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LUCAS MALVEZZI DE MACEDO

FORMULAÇÃO TÓPICA CONTENDO EXTRATO DE *ROSMARINUS OFFICINALIS* (ALECRIM) PARA CICATRIZAÇÃO DE LESÕES CUTÂNEAS

TOPICAL FORMULATION CONTAINING *ROSMARINUS OFFICINALIS* (ROSEMARY) EXTRACT FOR WOUND HEALING

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Ciências, Área de Concentração em Pesquisa Clínica

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Take my hand, stay, Joanne
Heaven's not ready for you
Every part of my aching heart
Needs you more than the angels do

If you could I know that you'd stay
We both know things don't work that way
I promised I wouldn't say goodbye
So I grin and my voice gets thin
(Lady Gaga "Joanne")

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RESUMO

Rosmarinus officinalis L., popularmente conhecido como alecrim, é uma planta da família das Lamiaceae. As propriedades terapêuticas de *R. officinalis* têm sido usadas na medicina popular em preparações orais para aliviar cólicas renais, dismenorreia e como antiespasmódico. Ao alecrim, são atribuídas diversas propriedades como: antifúngica, antiviral, antibacteriana, anti-inflamatória, antitumoral, antitrombótica, antinociceptiva, antidepressiva, antiulcerogênica e antioxidante. A atividade antioxidante mais importante dos extratos é atribuída ao ácido rosmarínico, ácido carnósico e carnosol. Utilizar uma planta de fácil acesso como fonte de compostos antioxidantes naturais é uma estratégia interessante econômica e farmacologicamente. O objetivo deste estudo foi o desenvolvimento de uma emulsão óleo em água com imcorporação do extratod e alecrim com finalidade cicatrizante. Para tal, as folhas do alecrim foram submetidas a quatro métodos extrativos em etanol 100%: maceração, infusão, soxhlet e ultrassom. O extrato de infusão apresentou os melhores resultados para DPPH chegando a atingir 100% de inibição na concentração de 10 mg/mL, FRAP com 111,94 ± 4,26 mg de EAG/g, e para a quatificação de compostos fenólicos e taninos foram de 52,50 ± 2,75 mg de EAG/g e 74,47 ± 5,73 de EAT/L, respectivamente, enquanto que para a quantificação de flavonoides a amostra com melhor resultado foi a maceração com 72,38 ± 3,84 mg de EQ/g. Os ensaios de viabilidade celular foram realizados pelo método de MTT, como resultado as amostras na concentração de 6,25-100 µg/mL apresentaram viabilidade ≤80%. O ensaio de viabilidade celular mostrou que os extratos em concentrações de 6,25-100 µg/mL não apresentaram potencial citotóxico. Os extratos que apresentaram efeito contra uma quantidade maior de cepas no teste de difusão de discos foram infusão e ultrassom e o teste de concentração inibitória mínima utilizou essas duas amostras apresentando inibição, respectivamente, frente a *Staphylococcus aureus* (≥1,5 e ≥3 mg/mL), *Streptococcus oralis* (≥6 e ≥12,5 mg/mL), *Escherichia coli* (não teve efeito e ≥25 mg/mL) e *Pseudomonas aeruginosa* (≥6 e ≥12,5 mg/mL). O teste de estabilidade seguiu o prescrito pelo Guia de Estabilidade para Produtos Cosméticos da ANVISA, e como resultado apresentou ausência da separação de fase, o pH somente apresentou aumento para a emulsão branca, e a densidade e viscosidade para todas as condições e para ambas formulações apresentaram aumento significativo ao longo de 90 dias. As análises no têxturometro apontaram para uma menor espalhabilidade para a emulsão com acréscimo do extrato, principalmente. A avaliação sensorial do produto resultou em uma aceitação de 84% dos voluntários de uma nota geral de 4,08. Com base nos resultados , conclui-se que o extrato de alecrim pode ser usado em uma emulsão como promissor ativo cicatrizante devido sua elevada atividade antioxidante e antimicrobiana.

