UNIVERSIDADE VILA VELHA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DESENVOLVIMENTO E AVALIAÇÃO DA ATIVIDADE CICATRIZANTE *IN VITRO* E *IN VIVO* DE UM BLEND DE ÓLEOS VEGETAIS

MARCIO GUIDONI

VILA VELHA

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Dissertação apresentada à Universidade Vila Velha como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para obtenção do grau de Mestre em Ciências Farmacêuticas.

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LISTA DE ABREVIATURAS E SIGLAS

% - Porcentagem

°C – Grau Celsius

µg - Micrograma

µL – Microlitro

µM – Micromolar

ABTS - Ácido 2,2'- azinobis(3-etilbenzenotiazolina- 6-sulfônico)

ALA - Ácido Alfa-Linolênico

ANVISA – Agência Nacional de Vigilância Sanitária

ARA - A*rachidonic Acid*

BF - *Boron Trifluoride*

BHT - *Butylated Hydroxytoluene*

CO_2 – Gás Carbônico

CP - *CellProfile*

DAPI - 4',6-diamino-2-phenylindole

DHA - *Docosahexaenoic Acid*

DMEM - *Dulbecco's modified Eagle's medium*

DMSO – Dimetilsulfóxido

DPPH - 2,2-diphenyl-1-picryl-hidrazyl

ELISA - Ensaio Imunoenzimático (*Enzyme Linked Immuno Sorbent Assay*)

EGF - *Epidermal Growth Factor*

ES – Espirito Santo

EPA - *Eicosapentaenoic Acid*

FAME - *Fatty Acid Methyl Esters*

FBS - *Fetal Bovine Serum*

FDA – *Food and Drug Administration*

FID - *Flame Ionization Detector*

FRAP - *Ferric Reducing Antioxidant Power*

g – Grama

GC – *Gas Chromatographic*

GC-FID - *Flame Ionization Detector*

GLA - Ácido Gama-Linolênico

H – Hora

H&E - *Hematoxylin and Eosin*

H_2O_2 - Peróxido de hidrogênio

IL – Interleucina

IL-1 – Interleucina 1

IL-6 – Interleucina 6

KOH – Hidróxido de Potássio

LPS – Lipopolissacarídeo

LTB_4 – *Leukotriene* B_4

M - Molar

mM - Milimolar

min – Minutos

mL – Mililitro

MIC - *Minimum Inhibitory Concentration*

MMP – *Matrix MetaloProteinasys*

MPO - *Myeloperoxidase*

MUFAs – *Monounsatured Fatty Acids*

MTT - (brometo de 3-4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

nm – Nanômetro

NBT - N*itroblue Tetrazolium*

NO - Óxido Nítrico

NPS - Nitroprussiato de Sódio

O_2^- - Ânion Superóxido

PAF - Fator de Ativação Plaquetária

PDGF - *Platelet Derived Growth Factor*

PGE_2 – *Prostaglandin* E_2

PMN – *Polymorpho Nuclear*

PUFAs – *Polyunsatured Fatty Acids*

SD - S*tandard Deviation*

SEM - S*tandard Error of Mean*

SFAs – *Satured Fatty Acids*

SNP - *Sodium Nitroprusside*

TCC - *Triphenyl Tetrazolium Chloride*

TGF-β - Fator de Crescimento Transformador Beta

TNF-α – Fator de Necrose Tumoral Alfa

TPTZ - (2,4,6-tris(2-pyridyl)-s-triazine)

USA – *United States of America*

UK – *United Kingdom*

UVV – Universidade Vila Velha

VOB – *Vegetable Oil Blend*

RESUMO

GUIDONI, Marcio, M.Sc, Universidade Vila Velha - ES, abril de 2018. **Desenvolvimento e Avaliação da Atividade Cicatrizante *In Vitro* e *In Vivo* de um Blend de Óleos Vegetais.** Orientador: Prof. Dr. Marcio Fronza.

Os óleos vegetais são bem conhecidos por seus efeitos benéficos para a saúde, especialmente na pele, prevenindo danos oxidativos, desidratação tecidual, melhorando a reepitelizacão e melhorando a cicatrizacão de feridas. O presente estudo teve como objetivo investigar os benefícios *in vitro* e *in vivo* para a cicatrização de feridas de uma fórmula a base de um mistura de óleo vegetal (VOB) e determinar sua composição de ácidos graxos. Os efeitos de VOB (1-200 µg/mL) na proliferação e migração de fibroblastos foram investigados através do teste de *scratch assay*. A atividade anti-inflamatória foi estudada em células de macrófagos RAW 264.7 estimulados com lipopolisacarídeos, investigando a liberação de óxido nítrico (NO), produção de anion superóxido (O_2^-), fator de necrose tumoral alfa (TNF-α) e interleucina 6 (IL-6). O ensaio do sequestro dos radicais ABTS, de redução férrica (FRAP), e os ensaios de (DPPH) foram utilizados para avaliar a atividade antioxidante da VOB. A atividade antibacteriana foi testada pelo teste de microdiluição, com cepas de *S aureus*, *E coli*. Além disso, o perfil de ácidos graxos de VOB foi analisado por cromatografia gasosa. Para o ensaio de cicatrização *in vivo* sessenta animais foram divididos aleatoriamente em três grupos com 20 animais. Quatro feridas excissionais foram criadas no dorso de cada animal e em seguida tratados topicamente com formulação VOB (VOB), controle positivo ou controle do veículo. Biópsias foram colhidas para análise histológica e bioquímica. *In vitro*, VOB estimula a proliferação e migração de células de fibroblastos, atingindo valores de 49,2 ± 5,1% e 57,5 ± 3,7%, respectivamente, comparados a 6,2 ± 2,0% exibidos no grupo controle. O VOB suprimiu a citocina pró-inflamatória IL-6, NO e O_2^- em células RAW 264.7 estimuladas com LPS de uma maneira dose dependente. *In vivo*, VOB apresentou maior re-epitelização quando comparado com grupo controle, modulando também a liberação de citocinas pró-inflamatórias como IL-1, IL-6 e TNF-α. Além disso, VOB mostrou uma robusta deposição de colágeno na matriz, apresentando uma cicatriz não-hipertrófica. O estudo sugere que a formula desenvolvida tem propriedades promotora na cicatrização de feridas cutâneas.

Palavras-chaves: óleos vegetais. ácidos graxos essenciais. cicatrização. colagênese. citocinas.

ABSTRACT

GUIDONI, Marcio, M.Sc, Universidade Vila Velha - ES, april of 2018. **Development and Evaluation of the In Vivo and In Vivo Healing Activity of a Blend of Vegetable Oils.** Advisor: Prof. Dr. Marcio Fronza.

Vegetable oils are well known for their beneficial effects on health, especially on the skin, preventing oxidative damage, tissue dehydration, improving re-epithelization and improving wound healing. The present study aims to investigate the in vivo and in vitro benefits of wound healing of a vegetable oil (VOB) mixture and to determine its composition of fatty acids. The effects of VOB (1-200 µg / mL) on proliferation and migration of fibroblasts were investigated using the scratch assay test. The anti-inflammatory activity was studied in murine macrophages cells stimulated with RAW 264.7 lipopolysaccharides (24-hour incubation), investigating the release of nitric oxide (NO), generation of superoxide anions (O_2^-), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). The ABTS radical scavenging ability test, the antioxidant reduction potential (FRAP), the (DPPH) assays were used to evaluate the antioxidant activity of VOB. The antibacterial activity was tested by the microdilution test. In addition, the VOB fatty acid profile was analyzed by gas chromatography. Sixty animals were randomly divided into three groups with 20 animals. Four full-thickness wounds were perforated on the skin on the back side of each animal and treated topically with VOB formulation, positive control and vehicle control. Wounds were collected for histological and biochemical analysis. VOB stimulates the proliferation and migration of fibroblast cells, reaching values of 49.2 ± 5.1% and 57.5 ± 3.7%, respectively, compared to 6.2 ± 2.0% displayed with control cells. VOB suppressed the proinflammatory IL-6 cytokine and that of NO and O_2^- in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. VOB presented higher re-epithelization when compared to the control group, also modulating the release of proinflammatory cytokines (IL-1, IL-6, TNF-alpha). In addition, VOB showed a strong deposition of collagen in the matrix, presenting a non-hypertrophic scar. The study suggests that the developed formula has positive properties in the healing of cutaneous wounds.

Key-words: vegetable oils. essential fatty acids. healing. collagenase. cytokines.

1 INTRODUÇÃO GERAL

1.1 Pele

A pele é o maior órgão do corpo humano, recobrindo cerca de 7500 cm^2 de indivíduo adulto. Esse órgão protege nosso corpo contra atrito, patógenos, perda excessiva de água e atua em sua termorregulação. Além disso, contém receptores que permitem a percepção da dor, tato, pressão e temperatura (ISAAC, 2010).

A pele apresenta uma estrutura com duas camadas distintas: a epiderme e a derme.

A epiderme é a camada mais externa, sendo formada pelo tecido epitelial. É subdividida em cinco camadas: estrato córneo, estrato lúcido, estrato granuloso, estrato espinhoso e estrato germinativo (ISAAC, 2010).

A camada mais externa é o extrato córneo, que é constituído por células mortas ricas em queratina. Suas células são muito achatadas, lembrando escamas. Essa camada funciona como uma barreira contra patógenos e agentes químicos. Sua espessura pode variar, sendo maior nas mãos e pés, que são partes que sofrem com o atrito e peso. O extrato córneo encontra-se em constante descamação (ARDA et al., 2014).

O estrato lúcido encontra-se abaixo do extrato córneo, entretanto, só é possível visualizá-lo em locais onde a pele é mais grossa. Suas células são mortas, transparentes, achatadas e anucleadas (THOMAS e BURKEMPER, 2013).

No estrato granuloso, as células são achatadas e apresentam grânulos de querato-hialina. As terminações nervosas chegam até esse estrato (THOMAS e BURKEMPER, 2013).

O estrato espinhoso apresenta células ligadas através de desmossomos, conferindo assim resistência ao tecido e um aspecto espinhoso (THOMAS e BURKEMPER, 2013).

O estrato germinativo, também chamado de camada basal, contém as células-tronco da epiderme e é a sua camada mais profunda. Esse estrato forma as células que darão origem a todas as camadas mais superiores. As células formadas nesse

estrato vão sendo “empurradas” para as camadas mais superiores, sofrendo modificações morfológicas e nucleares (THOMAS e BURKEMPER, 2013).

No estrato germinativoque estão contidos os melanócitos, células responsáveis pela produção da melanina. A melanina é responsável pela diferença de cor entre as pessoas. Uma pessoa de pele negra possui mais melanócitos ativos que uma pessoa de pele branca (JUNQUEIRA et al., 2013).

Após a epiderme, encontramos a derme. Ela é formada por tecido conjuntivo e nela estão localizados os nervos, vasos sanguíneos e linfáticos, folículos pilosos e as glândulas sudoríparas. A derme também pode ser dividida em camadas: a camada papilar e a camada reticular. A camada papilar, camada logo abaixo da epiderme, possui projeções que se encaixam na epiderme. A camada reticular é a camada mais espessa e é constituída por tecido conjuntivo mais denso (BURKEMPER, 2013, THOMAS e ARDA et al., 2014;).

Abaixo da derme, encontramos o tecido subcutâneo, conhecido também como tecido adiposo subcutâneo. Esse tecido não faz parte da pele, mas representa a região de união da pele com outros órgãos (JUNQUEIRA et al., 2013).

Algumas estruturas são associadas à pele: pelos, unhas, glândulas sebáceas e sudoríparas. Os pelos são estruturas compostas por três partes: a cutícula (camada mais externa), o córtex (células alongadas com pigmentos) e a medula (apenas em pelos mais grossos). Eles crescem em estruturas denominadas folículos pilosos. As unhas são compostas por células bastante compactadas e ricas em queratina dura. As glândulas sebáceas são responsáveis por liberar uma substância oleosa e geralmente estão localizadas nos folículos pilosos. As glândulas sudoríparas são glândulas tubulares que eliminam suor e estão localizadas em todo o corpo (exceto lábios e glande do pênis) (JUNQUEIRA et al., 2013).

1.2 Processo de cicatrização

Uma ferida pode ser definida como um rompimento da estrutura anatômica da pele, com a perda da funcionalidade celular normal. A cicatrização da ferida é uma complicada sucessão de eventos bioquímicos e celulares, para repor a integridade estrutural e funcional do tecido injuriado. A cicatrização de feridas cutâneas é um processo biológico multifacetado que pode ser dividido em três ciclos sobrepostos:

inflamação, reepitelização e remodelagem ou maturação do tecido (REIKE et al., 2012).

O processo se inicia imediatamente depois da injuria do tecido da pele, com a liberação de grânulos plaquetários, importante na cascata de coagulação. Simultaneamente, citocinas e fatores de crescimento são liberados, assumindo importante papel no recrutamento de células inflamatórias para o local da ferida, aumentando o fluxo dessas células para o debridamento (fase inflamatória), formação de tecido de granulação (angiogenese), proliferação de fibroblastos, formação de matriz extra-celular e colágeno, re-epitelização e finalmente a remodelação do tecido (ISAAC et al., 2010).

A fase inflamatória depende de inúmeros mediadores químicos, das células inflamatórias, como as polimorfonucleares (PMN), macrófagos e linfócitos. O macrófago é a célula inflamatória mais importante dessa fase (BEHM et al., 2012). Permanece do terceiro ao décimo dia, fagocita bactérias, debrida corpos estranhos, e direciona o desenvolvimento do tecido de granulação. Além da presença de células e mediadores inflamatórios, conta com o importante papel da fibronectina (CLARK, 1998). Sintetizada por uma variedade de células como os fibroblastos, queratinócitos e células endoteliais, ela adere, simultaneamente a fibrina, ao colágeno, e a outros tipos de células funcionando como cola, para estabilizar o coagulo de fibrina, as células e os componentes da matriz (BARRIENTOS et al., 2008).

