PEDRO HENRIQUE SOUZA CESAR

DESENVOLVIMENTO E CARACTERIZAÇÃO DE UM CURATIVO BIOPOLIMÉRICO CONTENDO PRODUTOS NATURAIS

LAVRAS-MG

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Profa. Dra. Silvana Marcussi

Orientadora

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DESENVOLVIMENTO E CARACTERIZAÇÃO DE UM CURATIVO BIOPOLIMÉRICO CONTENDO PRODUTOS NATURAIS

DEVELOPMENT AND CHARACTERIZATION OF A BIOPOLYMERIC WOUND DRESSING CONTAINING NATURAL PRODUCTS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agroquímica, para a obtenção do título de Doutor

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RESUMO

A desenvolvimento de novas tecnologias voltadas para o tratamento de ferimentos tem garantido um ganho maior de qualidade de vida para os pacientes nos centros de saúde. Com a promessa de oferecer trocas menos dolorosas, acelerar o processo de cicatrização e promover a proteção dos ferimentos contra o ataque de micro-organismos, os curativos biopoliméricos vem ganhando um espaço cada vez maior do mercado de investimentos. Muito embora estejam disponíveis um vasto catálogo de alternativas, o alto custo é a principal barreira que impede a sua ampla utilização. A incorporação de produtos naturais nas matrizes biopoliméricas, voltadas para o tratamento de feridas vem mostrando resultados promissores e atraindo atenção dos grupos de pesquisa. O própolis, uma mistura de resinas e óleos essenciais e polifenóis, é capaz de acelerar a recuperação dos ferimentos, atuar como um agente antimicrobiano e modular as respostas imunológica. Por sua vez, os retinóis como o ácido *all-trans* retinóico (atRA), são capazes de estimular a síntese de colágeno do tipo 1, aumentar a síntese de matriz extracelular, criando assim um ambiente favorável para a multiplicação, diferenciação e amadurecimento do novo tecido. Baseado nesses preceitos, este trabalho teve como objetivo o desenvolvimento de uma matriz biopolimérica de alginato de sódio e poli álcool vinílico em diferentes proporções incorporada com extrato alcoólico de própolis e atRA. Os materiais desenvolvidos foram analisados por microscopia exploratória de varredura, e avaliados segundo suas propriedades físico-químicas, toxicológicas e atestadas as atividades farmacológicas dos princípios ativos quanto suas propriedades antimicrobianas, antioxidantes e anti-inflamatórias. Como resultados, os materiais desenvolvidos apresentaram um bom desempenho mecânico, com características morfológicas esperadas para materiais do tipo. Os curativos obtidos foram considerados adequados para o contato com superfícies biológicas, em especial as células sanguíneas e seus componentes. O extrato de própolis obtido foi eficaz contra os micro-organismos *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19117, *Salmonella enteritidis* AOAC 100201 e *Pseudomonas aeruginosa* INCQS 0025. Tanto o atRA quanto o própolis apresentaram propriedades antioxidantes e anti-inflamatórias, que podem auxíliar no processo de cicatrização. Os curativos desenvolvidos neste trabalho apresentam um grande potencial de uso na prática clínica e sua facilidade de síntese e escalabilidade podem permitir seu uso diário.

Palvras-chave: Curativos, Biopolímeros, Própolis, Retinóis, Alginato de Sódio, Poli (álcool vinílico)

ABSTRACT

The development of new technology aimed to the treatment of wounds has been assuring to the patients in health centers an increase in quality of life. With the promise to offer less painful dressing changes, speeding up healing process and protecting wound from pathogens attack, the biopolymeric dressings has been increasing its share of the market. Although a great number of wound dressing options are available, their high cost consists in a major problem regarding its utilization. The incorporation of natural products in biopolymeric matrices, develop to treat have shown interesting results, attracting the attention of many research groups. Propolis, a mixture of resins, essential oils and polyphenols, is capable of speeding up wound recovery, acting as an antimicrobial agent and to modulate immune responses. Retinols, such as all-trans retinoic acid (atRA), are capable of stimulating type I collagen deposition, increase the synthesis of extracellular matrix, and consequently creating an adequate environment to multiplication, differentiation and maturation of the new tissue. Based on these principles, this work aimed the development of a biopolymeric matrix composed of sodium alginate and poli (vinyl alcohol) in different ratios incorporated with propolis and atRA. The developed materials were analyzed by scanning electron microscopy, and evaluated its physico-chemical features and toxicological potential. A screen of biological activities was performed regarding its antimicrobial, antioxidant and anti-inflammatory properties. As a result, the materials developed here presented a good mechanical behavior, with adequate morphological features expected for such material. The wound dressings obtained were suitable to be in touch with biological surfaces, in special to blood and its cell components. The propolis extract obtained was capable of inhibiting the growth of Staphylococcus aureus ATCC 25923, Listeria monocytogenes ATCC 19117, Salmonella enteritidis AOAC 100201 and Pseudomonas aeruginosa INCQS 0025 strains. Both atRA and propolis presented antioxidant and anti-inflammatory capacity, which could help in healing process. The wound dressings developed here possess a great potential of use in common medical practice and due to its simple synthesis and scalability, it may allow its easy fabrication and implementation in daily uses.

Key-words: Wound dressing, Biopolymers, Propolis, Retinoids, Sodium alginate, Poli (vinil alcohol)

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LISTA DE SIGLAS

atRA – Ácido *all-trans* retinóico

EGF – Epidermal growth fator

IGF-1 – Insulin-like growth fator 1

IL-X **–** Interleucina (X – número referente ao tipo específico)

PDGF – Platelet derived growth factor

PVA – poli (álcool vinílico)

AS – Alginato de sódio

TGF-α – Tumor growth fator alpha

TGF-β - Tumor growth fator beta

VEGF – Vascular endotelial growth factor

ÍNDICE

1 INTRODUÇÃO

Ferimentos podem ser compreendidos como qualquer interrupção na continuidade dos tecidos saudáveis, ocasionado de maneira acidental ou proposital por agentes químicos, físicos ou biológicos (ENOCH; LEAPER, 2008). Para profissionais da área médica e clínica, o cuidado com os ferimentos e uso adequado de bandagens e etapas de cura e limpeza são requisitos indispensáveis.

Tábuas de barro datando do ano 2.200 AC traziam em si um dos primeiros relatos a respeito do cuidado com ferimentos. Nessas tábuas havia a descrição de três procedimentos: Lavar o local, aplicar emplastos e cobertura (MAJNO, 1991). Reservadas a distância temporal e tecnológica, este procedimento não difere muito do que é praticado hoje. Atualmente, o cuidado básico aos ferimentos consiste em avaliar a situação da injúria, preparar o local para receber a cobertura, realizar suturas se necessário, limpar o local e aplicar alguma forma de curativo (DREIFKE; JAYASURIYA; JAYASURIYA, 2015a). A escolha entre deixar o ferimento aberto ou coberto depende do tipo de lesão, e do potencial de contaminação a que a injúria estaria exposta. Finalmente, uma complicada decisão toma frente, qual tipo de cobertura utilizar?

Em ferimentos simples, a manutenção da limpeza e uma cobertura simples são suficientes para que a pessoa ferida esteja protegida de agentes infecciosos e possa se recuperar tranquilamente. Por outro lado, ferimentos crônicos, complexos ou mais extensos e profundos como as úlceras de pressão ou ferimentos em pacientes diabéticos e portadores de doenças vasculares, é necessário o uso de coberturas adequadas (POWERS et al., 2016). Uma vez que se prima pelo bem-estar do paciente e a minimização da dor, associado a um tratamento rápido e eficaz, faz-se necessário escolher adequadamente um curativo.

É certo que não existe um tipo de cobertura que sirva para todas as enfermidades ou atenda a todos os pré-requisitos para ser um bom curativo, porém determinadas características devem ser alcançadas para se ter um tratamento adequado, sendo elas: permeabilidade ao vapor d'água e ao oxigênio, ausência de imunogenicidade, manutenção da umidade, capacidade de melhorar o resultado cosmético, atuar como barreira contra agentes infecciosos e facilidade de uso, remoção e troca (ZARRINTAJ et al., 2017).

Atualmente, existem no mercado uma infinidade de curativos disponíveis para tratar feridas. Estão disponíveis espumas, hidrogéis, hidrocolóides, curativos de alginato e diversos tipos de bandagens, contendo princípios ativos ou não. Dessa forma a escolha do método de cuidado e do curativo a ser utilizado se torna uma tarefa bem mais complexa do que deveria.

Ainda que a diversidade de curativos seja abundante, é necessário melhorar as propriedades regenerativas das bandagens, facilitar sua aplicação e reduzir o incomodo durante as trocas para o paciente (NISCHWITZ; HOFMANN; KAMOLZ, 2019). Mais ainda, deve-se investir em pesquisas que promovam a redução dos custos, de modo a torná-los acessíveis ao maior número de pacientes sem que haja perda de qualidade (KAPP; SANTAMARIA, 2017).

Os curativos do tipo espuma foram desenvolvidos para atender os principais requisitos de um curativo ideal (SUN; TAN, 2013). As espumas foram introduzidas na prática clínica na década e são conhecidos por ajudar a manter a temperatura e a umidade no local da ferida, condições ideais para a recuperação tecidual (FAN et al., 2016; KARRI et al., 2016). Além dessas características, as espumas não aderem ao ferimento fazendo com que as trocas sejam praticamente indolores (LIU et al., 2017). As espumas ainda atuam como uma barreira contra bactérias e de acordo com os princípios ativos presentes na sua composição, podem ser aplicadas sobre ferimentos infeccionados. Por outro lado, existem algumas desvantagens: os curativos do tipo espuma não são adequados para ferimentos secos ou com pouquíssimo exsudato (fluído liberado pelo processo inflamatório nos ferimentos), tampouco para ferimentos altamente exsudativos, podendo contribuir para a maceração do tecido ao redor do ferimento se aplicados (DHIVYA; PADMA; SANTHINI, 2015). Para o primeiro caso são recomendados hidrogéis e hidrocolóides, enquanto que no segundo caso recomenda-se o uso de curativos de alginato ou terapia oclusiva por vácuo (ROSENBAUM et al., 2018).

O uso de produtos naturais pela humanidade pode ser considerado tão antigo quanto sua própria existência (BUTLER; ROBERTSON; COOPER, 2014). A natureza é uma fonte riquíssima de produtos de interesse farmacêutico e atualmente mais de 25% das drogas prescritas são de origem vegetal (DINIC et al., 2015). Por muito tempo a indústria farmacêutica depositou a maior parte de seus investimentos em compostos sintéticos ou bioinspirados devido aos avanços da química orgânica e a facilidade de se obter compostos isolados, bem como a aparente segurança transmitida por medicamentes alopáticos. De acordo com a OMS 11% dos medicamentos considerados como essenciais para os centros de saúde do mundo todo, são de origem vegetal. Dentre os produtos naturais de interesse, o própolis e as vitaminas tem ganhado destaque devido ao grande número de aplicações e atividades biológicas que possui (PASUPULETI et al., 2017).

O própolis ou a própolis é formado por 30% de cera de abelha, 50% de resinas vegetais, 10% de óleos essenciais e aromáticos, e 5% de pólen e outras substâncias (ANJUM et al., 2018). São atribuídas ao própolis a ação bactericida, cicatrizante, anti-inflamatória e

imunomoduladora (TORETI et al., 2013). Este produto natural além de possuir um baixo custo e ampla aceitação popular, ainda conta com uma baixa imunogenicidade. Enquanto que as vitaminas são micronutrientes orgânicos essenciais aos organismos em pequenas quantidades. As vitaminas se dividem em hidrossolúveis (como as vitaminas C e B), e lipossolúveis (como as vitaminas A, D, E e K). Em especial, a vitamina A, representada genericamente por moléculas lipossolúveis compostas por uma cabeça contendo um anel aromático (porção apolar) e uma cauda poliênica (porção polar). Os principais grupos representativos da vitamina A são: retinol, retinal e ácido retinóico os quais apresentam um grupo terminal contendo as funções álcool, aldeído e ácido carboxílico (respectivamente) (BISWAS et al., 2018). Popularmente a vitamina A é conhecida como um nutriente essencial para visão, no entanto seu papel se estende para várias funções como controle da expressão de diversos genes, diferenciação celular, reepitelização e controle das respostas inflamatórias (FU et al., 2007).

Assim, este trabalho buscou associar os produtos naturais, própolis e vitamina A (altamente importante para a recuperação tecidual) a um curativo do tipo espuma, buscando produzir curativos de baixo e custo e altamente eficientes.

2 REFERENCIAL TEÓRICO

2.1 Ferimentos e recuperação tecidual

Ferimentos ou injúrias podem ser entendidos como uma interrupção na integridade de um tecido, tendo uma etiologia acidental ou intencional. Mediante a ocorrência da injúria, inicia-se a ativação de rotas metabólicas e processos celulares para conter e recuperar o tecido danificado (ENOCH; LEAPER, 2008). Os ferimentos podem ser separados em agudos (incluindo incisões cirúrgicas) ou crônicos. São denominados agudos quando os processos de recuperação ocorrem em um período de tempo que não ultrapassa algumas poucas semanas (UBBINK et al., 2015). Entretanto, se a injúria persiste e os processos de recuperação se estendem por tempo superior a 12 semanas e apresentam alguma complicação (processo inflamatório persistente). Estes ferimentos são chamados de crônicos (FRYKBERG; BANKS, 2015). Um ferimento pode ser considerado como curado (resolvido) quando o tecido conjuntivo (conectivo) foi reparado e o ferimento foi completamente reepitelizado por regeneração tecidual (GURTNER et al., 2008). Além disso, é necessário que o tecido tenha sua estrutura anatômica normalizada e seja funcional sem que haja nenhum tipo de intervenção como drenagens ou remoção de tecidos danificados. Didaticamente divide-se o processo de recuperação tecidual

em quatro fases de acordo com os tipos celulares e processos moleculares em: hemostasia, inflamação, proliferação e remodelação tecidual (GONZALEZ et al., 2016).

2.1.1 Hemostasia

A primeira medida fisiológica a ser tomada mediante uma injúria, onde há o rompimento da micro ou macrovasculatura, é a contenção do processo hemorrágico. A liberação de mediadores inflamatórios para o meio extracelular inicia a cascata de coagulação e promove a constrição da parede dos vasos sanguíneos através do aumento dos níveis de cálcio citoplasmático (KOLEDOVA, 2006). Após a formação do trombo e agregação plaquetária, o fluxo sanguíneo que estava impedido anteriormente em situação de acidose e hipóxia leva a um mecanismo de feedback negativo que promove a vasodilatação mediada pelo oxido nítrico, adenosina e outros metabólitos vasoativos. As plaquetas presentes no local da injúria liberam mediadores como PDGF (platelet-derived growth factor), IGF-1 (insulin-like growth factor 1), EGF (epidermal growth factor), TGF-β (transforming growth factor-β) e PF-IV (platelet factor-IV). Essas proteínas iniciam os processos de recuperação tecidual, ativam e atraem células especializadas para o sítio da inflamação (PAKYARI et al., 2013).

2.1.2 Inflamação

Durante a fase inflamatória, que se inicia logo nas primeiras 24 horas após a injúria, o objetivo principal é a prevenção de infecções. Esta fase pode ser entendida em duas etapas, sendo uma imediata e a outra tardia, diferindo entre si pelos tipos celulares e o perfil inflamatório dominante envolvido (EMING et al., 2007). Na fase imediata ocorre ativação das vias clássicas e alternativa do complemento. Os neutrófilos são as primeiras células a colonizar o local e são atraídos (quimiotaxia) principalmente por fragmentos da matriz extracelular, proteínas do complemento, TGF-β e produtos bacterianos (GURTNER et al., 2008). Devido a alterações nas moléculas de adesão, os neutrófilos se tornam "pegajosos" e aderem à superfície dos vasos adjacentes (marginação). Posteriormente, ocorre a rolagem dos neutrófilos seguindo o fluxo sanguíneo e a direção das selectinas e integrinas (PARK; BARBUL, 2004). Ao parar a rolagem, os neutrófilos migram para fora dos vasos (diapedese). Uma vez no local do ferimento, os neutrófilos fagocitam materiais estranhos e destroem as bactérias através da liberação de produtos tóxicos.

A etapa tardia, que tem seu início por volta de 48 horas, é marcada pela chegada dos macrófagos atraídos por mensageiros químicos liberados pelas plaquetas e células danificadas que continuam o processo fagocitário (RODERO; KHOSROTEHRANI, 2010). Essas células

são consideradas as mais importantes no processo de recuperação tecidual durante a fase inflamatória tardia pois, além de atuarem como células fagocíticas, ainda são liberadores primários de fatores de crescimento que darão início a fase proliferativa. Os macrófagos são como reservatórios enormes de fatores de crescimento, como o TGF-B e EGF (endothelial growth factor), que atuam estimulando a angiogênese e a formação de tecido de granulação (GUO; DIPIETRO, 2010). Os linfócitos são as últimas células a chegarem, e aparecem por volta de 72 horas após a injúria, atraídos pela IL-1 (Interleucina-1), proteínas do complemento e IgG (imunoglobulina G), atuando no controle da produção e remodelação de colágeno, produção/degradação de matriz extracelular, que irá atuar como um arcabouço que sustentará as células para a neovascularização, formação do tecido de granulação e reepitelização (PORTOU et al., 2015). Ao fim desta etapa é possível observar a presença de fibras colágenas na borda do ferimento, porém, estas não contribuem diretamente para o fechamento da ferida, pois são orientadas verticalmente e não horizontalmente onde podem realizar forças de contração (tração) (WILLENBORG; EMING, 2014).

2.1.3 Proliferação

Uma vez alcançada a hemostasia, o controle da resposta inflamatória e o local da injúria está livre de *debris* celulares, inicia-se a fase proliferativa por volta do terceiro dia, podendo durar por um período de duas a quatro semanas. Este estágio é marcado pela migração de fibroblastos, por uma intensa deposição de matriz extracelular, deposição de colágeno e formação de tecido de granulação que simultaneamente permitirão a reepitelização e o fechamento do ferimento (GONZALEZ et al., 2016).

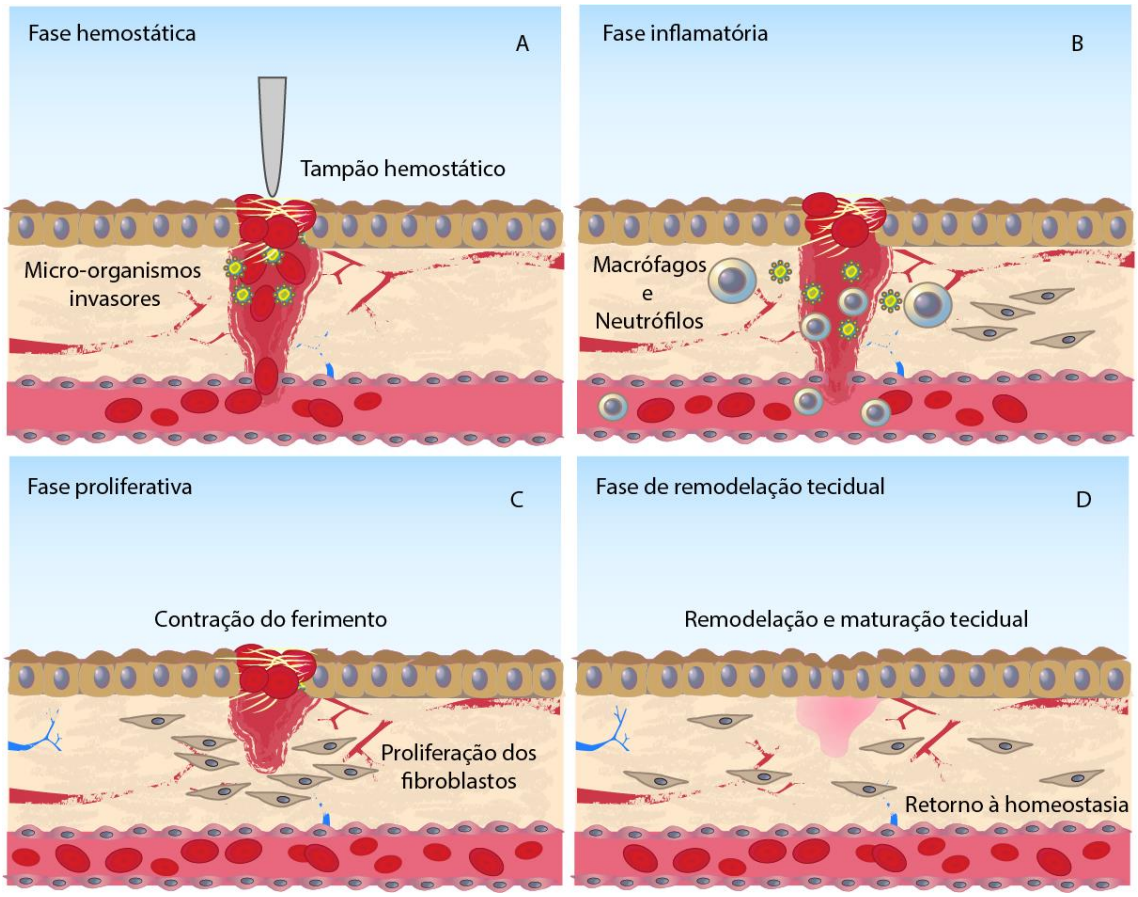
Uma vez no local da inflamação, os fibroblastos se proliferam e começam a produzir proteínas integrantes da matriz extracelular como fibronectina, hialuronan, proteoglicanos e pro-colágenos, que serão depositados no ambiente (PASTAR et al., 2014). Ao fim da primeira semana o leito da ferida já possui uma matriz acumulada que dará suporte para a migração de novas células e fornecerá pontos de ancoragem para a retração das bordas do ferimento. O resultado dessa fase é um tecido de coloração rosada, rico em vasos sanguíneos, tecido fibroso que substitui o tampão hemostático (trombo), sendo denominado tecido de granulação. Após a deposição da matriz, os fibroblastos passam por uma mudança de fenotípica e assumem um perfil de miofibroblastos. Essas células (miofibroblastos) emitem pseudópodes que os permitem se conectar com as células vizinhas, e às proteínas da matriz, fibronectina e ao colágeno.

