainly at the expense of oxygen radicals (H_2O_2), nitrogen,

and azurophilic enzymes (myeloperoxidase) (Swain et al., 2002; Kumar et al., 2005; Fonder et al., 2008; Reinke and Sorg, 2012).

Research related to healing provides evidence to the important role of neutrophils in the process of tissue repair (Aratani, 2018). Our results suggest that wounds treated with bacterial cellulose membrane associated with extracts of red propolis count with an increase of neutrophils in the crucial phase of tissue recovery, confirmed by MPO activity at 7 days follow-up and by the presence of inflammatory infiltrate and edema seen in histology. It is possible to observe the presence of neutrophils in a smaller quantity even in the late phase of tissue remodelling, suggesting that the inflammatory phase of the cicatricial process continued during the proliferation and remodelling phases. It is known that diabetic wounds face a prolonged inflammatory phase with intense neutrophilic infiltrate that can delay healing (Li et al., 2007).

According to Andrade et al. (2011), the greater the number of inflammatory cells recruited to the lesion site, the greater the MPO activity and total protein levels. Thus, MPO contributes to the physiological feedback of polymorphonuclear recruitment, contributing to the reduction in their influx into inflammation.

The increase in the number of neutrophils corroborates the increasein the levels of pro-inflammatory cytokines, among them the TNF-α and IL-1β (Andrade et al., 2011). These cytokines are at high levels in the case of diabetic wounds (Badr, 2012). The TNF-α is released primarily by macrophages within minutes after injury, locally and systemically, modulating innumerable immunological and metabolic events. It is a potent activator of neutrophils and mononuclear phagocytes and serves as a growth factor for fibroblasts and, consequently, angiogenesis (Sherwood and Toliver-Kinsky, 2004). In our study, we identified that TNF-α levels were significantly higher in the groups stimulated by red propolis at 7 days of treatment, while the presence of neutrophils was also significantly higher. It is known that red propolis can exert both pro-inflammatory and anti-inflammatory activity depending on the concentration and routes of administration, as well as the experimental conditions (Bufalo et al., 2014; Sforcin, 2016).

More than any other family of cytokines, the IL-1 family of ligands and receptors is associated with inflammation (Dinarello et al., 2010). Contrary to the increase in TNF-α in the groups treated with bacterial cellulose membrane

associated with red propolis, we observed a reduction in the release of IL-1β in these same groups at 7 days of treatment without statistically significant differences, suggesting a probable anti-inflammatory action of red propolis, possibly related to the presence of the markers found in the extracts, standing out the Formononetin present in high concentrations. *In vitro* study confirmed that this compound reduced the action of IL-1β and inhibited the activation of nuclear factor kappaB (NF-κB), protecting against apoptosis of pancreatic β cells caused by IL-1β, which may be used in the future for the treatment of diabetes mellitus (Wang et al., 2012).

Several other inflammatory mediators are released during the inflammation process, such as prostaglandins, chemokines, growth factors (TGF-β and PDGF), and nitric oxide (Gillitzer and Goebeler, 2001; Stroncek et al., 2009). Numerous studies have shown that the TGF-β superfamily plays a regulatory role in the tissue repair process, affecting all types of cells involved in the healing stages, inducing the proliferation of keratinocytes and fibroblasts, causing formation of new blood vessels and granulation tissue (Wang et al., 2006; Fan et al., 2015; Hozzein et al., 2015; Dos Santos Gramma et al., 2016). In our study, we measured TGF-β levels and showed a significant increase in the groups treated with bacterial cellulose membrane and red propolis at 7 daysfollow-up, with a subsequent reduction at 14 days. We believe that red propolis exerted a positive effect on the expression of TGF-β, influencing the reduction of inflammation, angiogenesis, and complete reepithelialization of the lesions at 14 days of treatment. This can be explained by the peculiar composition of red propolis, with emphasis again on the marker Formononetin previously described as responsible for increasing TGF-β levels and accelerating the closure of excisional wounds in mice (Huh et al., 2011).