A fase proliferativa ou de formação do tecido é responsável pelo fechamento da ferida propriamente dita. A primeira fase é a re-epitelização, que ocorre pela migração de queratinócito não-danificados da borda da ferida, em direção ao centro. Fatores de crescimento são responsáveis pelo elevado número de mitoses e hiperplasia do epitélio (KOLACZKOWSKA e KUBES, 2013). A segunda fase da proliferação inclui a fibroplasia e formação da matriz, que é extremamente importante na formação do tecido de granulação (coleção de elementos celulares, incluindo fibroblastos, células inflamatórias e componentes neovasculares e da matriz, como a fibronectina, as glicosaminoglicanas e o colágeno). A formação do tecido de granulação depende do fibroblasto, célula crítica na formação da matriz. Longe de ser apenas produtor de colágeno, o fibroblasto produz elastina, fibronectina, glicosaminoglicana e proteases, estas responsáveis pelo debridamento e remodelamento fisiológico (WERNER e GROSSE, 2003). A última fase da proliferação

é a angiogenese, essencial para o suprimento de oxigênio e nutrientes para a cicatrização.

A remodelação do tecido é a última das fases, ocorre no colágeno e na matriz; dura meses e é responsável pelo aumento da força de tensão e pela diminuição do tamanho da cicatriz e do eritema. Reformulações dos colágenos, melhoria nos componentes das fibras colágenas, reabsorção de água são eventos que permitem uma conexão que aumenta a força da cicatriz e diminui sua espessura (DOILLON et al., 1985).

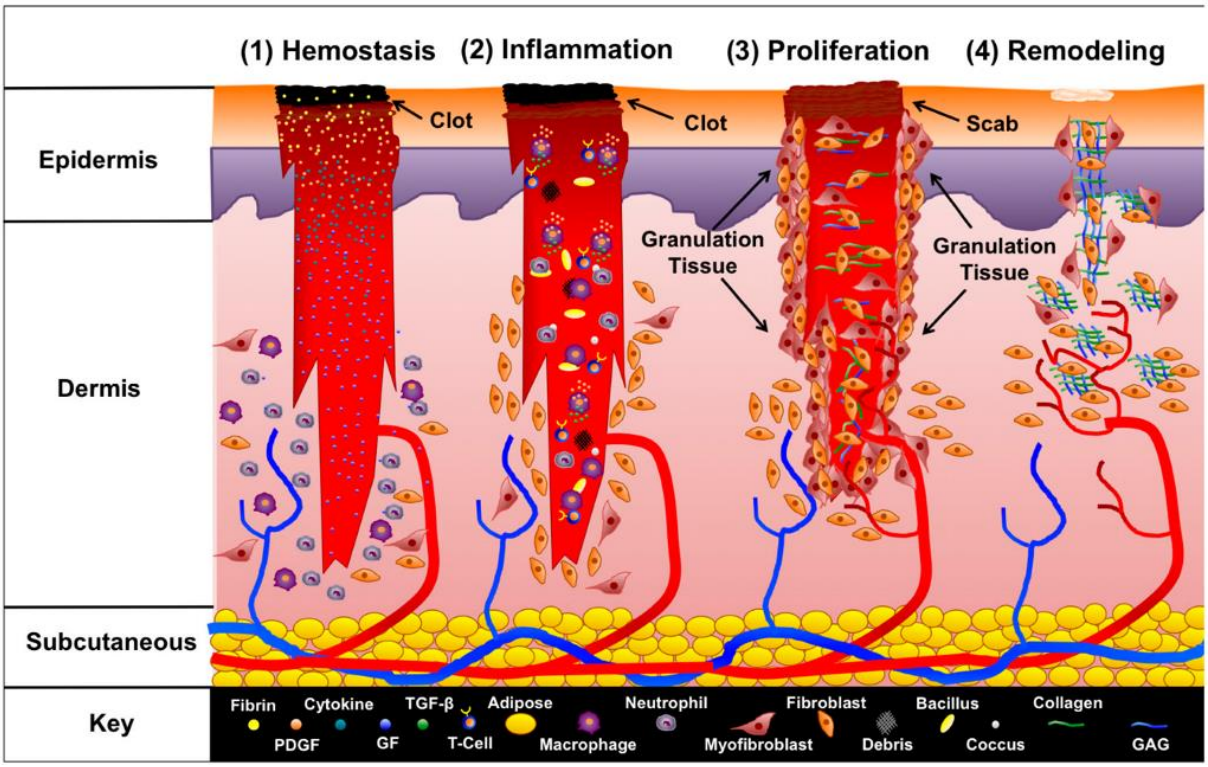

Figura 1. Representação esquemática das fases do processo de cicatrização na pele de acorod com Mellott AJ, et al., 2016.

1.3 Óleos Vegetais

Segundo a Agência Nacional de Vigilância Sanitária – (ANVISA, 2009), os óleos vegetais são produtos obtidos de espécies vegetais, constituídos principalmente de glicerídeos de ácidos graxos, podendo conter pequenas quantidades de fosfolipídios, constituintes insaponificáveis e ácidos graxos livres. Por serem susceptíveis a processos de decomposição e volatilização durante as etapas de processamento, os

óleos refinados e desodorizados tendem a apresentar menores quantidades destes compostos quando comparados aos óleos brutos (CMOLÍK et al., 2000).

Os ácidos graxos que esterificam com o glicerol apresentam cadeias alifáticas saturadas e insaturadas, em proporções variáveis. Os óleos e gorduras contêm diferentes tipos de ácidos graxos e que, dependendo do comprimento da cadeia e do grau de insaturação, podem representar o parâmetro de maior influência sobre as propriedades desses compostos (KNOTHE, 2005). Além destes, o fator genético e ambiental pode determinar as proporções dos ácidos graxos saturados e insaturados presentes nos óleos vegetais (MORETTO E FETT, 1998).

Os ácidos graxos são divididos de acordo com a localização das ligações químicas. Dois importantes grupos incluem os n-3 ou ômegas-3 e os n-6 ou ômega-6. Os principais ácidos graxos que compõem o grupo ômega 3 são: ácido alfa-linolênico, ácido eicosapentanóico e o ácido docosahexanóico. Os principais ácidos graxos que compõem o grupo ômega-6 são: ácido linolêico e ácido araquidônico. Os ácidos graxos dos grupos ômega 3 e 6 não são sintetizados pelo organismo humano, e nem um ácido graxo pode ser transformado em outro (MARQUES et al., 2004).

A capacidade antioxidante total de óleos vegetais comestíveis é determinada por sua composição físico-química e pode estar associada a atributos de qualidade dos óleos, especialmente sua bioatividade e possivelmente a sua estabilidade oxidativa (CASTELO BRANCO et al., 2011).

Os óleos vegetais podem ser utilizados para vários fins, dentre os quais, destacam-se: gêneros alimentícios (TURATTI, 2007), combustíveis renováveis (biodiesel) (KNOTHE, 2006), medicina popular (STEDER, 2016), cosméticos (NATTAYA, 2016), indústria petroquímica (DE SOUZA, 2015).

Em especial, presente trabalho, tem por objetivo, analisar as propriedades biológicas dos óleos vegetais, em relação a melhora do processo de cicatrização de feridas na pele, bem como modulação da atividade inflamatória tecidual local.

1.3.1 Óleo de Girassol

O girassol (*Helianthus annuus)* é uma planta originária do México e cresce bem na Europa central e na Rússia meridional, necessitando de muito sol e de umidade. As suas sementes possuem em seu óleo, ácido oléico e uma grande abundância de ácidos graxos não saturados, especialmente o ácido linolênico e o ácido linolêico, que melhoram a quimiotaxia de leucócitos polimorfonucleares após a injúria tecidual (MARQUES et al., 2004).

De acordo com PROTTEY et al. (1977) “ácidos linoleico e araquidônico, são importantes na manutenção da barreira cutânea para perda de água e como precursores de prostaglandinas, os quais estão ambos envolvidos na regulação da divisão celular e diferenciação da epiderme, e consequentemente no controle do processo de descamação da pele”. O ácido linoleico é o mais potente mediador pró-inflamatório que causa migração de granulócitos e macrófagos. De acordo com GLASGOW E ELING (1990) “o ácido linoleico é essencial para regulação dos eventos bioquímicos que precedem a mitogênese fibroblástica, desde que ele estimula alguns fatores de crescimento celular”. Os fatores de crescimento são proteínas que ligam-se a receptores na superfície da célula e como resultado ativam a proliferação e/ou diferenciação celular.

De uma forma geral o óleo de girassol, extraído à frio, pode ser usado como óleo de salada, como parte de dietas de pacientes portadores de esclerose múltipla e em formulações tópicas para tratamento de feridas cutâneas como queimaduras (ZANOSCHI et al., 1991), úlceras de pressão e sobre diversos processos cutâneos hiperqueratósicos (FERRANDO, 1986) pelo seu alto teor de ácidos graxos insaturados.

1.3.2 Óleo de Linhaça

O óleo de linhaça (*Linum usitatisimum*) é obtido por processamento a frio das sementes da linhaça. É rico em ácido graxos poli-insaturados, como os ácidos graxos essenciais, linolênico (ômega-3), linolêico (ômega-6) e ácido graxo mono-insaturado oleico (ômega-9) (FRANCO, 2011).

O óleo de Linhaça apresenta-se como uma ótima fonte de ácido linolênico (55%) atuando como anti-oxidante e imunoestimulante (FDA). O uso de óleo de linhaça no processo de cicatrização aumenta a migração de fibroblastos para a área da ferida. A presença de ácido linolênico demonstra uma potente atividade cicatrizante, o que pode-se concluir que este seja um grande candidato promotor da cicatrização (LEWINSKA, 2015).

O ácido linolênico atua sobre a membrana celular, aumentando a sua permeabilidade, facilitando a entrada de fatores de crescimento, promovendo a mitose e a proliferação celular. Estimula a neo-angiogênese e são quimiotáticos para leucócitos, podendo ser usados em qualquer fase da cicatrização. Além disso auxiliam no debridamento autolítico, são bactericidas para *S. aureus* (MANDELBAUM et al., 2008).

1.3.3 Óleo de Groselha Negra

O óleo de groselha negra (*Ribes nigrum)* é um óleo 100% puro e natural extraído mediante prensagem a frio e filtração, sendo a semente a parte utilizada para extração. Rica em ácido gama-linolênico (GLA), ácido alfa-linolênico (ALA), vitaminas C, antioxidantes naturais, antocianinas, bioflavonóides, evitando a produção de radicais livres (REED et al., 2014).

O ácido graxo ɣ- linolênico é conhecido por desempenhar um papel crucial na geração de derivados de prostaglandina. Em condições fisiológicas humanas normais, o ácido graxo ɣ- linolênico resulta da bioconversão do ácido linoléico, o ácido graxo mais essencial para os seres humanos (ZAREVUCKA et al., 2003).

O Óleo de Groselha Negra, e rico em ácidos GLA e ALA, que modulam a composição da membrana lipídica e produção de eicosanoides. Controlando a produção de mediadores pró-inflamatórios (IL-1, TNF-alfa) (WU et al., 1999).

1.3.4 Óleo de Oliva

O óleo de oliva (*Olea europaea*) é obtido do fruto maduro da árvore de oliva, por prensagem somente a baixas temperaturas (prensagem a frio). O óleo extra virgem é filtrado mecanicamente. O óleo de oliva é rico em ácido oleico (ômega-9),

possui propriedades lubrificantes, condicionantes, hidratantes e dermo-protetoras. Ajuda a restaurar o manto hidro lipídico da pele (CURI et al., 2002).

Estudo recente, demonstrou que os ácidos oléicos e linoleicos podem ser utilizados em feridas como agentes pró-inflamatórios durante a fase inflamatória do processo de cicatrização, contribuindo para acelerar o processo de reparo (HATANAKA et al., 2007).

Sabe-se que o curativo úmido oleoso, característico da aplicação de ácidos graxos, serve como barreira protetora contra microrganismos, evita a desidratação tecidual, mantém a temperatura corpórea e diminui os traumatismos durante a substituição dos curativos (CURI et al., 2002).

1.3.5 Óleo de Rosa Mosqueta

O óleo de rosa mosqueta é obtido das sementes da *Rosa aff. Rubiginosa*. O óleo é extraído por solventes orgânicos, refinado por *winterização* para eliminar triglicerídeos de maior saturação e finalmente estabilizado. A semente da rosa mosqueta é rica em ácidos graxos insaturados: ácido oleico (16%), linolênico (39%), linoleico (41%), além da vitamina A ácida (tretinoína). Tem ação renegeradora de tecidos, provome a neo-angiogenese, cicatrizante e emoliente (VARANI et al., 1991).

O ácido ascórbico ou vitamina C, presente neste óleo, além de sua propriedade antioxidativa, participa na biossíntese de catecolaminas. É importante na defesa do organismo contra infecções e fundamental na manutenção da integridade das paredes dos vasos sanguíneos. É essência para a formação das fibras colágenas da derme. O ácido ascórbico é capaz de estimular a proliferação celular, bem como a síntese de colágeno pelos fibroblastos dérmicos, sendo vantajoso e benéfico no processo de cicatrização (ARANHA et al., 2000).

A tretinoína é uma molécula multi-funcional que regula direta ou indiretamente processos biológicos em diversos tipos celulares, ou seja, tem atividade pleotrófica. Na pele do indivíduo adulto, os eventos induzidos incluem a expansão da epiderme e a produção de nova matriz extracelular. Tanto a epiderme como a derme são órgãos-alvo independentes. As duas populações celulares respondem com proliferação, crescimento e produção de fatores de crescimento (VARANI et al., 1991).

1.3.6 Óleo de Macadâmia

Óleo obtido das nozes da macadamia (*Macadamia ternifolia nut oil)* é a maior fonte de ácido plamitoleico (25%) e ácido oleico (40%). É facilmente absorvido pela pele, e mantém o teor hídrico cutâneo (MARO et al., 2011).

A aplicação tópica de ácidos graxos no tratamento de feridas, entre outros aspectos, pode servir como uma barreira contra microrganismos, além de manter a hidratação da lesão e evitar possíveis traumas. Entre diversos tipos de ácidos graxos, destaca-se o ácido palmitoléico, que pode ser encontrado na secreção natural da pele e também na composição do óleo de macadâmia. Este óleo, rico em ácidos palmitoléico e oleico, é utilizado para tratar ferimentos e também como cosmético, porém, pouco se sabe sobre os mecanismos de ação dessa substância e o efeito da mesma sobre células envolvidas no reparo tecidual. Queratinócitos formam a camadas celulares mais externas da epiderme que além de serem importantes na integridade da pele, também participam da resposta imune e cicatrização expressando citocinas, quimiocinas e fatores de crescimento (HATANAKA, 2007).