A angiogênese é iniciada logo nos primeiros momentos após o controle da hemorragia e aumenta sua intensidade durante a etapa proliferativa, estimuladas por fatores de crescimento

liberados pelas plaquetas e em resposta aos baixos teores de oxigênio no local. Os miofibroblastos também são responsáveis por promover a angiogênese (DEMIDOVA-RICE; DURHAM; HERMAN, 2012; EMING et al., 2007). Durante esse processo de formação de novos vasos, ocorre a degradação proteolítica da matriz nos locais onde os novos capilares irão brotar, ocorrendo a migração em direção ao estímulo (aumento no gradiente quimiotático: VEGF, PDGF, angiogenina, TGF-α e TGF-β). Os vasos recém-formados possuem a característica marcante de serem edematosos, devido a sua imaturidade celular. Assim, o tecido granular rico em vasos sanguíneos adquire sua coloração e umidade típicas devido ao extravasamento de líquido pelos capilares. As células epiteliais da margem do ferimento migram em direção ao centro da injúria logo nas primeiras horas, cobrindo a região com uma fina camada de epitélio, por um processo chamado epibolia. Doze horas após, observa-se um aumento pronunciado na atividade mitótica nas células mais basais do epitélio. Com a matriz formada e uma rede rica de capilares recém-formados, as células epiteliais tem suporte mecânico e nutricional para iniciarem o fechamento da ferida (MIRON et al., 2017; PASTAR et al., 2014).

Remodelação tecidual: A última etapa da recuperação tecidual, chamada de remodelação tecidual tem início conjuntamente com a formação do tecido de granulação e pode durar por longos períodos dependendo da gravidade da lesão. A síntese e a degradação de colágeno ocorrem constantemente, até que um equilíbrio seja alcançado, prazo que pode levar até 21 dias (VELNAR; BAILEY; SMRKOLJ, 2009). Para que a recuperação seja atingida, é necessário a maturação da matriz extracelular, que pouco a pouco vai adquirindo tonicidade e força. Estima-se que as fibras colágenas formadas recuperam até 80% da força original, quando comparadas ao tecido íntegro. A remodelação cessa à medida que há uma redução da atividade das metaloproteases e um aumento nos inibidores de metaloproteases no organismo. Juntamente, é necessário a redução no número de macrófagos e fibroblastos ativos no local, retorno da circulação normal no local, bem como o fim da formação de novos capilares (CHEN et al., 2015; MARTIN; NUNAN, 2015). Essas etapas culminam com o fechamento completo da ferida.

Figura 1 – Etapas da recuperação tecidual. A – Fase hemostática; B – Fase Inflamatória; C – Fase proliferativa; e D – Fase de remodelação tecidual



Fonte: Elaborada pelo próprio autor

2.2 Curativos

O termo curativo é dado ao conjunto de procedimentos utilizado no tratamento de feridas visando sua proteção, controle de umidade e recuperação da injúria. Em se tratando de ferimentos, independentemente de seu tamanho ou gravidade, o ideal é que os cuidados apropriados sejam tomados para evitar uma infecção (DHIVYA; PADMA; SANTHINI, 2015). Além disso, quando um ferimento está coberto por um curativo ocorre a continua ação de proteases, agentes quimiotáxicos, fatores de crescimento e do complemento, que atuam na cicatrização, são preservados, ao passo que ferimentos descobertos, tais fatores seriam degradados devido a exposição ao ambiente. A utilização de curativos ainda atua promovendo hipóxia local, considerado um importante estímulo para a promoção da angiogênese e redução do pH, o que leva a uma diminuição dos níveis de infecção (SARABAHI, 2012). Até os anos 1948, acreditava-se que um ambiente seco era ideal para a recuperação de ferimentos, até que

Oscar Gilje demonstrou que um ambiente úmido no leito da ferida era mais eficaz na recuperação de ulcerações. A partir dos anos 80, deu-se início ao uso de curativos mais modernos e mais adequados a cada tipo de ferida (MOGOŞANU; GRUMEZESCU, 2014).

Apesar dos avanços da indústria farmacêutica e de engenharia de materiais, ainda hoje muitos centros de saúde no Brasil não dispõem de materiais adequados para tratar injúrias de maneira satisfatória (ADAS et al., 2010; COTTA et al., 2006; OLIVEIRA et al., 2012). O uso por exemplo de gases e fibras que aderem ao machucado, que não atendem a um padrão de maciez e conforto para o paciente, reduzem a qualidade de vida, especialmente de indivíduos acamados. Geralmente são secos e não são capazes de manter a umidade ótima no local da injúria ou proteger contra a entrada de patógenos, além de causar dor durante o processo de troca.

Um curativo tido como ideal precisa ter como características a capacidade de manter umidade adequada na interface ferida/cobertura, controlar o excesso de exsudato, permitir trocas gasosas, fornecer isolamento térmico, ser impermeável a bactérias, ser isento de partículas grosseiras, tóxicas ou imunogênicas, e permitir uma troca sem provocar traumas (ABDELRAHMAN; NEWTON, 2011). Atualmente os curativos são desenvolvidos não somente como uma simples cobertura, mais ainda, são desenvolvidos para interagir com a interface do ferimento controlando assim sua umidade, liberando fatores de crescimento, anti-inflamatórios e antimicrobianos.

Os curativos modernos são geralmente feitos de polímeros sintéticos ou em combinação com polímeros naturais apresentando características adequadas para cada uso. São classificados em passivos, interativos e bioativos (DREIFKE; JAYASURIYA; JAYASURIYA, 2015b). Os passivos são produtos não oclusivos, tais como bandagens de tule ou rayon, utilizados para cobrir o ferimento enquanto medicamentos e a imunidade do paciente restauram a injúria. Os interativos são curativos semi-oclusivos ou oclusivos, disponíveis na forma de espumas, hidrogéis e hidrocolóides. Essas formas de cobertura atuam como uma barreira contra a penetração de micro-organismos patogênicos. Por último, os bioativos, são produzidos a partir de materiais que irão auxíliar no processo de cicatrização, esses curativos são conhecidos por serem biocompatíveis, biodegradáveis e não-imunogênicos. Os materiais são geralmente colágeno, ácido hialurônico, quitosana, alginatos ou elastina, podendo ser utilizados sozinhos ou combinados com outros polímeros (CHAUDHARI et al., 2016). Embora existam uma grande variedade de curativos comerciais disponíveis, em muitos centros de saúde o baixo orçamento e a dificuldade de se obter novas tecnologias tem feito com que o uso de coberturas

inadequadas seja comum. Em muitas situações, os pacientes são tratados com coberturas simples e que aderem no ferimento, liberando fibras e causando dor durante as trocas.

2.3 Introdução aos biomateriais

Biomateriais são amplamente utilizados na área médica e são alvos de estudos que vem ganhando destaque crescente nas últimas décadas. O conceito de biomaterial diverge quanto sua abrangência para diferentes autores, no entanto para o periódico Biomaterials (Elsevier) e a *American National Institute of Health*, tais entidades podem ser entendidas como componentes que interagem com os sistemas biológicos (fluidos e tecidos), e podem ser aplicados como ferramentas no diagnóstico, tratamento, reparação, substituição e modulação de algum processo biológico (ELSEVIER, 2017). A terminologia biomaterial, embora possa causar confusão, em seu sentido mais adequado não se restringe a materiais de origem biológica. Sendo assim, podem ser fabricados a partir de compostos sintéticos, naturais ou naturais quimicamente modificados (BERGMANN; STUMPF, 2013). As principais categorias de biomateriais são: cerâmicas, polímeros, metais e compósitos.

A introdução desses materiais no organismo humano data de milênios atrás, como se pode observar através de evidências como implantes de dente feito com nácar (material derivado das conchas), datando do ano 600, pelo povo Maia. No período Neolítico, o linho foi utilizado como material de escolha para realizar suturas, e na idade média, intestino de gato era utilizado pelos povos europeus com o mesmo fim. Na África e Índia, pinças de formigas eram utilizadas para manter as extremidades de ferimentos unidas (MIGONNEY, 2014).

Até meados do século passado o termo biomaterial ainda não havia sido cunhado, e sua aplicação era feita com pouco critério, sendo fundamentalmente do tipo tentativa e erro. A ausência da compreensão dos conceitos de biocompatibilidade e conhecimentos de esterilização tornavam o uso de implantes um fator de alto risco. De acordo com Bath, os biomateriais podem ser agrupados em quatro classes de acordo com o tipo de compatibilidade que apresentam quando aplicados no local pretendido (BHAT, 2002). Essas classificações são: biotolerante - são aqueles separados do tecido ósseo por uma camada de tecido mole; bioinerte - nesta classe é possível, em determinados casos, estabelecer contato direto com o tecido ósseo, porém sem que haja interação química entre o material implantado e o tecido; bioativo - possuem a propriedade de estabelecer interações químicas com o tecido ósseo, processo conhecido como osteointegração. Os materiais dessa classe aderem aos tecidos e a matriz extracelular e são implantados na superfície do tecido ósseo; e bioreabsorvíveis - são os materiais que, após um

tempo em contato com os tecidos, são degradados e absorvidos pelo organismo, sendo particularmente ideais em situações onde a retirada do implante é desaconselhada.

Independente da aplicação, os biomateriais devem ser desenvolvidos para permitir seu uso sem rejeição por tempo suficiente para exercer a função pretendida. Para tanto, o processo de fabricação dos biomateriais deve levar em conta os materiais a serem utilizados, as técnicas empregadas, sua estabilidade e a necessidade de esterilização. Para todas as etapas, existem um conjunto análises físicas, químicas e biológicas que devem ser atendidas para sua utilização. Por fim, os biomateriais precisam ser funcionais. Isto é, devem apresentar uma composição e design que permita o desempenho de suas funções idealizadas. Por exemplo, uma prótese óssea deve ser capaz de resistir as forças de tração e compressão às quais um osso saudável está submetido, com ausência de reações biológicas indesejadas.

2.4 O mercado investidor no setor de biomateriais

No mundo todo, o mercado de biomateriais tem experienciado grandes ondas de investimentos impulsionadas pela necessidade de se desenvolver tecnologias mais eficazes para lidar com as limitações do organismo em se recuperar e manter sua funcionalidade (JOSE PAULO SANT ANNA, 2014). Nos últimos 10 anos o mercado global de biomateriais movimentou mais de US$ 25 bilhões (dados de 2008), sendo que os Estados Unidos movimentaram 43% desse mercado, seguido pela Europa com 33%, Ásia (Pacífico) 3%, Brasil com 2% e 19% no restante do mundo (ZION MARKET RESEARCH, 2017). Em 2012 este mercado quase que dobrou seu valor, passando a representar uma economia de US$ 44 bilhões. Ao fim de 2017 era previsto que este valor chegasse a cifra de US$ 88,4 bilhões, e até 2021 este mercado deva chegar a US$ 149,17 bilhões (BHAVYA BANGA, 2017).

Entre as causas relacionadas a esse aumento exacerbado se encontra o grande número de casos de diabetes, problemas vasculares, osteoporose e um aumento no interesse em dispositivos que promovam a recuperação tecidual e atuam na liberação controlada de fármacos (JIN et al., 2015). No panorama global, dados demográficos indicam um envelhecimento populacional paralelo a um aumento na expectativa de vida, bem como avanços na área médica em tratar doenças tidas como intratáveis ou com soluções frágeis. Essa tendência é acompanhada pelo Brasil, tida como um fator capaz de influenciar positivamente os investimentos, assim a estimativa é que até 2022 o mercado brasileiro movimente US$ 5,18 bilhões, face aos US$ 1,89 bilhão no ano de 2017. Atualmente, dentre todos os segmentos na área de biomateriais (metálicos, cerâmicos, biovidros, polímeros e compósitos), os biomateriais

metálicos correspondem a 50%, entretanto a tendência é que no futuro os materiais poliméricos acabem por alterar essa fração, uma vez que apresentam características apropriadas quanto à flexibilidade, elasticidade, biocompatibilidade, degradação controlada e custo (PIRES; BIERHALZ; MORAES, 2015).

2.5 Curativos do tipo espuma

Os curativos do tipo espuma (indexado pelos termos *foam dressings*, *3D porous scaffold* e *sponge scaffold*) são arcabouços tridimensionais altamente porosos e absorventes, sendo considerados uma ótima escolha quando se pretende manter a temperatura e a umidade adequada no local da ferida (ZHANG; MA, 1999b). É de comum conhecimento nos centros de atendimento médico que a manutenção da umidade promove uma rápida cicatrização, no entanto os ferimentos já possuem uma tendência em perder umidade para o ambiente, já que o tecido perdeu sua integridade física (JUNKER et al., 2013). Da mesma forma, a manutenção da temperatura também é imperativa no processo de recuperação tecidual. No organismo humano, as células e enzimas estão adaptadas ao seu meio fisiológico e uma queda de apenas 2°C é suficiente para afetar negativamente o processo de recuperação (MELLING et al., 2001).

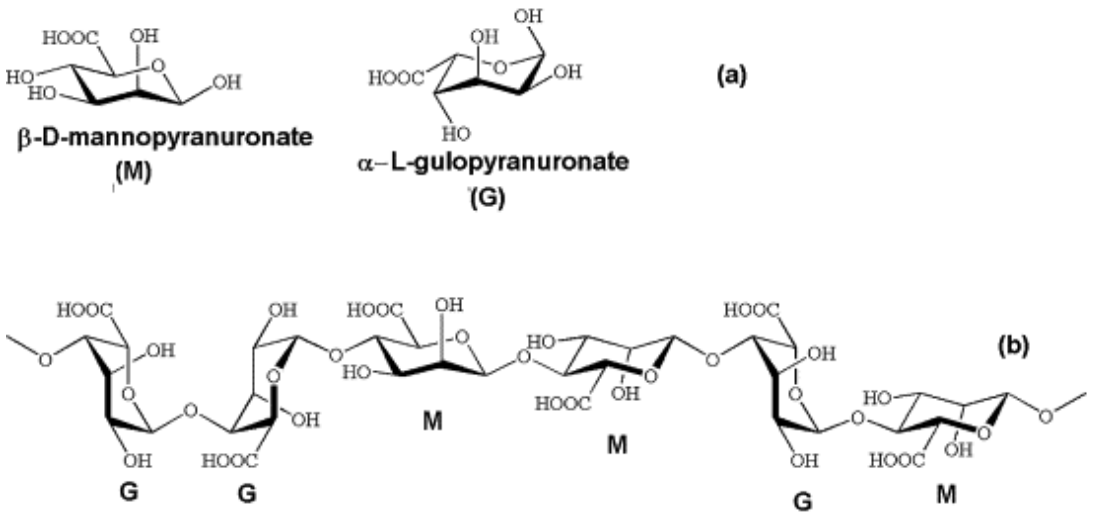
Muitas vezes os curativos do tipo espuma são referidos como ideais. São capazes de atender aos pré-requisitos básicos para tal (controle da umidade, manutenção da temperatura no ferimento, entre outros), acrescentando ainda, o seu baixo valor unitário (VATS et al., 2003). São indicados para ferimentos contendo exsudato (moderado à intenso sem prurido). Em caso de feridas contaminadas devem ser trocados diariamente (THOMAS, 2008). Além de ferimentos convencionais, os curativos do tipo espuma são utilizados para recobrir regiões recém operadas, ferimentos traumáticos, locais doadores de tecido, queimaduras de menor intensidade, úlceras diabéticas, de pressão ou de insuficiência venosa. Ao contrário, são evitados em ferimentos secos, necrosados ou com excesso de exsudato, pois podem favorecer a maceração dos tecidos vizinhos à injúria (SOOD; GRANICK; TOMASELLI, 2014a).

2.6 Descrição e aplicações do alginato de sódio e do álcool polivinílico

O alginato é um copolímero aniônico de ocorrência natural, obtido de algas marinhas ou bactérias modificadas geneticamente, através do tratamento com soluções básicas (tipicamente NaOH), processos de filtração e precipitação utilizando cloreto de cálcio ou de sódio. A composição básica dos alginatos é uma mistura dos carboidratos L-guluronato (G) e

D-manuronato (M) formando blocos do tipo GGGGG, MMMM ou GMGM em ligações α ou β (1,4) (TONG et al., 2017).

Figura 2 – Estrutura molecular dos monômeros de alginato de sódio e seus possíveis arranjos poliméricos. A – Copolímeros de manuronan e guluronan; B – Arranjo sequencial dos copolímeros (GG; MM; e GM)



Fonte: Davis (2003)

Os alginatos podem ser obtidos em diferentes escalas de massa molecular, onde o aumento da sua massa molecular (que varia entre 32.000 – 400.000 g/mol) interfere na rigidez e viscosidade dos biomateriais produzidos (LEROUX; GUILAK; SETTON, 1999). Para cada tipo de aplicação existe uma escala de massa molecular adequada. Em determinados casos uma alta viscosidade pode ser prejudicial, especialmente quando se trabalha com proteínas ou células no preparo do biomaterial, pois a força necessária para homogeneizar uma solução de alta viscosidade pode danificar essas estruturas (LEE; MOONEY, 2012).

Tradicionalmente os alginatos são aplicados nas áreas da indústria de alimentos como espessantes e na farmacêutica como gelificantes e agentes liberadores de fármacos. Os alginatos ainda são utilizados em outras atividades como a liberação local de medicamentos tem ganhado mais destaque. Estudos envolvendo a liberação de moléculas de baixo peso molecular incorporadas em géis de alginato tem mostrado que dado a sua estrutura típica nanoporosa (tamanho aproximado de 5 nm), a liberação dessas moléculas ocorre em poucas horas (SUN; TAN, 2013). Assim, faz-se necessário o desenvolvimento processos de reticulação ou a

produção de blendas específicas para a liberação controlada desses agentes (TØNNESEN; KARLSEN, 2002).

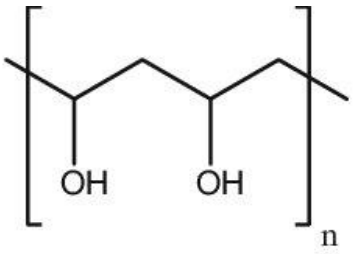
O tratamento de ferimentos agudos e crônicos com rapidez e bons resultados cosméticos é uma pressão feita por pacientes, e todo o corpo clínico, uma vez que além da parte estética ainda há as perdas econômicas do sistema público associadas ao manejo de feridas (LUPO, 2006). Nesse cenário os curativos feitos a partir de alginato, ou em associação a este representam recursos vantajosos para a prática clínica (BOATENG et al., 2008). Em contraste aos curativos convencionais onde a ferida se mantém em condições de baixa umidade, os sistemas de cobertura de alginato, fornecem um ambiente úmido que facilita a recuperação tecidual (JONES, 2006; QIN, 2008).

As coberturas de alginato são tipicamente produzidas na forma de gel, seguidas por um processo de liofilização para formar arcabouços porosos conhecidos como espumas, e coberturas fibrosas (SAKIYAMA-ELBERT; HUBBELL, 2001). As coberturas secas para serem utilizadas precisam ser reidratadas formando uma estrutura de gel e assim fornecer umidade para o ferimento. Dessa maneira, estas formas de cobertura atuam acelerando a formação do tecido de granulação, a rápida reepitelização e a recuperação, na medida em que minimizam a infecção local (LIAKOS et al., 2014). Muitas coberturas são comercializadas no momento, entretanto são onerosas para a aquisição nos centros públicos de saúde, entre esses produtos se encontram Algicell™ (Derma Sciences) AlgiSite M™ (Smith & Nephew), Comfeel Plus™ (Coloplast), Kaltostat™ (ConvaTec), Sorbsan™ (UDL Laboratories), e Tegagen™ (3M Healthcare).