5 Conclusion

This study demonstrated that bacterial cellulose membranes associated with extracts of red propolis accelerate the healing process of diabetic wounds, evidenced by the significant reduction in lesion size, complete epithelization and increase of TGF-β levels, as well as the control of prolonged inflammation. Therefore, it is possible that these effects are directly related to the presence of flavanoids from extracts of red propolis, which have been associated with antimicrobial and anti-

592 inflammatory activities, being used in folk medicine. Thus, we produced a
593 biotechnological dressing useful for treating chronic wounds due to diabetes.
594
595 **Conflict of interest**
596 The authors declare no conflict of interest.
597
598 **Acknowledgements**
599 The authors would like to thank the colleague Fabiana Agostini and research
600 fellow Paulo Henrique Ancilaggo for the important participation in the present work.
601 The authors thank the FAPERGS, CAPES and CNPq for financial support.
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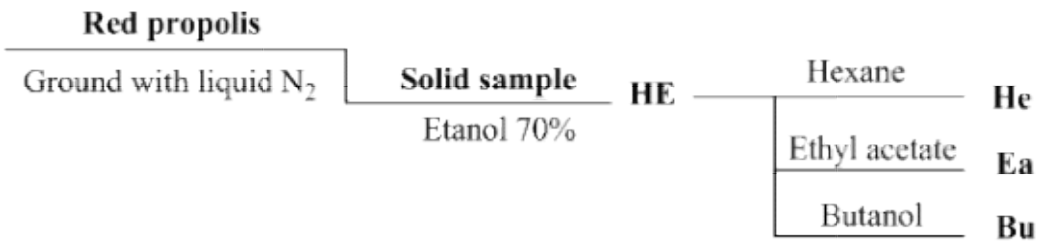
Scheme 1: Extraction and fractionation by solvent depletion, with increased polarity, of red propolis.



967 **TABLE CAPTIONS**

968

969 **Table 1:** Chemical composition by direct infusion in ESI(+)-QTOF.

970

971 **Table 2:** Parameters for the UFLC-ESI-QTOF method used for compounds
972 quantification.

973

974 **Table 3:** Concentration of isoflavones in $\mu g.g^{-1}$ of membranes with extracts of red
975 propolis: ethyl acetate (Ea), butanol (Bu), and hexane (He).

976 **Table 1.**

Entry	Membrane/extract	Precursoríon *m/z*	Identification	Diff. ppm	Reference
1	Ea, Bu	123.0451	Benzoicacid	3.21	(Alencar et al., 2007)
2	Ea, Bu	137.0602	Anisaldehyde	2.03	(Alencar et al., 2007)
3	Ea	255.0799	Daidzein	4.21	(Awale et al., 2008)
4	Ea, Bu, He	257.0813	Liquiritigenin, Isoliquiritigenin	0.21	(Frozza et al., 2013)
5	Ea, Bu, He	269.0812	Formononetin	0.34	(Frozza et al., 2013)
6	Ea	271.0951	Medicarpin	2.20	(Frozza et al., 2013)
7	Ea	273.1097	Vestitol; Isovestitol; Neovestitol	4.32	(Frozza et al., 2013)
8	Ea, He	285.0768	Biochanin A	0.56	(Piccinelli et al., 2011)
9	Ea, Bu	287.0993	Vesticarpan	4.72	(Piccinelli et al., 2011)
10	Ea, Bu	523.1741	Retusapurpurin	1.23	(Piccinelli et al., 2011)

977

978 **Table 2.**

Entry	R^2	LOD($\mu .L^{-1}$)	LOQ($\mu .L^{-1}$)
Biochanin A	0.9953	7.37	22.34
Daidzein	0.9992	6.95	21.06
Formononetin	0.9966	11.81	35.78
Isoliquiritigenin	0.9985	3.61	10.95
Liquiritigenin	0.9989	26.86	81.39

979
980

981 **Table 3.**

Membrane/extract	Liquiritigenin (μg.g^{-1})	Formononetin (μg.g^{-1})	Daidzein(μg.g^{-1})	Biochanin A (μg.g^{-1})	Isoliquiritigenin(μg.g^{-1})
0 day					
Hexane (He)	297.54±0.1	294.59 ±6.0	nd	276.13 ±2.48	nd
Ethyl acetate (Ea)	1692.48±12.6	4423.71 ±18.2	411.02 ±0.8	358.82 ±13.7	813.51 ±15.4
Butanol (Bu)	326.83 ±0.7	263.59 ±0.95	nd	nd	72.76 ±0.7
2 days					
Hexane (He)	281.20 ±0.1	245.41 ±7.3	nd	230.57 ±9.2	nd
Ethyl acetate (Ea)	1576.64 ±15.9	4421.13 ±15.1	316.58 ±14.25	295.06 ±15.2	770.18 ±18.2
Butanol (Bu)	268.51 ±1.8	250.27 ±2.47	nd	nd	53.29 ±2.2
7 days					
Hexane (He)	260.08 ±0.1	178.72 ±1.9	nd	155.01 ±1.1	nd
Ethyl acetate (Ea)	1446.89 ±21.3	3270.28 ±1.7	284.97 ±4.9	210.68 ±2.3	586.76 ±10.4
Butanol (Bu)	209.27 ±7.8	194.47 ±5.61	nd	nd	43.70 ±1.6
14 days					
Hexane (He)	250.76 ±0.1	153.49 ±3.8	nd	149.56 ±2.8	nd
Ethyl acetate (Ea)	1356.51 ±11.1	2907.77 ±16.6	229.61 ±4.8	194.86 ±9.4	481.66 ±10.7
Butanol (Bu)	173.23 ±3.9	173.61 ±2.1	nd	nd	40.22 ±1.0