Palavras chave: extração; antioxidante; atividade bacteriana; viabilidade celular; formulação; estabilidade

ABSTRACT

Rosmarinus officinalis L. known as rosemary is a plant of Lamiaceae family. Therapeutic properties of *R. officinalis* have been used in folk medicine in oral preparations to relieve renal colic, dysmenorrhea and as antispasmodic. Rosemary shows properties such as: antifungal, antiviral, antibacterial, anti-inflammatory, antitumor, antithrombotic, antinociceptive, antidepressant, antiulcerogenic and antioxidant. Activity of the extracts is attributed to rosmarinic acid, carnosic acid and carnosol. Employing an accessible plant which is easily grown, as a source of natural antioxidant compounds can be interesting economically and pharmacologically. The aim of this study was evaluated antioxidant, microbiological and cell viability activities, and quantify phenols, tannins and flavonoids in four rosemary extracts in order to incorporate them in a topical emulsion for wound healing. Rosemary leaves were subjected to extractive methods in 100% ethanol: maceration, infusion, soxhlet and ultrasound. The infusion extract showed the best results for DPPH reaching 100% inhibition in the concentration of 10 mg/mL, FRAP with 111.94 ± 4.26 mg GAE/g, and for quantification of phenolic compounds and tannins were 52.50 ± 2.75 mg GAE/g e 74.47 ± 5.73 TAE/L, respectively, while for flavonoids quantification the sample with best results was maceration with 72.38 ± 3.84 mg de QE/g. Cell viability test were performed using the MTT method, as result samples at concentration of 6.25-100 µg/mL showed ≤80% of viability. The extracts that showed effect against a greater number of strains in diffusion discs test were infusion and ultrasound and the minimum inhibitory concentration evaluation used these two samples showing inhibition, respectively, against *Staphylococcus aureus* (≥1,5 e ≥3 mg/mL), *Streptococcus oralis* (≥6 e ≥12,5 mg/mL), *Escherichia coli* (non-effect e ≥25 mg/mL) and *Pseudomonas aeruginosa* (≥6 e ≥12,5 mg/mL). The stability teste followed the prescribed by ANVISA Guia de Estabilidade para Produtos Cosméticos, ad as result it showed no phase separation, the pH only changed for white emulsion, and density and viscosity for all conditions and both emulsions showed significant increase over 90 days. The texturometer analyses pointed to lower spreadability for emulsion with extract addition, mainly. The sensory evaluation od product resulted in an 84% acceptance by volunteers and an overall score of 4.08. Based on the results, it is possible concluded that the rosemary extract can be used in an emulsion as healing active due to its high antioxidant and antimicrobial activities.

Key words: extraction; antioxidant; bacterial activity; cell viability; formulation; stability

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LISTA DE ABREVIATURAS E SIGLAS

% - Porcentagem

°C - Graus Celsius

$AlCl_3$ - Cloreto de alumínio

ANVISA - Agência Nacional de Vigilância Sanitária

cP - Centipoise

$FeCl_3$ - Cloreto férrico

CEP - Comitê de Ética em Humanos

DPPH - 1,1-difenil-2-picrilhidrasil

EAG - Equivalentes de ácido gálico

EAT - Equivalentes de ácido tânico

EC_{50} - Concentração efetiva a 50%

EQ - Equivalentes de quercetina

FRAP - Poder de redução do íon ferro

g - Grama

h - Hora

HCl - Ácido clorídrico

HaCaT - Queratinócitos humanos imortalizados

IBD - Associação de Certificação Instituto Biodinâmico

IC_{50} - Concentração inibitória média

INF - Infusão

INT - *2-(4-iodophenyl)-3- (4-nitrophenyl)-5-phenyltetrazolium chloride*

L - Litro

MAC - Maceração

MIC - Concentração inibitória mínima

min - Minuto

mg - Miligrama

mL - Mililitro

mM - Milimolar

mm - Micrômetro

MTT - (3-bromide (4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-tetrazolium)