O álcool polivinílico (PVA, PVOH ou ainda PVAI) é um copolímero sintético preparado a partir do poli(acetato de vinila) através de uma reação de hidrólise, que resulta no poli(álcool vinílico-co-acetato de vinila) um produto cristalino e hidrossolúvel (ALVES et al., 2011). O comportamento desse polímero em solução varia com o grau de hidrólise, peso molecular, distribuição dos blocos de partículas e cristalinidade. Para sua comercialização, o PVA deve apresentar um grau de hidrólise entre 86,5 – 98%. Além do grau de hidrólise, o peso molecular influência drasticamente suas propriedades mecânicas e biológicas. Ao se aumentar a massa molecular caminha-se para uma redução na solubilidade em água, aumento da viscosidade, resistência à tração, redução na flexibilidade e resistência à degradação pela água. Ao passo que, ao aumentar o grau de hidrólise, ocorre de maneira semelhante, a redução de sua solubilidade em água, resistência à degradação, aumenta sua viscosidade e perda flexibilidade

(LIMPAN et al., 2012). O PVA ainda apresenta ausência de toxicidade e imunogenicidade, e biocompatibilidade e biodegradabilidade (SUZUKI; SASAKI, 2015).

Figura 3 – Estrutura do monômero de poli (álcool vinílico)



Fonte: Amann (2011)

Para a manufatura de produtos biomédicos, o PVA em muitos casos é o polímero de escolha devido às propriedades que exibe. Dessa maneira, este material é aplicado sozinho ou associado a outros polímeros na produção de arcabouços para o cultivo de células, curativos do tipo espuma, hidrogéis e substitutos para cartilagens e tendões (KOKABI; SIROUSAZAR; HASSAN, 2007). Entretanto, apesar de algumas formas apresentarem alto grau de resistência à água, ainda é necessário um processo de reticulação (*cross-linking*) para evitar sua susceptibilidade em água (CHO; OH; LEE, 2005). Os processos de reticulação podem ser físicos ou químicos, onde os físicos são por etapas sucessivas de congelamento-descongelamento (freeze-thawing), uso de radiação-γ ou bombardeamento de elétrons (TILLET; BOUTEVIN; AMEDURI, 2011).

2.7 Uso de produtos naturais incorporados em biomateriais

2.7.1 O própolis

O própolis ou a própolis (CAS No. 9009-62-5) é um material de caráter resinoso e balsâmico produzido à partir de diferentes partes de plantas como brotos, cascas, botões florais e exsudados resinosos, os quais as abelhas ingerem, regurgitam e ainda adicionam secreções salivares e cera para a produção (BANKOVA; DE CASTRO; MARCUCCI, 2000). O nome própolis indica a sua utilidade, do grego *pro* = em favor de, e *polis* = cidade. O própolis é utilizado pelas abelhas para selar aberturas, revestir as paredes da colmeia atuando como isolante térmico e agente impermeabilizante, além de ser utilizado para embalsamar insetos

invasores que morrem no interior da colmeia, evitando sua decomposição e infecção do local por micro-organismos (PARK; ALENCAR; AGUIAR, 2002a).

Basicamente o própolis é formado por 30% de cera de abelha, 50% de resinas vegetais, 10% de óleos essenciais e aromáticos, e 5% de pólen e outras substâncias (DAUGSCH et al., 2008). No entanto, existem variações na presença de determinados componentes do própolis que os tornam geograficamente e farmacologicamente distintos. Por exemplo, dependendo da composição o própolis pode assumir uma coloração esverdeada, avermelhada, castanha ou quase preta. É importante mencionar que essas variações de coloração estão atreladas à flora local (PARK; ALENCAR; AGUIAR, 2002b). Apesar da alta complexidade do própolis e suas variações locais, sazonalmente as diferenças do própolis para um mesmo grupo de abelhas são pequenas e qualitativas, uma vez que esses insetos visitam basicamente as mesmas fontes vegetais ao longo do ano (BANKOVA et al., 1999).

Ao todo, mais de 300 compostos já foram identificados no própolis, e, no Brasil, os principais compostos biologicamente ativos encontrados foram compostos fenólicos (flavonoides, ácidos aromáticos e benzopiranos), terpenoides (di e triterpenos) e óleos essenciais (SILVA-CARVALHO; BALTAZAR; ALMEIDA-AGUIAR, 2015). Para a extração desses compostos os solventes mais utilizados são etanol, éter, acetona, tolueno, propileno glicol e água. A escolha do solvente varia com o interesse de aplicação. Para a indústria de alimentos e médica, são mais utilizados os extratos aquosos e etanólicos, enquanto que para a indústria cosmética os extratos glicólicos são mais recomendados (TRUSHEVA; TRUNKOVA; BANKOVA, 2007).

A utilização do própolis tem uma história antiga, tendo sido empregado no Egito antigo em rituais de mumificação, e também como agente cicatrizante e antisséptico por muitos outros povos, como gregos e romanos. Nos dias atuais já foram atribuídas ao própolis diversas propriedades farmacológicas tais como antimicrobiana, antitumoral, antioxidante, anti-inflamatória, cicatrizante e imunomoduladora.

2.7.2 Propriedades farmacológicas do própolis

Ação antimicrobiana

Dentre as atividades biológicas do própolis, a atividade antimicrobiana é a mais bem documentada, relatada por diversos autores utilizando variedades de todo o mundo. Muitos trabalhos evidenciam a capacidade do própolis de combater agentes infecciosos, especialmente contra bactérias Gram-positivas. Sua ação ocorre pela inibição da motilidade bacteriana,

inibição enzimática, rompimento do potencial de membrana, inibição do crescimento e citotoxicidade. Em um trabalho publicado por Mirzoeva, Grishanin e Calder (1997), a ação do própolis na redução da virulência, e citotoxicidade ocorreu pela ação dos seus diversos componentes, como por exemplo os derivados do ácido cinâmico (ácido cafeico) e flavonoides (quercetina e narigenina) atuando em conjunto.

O uso de extratos de própolis ou seus compostos isolados, em conjunto ou não com antibióticos, foram eficazes no combate à muitas cepas de bactérias como as dos gêneros *Staphylococcus*, *Helicobacter*, *Bacillus*, *Pseudomonas*, *Salmonela* e *Enterococci*, inclusive aquelas que se mostraram anteriormente resistentes (CARDOSO et al., 2016; KUJUMGIEV et al., 1999; ORSI et al., 2006). A *Helicobacter pylori* (*H. pylori*) é considerada o principal patógeno humano associada a muitas doenças como gastrite crônica, ulceras e câncer na região gástrica. Dada a ação bactericida do própolis, seu uso foi implementado no combate à *H. pylori* em um trabalho conduzido por Cui e colaboradores (2013). Uma varredura dos principais fenólicos presentes no própolis mostrou que o composto derivado do ácido cafeico, o CAPE (caffeic acid phenetyl ester), foi capaz de inibir uma enzima essencial para esta bactéria, a deformilase peptídica (PDF), que é considerada o principal alvo no combate a infecção por *H. pylori*. Os extratos de própolis apresentaram ainda ação anti-cariogênica (contra *Streptococcus mutans* e *Enterococcus faecalis*) e no tratamento de infecções de pele em queimaduras (CARDOSO et al., 2016; KAYAOGLU et al., 2011; MACHADO et al., 2016). Além disso, a administração conjunta do própolis com antibióticos comerciais aumentou a potência destes no combate a bactérias gram-negativas e gram-positivas resistentes, permitindo a redução da dose dos antibióticos e promovendo um ganho de qualidade no tratamento para os pacientes (SCAZZOCCHIO et al., 2006b; WOJTYCZKA et al., 2013).

As ações antifúngica, antiviral e antiprotozoária do própolis foram demonstradas em muitos trabalhos. Em especial, o potencial do extrato alcóolico de própolis de várias localidades do mundo, com propriedades antifúngica, antibacteriana e antiviral foi descrito. No trabalho em questão, confirmou-se os seus efeitos antifúngicos contra *Candida albicans*, e antiviral contra Influenza A (KUJUMGIEV et al., 1999). Um dado interessante nesse artigo destaca que embora grande parte dos efeitos farmacológicos do própolis sejam atribuídos à flavonoides, derivados ésteres de ácidos fenólicos e terpenóides, a ausência ou um baixo teor em algum desses compostos não interfere qualitativamente na ação bactericida, fungicida ou antiviral. Um estudo utilizando extratos etanólicos de diversas amostras de própolis brasileiro contra *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. kruzei*, *C. tropicalis* e *C. parapsilosis*, conhecidas por serem

agentes promotores de candidíase oral e de contaminação de cateteres, observou-se uma forte ação inibitória sobre a formação de biofilme e redução no número de unidades formadores de colônias desses micro-organismos (FREIRES et al., 2016). Falcão e colaboradores (2014) utilizaram duas amostras de própolis provenientes de regiões geográficas distintas de Portugal e compararam suas atividades antifúngica e antiprotozoária com os extratos das principais fontes de matéria prima para a produção do própolis. Essas amostras de própolis tiveram um bom desempenho antiparasitário contra *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi* e *Leishmania infantum*, bem como antifúngico para *Candida albicans*, *Trypanosoma rubrum* (um dos principais agentes causadores de micoses em humanos) e *Aspergillus fumigatus*.

Anti-inflamatória e Imunomodulatória

A resposta inflamatória é um evento que ocorre naturalmente em resposta a um dado estímulo que pode ser a invasão de um micro-organismo, uma injúria ou um estresse. A resposta inflamatória ou inflamação é uma cascata complexa de fatores químicos (citocinas, quimiocinas e radicais livres) com mobilização de células especializadas que migram para os locais de inflamação e buscam resolver esta reação (HANADA; YOSHIMURA, 2002). Em determinados casos em que a resposta inflamatória perdura por longos períodos, pode ocorrer o desenvolvimento de patogenias como aterosclerose, asma, câncer, Alzheimer e Parkinson. Pesquisas conduzidas por diversos autores verificaram que diferentes extratos de própolis bem como seus constituintes isolados eram capazes de atuar sobre a resposta inflamatória. Na literatura existem muitos trabalhos que ligam os compostos fenólicos do própolis como os principais responsáveis por atuarem nas respostas inflamatórias. Funakoshi-Tago e demais autores (2015) investigaram os efeitos anti-inflamatórios dos flavonoides isolados do própolis nepalês e observaram que os compostos 3′,4′-diidroxi-4-metoxidalbergiona, 4-metoxidalbergiona, cearoina, e crisina reduziam a expressão de citocinas pró-inflamatórias como IL-6, TNF-α e IL-13 em mastócitos. A liberação de ácido araquidônico é crucial para desencadear os processos inflamatórios. O composto CAPE, presente no própolis de diversas localidades, o ácido cafeico, quercetina e narigenina, entre outros compostos fenólicos, foram capazes de inibir a produção de eicosanoides. Mais precisamente, esses compostos são capazes de suprimir a via das lipoxigenases que convertem o ácido araquidônico em mediadores inflamatórios, sendo que o CAPE é o modulador mais potente (ABDEL-LATIF et al., 2009). Esse composto também se mostrou capaz de acelerar a recuperação da esteatose hepática

induzida por uma dieta com alto teor de gordura em ratos. Esse efeito se deve principalmente pela redução de fatores pró-inflamatórios e um decréscimo na via do COX-2 (BEZERRA et al., 2012). Um estudo conduzido com extrato etanólico e aquoso de própolis derivado do norte da China demonstrou a capacidade deste em promover uma redução na formação de edema e nos níveis de interleucina-6 em um modelo animal de artrite reumatoide induzida por FCA (Freund's complete adjuvant) (HU et al., 2005).

Além do controle das respostas inflamatórias, o própolis também é capaz de modular como o sistema imune reage frente às diversas alterações que está submetido. Até a década de 1990, estudos dessa natureza eram escassos e continham poucas informações. Atualmente com o avanço das técnicas de análise e pesquisa, houve um salto no número de publicações a respeito da ação Imunomodulatória (ORŠOLIĆ; BAŠIĆ, 2003).

O própolis verde, originário do Brasil, ao ser administrado em um grupo de camundongos BALB/c desempenhou uma ação modulatória nos passos iniciais da resposta imune. No ensaio em questão macrófagos foram isolados do peritônio e foram estimulados com LPS. Foi observado que os macrófagos do grupo que recebeu o extrato etanólico de própolis apresentavam uma expressão maior de receptores *Toll-like* (TLR) 2 e 4. Tais receptores desempenham um papel importante contra infecções, uma vez que eles são capazes de reconhecer padrões moleculares associados à patógenos (PAMPs) e atuar como a primeira barreira de defesa contra micro-organismos (ORSATTI et al., 2009). O trabalho publicado por Machado et al. (2012) mostrou que o extrato aquoso do própolis comercial Green (ApisFlora©), bem como de amostras de própolis de outras localidades do Brasil possuem um potencial antioxidante e anti-inflamatório utilizando um modelo de inflamação pulmonar induzido por LPS, reduzindo o número excessivo de células colonizando o local tais como neutrófilos e macrófagos, e controlando os níveis das citocinas pró-inflamatórias IL-6 e TNF-α, e aumentando a expressão de TGF-β e IL-10, sendo que a primeira ação mencionada é responsável por manter a homeostasia pulmonar impedindo que nesse órgão vital ocorra uma inflamação exacerbada. A ação anti-inflamatória do própolis não se limita à sua ingestão, alguns trabalhos atestaram a ação anti-inflamatória do seu extrato etanólico na redução do formação de edema em pata (própolis brasileiro) e orelha de ratos (própolis chileno) (NAITO et al., 2007). Sua aplicação também se mostrou eficaz no controle de irritações psoriáticas, reduzindo a peroxidação lipídica na pele e a ação de macrófagos no sítio da irritação (ORŠOLIĆ; BAŠIĆ, 2003).

Cicatrizante

A cicatrização é uma cascata de eventos bem ordenados em que após uma injúria, visa restaurar a integridade do tecido danificado. Esses eventos podem ser divididos didaticamente em inflamação local, coagulação, reconstrução tecidual, contração e remodelação (BRADSHAW et al., 2001). Há relatos sobre a aplicação do própolis no contexto de recuperação tecidual desde a antiguidade clássica, muitas culturas fazem o uso etnofarmacológico do própolis, e atualmente vem sendo investigado o seu uso no tratamento de diversos tipos de ferimentos com grande sucesso terapêutico. Os principais compostos isolados do própolis relacionados à essas atividades são ácidos graxos, terpenóides, β-esteroides, flavonoides, vitaminas e sais minerais, que atuam em conjunto para a promoção da recuperação da injúria (SILVA-CARVALHO; BALTAZAR; ALMEIDA-AGUIAR, 2015).

O própolis é amplamente utilizado para a recuperação de diversos tipos de ferimentos tais como queimaduras e ulceras externas, para a aceleração no tempo de recuperação tecidual, aumentando a velocidade de contração e a resolução da inflamação local (RAMOS; MIRANDA, 2007). O trabalho conduzido por Miranda et al. (2015) demonstrou que além da capacidade leishmanicida promovida pelo própolis, também foi observada uma aceleração na recuperação tecidual nas feridas causadas por leishmaniose cutânea. No trabalho em questão, foi utilizado um agente carreador de óxido nítrico (NO) à base de Rutênio. Os dados obtidos do experimento demonstravam que o própolis tornava o NO mais disponível no local da lesão, bem como estimulava a síntese de colágeno, o recrutamento celular, o controle da inflamação e ainda fornecia antioxidantes. O sucesso desse trabalho levou os autores a sugerirem tal abordagem ao invés do tratamento padrão com glucantime.

A diabetes é uma condição que afeta a vida dos indivíduos com certas restrições e cuidados, especialmente no contexto da recuperação tecidual de ferimentos em que há uma inabilidade ou letargia em recuperar a lesão. Nesses casos especiais como feridas crônicas ou em úlceras de pé, o uso do extrato etanólico de própolis em um modelo de ratos diabéticos induzidos por estreptozotocina, observou-se uma normalização no tempo de recuperação dos tecidos, bem como da resolução da ferida (ferimentos crônicos) (MCLENNAN et al., 2008). Em queimaduras, ao ser comparado com a sulfadiazina de prata, apresentou tempo de recuperação 2 dias mais rápido, bem como um potencial microbicida comparável a este controle (DE ALMEIDA et al., 2013). E em tecidos delicados como a córnea, onde poucos fármacos são aprovados e aconselhados para uso, uma solução ocular feita a partir do extrato etanólico (70%) de própolis foi capaz de reduzir a inflamação local em ratos *Wistar*, reduzindo o tempo

de recuperação esperado para esse tipo de ferimento (MARTIN et al., 2013). Acredita-se que sua ação reparadora e cicatrizante deve-se ao conjunto de atividades desempenhada pelos seus constituintes como antimicrobiana, anti-inflamatória e imunomoduladora (FITZMAURICE; SIVAMANI; ISSEROFF, 2011).

2.7.3 Propriedades cicatrizantes e anti-inflamatória da vitamina A

Vitaminas são micronutrientes orgânicos essenciais para que o organismo possa manter um funcionamento adequado, entretanto, são necessárias em pequenas quantidades. Ao todo 13 vitaminas são reconhecidas, sendo divididas entre lipossolúveis (A, D, E e K) e hidrossolúveis (C e complexo B). O nome de uma vitamina é genérico, não sendo relacionado com a estrutura da molécula em si, mas sim, com sua atividade biológica. Dessa forma, diversas moléculas desempenham o papel da Vitamina A ou D, por exemplo. As vitaminas atuam na regulação do crescimento, diferenciação celular, reprodução, respostas imunológicas e processos metabólicos (COMBS; MCCLUNG, 2017).

As moléculas pertencentes ao grupo da Vitamina A, são micronutrientes lipossolúveis que atuam na manutenção da epiderme, promovendo a maturação e diferenciação celular, bem como a recuperação tecidual através da estimulação da angiogênese, recrutamento celular, síntese de colágeno e reepitelização. O ácido retinóico (uma forma de vitamina A) é conhecido por aliviar os sintomas clínicos da acne comum, psoríase e retardar o envelhecimento causado por exposição aos raios solares (ROSS, 2010; VARANI et al., 2000). Os primeiros trabalhos a respeito dos efeitos do uso tópico da vitamina A foram conduzidos por Montagna (1954) e Sobel et al., (1959), e forneceram os primeiros relatos da absorção cutânea levando a efeitos locais e sistêmicos. Posteriormente, outros autores trabalharam com os mecanismos de absorção cutânea realizando testes com formas naturais e sintéticas dos retinóis (SAURAT; SORG; DIDIERJEAN, 1999). Estudos experimentais e clínicos demonstraram que a deficiência da vitamina A é responsável por retardar a recuperação tecidual, ao contrário, a suplementação pela dieta ou aplicação tópica da vitamina A e outros retinóis é capaz de potencializar os mecanismos de recuperação (POLCZ; BARBUL, 2019).

Retinóis na recuperação tecidual

Os retinóis, de modo geral, são reguladores do crescimento e diferenciação celular. O mecanismo pelo qual ocorre a ação reguladora dos retinóis é mediado principalmente por duas famílias de receptores nucleares, compreendidos na superfamília dos receptores de hormônios

esteroides. Esses receptores, denominados receptores de ácido retinóico (RAR) e Receptores X de retinóis, se ligam e estimulam a transcrição de genes necessários aos processos de diferenciação e proliferação dos tecidos. Algumas das ações terapêuticas relacionadas aos retinóis estão relacionadas à expressão e secreção de fatores de crescimento como TGF-β e suas isoformas, e IGF-1 (MANICASSAMY; PULENDRAN, 2009).

Logo após o controle da hemorragia local em um ferimento, inicia-se o processo concomitante de controlar a inflamação, e a formação do tecido de granulação. As plaquetas liberam alfa grânulos os quais secretam fatores de crescimento que são responsáveis por regular a interação entre as células locais, dada a natureza ubíqua desses fatores de crescimento. Os retinóis regulam então a expressão desses fatores de crescimento, aumentando em até 50 vezes em alguns tipos celulares a sua produção, como no caso dos queratinócitos (POURJAFAR et al., 2017).

A administração de corticoides para o controle de doenças inflamatórias está ligada a uma redução na velocidade de cicatrização, pois muitos processos na cascata de cicatrização possuem natureza pró-inflamatória. Wicke e colaboradores conduziram uma série de ensaios randomizados para avaliar a ação dos retinóis na recuperação tecidual, mediante o uso de corticoides (WICKE, 2000). No trabalho em questão, os animais (ratos wistar) tratados com corticoide apresentavam uma redução severa nos níveis de TGF-β e IGF-1, e consequente demora na recuperação. A administração conjunta com os retinóis atRA e o ácido 9-cis-retiníco promoveu uma aceleração da cura dos ferimentos e um aumento dos níveis de TGF-β e IGF-1.

A tendência dos ferimentos em diabéticos se prolongar é um desafio para os profissionais de saúde. Os ferimentos em pacientes diabéticos demoram muito a iniciar as respostas inflamatórias, com baixa migração celular para os locais feridos, além de letargia na formação do tecido de granulação e contração da ferida. Kitano e colaboradores administraram topicamente na forma de gel 0,1% de atRA durante 5 dias. Em relação ao grupo controle, o grupo que recebeu o gel de atRA apresentou uma rápida contração do ferimento com maior hipertrofia da derme e epiderme. Histológicamente, os animais apresentavam uma colonização celular mais intensa, com melhor desenvolvimento da vasculatura, e deposição de colágeno. (KITANO et al., 2001). Assim como em diabéticos, pacientes com ulcerações, seja por insuficiência venosa ou reumática apresentam ferimentos com um grau elevado de complexidade e demora na resolução (CASTLEBERRY et al., 2016). Um grupo de pesquisadores utilizou uma solução de ácido retinóico a 0,05% em um grupo de pacientes com ulcera de perna, aplicada no leito da ferida. Após uma semana, os primeiros resultados

demonstraram a formação inicial de tecido de granulação e reepitelização. Em uma visão histológica dos ferimentos, ficou evidente uma maior quantidade de tecido de granulação, maior vascularização, deposição de colágeno e matriz extracelular (PAQUETTE; BADIAVAS; FALANGA, 2001). Em suma, a aplicação da solução de retinol permitiu uma mudança no status da ferida de crônica para aguda.