FIGURES CAPTIONS

Figure 1 – HRMS spectrum for the membrane embedded with ethyl acetate extract (Ea).

Figure 2 – Macroscopic changes in skin excisional wounds during wound closure.

Figure 3 – Treatment with bacterial cellulose membrane associated with red propolis induces changes in inflammatory parameters.

Figure 4 – Extracts of red propolis associated with the bacterial cellulose membrane reduce the area of the lesions.

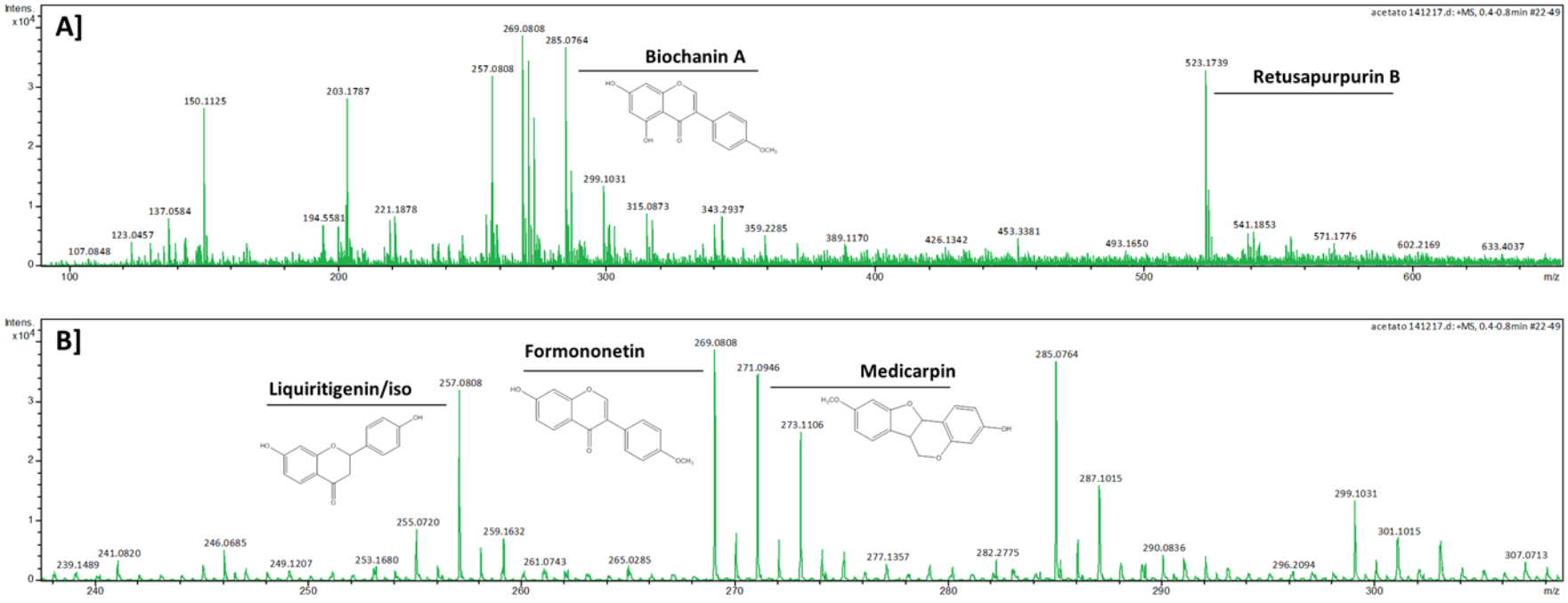
Figure 5 – Increase in the percentage of wound contraction with the use of extracts of red propolis associated with the bacterial cellulose membrane.