N - Normal

nm - Nanômetro

ROS - Espécies reativas de oxigênio (ROS)°

rpm - Rotações por minuto

SisGen - Sistema Nacional de Gestão do Patrimônio Genético

SOX - Soxhlet

TPTZ - 2,4,6- tripiridil-s-triazina

UFC - Unidades formadoras de colônia

ULT - Ultrassom

v/v - volume/volume

µg - Micrograma

µL - Microlitro

µm - Micrometro

SUMÁRIO

1 INTRODUÇÃO

Rosmarinus officinalis L., popularmente conhecida como alecrim, é uma planta da família das Lamiaceae. Trata-se de uma planta aromática, de folhas perenes e pontiagudas e cultivada no mundo todo. O alecrim possui propriedades terapêuticas e tem sido usado na medicina popular em preparações orais para aliviar cólicas renais, dismenorreia e como antiespasmódico (al-Sereiti, Abu-Amer et al. 1999, Begum, S et al. 2013, Ribeiro-Santos, Carvalho-Costa et al. 2015). Ao alecrim, são atribuídas diversas propriedades como: antifúngica, antiviral, antibacteriana, anti-inflamatória, antitumoral, antitrombótica, antinociceptiva, antidepressiva, antiulcerogênica e antioxidante (Begum, S et al. 2013, Ojeda-Sana, van Baren et al. 2013, Ribeiro-Santos, Carvalho-Costa et al. 2015).

A atividade antimicrobiana é devido a ação dos metabólitos secundários encontrados no *Rosmarinus officinalis* agem diretamente na célula bacteriana, essa ação é devido a sinergia entre os compostos bioativos, como apontado por Moreno, Scheyer et al. (2006) e Ivanovic, Misic et al. (2012) que mostraram a ação conjunta do fenol ácido rosmarínico e diterpeno ácido carnósico. A atividade antimicrobiana do extrato do alecrim vem sendo estudada em alimentos (Fernández-López, Zhi et al. 2005, Govaris, Florou-Paneri et al. 2007, Gómez-Estaca, López de Lacey et al. 2010).

Em contrapartida o poder antioxidante dos compostos do alecrim tem recebido pouca atenção (Loussouarn, Krieger-Liszkay et al. 2017). Entretanto, alguns estudos afirmam que seus extratos apresentam alta atividade antioxidante (Robards, Prenzler et al. 1999, González-Trujano, Peña et al. 2007, Begum, S et al. 2013, Kheiria, Sotomayor et al. 2013).

A atividade antioxidante mais importante dos extratos de alecrim é atribuída ao ácido rosmarínico, ácido carnósico e carnosol. A área geográfica em que o alecrim cresce influencia na quantidade de ácido carnósico e rosmarínico presente no extrato, levando também a uma variação na sua atividade antioxidante (Kheiria, Sotomayor et al. 2013).

Os antioxidantes naturais podem agir sinergicamente no combate aos radicais livres, inibindo a geração de espécies reativas de oxigênio (ROS) e neutralizando os radicais livres (Hyun, Shrestha et al. 2015). As espécies reativas de oxigênio estão envolvidas em um grande número de doenças degenerativas tais como câncer, diabetes, cirrose, aterosclerose e também no processo de cicatrização (Aboutwerat, Pemberton et al. 2003, Pattanayak, Nayak et al. 2011).

Cicatrização é um processo dinâmico complexo que resulta na restauração da continuidade e função anatômica da pele; considerada um processo biológico regular do corpo, uma vez que a pele tem a habilidade natural de promover sua regeneração. A cicatrização depende de muitos

fatores e pode ser comprometida sob certas condições como pacientes com diabetes ou queimaduras extensas e de elevado grau (Lazarus, Cooper et al. 1994, Beldon 2010, Groeber, Holeiter et al. 2011, Pereira and Bártolo 2016).

Os radicais livres, formados durante o processo de cicatrização, tem efeitos fisiológicos na migração celular, angiogênese e no combate aos microrganismos que se alojam na ferida (Sen 2009). No entanto, quando a concentração de radicais livres se torna alta causando danos às células e tecidos vizinhos à ferida (Houghton, Hylands et al. 2005).

Os antioxidantes de origem natural poderiam ser incorporados em uma formulação de uso tópico com finalidade de neutralização dessas espécies reativas danosas às células localmente. A aplicação tópica é vantajosa, já que não há efeito de primeira passagem, minimizando chances de efeitos colaterais sistêmicos e interações medicamentosas, além de não exigir um monitoramento laboratorial durante o tratamento (Havlickova and Friedrich 2008).