3 OBJETIVOS GERAIS

O objetivo neste trabalho é a produção de um biomaterial do tipo espuma, composto de PVA (Ácido polivinílico) e alginato de sódio contendo extrato de própolis e ácido *all-trans* retinóico, visando sua aplicação como curativo

3.1 Objetivos específicos

I – Produção de arcabouços de PVA e alginato de sódio através de um método simples e replicável;

II – Incorporação de extrato etanólico de própolis verde e ácido *all-trans* retinóico na estrutura do biomaterial

III – Caracterização do biomaterial acerca de suas propriedades físicas;

IV – Avaliação da liberação de própolis e vitamina A ao longo do tempo, objetivando estimar o tempo de uso

V – Avaliação toxicológica *in vitro* e microbiológica do material e dos princípios ativos sobre linhagens de micro-organismos comuns em ferimentos;

VI – Avaliação toxicológica e cicatrizante *in vivo* em ratos

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ARTIGOS DESENVOLVIDOS

4 ARTIGO I - DEVELOPMENT AND CHARACTERIZATION OF A POLY (-VINYL ALCOHOL) AND SODIUM ALGINATE FOAM WOUND DRESSING LOADED WITH PROPOLIS AND VITAMIN A

FORMATADO DE ACORDO COM AS NORMAS DO PERIÓDICO CARBOHYDRATES POLYMERS

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Development and characterization of a Poly (-vinyl alcohol) and Sodium alginate foam wound dressing loaded with propolis and vitamin A

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Abstract

Propolis is a complex mixture of polyphenols, essential oils, resins and waxes, with antibacterial, anti-inflammatory and healing properties. Vitamin A, in the form of all-trans retinoic acid, is implicated in wound healing by stimulating angiogenesis, cell recruitment, extracellular matrix deposition, and reepithelization. The incorporation of both agents to a polymeric wound dressing composed of poly (-vinyl alcohol) and sodium alginate may result in improved healing allied to controlled release, fluid uptake and wound protection. In the present work we have physically characterized this wound dressing and analyzed its releasing kinetics. The anti-inflammatory capacity was also assayed. SEM images showed a highly porous structure with a diverse morphology related to the content of the polymers. FT-IR spectra displayed a highly cross-linked structure with both polymers connected by hydrogen bonds. The wound dressings developed were able to retain great volumes of PBS and to retain their proper structure in a degradation model by at least 2 days of use. Propolis and vitamin A releasing behavior were maintained for a period of 6 hours. The concentrations of the biologically active substances were capable of promoting anti-inflammatory action in an erythrocyte membrane stabilization model. The wound dressings obtained here showed adequate physical properties, though the resistance to degradation of the wound dressings should be improved. The fabrication process did not affect the anti-inflammatory capacity. Further tests are needed to ensure the biocompatibility and to assess other the biological activities of therapeutic agents.

Keywords: biomaterials engineering, biomedical materials, natural products, tissue healing, tissue regeneration.

Introduction

Wound healing is a complex and dynamic process aimed to restore the functionality of the affected tissue. Many strategies have been employed to improve the quality of wound healing in order to minimize scarring, reduce inflammation and manage the stages of wound healing (GONZALEZ et al., 2016). Immediately after an injury, it is advised to clean the affected site and proceed to proper wound caring. According to the type and severity of the wound, it is desirable that the wound dressing provides and moist and protected barrier, able to prevent infections and contaminations, besides being easy to apply and to change.

Since the beginning of human concept of wound care, many natural products have been used to speed up healing and treating inflammation (SAMUELSEN, 2000). One such natural product is propolis, a complex mixture of resins, waxes, pollen, polyphenols and essential oils (HAVSTEEN, 2002). Propolis has a long story in human history and currently has been to object of study of many research groups. This fact is due to the amount of medicinal properties attributed to its use. Regarding the concept of wound healing, propolis is able to provide anti-bacterial properties, anti-oxidant, healing and anti-inflammatory action (ORYAN; ALEMZADEH; MOSHIRI, 2018; PARK et al., 2004; RAMOS; MIRANDA, 2007). Vitamin A by its turn is commonly used to restore damaged skin restoring its elastic properties and acting by promoting the formation of well-ordered collagen crosslinked fibers in wounds (COMBS et al., 2017a; MORA; IWATA; VON ANDRIAN, 2008; MUSALMAH et al., 2005). However, note should be taken that conflicting data about its application exist (COMBS et al., 2017b; KITANO et al., 2001). This vitamin is a simplistic name for a group of retinoids. In the present work, vitamin A is used in the form of the bioactive *all-trans* retinoic acid.

Polymeric wound dressings (scaffolds) loaded with drugs or natural products aimed to improve healing has been used with great success. The scaffolds produced can be tailored to match the needs and specifications of each wound (DHIVYA; PADMA; SANTHINI, 2015; EAGLSTEIN, 2001). The choose of the composing polymers, method of preparation, nature of the material and form of incorporation of the bioactive substance, are just a few of the examples. In the present work we have developed different blends of a lyophilized polymeric matrix composed of Poly (-vinyl alcohol) (PVA) and Sodium Alginate (SA), loaded with propolis and vitamin A. The result obtained is a foam wound dressing, able to retain great quantities of fluids and with controlled release of the bioactive agents. Here we have performed a series of characterizations aimed to comprehend the structure and properties of the material and the effect of the bioactive products.

Material and Methods

Propolis and Vitamin A

Green propolis was obtained commercially from Apis Flora® (Ribeirão Preto, SP – Brazil) in the form of a lyophilized powder. Vitamin A was acquired in the form of *all-trans* retinoic acid. Each mg of the vitamin A contains 3333 IU (each IU corresponds to the action of 0.3 g of *all-trans* retinoic acid)

Green propolis extraction methods

Ultrasound assisted extraction: 10 g of green propolis powder was solubilized in 100 mL of 80% ethanol, the solution was kept in the dark and submitted to the following parameters. In a probe ultrasound: freq – 60 kHz; Π – 300 W; t – 40 min (TRUSHEVA; TRUNKOVA; BANKOVA, 2007). After sonication the extract was kept in constant agitation in an orbital shaker for three days. The extract was vacuum filtered and stored. The remaining material was submitted to a second extraction process in the same conditions. Both extracts were put on a rotary evaporator to remove ethanol, then freeze-dried. The remaining powder was weighed and solubilized in ethanol 80% to a final concentration of 20 μg/μL.

Scaffolds preparation

The scaffolds were prepared according to the following method: Initially the PVA and Sodium alginate polymeric solution was prepared in deionized water at 95°C for PVA and 40°C for SA; constant stirring at 700 RPM and 3.5% (m:m), in separated beakers. On a new beaker it was added the Tween 80 at 8% of the desired polymeric solution and it spread in bottom to enhance solubility. The next step was the addition of the polymeric solutions according to the desired proportion (100:0, 75:25, 50:50 and 0:100; PVA:SA) under constant stirring and room temperature. Once the solution is uniform, we procced to the cross-linking step by lowering the pH with HCl to ~2 and then adding 0.75% (polymeric solution) of Glutaraldehyde (GA). In this step, in order to form longer polymeric chains, it is advised to reduce the stirring to 120 RPM for 30 minutes. Finally, this solution is poured in a silicon mold and frozen at -80°C and then freeze-dried at 0.180 mBar and -50°C for 24 h. Vitamin A (at 0.5%) has been added solubilized tween 80, in low light conditions. Propolis was added prior to reticulation step with temperatures below 40 °C in a concentration of 10% in relation to the polymeric solution.

Scanning electron microscopy of the samples

Samples morphology and pore measurements were analyzed in a Leo Evo 40 electron microscopy. Images were treated in Adobe Photoshop® CC 17 and Adobe Illustrator® CC 17

to enhance contrast and compose the figure. Pore sizes were calculated using the software ImageJ. For each image 50 pores were sampled and measured the longest diameter in three different samples.

Fourier transformed infrared (FTIR) spectroscopy

Infrared spectroscopy was conducted using an IR spectrometer Varian-660 IR (Pike) by ATR. The scaffolds were analyzed in a range between 4000 and 400 cm^{-1}, 16 scans and a resolution of 4 cm^{-1}

Porosity, swelling rate and degradation rate in phosphate buffered saline (PBS)

Porosity was measure the ethanol displacement method according to Zhang and Ma (1999) (ZHANG; MA, 1999a). Total porosity was calculated according to the following equation:

$$P = \frac{(Wf - Wi)}{\rho Vi} \times 100$$

Where W_f corresponds to the weight after stabilization, W_i corresponds to the initial weight before addition of ethanol, V_i is the initial volume of the sample and ρ is the density of pure ethanol (0.789 g mL^{-1}, Sigma-Aldrich). Due to the great variations observed for this method, this test was conducted in triplicates and repeated in three different days.

Swelling ratio was calculated as the maximal quantity of PBS absorbed by units of time. Data were collected and displayed as a function of time. Briefly, the scaffolds were placed in petri dishes containing PBS. After the determined periods of time (from 0 to 10 minutes), the sponges were collected with a pair of tweezers and the excess water on the surface was removed. Samples were weighed and returned to the PBS solution (KUMAR et al., 2010). Due to the great variation observed for this method, the test was conducted in triplicates and repeated in three different days.

Degradation rate was conducted in PBS containing lysozymes at physiological concentration (10.000 U mL^{-1}), at 37°C. Samples were cut into squares weighing 100 mg and placed into vials containing PBS and lysozymes. During collection days (5^{th}, 10^{th} , 15^{th} and 20^{th} days), samples were rinsed thoroughly with deionized water, then frozen at -80°C, freeze-dried, weighed and put back in a fresh medium (MADHUMATHI et al., 2010). The loss of mass was recorded and plotted as a percentage in function of time according to the equation:

$$DR(\%) = \frac{Wi - Wf}{Wi} \times 100$$

Whereas W_i and W_f means initial weight and final weight, respectively.

Propolis releasing kinetics

Propolis release from polymeric scaffold to the solution was measured by using a Franz's cell model. The donor chamber contained the scaffold immersed in 5 mL PBS at 32°C, to simulate the temperature of a damaged tissue. The receiving chamber was separated from the donor chamber by a Strat-M membrane, and contained 30 mL of PBS (FABRI et al., 2011). The bottom solution was agitated at 100 RPM to simulate the dynamics of skin. In specified time intervals (from 0 minutes up to 4 hours) 3 mL aliquots were collected and analyzed in a spectrophotometer at 299 nm using a quartz cuvette. Immediately after each sample collection, another 3 mL of fresh buffer was added to the receiving chamber. This analysis was conducted only for propolis due to the hydrophobic nature of vitamin A. Initial analysis showed that vitamin A interfere to propolis releasing rate. Data were compared to an absorbance x concentration curve, and it was calculated the concentration of propolis (%) by unit of time.

Vitamin A releasing kinetics

Vitamin A releasing kinetics were conducted in a Franz cell model in the same conditions for 4 hours to prevent drug degradation by temperature. The releasing medium of the donor chamber and the receiving chamber was composed of PBS (pH 7.4) added with 2% tween 80, to ensure sinking of vitamin A. The samples were analyzed in spectrophotometer at 350 nm using a quartz cuvette. Immediately after each sample collection, another 3 mL of fresh buffer was added to the receiving chamber. Samples containing vitamin A and propolis were also assayed to comprehend the effects in propolis release. Data were compared to an absorbance x concentration curve, and it was calculated the concentration of propolis (%) by unit of time. The reciprocal effect of vitamin A and propolis upon their releasing behavior was observed, by using blanking the spectrophotometer with the respective standard.

Erythrocytes membrane stabilization assay

Human erythrocytes membrane was used as a model for anti-inflammatory action. It is known that erythrocytes membrane resembles lysosomal membranes, which during inflammatory reactions are mobilized and their content released causing a series of physiological alterations (KLESZCZYŃSKA et al., 2005).Thus, the methodology proposed by Shinde et al. (1999), relates the inhibition of hemolysis induced by heat to anti-inflammatory potential. Briefly, fresh whole blood was collected and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the remaining content was used and diluted in PBS to a 2% concentration.

The anti-inflammatory assay was conducted as such: The scaffolds were cut into 0.5 cm x 0.5 cm x 0.2 cm pieces and placed inside 5 mL caped tubes. Each tube contained 3.5 mL of

erythrocytes rich solution at 2%. Samples containing the wound dressings were put into a thermostatic bath at 54 °C for 20 minutes and three tubes containing only erythrocytes solution were maintained in a refrigerator at 6 °C to serve as negative controls and other three without the scaffolds at 54°C to serve as positive controls. Salicylic acid (200 μg mL^{-1}) and sodium diclofenac (100 μg mL^{-1}) were used as anti-inflammatory parameter controls. The tubes were centrifuged at 3000 rpm for 5 minutes, resulting in the pellet formation. The supernatant was collected and its absorbance at 540 nm was measured. The percentage of hemolysis was obtained by the formula:

$$Anti - inflammatory\ \% = 1 - \left(\frac{A2 - A1}{A3 - A1}\right) x100$$

Where A1 = absorbance of the negative control

A2 = sample absorbance

A3 = absorbance of the positive control

Statistics

All tests were conducted in triplicates and, when specified, repeated in three different days to reduce Standard deviation of data. Data were analyzed by one-way ANOVA and difference between means was assessed by Scot-Knott's test. $P<0.05$ was considered statistically significant.

Results and Discussion

Scanning electron microscopy (SEM)

The SEM images shows the front view (Figure 1) and cross section (Figure 2) of the scaffolds. The great majority of the pores found on the wound contacting surface were smaller than 100 nm in all three scaffolds. One of the main concerns while producing a topical wound dressing is absence of adherence in the granulating tissue in order to avoid further damage and pain during changes. Therefore, the presence of smaller pores reported here are linked to reduce the likelihood of the newly formed epithelium to migrate into the foam material (PYUN et al., 2015). According to the SEM images obtained, the addition of sodium alginate reduces the pore size and increases quantity of pores. Data found in previous works corroborate with this statement, in which an increase in viscosity achieved by adding more SA, results in a finer pore structure (FAN et al., 2010; FLORCZYK et al., 2011).

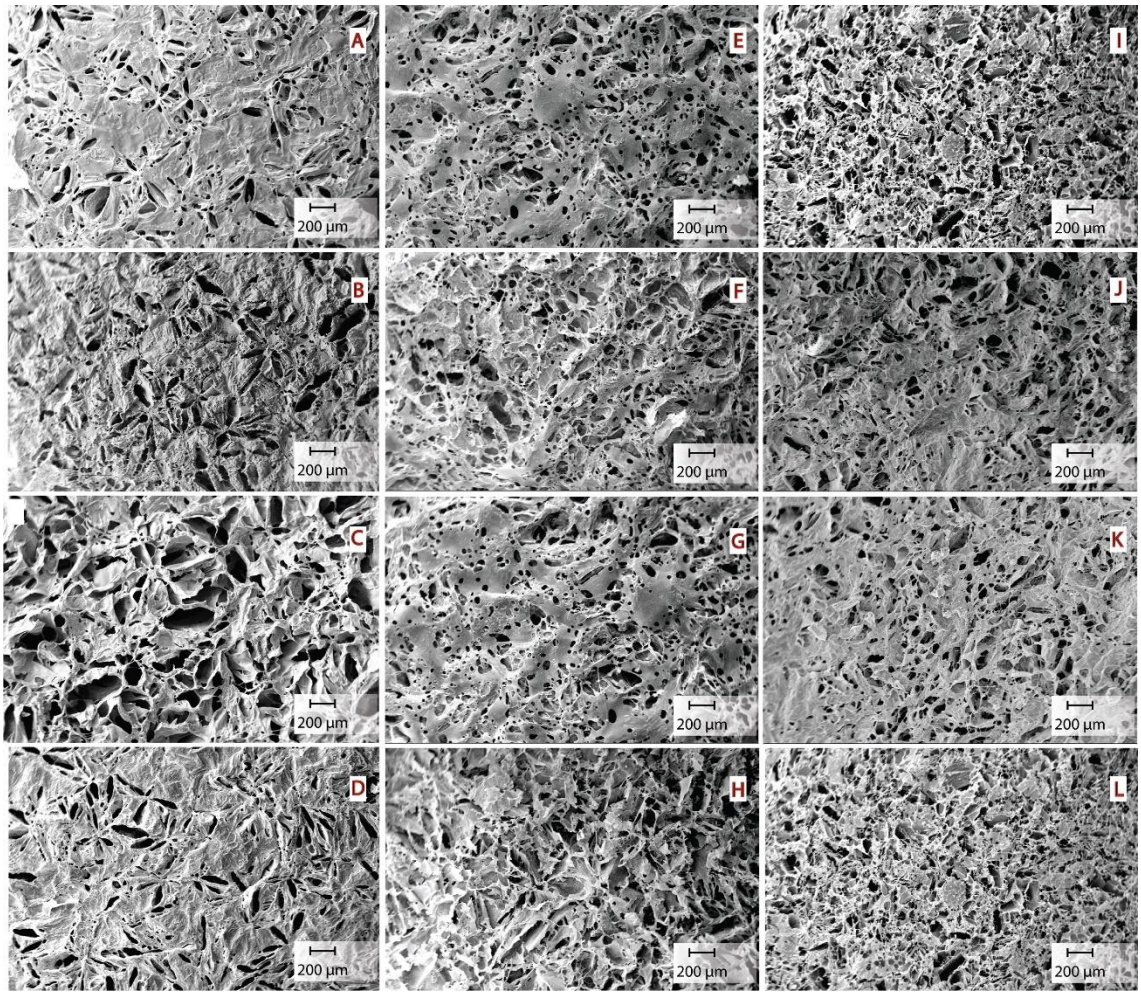


Figure 1 – Wound contacting surface Scanning electron microscopy photographs. A – 100:0 (PVA:SA); B - 100:0 P (propolis) C - 100:0 A (vitamin A); D - 100:0 PA (propolis + vitamin A); E – 75:25; F - 75:25 P; G - 75:25 A; H - 75:25 PA; I – 50:50; J - 50:50 P; K - 50:50 A; and L - 50:50 PA.

Table 1 – Morphological measurements of the wound dressings

Sample	Mean diameter (µm)	ϴMin (µm)	ϴMax (µm)
A (100:0)	110.31±49.31 **b**	68.38	240.20
B (100:0 P)	128.67±49.10 **a**	23.97	244.89
C (100:0 A)	150.67±77.81 **a**	43.04	354.60
D (100:0 PA)	157.22±63.82 **a**	30.79	327.79
E (75:25)	89.95±59.03 **c**	29.21	241.44
F (75:25 P)	83.97.45±59.24 **c**	35.56	241.65

G (75:25 A)	103.84±42.33 **b**	41.76	194.92
H (75:25 PA)	105.49±48.59 **b**	47.60	251.65
I (50:50)	55.41±14.41 **d**	34.60	79.06
J (50:50 P)	87.67±34.78 **c**	31.25	183.13
K (50:50 A)	96.97±43.93 **b**	33.22	215.47
L (50:50PA)	55.13±27.96 **d**	11.58	147.89

Ratios (PVA:SA), P (propolis) and A (vitamin A). Values were obtained from the observation of 50 pores in three repetitions. All values are expressed in μm. Mean diameter are followed by the standard deviation of data. The values followed by the same letter do not differ statistically from each other using Scott-Knott test ($p<0.05$). The letters are displayed in decreasing order.

Another difference observed is related to pores architecture, since the addition of SA, results in rounder interconnected pores. Cross-section images (figure 2) shows deep pores displayed in a columnar fashion for all 100:0 wound dressings irrespective the content of propolis and/or vitamin A.