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Capitulo I

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The *in vitro* biological effects of a vegetable oil blend for pharmaceutical applications in skin care

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ABSTRACT

Vegetable oils are well known for their beneficial health effects specially in cutaneous skin offering a natural barrier against microorganisms, preventing oxidative damage, tissue dehydration, enhancing reepithelization and improving scar healing. The present study aims to investigate the *in vitro* benefits for cutaneous wound healing of a vegetable oil blend (VOB) and determine its fatty acid composition. The effects of VOB (1-200 µg/mL) on fibroblasts proliferation and migration were investigated through scratch assay. The anti-inflammatory activity was studied in lipopolysaccharide stimulated murine macrophages RAW 264.7 cells (24 h incubation), by investigating the release of nitric oxide (NO), superoxide anion generation (O_2^-), tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6). ABTS radical scavenging capacity assay, ferric reducing antioxidant potential (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and NO scavenging assays were used to evaluate the antioxidant activity of VOB. Antibacterial activity was tested by the microdilution test. In addition, VOB fatty acid profile was analysed by gas chromatography. VOB stimulate proliferation and migration of fibroblasts cells especially at 100 and 200 µg/mL VOB concentration when compared to control ($p < 0.05$) reaching values of 49.2 ± 5.1% and 57.5 ± 3.7%, respectively compared to 6.2 ± 2.0% exhibited with control cells. The VOB suppressed the pro-inflammatory IL 6 cytokine and the of NO and O_2^- in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. The predominant monounsaturated fatty acid was oleic acid (C18:1n-9; 63.39%) and linoleic acid (C18:2n-6; 4.79%) and linolenic acid (C18:3n-6; 5.09%) the major polyunsaturated fatty acid found in the VOB, respectively. These results suggest that the proposed VOB can accelerate wound healing process with cell proliferation, antioxidative and anti-inflammatory activities.

Keywords: Vegetable oil blend; fatty acids; antioxidants; anti-inflammatory; nitric oxide; scratch assay.

1. Introduction

Due to a widespread lifestyle shift caused by a multitude of factors in the last years, there is a growing demand for natural products. The world is considered a rich source of natural products and, the vegetable oils that are obtain from many renewable resources are getting even more attention than ever due to the numerous healthy benefits and attracting a great interest for the development of natural and eco-friendly cosmetics (Lu et al., 2011, Sarkar et al., 2017). The medicinal value claim on the bioactive fatty acids constituents that have multiple skin benefits and may produce definite physiological action on the human body (Balboa et al., 2014, Begashaw et al., 2017, Zielińska and Nowak, 2017).

Vegetable oils are composed primarily by triacylglycerols and sparse amount of diacylglycerols and monoacylglycerols. They also contain phospholipids, free sterols, tocopherols and tocotrienols, triterpene alcohols, hydrocarbons and fat-soluble vitamins in small amounts. Fatty acids composition of vegetable oils is classified according to the presence or absence of double bonds as saturated (SFAs-without double bonds), monounsaturated (MUFAs-with one double bond) and polyunsaturated fatty acids (PUFAs- with more than two double bonds). Depending on chain length and degree of unsaturation, great influence on the chemical biological properties of these compounds may be observed. In addition, the genetic and environmental factors can determine the proportions of saturated and unsaturated fatty acids present in vegetable oils (Orsavova et al., 2015, Sarkar, 2017).

The wound healing is a natural process in which a body response after injury to protect itself from outer environments while the skin repair occurred simultaneously (Napavichayanun and Aramwit, 2017). Wound healing runs in three basic phases, inflammatory, proliferative and maturation phase (Gurtner et al., 2008). After skin injured, the wound healing process begins immediately with the clotting formation at the wound site by platelet aggregation and vasoconstriction. The pro-inflammatory cytokines and chemokines including interleukin-6 (IL 6), and tumor necrosis factor (TNF-α) are released to activate inflammatory cells (Schreml et al., 2010, Napavichayanun and Aramwit, 2017). Neutrophils, macrophages, and fibroblasts are infiltrated to the wound site. Then, nitric oxide, oxygen free radicals, and matrix metalloproteinase (MMP) are generated to prepare for proliferation phase (Fullerton and Gilroy, 2016). The proliferation phase involves epithelialization and angiogenesis in which

transforming growth factors (TGF) and epidermal growth factor (EGF) are important factors to stimulate proliferation, migration, and differentiation of fibroblasts and keratinocytes, formation of extracellular matrix and collagen, and finally tissue remodelling (Schreml et al., 2010).

Vegetable oils are frequently used to treat wounds, mainly in underdeveloped countries, and the most abundant fatty acids being responsible for their therapeutic effect are oleic, linoleic and linolenic acid (McDaniel et al., 2008, Vaughn et al., 2017, Ferreira et al., 2012). The non-saponifiable lipids are usually responsible for the anti-inflammatory and antioxidant effects of vegetable oils. The fatty acids are considered necessary for the maintenance of epidermal integrity and the water barrier in the skin. They are metabolic precursors of arachidonic acid and prostaglandins in the epidermis and are important for regulation of cell division and epidermis differentiation (Pieper and Caliri, 2003; Sarkar et al., 2017).

Therefore, the aim of this work was to evaluate the pharmacotherapeutic potential of the vegetable oil blend (VOB), obtained from a direct mixture of six vegetable oils: flaxseed oil, blackcurrant oil, olive oil, rosehip oil, macadamia oil, sunflower oil related to its *in vitro* antioxidant, anti-inflammatory, antibacterial and capability to stimulate the proliferation and migration of fibroblasts thus improving tissue reepithelization.

2. Material and methods

2.1. Chemicals and biochemical

Lipopolysaccharide (LPS), penicillin, streptomycin, 2,2-diphenyl-1-picryl-hidrazyl (DPPH), butylated hydroxytoluene (BHT), triphenyl tetrazolium chloride (TTC), nitroprusside, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), tocopherol, collagen solution, platelet derived growth factor-BB (PDGF) and Prolong Gold antifade reagent with 4',6-diamino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co, MO, USA. TNF-α and IL 6 ELISA kit were from eBioscience, San Diego, California, USA. All solvents and reagents were of analytical grade and obtained from various commercial sources. The vegetable oils were purchased from SM Produtos Farmacêuticos, São Paulo, Brasil.

2.2. Cell lines

Mouse macrophages RAW 264.7 (American Type Culture Collection - ATCC® TIB-71™) and murine fibroblasts (L929 cell line, ATCC®-CCL1™) (Cell Line Service, Rio de Janeiro, Brazil) were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10 % fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin, at 37 °C, in a humidified atmosphere containing 5 % CO_2 (all Sigma-USA).

2.3. Preparation of the vegetable oil blend

The vegetable oil blend (VOB) was prepared by the direct mixture of flaxseed oil (15%), blackcurrant oil (10%), olive oil (20%), rosehip oil (10%), macadamia oil (15%), and sunflower oil (30%). The VOB was stored in an amber glass bottle in the absence of light and moisture at room temperature.

2.4. VOB fatty acid profile

VOB fatty acid methyl esters (FAME) were prepared by methylation with boron trifluoride (BF3) in methanol according to Joseph and Ackman (1992). The FAME composition was determined by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan), coupled with flame ionization detector (FID). Fatty acids were identified by comparing the retention time using a known standard of FAME (GLC85 reference standard, NU-CHEK PREP INC., Elysian, USA). The internal standard used was methyl tricosanoate (C23:0 reference standard, NU-CHEK PREP INC., Elysian, USA). FAME were separate on a capillary column DB-5 Agilent (30 m x 0.25 mm d.i. x 0.25 µm). Nitrogen was used as a carrier gas at 0.6 mL min^{-1}. The chromatographic conditions was: injector 250 °C, split 1:50, injection volume 1 µL; oven: 100 °C for 0.5 min, followed by an increment of 3 °C min^{-1} to 260 °C; FID was maintained at 280 °C.

2.5. DPPH radical scavenging assay

The DPPH scavenging activity of VOB was evaluated from the bleaching of the purple methanol solution of free radical DPPH according to Benevides et al., 2017. The antioxidant activity was expressed as IR_{50} value (µg mL^{-1}) obtained from three independent experiments.

2.6. Ferric reducing antioxidant potential assay (FRAP)

Antioxidant capability of VOB was estimated as described by Pulido et al., (2000) with modifications Benevides et al., (2017). FRAP reagent was mixed with VOB or ethanol (for the reagent blank), incubated at room temperature for 10 min and then the absorbance was measured at 595 nm using a microplate reader (Molecular Devises, Spectra Max 190, USA). The results were expressed as IR_{50} value (µg mL^{-1}). Experiments were carried out at least in triplicate.

2.7. Nitric oxide radical scavenging assay

The compound sodium nitroprusside (SNP) is known to spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent (Green et al., 1982; Benevides et al., 2017). Briefly, the reaction mixture containing sodium nitroprusside in phosphate buffered saline with or without the VOB was incubated at room temperature for 30 minutes. After, 150 µL of incubated solution was mixed with 150 µL of Griess reagent and the absorbance of chromophore formed measured at 540 nm in an ELISA plate reader (Molecular Devices Spectra MAX 190). The results were expressed as IR_{50} value (µg mL^{-1}). Experiments were carried out at least in triplicate.

2.8. ABTS free radical scavenging

The antioxidant activity was determined according to (RE et al., 1999) with modifications. In 96-well microplates 270 µL of ABTS•+ radical cation was mixture with 30 µL of VOB at different concertation (ethanol for blank) and allowed for reaction in the dark during 10 minutes. After the absorbance was measured at 734 nm using a microplate reader (SpectraMax 190 Microplate Reader, Molecular Devices, California, USA). The results were expressed as IR_{50} value (µg mL^{-1}). Experiments were carried out at least in triplicate.

2.9. Cytotoxicity

Cellular viability was performed using the MTT assay according to Marques et al. 2017. Macrophages RAW 264.7 and L929 fibroblasts cells were incubated for 24 h in the presence or absence of VOB with concentrations up 1000.0 µg mL^{-1}. Experiments were carried out at least in triplicate and results expressed as percentage of viable cells.

2.10. Minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration (MICs) of VOB were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and the Gram-negative bacteria *Escherichia coli* (ATCC 8739) by the standard NCCL method (NCCL, 2008), in a 96-well microtiter plate according to Benevides et al., 2017. Different concentrations of VOB ranging from 62.5 to 2000.0 µg mL^{-1} were tested. The experiments were carried out at least in triplicate.

2.11. Nitric oxide analysis in the supernatant of macrophage cell culture

Nitric oxide (NO) production was determined by measuring the amount of nitrite in LPS stimulated macrophages supernatant according to the Griess reaction (Green et al., 1982) with minor modifications (Dos Santos Gramma et al., 2016). RAW 264.7 cells were treated by LPS (1 µg mL^{-1}) with or without VOB (10.0 - 200.0 µg mL^{-1}) for 24 h. Next, the culture supernatant was mixed with Griess reagent (1:1) and incubated for 10 min. The absorbance at 540 nm was measured in an ELISA plate reader (SpectraMax 190; Molecular Devices, USA) and the inhibitory rates were calculated by using a standard calibration curve prepared with sodium nitrite by comparing to LPS stimulated control group.

2.12. Quantitative colorimetric Nitroblue Tetrazolium Assay

The determination of intracellular superoxide anion production was evaluated in LPS-activated murine macrophages RAW 264.7 according to Choi et al. (2006) with modifications. Briefly, RAW 264.7 cells were seeded a density of 2 x 10^5 cell mL^{-1} in 96-well plates and cultured in a 37 °C humidified incubator with 5% CO_2 in air for 24 h. Then cells were stimulated with LPS 1 µg mL^{-1} in the presence or absence of increasing concentrations (10.0 - 200.0 µg mL^{-1}) of VOB for 24 h. After incubation the supernatant was removed and 100 µL nitroblue tetrazolium (NBT) (1 mg mL^{-1}) was added each well. The cells were washed with methanol and dried for 20 minutes at 37 ºC. After incubation period of 2 h, the formazan crystals formed were dissolved with dimethyl sulfoxide (DMSO) and potassium hydroxide (KOH). The optical density was measured at 620 nm, using a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). The experiments were carried out at least in triplicate.

2.13. Measurement of Cytokines

Quantification of TNF-α and IL6 production in the supernatant of LPS-activated murine macrophages RAW 264.7 after VOB expired was determined by Enzyme-Linked Immunosorbent Assay (ELISA) techniques using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturer's instructions (eBioscience, San Diego, California, USA). Optical densities were measured at 450 nm in a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). Cytokine levels were expressed in pg, sensitivities were >10 pg mL^{-1}.

2.14. In vitro wound healing (scratch) assay

The *in vitro* scratch wound assay which mimics cell proliferation and/or migration was carried out as previously described (Fronza et al., 2009, Liang et al., 2007). Briefly, fibroblasts were

cultured to nearly confluent cell monolayers and them an artificial linear wound was introduced. After, the monolayers were treated for 16 h with different concentrations of VOB (1.0 - 200.0 μg mL^{-1}). PDGF was used as positive control. After incubation, cells were fixed stained with DAPI and the cellular migration into the wounded area was quantified using CellC® software. Results were expressed as percentage of cells that migrate and/or proliferate into the injured area by comparison to the untreated control group.

2.15. Statistical Analysis

Data were analyzed by ANOVA and the post hoc Tukey's test, using GraphPad software (San Diego, CA, 176 USA). All data were expressed as the mean ± standard error of mean (SEM) or standard deviation (SD), and $p < 0.05$ values or less were considered to indicate statistically significant difference.

3. Results

3.1. Fatty acid composition of VOB

Characterizations of fatty acids composition in percentage of total methyl ester of VOB were done by GC-FID. The VOB FAME composition is presented in Table 1 showing the presence of monounsaturated fatty acid (66.14 %) with oleic acid (C18:1n-9; 63.39 %) as the major lipid followed by the linoleic acid (C18:2n-6; 4.79 %) and linolenic acid (C18:3n-3; 5.09 %) as the major polyunsaturated fatty acids (10.72 %), respectively.

Table 1. Relative percentages of FAME in the vegetable oil blend (VOB).