Figure 6 – Microscopic analysis from mice tissue samples stained with H/E.

Figure 7 – Association of bacterial cellulose membrane and red propolis increases the activity of myeloperoxidase (MPO) in the inflammatory period.

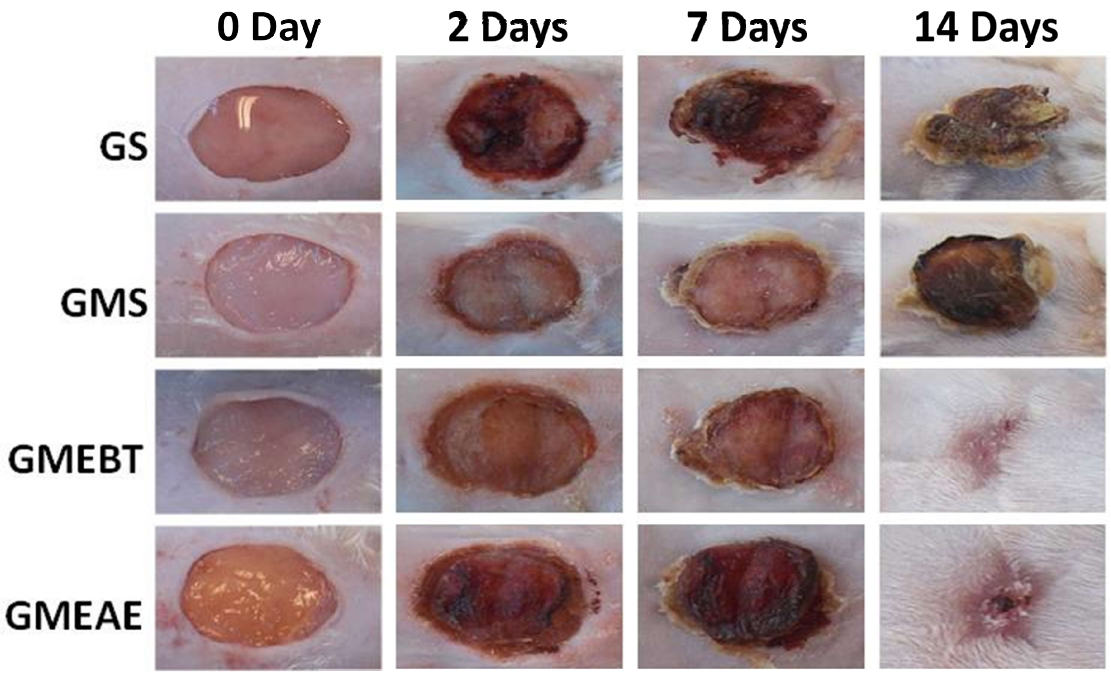
Figure 8 – Changes in the levels os pro-inflammatory citokynes during wound healing.

