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Filme à base de polissacarídeo carregado com vitamina C e própolis: um promissor dispositivo para acelerar a cicatrização de feridas diabéticas

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Pelotas, 2019

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica e Bioprospecção, da Universidade Federal de Pelotas, como requisito parcial para obtenção do grau de Mestre em Bioquímica e Bioprospecção.

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Dedico essa dissertação
aos meus pais Rosi e Beto,
e a minha namorada Renata.

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“… A alegria da vida vem de nossos encontros com novas experiências e, por isso, não há alegria maior do que ter um horizonte cambiante, cada dia com um novo e diferente sol”

(Christopher McCandless)

RESUMO

VOSS, Guilherme. **Filme à base de polissacarídeo carregado com vitamina C e própolis: um promissor dispositivo para acelerar a cicatrização de feridas diabéticas.** 2019. 62f. Dissertação (Mestrado em Bioquímica e Bioprospecção) - Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2019.

A cicatrização de feridas pode ser um processo doloroso e demorado em pacientes com diabetes *mellitus*. Em vista disso, o uso de dispositivos de cicatrização de feridas pode ajudar a acelerar esse processo. No presente estudo, filmes de celulose carregados com vitamina C (VitC) e/ou própolis (Prop), dois compostos naturais com propriedades farmacológicas atraentes, foram projetados. Conforme avaliado, a VitC pode ser liberada de maneira controlada dos filmes contendo celulose (Cel) e álcool polivinílico (PVA), tais como Cel-PVA/VitC e Cel-PVA/VitC/Prop. Estudos de atividade antibacteriana *in vitro* mostraram uma redução na contagem de bactérias (*Escherichia coli* e *Staphylococcus aureus)* após o tratamento com os filmes Cel-PVA/VitC, Cel-PVA/Prop e Cel-PVA/VitC/Prop. Além disso, as propriedades antibacterianas e cicatrizantes dos filmes à base de celulose foram avaliadas em um modelo animal de diabetes induzido estreptozotocina (STZ) (100 mg/kg). Após o período de sensibilização, os animais foram tratados com os filmes durante 15 dias. Os camundongos diabéticos exibiram comprometimento da cicatrização, enquanto o tratamento com o filme Cel-PVA/VitC/Prop aumentou a velocidade de cicatrização da ferida. Observou-se uma redução acentuada nas contagens bacterianas presentes no ambiente da ferida de camundongos diabéticos após o tratamento com Cel-PVA/VitC, Cel-PVA/Prop e Cel-PVA/VitC/Prop. A análise histológica demonstrou que o grupo de camundongos diabéticos não tratados não apresentou cicatrização completa, enquanto o grupo tratado com os filmes Cel-PVA/VitC e Cel-PVA/VitC/Prop apresentou boa resposta cicatricial. Desta forma, esses novos filmes podem representar uma nova abordagem terapêutica para acelerar a cicatrização de feridas diabéticas.

Palavras-chave: Celulose, cicatrização de feridas diabéticas, doença metabólica, própolis, diabetes *mellitus*.

ABSTRACT

VOSS, Guilherme. **Polysaccharide-based film loaded with vitamin C and propolis: A promising device to accelerate diabetic wound healing.** 2019. 62f. Dissertation (Master in Biochemistry and Bioprospecting) - Biochemistry and Bioprospecting Postgraduate Program. Federal University of Pelotas, Pelotas, 2019.

Wound healing can be a painful and time-consuming process in patients with diabetes *mellitus*. In view of this, the use of wound healing devices can help expedite this process. In the present study, cellulose films, loaded with vitamin C (VitC) and/or propolis (Prop), two natural compounds with attractive pharmacological properties, were designed. As assessed, VitC can be released in a controlled manner from films containing cellulose (Cel) and polyvinyl alcohol (PVA), such as Cel-PVA/VitC and Cel-PVA/VitC/Prop. Studies of in vitro antibacterial activity showed a reduction in bacterial counts (*Escherichia coli* and *Staphylococcus aureus*) after treatment with the films Cel-PVA/VitC, Cel-PVA/Prop and Cel-PVA/VitC/Prop. In addition, the antibacterial and healing properties of the cellulose-based films were evaluated in an animal model of diabetes induced with streptozotocin (STZ) (100 mg/kg). After the sensitization period, the animals were treated with the films for 15 days. Diabetic mice exhibited impairment of healing, while treatment with the Cel-PVA/VitC/Prop film increased the healing rate of the wound. There was a marked reduction in bacterial counts present in the wound environment of diabetic mice following treatment with Cel-PVA/VitC, Cel-PVA / Prop and Cel-PVA / VitC / Prop. Histological analysis showed that the group of untreated diabetic mice did not show adequate healing, whereas the group treated with the films Cel-PVA / VitC and Cel-PVA/VitC/Prop showed good cicatricial response. In this way, these new ecological films may represent a new therapeutic approach to accelerate the healing of diabetic wounds.

Keywords: Cellulose, diabetic wound healing, metabolic disease, propolis, diabetes *mellitus.*

LISTA DE FIGURAS

Revisão Bibliográfica

LISTA DE FIGURAS

Artigo Científico

LISTA DE TABELAS

Artigo Científico

LISTA DE ABREVIATURAS

DM	Diabetes *mellitus*
E. Coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
FTIR	Espectro de Infravermelho por Transformada de Fourier - do inglês *Fourrier transform infrared*
STZ	Estreptozotocina – do inglês *Streptozotocin*
DNA	Ácido desoxirribonucleico
NAD^+	Nicotinamida adenina dinucleotídeo oxidado
VitC	Vitamina C
Prop	Própolis
PVA	Álcool polivinílico - do inglês *poly(vinyl alcohol)*
XRD	Difração de Raios-X - do inglês X-*ray diffraction*
SWF	Fluido de ferida simulado – do inglês *Simulated wound fluid*
HE	Hematoxilina e eosina – do inglês *Hematoxylin and eosin*
MT	Tricromo de Masson - do inglês *Masson's trichrome*
Cel-PVA	Celulose:PVA
Cel-PVA/VitC	Celulose:PVA/Vitamina C
Cel- PVA/ Prop	Celulose:PVA/Própolis

LISTA DE SÍMBOLOS

<	Menor
>	Maior
≤	Menor ou igual
≥	Maior ou igual
Kg	Kilograma
g	Grama
mg	Miligrama
min	Minuto
H	Horas
mL	Mililitros
µM	Micromolar
µL	Microlitro
M	Molaridade
cm	Centímetro
mA	Miliampère
mm	Milímetros
dL	Decilitro
%	Porcentagem
β	Beta
γ	Gama
κ	Kappa

SUMÁRIO

1. Introdução

A cicatrização de feridas é considerada um dos maiores desafios da pesquisa médica, envolvendo processos complexos com diversas fases distintas (GURTNER et al., 2008). Estas fases compreendem desde as escaras até a restauração do tecido lesado e são divididas em fase inflamatória, proliferativa e de maturação (SERRA et al., 2017). Entre os fatores sistemáticos que podem prejudicar a cicatrização de feridas encontram-se algumas doenças, tais como doença arterial coronariana, doença vascular periférica, câncer e diabetes *mellitus* (DM) (HESS, 2011). Desta forma, a DM quando associada ao desenvolvimento de feridas afeta severamente a qualidade de vida dos pacientes, levando a internação prolongada e amputações de membros, principalmente inferiores (FRYKBERG; BANKS, 2015). Portanto, o tratamento adequado das feridas implica em proporcionar um ambiente de cicatrização favorável no local da lesão e, também, superar os fatores locais e sistêmicos que prejudicam o processo de cicatrização (FRYKBERG; BANKS, 2015).

Considerando o desenvolvimento do campo de estudos da cicatrização de feridas, destacam-se os materiais de curativos os quais desempenham um papel fundamental para corrigir os problemas acima mencionados. Os curativos são biomateriais utilizados para cobrir as feridas, os quais atuam como uma barreira física capaz de absorver os exsudatos, manter o equilíbrio de umidade, permitir a troca gasosa e evitar que as feridas sejam infectadas (RIZZI et al., 2010). Atualmente, curativos formulados a partir de biopolímeros (por exemplo, proteínas e polissacarídeos) têm recebido grande atenção devido às suas propriedades em comparação com outros curativos (CHATTOPADHYAY; RAINES, 2014; BIRCH et al., 2015).

Os biopolímeros são gerados a partir de fontes renováveis e, em geral, custam menos que os polímeros sintéticos ou outros materiais (por exemplo, cerâmicas ou metais). Além disso, os biopolímeros apresentam propriedades atrativas, como não toxicidade, biocompatibilidade, biodegradabilidade e fácil processamento e moldabilidade (AGRAWAL et al., 2014, FACCHI et al., 2017). Entre os biopolímeros, a celulose tem sido extensivamente utilizada na formulação de materiais de curativos devido à sua excelente biocompatibilidade,

boas propriedades mecânicas e estrutura química (LI et al., 2015, WANG et al., 2016).

Neste estudo, fibras de celulose foram misturadas com álcool polivinílico (PVA), um polímero hidrofílico semicristalino, solúvel em água e não-tóxico, para formar um filme composto visando seu uso como um curativo. As fibras de celulose foram extraídas da casca de arroz, um resíduo agrícola produzido em todo o mundo (DE OLIVEIRA et al., 2017). Além disso, os filmes compostos foram carregados com vitamina C (L- ácido ascórbico) e própolis. A vitamina C é um conhecido antioxidante natural envolvido em todas as fases da cicatrização de feridas, principalmente na fase de formação de colágeno (MOHAMMED et al., 2015). Além disso, alguns estudos relatam a participação da vitamina C na modulação do sistema imune (MANNING et al., 2013). Já a própolis, é uma substância resinosa produzida por abelhas, amplamente utilizada devido às suas propriedades antibacteriana, antifúngica e antiviral que, segundo alguns estudos, é capaz de melhorar alguns mecanismos biológicos envolvidos na cicatrização de feridas (por exemplo, epitelização) (MARTINOTTI; RANZATO, 2015; FREIRES et al., 2016). A ação anti-inflamatória também é atribuída a própolis (DE ALMEIDA et al., 2013, FREIRES et al., 2016). Portanto, este estudo buscou investigar as propriedades antibacterianas e de cicatrização de feridas desses filmes à base de celulose carregados com vitamina C e própolis para gerar um curativo avançado e ecologicamente correto.

2. Objetivos

Objetivo geral

Avaliar as propriedades antibacterianas e de cicatrização de filmes à base de celulose e PVA carregados com vitamina C e própolis em feridas diabéticas induzidas em camundongos.

Objetivos específicos

- Caracterizar o filme a base de celulose com espectro de infravermelho por transformada de Fourier (FTIR);
- Avaliar o perfil de liberação de vitamina C;
- Avaliar a atividade bactericida dos filmes a base de celulose e PVA;
- Avaliar a cicatrização de feridas diabéticas tratadas com os filmes à base de celulose e PVA em modelo animal;
- Quantificar a proliferação bacteriana no local das feridas tratadas com os filmes a base de celulose e PVA e realizar avaliação histológica no tecido do dorso de camundongos.

3. Revisão Bibliográfica

Cicatrização

A pele é o maior e o mais complexo órgão do corpo, e é responsável por múltiplas funções como barreira protetora, termo regulação, síntese de vitamina D, sede de receptores sensoriais e reservatório de água, minerais e gorduras (ZIMMERMAN et al., 2014). Estruturalmente, a pele é composta por duas camadas (Figura 1), a derme e a epiderme. A epiderme, de acordo com Almeida (2009) é constituída por um epitélio estratificado pavimentoso e queratinizado, caracterizado por células dispostas em várias camadas. Esta serve como barreira que protege o organismo contra os estresses ambientais, tais como: invasão por microrganismos, perda de água e injúrias mecânicas e térmicas. Por último, logo abaixo se encontra a derme, a qual fornece sustentação para a epiderme com um tecido conjuntivo rico em vasos, glândulas sebáceas e sudoríparas, folículos pilosos e corpúsculos tácteis (AZZI et al., 2005).

Existem três tipos celulares principais na epiderme, os queratinócitos, com função de barreira protetora, os melanócitos, responsáveis pela pigmentação da pele e células de Langerhans, que servem como mediadoras da resposta inflamatória. Já a derme é uma camada basal conjuntiva espessa, com diversos tipos celulares: os fibroblastos, fibrócitos, fibras colágenas e elásticas que proporcionam resistência e elasticidade a pele, capilares sanguíneos que nutrem a epiderme sem penetrá-la, terminações nervosas sensoriais e anexos da pele (BLANES, 2004).

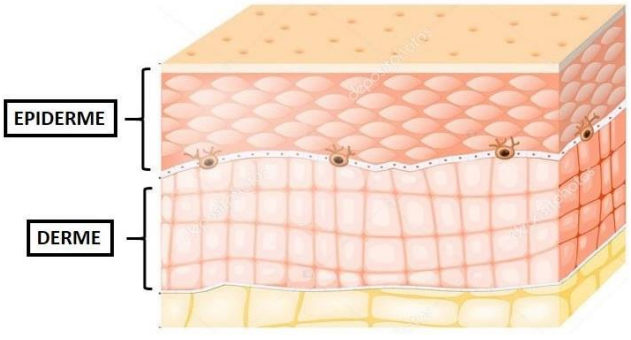

Figura 1. Divisão estrutural da pele (Adaptado de MARTINI et al., 2009).

No momento em que ocorre o rompimento da estrutura e das funções fisiológicas normais da pele, é desencadeada imediatamente a frente de defesa

do organismo. Desta forma, uma série de eventos dinâmicos vão regenerar o tecido lesionado reestabelecendo a sua continuidade e iniciando o processo de cicatrização que pode ser dividido em três principais fases: inflamatória, proliferativa e de maturação (OBARA et al., 2003).