FAME	**%**
SATURED	
10:0	0
12:0	0.32
14:0	0.32
15:0	0.02
16:0	15.05
17:0	0.12
18:0	5.98
20:0	0.89
22:0	0.66
Total	**23.39**
MONOUNSATURATED	
16:01	2.62
18:1n-9	63.39
20:1n-9	0.08
22:1n-9	0.05
Total	**66.14**
POLYUNSATURED	
18:2n-6	4.79
18:3n-6	5.09
20:4n-6	0.06
20:2n-6	0.08
20:3n-6	0.68
20:3n-3	0.02
Total	***10.72***

3.2. VOB in vitro anti-oxidative effect

Natural antioxidants play an important role in providing the stability to vegetable oils hampering their oxidation. Antioxidant activity should not be concluded based on a single antioxidant test model, therefore, antioxidant activity of VOB was evaluated using four different chemical assays and the results are present at table 2. VOB exhibited only slight antioxidant activity (IR_{50} 233.7 ± 1.48 µg mL^{-1}) estimated by its ability to reduce of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) comparing to tocopherol (IR_{50} 9.68 ± 2.30 µg mL^{-1}). VOB did not exhibited any scavenging activity of DPPH, ABTS and NO radical (Table 2) up to 2000 µg mL^{-1}.

Table 2. *In vitro* antioxidant activity of vegetable oil blend determined by DPPH, ABTS, FRAP and NO radical scavenging assay.

	Antioxidant activity (IR_{50} µg mL^{-1})			
Sample	**DPPH**	**FRAP**	**ABTS**	**NO**
VOB	> 2000.0	233.7 ± 1.5 [a]	> 2000.0	> 2000.0
Tocopherol	9.7 ± 2.3.0	4.8 ± 0.2[b]	6.5 ± 0.5	-

VOB: vegetable oil blend. Different letters in the same column correspond to significant differences ($p < 0.05$). Tests (n=3) were performed in triplicate and expressed as mean ± standard error.

3.3. VOB did not exhibit in vitro cytotoxicity

Next the colorimetric MTT assay was performed to determine the appropriated concentration of the blend which would not affect cellular viability. VOB was tested in the non-cancerous L929 fibroblasts and murine macrophages RAW 267.7 cells. VOB did not exhibited any cytotoxic effect against fibroblast and macrophages compared to basal control (only cell culture medium, considered as 100 % viability), showing 112 % and 108% viability at the highest tested concentration of 500.0 µg mL^{-1}, respectively (Figure 1).

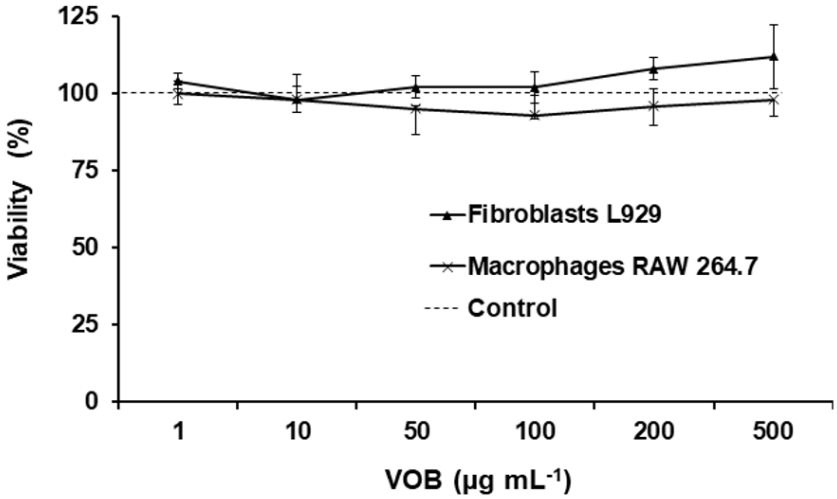

Figure 1. Cellular viability of fibroblasts L929 and macrophages RAW 264.7 after 24 h exposure with 1 to 500.0 µg mL^{-1} vegetable oil blend (VOB) concentration by the colorimetric MTT assay. Values are expressed in percentage of cellular viability compared to basal control cells and represent means ± SD of three independent experiments.

3.4. In vitro cell migration/proliferation

To evaluate *in vitro* cell migration/proliferation the scratch assay was performed using fibroblasts. After 16 h in cultured with VOB at different concentrations, the results showed a positive dose-dependently enhanced in the fibroblast migration/proliferation in the artificial gap, especially at 100 and 200 µg mL^{-1} VOB concentration reaching values of 49.2 ± 5.1% and 57.5 ± 3.7%, respectively, when compared 6.2 ± 2.0% exhibited with control cells ($p < 0.05$). The significant stimulatory effects of VOB was comparable to PDGF (59.4 ± 6.8%) used as positive control (Figure 2).

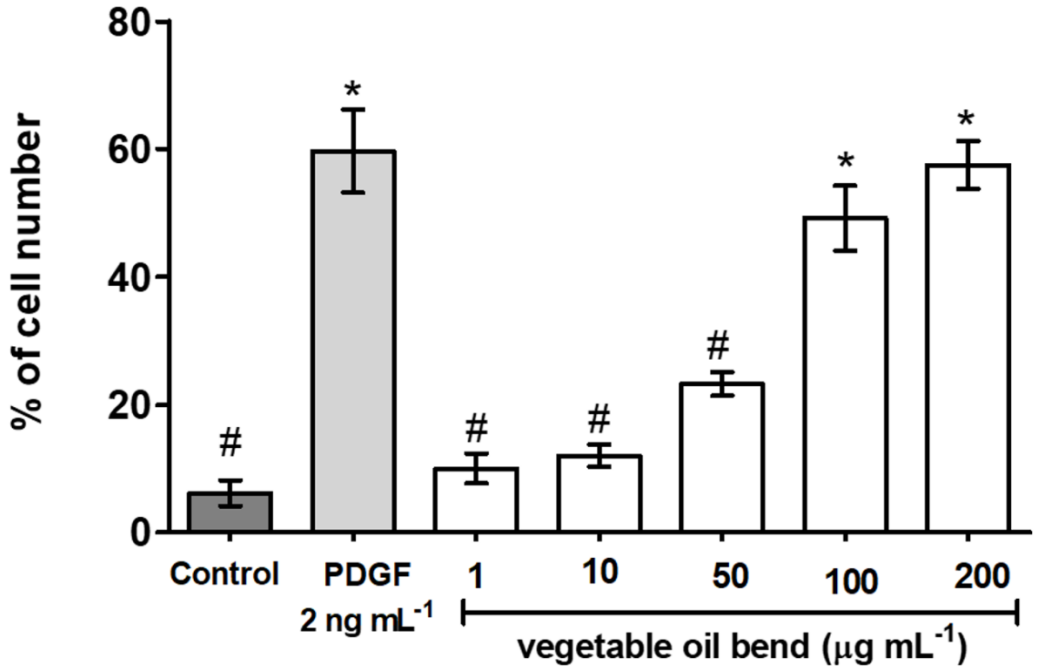

Figure 2: Effect of vegetable oil blend (1 to 200 µg mL^{-1}) on the proliferative/migratory activity of fibroblasts in the scratch assay after 16 h of incubation (37 ºC; 5% CO_2) in DMEM medium supplemented with 10% FBS. PDGF was used as positive control at 2 ng mL^{-1} concentration. Results were expressed as percentage of cell numbers in the injured area, compared to the control group (DMEM medium only). Bars represent the mean ± SD of two experiments. Significant difference ($p < 0.05$) *compared to control group and # compared to positive control group.

3.5. In vitro NO in macrophages

NO overproduction is a typical event that occurs in LPS-stimulated macrophages and is commonly used as an indicator of a typical inflammatory response. The amount of nitrite (NO_2^-), a stable metabolite of NO, was measured in the RAW 264.7 cell supernatants using the Griess assay (Figure 3). LPS induced NO production were significantly blocked by VOB in dose-dependent manner. Reductions of 20.1 ± 1.2 % and 22.9 ± 0.9% were observed in the macrophages supernatant after treatment with 100 and 200 µg mL^{-1} VOB concentrations, respectively.

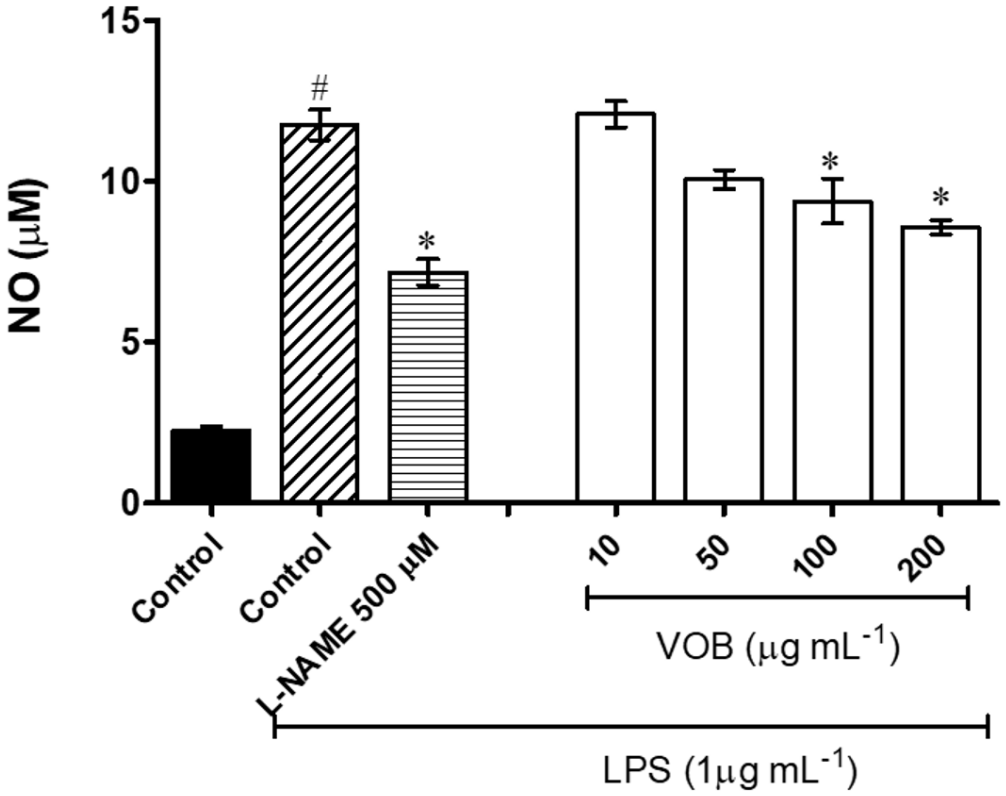

Figure 3: Inhibitory effects of vegetable oil blend (VOB) on nitrite production of LPS-activated RAW 264.7 cells. LPS (1 μg mL^{-1}) with or without VOB (10 to 200 μg mL^{-1}) was added to RAW 264.7 cells and nitrite concentration was measured by Griess reaction assay. Data represent the mean ± SD of triplicate experiments. * ($p < 0.05$) indicates significant differences from the LPS-treated group. # ($p < 0.05$) indicates significant differences from unstimulated control group.

3.6. Effect of VOB on the production Superoxide Anion in LPS-stimulated RAW 264.7 macrophage cells

The colorimetric NBT assay was used to measure the intracellular production of superoxide anion (O_2^-) in LPS-stimulated RAW 264.7 macrophage cells. As shown in figure 4, O_2^- production induced by the LPS decreased considerably after VOB were added. O_2^- production was statistically significant ($p<0.05$) decreased after VOB 100 and 200 μg mL^{-1} treatment compared to control group.

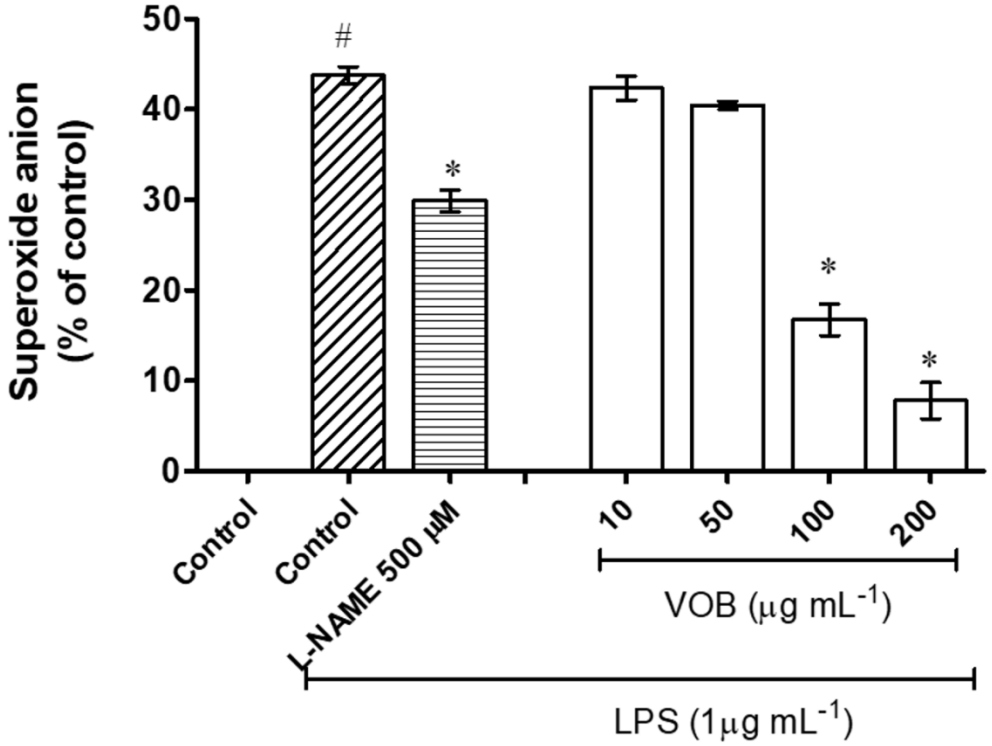

Figure 4. Inhibitory effects of vegetable oil blend (VOB) on intracellular superoxide anion production in LPS-activated RAW 264.7 cells using the colorimetric NBT assay. The level of superoxide anion in control cells was arbitrarily expressed as zero (0). Data represent the mean ± SD of triplicate experiments. * ($p<0.05$) indicates significant differences compared with LPS-treated cells. # ($p < 0.05$) compared with control.