Figure 1



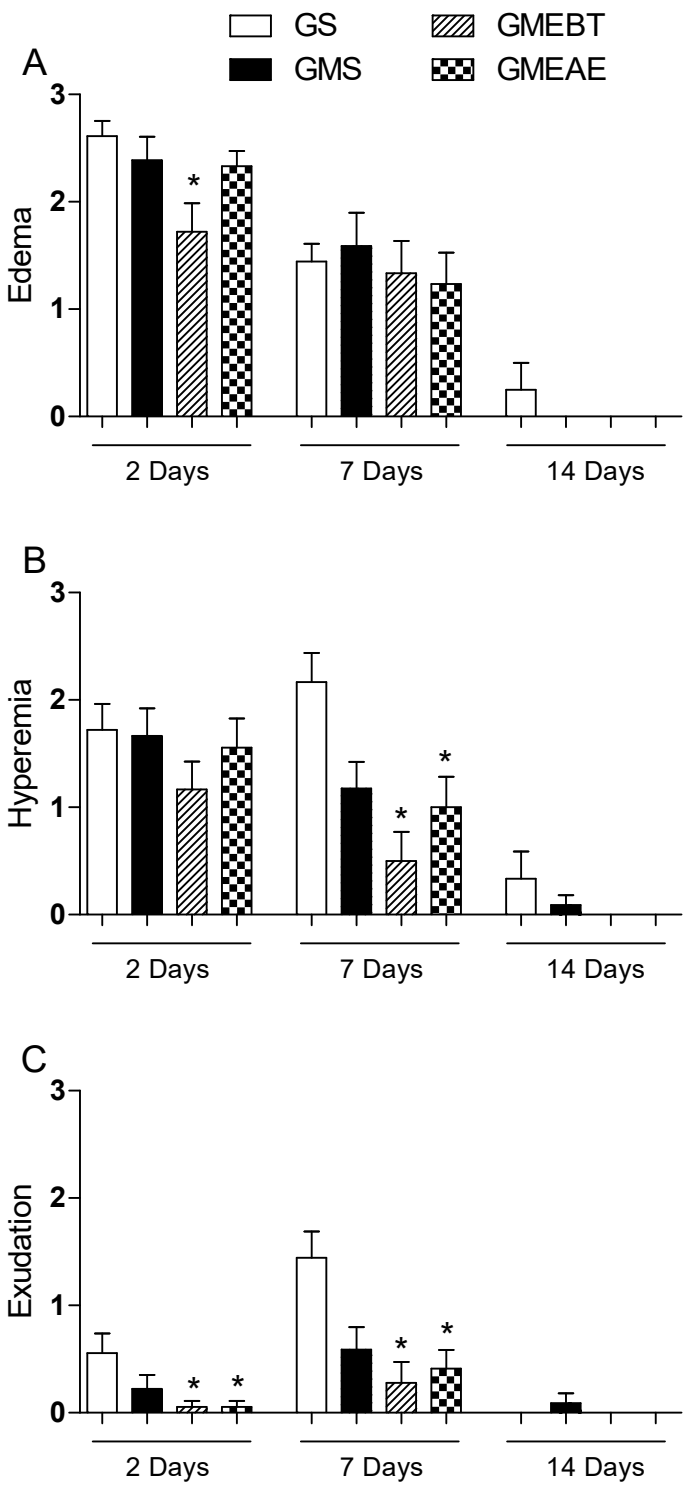
In A) in full mode, and in B) expanded between 240 to 300uam.

Figure 2



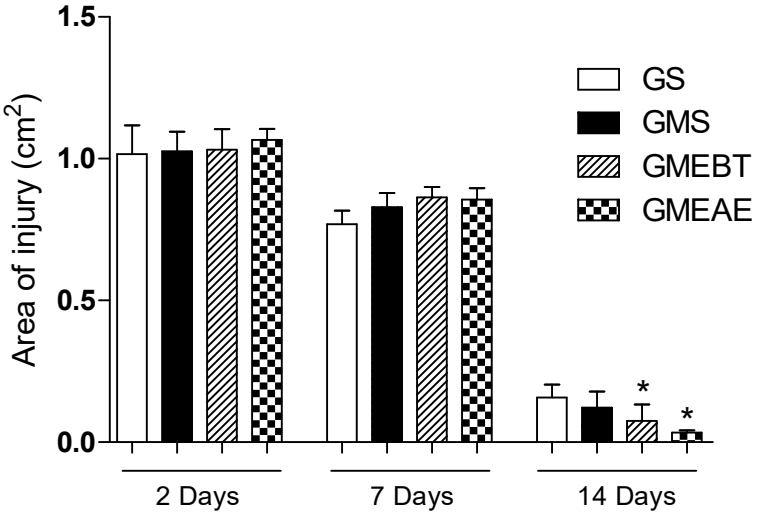
The wound sites were photographed at the indicated intervals Different aspects of the cicatricial process (edema, hyperemia, exudation, crusts and epithelial tissue) were evaluated on days 0, 2, 7 and 14 after the surgical procedure.