Ainda existem poucas referências na literatura sobre a incorporação do extrato de alecrim em formulações para uso tópico. Dessa forma, o presente trabalho pretende a incorporação do extrato de alecrim contendo ácido carnósico e ácido rosmarínico em formulações com finalidade tópica, a fim de melhorar a cicatrização de feridas.

O presente trabalho apresenta revisão de literatura entitulada “Rosemary (*Rosmarinus officinalis* l., syn *Salvia rosmarinus* Spenn.) And its topical application: a review” (De Macedo, L. M.; Santos, E. M.; Militão, L.; Tundisi, L. L.; Ataide, J. A.; Souto, E. B.; Mazzola, P. G.). Plants, v. 9, p. 651, 2020. DOI: https://doi.org/10.3390/plants9050651). Seguido pelo artigo de resultados intitulado “Development and evaluation of formulation (oil-in-water) containing *Rosmarinus officinalis* extract for wound healing effect” (De Macedo, L. M; Santos, E. M; Ataide, J. A.; Jozala, A. F.; Lancellotti, M.; Mazzola, P. G., a ser submetido em periódico). Por último os itens e subitens utilizados tradicionalmente em dissertações. As referências do artigo de revisão e do artigo de resultados encontram-se ao final dos respectivos capítulos nos formatos exigidos pelas revistas. As demais referências encontram-se no *item 9*, ao final do trabalho.

2 OBJETIVO

2.1 Objetivo Geral

Obter extrato de alecrim e a elaborar formulação de uso tópico – emulsão óleo em água (O/A), contendo ácidos carnósico e rosmárico e a avaliação de seu efeito cicatrizante em lesões tópicas.

2.2 Objetivos específicos

- Preparar extratos a partir de folhas de alecrim por métodos de extração por ultrassom, maceração, soxhlet e infusão.
- Avaliar a atividade antioxidante, antimicrobiana e de viabilidade celular, *in vitro*, dos extratos de alecrim.
- Desenvolvimento e avaliação da estabilidade preliminar e acelerada de formulações água em óleo
- Avaliação de formulações em Texturômetro
- Análise sensorial

3 Capítulo I - Revisão de Literatura

"ROSEMARY (*ROSMARINUS OFFICINALIS* L., SYN *SALVIA ROSMARINUS* SPENN.) AND ITS TOPICAL APPLICATION: A REVIEW"

Lucas Malvezzi de Macedo, Érica Mendes dos Santos, Lucas Militão, Louise Lacalendola Tundisi, Janaína Artem Ataíde, Eliana Barbosa Souto and Priscila Gava Mazzola

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Abstract: Topical application is an important administration route for drugs requiring local action on the skin, thereby avoiding their systemic absorption and adverse side effects. *Rosmarinus officinalis* L.(syn. *Salvia rosmarinus* Spenn.), popularly known as rosemary, is an aromatic plant with needle-likeleaves belonging to the *Lamiaceae* family. Rosemary has therapeutic properties and has been used in the folk medicine, pharmaceutical, and cosmetics industries, mainly for its antioxidant and anti-inflammatory properties, which are attributed to the presence of carnosol/carnosic and ursolic acids. The therapeutic use of rosemary has been explored for the treatment of inflammatory diseases; however, other uses have been studied, such as wound healing and skin cancer and mycoses treatments, among others. Besides it therapeutic uses, rosemary has potential applications in cosmetic formulations and in the treatment of pathological and non-pathological conditions, such as cellulite, alopecia, ultraviolet damage, and aging. This review aims to critically discuss the topical applications of rosemary found in the literature while also offering relevant information for the development of topical formulations of its bioactive compounds.

Keywords: rosemary; antioxidant; anti-inflammatory; flavonoids; polyphenols; terpenes

Introduction

The use of herbal drugs to treat a broad spectrum of diseases and/or to modify non-pathological states [1-4] has increased worldwide. It is known that the secondary metabolites of plants have therapeutic effects; many have been used in the treatment of different diseases, such as obesity [5] and brain [6] and skin diseases [7] as well as in the treatment of non-pathological states, such as aging [8].