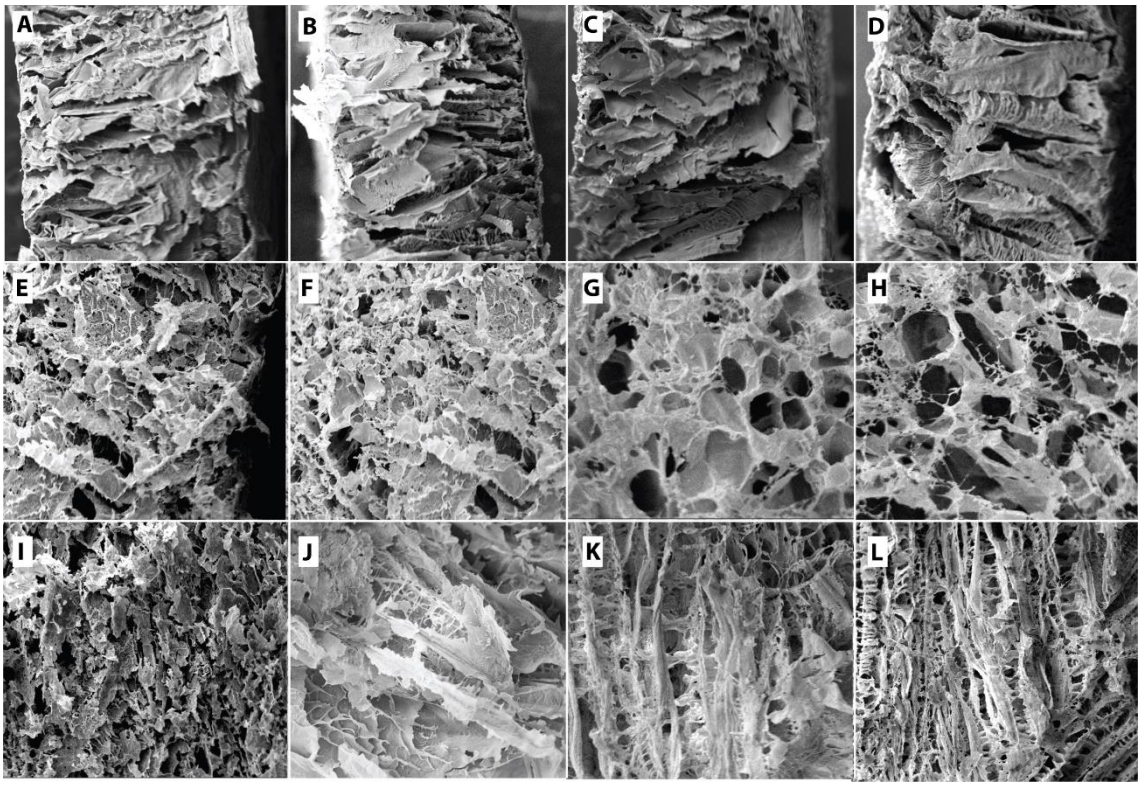


Figure 2 – Cross section SEM photographs. A – 100:0 (PVA:SA); B - 100:0 P (propolis) C - 100:0 A (vitamin A); D - 100:0 PA (propolis+vitamin A); E – 75:25; F - 75:25 P; G - 75:25

A; H - 75:25 PA; I – 50:50; J - 50:50 P; K - 50:50 A; and L - 50:50 PA. Images are not in the same scale.

Pure PVA scaffold displays fewer pores inside the cells, which is a strong indicator of poor interconnectivity, though it should be noted that the polymeric structure is highly permeable. This effect could result in an impaired fluids and gases exchange and it may reflect on reduced propolis release (MI et al., 2001). Pore sizes, structure and distribution are linked to many factors such as polymeric composition, solute/solvent ratio, freezing speed and temperature. Initial experiments were performed in different freezing temperatures and polymeric ratios (data not shown). The results of the freezing test showed that temperatures higher than -25°C are unable to form foam scaffolds with good structural organization and highly porous, if the solvent is water (MA; CHOI, 2001). The more water soluble the polymer, the smaller is the pore sizes (LOH; CHOONG, 2013). Thus, as can be seen from table 1, the addition of SA to the PVA polymeric solution can be used to control both porosity and pore sizes. Additionally, with the addition of SA (75:25 or 50:50 scaffolds), cross-sections electron micrographs show rounder shapes with cells unevenly distributed with high interconnectivity. Wound dressings composed of equal parts of sodium alginate and PVA fail to follow a pattern of pore structure. Wound dressing I, presents crackled columns with interconnected pores resembling leaves. In wound dressing J, the crackled pattern becomes more integer and less interconnected. For both K and L wound dressings, the columns streams from bottom to top and are divided by horizontally oriented interconnections. The addition of propolis to vitamin A seemed to promote a reduction in pore size, as compared to vitamin A alone. Besides the biological effect expected for vitamin A on *in vivo* studies, its addition seemed to be very advantageous. Specially for the 75:25 foam, the addition of vitamin A resulted in a more malleable and firm structure, alongside with a porosity adequate enough to avoid migration of growing cells inside the scaffold.

Fourier Transform Infrared (FTIR) analysis

FTIR spectroscopy of the raw polymers, glutaraldehyde (GA) and the wound dressings were carried out to detect any changes in peaks that could indicate the nature of the interaction among those parts (Figure 3). The FTIR spectra obtained for the blends display similar features with the raw material. GA presents an intense O-H peak around 3300 cm^{-1} (blue region) due to the presence of water in its solution, while both PVA and SA, presents more discrete stretching bands in the same region. This peak is also present in all samples, since both polymers are

connected via intra and intermolecular O-H bonds (ANDRADE et al., 2008; PAPAGEORGIOU et al., 2010). Crosslinking with GA promotes the formation of acetal bridges, represented by the dashed line in 1731 cm^{-1} (MANSUR et al., 2008). The two peaks marked in red are characteristic of the presence of aldehyde groups from GA. The peaks localized in 1596 and 1404 cm^{-1} are characteristic of the metallic complex formed between carboxylate and sodium in SA (raw). These peaks are lost upon crosslinking with GA due to the formation of acetal bridges.

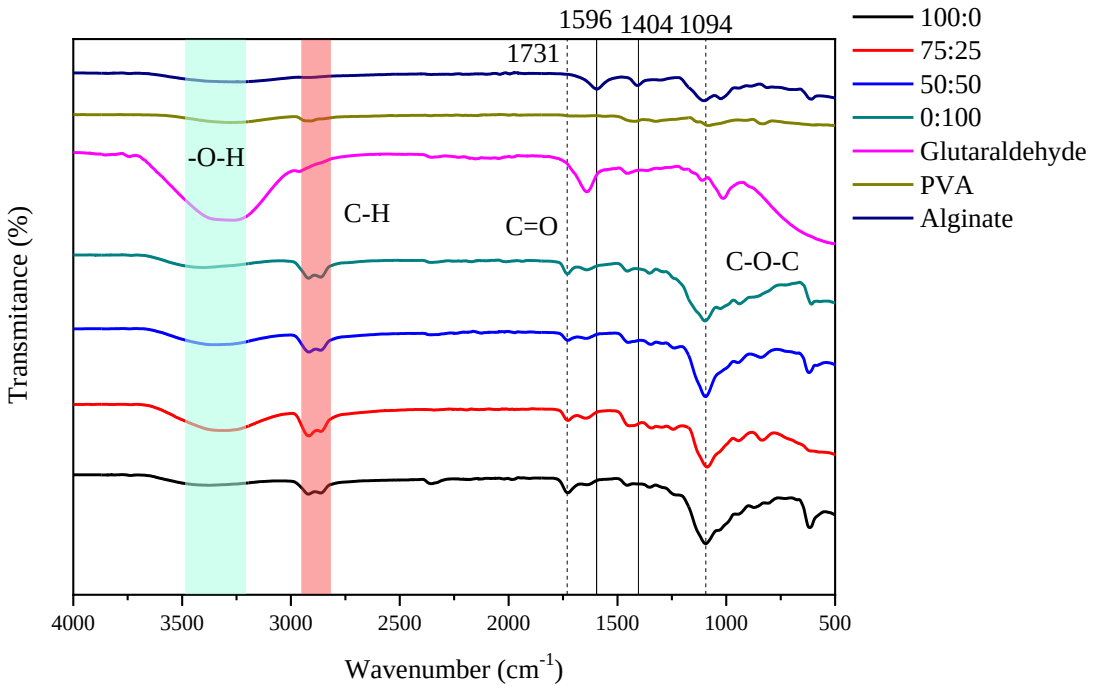


Figure 3– FTIR spectra from raw Poly -vinyl alcohol (PVA), Sodium Alginate (SA) and Glutaraldehyde (GA) stacked with the scaffolds spectra (100:0 PVA:SA (v:v); 75:25, 50:50 and 0:100)

Porosity, swelling rate and degradation rate in phosphate buffered saline (PBS)

Porosity is a measure of the empty space in a material and it is related to fluids transport, swelling rate and structural integrity. It is known that highly porous scaffolds improve wound healing by ensuring sufficient gas exchange and metabolic waste products elimination (KUMAR et al., 2010; YOU et al., 2017). Fluids uptake by the scaffold is essential to avoid tissue maceration since the content of this exudate contains matrix metalloproteinases and other biochemical components that may degrade the growing tissue (DHIVYA; PADMA; SANTHINI, 2015). In our work, the porosity means of each scaffold had a very similar behavior in relation to the presence of propolis, vitamin A and both (Figure 4). However, the addition of

propolis or vitamin A promotes an increase in porosity in relation to the control scaffold. Although the pore size influences the porosity, the addition of sodium alginate (small pores) do not affect total porosity, since the distribution of pores in the wound dressing is higher than the scaffold 100:0 (larger pores).

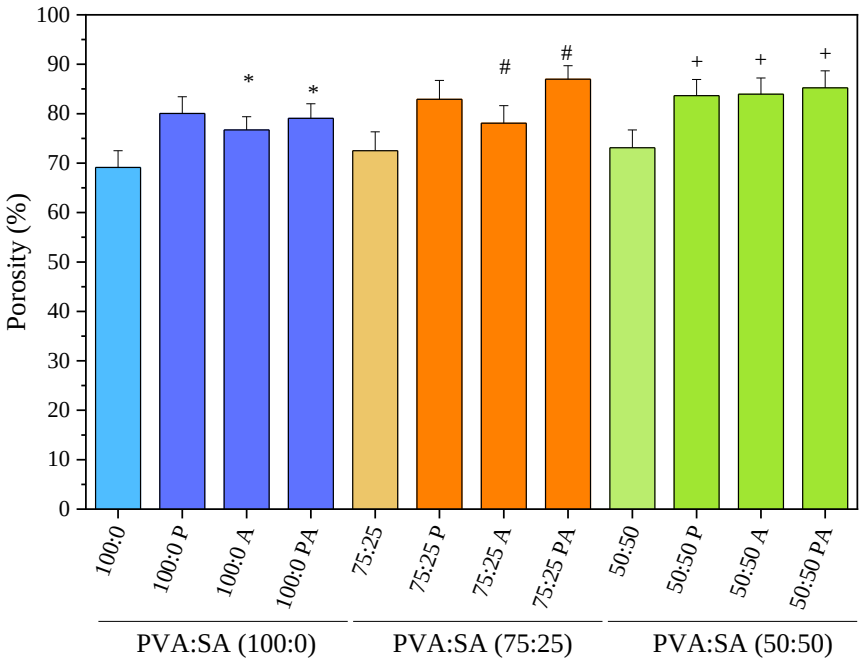


Figure 4 – Porosity of the three scaffolds and the effect of the addition of propolis and/or vitamin A. Porosity was calculated in triplicates and the experiment was repeated twice to ensure its validity. Data was submitted to ANOVA ($p<0,05$). * - Means differ from the control 100:0; # - Means differ from the control 75:25; + - Means differ from the control 50:50. The proportions correspond to PVA:SA; P (propolis); A (vitamin A); PA (propolis+vitamin A). The values followed by the same symbol do not differ statistically from each other using Scott-Knott test ($p<0,05$). The letters are displayed in decreasing order.

The swelling behavior of a wound dressing is of paramount importance. As mentioned above, the proper control of fluids is necessary to avoid tissue maceration, but also to provide adequate hydration to the wound site (KUMAR et al., 2012). Swelling behavior are influenced by the hydrophilic nature of the polymers composing the material, as well as the flexibility and structure of the pores (CHANG et al., 2003; HUANG; YANG, 2008). As can be perceived in figure 4, all wound dressings exhibited, in general, a good swelling as they were capable of retaining more than twice their own weight in PBS.

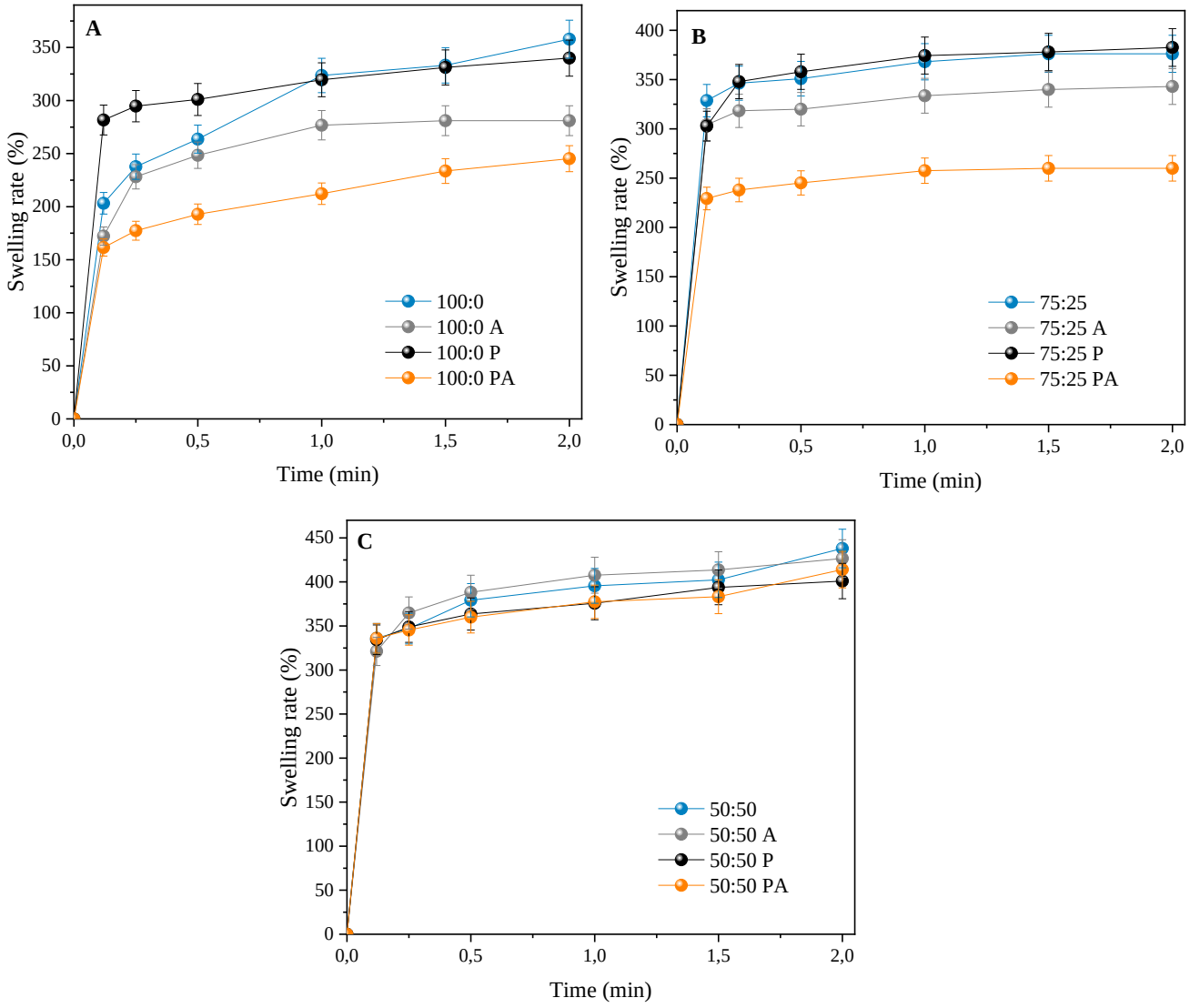


Figure 5 – Swelling rate of the different scaffolds developed and the effect of propolis, vitamin A or both upon water uptake. Data is followed by standard deviation. The proportions correspond to PVA:SA; P (propolis); A (vitamin A); PA (propolis+vitamin A).

The addition of the hydrophobic vitamin A reduced the capacity of retaining PBS, except for the blend 50:50 containing propolis and vitamin A, for which this effect was not observed. As the concentration of SA was increased, the liquid holding capacity also increased. This effect is due to the more hydrophilic nature of SA in comparison to PVA. Exudates may arise from different factors and has different characteristics regarding their constitution, color and viscosity (VOWDEN; VOWDEN, 2003). The scaffolds developed here were tested for PBS uptake, reflecting a case where the exudate is waterier and thinner. These cases are related,

for example, to *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection, that causes fibrinolysis and hence watery and thin exudates (FONDER et al., 2008). Further, many types of wounds where occurs blood vessels trauma, or maceration of the skin tissue, display this characteristic exudate. According to existing literature, the levels of PBS uptake would be enough to manage most types of wounds, as the ones mentioned previously (ATTINGER et al., 2006; JAYAKUMAR et al., 2011; SOOD; GRANICK; TOMASELLI, 2014b). Wound dressings containing alginates, in general, are very recommended for light to moderate exudative wounds. Alginates are considered good hemostatic agents, besides presenting a mildly antibacterial action (FRIEDMAN; SU, 1984; MERTZ; OVINGTON, 1993). Allied to these factors, they also are soft, nonwoven fibers and are capable of promoting excellent fluids management (KANNON; GARRETT, 1995). It should be noted that the addition of propolis could avoid the infection and complications on the wound site, inhibiting the evolution of the wound site to an infected state and alteration in exudate viscosity.

The third parameter analyzed in this section is the degradation rate in PBS containing lysozymes. Wound dressings should be designed to withstand the conditions of the proposed use, thus we used lysozymes concentration and medium temperature similar to the ones found in the affected site (MORGADO et al., 2014). In figure 5, we provide the degradation kinetics in a period of 20 days.

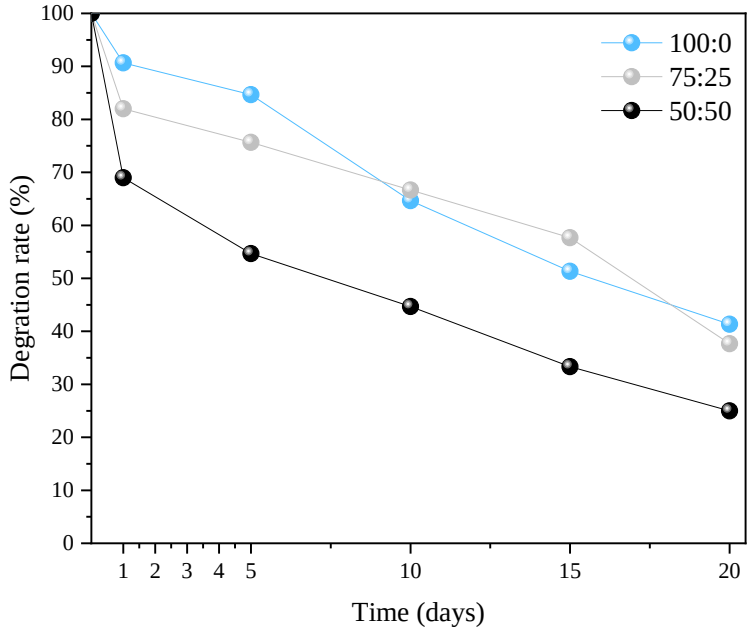


Figure 6 – Degradation rate assay was performed in PBS with lysozymes during 20 days. Data was obtained from triplicates means and expressed in percentage.

Degradation rates influences the release of the bioactive molecules due to an increase in superficial area. Though degradation is important to the release of propolis and vitamin A, conversely an accelerated degree of degradation results in loss of swelling capacity and structural integrity. Besides that, in a real scenario, faster degradation means that the patient should be submitted to more wound dressing changes, resulting in more pain and loss of quality of life. In special, the scaffolds containing SA are more susceptible to enzymatic degradation due to the presence of glycoside bonds between the monomeric units in SA. We have observed that a loss of 25% in mass resulted in a significant loss in mechanical integrity, therefore, wound dressings with higher losses of mass should be discarded. After 24 hours, only 100:0 and 75:25 scaffolds retained its physical integrity and usability. After 48 hours, the wound dressing 50:50 is considered unsuited for use. Wound dressing 75:25 was suitable for use up to five days, while 100:0 lasted for almost a week. This point is of great concern since the faster degradation of the wound dressings could release debris to the wound site and promote maceration of the interfacing tissues. Thus, we believe that the scaffolds 100:0 and 75:25 (50:50 was discarded due to failure in resisting degradation), developed here should be used by no more than three to five days.

Propolis release kinetics

An appropriated and sustained release profile is vital for the performance of the scaffold. Results displayed in figure 6, shows incomplete release of propolis even after 24 h. One possible reason may be related to the incorporation of propolis to the very structure of the scaffold and the complexity of the internal structure to release propolis completely.