3.7. Determination of cytokine production

Concerning the cytokines production, the effects of VOB on LPS induced inflammation in RAW 264.7 macrophage were evaluated by measuring the production of TNF-α and IL-6 cytokines. As observed in Figure 5, stimulation with LPS for 24 h significantly induced the release of cytokines pro-inflammatory (TNF-α and IL 6) indicating that an inflammatory response was induced in the RAW 264.7 cells. Interesting, VOB was capable to significant reduce the production of IL 6 at 100 and 200 µg mL^{-1}, however did not exhibited any influence on TNF-α production.

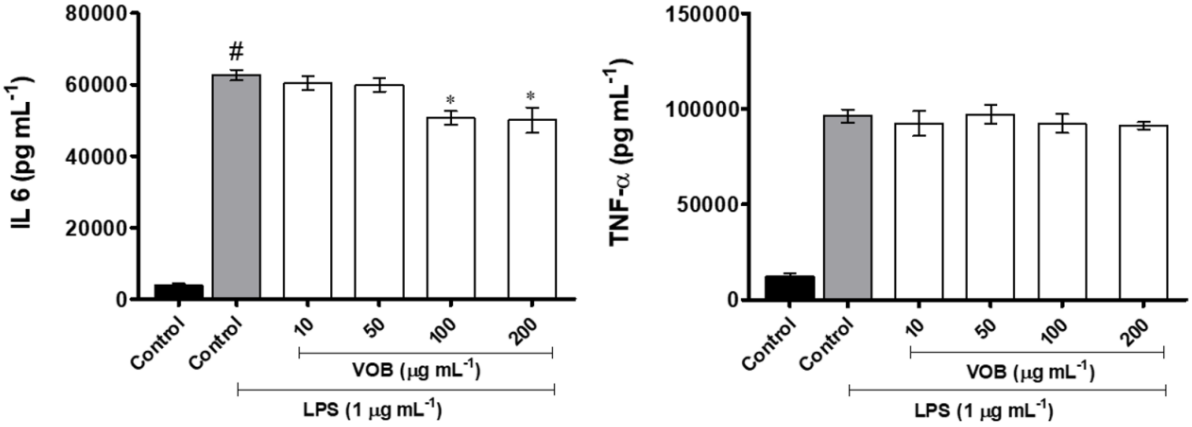

Figure 5. Effect of vegetable oil blend (VOB) on proinflammatory cytokine production in LPS-stimulated macrophages. (A) IL 6 and (B) TNF-α production was measure using ELISA Kits as described in material and methods section. Results were expressed as mean ± SD of two independent experiments. *($p < 0.05$) indicates significant differences compared with LPS-treated cells. # ($p < 0.05$) compared with control.

3.8. Antibacterial activity of VOB

Wound bacterial colonization present a continuous challenge in the treatment of skin injuries and wound healing (Vittorazzi et al., 2016). Therefore, antibacterial activity of VOB was preliminary assayed against the common Gram positive *Staphylococcus aureus* bacteria and the Gram negative *Escherichia coli* bacteria frequently hosted in cutaneous wound. VOB exhibited only a slight activity against the Gram Positive *S. aureus* bacteria with MIC value of 2000 µg mL^{-1} and did not exerted any antibacterial activity against *E. coli* up to 2000 µg mL^{-1} (Table 3).

Table 3. Minimum Inhibitory Concentration (MIC) of vegetable oil blend (VOB) determined by the microdilution method.

Sample	MIC (µg mL^{-1})	
	S. aureus	***E. coli***
VOB	2000	-
Ampicilina	0.8	4

4. Discussion

In the present study, it was found that blending may be consider a physical and economical, as well as, the simplest procedure to change fatty acid composition, enhancing the

commercial application and make new specific products with a desire biological property, in general, with affordable prices (Hashempour-Baltork et al., 2017). In general, the natural vegetable oils have been used as topical therapy worldwide. They are easily accessible and a relatively inexpensive option for skin care including its therapeutic potential to positively influence the cutaneous wound healing (Vaughn et al., 2017). Besides the emollients properties, many natural oils possess specific compounds with antimicrobial, antioxidant and anti-inflammatory activities. Moreover, it is possible to metabolize lipids derived from topically applied emollients and to utilize them as nutritional building blocks for the formation of a healthy and functional epidermal barrier (McDaniel et al., 2008; Sarkar et al., 2017; Vaughn et al., 2017).

Unique characteristics of vegetable blend oils are important when considering their use for topical skin care. Differing ratios of essential fatty acids are major determinants of the barrier repair benefits of natural oils. Oils with a higher linoleic acid to oleic acid ratio have better barrier repair potential (Vaughn et al., 2017). In particular the quantities of fatty acids with healing effect are highlighted as oleic fatty acid (C18: 1n-9), linoleic acid (C18: 2n-6) and linolenic acid (C18: 3n-3), as previous described (Cardoso et al., 2004). The fatty acid profile of VOB exhibited predominant monounsaturated fatty acid, oleic acid (C18:1n-9; 63.39 %), and polyunsaturated fatty acid, linoleic acid (C18:2n-6; 4.79 %), linolenic acid (C18:3n-3; 5.09 %), respectively. Thus, the fatty acid composition reinforces the potential therapeutic applicability for improving the natural skin-barrier function.

The fatty acids of the family omega-3 (linolenic acid) and omega-6 (linoleic acid) are of great importance for the inflammatory process, since they are not synthesized by de novo synthesis and are precursors of the polyunsaturated fatty acids, such as eicosapentaenoic, docosahexaenoic and arachidonic (Kiecolt-Glaser et al., 2016, Mišurcová et al., 2011). Linoleic acid exhibited an important chemotactic role for macrophages, contributing to the autolytic debridement in the wound bed by increasing the production of metalloproteins, inducing the granulation and being able to accelerate the healing process (Ferreira et al., 2012). In the light of this VOB also demonstrated great stimulatory effects on the proliferative and migratory activity of fibroblasts contributing therefore to granulation tissue formation and reepithelialization of the skin.

The inflammatory phase of wound healing normally leads to the release of biologically active mediators and oxygen-free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl anion and, the excess of these agents is well known to causes tissue damage and hampered the tissue repair (Fullerton and Gilroy, 2017; Schreml et al., 2010). In macrophage

cells stimulated with LPS that is known to be an endotoxin, which induces the release of pro-inflammatory cytokines such as IL-6 and IL-1, and other inflammatory mediators such as NO (Benevides et al., 2017, Vittorazzi et al., 2016). VOB was able to significant suppressed the production of these hazards of healing process. The overproduction of NO has been reported to contribute to the pathogenesis of inflammatory diseases, including rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and unhealed wounds. Activated macrophages release into the extracellular medium, several reactive oxygen species, including singlet oxygen, superoxide anion among others. VOB has been shown to be highly effective in inhibiting the production of these radicals, especially NO and anion superoxide and inflammatory cytokines which proves to be a good alternative as an antioxidant and anti-inflammatory agent (Dhavamani et al., 2014, Badea et al., 2015)

Another significant problem with wounds is the high risk of infection. Therefore, if an agent active against these microorganisms causing the infection is used in the healing process, it will then help to reduce the risk of infection and the overall time for wound healing can be reduced significantly (Misic et al., 2014). Linoleic acid was proven to be able to inhibit the growth of *Staphylococcus aureus* by altering the synthesis of proteins, cell walls, nucleic acids and cell membranes during the division (Ferreira et al., 2012). Although, VOB elicited only a discrete antibacterial action, it can assume that external use of VOB may protect the cutaneous wounds from pathogenic bacteria and their harmful effects on wound healing.

In addition, skin chronic wounds present a painful, unsightly, and unpleasant sensory experience. VOB could be used to stimulate wound hydration diminishing trauma during dressing changes, besides a hydrated wound is registered to collaborate with the process of reepithelialization, granulation, tissue formation, angiogenesis, fibroblast migration, collagen synthesis, and remodeling of injured tissue (Okuma et al., 2015). Therefore, VOB should be used as natural synergetic compound to threat skin wound.

5. Conclusion

This work highlights the role of a high effective and low-cost vegetable oil blend which may be an effective therapy against skin inflammatory disorders or treat patients with skin injuries. In conclusion, the authors encourage that more work should be done to improve our understanding, and better explain the mechanism by which VOB improves proliferation and migration of fibroblasts and alters proinflammatory and oxidative mediators in macrophages.

Acknowledgment

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Capitulo II

Manuscrito escrito seguindo as normas da revista *Industrial Crops and Products*

Development and evaluation of a vegetable oil blend formulation for cutaneous wound healing

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Highlights

A vegetable oil blend (VOB) was assayed for skin care applications.

The VOB accelerated wound closure *in vivo*.

The VOB affected pro-inflammatory mediators.

The VOB increased the deposition and organization of collagen fibres.

The data indicated benefits of VOB use in skincare applications.

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Abstract

Vegetable oils have been used for years in the treatment of cutaneous wounds. They are an important source of unsaturated fatty acids, which have anti-inflammatory properties and serve to promote healing. This study aimed to evaluate the *in vivo* capacity of a vegetable oil blend formulation (VOB) developed to accelerate cutaneous wound closure and to investigate the dynamic wound healing processes. Sixty animals were randomly divided in three groups of 20 animals each. Four full-thickness wounds were punched in the back side skin of each animal and topically treated with the VOB formulation, Dersani® ointment (positive control) or the vehicle control. After 2, 7, 14 and 21 days post-wounding, five rats from each group were euthanized, and the rates of wound closure and re-epithelialization were evaluated. The wounds were harvested for histological and biochemical analysis. VOB resulted in faster and greater re-epithelialization in the *in vivo* full-thickness excisional wounds, exhibiting significant wound area reduction of 8.9, 8.0, 35.1, 45.2 and 47.0% after 2, 5, 10, 14 and 21 days post-wounding, respectively, when compared with the vehicle control. Histological and biochemical analyses of the skin biopsies showed that the VOB-treated wounds exhibited increased granulated tissue and a regulated inflammatory response by modulation of the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1. Moreover, VOB-treated wounds showed a marked and robust increase in the deposition and organization of collagen fibres in the wound site and improved the quality of the scar tissue. Altogether, these data revealed that VOB accelerates wound healing processes and might be beneficial for treating wound disorders.

Keywords: Fatty acids, vegetable oil, wound healing, cytokines, collagen, inflammation.

1. Introduction

In response to a tissue injury, the skin is repaired through a sequence of coordinated events involving various cell types, including leukocytes, platelets, fibroblasts and epithelial cells (Cerveró-Ferragut et al., 2017; Thakur et al., 2011). The wound healing process is divided into three overlapping phases: inflammatory, proliferative, and remodelling (Cerveró-Ferragut et al., 2017). The healing process begins immediately after the skin is broken through the creation of the "platelet plug" to control the homeostasis (Napavichayanun and Aramwit, 2017).

The first cells to appear at the wound site are the polymorphonuclear leukocytes, also called the inflammatory infiltrate, which reach the site of inflammation through diapedesis (Lindley et al., 2016). Together with macrophages and resident cells, they produce and release different pro-inflammatory cytokines and chemokines, including interleukin-1 and 6 (IL-1, IL-6) and tumour necrosis factor (TNF-α) for the activation of further inflammatory cells, giving rise to the proliferative phase (Okuma et al., 2015; Li et al., 2017). The proliferative phase involves epithelization and angiogenesis, in which transforming growth factor (TGF) and epidermal growth factor (EGF) are important for the proliferation, migration and differentiation of fibroblasts and keratinocytes (Cerveró-Ferragut et al., 2017; Maver et al., 2015; Napavichayanun and Aramwit, 2017). Tissue maturation and remodelling is the last phase of wound healing, where collagen synthesis comes into equilibrium with collagen breakdown. This phase is significant for strengthening the collagen matrix and the subsequent scar. Collagen re-formulations, the enhancement of collagen fibre components, and water reabsorption are events that allow for connections, increase the strength of the scar and decrease its thickness (Greaves et al., 2013; Napavichayanun and Aramwit, 2017; Serhan et al., 2015).

Vegetable oils are considered a rich source of fatty acids that have been used prominently in the medical and cosmetic fields (Zielińska and Nowak, 2017). The fatty acids contained in these oils create an occlusive film on the skin, reducing the transepidermal water loss (TEWL), which contributes to the maintenance of correct hydration of the skin (Knowles and Watkinson, 2014; Zielińska and Nowak, 2017). Linoleic (C18:2n-6) and alpha-linolenic (C18:2n-3) fatty acids are essential for normal cellular functions and act as precursors for the synthesis of long chain polyunsaturated fatty acids such as arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C20:6-n3), which are involved in numerous cellular functions, such as membrane fluidity and the synthesis of eicosanoids such as prostaglandins, leukotrienes and thromboxanes (Ferreira et al., 2012; Manhezi et al., 2008).

Thus, they have the ability to modify inflammatory and immunological reactions, altering leukocyte functions and accelerating the process of tissue granulation. Vegetable oil formulations have been used with great success in the treatment of wounds and pressure ulcers in bedridden patients due to their low cost and widespread availability (Ferreira et al., 2012; Manhezi et al., 2008).

Despite several reports in the literature describing the biological properties of vegetable oils, few reports are available about *in vivo* studies of the use of VOB for cutaneous wound healing. Thus, this study aimed to evaluate the efficacy of a developed VOB formulation in cutaneous wound healing processes using the full-thickness excisional wound model in rats and to compare it to a commercially available reference product (Dersani® ointment) indicated for the treatment of any type of cutaneous lesion.

2. Materials and methods

2.1 Chemicals and biochemicals

Dersani® ointment was purchased from a local pharmacy. TNF-α, IL-1α, IL-6, and TGF-β ELISA kits were purchased from eBioscience (San Diego, CA, USA). Bradford protein assay reagents were purchased from Thermo Scientific (Rockford, IL, USA). Ketamine and xylazine were purchased from Vetbrands (Paulinia, SP, Brazil). Other reagents used were of analytical grade and were obtained from various commercial sources.

2.2 Vegetable oils

The following vegetable oils were purchased from SM Farmaceutica (Campinas, SP, Brazil) and were accompanied by quality control reports attesting to a degree of purity above 99.2%: sunflower oil (*Helianthus annuus)*, olive oil (*Olea europaea*), rosehip oil (*Rosa aff. rubiginosa),* linseed oil (*Linum usitatissimum*), black currant oil (*Ribes nigrum)*, and macadamia oil (*Macadamia ternifolia* nut oil*)*.