Fase inflamatória

Conhecida também como fase exsudativa, a fase inflamatória envolve um conjunto de reações locais e sistêmicas, resultado da interação de células inflamatórias e vasculares. A resposta inflamatória é uma reação local não específica à lesão do tecido e/ou invasão bacteriana. É uma parte importante dos mecanismos de defesa do corpo, e uma fase essencial do processo de cicatrização. A vasoconstrição mediada por componentes vasoativos é a primeira etapa da resposta inflamatória e esta é desencadeada a fim de conter a hemorragia local. Simultaneamente, ocorre a deposição e ativação plaquetária com infiltração de fibrina e eritrócitos (BLANES, 2004).

Por sua vez, a ativação plaquetária libera citocinas que são responsáveis pela migração de células inflamatórias para o local lesionado. Nesta fase, predominam neutrófilos e macrófagos, os quais vão fagocitar bactérias e restos celulares. Além, disso os macrófagos produzem diversos fatores de crescimento, estes por sua vez, atraem outras células de defesa para o local lesionado e induzem a proliferação de fibroblastos e queratinócitos, que são as principais células de regeneração da pele (MIDWOOD et al., 2004).

Fase proliferativa

A fase proliferativa caracteriza-se pela reepitelização, angiogênese e fibroplasia. Os queratinócitos migram da borda para o centro da ferida e multiplicam-se, reestruturando as camadas da epiderme e promovendo a reepitelização tecidual. A angiogênese é a fase onde ocorre a formação de vasos novos e finos a partir de vasos íntegros adjacentes, estes com a função de fornecer oxigênio e nutrientes para cicatrização (DESMOULIÈRE et al, 2005). Simultaneamente, ocorre a formação do tecido de granulação, que é um tecido frouxo obtido a partir da deposição e proliferação de fibroblastos. Estes fibroblastos secretam elastina, fibronectina, glicosaminoglicana, proteases e

colágeno (principalmente colágeno tipo III), os quais são responsáveis pela regeneração tecidual (ISAAC et al., 2010).

Fase de maturação

A fase de maturação caracteriza-se por eventos tais como, deposição, agrupamento, remodelação do colágeno e regressão endotelial. Além disto, ocorre também a diminuição de todos os elementos celulares, inclusive as células inflamatórias (Figura 2). Nesta fase o colágeno tipo III, presente em maior quantidade no tecido de granulação, é degradado dando lugar à produção fibroblástica de colágeno tipo I (TAZIMA et al., 2008). Nesta fase, a cicatriz torna-se mais clara e plana.

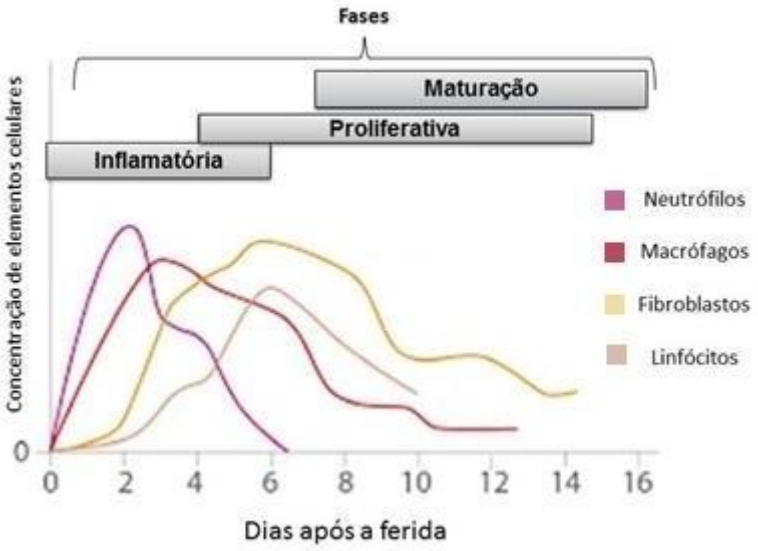

Figura 2. Evolução da concentração de elementos celulares e fibroblastos nas fases sequenciais do processo de cicatrização (Adaptado de TAZIMA et al., 2008).

O processo de maturação da ferida é lento, podendo levar meses para reorganizar as fibras colágenas e atingir as quantidades adequadas de colágeno tipo I e III, que irão aumentar a força da cicatriz, diminuir sua espessura e fornecer elasticidade (CHILDS; MURTHY, 2017). Tento em vista os processos envolvidos na cicatrização de feridas, ainda deve-se levar em consideração a existência de doenças que podem influenciar nestes processos dificultando a adequada cicatrização de tecidos lesados, como por exemplo, a DM.

Proliferação bacteriana em feridas

Pacientes com doenças crônicas, como no caso da DM, apresentam comumente complicações relacionadas à cicatrização de feridas, como taxa de cura mais lenta e agravamento da ferida (FRYKBERG; BANKS, 2015). As alterações cutâneas ocasionadas pela má cicatrização das feridas geralmente aparecem subsequentemente ao desenvolvimento da DM, podendo estar associada à proliferação bacteriana, resultando em complicações graves que diminuem significativamente a qualidade de vida do paciente (PAVLOVIĆ et al., 2007).

As feridas na sua fase inicial podem estar isentas de bactérias patogênicas, porém podem tornar-se rapidamente colonizadas pela exposição a bactérias da pele e do ambiente (BENBOW, 2010). Portanto, quando a proliferação de bactérias se torna uma infecção clínica, a cicatrização de feridas é prejudicada, podendo até mesmo agravar o quadro do paciente. Assim, a infecção da ferida ocorre como resultado do desequilíbrio entre o sistema imunológico do paciente, as bactérias e as condições no interior da ferida, o que pode precipitar a proliferação bacteriana (FRYKBERG; BANKS, 2015). Portanto, a infecção ocorre quando as condições na ferida são ideais para que as bactérias se multipliquem e também quando há baixa resistência do paciente (BENBOW, 2010).

Acredita-se que as infecções associadas a DM são predominantemente polimicrobianas, e vários gêneros bacterianos podem contaminar a ferida destes pacientes, como por exemplo, *Staphylococcus, Pseudomonas, Streptococcus, Enterococcus, Corynebacterium, Acinetobacter, Prevotella, Porphyromonas* e membros da família *Enterobacteriaceae*. Entre as espécies gram-positivas e gram-negativas que se encontram predominantemente em feridas diabéticas, destacam-se o *Staphylococcus aureus* e *Pseudomonas aeruginosa* (MENDES et al., 2014; SPICHLER et al., 2015).

As feridas crônicas são consideradas um dos problemas de saúde mais agravantes relacionados à DM. Cerca de 15% a 20% de pacientes diabéticos (WORLD HEALTH ORGANIZATION – WHO, 2016) desenvolvem feridas ao longo da vida. Portanto, o tratamento de feridas infectadas é considerado um tema de grande importância médica, particularmente, em um período em que o uso indiscriminado de antimicrobianos tem dificultado o tratamento hospitalar

dos pacientes, com repercussão no aparecimento cada vez maior de microorganismos resistentes a essa classe de fármacos (ALVES et al., 2008).

Frente a isto, em situações em que ocorre um aumento na proliferação bacteriana em feridas diabéticas, umas das alternativas para tentar contornar esta situação é a utilização de curativos com capacidade de reduzir a proliferação bacteriana. Estes podem atuar como adjuvantes na terapia sistêmica com antibioticoterapia, como por exemplo, o curativo impregnado com ativos (JAYAKUMAR et al., 2011).

Staphylococcus aureus

O *S. aureus* é uma bactéria esférica, do grupo dos cocos gram-positivos, medindo aproximadamente 0,5 a 1,5 µm de diâmetro, é considerado imóvel, não esporulado e geralmente não encapsulado. Apresenta-se em diversas formas, que vão desde isolados, aos pares, em cadeias curtas, ou agrupados irregularmente (com aspecto semelhante a um cacho de uvas), devido à sua divisão celular (Figura 3) (CASSETTARI, et al., 2005).

Figura 3. Microscopia da bactéria *S. aureus* (Adaptado de Kilham, 2015).

O gênero *Staphylococcus* pertence à família *Micrococcae*, possui 33 espécies, sendo que 17 delas podem ser isoladas de amostras biológicas humanas. Comumente, esse gênero faz parte da microbiota da pele humana normal, sendo que a espécie de maior interesse médica é o *S. aureus* (KONEMAN, et al., 2001; CASSETTARI, et al., 2005). Embora esta bactéria seja

considerada um dos principais patógenos envolvidos em feridas diabéticas, ainda pouco se sabe sobre a patogênese dessa doença (BRAUNWALD et al., 2002). Estudos demonstram que pacientes diabéticos apresentam maior colonização bacteriana na pele e fossas nasais por *S. aureus* do que indivíduos não diabéticos (BANNERMAN et al., 2003; CASSETTARI, et al., 2005). O surgimento de cepas de *S. aureus* resistente a diversos antimicrobianos iniciou entre as décadas de 50 e 70, começando com a resistência à penicilina e linhagens multirresistentes, que tornaram o tratamento de infecções estafilocócicas especialmente problemático (COUTO; PEDROSA, 1999). Mais tarde, foi isolado *S. aureus* resistente à vancomicina, um dos antibióticos de última escolha para pacientes com feridas crônicas nos pés, um dos maiores problemas relacionados à DM (DAVIS, 2005). Embora, a maior suscetibilidade de pacientes diabéticos a infecções por diferentes tipos de bactérias esteja bem estabelecida, a cronicidade associada a essas infecções ainda é pouco conhecida.

Escherichia coli

A *E. coli*, pertence à família *Enterobacteriaceae* e ao gênero *Escherichia,* o qual compreende diversas espécies (CAMPOS; TRABULSI, 2002). Esta bactéria encontra-se em formato de bastonete curto, é gram-negativa, não esporulada, medindo em torno de 1,1 a 1,5 µm por 2 a 6 µm, sendo a maioria móvel devido à existência de flagelos peritríqueos (Figura 4) (OLIVEIRA et al., 2004). A temperatura ótima de crescimento destas bactérias é em torno de 37ºC, o que favorece seu crescimento em feridas diabéticas (QUINN et al., 2005).

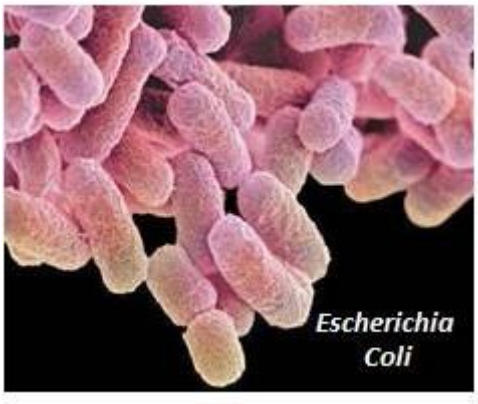

Figura 4. Microscopia da bactéria *E. coli* (Adaptado de < https://diariodebiologia.com/2011/07/surpreendentes-imagens-de-microscopia-eletronica/>).

As cepas de *E. coli* são frequentemente isoladas de infecções de pele, porém, seu potencial de virulência ainda não foi completamente estudado (PETKOVŠEK et al., 2009). Sabe-se que as infecções causadas por bactérias, como no caso da *E. coli* ocasionam descompensação em pacientes com DM, alterando o controle metabólico e aumentando consequentemente as necessidades diárias de insulina para os que fazem uso da mesma. Além disto, em pacientes sem maiores complicações crônicas, existe controvérsia quanto a maior predisposição do indivíduo ao desenvolvimento de infecções (KONOP et al., 2018). Desta forma, quando o processo infeccioso é instalado, pode ocorrer um agravamento do controle metabólico o que resulta em um paciente com maiores problemas de cicatrização das feridas contaminadas, tornando assim a busca por novas alternativas terapêuticas de extrema importância (AHMED et al., 2018).

Diabetes *melittus*

A primeira referência sobre a DM data de 1500 a.C., com descrições de uma doença caracterizada pela emissão frequente e abundante de urina. Os primeiros estudos começaram por volta de 1869, quando Langerhans publicou sua tese de doutorado na qual demonstrava a presença no pâncreas de grupos de células vascularizadas, independentes do pâncreas exócrino (HERRERA, 1979).

Anormalidades associadas com essa doença envolvem o metabolismo de carboidratos, proteínas e lipídeos, podendo afetar tanto seres humanos quanto animais. Desta forma, comorbidades como dislipidemias, hipertensão, obesidade provenientes do sedentarismo e ingestão alimentar excessiva podem resultar no acometimento da DM. Devido a estes fatores, sua prevalência tem aumentado dramaticamente ao longo das últimas décadas (ISLAN, 2011).

Segundo dados da Organização Mundial de Saúde (OMS) (2018), a DM é considerada uma das principais doenças crônicas que afetam o homem contemporâneo e tem sido conceituada como uma síndrome patológica que se manifesta clinicamente através de desordens endócrino-metabólicas associadas a um descontrole dos níveis glicêmicos no sangue. No ano de 2015, a Federação Internacional de Diabetes (*International Diabetes Federation*) estimou que 8,8% (intervalo de confiança de 95%: 7,2 a 11,4) da população mundial com 20 a 79 anos de idade vivia com DM. Portanto, se as tendências atuais persistirem, o número de pessoas com esta doença foi projetado para ser superior a 642 milhões em 2040. Ainda, cerca de 75% dos casos são de países em desenvolvimento, nos quais deverá ocorrer o maior aumento dos casos de DM nas próximas décadas. Este aumento da prevalência da DM está associado a fatores, como estilo de vida sedentário e maior frequência de excesso de peso (BEAGLEY et al., 2014).

No homem, as células beta (β) presentes no pâncreas são acometidas por disfunções que podem resultar na deficiência absoluta ou relativa da secreção de insulina dando origem à síndrome da DM (PÖPPL; GONZÁLEZ, 2005). A insulina é um hormônio de estrutura polipeptídica formado a partir de um precursor denominado pró-insulina. Diversos estímulos estão envolvidos na modulação da secreção de insulina pelas células β, sendo o nível de glicose sanguínea o mais importante deles. Diante de uma hiperglicemia ocorre síntese e liberação de insulina por exocitose. Este hormônio então se liga aos seus receptores e ativa os sistemas de transporte de glicose através da membrana celular (CISTERNAS, 1999).