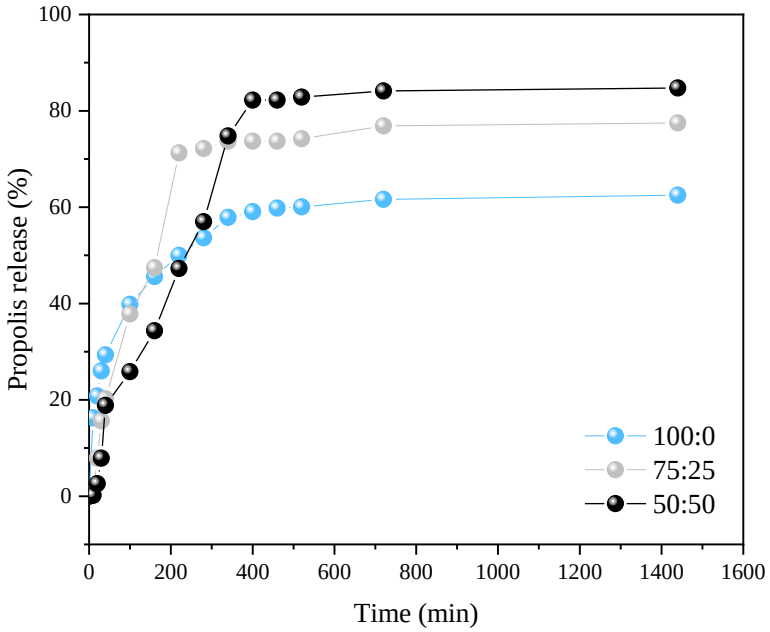
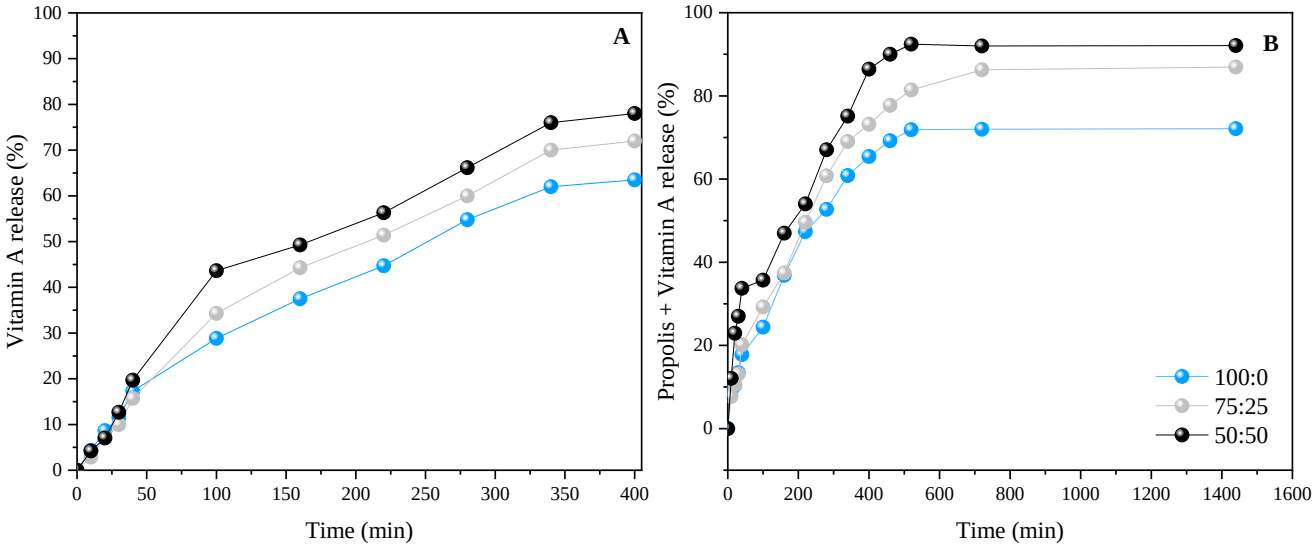


Figure 7 – Propolis release rate conducted in a Franz's cell diffusion model and evaluated by spectrophotometry at 299 nm. Diffusion was observed during 24 hours until stabilization. The proportions correspond to PVA:SA; P (propolis).

The releasing behavior showed direct relationship with the results obtained for swelling rate (more internal contact surface) and degradation rate (loss of mass accompanied by release of propolis). In this sense, the scaffold 50:50 released more propolis than 75:25 and 100:0. A continuous and controlled release of propolis from the polymeric dressing offer some potential advantages. The prolonged action of propolis could aid in many aspects of wound healing such as maintaining bactericidal action, providing bioactive molecules with anti-inflammatory action and anti-oxidant action.

Vitamin A releasing kinetics and its effect on propolis release

Vitamin A release was slower than the release of propolis, though its addition to the blends promoted an increase in propolis diffusion to the medium. The kinetics observed for vitamin A followed the same pattern as observed for propolis. This fact was addressed to the degradation rate of wound dressings. Vitamin A was quantified up to 400 minutes, reaching 76% of release for the sample composed of 50:50 PVA:Sodium alginate, 70% for 75:25 and 62% for 100:0 (Figure 8 A), further readings showed decrease in vitamin A quantity. Though it is worth mentioning that wound dressings aged 15 days presented only minor signs of Vitamin A degradation, what means that the degradation is more pronounced in solution, while it is preserved on the polymeric matrix. Vitamin A in combination with propolis resulted in a higher release of both propolis (B) and vitamin A (C), with propolis releasing values of 92% for 50:50, 86% for 75:25 and 72% for 100:0 scaffolds (Figure 8B) and vitamin A (Figure 8 C) releasing values of 68%, 78.6% and 82.3%, respectively.



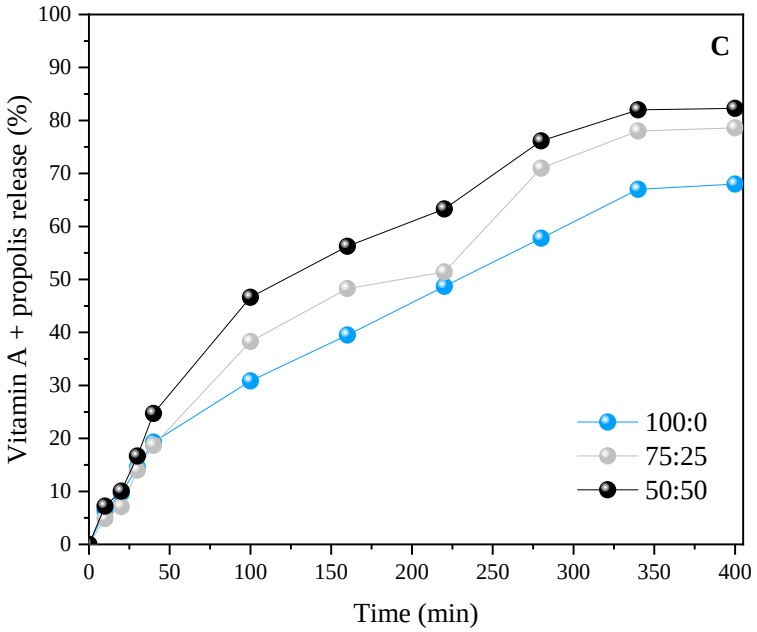


Figure 8 – Vitamin A releasing from the scaffolds and the effect of Vitamin A in combination to propolis on the releasing kinetics. A – vitamin release over time. B – effect of the vitamin A upon propolis release. C – Effect of propolis upon vitamin A release.

The addition of vitamin A and Tween 80 used here is theorized to aid in the release of polyphenols, in special for the more hydrophobic and with higher molecular mass (ZILLICH et al., 2013). According to Lu, Kelly and Miao (2016) the use of oil-in-water emulsions (higher content of water in relation to oil) improved the release and skin absorption rate of polyphenols like quercetin, rutin, and resveratrol through skin (LU; KELLY; MIAO, 2016). The absorption of resveratrol through skin may be also improved by five to ten-fold following the formation of a microemulsion with tween 80 (YUTANI et al., 2012). Unpublished data discloses the HPLC spectra for propolis that allowed the identification of gallic acid, catechin, chlorogenic acid, caffeic acid, vanillin, p-coumaric acid, ferulic acid, salicylic acid, resveratrol, quercetin and rutin. Being quercetin and rutin the majoritarian compounds. We can correlate this hypothesis to the higher protection offered by those samples containing vitamin A in heat induced hemolysis assay. Furthermore, the extended action could improve the patient common compliance by reducing the discomfort of frequent dressing changes (HECKE; GRYPDONCK; DEFLOOR, 2007).

Heat induced hemolysis and correlation with anti-inflammatory action

During inflammatory reactions lysosomes release its content, rich in enzymes that play many physiological roles during inflammation (GE et al., 2015). Non-steroidal anti-

inflammatories exerts their pharmacological action by inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes (CARRILLO et al., 2016). Since erythrocytes membrane are very similar to lysosomes, these cells can be used as model to study anti-inflammatory action of many substances. The inflammatory reaction is stimulated by heat to induce its membrane destabilization (content release simulation) and if a particular agent is able to minimize this heat hemolysis, we can relate this action to a possible anti-inflammatory potential. Here we evaluated the potential of vitamin A and propolis wound dressings to minimize this action and the results were compared to sodium diclofenac and acetyl salicylic acid.

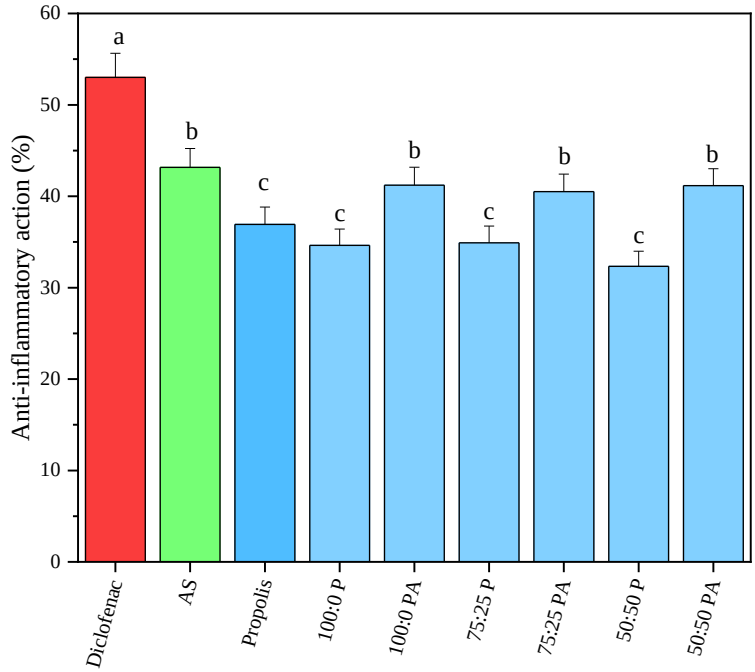


Figure 9 –Anti-inflammatory activity of propolis and vitamin A wound dressings, evaluated by erythrocytes membrane stabilization submitted to high temperature. Data followed by the same letters are considered statistically equal ($p<0.05$). AS: acetyl salicylic acid. The proportions correspond to PVA:SA; P (propolis); PA (propolis + vitamin A). The values followed by the same letter do not differ statistically from each other using Scott-Knott test ($p<0.05$). The letters are displayed in decreasing order.

As can be seen in figure 9, both propolis and vitamin A promoted the stabilization of erythrocytes membrane. Anti-inflammatory action was higher when both agents were used in combination. This pharmacological action was preserved even after fabrication process. The

activity of both vitamin A and propolis was smaller than the one observed for diclofenac and equal to AS (~38.5% of anti-inflammatory action). Management of inflammation in initial phases is necessary to minimize scaring, excessive pain and fluid release, even a moderate anti-inflammatory action as observed here could aid in the treatment. The anti-inflammatory action is highly desirable once it promotes the adequate progress of each phase during healing, avoiding the development of chronic wounds (MARTINOTTI; RANZATO, 2015; ORYAN; ALEMZADEH; MOSHIRI, 2018).

Conclusions

In this work we have characterized PVA:Sodium Alginate scaffolds obtained by freeze drying loaded with propolis and vitamin A. With a simple preparation methodology, the scaffolds developed presented adequate physical properties such as propolis releasing profile, pore size and swelling rate. Propolis and vitamin A releasing behavior were maintained for a period of 6 hours. The concentrations of the biologically active substances were capable of promoting anti-inflammatory action in an erythrocyte membrane stabilization model. The wound dressings obtained here showed adequate physical properties, though the resistance to degradation of the wound dressings should be improved. The fabrication process did not affect the anti-inflammatory capacity. Further tests are needed to ensure the biocompatibility and to assess other the biological activities of therapeutic agents.

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Conflict of interest

The authors declare that they have no conflict of interest.

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5 ARTIGO II - POLY (-VINYL ALCOHOL) AND SODIUM ALGINATE FOAM WOUND DRESSING LOADED WITH PROPOLIS AND VITAMIN A: FABRICATION PROCESS, BIOLOGICAL AND BIOCOMPATIBILITY ASSESSMENT

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Poly (-vinyl alcohol) and sodium alginate foam wound dressing loaded with propolis and vitamin A: Fabrication process, biological and biocompatibility testing

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Abstract

Propolis is an important compound produced by bees rich in polyphenols and other biologically active substances with healing, antioxidant, bactericidal and anti-inflammatory properties. Vitamin A is a lipophilic micronutrient that acts in epidermis differentiation and maturation, as well as in promoting angiogenesis and cellular recruitment. The use of polymeric wound dressings containing bioactive substances is an advantageous strategy to treat many types of wounds. In our work, we have developed three poly (-vinyl alcohol) and sodium alginate (100:0, 75:25 and 50:50, v:v) wound dressing containing propolis and vitamin A. In this article we have tested two different propolis extraction process and its biological properties in combination with vitamin A. The wound dressings were obtained by freeze-drying and reticulated with glutaraldehyde. Biocompatibility tests were performed with the wound dressing and its components. Propolis ultrasound assisted extraction (UAE) method was more efficient in comparison to maceration, yielding more polyphenols. The UAE was rich in rutin (26.4 mg/g of extract) and quercetin (17.21 mg/g), presenting a high antioxidant activity by the DPPH method. Both propolis extracts were tested against Gram-negative and Gram-positive bacteria, although only the UAE was effective against all strains. The presence of vitamin A did not affect propolis performance in none of the biological activities. The scaffolds containing propolis extract were tested and the bactericidal effect maintained even after the fabrication process. The scaffolds and its components, alongside with propolis and vitamin A, did not presented any toxic effect in the conditions assayed. Wound dressings developed here have the potential to treat moist wounds, avoiding bacterial infections and is expected to promote a faster healing attributed to the presence of vitamin A and polyphenols.

Keywords: biomedical materials, natural products, tissue healing, tissue regeneration.

Introduction

Propolis is an important compound produced by bees composed mainly by resins (50%), waxes (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) (SILVA-CARVALHO; BALTAZAR; ALMEIDA-AGUIAR, 2015). Bioactive compounds found in propolis such as flavonoids, terpenes, beta-steroids, alcohols, vitamins and aromatic aldehydes are responsible for its antibiotic, antioxidant, cytoprotective and anti-inflammatory action (MARCUCCI, 1995). Propolis have been used in traditional medicine for thousands of years, and has gained scientific notoriety over the course of time. Due to its medicinal properties, propolis is considered one of the best natural products to treat wounds (WAGH, 2013).

Vitamin A is a micronutrient with lipophilic character, composing the family of retinoids (COMBS et al., 2017c). Vitamin A in the form of *all-trans* retinoic acid has been used to treat skin conditions like psoriasis and acne, but also to treat serious health issues such as in the treatment of acute promyelocytic leukemia (CASTRO et al., 2011; MIRZA et al., 2006). Topical application of all-trans retinoic acid was shown to improve wound healing in diabetic rats, and to increase collagen synthesis in diabetic human skin in organ culture (BRAUNGART; DEGITZ; MAGDOLEN, 2001; LATEEF et al., 2005). Vitamin A plays an essential role in angiogenesis, reepithelization and fibroplasia. Literature data have shown that local and systemic supplementation with vitamin A promoted and increase in collagen synthesis (ABDELMALEK; SPENCER, 2006; KARUKONDA et al., 2000). Animals deprived of vitamin A healed poorly after surgery, in comparison to the control group. In the study, it was observed a delay in wound closure and poor dermatologic aspect (ARRUDA; SIQUEIRA; DE VALÊNCIA, 2009).

In the broad sense of the word, wounds may be defined as an interruption in any tissue integrity, being the etiology accidental or intentional (i.e. surgical process)(ENOCH; LEAPER, 2008). Immediately after an injury, the organism initiates specific routes aimed to control hemorrhagic process, pathogens infection and production of a scaffold matrix to harbor cells and support angiogenesis (ARRUDA; SIQUEIRA; DE VALÊNCIA, 2009). The resolution of the wound healing cascade is marked by the complete closure of the injury.

Although our body possess the biological machinery to heal and repair many different injuries, an open and untreated wound pose as a serious health problem. An untreated wound is constantly exposed to pathogens and mechanical damage; besides those factors, the overall healing may be defective with excessive scaring tissue formation, fibrosis or a delay in resolving the event (GUO; DIPIETRO, 2010). Another important factor to take into account is

related to risk groups such as diabetics and bedridden patients. In both cases wound healing is a difficult process to resolve, therefore the development of technologies and devices able to promote a faster wound healing and gains in quality of life are fundamental. In our work, we have developed a wound dressing composed of poly(-vinyl alcohol) and sodium alginate incorporated with propolis and vitamin A. Here we will describe the best propolis extraction method, its bactericidal and antioxidant actions, and its incorporation in the material. Finally, we will provide information related to an initial biocompatibility testing of the material.

Materials and Methods

Reagents, Propolis and Vitamin A

Green propolis was obtained commercially from Apis Flora® (Ribeirão Preto, SP – Brazil) in the form of a lyophilized powder. Vitamin A was acquired in the form of an oil containing *all-trans* retinoic acid. Each mg of the vitamin A was acquired in the form of *all-trans* retinoic (3333 IU. Each IU corresponds to 0,3 g of *all-trans* retinoic acid). Thrombin (in the form of Factor IIa) was purchased from Sigma. High-density polyethylene was obtained from sample bottle and cooper wire was purchased from a general store.

Green propolis extraction methods

Ultrasound assisted ethanolic extraction:

10 g of green propolis powder was solubilized in 100 ml of 80% ethanol, the solution was kept in the dark and submitted to the following parameters. In a probe ultrasound: freq – 60 kHz;П – 300 W; t – 40 min. After sonication the extract was kept in constant agitation in an orbital shaker for three days. The extract was vacuum filtered and stored. The remaining material was submitted to a second extraction process in the same conditions. Both extracts were put on a rotary evaporator to remove ethanol, then freeze-dried. The remaining powder was weighed and solubilized at 20 μg/μL in ethanol 80%.

Methanolic extraction:

10 g of propolis was added to 100 mL of methanol 50%, under reflux, in a heat plate at 80°C, boiling for 15 minutes. After this step the extract was filtered in a filter paper (Whatman filter No.1) and stored. The residue was submitted to the processing step two more times. The extracted material was gathered and put on a rotary evaporator to remove methanol. The extract was then submitted to a freeze-drying process, weighed and solubilized in ethanol 80% at the ratio of 20μg/μL.

Scaffolds preparation

The scaffolds were prepared according to the following method: Initially the PVA and Sodium alginate polymeric solution was prepared in deionized water at 95°C for PVA and 40°C for SA; constant stirring at 700 RPM and 3.5%, in separated beakers. On a new beaker ir was added the Tween 80 at 8% of the desired polymeric solution and it spread in bottom to enhance solubility. The next step was the addition of the polymeric solutions according to the desired proportion (100:0, 75:25, 50:50 and 0:100) under constant stirring and room temperature. Once the solution is uniform, we procced to the cross-linking step by lowering the pH with HCl to ~2 and then adding 0,75% of Glutaraldehyde (GA). In this step, in order to form longer polymeric chains, it is advised to reduce the stirring to 120 RPM for 30 minutes. Finally, this solution is poured in a silicon mold and frozen at -80°C and then freeze-dried at 0.180 mBar and -50°C for 24 h. Vitamin A (at 0.5%) has been added solubilized tween 80, in low light conditions. Propolis was added prior to reticulation step with temperatures below 40 °C in a concentration of 10% in relation to the polymeric solution.

Determination of phenolic composition of extracts

Total phenolic content was measured by Folin-Denis method using tannic acid as a standard, for both extracts, Ultrasound assisted ethanolic extraction (UAE) and methanolic extract (ME) (AOAC, 2016).

The high-performance liquid chromatography (HPLC) was performed, only for UAE, using a Shimadzu UHPLC chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with two LC-20AT high-pressure pumps, an SPD-M20A UV-vis detector, a CTO-20AC oven, a CBM-20A interface, and an automatic injector with an SIL-20A auto sampler. Separations were performed using a Shim-pack VP-ODS-C18 (250 mm×4.6 mm) column, connected to a Shim-pack Column Holder (10 mm×4.6 mm) pre-column (Shimadzu, Japan). The phenolic standards used were gallic acid, catechin, epigallocatechin gallate, epicatechin, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, resveratrol and quercetin all purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions were prepared in methanol (HPLC grade; Sigma-Aldrich, USA).

The mobile phase consisted of the following solutions: 2% acetic acid in water (A) and methanol:water:acetic acid (70:28:2 v/v/v) (B). Analyses were performed for a total time of 65 min at 40 °C, flux of 1 ml min^{-1}, wavelength of 280 nm, and injection volume of 20 µL in a gradient-type system (100% solvent A from 0.01 to 5 min; 70% solvent A from 5 to 25 min; 60% solvent A from 25 to 43 min; 55% solvent A from 43 to 50 min; and 0% solvent A for 10 min) until the end of the run. Solvent A was increased to 100%, seeking to maintain a balanced

column. Acetic acid and methanol (HPLC grade; Sigma-Aldrich, USA) were used in the preparation of the mobile phase (Marques et al., 2016).