2.3 Preparation of the VOB formulation and controls

The vegetable oil blend formulation (VOB) was prepared by simple mixing of the vegetable oils in the following proportions: sunflower oil (*Helianthus annuus*) 30%, olive oil (*Olea europaea*) 20%, rosehip oil (*Rosa aff. Rubiginosa)* 10%, linseed oil (*Linum usitatissimum*) 15%, black currant oil (*Ribes nigrum*) 10%, macadamia oil (*Macadamia ternifolia* nut oil) 15%. To prepare the ointment, the VOBs were heated to 45 °C, and 10% Compritol ATO 888 (glyceryl dibehenate, tribehenin) was added. Upon returning to room temperature, the VOB acquired an ointment texture. The commercially available Dersani®

ointment was used as a positive control (caprylic acid, capric acid, soy lecithin, vitamin A, vitamin E, caproic acid, and sunflower oil - linoleic acid). Mineral oil was formulated with the same gelling agent used in the VOB formulation to obtain the same ointment consistency and was then used as the vehicle control.

2.4 Formulation stability testing

The stability of the formulation and the expiration date were determined by using the accelerated stability method according to the Brazilian Health Regulatory Agency (ANVISA - Agencia Nacional de Vigilância Sanitária, 2008). The VOB was conditioned in a transparent, neutral glass bottle with a cover that guaranteed a good seal, therefore avoiding the loss of gases and evaporation to the medium. The VOB was submitted to heating in an oven at 37 ºC ± 2 ºC, alternating with cooling in the refrigerator at 5 ºC ± 2 ºC, with cycles of 24 h each over 4 weeks. Organoleptic characteristics such as colour, odour, and appearance and physical-chemical parameters such as pH and viscosity were evaluated (ANVISA - Agencia Nacional de Vigilância Sanitária, 2008). Moreover, the VOB fatty acid profile was analysed by gas chromatography before and after the accelerated stability test.

2.5 VOB fatty acid profile

The fatty acid content of the VOB was analysed by a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) coupled with a flame ionization detector (FID). VOB fatty acid methyl esters (FAME) were prepared by methylation with boron trifluoride (BF_3) in methanol according to Joseph and Ackman (1992). Fatty acids were identified by comparing the retention times to a known FAME standard (GLC-85 reference standard, NU-CHEK PREP INC., Elysian, USA). The internal standard used was methyl tricosanoate (C23:0 reference standard, NU-CHEK PREP INC., Elysian, USA). FAME were separated on a capillary column DB-5 Agilent (30 m x 0.25 mm d.i. x 0.25 µm). Nitrogen was used as a carrier gas at 0.6 mL min^{-1}. The chromatographic conditions were as follows: injector 250 °C, split 1:50, injection volume 1 µL; oven: 100 °C for 0.5 min, followed by an increment of 3 °C min^{-1} to 260 °C; FID was maintained at 280 °C.

2.6 Animals

All experiments involving the use of animals were conducted in agreement with the Brazilian Animal Care Committee and were approved by the Committee of Ethics, Bioethics and Animal Welfare of the Universidade Vila Velha (UVV) (CEUA-UVV protocol 381/2016). A total of sixty healthy adult male Wistar rats (*Rattus norvegicus*) weighing between 270-330 g and aged 6-8 weeks were obtained through Central Biotério of the Universidade Vila Velha, Vila Velha, Brazil. The animals were kept under standard temperature controlled conditions

(22 ± 2 °C) with 12 h light/dark cycles and with free access to food and water. Two weeks prior to the experiment to produce the lesions, the animals were housed in separate cages in order to avoid injury by contact with other individuals, which could interfere with the progress of the experiment.

2.7 In vivo wound healing experiment

Prior to the production of the wounds, the animals were weighed and anaesthetized with 4% hydrated chloride. After shaving and cleaning the skin with 70% ethanol, four full-thickness excision wounds were created on the dorsum region of each rat with a sterile 150 mm punch biopsy. Then, the animals were randomly divided into three groups (n=20) and evaluated for 21 days according to the standard protocols (Dos Santos Gramma et al., 2016; Caetano et al., 2015). The animals were treated daily with VOB, Dersani® ointment (positive control), or the vehicle control. The wounds were covered with gauze and tape to keep them protected. Five animals from each group were euthanatized on days 2, 7, 14 and 21 after the surgical procedure, and the wounds and their surrounding areas were collected and stored for future histological and biochemical investigations (Dos Santos Grama et al., 2016; Caetano et al., 2015).

2.8 Wound area studies

The wound areas were calculated using ImageJ software (NIH, USA). The morphometric analysis of the wounds was performed using images of the wounds at zero, 2, 5, 7, 10, 14 and 21 days post wounding. The rate of wound closure that represents the percentage of wound reduction from the original wound size was calculated using the following formula: ((wound area day 0 - wound area at day 2, 5, 7, 10, 14 and 21)/wound area day 0) x 100. Values were expressed as the percentage of healed wounds.

2.9 Histological processing

Two wound biopsies from each animal in each group and treatment time were conditioned for 24 h in 3.7% phosphate-buffered formaldehyde, followed by histological processing and paraffin inclusion. Serial histological sections with thicknesses of 3-5 µm were mounted on glass slides and stained with haematoxylin and eosin (H&E) (for evaluation and quantification of the inflammatory infiltrate) and with a solution of Sirius Red F3BA saturated in aqueous picric acid (for quantification of collagenase) (Fronza et al., 2014; Dos Santos Grama, et al., 2016).

2.10 Evaluation of inflammatory infiltrate

Paraffin-wound sections stained with haematoxylin-eosin (H&E) were photographed using image capture software (Honestec VHS to DVD 3.0 SE) in a blinded fashion at 100x using a digital camera attached to a light microscope (Model Leica Mikroscope Type 501095).

Approximately thirty different fields (from the superficial dermis, the deep dermis, and a non-wound area) were examined, and a region of interest was acquired for each field. The images from each group on their respective day were loaded and analysed using the open source software CellProfiler (CP) (version 2.1.1), designed for the quantitative analysis of biological images, in order to identify the size and shape of the cells in the inflammatory infiltrate (Carpenter, 2009). Five different microscopic slides were used for each treatment at each time point (n=20 wounds/group), and the data were reported as the average of the total number of inflammatory cells per group (Dos Santos Gramma et al., 2016).

2.11 Evaluation of collagenesis by imaging

The morphometric analysis corresponding to the area occupied by the collagen fibres was determined by analysing the colour density by digitally converting the images first to greyscale and then to black and white using the CellProfiler (CP) software (version 2.1.1) (Carpenter, 2009). Four different fields of each wound tissue stained with Sirius red were photographed using image capture software (Honestec VHS to DVD 3.0 SE) at 100x using a digital camera attached to a light microscope (Model Leica Microscope Type 501095). The distribution of collagen (red colour) was quantified using the Image Math and Measure Image Intensity functions of the CellProfiler (CP) software (version 2.1.1). Five different rats were used for each treatment at each time (n=20 wounds/group), and the results were reported as the average distribution of collagen per treatment (Fronza et al., 2014; Dos Santos Gramma et al., 2016).

2.12 Cytokine measurements

Two wound specimen biopsies collected from each animal at days 0, 2, 7, 14 and 21 post-wounding were immediately frozen at -80 °C. Next, fragments of these biopsies were homogenized on ice using Lysing Matrix A tubes and a Fast Prep-24 homogenizer (MP Biomedicals, Santa Ana, CA) and then centrifuged (1500 g). The homogenate fluid obtained was used to measure the IL-1, IL-6, TNF-α, and TGF-β using the enzyme-linked immunosorbent assay (ELISA) following the manufacturer's specifications for each assay (eBioscience, San Diego, California, USA). Optical densities were measured at 450 nm in a microplate reader (Molecular Devices Spectra MAX 190, USA). Cytokine levels were expressed in pg; sensitivities were > 10 pg mL^{-1}.

2.13 Total protein quantification

The total protein contents of the homogenate fluid obtained from the tissue sections of the wounds treated with the VOB, the positive control, and the vehicle control were estimated using the Coomassie protein assay reagent (Rockford, USA) according to the manufacturer's

instructions. Experiments were performed in 96-well plates, and protein concentrations were calculated by regression analysis using a standard curve with bovine serum albumin (BSA) by colourimetric measurements at 595 nm in an ELISA plate reader (Molecular Devices Spectra MAX 190).

2.14 Biochemical measurement of myeloperoxidase (MPO)

The density of the neutrophilic infiltrate in the wounds of the animals was determined through the myeloperoxidase (MPO) assay, as previously described by Dos Santos Gramma et al., 2016. The results were reported as the total number of neutrophils x 10^3/mg tissue by comparing the absorbance of the tissue homogenate to a standard curve generated using mouse peritoneal neutrophils.

2.15 Statistical analysis

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data are presented as the mean ± standard error of mean (SEM) or standard deviation (SD), and statistical comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-test or two-way-ANOVA when appropriate. The level of significance was $p < 0.05$.

3. Results

3.1 Accelerated stability test

Different physicochemical properties of the VOB formulation were evaluated after the accelerated stability test. The results indicated that no changes in colour, appearance, odour or viscosity were observed. The pH of the VOB was also determined in order to ensure that the formulation would not produce any irritation of the skin. The freshly prepared VOB exhibited a pH of 3.85 ± 0.2, and after the accelerated stability test, the pH was found to be 3.87 ± 0.3. These results indicate that the pH and organoleptic characteristics of the VOB were markedly stable during the test period.

3.2 Fatty acid composition of the VOB formulation

Characterizations of the fatty acid composition in the freshly prepared VOB and after the stability testing were expressed as the percentage of total methyl esters and were analysed by GC-FID (Table 1). Table 1 shows that although the VOB that underwent the stability test was stressed at high temperatures and cooling, the fatty acid profile of the sample did not change, and the characteristics of the polyunsaturated fatty acids were preserved.

Table 1. Relative percentages of FAME in the freshly prepared vegetable oil blend (VOB) and after the stability testing.

FAME	VOB ointment freshly prepared (%)	VOB ointment stability testing (%)
Saturated		
10:00	0.04	0.03
12:00	0.31	0.27
14:00	0.28	0.26
15:00	0.05	0.06
16:00	9.64	9.84
17:00	0.08	0.08
18:00	3.79	3.91
20:00	1.05	1.21
22:00	6.67	8.95
Total	21.91	24.61
Monounsaturated		
16:1n-7	1.94	1.96
18:1n-9	32.88	31.24
20:1n-9	0.45	0.44
Total	35.27	33.64
Polyunsaturated		
18:2n-6	34.53	34.21
18:3n-3	7.57	7.09
Total	42.1	41.3

3.3 In vivo wound closure assessment

Topical application of the VOB allowed faster and greater re-epithelialization in *in vivo* full-thickness excisional wounds compared to the vehicle control and the positive control groups, as shown in Figure 1. In addition to accelerating the wound closure, the VOB led to a rapid recovery of the mature epidermal structure, with the lesions becoming progressively less inflamed and producing fewer scars than those wounds treated with either the positive control or the vehicle control. Significant wound area reductions of 8.9, 8.0, 35.1, 45.2 and 47.0% were observed in the VOB treated group compared to the vehicle control group after 2, 5, 10, 14 and 21 days post-wounding, respectively. On the other hand, wounds treated with the positive control exhibited significant reductions of 7.2, 9.6 and 20.1% in the wound area after 2, 5 and 14 days, respectively. Thus, it is evident that the wound healing rate of the VOB-treated lesions

was similar to or even greater than that of the positive control, especially during the re-epithelization stage after 10 days and at the maturation stage on the 21st day.

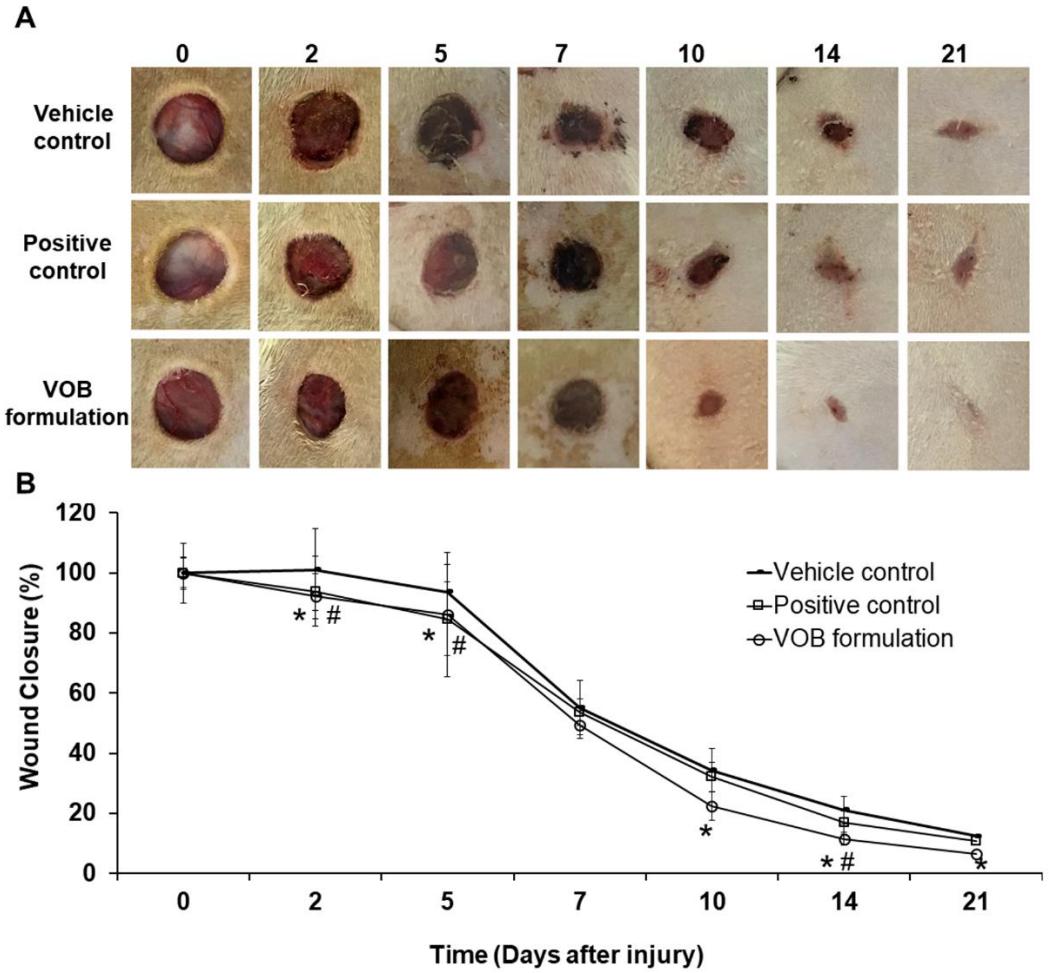

Figure 1. Topical application of the VOB formulation accelerates excisional wound closure. (A) Photographic representation of the wounds on the indicated days post-wounding. (B) Percentage of wound closure after daily topical application of the VOB, positive control and vehicle control at days 0, 2, 5, 7, 10, 14, and 21. Data are expressed as percent area reduction from the original wound size (Day 0). Mean values ± SEM (n=20 wounds/group), * $p < 0.05$, VOB formulation compared to vehicle control; # $p < 0.05$, Positive control compared to vehicle control.