A DM é dividida principalmente em dois tipos: tipo 1 e 2. Nos pacientes diabéticos tipo 1 existe uma deficiência na produção de insulina, que é decorrente da destruição autoimune das células β do pâncreas, desta forma, seu tratamento exige uso de insulina exógena (KING; BOWE, 2016). Já no paciente

portador da DM tipo 2, os tecidos do corpo se tornam resistentes à ação da insulina, com disfunções concomitantes nos receptores deste hormônio. Neste caso, é frequente a utilização de agentes hipoglicemiantes orais, e a insulina pode ou não ser utilizada (BARRETT et al., 2013).

Diabetes x Cicatrização

Diversos fatores interferem no metabolismo do processo de cicatrização em portadores da DM, tornando-o lento e sujeito a complicações (GOOVA et al., 2001). A dificuldade de cicatrização está relacionada com a DM, no qual ocorre uma diminuição dos níveis de oxigênio que chega aos nervos através de pequenos vasos sanguíneos. Além disto, pode ocorrer a formação de um processo inflamatório, levando ao mau funcionamento dos nervos e causando consequentemente a neuropatia diabética (DEVECI et al., 2005). Ademais, ocorre uma diminuição da angiogênese, alteração na proliferação de queratinócitos, fibroblastos e células endoteliais, diminuição da migração de fibroblastos, defeitos na deposição de colágeno e diminuição da produção de fatores de crescimento também prejudicando a cicatrização (SPRAVCHIKOV et al., 2001). Os efeitos ocasionados pela DM envolvem a participação de diferentes processos bioquímicos, como o aumento do estresse oxidativo e a alta expressão de genes pró-inflamatórios e de fatores de transcrição (MAKRANTONAKI et al., 2016).

Além das alterações diretamente relacionadas com as fases da cicatrização, a hiperglicemia crônica induz modificações nas células endoteliais, dando origem a microangiopatia diabética, uma enfermidade que ocasiona uma diminuição do fluxo do sanguíneo. Esta diminuição do fluxo de sangue, causada pelos danos endoteliais, diminui a oxigenação e nutrição dos tecidos dificultando o processo de cicatrização como um todo, além de estar associada a lesões isquêmicas (AKBARI; LOGERFO, 1999).

Outro problema desencadeado em portadores da DM é a neuropatia diabética, onde a redução do fluxo sanguíneo em capilares perineurais promove lesões isquêmicas no entorno do epineuro e do perineuro. Associadas às alterações metabólicas, estas lesões levam à redução da condução de impulsos sensitivos e motores nos nervos periféricos, desencadeando dor, formigamento

ou perda de sensibilidade, principalmente nas mãos, braços, pés e pernas (GUIMARÃES et al., 2009). Com a sensibilidade dolorosa e o reflexo protetor diminuídos, os pacientes diabéticos ficam expostos a lesões e traumatismos que ocorrem sem que os doentes percebam (OLIVEIRA, 2007).

Frente a isto, portadores da DM que não controlam os níveis séricos de glicose, desencadeando uma hiperglicemia, apresentam maior predisposição a desenvolver problemas, principalmente em tecidos cujas células não dependem da insulina para captar a glicose, como é o caso do sistema nervoso, do coração, dos rins e dos capilares sanguíneos, resultando em uma alta concentração de glicose no meio intracelular (AHMED, 2005). Ainda, a hiperglicemia ocasiona alterações no metabolismo que causam danos às células e seus constituintes. Acredita-se que a glicose elevada atue em diversas vias metabólicas, dentre elas, a glicação proteica, a via do poliol e a via da proteína cinase C, que estão relacionadas com a cicatrização (DeFronzo et al., 2015).

Modelo animal experimental de diabetes *mellitus*

Estudos envolvendo modelos animais são de grande importância, tendo em vista as diversas limitações de investigação da diabetes diretamente em seres humanos. Existe uma semelhança fisiológica entre roedores e os seres humanos, tornando o modelo experimental de DM uma importante ferramenta para o melhor entendimento desta patologia (CHATZIGEORGIOU et al., 2009). Os modelos experimentais utilizados para induzir a DM em animais de experimentação podem ser de dois tipos: modelos induzidos quimicamente ou modelos espontâneos (ETUK, 2010; MORDES et al., 2004; SAKATA et al., 2012).

A indução química da DM em animais de experimentação ocorre após a destruição seletiva das células β pancreáticas. O aloxano e a STZ (Figura 5), são as duas substâncias amplamente utilizadas para desenvolver estes protocolos. Ambas exercem ação diabetogênica nos animais. A dose necessária dos indutores para a indução depende da espécie, via de administração e do estado nutricional (LENZEN, 2008).

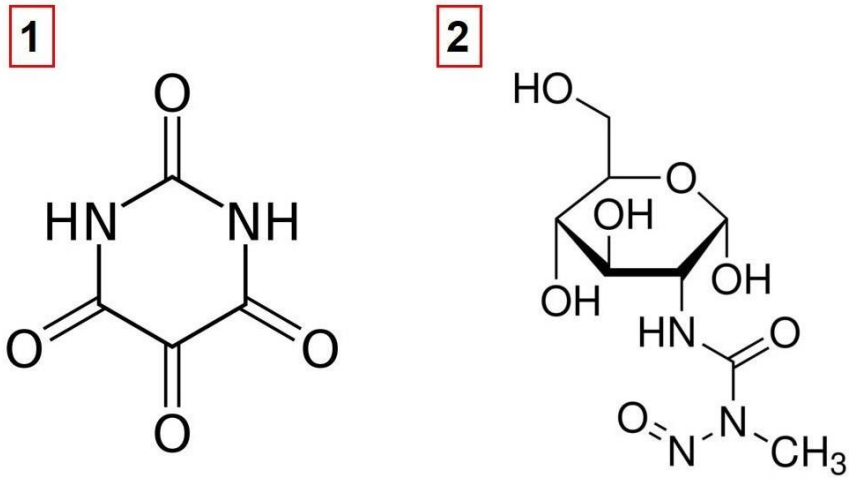

Figura 5. Estrutura química do aloxano (1) e estreptozotocina (2).

O alonaxo inibe a glicocinase, formando espécies reativas de oxigênio que acometem as células β pancreáticas, desencadeando o estado da DM. Este modelo é menos utilizado devido às dificuldades em induzir a DM e manter os animais em condições favoráveis para a realização do experimento (LENZEN, 2008).

Outro indutor de DM é a STZ, um glicosídeo nitrosurea (N-metil-N nitrosouréia) natural isolado do *Streptomyces achromogenes.* Essa substância causa dano nas bases do ácido desoxirribonucleico (DNA), diminuindo a nicotinamida adenina dinucleotídeo (NAD^+), a qual inibe a biossíntese e a secreção de insulina promovendo a destruição irreversível das células β pancreáticas, resultando na síndrome diabética (AKBARZADEH et al., 2007). Desta forma, a STZ apresenta vantagens quando comparada com outros indutores químicos de DM, principalmente pela facilidade de indução e por manter os animais em condições adequadas para a realização do protocolo experimental (AKBARZADEH et al., 2007).

Biopolímeros constituindo curativos

A escolha adequada do material a ser utilizado na constituição de um curativo dérmico é um dos primeiros passos para se atingir uma favorável cicatrização da

pele lesada (GURTNER et al., 2008). Os biopolímeros são polímeros produzidos por organismos vivos; em outras palavras, são biomoléculas poliméricas, classificadas estruturalmente como polissacarídeos, poliésteres ou poliamidas (KHACHATOORIAN et al., 2003; MOHANTY et al., 2005).

Entre os materiais bioativos, são amplamente explorados os biopolímeros de diferentes origens. O uso destes biopolímeros como material de tratamento de feridas tem um amplo escopo, devido às suas excelentes propriedades de biocompatibilidade, capacidade de suportar o crescimento celular, potencial regenerativo, biodegradabilidade e durabilidade (SAHANA; REKHA, 2018). Estes compostos encontram-se disponíveis na natureza em grandes quantidades, podendo ser obtidos com elevado grau de pureza e a custos relativamente baixos, sendo possível em vários casos ser realizado nos mesmos, modificações químicas o que irá tornar suas propriedades mais apropriadas para aplicações específicas (BURD, HUANG, 2008).

Alguns exemplos de biopolímeros que podem ser empregados na constituição de curativos são os polissacarídeos, quitina, quitosana, alginato, pectina, xantana, celulose e seus derivados (metilcelulose, carboximetilcelulose) (MUZZARELLI, 2009; MURAKAMI et al., 2010; YANG et al., 2010; BELLINI et al., 2012; FAN et al., 2013; BELLINI et al., 2015). A celulose, principal componente presente na estrutura das plantas, é o polímero natural mais abundante na terra, com uma elevada taxa de regeneração via fotossíntese. Este polímero é um polissacarídeo de fórmula molecular $(C_6H_{10}O_5)_n$, composto pela união de moléculas de β-D-glucopiranose através das ligações β-$_{1,4}$-glicosídicas (Figura 6) (KLEMM et al., 2005; VIERA, 2013).

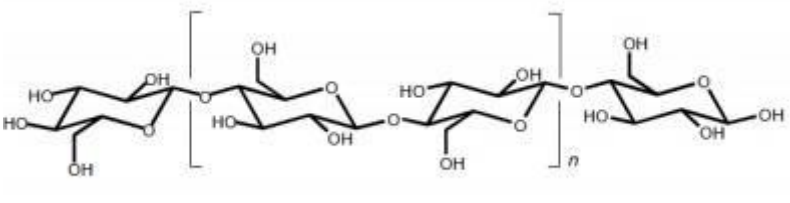

Figura 6. Estrutura química da celulose.

A aplicação de materiais feitos a partir de celulose no tratamento de feridas se dá principalmente devido às suas propriedades de retenção de umidade, tendo em vista que feridas úmidas são conhecidas por curar mais rapidamente devido ao fornecimento adequado de fatores de crescimento e outras moléculas para os tecidos em cicatrização (SULAEVA et al., 2015). A celulose contribui também na absorção de exsudatos, resultando na absorção de restos celulares (como tecido necrótico e revestimento fibrinoso) e a estrutura de celulose porosa auxilia na regeneração tecidual (KUCIŃSKA-LIPKA et al., 2015). Ainda, a celulose pode ser facilmente misturada com outros materiais, particularmente os antimicrobianos, para prevenir as infecções no local da ferida (LIN et al., 2013).

Incorporação de agentes biologicamente ativos em curativos

Uma estratégia que tem ganhado grande atenção na produção de curativos dérmicos e sido fundamental na prevenção e terapia de infecções é a incorporação de agentes bioativos diversos nos curativos constituídos de biomateriais (JAYAKUMAR et al., 2011). Neste contexto, o uso de vitamina C e própolis incorporados neste tipo de formulação contendo celulose é vantajoso, uma vez que o tempo de contato do filme com os microrganismos, que normalmente estão presentes neste tipo de lesão, pode ativar os mecanismos de transporte passivo através da membrana celular (DONSI et al., 2010).

Vitamina C

A vitamina C (ácido ascórbico) é um constituinte normal da pele encontrado em níveis elevados tanto na derme quanto na epiderme. Além disto, a vitamina C atua como um antioxidante natural, apresentando propriedades anti-inflamatórias, fotoprotetoras, além de ser um bioestimulador conhecido da síntese de colágeno e síntese de hidroxiprolina e hidroxilisina (BLASCHKE et al., 2013). O efeito antioxidante desta vitamina desempenha um papel importante auxiliando na proteção contra espécies reativas de oxigênio derivadas da atividade metabólica (SIES, 2014). Devido a esta atividade, ela protege componentes biológicos, como lipoproteínas de baixa densidade, contra modificações oxidativas e, assim, pode assumir um papel ateroprotetor (PADAYATTY et al., 2003).

Ainda, a vitamina C é útil para construir uma resistência frente às doenças. Sendo essencial na dieta dos seres humanos esta vitamina está presente no cérebro dos mamíferos, juntamente com várias aminas neurotransmissoras. Frente a isto, esta vitamina tem sido utilizada para o tratamento de diversas enfermidades, tais como resfriados, infertilidade e câncer (KARIMI-MALEH et al., 2014).

Própolis

A própolis é uma mistura complexa produzida pelas abelhas a partir de diversas substâncias coletadas de diferentes partes das plantas como botões florais, exsudatos e brotos. As abelhas utilizam a própolis para proteger a colmeia contra agente invasores, embalsamar animais, manter a temperatura agradável e para o preparo de locais assépticos para a postura da abelha rainha (KUMAZAWA et al.,2004).

A composição da própolis é de 50% de resina constituída por flavonóides e ácido fenólico, 5% pólen, 30% cera, 10% óleos essenciais e 5% outros compostos, tendo variações conforme a vegetação na qual é colhida (PIETTA et al., 2002; VARGAS et al., 2004). Muitas propriedades biológicas têm sido atribuídas à própolis, como atividade antioxidante, antimicrobiana e anti-inflamatória (DOBROWOLSKI et al., 1991; NAGAI et al., 2003; KUMAZAWA et al., 2004). A própolis também é amplamente utilizada para prevenir e tratar resfriados, feridas, úlceras, reumatismo, entorses, doenças cardíacas e DM (HU et al., 2005; ZHU et al., 2011; LI et al., 2012).

Estes efeitos terapêuticos têm sido atribuídos aos diversos compostos fenólicos que compõem a própolis, sendo os flavonoides considerados os principais compostos, e encontram-se, ainda, alguns ácidos fenólicos e seus ésteres, aldeídos fenólicos, álcoois e cetonas (PARK et al., 1998). Por estas razões, a própolis está sendo amplamente utilizada na medicina popular, com uso extensivo em alimentos e bebidas para melhorar a saúde e prevenir doenças. A aplicação médica da própolis levou a um aumento no interesse em sua composição química e potencial uso clínico em humanos (SALATINO et al., 2011; TORETI et al., 2013).

Baseado nas considerações apresentadas acima, o desenvolvimento de novos biomateriais projetados para o tratamento de feridas diabéticas é de suma importância. Ainda, ressalta-se que filmes a base de celulose carregados com própolis e vitamina C podem ser promissores na cicatrização de feridas devido às suas diversificadas propriedades farmacológicas descritas.