The phenolic compounds in the extract were identified by comparison with the standards retention times. Quantification was performed by the construction of analytical curves obtained by linear regression using Origin Pro2018 software (OriginLab, Northampton, MA, USA) and considering the coefficient of determination (R^2) equal to 0.99. Addition of standards to the extracts was also used as an identification parameter.

DPPH assay to determine antioxidating activity of extracts

The antioxidant capacity of propolis ultrasound assisted extraction and vitamin A was assessed by the scavenging of DPPH free radical according to the manual provided by EMBRAPA (2007). Initially, it was prepared in ultrapure water a 50% methanolic solution and a 70% acetone solution. Both solutions were mixed in equal parts of 40 mL plus 100 mL of ultrapure water and homogenized. In another recipient, 2,4 mg of DPPH reagent was dissolved in methanol to a final volume of 100 mL and a final concentration of 60 μM. The reagent was stored in amber glass, and stored away from heat and light. This solution should be used in the same day. 100 μL of the extract solution and fragments of 100 mg from each scaffold containing propolis and/or vitamin A were used as samples to assess antioxidant capacity. The samples were added to 3.9 mL of DPPH solution and taken to the spectrophotometer until it was stabilized. Methanol was used as a blank. Control sample was made by adding 100 μL of ascorbic acid solution at 0.5 mM to 3.9 mL of DPPH solution. The total antioxidant capacity was calculated by:

$$Antioxidant\ capacity = 1 - \frac{Control\ ABS - Sample\ ABS}{Control\ ABS} \times 100$$

The assay was carried out in triplicates and data were plotted as the mean percentage and standard deviation of data.

Bactericidal activity

Standardization of bacterial inoculum

The following strains were employed *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19117, *Salmonella enteritidis* AOAC 100201 and *Pseudomonas aeruginosa* INCQS 0025, belonging to the Food Microbiology Laboratory, located at the Food Science Department at Universidade Federal de Lavras. The stock cultures were stored at -18°C in freezing culture medium (15% glycerol; 0.5% bacteriological peptone and 0.3% of yeast

extract; and 0.5% of NaCl, final pH of 7.2 ±0,2). The stock cultures were reactivated by aliquots transfer of 100 µL to tubes containing 10 mL of broth Brain Heart Infusion (BHI) and incubated at 37°C for 24h. Inoculum standardization was accomplished with a growth curve. Optic density (DO 600nm) was monitored along with plate counting, on trypticase soy agar (TSA) with incubation at 37° C for 24h. The cultures were standardized in 10^8 CFU/mL (approximately).

Disc Diffusion Assay

Here was employed the disc diffusion technique (CLSI M100-ED29, 2019) with modifications. Aliquots of 100 µL from the standardized suspensions for each bacterial culture were inoculated in BHI agar. Bacterial strains were plated at 10^7 CFU mL^{-1} to simulate infection conditions. After the spreading, filter paper disc (5 mm Ø) with 5 µL of propolis extracts at 20 $\mu g.\mu L^{-1}$ were placed equidistantly. In order to compare the results of this activity properly, controls containing 70% ethanol and chloramphenicol (1000 mg/L) were also tested. For each strain were employed 3 discs per plate, in three replicates and three assay repetitions. Plates were incubated at 37 °C for 24 h and the inhibition halos were measured with a digital pachymeter. This assay was performed once more after the development of the wound dressings in order to confirm the manutention of the antimicrobial action. In brief, the wound dressings were cut into 5 mm in diameter disks and proceeded as above mentioned.

Minimum bactericidal concentration

The minimum bactericidal concentration against bacterial cells (MBC) of extracts was determined using microdilution technique with 96-well polystyrene microplates according to CLSI M100-Ed29 (Clinical and Laboratory Standards Institute, 2019) with modifications. Antimicrobial solutions were prepared in trypticase soy broth (TSB). Analyses were done at concentrations of 0.08 mg mL^{-1} (0.45%; v/v); 0.16 mg mL^{-1} (0.89%; v/v); 0.32 mg mL^{-1} (1.78%; v/v); 0.65 mg mL^{-1} (3.57%; v/v); 1.3 mg mL^{-1} (7.14%; v/v) and 2.6 mg mL^{-1} (14.28%; v/v). 140 µL aliquots of the solutions were added to the wells and 10 µL of the standardized cultures were inoculated and incubated at 37 ºC for 24 hours. After incubation, aliquots of 10 µL the cultures were drop plated in TSA and incubated at 37°C for 24 hours. The concentration of propolis extract solution in which growth was not observed was considered the CBM.

Biocompatibility potential of the wound dressings

Hemolysis

Hemolysis evaluation was conducted according to ASTM guide F756 – 17 (ASTM, 2017). Total blood of a healthy donor was collected in a vacutainer containing heparin. Blood sample was centrifuged at 700xg for 15 minutes, to separate erythrocytes from plasma. With the aid of a pipette, plasma was removed leaving only the erythrocytes layer. No washing steps were adopted. From this solution, we have adjusted the hematocrit to 0,15% in PBS (1,15 mM de $NaH_2PO_4.H_2O$, 4 mM de $Na_2HPO_4.H_2O$ and 0,15 M NaCl, pH~7,4). We have evaluated glutaraldehyde, propolis, vitamin A/tween 80 emulsion (0,6 µL/µL Tween 80), the scaffolds 100:0, 75:25 and 50:50 (containing propolis, vitamin A, both and plain). All scaffolds were used as 0.5 x 0.5 x 0.2 cm sample. As control samples we have used sterilized cooper wire (sanded to remove polymer coating), synthetic rubber, HDPE and PBS. The table 1 below summarizes the specifications of each sample.

Table 1 – Controls preparation for hemolysis assay

Sample	Dimensions/volume	Erythrocytes solution
(C-)	300 µL PBS	1,2 mL
(C+)	30 µg B. moojeni +270 µg PBS	1,2 mL
Propolis	150 µL + 150 µL PBS	1,2 mL
Vit A emulsion	50 µL + 250 µL PBS	1,2 mL
Cooper Wire	~ 0.03 cm^3 ~ 0,268 g	1,2 mL
Synthetic rubber	0.5 x 0.5 x 0.2 cm	1,2 mL
High density polystyrene	0.5 x 0.5 x 0.2 cm	1,2 mL

The test was conducted in 2 hours incubation period, at 37° C (hemostatic bath). At fixed 30 minutes intervals, samples were gently reversed twice. After the incubation period, samples were centrifuged at 700xg for 10 minutes. At the end of the centrifugation, a small erythrocytes pellet will form. The supernatant was collected and analyzed in spectrometer at 540 nm. The color effect of each sample was neutralized by adding the sample to a vial containing PBS and discounting the value from the sample with the erythrocytes. Negative control was considered mechanical hemolysis, and was subtracted from all results. For positive control 30 µg of *Bothrops moojeni* venom was used (CESAR et al., 2019). The assay was carried out in triplicates. Total hemolysis was given by the formula

$$\boldsymbol{Hemolysis}\ (\%) = \frac{\boldsymbol{Sample\ A - Sample\ A(PBS) - C(-)A}}{\boldsymbol{C(+) - C\ (-)A}} \times \mathbf{100}$$

Clotting activity

The clotting activity was performed similar to Sagnella and Mai-Ngam (SAGNELLA; MAI-NGAM, 2005) with modifications. Initially 200 μL of plasma was added to test tubes in a thermostatic bath at 37°C. To each tube was added the following samples: propolis UAE (30 μL), vitamin A emulsion (5 μL + 25 μL PBS), and the scaffolds in the same dimensions of the hemolysis test. Each tube was observed individually and timed. Plasma displaying a sticky and more dense aspect upon addition of the sample was considered as clotted and the time needed to change plasma viscosity was noted. Controls containing only plasma, thrombin (5 μL - 0.2 μg/μL, in the form of Factor IIa), cooper wire and high-density polystyrene were also assayed. Samples that were unable to clot in 240 seconds were considered as normal if: 1) following addition of 30 μL of 0,5 mM $CaCl_2$ presented the formation of a clot in at least 15 seconds; and 2) following addition of 5 μL of thrombin presented clotting formation in at least 15 seconds. Samples unable to clot in 240 seconds, and fail to clot upon addition of $CaCl_2$ or thrombin in the stipulated times, were considered as nonclotting.

Activity upon thrombi

The action of the material and its components upon blood thrombi was conducted as follows. Briefly, 100 μL of total blood was added to a 96-wells microplate and left to clot (15 - 20 minutes). To the wells was added propolis ultrasound assisted extract (10 μL + 20 μL PBS), vitamin A emulsion (5 μL + 25 μL PBS), thrombin (5 μL - 0.2 μg/μL, in the form of Factor IIa). Due to the limitations of the samples such as the porosity of the material and the minimized contact surface with thrombi we have performed the use of extracts according to specification in ISO 10993-12. The scaffolds extraction was prepared as: 0.2 g of material to 3 mL of PBS, at 37°C, for 24 h, under agitation in a hemostatic bath, 30 μL was used in each well. The microplates were covered and stored in a cell culture chamber with high humidity to prevent evaporation and drying of the thrombi. The quantification of thrombolysis was measured as the quantity of fluid obtained by aspiration with a pipette from each well. The mean value of the replicates was subtracted from the initial volume and calculated the percentage of thrombolysis. If the quantity of fluid were less than the initial volume, samples were considered thrombotic (CINTRA et al., 2012). To quantify the loss of fluid by evaporation three control wells without any sample were used and its volume of liquid was quantified and subtracted the loss from all

samples. Due to the variations of this test, the method was conducted three times in different days.

Statistical analysis

Data were submitted to analysis of variance, and the means of all parameters analyzed were compared using the Scott Knott test at a 5% probability level. Statistical analysis was performed using the statistical program "R".

Results and discussion

Extraction and yielding efficiency of phenolic compounds from propolis

In our work we choose propolis as the main biological effector due to the pharmacological properties attributed to its use (MARTINOTTI; RANZATO, 2015; NAITO et al., 2007; ORYAN; ALEMZADEH; MOSHIRI, 2018). Here we have prepared a polymeric wound dressing embedded with propolis ethanolic extract and vitamin A. The first preoccupation during the conduction of this experiment was the preparation of a suitable propolis extract, able to yield the highest level of phenolic compounds. The most efficient method was Ultrasound assisted ethanolic extraction which resulted in 86.22±1.8 (mg of phenolic content in 100g of propolis) compared to methanolic extraction (18.98±1.2), quantified by Folin-Denis method.

Phenolic content analysis

The extract obtained from propolis by Ultrasound assisted ethanolic extraction (UAE) was submitted to a HPLC analysis, the following phenolic compounds were identified: gallic acid, catechin, chlorogenic acid, caffeic acid, vanillin, p-coumaric acid, ferulic acid, salicylic acid, resveratrol, quercetin and rutin (Figure 1).

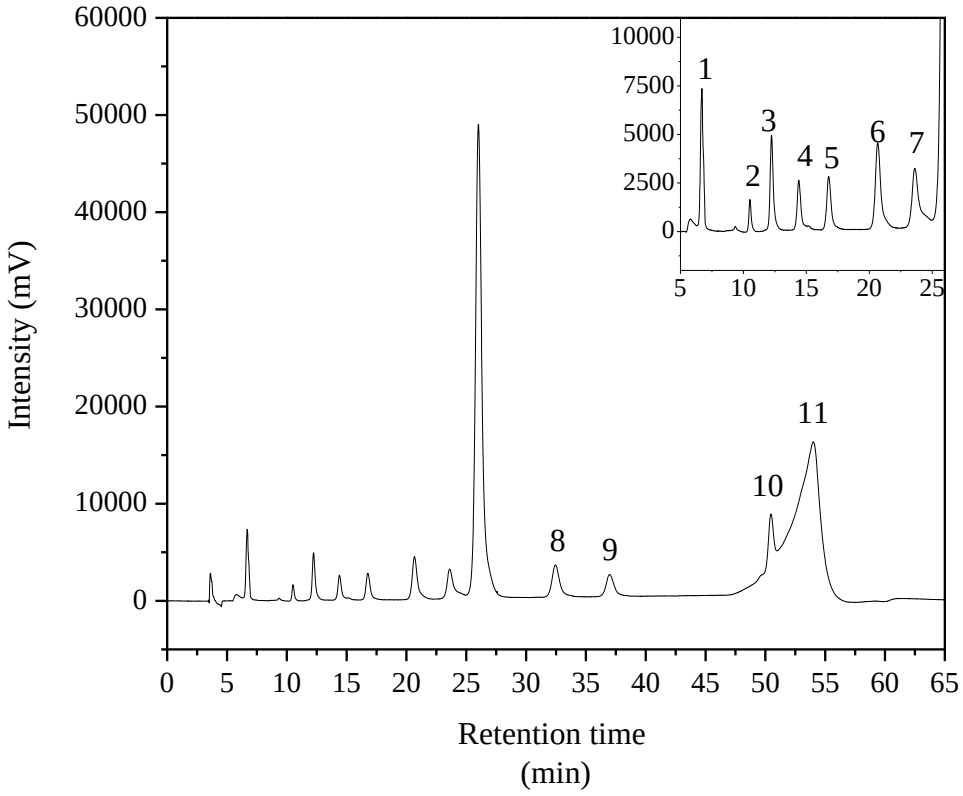

Figure 1 - Phenolic compounds identification of the peaks obtained by Ultrasound assisted ethanolic extraction (UAE) by comparing the retention times of each compound with phenolic patterns. Peaks 1 (gallic acid), 2 (catechin), 3 (chlorogenic acid), 4 (caffeic acid), 5 (vanillin), 6 (p-coumaric acid), 7 (ferulic acid), 8 (salicylic acid), 9 (resveratrol), 10 (quercetin) and 11(rutin).

The phenolic compounds identified were quantified and expressed in mg g^{-1} in table 2 followed by the retention times.

Table 2 - Phenolic compounds content in ethanolic extract of propolis (UAE) sorted by retention time.

Phenolic compounds (peak)	**Concentration** (mg g^{-1})	**Retention time** (min)
Gallic acid	1.99	6.58
Catechin	0.24	10.47
Epigallocatechin gallate	2.16	12.11
Caffeic acid	1.14	14.32
Vanillin	1.48	16.69

p-Coumaric acid	3.87	20.60
Ferulic acid	2.21	23.27
Salicylic acid	6.88	32.11
Resveratrol	4.10	36.37
Quercetin	17.21	50.79
Rutin	26.4	53.49
Σ Phenolic compounds	57.5	

Rutin and quercetin were the major compounds found in the extract, both of which are related to many pharmacological effects that will be discussed further. One peak around 26 minutes remained unidentified. According to the supplier this propolis is rich in Artepillin C (27 mg g^{-1}), however we cannot address this compound to the 26 minutes peak, because we don't have its standard for proof.

Microbicidal evaluation

Bacterial growth inhibition evaluated by disc diffusion

Although the content of phenolic compounds is a preponderant factor while choosing the best extraction technique, the composition and the type of polyphenols extracted also plays an important role. In our work, the bactericidal activity for both extracts were evaluated against two strains, two Gram-positive and two Gram-negative (*Staphylococcus aureus,* ATCC 25923 and *Pseudomonas aeruginosa,* ATCC 27853, respectively). It is broadly known that infection is one of the major problems faced during the wound healing process, responsible for delaying wound closure, and consisting of a life-threatening issue for bedridden patients. Due to its importance, this activity was chosen to establish the proper concentration of propolis to fabricate the wound dressings. The results obtained for each bacterial strain are displayed in table 3 bellow.

Table 3 – Antimicrobial activity of different propolis extract upon *Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes and Salmonella enteritis*

	Inhibition halo (cm)*

Antimicrobial agent	*S. aureus*	*P. aeruginosa*	*L. monocytogenes*	*S. enteritis*
UAE	2.2±0.2 **c**	1,6±0,3 **d**	1.7±0.2 **d**	2.0±0.2 **c**
ME	1.1±0.1 **e**	1.2±0.2 **e**	1.0±0.1 **e**	1.0±0.2 **e**
Ethanol 80%	0.1±0.0 **f**	0 **f**	0.1±0.0 **f**	0 **f**
Chloramphenicol	3.5±0.3 **a**	1.8±0.2 **d**	3.2±0.2 **a**	2.9±0.2 **b**

*Values are presented as the mean of three different experiments performed in triplicates followed by S.D. The values followed by the same letter do not differ statistically from each other using Scott-Knott test ($p<0,05$). The letters are displayed in decreasing order.

Based on the results obtained for this assay, UAE presented the best antimicrobial performance, in comparison to the ME. The effect of ethanol 80% on bacterial growth is negligible.

Minimum Bactericidal Concentration (MBC)

The bactericidal action of propolis upon the strains tested was evaluated and the MBC calculated. All concentrations were evaluated in triplicates for *S. aureus*, *P. aeruginosa, Listeria monocytogenes* and *Salmonella enteritidis.*

Tabela 4: Minimum Bactericidal Concentration assay for each bacteria strain

UAE concentration (mg/mL)	Observed action*			
	S. aureus	*P. aeruginosa*	*L. monocytogenes*	*S. enteritidis*
2.6	+	+	+	+
1.3	+	+	+	+
0.65	+	-	+	+
0.32	-	-	-	-
0.16	-	-	-	-
0.08	-	-	-	-
0.04	-	-	-	-

+ :Observed bactericidal effect (no colony growth)

- :Not observed (with colony growth)

*Data were confirmed based on three independent assays.

UAE: propolis ethanolic extract obtained by ultrasound assisted ethanolic extraction.

In this test, the minimum bactericidal dose able to kill all bacterial strains was of *S. aureus* (1.3 mg mL^{-1}), *P. aeruginosa* (2.6 mg mL^{-1}), *L. monocytogenes* (1.3 mg mL^{-1}), *S. enteritidis* (1.3 mg mL^{-1}). In order to confirm if this concentration was inhibitory, indeed, we have performed a drop plate assay, which further confirmed the bactericidal effect of the aforementioned concentrations. The concentration of 1.3 mg mL^{-1} (200 μg), was used as a starting point in the development of the wound dressings. Empirically we proposed that the concentration of extract from propolis in a 1.5 mL polymeric solution should be of 25 times the ratio in a microplate well (each 1.5 mL wound dressing contains 5.2 mg of propolis; 20 μg μL^{-1}), taking into account that the extract should be present in a concentration enough to gradually released from the wound dressing and exert its biological activities. Thus, in a single wound dressing we would find 6 mg of propolis extract. After the preparation of the foam wound dressings, we cut them into small disks of 5 mm in diameter and proceeded to another disk diffusion assay to check if the fabrication process had any negative impact on propolis activity. The results observed are displayed in the table 5.

Table 5 – Disc diffusion assay using wound dressing samples

Wound dressing*	*S. aureus*	*P. aeruginosa*	*L. monocytogenes*	*S. enteritidis*
100:0	1.7±0.1 **b**	1.4±0.2 **d**	1.6±0.2 **c**	1.7±0.1 **b**
75:25	1.9±0.1 **a**	1.7±0.1 **b**	1.9±0.2 **a**	1.8±0.1 **a**
50:50	1.9±0.2 **a**	1.8±0.2 **a**	1.8±0.2 **a**	1.8±0.2 **a**

*100:0 – 100% PVA : 0% Sodium Alginate; 75:25 - 75% PVA : 25% Sodium Alginate; 50:50 - 50% PVA : 50% Sodium Alginate. The values followed by the same letter do not differ statistically from each other using Scott-Knott test ($p<0,05$). The letters are displayed in decreasing order.

Microbicidal activity were maintained even after the process of fabrication. The concentration proposed from the Minimum bactericidal concentration was adequate to maintain the bactericidal effect against all strains tested. Though small differences were found among the three developed dressing formulations.

Antioxidant activity

Antioxidant activity is an important parameter to assess in our study, since this property grants many advantages during wound healing process. The DPPH assay is based on the radical

scavenging capability of the sample in reducing α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, $M = 394.33$) stable free radical, this methodology is a simple, low cost and reliable option to assess natural products antioxidant capacity. Figure 3 shows the performance of the propolis extract and of the wound dressing containing propolis alone and in combination with vitamin A.

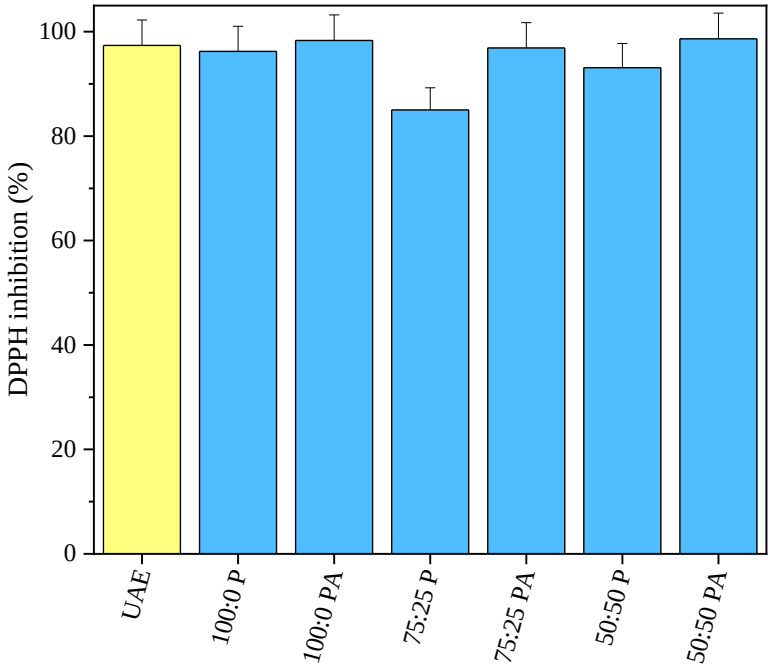

Figure 3 –Antioxidant activity measured by the method of DPPH. Poly vinyl alcohol (PVA): Sodium Alginate, percentage ratios (100:0; 75:25; 50:50). The mixtures of PVA and sodium alginate were evaluated with addition of propolis ethanolic extract - UAE - ultrasound assisted ethanolic extraction (P) and UAE plus vitamin A (PA). Data are presented as the average of three repetitions ± Standard deviation.