3.4 Vegetable oil blend (VOB) positively influenced the inflammatory phase

Histological analysis of the wound biopsies stained with H&E were used for quantitative analysis of the cellular density at the wound site (Figure 2, A and B). Analysis of the inflammatory cellular infiltrate in the wound biopsies treated with the VOB exhibited a significant reduction in the inflammatory infiltrate at the wound site after 2, 7 and 14 days, when compared to the vehicle control and the reference drug groups ($p < 0.05$). Reductions of 24.2, 14.4 and 26.6% were observed in the cellular densities on days 2, 7 and 14, respectively,

compared with the vehicle control group. No significant differences were observed in the wounds treated with the positive control compared to those treated with the vehicle control. The cellular density after 21 days post wounding were similar between all groups and decreased to nearly the physiological cell number (Figure 2B).

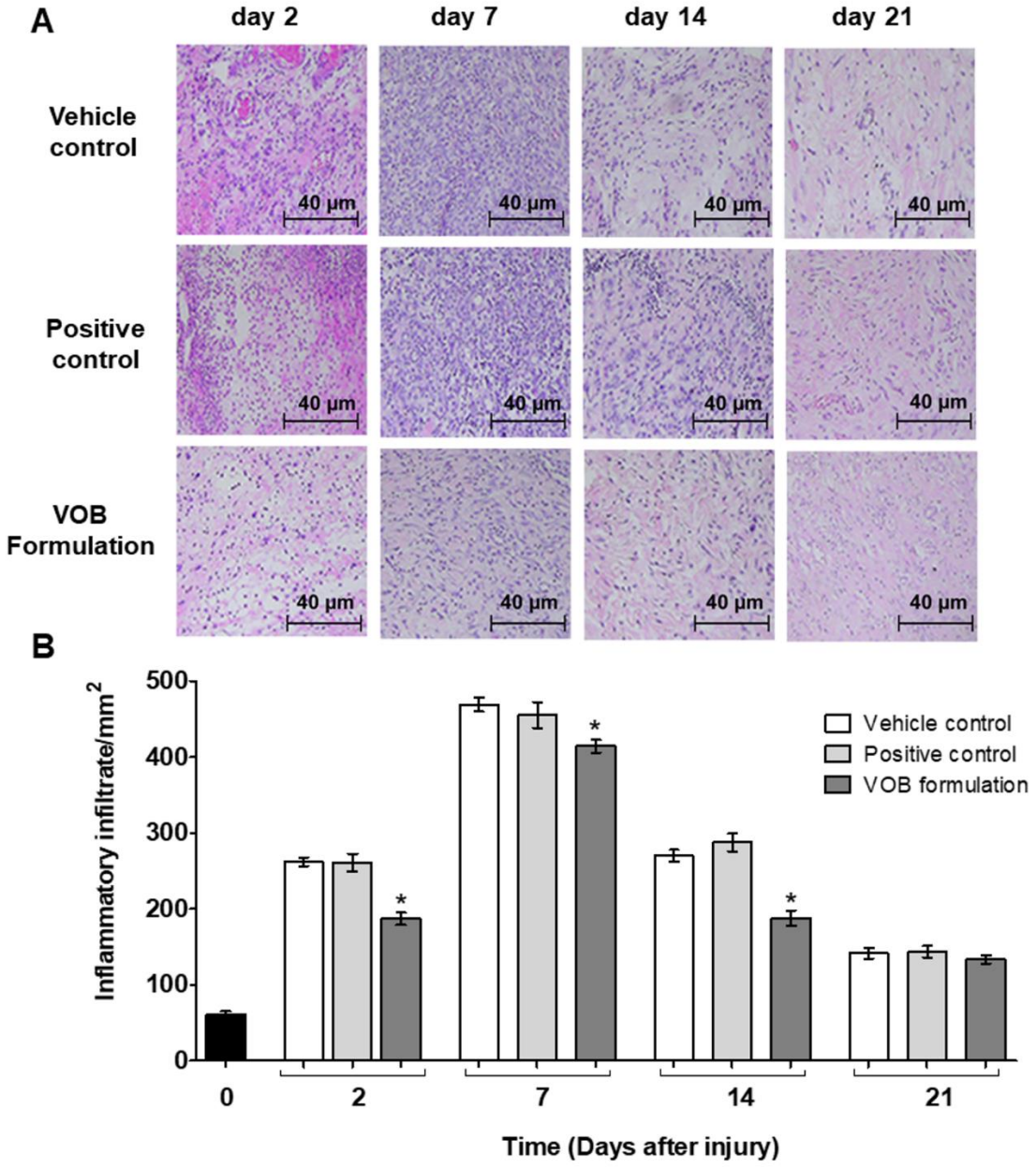

Figure 2. Vegetable oil blend formulation (VOB) affects polymorphonuclear recruitment at the wound site. (**A**) Representative photomicrography of the wound sections stained with H&E (400x). (**B**) Quantitative analysis of inflammatory infiltrate. The values

represent the mean ± SEM (n=20 wounds/group), *p<0.05 compared to the vehicle control group.

Subsequently, the presence and involvement of neutrophils in the inflammatory process were estimated by investigating the activity of the enzyme myeloperoxidase (MPO). Very low MPO levels were identified in the animals with preserved skin (day zero) (Figure 3). However, on days 2 and 7 post-wounding, the MPO levels presented considerably increased levels of MPO in all groups, although no significant difference was observed among the tested groups. At 21 days post-wounding, the concentrations of MPO decreased back to the physiological levels observed on day zero.

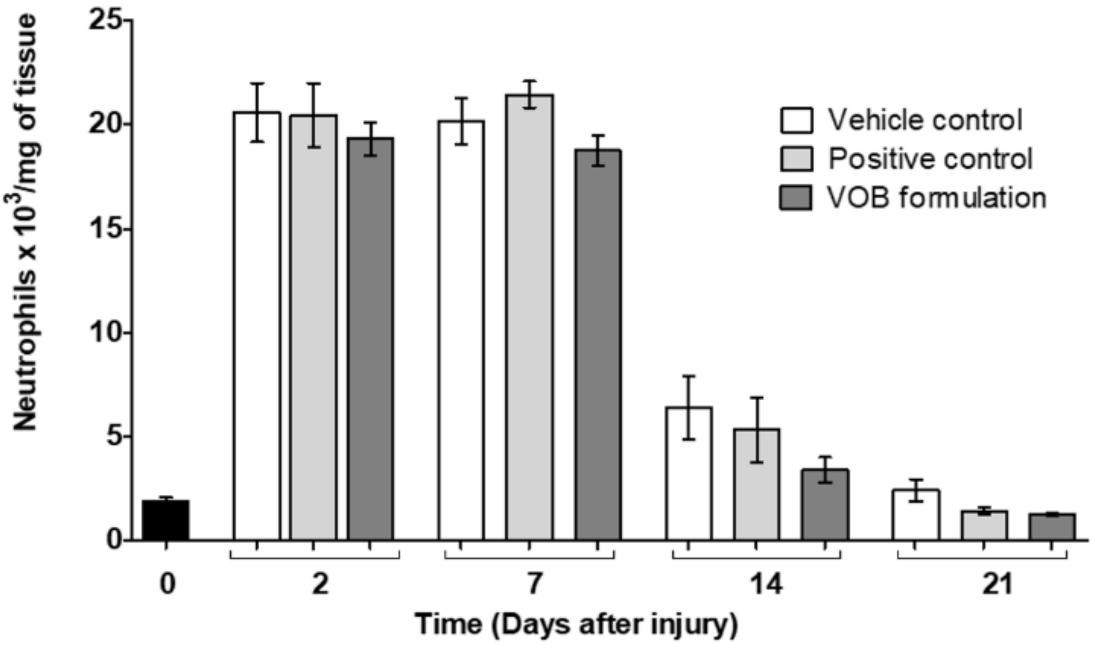

Figure 3. Tissue neutrophil accumulation determined by myeloperoxidase (MPO) concentrations in the wound biopsies treated with the VOB formulation, the positive control and the vehicle control at 0, 2, 7, 14, 21 days. Values represent mean ±SEM (n=10 wounds/group).

To investigate whether the decrease in inflammatory infiltrate might have some correlation to the oedema and the inflammatory phase of wound healing, the total protein contained in the tissue biopsy homogenates was analysed. The wounds of the VOB-treated group exhibited a significant reduction in the total protein content at 7 days post-injury when compared to the

vehicle control group (Figure 4). No significant differences were observed in the positive control group compared to the vehicle control group during the entire experimental time.

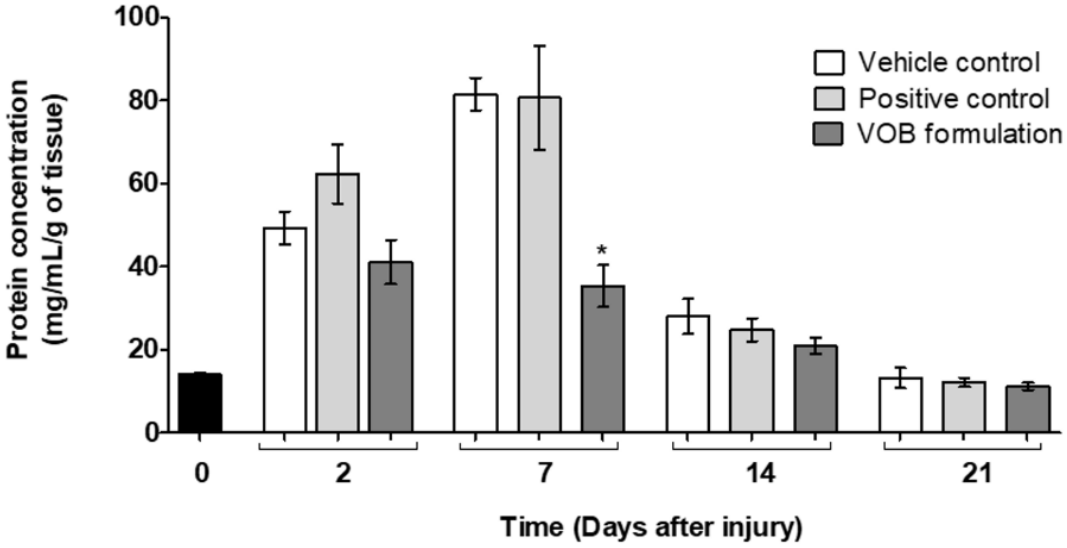

Figure 4. Total protein content in the wound tissue treated with the VOB formulation, positive control and vehicle control on days 0, 2, 7, 14 and 21 post wounding. Total protein was measured according to the Coomassie assay in homogenates prepared from the wound biopsies. Values represent mean ± SEM (n=10 wounds/group). * $p<0.05$ compared to vehicle control group.

3.5 Cytokine quantitation in the skin wound biopsies

Cytokines are mainly produced by macrophages and lymphocytes, although they can also be produced by polymorphonuclear leukocytes (PMN), endothelial cells and epithelial cells. Therefore, following the investigation of the influence of the VOB on the production and release of different cytokines and growth factors, such as TNF-α, IL-1α, IL-6 and TGF-β, the wound biopsy homogenates were examined. In fact, the VOB altered the release of pro-inflammatory cytokines at the wound site. As observed in Figure 5 A, the concentrations of the TNF-α detected in the homogenate tissues prepared from the wound biopsies following exposure to the VOB after 2, 7, and 14 days were significantly reduced compared to the vehicle control group ($p <0.05$). The positive control group exhibited a significant decrease in TNF-α concentrations only after 14 days. IL-1α cytokine concentrations after 2 and 7 days also showed a significant decrease after VOB treatment compared to the vehicle control treatment ($p <0.05$) (Figure 5 B). No significant effects were observed in the positive control group. Concentrations

of IL-6 cytokine were significantly suppressed after 2 and 21 days post-injury in the group treated daily with VOB compared to the vehicle control group, whereas the positive control group only presented significant effects at day 21 (Figure 5 C). No significant effect on the production of TGF-β was observed after topical treatment of the wounds with VOB and the positive control during the experimental procedure. Although no significant differences were observed in the TGF-β production, VOB treatment positively influenced the production of TGF-β after 2, 7 and 14 days.

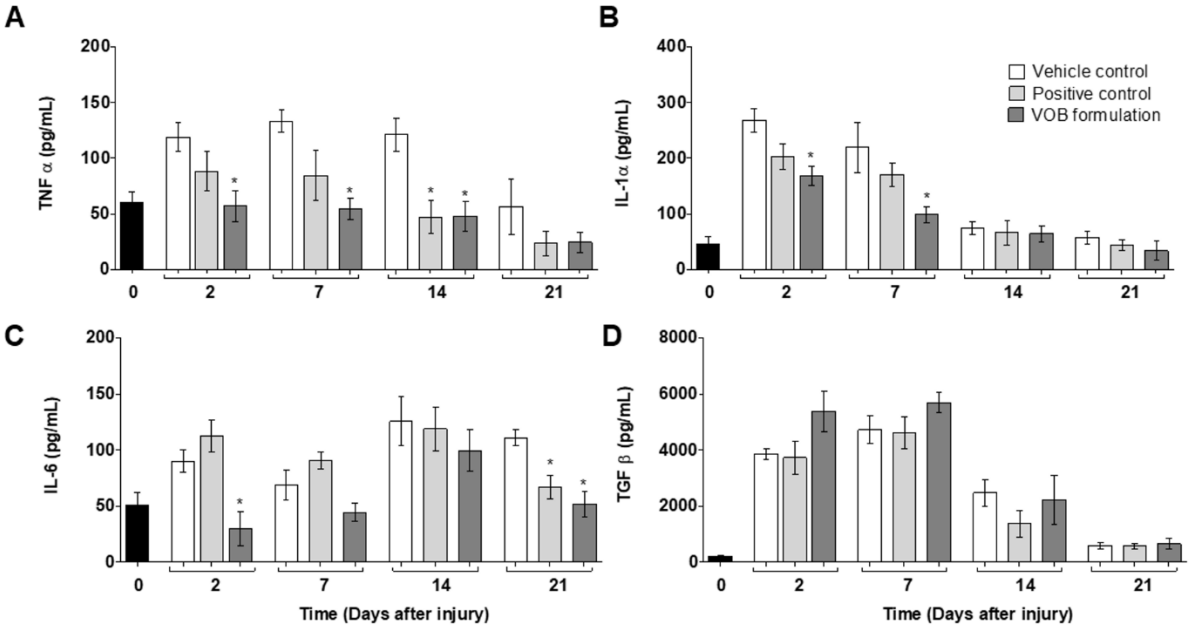

Figure 5. VOB modulates cytokine production in the skin wound biopsies. Tissue homogenates were prepared from the wound biopsies obtained from animals treated with the VOB formulation, positive control or vehicle control at days 0, 2, 7, 14 and 21 post wounding. (A) TNF-α, (B) IL-1 α, (C) IL-6, (D) TGF-β were assayed by ELISA. Data are means ± SEM (n=10 wounds/group). * $p<0.05$ compared to vehicle control group.