4. Artigo científico

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio artigo.

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Polysaccharide-based film loaded with vitamin C and propolis: A promising device to accelerate diabetic wound healing

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ABSTRACT

Wound healing can be a painful and time-consuming process in patients with *diabetes mellitus*. In light of this, the use of wound healing devices could help to accelerate this process. Here, cellulose-based films loaded with vitamin C (VitC) and/or propolis (Prop), two natural compounds with attractive properties were engineered. The starting materials and the cellulose-based films were characterized in detail. As assessed, vitamin C can be released from the Cel-PVA/VitC and Cel-PVA/VitC/Prop films in a controlled manner. In vitro antibacterial activity studies showed a reduction of bacteria counts (*Escherichia coli* and *Staphylococcus aureus*) after Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop treatments. Moreover, we examined the antibacterial and wound healing properties of the cellulose-based films in a streptozotocin (STZ)-induced diabetic animal model. Diabetic mice exhibited impaired wound healing while the Cel-PVA/VitC/Prop treatment increased the wound closure. A marked reduction in bacterial counts present in the wound environment of diabetic mice was observed after Cel-PVA/VitC, Cel-PVA/Prop and Cel-PVA/VitC/Prop treatment. Histological analysis demonstrated that the non-treated diabetic mice group did not exhibit adequate wound healing while the treated group with Cel-PVA/VitC and Cel-PVA/VitC/Prop films presented good cicatricial response. Furthermore, these novel eco-friendly films may represent a new therapeutic approach to accelerate diabetic wound healing.

1. Introduction

Wound healing is one of the greatest challenges in medical and biomedical research since it involves a complex process with very particular stages (Gurtner et al., 2008). These stages are related and each one is controlled by specific local and systemic factors that can be affected by numerous variables (Gurtner et al., 2008). For instance, chronic diseases (e.g. coronary artery disease, peripheral vascular disease, cancer, *diabetes mellitus*, among others) are an example of a systematic factor that can impair wound healing (Hess, 2011). Patients with chronic diseases commonly show complications related to wound healing, such as slower heal rate and faster wound worsening (Frykberg and Banks, 2015). These issues related to wound healing can result in severe complications that greatly decrease the patient's quality of life. Therefore, efficient wound management implies providing a favorable healing environment at the site of injury and, also, overcoming the local and systemic factors that impair the healing process (Frykberg and Banks, 2015).

In the light of recent developments in the wound management field, the wound dressing materials have played a key role to fix the aforementioned tasks. Wound dressings are biomaterials utilized to cover the wounds (physical barrier) capable to absorb exudates, maintain the

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0378-5173/

moisture balance, allow gas exchange and prevent the wounds from being infected (Rizzi et al., 2010). Moreover, ideal wound dressings should present similar properties (physicochemical and biological) to the treated area and degradation rate on a time scale compared to wound healing (Chen et al., 2017). Currently, wound dressings formulated from biopolymers (e.g. proteins and polysaccharides) have gained great attention due to their properties compared to other dressings (Birch et al., 2015; Chattopadhyay and Raines, 2014). Biopolymers are generated from renewable sources and, in general, cost lower than synthetic polymers or other materials (e.g. ceramics or metals). In addition, biopolymers show attractive properties such as non-toxicity, biocompatibility, biodegradability and easy processing and mouldability (Agrawal et al., 2014; Facchi et al., 2017). Among the biopolymers, cellulose has been extensively utilized in the formulation of wound dressing materials due to its excellent biocompatibility, good mechanical properties, and chemical structure (Li et al., 2015; Wang et al., 2016). As reported, wound dressings based on cellulose or cellulose-derivatives are utilized as efficient platforms for treating a wide variety of injured tissues (Khamrai et al., 2017; Li et al., 2015).

In this study, cellulose fibers were blended with poly(vinyl alcohol) (PVA), a hydrophilic semi-crystalline, water soluble, and non-toxic synthetic polymer, in order to form a composite film aiming its use as a wound dressing. Herein, the cellulose fibers were extracted from rice husk, a worldwide produced agricultural waste (de Oliveira et al., 2017). Furthermore, the composite films were loaded with vitamin C (L-ascorbic acid) and propolis. Vitamin C is a well-known natural antioxidant involved in all phases of wound healing, mainly in collagen formation phase (Mohammed et al., 2015). Moreover, some studies report the participation of vitamin C in the immune system modulation (Manning et al., 2013). Propolis, a resinous substance produced by bees, is a natural antibacterial, antifungal, and antiviral agent that according to some authors is able to improve some biological mechanisms involved in wound healing (e.g. epithelialization) (Freires et al., 2016; Martinotti and Ranzato, 2015). Anti-inflammatory action is also attributed to propolis (de Almeida et al., 2013; Freires et al., 2016). Therefore, this study attempt to investigate the antibacterial and wound healing properties of these cellulose-based film loaded with vitamin C and propolis in order to generate an advanced and eco-friendly wound dressing device.

2. Experimental

2.1. Materials

PVA (98% hydrolyzed and M_w of 124,000 g/mol), glutaraldehyde (25% v/v in water) and streptozotocin (STZ) from Sigma-Aldrich (USA). Vitamin C (L-ascorbic acid, analytical grade) from Sinopharm (China). Brazilian propolis (lyophilized, main constituents: kaempferide, isosakuranetin, and trace amounts of kaempferol) from Catedral Pharmaceuticals (Brazil). Ethanol (P.A.), sodium hydroxide (NaOH, P.A.), hydrochloric acid (HCl, 30–34% v/v) and hydrogen peroxide (H_2O_2, 35% w/v) from Synth (Brazil). Rice husk (RH) was kindly donated by a local rice mill (Pelotas-RS, Brazil). All chemicals were used as-received.

2.2. Extraction of cellulose from rice husk

RH was previously ground and, then, washed with Distilled water at room temperature under mechanical stirring in order to remove impurities. This process was done twice. The washed RH was dried overnight in an oven at 60 °C. The dried RH was subsequently treated using an aqueous NaOH solution (5% w/v). In this case, the RH:NaOH solution ratio was fixed in 1:30 (w/v). This system (RH/NaOH solution) was kept under vigorous mechanical stirring at 80 °C for 4 h. After that, the cellulose pulp was recovered using vacuum filtration. It was thoroughly washed with Distilled water till the neutral pH is attained and dried at 60 °C overnight. The treatment with NaOH solution was performed twice in order to ensure the complete removal of hemicellulose and lignin. Next, the dried cellulose pulp was bleached using an alkaline H_2O_2 solution (30% w/v, pH 9). Again, the cellulose pulp: H_2O_2 solution ratio was fixed in 1:30 (w/v). The bleaching reaction was kept under mechanical stirring at 50 °C for 3 h. Thereafter, the reaction system was allowed to cool up to room temperature and an excess of Distilled water was added to it. The bleached cellulose fibers were recovered by filtration, exhaustively washed with Distilled water up to the neutral pH is attained and, then, dried at 50 °C overnight. The dry cellulose fibers were milled and sieved using a 32-mesh sieve. The cellulose yielding was 32% in respect to the initial dry RH weight.

2.3. Synthesis of cellulose-based films

Films were prepared by the solvent casting method using the extracted cellulose and PVA. Firstly, PVA (0.1 g) was solubilized in Distilled water (50 mL) at 80 °C for 4 h under magnetic stirring. Next, the PVA solution was cooled to room temperature and, then, cellulose (0.3 g) was added to it (cellulose:PVA mass ratio is 3:1). The cellulose-PVA mixture remained under magnetic stirring for 15 min and, then, it was placed in an ultrasonic bath for 30 min at 30 °C in order to ensure the system homogeneity. Next, the pH of the cellulose/PVA solution was adjusted to 4 using HCl solution (1.0 M) and 25 µL of glutaraldehyde (crosslink agent) was added. The filmogenic mixture was poured into a Petri dish (85 × 10 mm), which was subsequently placed in an oven (at 40 °C) for 24 h in order to evaporate the solvent. Finally, the resulting cellulose-PVA film was peeled off from the Petri dish, purified with Distilled water and, then, dried at 40 °C for 24 h in an oven. This film sample was labeled as Cel-PVA, respectively.

Furthermore, cellulose-PVA film samples containing vitamin C (VitC), propolis (Prop) or both of them (VitC and Prop) were synthesized separately using the above-described casting method with minor modifications. To synthesize the VitC-containing film, a specific amount of this compound (4 mg) was added to the PVA solution just after the cellulose-adding step. Similarly, to the synthesis of the Prop-containing film, 4 mg of this compound was added to the PVA solution just after the cellulose-adding step. To synthesize the film sample containing both compounds (VitC and Prop), 4 mg of each compound added to the PVA solution just after the cellulose-adding step. These film samples were labeled as Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop.

2.4. Characterization

Fourier transform infrared (FTIR) spectra were obtained using a Shimadzu IR Affinity-1 spectrometer (Japan) by co-adding 64 scans in the 4000–400 cm^{-1} range at 4 cm^{-1} resolution. Before the spectra acquisition, the samples were ground with spectroscopic grade KBr and pressed into discs. Powder X-ray diffraction (XRD) measurements were performed in a Siemens D-500 diffractometer (Germany) using the Cu-K_α radiation ($\lambda \approx 1.54$ Å) at a voltage of 30 kV and current of 20 mA. Diffraction patterns were recorded at a scan rate of 4°/min in a 2θ range of 10°–70°. Prior analysis the dry samples were powdered using an Anton Parr BM500 (USA) ball mill equipment. Mechanical properties were investigated by tensile tests using a texturometer equipment (Stable Microsystems TA.XT2, UK) with sample films 80 × 25 mm (length x width) in an atmosphere of 50% relative humidity with a test speed of 0.8 mm/min according to the ASTM D882-12 method (Tabari, 2017). The scanning electron microscopy (SEM) images were obtained using a JEOL JSM-6610LC microscope (USA) using an acceleration voltage of 8–10 kV. The dry samples were cut into rectangular pieces (about 4 × 2 mm), which were attached to sample holders with carbon tapes. Before the SEM visualization, these samples were gold-coated by sputtering. The liquid uptaking ability of the synthesized films was investigated by swelling experiments. Briefly, dry samples were weighed and then placed into vials filled with 50 mL of simulated

wound fluid (SWF) (2% w/v of bovine albumin, 0.02 M calcium chloride, 0.4 M sodium chloride, pH 7.4) (Said et al., 2014) at 37 °C. At pre-determined time intervals, the samples were withdrawn, blotted carefully with tissue paper to remove the surface-adhered liquid droplets and reweighed. The percentages of swelling at different times were calculated as follows:

$$swelling\ (\%) = \left[\frac{w_s - w_d}{w_d}\right] x100 \quad (1)$$

where w_s (g) and w_d (g) are the sample weights in the swollen state and in the dry state (n ≈ 3).

2.5. In vitro vitamin C release experiments

Release profiles of vitamin C from Cel-PVA/VitC and Cel-PVA/VitC/Prop were investigated in SWF (50 mL, pH 7.4). For this, film samples (100 mg) were soaked into the releasing media at 37 °C, which was kept under mild stirring. At pre-determined time intervals, an aliquot (4 mL) of the releasing media was taken out and its absorbance at a wavelength of 264 nm was measured using a UV–Vis Micronal BS82 spectrometer (Brazil). An equivalent volume (4 mL) of fresh releasing media was refilled in the system. The absorbance data were converted into concentration using a calibration curve, which was previously built using standard concentrations of vitamin C in SWF.

2.6. Biological experiments

2.6.1. In vitro antibacterial activity

The antibacterial activity of the cellulose-based films was tested using the Pour plate method with minor modifications. Briefly, overnight cultures of *Escherichia coli* (*E. coli*) (ATCC 25922), *Staphylococcus aureus* (*S. aureus*) (ATCC 6538) were diluted to adjust a microbial count of 1.5×10^8 CFU/mL. Moreover, film samples were aseptically sectioned (3 cm × 3 cm) and, then, distributed in sterile Petri dishes. These samples were inoculated with 1 mL of tested bacterial solution. After inoculation, 20 mL of the sterile standard counting agar (PCA) medium was added to the Petri dishes, which were incubated in a bacteriological oven for 48 h at 37 °C under aerobic conditions. Petri dishes without the films samples (inoculum only) were used as control. After the incubation period, bacterial colonies were counted using a colony counter and the mean log CFU/cm^2 was calculated. The bacterial reduction to evaluate the effectiveness of the tested materials was calculated as the difference between the log CFU of the inoculum and the log CFU recovered from the films samples.

2.6.2. Diabetic wound healing study in mice model

The experiments were carried out using male adult *Swiss* mice (25–30 g) from our own breeding colony. Animals were kept in a separate animal room, in a 12 h light/dark cycle (with lights on at 6:00 a.m.), at a room temperature of 22 ± 2 °C, with free access to food and water. The present experimental study was approved by the Ethical Research Committee of the Federal University of Pelotas, affiliated to the National Council for the Control of Animal Experimentation (CONCEA).

The diabetic mice were induced as described previously (Morikawa et al., 2005). In brief, mice were intraperitoneally (i.p.) injected with STZ, at the dose of 100 mg/kg, for three consecutive days. STZ was freshly dissolved in a citrate buffer (0.1 M; pH 4.4). Control animals received an injection of citrate buffer (10 mL/kg; i.p.) for three consecutive days. One week after STZ injection, the fasting blood glucose levels (day 0) were measured using the Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, IN). Blood glucose levels over 200 mg/dL confirmed a diabetic phenotype in the animals. After confirmation of diabetic induction, mice were anesthetized with isoflurane inhalation. The dorsal skin of each mouse was shaved, and a 9-mm wound was created on the back of each mouse with a biopsy puncher. Diabetic and non-diabetic animals received the application at the wound site of Cel-PVA, Cel-PVA/VitC, Cel-PVA/Prop, Cel-PVA/VitC/Prop films or no treatment (control and STZ groups). The films were fixed at the wound site with the use of a bandage that left they in direct contact with the lesion. The measurement of wound closure and the application of treatments at the wound site were performed on days 3, 6, 9, 12, and 15. The extent of wound healing was expressed as the percentage (%) of wound area closure. On 15th day, blood glucose levels were monitored using the Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, IN).