Figure 3



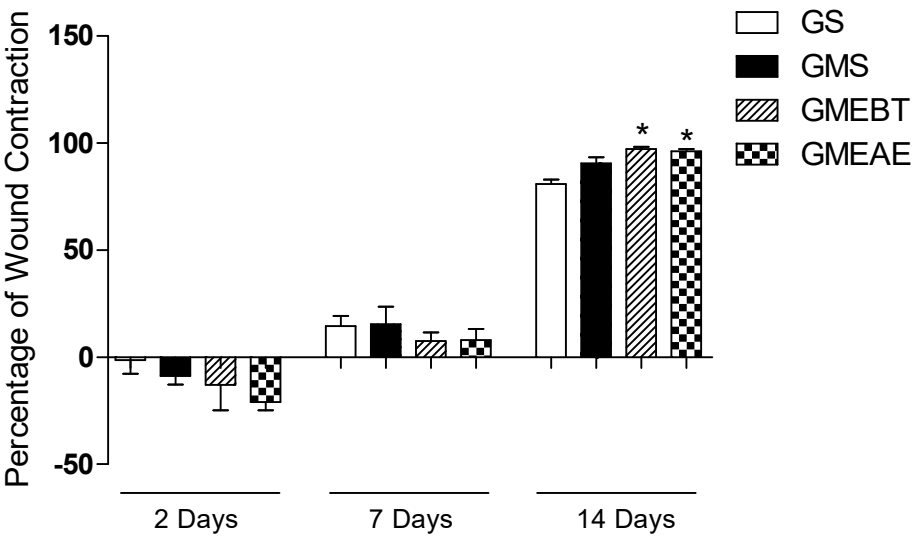
In **(A)** Edema, **(B)** Hyperemia and **(C)** Exudation. Data are expressed as the mean ± S.E.M. *P<0.05 indicates statistical difference compared to the saline control group.

Figure 4



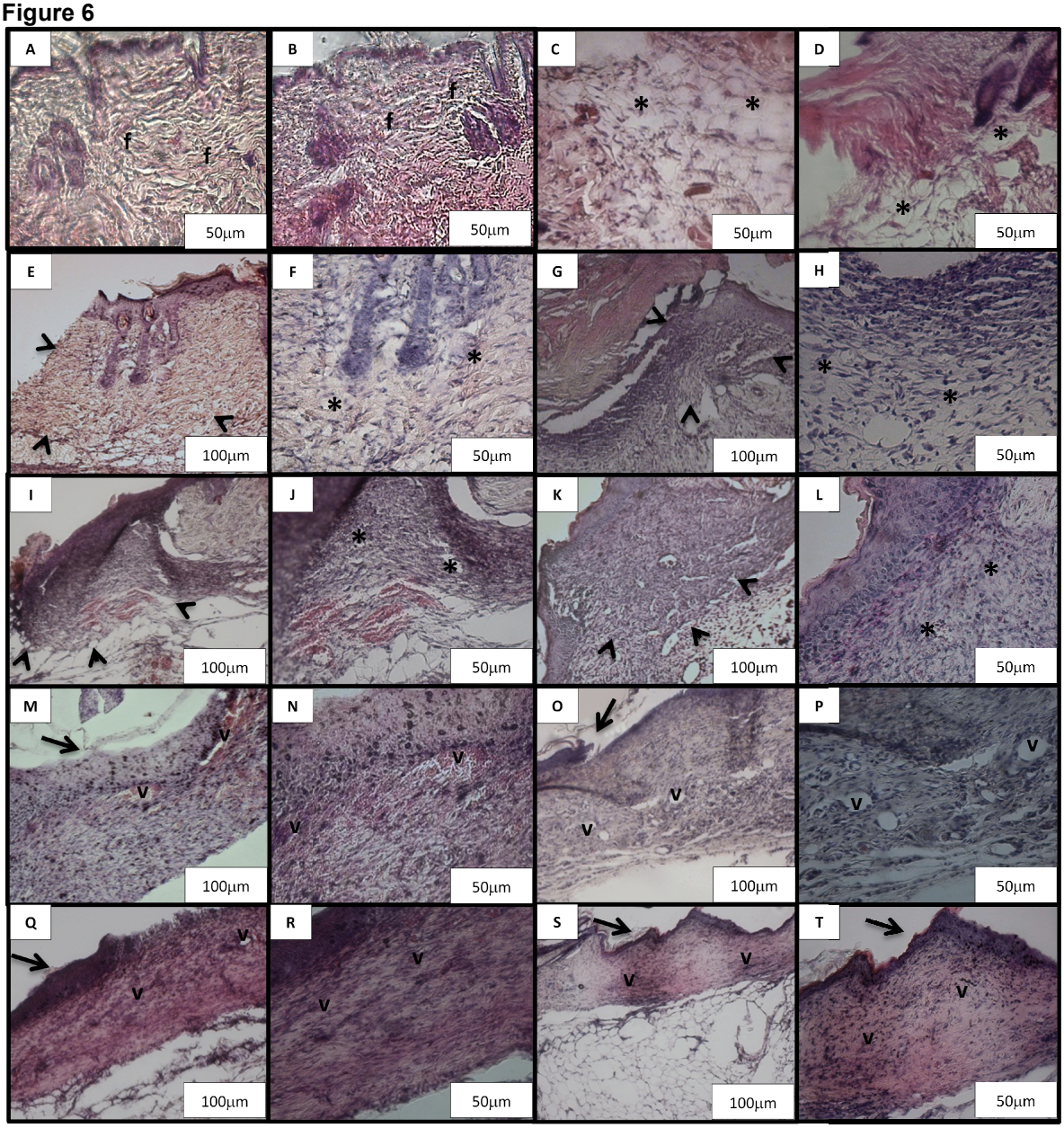
The areas of the lesions (cm^2) were measured on days 2, 7 and 14 after the surgical procedure. Results are expressed as the mean ± S.E.M. Of the average area of the wounds. *P<0.05 indicates statistical difference compared to the saline control group.