Rosmarinus officinalis L., commonly known as rosemary, belongs to the Lamiaceae family. The genus Rosmarinus has been merged into the genus Salvia in a recent phylogenetic analysis. This means that the *Rosmarinus officinalis* is no longer the correct name of the species studied. Since the name Salvia officinalis was already occupied when the merger was done, this species needed a new specific epithet in Salvia, so it is now known under the name *Salvia Rosmarinus* [9-11]. It is an aromatic plant with needle-like leaves that is cultivated

worldwide. Rosemary has therapeutic properties and has been used in folk medicine as an oral preparation to relieve renal colic, dysmenorrhea, and muscle spasms [12-14]. Rosemary has antifungal, antiviral, antibacterial, anti-inflammatory, antitumor, antithrombotic, antinociceptive, antidepressant, antiulcerogenic, and antioxidant activities [13-15]. Several medicinal applications for *R. officinalis* have been identified, such as treatment of disorders associated with the nervous, cardiovascular, gastrointestinal, genitourinary, menstrual, hepatic, and reproductive systems and with respiratory and skin conditions [13]. Owing to its diverse properties, rosemary has also been used widely in the food and cosmetics industries [16].

Many biomolecules have been identified to be responsible for the biological effects of rosemary essential oil and crude extract. However, specific compounds causing these effects have rarely been identified; this is due to the synergistic actions of several metabolites present in rosemary [17]. Therefore, it is difficult to associate a therapeutic or cosmetic activity with an isolatedbiomolecule. del Baño et al. characterized the distribution of rosemary flavonoids (eriocitrin, luteolin 3'-O-β-D-glucuronide, hesperidin, diosmin, isoscutellarein 7-O-glucoside, hispidulin 7-O-glucoside, and genkwanin) in the leaves, flowers, roots, and stems during different stages of the plant's growth [18].It was also reported a high concentration of flavonoids, polyphenols, and terpenes in *R. officinalis* leaves [19]. Rosemary contains an abundance of secondary metabolites, and their identification by ultra- and high-performance liquid chromatography and gas chromatography has revealed high contents of profile phenolic compounds (diterpenoids and flavonoids) and volatile compounds [20,21].

The biological activities of secondary metabolites and extracts of *R. officinalis* were reported in studies investigating various effects such as its antitumor, antioxidant, anti-infectious, anti-inflammatory, and analgesic activities and effects on the central nervous system, endocrine system, disorders such as cardiac remodeling after myocardial infarction, body weight changes, dyslipidemia, cerebral ischemia, hepato-nephrotoxicity, stress, and anxiety [22,23]. The anti-inflammatory activity of rosemary has been attributed to the presence of carnosol and carnosic, rosmarinic, ursolic, oleanolic, and micromeric acids, which act synergistically [24-26]. Specifically, the anti-inflammatory effect was also attributed to the synergic effects of ursolic and micromeric acids present in rosemary extract. The attribution of anti-inflammatory effects of the *R. officinalis* extract was due to the presence of ursolic, oleanolic, and micromeric acid acting in combination [24].

The skin is the largest organ in the human body; sensation, regulation, and protection are amongits most critical functions [27]. To enhance the permeation of bioactive compounds

through the skin, many approaches have been proposed. Of these approaches, nanocarriers including nanoemulsions, lipid nanoparticles, and liposomes have become popular owing to their lipid composition, enhanced biocompatibility, and biodegradability [28-32]. The release profile of the loaded bioactive compoundcan be modulated by altering the physicochemical composition of the nanocarrier matrix [33].

The aim of this review was to survey the publications related to the topical applications of rosemary and to discuss the formulations available for the delivery of the secondary metabolites of *R. officinalis*.

Methods

The Web of Science, Google Scholar, and SciELO databases were selected for research on the topical uses of rosemary, using "*Rosmarinus officinalis*" and "rosemary" as keywords. The authors areaware of the nomenclature update; however, literature still shows *Rosmarinus officinalis* as the main name for rosemary.