2.6.2.1. Open-field test. The open-field test evaluates the general locomotor and exploratory behaviors of mice. The open-field was made of plywood and surrounded by 30 cm-high walls. The floor of the open-field, 45 cm long and 45 cm wide, was divided by masking tape markers into 9 squares (3 rows of 3). On the 15th day, each animal was placed at the center of the open field and observed for 4 min to record the locomotor (number of segments crossed with the four paws) and exploratory (number of rearing on the hind limbs) activities.

2.6.2.2. Quantification of bacteria in treated wounds. On the 15th day, wounds samples from the non-diabetic and diabetic group treated mice were collected for quantification of bacteria. Briefly, the samples were collected with the aid of the swab in an area equivalent to 1 cm^2 of the wound. After wounds collected, the tip of the swabs from each group was transferred to microtubes containing 1 mL of sterile saline solution and vortexed. Immediately, seeding was performed using the calibrated bacterial loop of 10 μL, and Petri dishes containing BHI agar were also used. The inoculums present in the loops were seeded in a straight line in the center of the plate and the scattering was completed with a series of passages at a 90° angle through the original line. The plates were incubated inverted for 24 h at 35 ± 2 °C in a bacteriological oven. The counts were performed with the aid of a colony counter.

2.6.2.3. Histological analysis of wounds. Mice were euthanized on the 15th day, and the wound area was removed to the histological analysis. Wound fragments were fixed in 10% buffered formalin solution. For light microscopy examination, tissues were embedded in paraffin, sectioned at 3–4 μm and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT) for better collagen observation. Two different pathologists using an optical microscope examined all the slides.

2.7. Statistical analysis

The D'Agostino and Pearson omnibus normality test evaluated the normality of data. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test when appropriated. Data were expressed as mean ± standard error of the mean (S.E.M.). Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results and discussion

3.1. Characterization of extracted cellulose

The characterization data and discussion related to the extracted cellulose from rice husks are provided in the Supporting information file.

3.2. Characterization of cellulose-based films

Fig. 1a depicts the FTIR spectra of the pure components of the cellulose-based films. PVA spectrum exhibited a broad band centered at 3457 cm^{-1} associated with the stretching of O–H bonds, bands in the

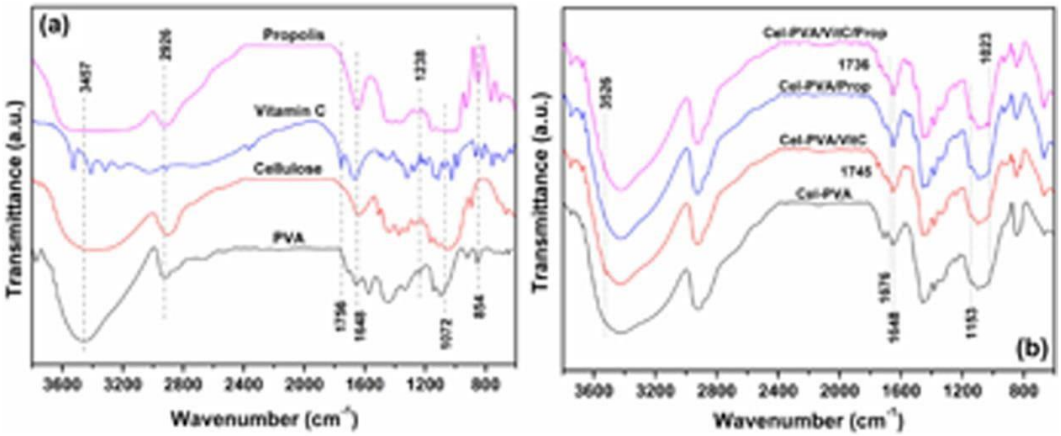

Fig. 1. (a) FTIR spectra of PVA, extracted cellulose, pure vitamin C and propolis. (b) FTIR spectra of the cellulose-based films (Cel-PVA, Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop).

region 2941–2910 cm^{-1} assigned to the C–H stretching vibration (CH_2 groups) and a band at 1710 cm^{-1} related to the C=O bond of residual acetate groups. The bands in the region 1140–1070 cm^{-1} were assigned to the C–O stretching vibration (Alves et al., 2016). The cellulose spectrum exhibited a band at 1640 cm^{-1} assigned to the O–H bending of absorbed water, intense bands in the region 1170–900 cm^{-1} associated to C–O–C and C–O motions of the β-glycosidic linkages and a sharp band at 898 cm^{-1} attributed to the C–H rock vibrations. Vitamin C spectrum showed sharp bands in the region 3530–3210 cm^{-1} associated with free and linked (H-bonds) hydroxyl groups and bands at 1756, 1672, 1321, and 1141 cm^{-1} assigned to the stretching vibration of C=O, C=C, C=C–OH (enol group) and C–O–C bonds (Desai and Park, 2005). Propolis showed a broad band in the region 3550–3100 cm^{-1} that correspond to O–H stretching and wagging vibrations (hydroxyl groups of phenolic compounds) (Oliveira et al., 2015). The bands in the region 2930–2880 cm^{-1} were assigned to the aliphatic and aromatic C–H stretching vibration. Moreover, the bands at 1648, 1238, 1072, and 854 cm^{-1} were associated with the stretching vibration of C=O and C=C (aromatic ring), C–O (polyols and ester groups of flavonoids) and angular deformation outside the plane of the aromatic C–H bond (Mot et al., 2011). Fig. 1b depicts the FTIR spectra of the cellulose-based films (Cel-PVA, Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop). Cel-PVA spectrum showed the typical bands of pure PVA and cellulose with some discrepancies. The broad band centered at 3428 cm^{-1} was assigned to the O–H stretching that compared to PVA spectrum was shifted to lower wavenumber region whereas compared to cellulose spectrum it was shifted to a higher region. The cellulose/PVA blending as well as the crosslinking with glutaraldehyde decreased the intramolecular H-bonds of PVA and increased the extension of intermolecular H-bonds between the polymers. Furthermore, the bands associated with the C–H stretching and bending vibrations (2920–2880 cm^{-1} and 1458 cm^{-1}) increased in intensity due to the alkyl groups of glutaraldehyde. The bands assigned to the C–O bonds (1140–1050 cm^{-1}) also increased in intensity indicating the acetal linkage between glutaraldehyde and the polymers. These data confirm the crosslinking of cellulose/PVA blending by glutaraldehyde (Yang et al., 2014). The spectra of vitamin C and/or propolis-loaded Cel-PVA films showed slight modifications as compared to the pristine film spectrum (Fig. 1b). Cel-PVA/VitC spectrum revealed the appearing of two bands at 3526 and 1745 cm^{-1} assigned to vitamin C (O–H and C=O stretching vibrations) and a shoulder-like band at 1672 cm^{-1} assigned to the stretching vibration of the C=C bond. These finds, confirm the stability of vitamin C after encapsulation. On the other hand, no band related to only propolis was encountered in the Cel-PVA/Prop spectrum likely due to the overlapping of such band by the Cel-PVA bands. However, it is clear that some bands present higher intensity due to the presence of propolis or they were shifted, which suggest an interaction between Cel-PVA and propolis (Oliveira et al., 2015). More intense bands at 1648 and 1460 cm^{-1} can be associated with the aromatic ring vibration of propolis (Oliveira et al., 2015). Finally, the Cel-PVA/VitC/Prop spectrum exhibited changes compared to the Cel-PVA spectrum that confirms the encapsulation of both compounds.

Fig. 2a shows a digital photograph of the Cel-PVA film demonstrating its homogeneity and the good dispersion of cellulose within the PVA matrix. The SEM image recorded from the film surface allows noticing the cellulose fibers distributed through a continuous phase (i.e. the PVA matrix) (Fig. 2b). The entire surface is covered by the fibers following a non-oriented distribution. Additionally, the absence of aggregates confirms the good interaction between the cellulose fibers and PVA.

As aforementioned, an ideal dressing device must be able to remove the excess of exudates and also to reduce maceration of surrounding healthy skin preserving an appropriate amount of moisture that is necessary for wound healing. Herein, the liquid uptake capacity of the cellulose-based films was investigated using SWF (pH 7.4) at 37 °C as swelling media. The results presented in Fig. 3a suggesting all films possess high absorptive capacity (equilibrium swelling ratio > 200%). Moreover, all film samples exhibited a fast swelling rate just before their immersion in SWF. The Cel-PVA film showed the highest absorptive capacity (410%) indicating that the presence of vitamin C or propolis into the film affects this property. Cel-PVA and Cel-PVA/Prop have achieved maximum swelling in 50 min, while for Cel-PVA/VitC and Cel-PVA/VitC/Prop the maximum swelling was achieved in 35 min indicating the influence of vitamin C on the liquid absorption mechanism. Vitamin C contains hydroxyl groups in its structure favoring the formation of H-bonds with the Cel-PVA matrix. H-bonds between the vitamin C and Cel-PVA impair the uptake capacity because they reduce the amount of free hydrophilic groups within the film matrix that interact with the water molecules. In addition, H-bonds act as additional crosslink points within the film restricting the matrix expansion and preventing the water influx. The addition of propolis into the Cel-PVA/VitC film attenuates the effect of vitamin C as observed in Fig. 3a. It is worth to note that all swelling curves remained constant after the equilibrium up to the end of the experiment suggesting all film samples are stable into SWF.

Wound healing devices must present good mechanical properties since they promote physical protection against forces experiences in

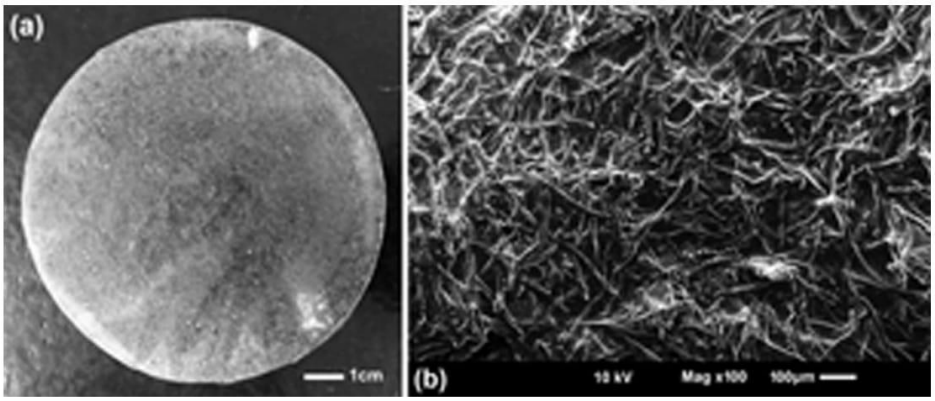

Fig. 2. (a) Photographic and (b) SEM images of as-prepared Cel-PVA film.

daily living. Moreover, good mechanical properties make easy the device handling. Wound healing devices based on cellulose can exhibit enhanced mechanical properties since cellulose fibers show high tensile strength and low density. Cellulose has a unique supramolecular structure in which microfibrils are held together by H-bonds (Cho et al., 2017). Herein, the mechanical properties of the cellulose-based films were investigated by tensile testing. As illustrated in Table 1, the cellulose-based films showed high Young modulus (10.4–15.5 GPa) and tensile strength (370.1–495.6 MPa). On the other hand, low elongation at break point (3.2–3.5%) was verified. These high mechanical properties can be ascribed to the good dispersion of cellulose fibers on PVA and to the H-bonds formed between these two polymers. H-bonds act as additional crosslinking point stiffening the polymer matrix. The vitamin C-loaded film exhibited an increment in Young modulus (~49%) and tensile strength (~34%) as compared to the Cel-PVA film. As suggested, vitamin C interacts with the polymer matrix increasing the film rigidity. This interaction between vitamin C and the polymer matrix is harmed by the presence of propolis, which decreases the mechanical properties of Cel-PVA/VitC/Prop as compared to the Cel-PVA/VitC. However, the presence of propolis within the polymer matrix did not change the mechanical properties of Cel-PVA/Prop film significantly. Overall, these finds corroborate with the swelling data. It should be mentioned that the cellulose-based films prepared in this work exhibited comparable or even superior mechanical properties than other polysaccharide-based films designed for wound healing purpose (Barud et al., 2011, Ifuku et al., 2013; Mushi et al., 2014). In a recent review paper, Tan et al. discuss the reinforcement effect caused by the incorporation of cellulose from different sources in a PVA matrix (Tan et al., 2015). Overall, Cel-PVA composites exhibited Young modulus values ranging from 1 to 4 GPa when small amounts of Cel are incorporated in a PVA matrix. Considering this information, it could be suggested that the mechanical properties of Cel-PVA composites can be tailored just adjusting the Cel:PVA mass ratio.