Based on the results obtained, it is possible to conclude that propolis and vitamin A have maintained their antioxidant properties even after the process of fabrication of wound dressing. Propolis had a high antioxidant action by being able to reduce 97% ($p<0.05$) of the free radical DPPH. With only the exception of the wound dressing 75:25 containing only propolis (85% of DPPH scavenging) all other dressings were able to reduce at least 93% of free radicals in solution. Due to the hydrophobic nature of vitamin A, it was not possible to assess its antioxidant potential through DPPH assay. Although there is a slightly change in antioxidant

potential when propolis extract and vitamin A are in combination, those results are not statistically different from propolis extract alone.

Biocompatibility potential of the wound dressings

Effect on erythrocytes, plasma and thrombi

One of the main concerns regarding wound dressings it is their behavior when in contact with living tissues and cells. In order to foresee this behavior a screen of biocompatibility tests is needed. Figure 5 bellow summarizes the compatibility results.

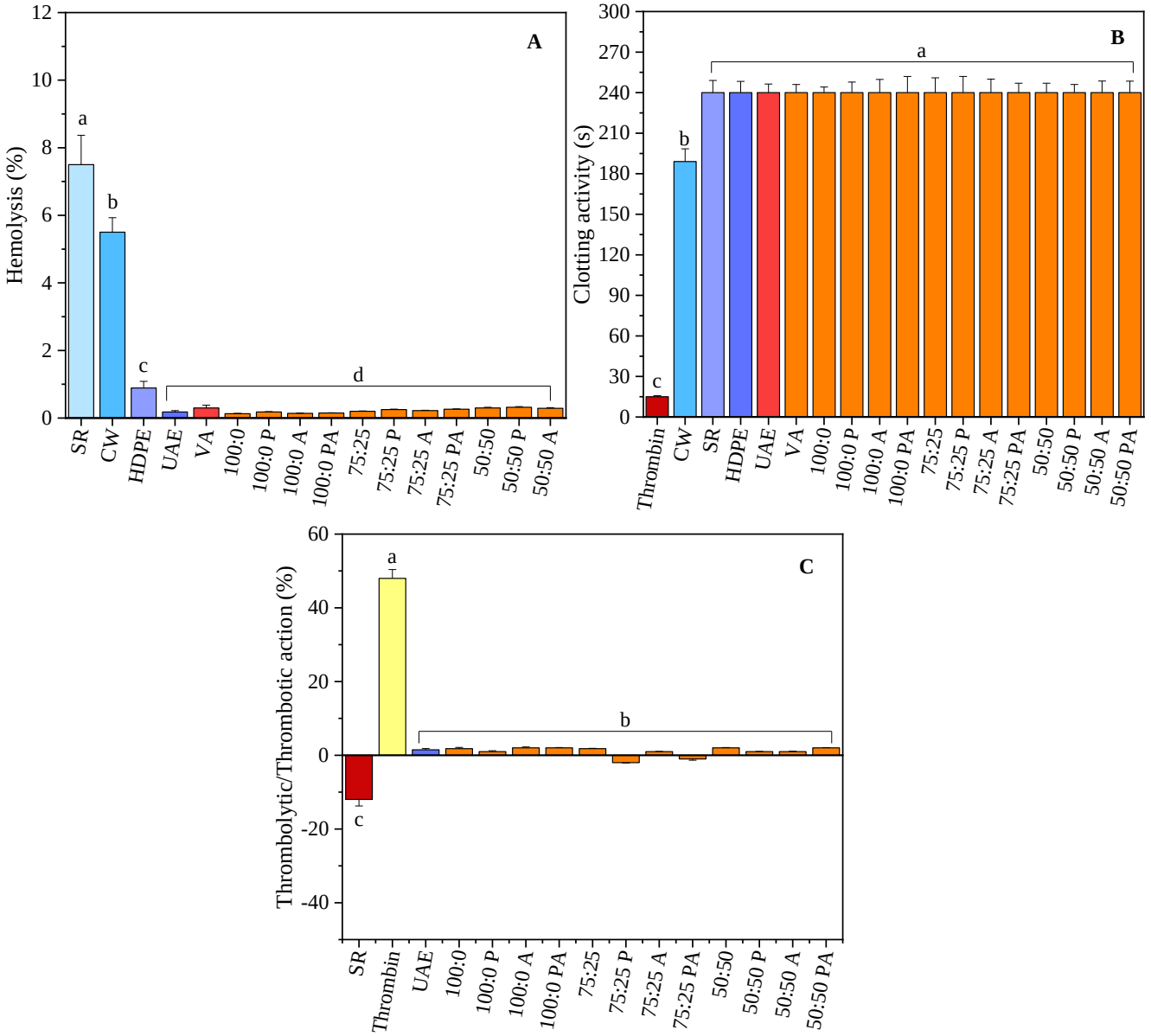

Figure 5 – Biocompatibility test screening. **A** Hemolysis assay: C (-) – PBS (mechanical hemolysis); SR – Synthetic rubber; CW - Cooper wire; HDPE – High density polystyrene; UAE – Ultrasound assisted propolis extract; VA – Vitamin A emulsion. Poly vinyl alcohol (PVA): Sodium Alginate (SA), percentage ratios (100:0; 75:25; 50:50). The mixtures of PVA and sodium alginate were evaluated with addition of propolis UAE (P) and vitamin A (A). **B** Clotting time assay: Thrombin (in the form of Factor IIa 0.2 μg μL^{-1}). After 240 s, $CaCl_2$ was added to three replicates of each sample to induce clotting and check any possible chelating action. To other three replicates, thrombin was added after the same time to check any possible non-clotting mechanism. **C** Activity upon thrombi: The control SR and the wound dressings were used as an extract as proposed in ISO 10993-12. Data are presented as the average of three

repetitions ± Standard deviation. The values followed by the same letter do not differ statistically from each other using Scott-Knott test ($p<0,05$). The letters are displayed in decreasing order.

In this present work we have tested the biocompatibility of the wound dressings containing propolis and vitamin A, as well as the chemicals used in the fabrication process. In hemolysis assay, according to the ASTM guide with adaptations, samples presenting hemolysis levels up to 2% are considered as non-hemolytic, from 2 to 5% considered as slightly hemolytic and above this range samples are considered as hemolytic. All samples and extracts were considered as non-hemolytic in the concentrations tested (even glutaraldehyde, known to be cytotoxic), since none of the readings were higher than 2% ($p<0,05$).

During clotting formation assay, samples that induce the formation of a fibrin clot under 60 seconds are considered as pro-coagulant (marked by a rigid thrombus, with low reminiscing fluid). Conversely, those samples that do not promote coagulation under 240 seconds, or no clotting is observed at all (even after the addition of $CaCl_2$ or thrombin), are considered as non-coagulant. The samples tested (including their extracts) did not promote clotting formation nor promoted any chelating (tested by addition of $CaCl_2$) or proteases inhibition (tested by previous incubation with thrombin, then addition to plasma) when incubated with plasma. We have also tested the extract of the materials upon blood thrombi, in order to understand the capacity to induce the formation of thrombus (thrombosis) or to disrupt (thrombolysis). Material leachable are often the cause of many incompatibility cases, especially in implantable or external communicating devices. In the present work the wound dressings extract did not promote any thrombotic nor thrombolytic effects.

Discussion

Propolis is an important natural product produced by bees composed mainly by resins (50%), waxes (30%), essential oils (10%), pollen (5%), and other organic compounds (5%)(CUNHA et al., 2004). Bioactive compounds found in propolis such as phenolic compounds, flavonoids, terpenes, beta-steroids, alcohols, vitamins and aromatic aldehydes are responsible for its antibiotic, antioxidant, healing and anti-inflammatory actions (MARTINOTTI; RANZATO, 2015). In order to take advantage of all its medicinal properties we have tested two extraction protocols and selected the more suitable to our objectives. The vast majority of research articles using propolis report the use of ethanolic extracts by maceration. Although this process is efficient in extracting phenolic compounds, it is time consuming and requires manual agitation or automated orbital shaking, by at least 5 to 7 days

to yield a considerable amount of bioactive substances. In our work, we have evaluated the methods of ultrasound assisted ethanolic extraction (UAE) and methanolic extraction (ME).

The higher extraction efficiency observed for the UAE was due to intense sonication that promotes successive cycles of adiabatic contraction and expansion (SAGNELLA; MAI-NGAM, 2005). This process increases the contact surface with the solvent and its permeation deeply into the propolis bulk. Sonication extraction also generates small air bubbles that shock with propolis causing the formation of cavities, increasing further the contact interface with the solvent (TRUSHEVA; TRUNKOVA; BANKOVA, 2007; YANG et al., 2013).

As expected, in the preliminary bactericidal assay, UAE had the best performance against both strains tested, a result we attribute to the superior total phenolic content. UAE also presented the highest antioxidant potential, when compared to ME. Due to the reduced extraction time observed for the sonication extraction and its microbicidal and antioxidant performance, the UAE was chosen for all analysis.

Polyphenols found in propolis are the main biological active molecules, capable of promoting anti-inflammatory, antioxidant, wound healing and microbicidal activities. Rutin, the most abundant compound found here, have been addressed to many therapeutic properties (GANESHPURKAR; SALUJA, 2017). A great number of studies have reported the action of rutin upon several bacterial strains, besides being implicated in the enhancement of other polyphenols action, and of commercially available antibiotics such as ampicillin, ciprofloxacin and erythromycin (AMIN et al., 2015; ARARUNA et al., 2012; GANESHPURKAR et al., 2013). In fact, this synergistic bactericidal action have been reported to occur to phenolic compounds found here such as quercetin, and the phenylpropanoids p-coumaric, ferulic, caffeic and chlorogenic acids (HEMAISWARYA; DOBLE, 2010). The healing activity of rutin has been previously explored by Almeida and colleagues (2012), this research team developed a hydrogel containing rutin which was tested in a cutaneous wound lesion in rats. The results found highlights that action of rutin in promoting wound contraction in relation to the control group. Also, rutin loaded hydrogels greatly reduced oxidative damage markers (TBARS assay and protein carbonylation) (ALMEIDA et al., 2012). Tran et al (TRAN et al., 2011) developed an *in situ* forming hydrogel containing rutin, and tested on topical wound in rodents. Similar to the previous cited work, the use of this hydrogel resulted in a faster wound contraction and reepithelization. Besides that, fibroblasts cultured with the rutin loaded hydrogel had a cell viability close to 100% as compared to the control (TRAN et al., 2011).

The second most prevalent polyphenol, quercetin, is considered to be a strong antioxidant agent by its ability to bind transition metal ions and scavenge free radicals such as peroxynitrite and superoxide anion, and to inhibit lipid peroxidation (VELLOSA et al., 2011). Quercetin not only contributes to reduce the levels of these highly reactive species but also increases the levels of glutathione, which further helps in the elimination of reactive oxygen species (LI et al., 2016). Xiao et al. (2011) have demonstrated in their work that quercetin also exerts anti-inflammatory action (this property was also found in our; data not shown). The group have reported that the regulatory actions of quercetin upon cyclooxygenase-2 (COX-2) occurs by the inhibition COX-2 mRNA and protein expression and reduction of prostaglandins production (XIAO et al., 2011). Inflammation is a normal part of the wound healing process marked by intense cell migration, production of exudates and elimination of bacteria. Although, if the organism fails to control infection, wound healing cascade cannot proceed to reepithelization. The afore mentioned microbicidal action of polyphenols along with anti-inflammatory action results in a powerful combination to promote wound healing.

Resveratrol has been shown to increase the activity of endothelial nitric oxide synthase (eNOS), and to promote an increase in the expression of vascular endothelial growth factor (VEGF), a molecule that plays an important role in angiogenesis (KHANNA, 2001; PENUMATHSA et al., 2007). In the context of wound healing, the use of resveratrol has been linked to improving recovery by stimulation of neovascularization, promotion of collagen synthesis, control of inflammation and antioxidant activity. Epigallocatechin gallate (ECG) is a polyphenol with high antioxidant capacity and have described its action in promoting keratinocytes growth and differentiation within a short period by using concentrations inside the physiological range (HSU, 2003). During wound healing the quality of scaring formation is indicator of normal healing process. Kapoor and colleagues (2004) have demonstrated that the administration of ECG improved significantly the quality of scaring by promoting the formation of better oriented and mature collagen fibers (KAPOOR et al., 2004). During wound healing cascade, angiogenesis, is a critical step for the progressions of all healing phases, in special the formation of granulation tissue. Angiogenesis, promotes the transportation of oxygen and nutrients to the wound site. Immunohistochemical analysis have shown that ECG also increased the number of new blood vessels, this action was mainly related to the upregulation of VEGF.

Vitamins are essential nutrients that a particular organism cannot produce to keep its adequate functioning. Vitamins acts by regulating and promoting cellular growth, differentiation, immunologic responses and metabolic process. Vitamin A is a generic term for

a collection of molecules presenting a beta-ionone ring and an isoprenoid chain, called retinyl group (O'LEARY; O'BRIEN; CRYAN, 2017). Retinoids increase collagen content in dermis by inhibiting collagen degradation and increasing collagen synthesis. As an indirect effect of the administration of retinoids, the increased collagen content promotes normalization of elastic tissue organization and a reduction in scaring (RITTIÉ; FISHER; VOORHEES, [s.d.]). In the present work, vitamin A (*all-trans* retinoic acid) was added due to its healing properties but the extension of its effects will be further discussed in animal studies. Our main goal in this work was to evaluate its effect in the biocompatibility (wound dressings should be tested in its final form according to ISO 10993) and antioxidant profile.

Although the moderate presence of resident bacteria in wound healing may be beneficial, it is a common worry that open wounds should be treated and covered to protect the wound of further infection. During wound healing, the reepithelization step is dependent of the infection state of the tissue (EDWARDS; HARDING, 2004). If the levels are not satisfied wound healing progression may be impaired and could develop to chronic wounds (PASTAR et al., 2014). Many chronic ulcers fail to recover completely due to the presence of *P. aeruginosa* and *Staphylococcus* spp. biofilms (both tested here)(GUO; DIPIETRO, 2010). The antibacterial activity of propolis is the object of many studies, and has been proved to be an important aid in the control of infection (SCAZZOCCHIO et al., 2006a). In general, propolis extracts are more efficacious against Gram-positive bacteria, rather than Gram-negative. This effect was observed in our work, and the concentrations used are in line with existing literature (WAGH, 2013). Silva et al (2012) used green propolis ethanolic extract from different geographical locations upon different strains of *S. aureus* and *P. aeruginosa*, the MBC concentrations ranged from 0.59 to 1.72 mg mL^{-1} for *S. aureus*, while *P. aeruginosa* ranged from 1.56 to 2.81 mg mL^{-1}(SILVA et al., 2012). In another work, using hydrogels loaded with propolis extract, the MBCs for *S. aureus* (same strain used in our work, ATCC 25923) was of 1.5 mg/mL, 2.5 times higher than the value obtained here (DE LIMA et al., 2016). The process of fabrication of the wound dressing maintained the effectivity of propolis, although it slightly reduced the halos size. However, it is important to note that disc diffusion method is highly dependent of the diffusivity of the compounds, and the presence of another barrier (wound dressing) would affect the results (LOKE et al., 2000).

During normal wound healing, low levels of oxidative species are highly necessary to fight infection, to regulate the production of several cytokines, and to act as intracellular signaling molecules (Y.A. BARKU, 2019). Many wound healing steps depends on the

production of nitrogen oxidative species and reactive oxygen species such as angiogenesis, reepithelization and tissue maturation (MULIYIL; NARASIMHA, 2014). However, in certain conditions like diabetes, poor nutritional state, infected tissues and areas with constant damage (pressure sores) the production of ROS and NOS are out of tune and may develop or aggravate chronic wounds (GOPINATH et al., 2004). High levels of ROS and NOS promotes the oxidation of DNA, membrane lipids, proteins and cause an imbalance on the wound site redox potential (SALGUEIRO et al., 2013; SGAMBATO et al., 2001). In these cases, the natural free radical detoxifying system may be depleted or acting with a low capacity. One of the proposed uses of foam wound dressings is aimed to treat pressure sores, by acting as a cushion. Thus, the association of propolis with a high antioxidant capacity to the wound dressing, as performed here, is essential to control the elevated concentration of ROS and NOS on the wound site and minimize further damage to repairing tissues.

One of the major concerns when working with biomaterials is the hemocompatibility of the device. Very often, biomaterials present excellent physical and chemical properties but fail in biocompatibility. Thus, a harmonic relation between mechanical performance and biological response must be achieved. Wound healing is a complex series of overlapping steps that include hemostasis, marked by the formation of clot to control hemorrhage, followed by inflammatory response, proliferative phase and finally remodeling phase. It is known that negative interferences in any of the wound healing phases may result in impaired recovery, increase in the susceptibility to infection and propensity to develop chronic wound.

According to ISO 10993-1 (2009), biomaterials in contact with breached or compromised surfaces are only advised to be tested for cytotoxicity, carcinogenicity, sensitization, irritation, pyrogenicity, acute, sub chronic and chronic toxicity. However, in our work we reached a consensus that if the material is in contact with blood and clots, it should be tested for hemocompatibility. Induction of hemolysis could activate inflammatory responses by release of intracellular mediators (ATP and hemoglobin), or interfere with clot integrity and clotting cascade activation by oxidative damage, shear stress, release of antigens and surface charge. Thus, if the wound dressing induced any of those effects, it could lead to increased inflammation and delay in wound healing. In the present work, all materials (including its components) tested presented tolerable levels of toxicity *in vitro* in the conditions and methodologies tested.

Conclusions

An effective biomaterial for wound dressing should be capable of performing an adequate biological response and be biocompatible. In our work, we have developed a wound dressing composed of PVA and sodium alginate blends incorporated with propolis extract and vitamin A. The choice of the polymers were based in their physical-chemical properties and their biological behavior. The foam wound dressings developed here maintained the function even after the fabrication process, being able to kill different bacterial strains (Gram-positive and Gram-negative) and to exert a great antioxidant capacity. The results suggest that the material developed for wound covering will be effective in controlling wound contamination and excessive oxidation, due to the presence of polyphenols. External data suggests that the presence of such polyphenols and vitamin A greatly improves wound healing by stimulating angiogenesis, immune responses, tissue maturation and the dermatologic aspect. The wound dressings were also tested for biocompatibility, and in the conditions tested no toxicity were detected. In further publications, we will describe the physical characterization of the material, and the *in vivo* performance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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6 ANEXOS

ANEXO I – REGISTRO DE DEPÓSITO DE PATENTE

	(BR/RJ) ; LUCIO FABIO CASSIANO NASCIMENTO (BR/RJ)
(21) BR 10 2019 014520-0	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 13/07/2019 (71) UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP (BR/SP)
(21) BR 10 2019 014530-7	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) UNIVERSIDADE FEDERAL DE LAVRAS (BR/MG) ; FUNDAÇÃO DE AMPARO À PESQUISA DE MINAS GERAIS (BR/MG)
(21) BR 10 2019 014562-5	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) JAMIL RIBEIRO CADE (BR/SP)
(21) BR 10 2019 014568-4	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) VERISURE SÀRL (CH)
(21) BR 10 2019 014575-7	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) BIOSENSE WEBSTER (ISRAEL) LTD. (IL)
(21) BR 10 2019 014583-8	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) FÚLVIA SORAES CAMPOS DE SOUSA (BR/BA)
(21) BR 10 2019 014587-0	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) HUMBERTO CANOBRE (BR/SP) ; MAURICIO ELIAS DANHESSI (BR/SP)
(21) BR 10 2019 014592-7	Código 2.1 - Pedido de Patente ou Certificado de Adição de Invenção depositado (22) 15/07/2019 (71) ASSOCIACAO PARANAENSE DE CULTURA - APC (BR/PR)

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Lavras, 25 de setembro de 2019

DECLARAÇÃO

Declaramos para os devidos fins que **PEDRO HENRIQUE SOUZA CÉSAR** figura como inventor da seguinte tecnologia:

- Pedido de patente intitulado: "**PRODUÇÃO DE ESPUMAS BIOPOLIMÉRICAS LIOFILIZADAS CONTENDO PRODUTOS NATURAIS**", de titularidade da UFLA, depositada no INPI em 15/07/2019, sob o nº BR 10 2019 014530 7.

Atenciosamente,

BRUNO GOMES DE CARVALHO
Coordenador Geral do Nintec/UFLA