3.6 Collagenesis

The synthesis, degradation and deposition of collagen in the injury site is considered an important step in the proper wound healing process. Throughout the experimental period, the intense production of collagen and the formation of new tissue at the wound site was observed, especially in the group treated with the VOB (Figure 6). At days 2, 7 and 14 post-injury, the collagen production in the VOB-treated group was significantly higher compared to the vehicle-control group, whereas after 21 days, the collagen concentration was significantly lower ($p<0.05$). No significant effects on the collagen amount were observed after wound treatment

with the positive control. Moreover, the VOB-treated wounds elicited more organized and dense collagen fibres compared to the wounds treated with the positive control or the vehicle control (Figure 6A).

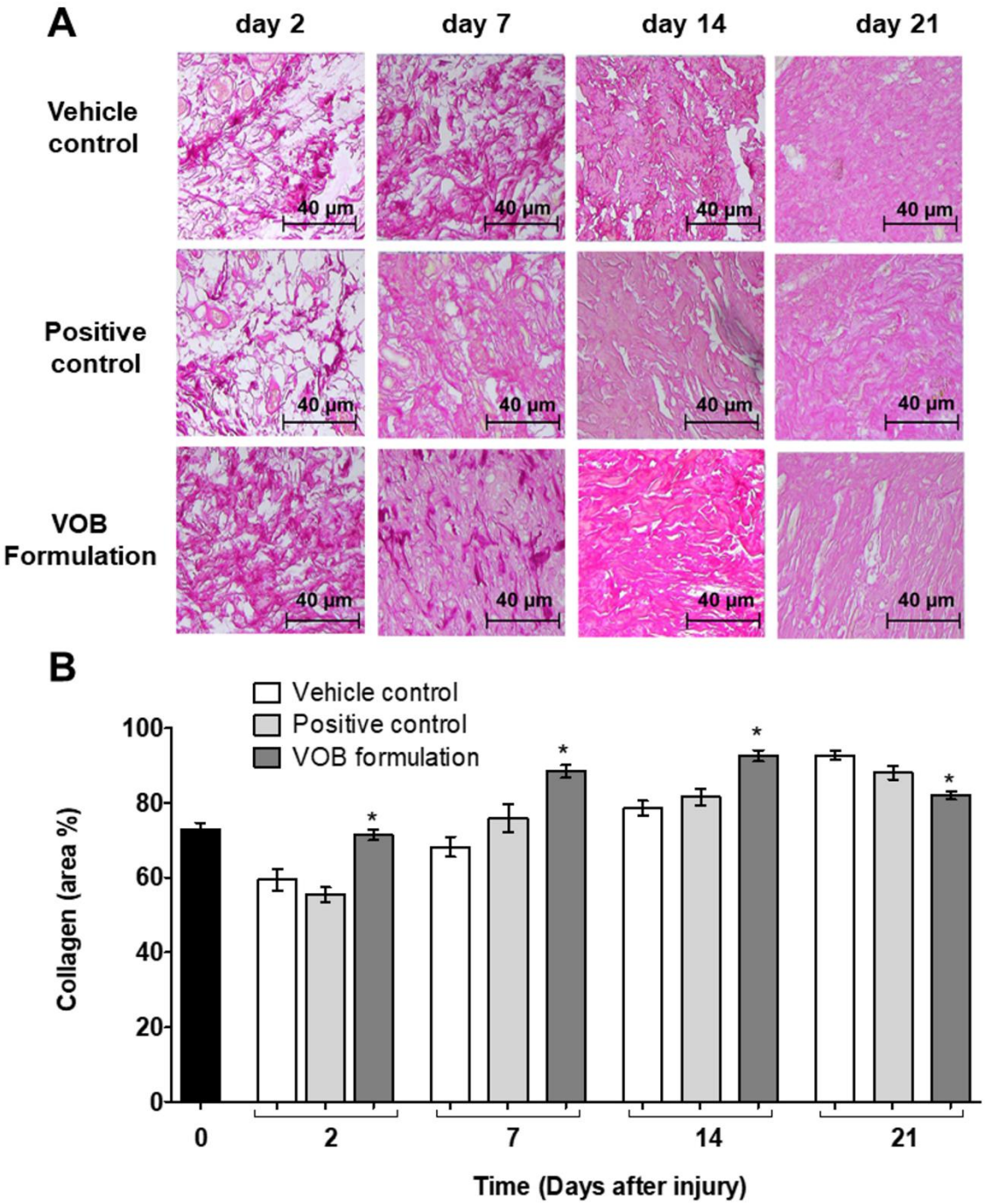

Figure 6. Effects of VOB formulation on the collagen content in the wound tissue biopsies at days 0, 2, 7, 14, and 21 after injury. (**A**) Representative photomicrograph of wound

tissue sections stained with Picrosirius red (100x). (**B**) Collagen content determined by digital densitometry using CellProfiler (CP) software. Data represent the means ± SEM (n=20 wounds/group), *$p<0.05$ compared to the vehicle control group.

4. Discussion

Wound healing is a complex and well-coordinated process involving different cell types and interactions with soluble mediators, growth factors and cytokines to build up the repair of the injured tissue. Poor wound healing after trauma, surgery, or chronic disease conditions affects millions of people each year and is considered a challenge to healthcare systems worldwide (Pieper and Caliri, 2003). The use of traditional therapies for skin wound care has greatly increased over the last decade. Vegetable oils, which are natural sources of fatty acids, have always been an effective, low-cost alternative for the treatment of skin wounds. However, the distinct lipid profile of each isolated vegetable oil does not produce a broad effect throughout the healing process. Therefore, the VOB formulation developed here, which has a unique fatty acid profile with very high levels of monounsaturated (35.27%) and polyunsaturated fatty acids (42.1%), demonstrated the provision of a broad spectrum of action in all phases of the healing process, contributing to the adequate healing of the skin.

Polyunsaturated fatty acids (PUFAs), in addition to their structural function, can modulate cell-cell interactions and intracellular signalling. Thus, altering the fatty acid composition of membrane phospholipids can modulate their fluidity by modifying the binding of cytokines to their receptors (Hatanaka and Curi, 2007; Maver et al., 2015). In addition, PUFAs are primary precursors of important lipid mediators of the inflammatory process, such as arachidonic acid, prostaglandins, thromboxanes and leukotrienes. Previous reports have shown that increasing the availability of n-3 polyunsaturated fatty acids results in a decreased proportion of arachidonic acid (20:4n-6) and an increased proportion of n-3 fatty acids in immune cell phospholipids, including neutrophils, monocytes, T lymphocytes and B lymphocytes (Calder and Grimble, 2002). Eicosanoids are also involved in modulating the intensity and duration of inflammatory and immune responses. The effects of PGE_2 and LTB_4 have been widely studied, demonstrating that PGE_2 has many pro-inflammatory effects that increase the vascular permeability and vasodilation; suppress lymphocyte proliferation and

natural killer cell activity; and inhibit the production of tumour necrosis factor (TNF-α), interleukin-1 (IL-1), and IL-6 (Calder and Grimble, 2002; Schreml et al., 2010).

During the healing process, excessive production of cytokines by inflammatory cells can lead to tissue injury and cell death. Polyunsaturated fatty acids can trigger the proper activation of these cells and therefore contribute to the healing process (Alam et al., 2017). Therefore, the present results suggests that the VOB formulation may modulate the inflammatory response by inhibiting the chemotaxis of inflammatory cells and controlling the production and release of pro-inflammatory cytokines, especially IL-1, IL-6 and TNF-α, in the wound site and could therefore control the degree and duration of the inflammatory response, contributing to successful wound closure.

Studies have shown that growth factors, especially TGF-β, play a key role in regulating the repair process of damaged tissues, affecting several cells involved in wound healing (Ghatak et al., 2017; Meyer et al., 2017). However, the production of TGF-β is vital for the control of the production of extracellular matrix components, and the massive and constant presence of TGF-β at the wound site can interfere with matrix deposition, in which case it does not provide benefits for healing and may even produce hypertrophic scarring (Cheng et al., 2017). Therefore, the VOB was shown to be effective in controlling the concentration of TGF-β, in modulating the presence of this growth factor in the initial and final stages, and in promoting an organized re-epithelization of the tissue formation and avoiding the formation of atrophic scars.

The proliferative and remodelling phases are important for full tissue recovery. During these phases, there is a large, gradual deposition of collagen newly synthesized by fibroblasts and keratinocytes (Miron et al., 2017). Collagen is the most important protein in the connective tissue that forms the skin, and proper healing depends directly on the process of the production, regulation and deposition of this protein (Abdullah et al., 2017; Miron et al., 2017). Therefore, the production of well-organized collagen fibres observed in the VOB group compared to the positive control and vehicle control groups is due to the VOB's ability to promote the proliferation and migration of responsive cells by the production of this protein.

5. Conclusion

In conclusion, under *in vivo* experimental conditions, VOB accelerates the healing of wounds and promotes a rapid and controlled remodelling of the skin, contributing to the formation of an aesthetically acceptable scar. VOB prevents the overexpression of the

inflammatory phase by decreasing the release of pro-inflammatory cytokines, thereby reducing migration of polymorphonuclear cells to the wound site and promoting proper deposition of the extracellular matrix. In this context, the developed VOB formulation may be a promising and economically viable option for a topical application for wound healing and invasive aesthetic procedures.

Conflict of interest

The authors disclosed no conflicts of interest

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CONCLUSÕES GERAIS

Este trabalho especifica uma nova formulação de origem vegetal, de baixo custo, a base de seis óleos vegetais, estabilizada em um sistema tipo "gel", o que permite uma melhor aplicabilidade sobre a pele lesada, altamente estável com comprovada eficácia na cicatrização de feridas. Todos os ingredientes que fazem parte da formulação são de origem vegetal, sem adição de qualquer insumo quimicamente modificada, o que permite que este produto se encaixe nas normas de Ecocertificação. Segundo as normas dos Órgãos Regulatórios Brasileiros VOB se encaixa também no perfil de cosmético grau 2, sendo uma opção interessante para aplicações secundárias tais como: hidratante, anti-eritematoso, calmante para pele, preventivo da formação de estrias, dentro outros. Nossos estudos *in vitro* e *in* vivo demonstraram ainda um promissor efeito antioxidante e bacteriostático, um potente efeito anti-inflamatório quando comparado ao placebo e ao produto similar disponível no mercado. Devido a seu perfil graxo único, rico em ácidos graxos poli-insaturados, VOB demonstra efeito positivo no reparo específico do tecido dérmico, o que se torna promissor seu uso em tratamentos estéticos da pele. Porém mais estudos devem ser conduzidos no sentido de aprofundar o conhecimento do uso de óleos vegetais em tratamento de feridas cutâneas.

ANEXOS

Anexo 1- Parecer CEUA

Universidade Vila Velha

Comissão de Ética, Bioética e Bem Estar Animal (CEUA-UVV)

PARECER DO RELATOR

Parecer Nº381 /2016

Pesquisador (a) Responsável: Dr. Márcio Fronza

Tipo de Pesquisa: **ESTUDO DE PRÉ-FORMULAÇÃO E ATIVIDADE CICATRIZANTE DE ÓLEOS VEGETAIS EM ÚLCERAS CUTÂNEAS EM RATOS**.

Instituição onde será desenvolvido: Laboratório de Pesquisa "19", Campus Nossa Senhora da Penha- Biopráticas- UVV

Situação: **APROVADO**

Ao analisar o projeto de pesquisa: **"ESTUDO DE PRÉ-FORMULAÇÃO E ATIVIDADE CICATRIZANTE DE ÓLEOS VEGETAIS EM ÚLCERAS CUTÂNEAS EM RATOS"**, tendo como pesquisador(a) responsável **Prof. Dr. Márcio Fronza**, que irá utilizar a(s) seguinte (s) espécie (s) animal (s) **camundongo heterogênico linhagem Swiss**, totalizando **60 (sessenta)** do gênero **masculino**, esta CEUA-UVV considera que o projeto se encontra adequado e satisfatoriamente de acordo com as exigências das Resoluções que regem esta Comissão e ao CONCEA. Assim, mediante a importância social e científica que o projeto apresenta, a sua aplicabilidade e conformidade com os requisitos éticos, sou de parecer favorável à realização do projeto classificando-o como **APROVADO**, pois o mesmo **atende** aos Requisitos Fundamentais da Normas de Conduta para a Utilização de Animais no Ensino, Pesquisa e Extensão na Universidade Vila Velha.

Vila Velha , **19 de outubro de 2016.**

Prof. João Luiz Rossi Junior

Relator da CEUA-UVV.

Universidade Vila Velha

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Anexo 2 - Comprovante de submissão manuscrito 1

Submitted Manuscripts

STATUS	ID	TITLE	CREATED	SUBMITTED
ADM: Admin, BJPS • Awaiting EIC Decision • Awaiting Reviewer Selection	BJPS-2018-0033	The in vitro biological effects of a vegetable oil blend for pharmaceutical applications in skin care View Submission	12-Jan-2018	23-Jan-2018