3.3. *In vitro* vitamin C release

To investigate the potential of using Cel-PVA film as a carrier for the controlled release of vitamin C we investigated the release profiles of Cel-PVA/VitC and Cel-PVA/VitC/Prop in SWF (pH 7.4) at 37 °C. The cumulative vitamin C release profiles from these film samples are shown in Fig. 3b. As observed, both films were able to control the vitamin C release profile preventing an initial burst releasing during the first minutes. Burst release leads to higher initial drug delivery, which may cause some inconveniences in patients. Moreover, the burst release reduces drastically the effective lifetime of the carrier device (Huang and Brazel, 2001). In general lines, the Cel-PVA/VitC and Cel-PVA/VitC/Prop films showed a comparable release profile. During the first 60 min the vitamin C was released fast, then, the release rate tends to slow down. Next, the release profile became stable up to the end of the experiment (360 min). After immersion for 360 min, the cumulative released amount of vitamin C from Cel-PVA/VitC and Cel-PVA/VitC/Prop was ca. 64% and 76%, respectively. As aforementioned, the

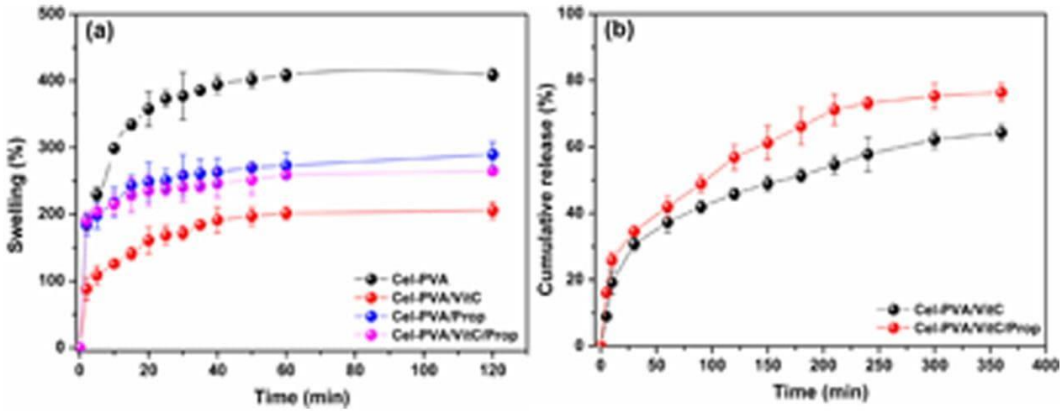

Fig. 3. (a) Swelling kinetics curve of the cellulose-based films and (b) *in vitro* vitamin C release profile from Cel-PVA/VitC and Cel-PVA/VitC/Prop films in SWF at 37 °C (Replicates: n ≈ 3).

Table 1
Mechanical properties of cellulose-based films.

Sample	Composition (w-%)				Mechanical Properties		
	[Cel]	[PVA]	[VitC]	[Prop]	Tensile strength (MPa)	Elongation at break (%)	Young Modulus (GPa)
Cel-PVA	66.7	33.3	–	–	370.1 ± 43.3	3.5 ± 0.5	10.4 ± 1.4
Cel-PVA/VitC	66	33	1	–	495.6 ± 51.6	3.2 ± 0.6	15.5 ± 1.6
Cel-PVA/Prop	66	33	–	1	385.3 ± 37.2	3.4 ± 0.6	11.2 ± 0.9
Cel-PVA/VitC/Prop	65.3	32.7	1	1	421.5 ± 30.9	3.3 ± 0.7	12.8 ± 0.8

encapsulated vitamin C interacts strongly with the Cel-PVA matrix likely due to the H-bonds that take place between the drug and the carrier. The swelling experiments showed that this interaction harms the liquid uptake and as consequence the diffusion of vitamin C outward the polymer matrix is impacted. On contrary, the presence of propolis into the Cel-PVA matrix reduces the density of H-bonds between vitamin C and the carrier favoring the liquid uptake and the drug release.

The release kinetics and mechanism were examined by fitting the release data to various kinetic models such as zero-order (Eq. (2)), first-order (Eq. (3)), Higuchi (Eq. (4)), Hixson-Crowell (Eq. (5)), and Korsmeyer-Peppas (Eq. (6)) (Pereira et al., 2013).

$$Q = k_0 t \tag{2}$$

$$\ln Q_t = \ln Q_0 - k_1 t \tag{3}$$

$$Q = k_H t^{1/2} \tag{4}$$

$$Q_0^{1/3} - Q^{1/3} = k_{HC} t \tag{5}$$

$$\log\left(\frac{Q}{Q_\infty}\right) = \log k_{KP} + n \log t \tag{6}$$

where Q (mg) is the amount of vitamin C released at time t, Q_0 (mg) is the initial amount of vitamin C encapsulated into the film samples, Q_t (mg) is the amount of vitamin C remained into the film samples, and Q_∞ (mg) is the amount of vitamin C released at equilibrium. k_0 (mg L^{-1} min^{-1}), k_1 (min^{-1}), k_H ($min^{-1/2}$), k_{HC} ($min^{-1/3}$ and k_{KP} (min^{-n}) are the release rate constants, and n (dimensionless) is the release exponent associated with the mechanism of release (Korsmeyer et al., 1983). The kinetic parameters, as well as the coefficients of determination (R2), values obtained from the zero-order, first-order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas plots (Fig. S3a–e, Supporting information) are presented in Table 2.

The R^2 values of Higuchi model were found to be higher when compared to the zero-order, first-order, and Hixson-Crowell models (Table 2) indicating that the Higuchi model fits better the experimental data related to the vitamin C release from Cel-PVA/VitC and Cel-PVA/VitC/Prop. Higuchi model deals with the release kinetics of systems with different geometries. It is based on some hypothesis: (i) initial drug concentration into the polymer matrix is much higher than its solubility; (ii) the drug particle size is much smaller in the comparison to the polymer matrix thickness; (iii) the drug diffusion is constant; (iv) ideal sink conditions are always maintained; and (v) boundary effects are neglected (Higuchi, 1963). Moreover, depending on this model, if the drug diffusion is the rate-limiting step, the solid/liquid interface will move towards the interior over time (Higuchi, 1963). As evidenced, the k_H value calculated to Cel-PVA/VitC/Prop sample is slightly higher than that calculated to Cel-PVA/VitC suggesting that the presence of propolis enhances the vitamin C release rate.

The release mechanism was investigated by fitting experimental data to the Korsmeyer-Peppas model, which is based on diffusion laws. This model establishes an exponential correlation between drug release and time, and it predicts the first 60% of drug release (Korsmeyer et al., 1983). According to the data shown in Table 2, the Korsmeyer-Peppas model fits satisfactorily the experimental data (R^2 values ≥0.974). The n exponent values calculated to the Cel-PVA/VitC and Cel-PVA/VitC/Prop were 0.46 and 0.41 indicating that the vitamin C release mechanism from both samples is controlled by a Fickian diffusion process (Fu and Kao, 2010). Fickian diffusion refers to the drug transport process in which the polymer relaxation time is much greater than the characteristic solvent diffusion time (Fu and Kao, 2010). This release mechanism corroborates the data obtained with the Higuchi model.

3.4. Biological experiments

3.4.1. In vitro antibacterial activity

The antibacterial activity of the cellulose-based films was evaluated against two bacteria strains, *E. coli* (gram negative) and *S. aureus* (gram positive). In Fig. 4, it can be observed that Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop films presented antimicrobial activity against the two microorganisms tested.

For *E. coli*, reductions of 36.7, 45.4 and 56.6% in bacteria counts were observed after Cel-PVA/VitC, Cel-PVA/Prop, and Cel-PVA/VitC/Prop treatments, respectively, when compared to the Cel-PVA film. Similarly, a significant reduction in *S. aureus* counts after treatments with Cel-PVA/VitC (28.9%), Cel-PVA/Prop (39.1%), and Cel-PVA/VitC/Prop (46.1%) films was also pointed out.

The use of vitamin C and propolis incorporated in this type of cellulose-containing formulation is advantageous since the time of contact of the film with the microorganisms can activate the mechanisms of passive transport through the cell membrane (Donsi et al., 2010). Vitamin C has anti-inflammatory activity and also healing on wounds and promotes immune defense (Naidu, 2003). Propolis is widely used in drugs with antibacterial action. The synergism between their constituents, such as flavonoids, phenolic acids, and other compounds, confer its antimicrobial activity (Salomao et al., 2008).

3.4.2. Diabetic wound healing study in mice

Considering that impaired wound healing is a complication caused by *diabetes mellitus*, the current study was conducted with the objective of investigating the antibacterial and wound healing properties of the cellulose-based film loaded with vitamin C and/or propolis in a STZ-

Table 2
Kinetics parameters and coefficients of determination (R^2) values from various drug release models for the vitamin C release in SWF (pH 7.4) at 37 °C.

Sample	Zero-order		First-order		Higuchi		Hixson-Crowell		Korsmeyer-Peppas		
	k_0	R^2	k_1	R^2	k_H	R^2	k_{HC}	R^2	k_{KP}	n	R^2
Cel-PVA/VitC	0.001	0.829	0.002	0.923	3.076	0.978	0.003	0.865	0.098	0.46	0.974
Cel-PVA/VitC/Prop	0.002	0.845	0.004	0.934	3.791	0.987	0.005	0.883	0.132	0.41	0.989

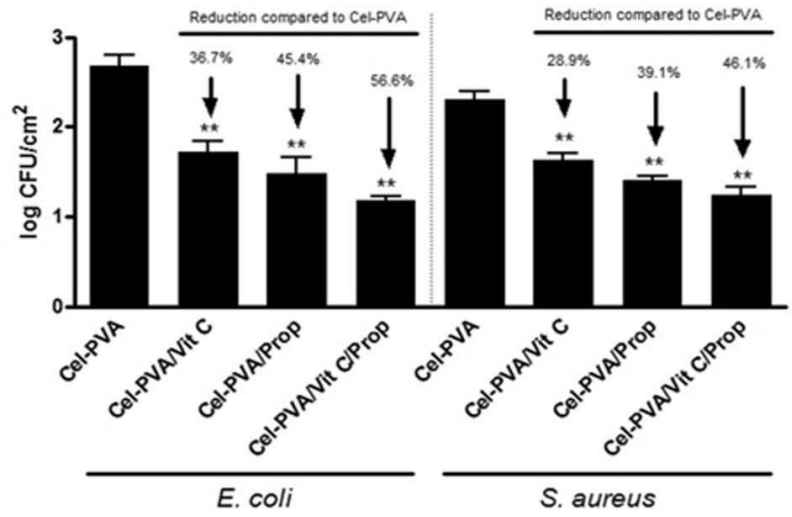

Fig. 4. Antibacterial activity by cellulose-based films loaded with vitamin C and/or propolis against *E. coli* and *S. aureus*. The results were expressed in log CFU/cm^2. Data are reported as mean ± standard error of the mean (S.E.M.). (**) $P < 0.05$ denotes significant levels when compared to Cel-PVA group (Replicates: n ≈ 3). (One-way analysis of variance/Newman-Keuls test.)

Table 3
Effect of treatments on glucose levels and behavioral parameters in the open field test in mice.

	Glucose (mg/dL)		Open Field Test	
	Day 0	Day 15	Number of Crossings	Number of Rearings
Control	153 ± 5	146 ± 7	99 ± 4*	41 ± 3*
Cel-PVA	156 ± 4	144 ± 6	95 ± 3*	42 ± 4*
Cel-PVA/VitC	124 ± 8	128 ± 7	89 ± 3*	40 ± 2*
Cel-PVA/Prop	126 ± 5	125 ± 5	100 ± 3*	51 ± 3*
Cel-PVA/VitC/Prop	133 ± 3	117 ± 5	104 ± 4*	42 ± 2*
STZ	444 ± 20	428 ± 12	30 ± 12$^{\#}$	10 ± 4$^{\#}$
Cel-PVA + STZ	430 ± 29	460 ± 21	48 ± 5$^{\#}$	20 ± 4$^{\#}$
Cel-PVA/VitC + STZ	423 ± 30	459 ± 20	49 ± 9$^{\#}$	21 ± 4$^{\#}$
Cel-PVA/Prop + STZ	403 ± 25	432 ± 20	51 ± 5$^{\#}$	15 ± 2$^{\#}$
Cel-PVA/VitC/Prop + STZ	444 ± 34	457 ± 18	55 ± 8$^{\#}$	17 ± 5$^{\#}$

One-way ANOVA followed by Newman-Keuls test. Data presented are mean values ± S.E.M. ($^{\#}$) $P < 0.05$ denotes significance levels when compared to control group and (*) $P < 0.05$ denotes significance levels when compared to STZ group.

induced diabetic mice model. As expected, on day 0 (ANOVA: $F_{(9,50)} = 57.61$, $P < 0.0001$) and day 15 (ANOVA: $F_{(9,50)} = 149.8$, $P < 0.0001$), the animals that received STZ injections showed higher levels of blood glucose than controls. Topical application of films did not affect hyperglycemia in diabetic mice ($P > 0.05$) (Table 3). No obvious variation in glucose levels was observed after treatments with films on wound area closure in non-diabetic mice ($P > 0.05$).

The films loaded with vitamin C and/or propolis were applied on an excisional 9-mm wound on the dorsal back of diabetic and non-diabetic mice groups. Photographs of the wound region were taken on days 0 and 15 in order to determine changes in wound size as a function of the treatment-time (Fig. 5a). To determine the wound healing ability of the cellulose-based films, the fraction exposed of the wound was determined on day 0 and, then, compared with the fraction exposed on day 15 (Fig. 5b). Overall, our findings revealed that diabetic mice have poor wound healing capacity when compared to the control group. The data analysis of wound area closure revealed that Cel-PVA/VitC/Prop treatment improved the wound closure in diabetic mice when compared to STZ group (Fig. 5a and b) (ANOVA: $F_{(9,50)} = 5.066$, $P < 0.0001$). No discrepancy was observed after treatments with films on wound area closure in non-diabetic mice ($P > 0.05$).

Indeed, one of the most prevalent chronic complications associated with this complex metabolic disorder is diabetic foot ulcer that results from impaired wound healing (Moura et al., 2014). In general, optimum wound healing comprises a series of well-orchestrated cellular and biomolecular events in several overlapping phases, including inflammation, cell migration/proliferation, tissue remodeling, and oxidative stress (Singer and Clark, 1999). Antioxidants have been postulated to help control wound oxidative stress and thereby accelerate wound healing. It is well documented that vitamin C reacts with and deactivates biologically significant radicals and oxidants (Corti et al., 2010). Nonetheless, vitamin C fortifies collagen biosynthesis and the synthesis of ceramides to form strong barrier lipids in the epidermis. Indeed, vitamin C is a cofactor in numerous enzymatic responses, such as collagen synthesis, which is indispensable in wound healing and counteracting capillary bleeding. Collagen production in human skin *in vivo* is stimulated by locally applied vitamin C (Ponec et al., 1997). In the skin wound healing context, propolis is also receiving the attention of researchers. Propolis promotes skin wound healing by facilitating the formation of granulation tissue, stimulating epithelial regeneration and modulating extracellular matrix deposition (Olczyk et al., 2013). In addition, it was reported that propolis could alleviate cell damage in fibroblast cells by suppressing intracellular reactive oxygen species production induced by excessive light (Murase et al., 2013). Cao et al. (2017) reported that oxidative stress had a strong negative impact on the vitality and collagen expression of skin fibroblasts, whereas propolis ethanol extracts efficiently reduced the undue accumulation of reactive oxygen species, protecting skin cells from oxidative injury. Additionally, biological activities of propolis on wound repair and tissue regeneration might be correlated to its antimicrobial, anti-inflammatory, and immunomodulatory properties (Martinotti and Ranzato, 2015). In line with these studies, effects of topical application of cellulose-based films loaded with vitamin C and/or propolis on wounds of diabetic and non-diabetic mice were evaluated. Here, it was verified an increase of the wound closure in diabetic mice treated with topical application of Cel-PVA/VitC/Prop film. However, Cel-PVA/VitC or Cel-PVA/Prop films did not exert this healing property. Taking into account these results, we can suggest that there was a synergic effect of propolis and vitamin C and the Cel-PVA/VitC/Prop film is a promissory biomaterial for wound healing applications. In addition, it is worth mentioning that the use of this biofilm allows the application as a bandage, which makes its use practical and easy.