Figure 5



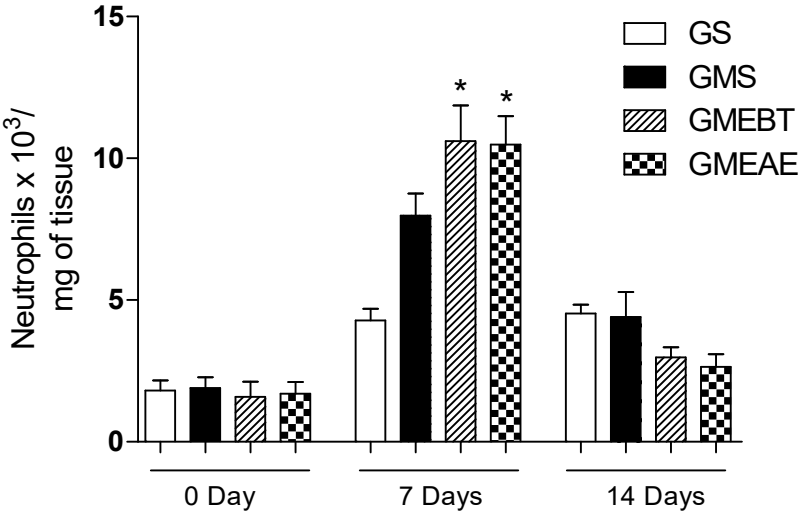
Percentage of wound contraction on days 0, 2, 7 and 14 after the surgical procedure. The contraction is calculated with respect to the wound area on Day 0 (at the time of induction). Data are expressed as the mean ± S.E.M. *P<0.05 indicates statistical difference compared to the saline control group.