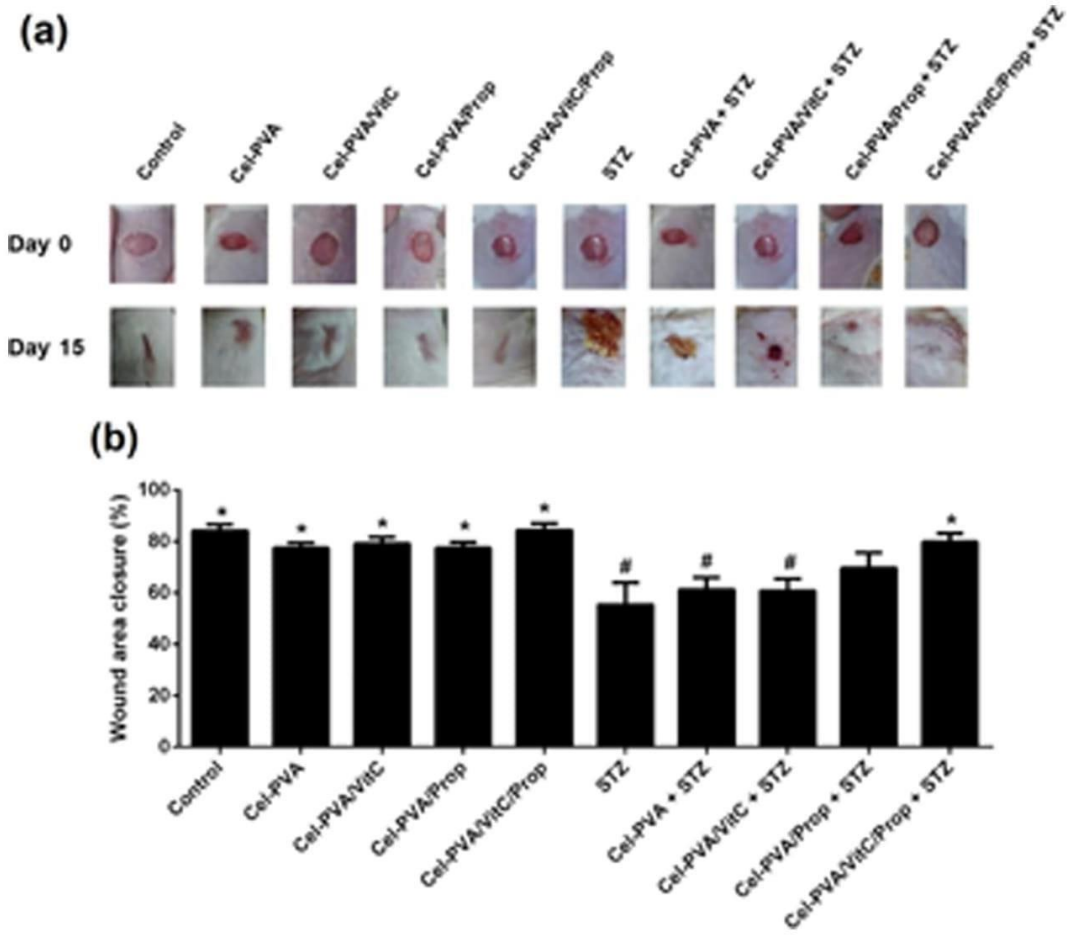

Fig. 5. Wound healing by cellulose-based films loaded with vitamin C and/or propolis in diabetic (STZ) and non-diabetic mice. (A) Photographic images of the extent of wound healing of one animal per group; (b) wound area closure (%). Data of wound area closure are reported as mean ± standard error of the mean (S.E.M.) of six animals per group (one-way analysis of variance/Newman-Keuls test). (#) $P < 0.05$ denotes significance levels when compared to control group and (*) $P < 0.05$ denotes significance levels when compared to STZ group.

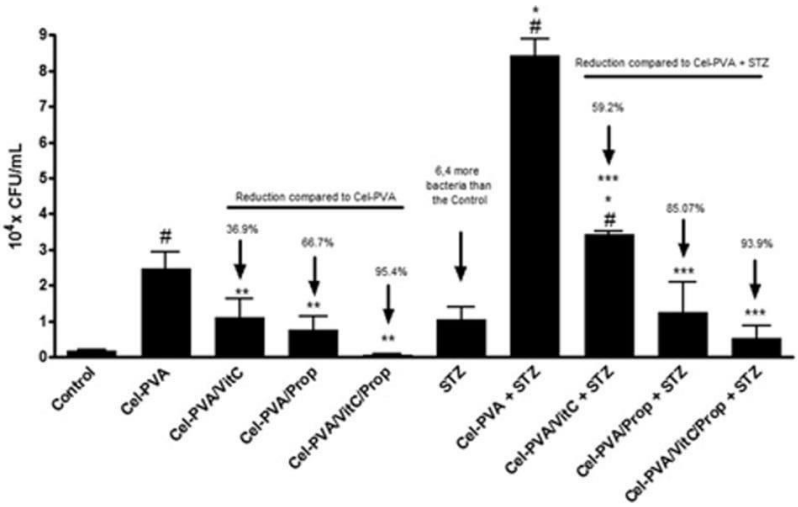

Fig. 6. Quantification of total number of bacteria in the wounds of non-diabetic and diabetic (STZ) mice treated with cellulose-based films loaded with vitamin C and/or propolis. The results were expressed in CFU/mL. Values are means ± standard error of the mean (S.E.M.) (one-way analysis of variance/Newman-Keuls test). (**) $P < 0.05$ denotes significant result when compared to Cel-PVA group. ($^{\#}$) $P < 0.05$ denotes significance levels when compared to control group and (***) $P < 0.05$ denotes significance levels when compared to Cel-PVA + STZ treatment (Replicates: n ≈ 3).

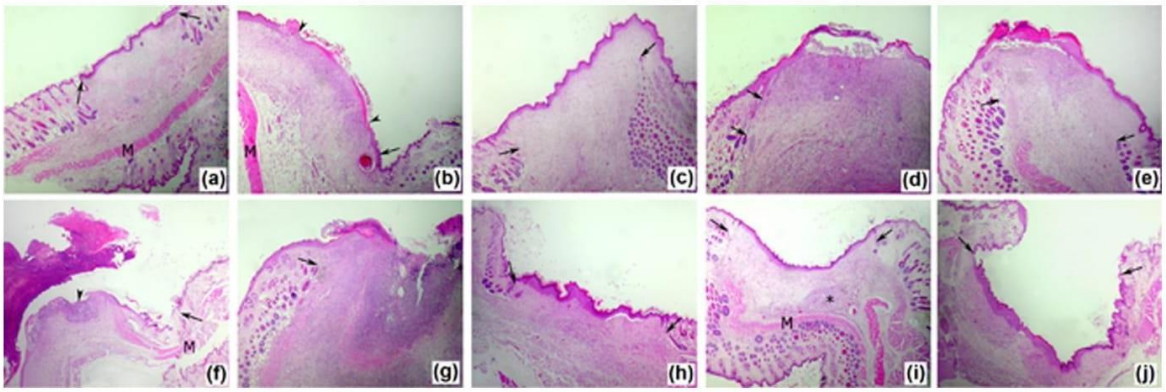

Fig. 7. Histological profiles of wound area in non-diabetic and diabetic (STZ) mice after 15 days of treatment with Cellulose-based films using HE staining (magnification 40×). *Notes:* (a) control of non-diabetic group – scar area (between arrows) and muscle layer (M). (b) Cel-PVA – ulceration areas (between head arrows), normal skin (right corner until the arrow) and M (muscular layer). (c) Cel-PVA/VitC – scar tissue (between arrows). (d) Cel-PVA/Prop – healing area (right side of the arrows) where thickening of the dermis is observed. (e) Cel-PVA/VitC/Prop – scar area (between the arrows) in the dermis with moderate mixed inflammatory infiltrate. (f) Control of diabetic (STZ) group – scar area (left side of the arrow), ulceration area with adjacent necrosis and intralesional bacteria (arrowhead) and muscular layer (M). (g) Cel-PVA + STZ – scar tissue (between arrows). (h) Cel-PVA/VitC + STZ – scar tissue (between arrows), inflammatory process and angiogenesis (*), and M (muscular layer). (i) Cel-PVA/Prop + STZ – scar tissue (arrow's right side). (j) Cel-PVA/VitC/Prop + STZ – scar tissue (between arrows).

3.4.2.1. Open field test. The probable effect of the tested treatments on locomotor and exploratory activities of mice was evaluated in the open field test. Statistical analysis showed that STZ injections decreased the number of crossings (ANOVA: $F_{(9,50)} = 18.95$, $P < 0.0001$) and rearings when compared to the control group (ANOVA: $F_{(9,50)} = 18.44$, $P < 0.0001$) (Table 3). Considering the background regarding the complications of diabetes, we believed that the reduction in locomotor and exploratory activities in diabetic animals is a result of a hyperalgesic condition. Peripheral neuropathy is one of the most frequent and serious complications of *diabetes mellitus* and it is characterized by a distal symmetrical sensorimotor polyneuropathy. As presented in Table 3, topical application of cellulose-based films loaded with vitamin C and/or propolis did not alter the number of crossings and rearings reduced by STZ exposure ($P > 0.05$). No alteration in the number of crossings and rearings was observed after the treatment of non-diabetic mice ($P > 0.05$). The current findings suggest that the Cel-PVA/VitC/Prop film effect is local. It can be hypothesized that Cel-PVA/VitC/Prop film is effective in treating the impaired wound healing (a complication of diabetes), not altering the pathological condition. These findings can be a result of the topical administration that permits the relatively rapid action of the vitamin C/propolis, albeit at a limited site.

3.4.2.2. Quantification of bacteria in treated wounds. Fig. 6 demonstrates the reduction of bacterial counts present in the wound in the groups of non-diabetic and diabetic mice after treatment with the Cel-PVA, Cel-PVA/VitC, Cel-PVA/Prop and Cel-PVA/VitC/Prop films.

The quantification of bacteria in mice injected with STZ was 6.4 times greater than that found in the control group. It can be attributed to the impaired immune status found in this group and the induction of *diabetes mellitus*. Here, cellulose-based films were used in the lesions to protect the site against infections and improve wound closure. When the films containing vitamin C and propolis (Cel-PVA/VitC and Cel-PVA/Prop) were placed in contact with the wound, there was a significant reduction in bacterial counts when compared to the Cel-PVA and Cel-PVA + STZ groups. In non-diabetic mice, Cel-PVA/VitC and Cel-PVA/Prop treatments reduced the bacterial counts by 36.9% and 66.7%, respectively, when compared to the Cel-PVA group. Taking into account the diabetic mice group, a reduction of 59.2% and 85.1% was observed after the Cel-PVA/VitC and Cel-PVA/Prop treatments, when compared to the Cel-PVA + STZ group. In parallel, Cel-PVA/VitC/Prop treatment of non-diabetic (95.4%) and diabetic (93.9%) mice showed a reduction in the bacterial counts, when compared to the Cel-PVA and Cel-PVA + STZ groups, respectively.

The Cel-PVA/VitC/Prop film demonstrated to have a high antimicrobial activity, and this may be due to the synergism between the two assets. The constituents of the film were able to accelerate healing by lowering the rate of bacterial contamination by being excellent bactericides and assisting in the formation of early granulation tissue in topically treated wounds. The pharmacological activities with biological, physiological, and medicinal benefits of propolis have been extensively studied and reviewed in the literature (Bankova, 2005). It is well-known that the chemical composition of propolis is qualitatively and quantitatively variable, depending on the geography and ecology of the regional plants (Bankova, 2005). Research has shown that healing time of diabetic lesions is high and due to the presence of microorganisms in the wound, there is an increase in infections and long periods for complete healing (Doupis and Veves, 2008). However, in spite of this bacterial increase observed in the Cel-PVA + SZT group, the treatments with Cel-PVA/VitC + STZ, Cel-PVA/Prop + STZ and Cel-PVA/VitC/Prop + STZ significantly reduced the number of bacteria, demonstrating the contribution of the active ingredients of the formulations to the antibacterial activity.

The antibacterial activity of propolis is greater against Gram-positive bacteria owing to the presence of flavonoids, acids, and aromatic esters on its composition. These compounds have effects on the cell walls of nuclear organisms through a mechanism not yet known (Brown et al., 2015). The intense inflammatory reaction in wounds may impair the repair process by promoting edema, an excessive amount of exudate favoring bacterial growth and, consequently, inhibition of fibroblast proliferation and collagen deposition (de Oliveira et al., 2007).

3.4.2.3. Histological analysis of wounds. The morphological alterations occurred on the wound area of diabetic (STZ) and non-diabetic mice groups treated with the cellulose-based films for 15 days was examined by histological analysis (Fig. 7). As assessed, the wound of the control animal (non-diabetic group) showed complete healing of the induced lesion. From histological viewpoint, it was observed dermis thickening, acanthosis, and absence of cutaneous annexes (Fig. 7a). In contrast, the wound of the non-diabetic mice treated with Cel-PVA film was unable to develop the healing process effectively (Fig. 7b). Moreover, we observed an area of ulceration with dermal hyperplasia. Neovascularization, lymphatic vessels with dilatation and severe inflammatory infiltrate of polymorphonuclear and mononuclear cells

were present in the mice of this non-diabetic group. The wound of non-diabetic mice treated with Cel-PVA/VitC film (Fig. 7c) presented the finest healing process in comparison to the control group. Here, the analysis of the scar tissue showed acanthosis and neovascularization in the adjacent dermis. As highlighted in Fig. 7d, the wound of the non-diabetic mice treated with Cel-PVA/Prop film presented ulcerations, dermal thickening with severe mixed (polymorphonuclear and mononuclear) inflammatory infiltrate with angiogenesis extending until the subcutaneous. Finally, the non-diabetic mice treated with Cel-PVA/VitC/Prop film (Fig. 7e) presented crusts on the wound surface. In addition, the dermis was thickened with neovascularization and discrete mixed inflammatory infiltrate cells.

Taking into account the STZ-induced diabetic mice group, the histological analysis revealed that the untreated animals were unable to accomplish the cicatrization process (Fig. 7f). In this case, the wound area presented a noticeable ulceration with intralesional bacterial colonies in the dermis and, also, crust formation. There was a severe lymphatic vessel dilatation, with a marked mixed inflammatory infiltrate in an adjacent area of the wound. Conversely, the diabetic mice treated with Cel-PVA film presented an efficient cicatrization process with minimal acanthosis and discrete dermal thickening. Additionally, the neovascularization was prominent in this group (Fig. 7g). A similar analysis was done to the diabetic mice treated with Cel-PVA/VitC film (Fig. 7h); however, in this case, it was observed a thickening of the dermis. These animals showed acanthosis with rare cellularity within the dermis. In addition, a focal neovascularization that extended until the subcutaneous layer of the wound was present. The wound region of the diabetic mice treated with Cel-PVA/Prop film did not develop the scar tissue properly (Fig. 7i). As observed, the dermis was thickened with an inflammatory response composed of neutrophils and mononuclear cells and prominent neovascularization. Surprisingly, the wound of diabetic mice treated with Cel-PVA/VitC/Prop presented the best scar tissue when compared with the other groups. Furthermore, discrete acanthosis and prominent neovascularization with slight thickening of the dermis (Fig. 7j) was observed in the wound area.

In additional histological analyses, all the slides were also stained with Masson's trichrome (MT) in order to investigate the collagen formation in the wound area in diabetic (STZ) mice after 15 days of treatment with the Cellulose-based films (Fig. 8). The diabetic control group (Fig. 8a and b) presented severe edema in the dermal layer. In addition, a prominent angiogenesis and slight fibroblast proliferation with paltry collagen presence were noticed. Diabetic mice treated with Cel-PVA film presented abundant fibroblast proliferation enlaced with thick collagen bands (Fig. 8c and d), while the diabetic mice treated with Cel-PVA/VitC showed a formed scar tissue with rough collagen bands enlaced with few fibroblasts (Fig. 8e and f). The wound area of the diabetic mice treated with Cel-PVA/Prop film showed an expressive amount of fibroblasts within the lesion area with a discrete amount of collagen evidenced by the paltry collagens deposition and thin bands (Fig. 8g and h). Finally, diabetic mice treated with Cel-PVA/VitC/Prop presented a homogeneous collagen and fibroblasts distribution within the scar tissue (Fig. 8i and j). From these results, it can be suggested that the vitamin C/propolis association is beneficial to stimulate tissue regeneration and revascularization of wounds in diabetics.

It is important to note that propolis has been found to accelerate wound healing in different animal models that mimic diabetic wounds (Lotfy et al., 2006). The topical application of propolis has further been shown to enhance the cutaneous wound healing of diabetic ulcers experimentally induced in mice by promoting TGF-β/SMAD-mediated collagen. In addition, oral supplementation of diabetic mice with propolis restore the proliferation and chemotactic capacity of B- and T-lymphocytes toward chemokines by interfering with the lipid inflammatory pathway (Al Ghamdi et al., 2015). At the same time, the administration of vitamin C expedites wound healing in rats. Kamer et al. suggest that vitamin C could confer benefits to tissue healing by significantly enhancing tissue hydroxyproline levels, neovascularization, fibroblast maturation, and collagen deposition (Kamer et al., 2010). Mohammed et al. highlights a comprehensive role of vitamin C in all phases of wound healing with regard to collagen synthesis, cellular apoptosis, antioxidant processes, and bone formation (Mohammed et al., 2015). In the inflammatory phase, vitamin C is required for timely neutrophil apoptosis and clearance (Vissers and Wilkie, 2007). Vitamin C differentially interacts with the integral processes of synthesis, maturation, secretion, and degradation of collagen during the proliferative phase (Mohammed et al., 2015).

Taken together, our results indicate that Cel-PVA/VitC/Prop film allowed an enhanced wound closure rate. Vitamin C and propolis association seem to have a better effect. Here, it was used a protocol of topical treatment every 3 days, and it could justify a minimal effect of the isolated treatments. In addition, our results revealed that the presence of propolis into the Cel-PVA matrix reduces the density of H-bonds between vitamin C and the carrier favoring the liquid uptake and the drug release, and it can explain the positive effect observed when Cel-PVA/VitC/Prop was used in biological experiments. It is worth mentioning that the biomaterials used in this study contain a slight amount of the active compounds (vitamin C and propolis), which suggest that even at low concentration, the association of these compounds was effective to accelerate the diabetic wound healing. Additionally, as demonstrated by histological analysis of wounds, the films loaded with vitamin C or vitamin C and propolis shown more effectiveness in the wound cicatrization process. Taken together, these results allow inferring that the combination of these biocompounds (cellulose, vitamin C, and propolis) with PVA may result in a composite material with the potential to accelerate wound healing process in diabetic model in mice.

4. Conclusion

Cellulose was extracted from rice husk, a worldwide agriculture residue, using a facile protocol. This cellulose (Cel) was blended with poly(vinyl alcohol) (PVA) in order to form films via casting method. Moreover, vitamin C and/or propolis were encapsulated in the Cel-PVA matrix. The film formation, as well as, the encapsulation of those biocompounds was confirmed FTIR analysis. As assessed, the presence of vitamin C and/or propolis in the film affects their mechanical, swelling, and release properties. It can be suggested that the interaction between the biocompounds the film matrix promotes these changes. In vitro release experiments revealed that the Cel-PVA/VitC and PVA/VitC/Prop films enable the release of vitamin C in a controlled manner. For both films, the Higuchi model explains the vitamin C release kinetics while the release mechanic corroborates to the Korsmeyer-Peppas model. In addition, the films loaded with vitamin C and/or propolis showed antibacterial activity against *E. coli* and *S. aureus*. This antibacterial activity was also verified against bacteria localized in the wounds of diabetics and non-diabetics mice. *In vivo* experiments performed in diabetics and non-diabetics mice groups suggest a synergistic effect of vitamin C and propolis that accelerates the wound healing process. Histological analysis corroborates this statement. Moreover, our results demonstrate that Cel-PVA/VitC/Prop film enhances wound healing in diabetic mice without altering its pathological condition. Finally, all these findings suggest that Cel-PVA/VitC/Prop may represent a new therapeutic approach for advanced wound healing applications. Hence, further studies are required to elucidate the other mechanisms involved in this potential action of this new biomaterial.

Conflicts of interest

There are no conflicts to declare.

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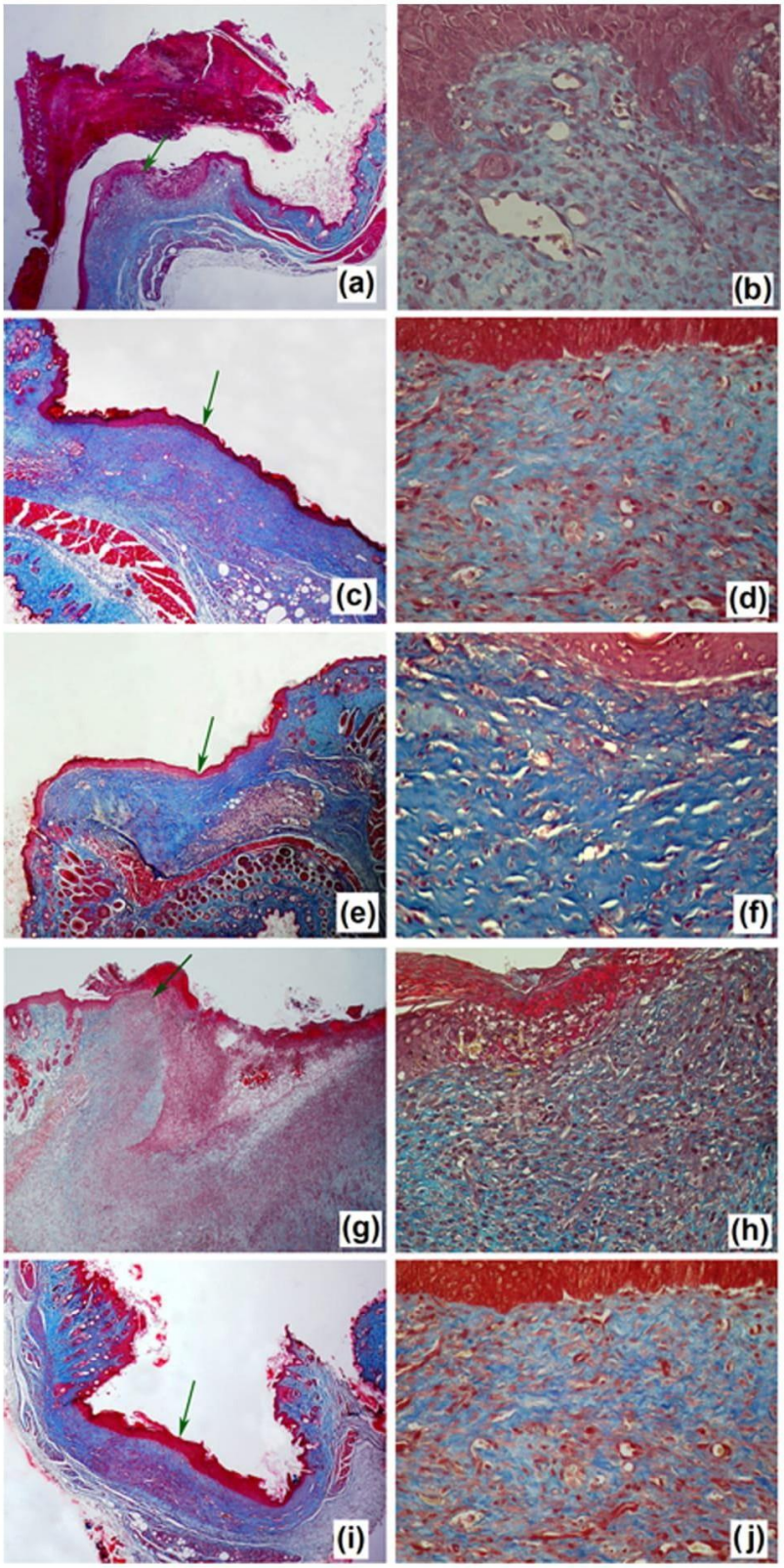

Fig. 8. Histological profiles of wound area in diabetic (STZ) mice after 15 days of treatment with the Cellulose-based films using MT staining. (a and b) Control of diabetic (STZ) group. (c and d) Cel-PVA treatment, (e and f) Cel-PVA/VitC treatment, (g and h) Cel-PVA/Prop treatment, (i and j) Cel-PVA/VitC/Prop treatments. Notes: The images presented in the left column have low magnification (40×), while the images presented in the right column have high magnification (400×). Green arrows indicate the selected regions of each image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2018.10.009.

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5. Conclusões

- Os filmes contendo vitamina C apresentaram liberação controlada;
- Os filmes carregados com vitamina C e/ou própolis apresentaram atividade antibacteriana *in vitro* contra *E. coli* e *S. aureus.* Este efeito também foi observado contra bactérias localizadas em feridas de camundongos diabéticos e não diabéticos;
- Os experimentos *in vivo* realizados em grupos de camundongos diabéticos e não diabéticos sugerem um efeito sinérgico da vitamina C e própolis na aceleração do processo de cicatrização de feridas;
- A análise histológica demonstrou que o filme contendo vitamina C + própolis melhorou a cicatrização de feridas em camundongos diabéticos sem alterar sua condição patológica;
- Finalmente, todas essas descobertas sugerem que o filme com vitamina C e própolis pode representar uma nova abordagem terapêutica para aplicações avançadas de cicatrização de feridas.

Perspectivas

Mais estudos serão necessários para investigar os possíveis mecanismos de ação envolvidos nas propriedades cicatrizantes e antibacterianas dos filmes contendo vitamina C e/ou própolis. Além disso, nosso grupo de pesquisa tem se dedicado ao estudo de doenças inflamatórias de pele, utilizando compostos orgânicos de selênio para o tratamento. Um dos modelos que se destacam é o de dermatite atópica induzida com 2,4-dinitroclorobenzeno em camundongos. Assim, buscaremos investigar o efeito destes compostos nas lesões semelhantes à dermatite atópica, a fim de identificar uma nova alternativa terapêutica para esta doença.

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Anexo A- Aprovação do Comitê de Ética em Experimentação e Bem-Estar Animal

UFPel
CEEA
Comissão de Ética em Experimentação Animal

Pelotas, 28 de novembro de 2016

Certificado

Certificamos que a proposta intitulada **"Avaliação das ações anti-inflamatória e cicatrizante de compostos sintéticos inéditos em feridas diabéticas"**, registrada com o nº23110.008659/2016-38, sob a responsabilidade de **Ethel Antunes Wilhelm** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 21/11/2016.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	05/12/2016 a 05/12/2021
Espécie/linhagem/raça	*Mus muscullus*/Swiss
N° de animais	96
Idade	60 dias
Sexo	Machos
Origem	Biotério Central

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 8659-2016**).

M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Ciente em: ____/____/2016

Assinatura do Professor Responsável: _____