Figure 6



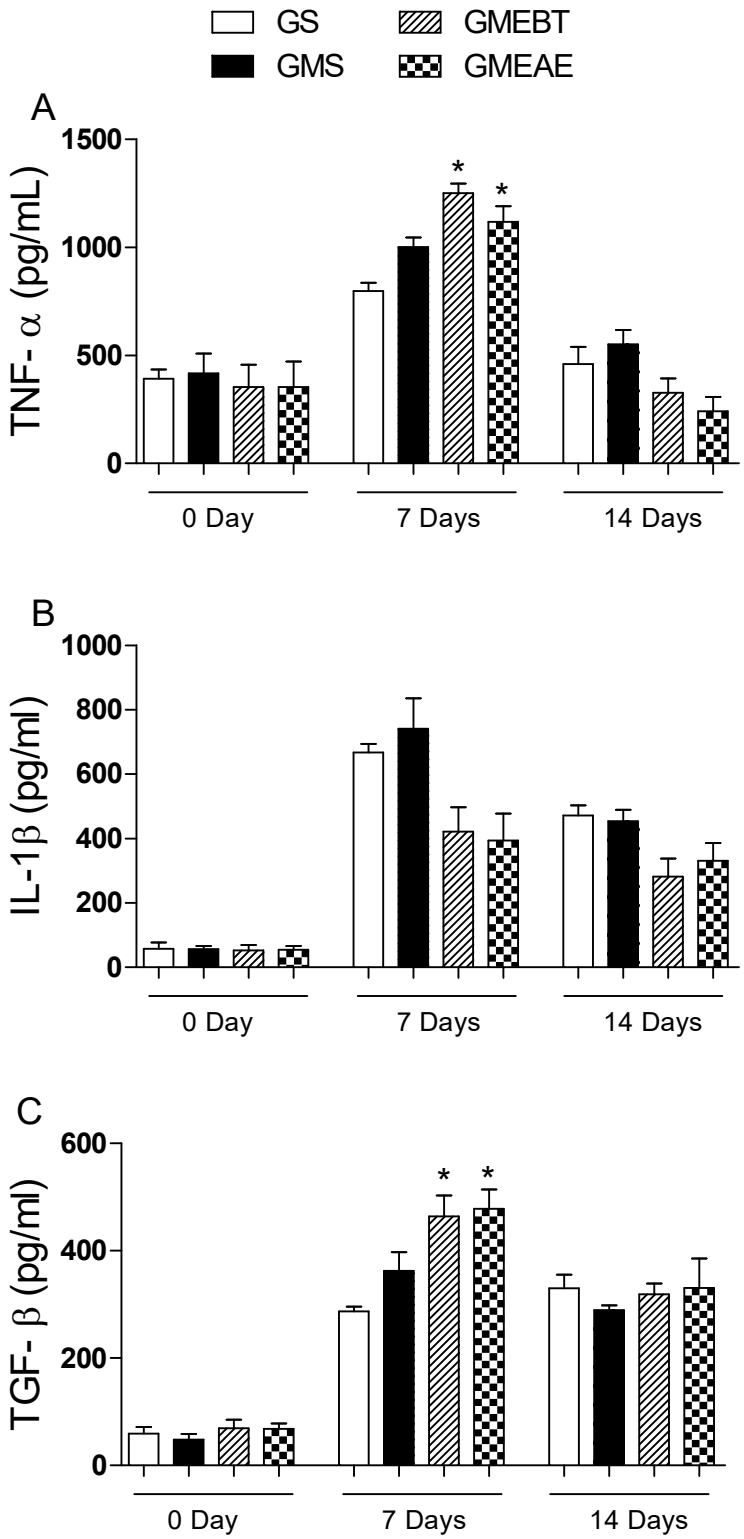
Day 0: Mild inflammatory response was observed immediately after lesion induction - 200x. Figure 6A (GS) and 6B (GMS) show regions of fibers (f) in connective tissue and edema is represented with asterisk (*) in Figure 6C (GMEBT) and 6D (GMEAE) . Day 7: Edema is distributed between the connective tissue (*) in all groups. Leukocyte recruitment (arrow head) are more expressive in groups GMEBT (I) 100x and (J) 200x, and GMEAE (K) 100x and (L) 200x, compared to groups without red propolis GS (E) 100x and (F) 200x, and GMS (G) 100x and (H) 200x. Day 14: Groups treated with bacterial cellulose membrane associated with red propolis GMEBT (Figure 6Q 100x and 6R 200x.) and GMEAE (Figure 6S 100x and 6T 200x) exhibited greater wound healing process compared to GS (Figure 6M 100x and 6N 200x) and GMS (Figure 6O 100x and 6P 200x). Repair tissue (arrows) and new blood vessels (*) are seen during wound healing process.

Figure 7



The animals were sacrificed on days 7 and 14 after the surgical procedure and lesion samples were removed to determine myeloperoxidase activity. The samples of day 0 were collected at the time of the surgical procedure. Data are expressed as the mean ± S.E.M. *P<0.05 indicates statistical difference compared to the saline control group.

Figure 8



The levels of pro-inflammatory cytokines: **(A)** TNF-α and **(B)** IL-1β and an anti-inflammatory cytokine: **(C)** TGF-β were measured by ELISA in the wound tissues on days 0, 7 and 14 after the surgical procedure. The samples of day 0 were collected at the time of the surgical procedure. Data are expressed as the mean ± S.E.M. *P<0.05 indicates statistical difference compared to the saline control group.

4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

O biocurativo desenvolvido a partir de membrana de celulose bacteriana associada à extratos de própolis vermelha se mostrou eficaz para o tratamento de feridas em camundongos diabéticos. Evidenciamos uma redução significativa no tamanho das lesões nos grupos tratados com este biocurativo quando comparados aos grupos controles, bem como uma melhora expressiva nos parâmetros inflamatórios. A própolis vermelha limitou a inflamação prolongada, sugerindo um efeito modulador durante este processo. Desta forma, entendemos que o uso de um conhecimento tradicional como a própolis vermelha associado às novas descobertas na área de biocurativos é benéfico para o reparo tecidual neste modelo experimental.

Perspectivas futuras:

- Dosar outros mediadores inflamatórios como o óxido nítrico envolvidos no processo cicatricial;
- Realizar análise histomorfométrica da densidade das fibras de colágeno tipo I e III, marcadas por Picrosirus red;
- Isolar os compostos da própolis vermelha para testá-los separadamente neste mesmo modelo;