Pharmaceutical Activities

Rosemary (Table 1) has attracted attention because it contains secondary metabolites with therapeutic potential, such as carnosol and carnosic, rosmarinic, ursolic, oleanolic, and micromeric acids (Figure 1). These compounds have been applied topically and studied for their anti-inflammatory capacity, wound-healing potential, effects on tissue survival, anti-skin-cancer effects, antinociceptive effects, antifungal effects, and UV-protective activity. The triterpenes ursolic, oleanolic, and micromeric acids exhibit the strongest anti-inflammatory activity of all the secondary metabolites [24]. In additionto the gross extracts, it is possible to use rosemary essential oil for topical applications [32]. The majorconstituents of the oil are β-pinene, 1, 8-cineole, borneol, camphor, limonene, and verbenone [34].

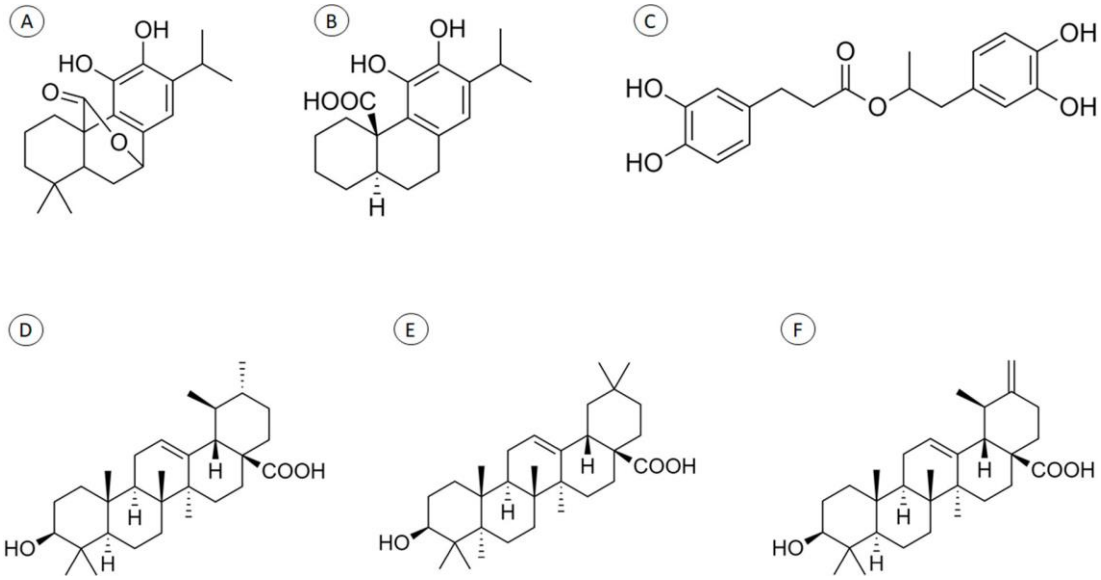

Figure 1. Chemical structure of some Rosmarinus officinalis secondary metabolites: carnosol (**A**), carnosic acid (**B**), rosmarinic acid (**C**), ursolic acid (**D**), oleanolic acid (**E**), and micromeric acid (**F**).

Table 1. Results collected about *Rosmarinus officinalis* uses.

Topic	Results	Reference
Anti-inflammatory activity	A. Carnosic acid inhibit NO; B. Carnosic acid platelet inhibition; C. Carnosol reduce atopic dermatitis; D. Rosemary extract showed anti-inflammatory activity similar to indometacin	[1-6]
Skin cancer	A. Rosemary extract reduces number, weight and incidence of tumors, and an increase in the latency period; B. Rosmarinic acid showed chemoprotective activity; C. Carnosic acid showed protective	[7-10]

	effect against melanoma	
Wound healing	A. Rosemary oil showed healing, angiogenesis and improvements in granulation tissue; B. Rosemary oil accelerate wound healing in diabetic and non-diabetic animals; C. Rosemary cream accelerate wound healing	[11-13]
Skin flaps survival	A. Rosemary oil showed improvement in tissue survival and viability, and tissue necrosis was lower	[14]
Transdermal effects	A. Monoterpenes, presented in rosemary oil, promoted cutaneous absorption	[15]
Antifungal activity	A. Rosemary oil was capable to inhibit *C. albicans* growth; B. Rosemary extract was responsible for inhibit fungal growth	[16,17]
Ginoid lipodystrophy (GLD, cellulite)	A. A cream with carnosic acid was responsible for an improvement of cellulites appearance	[2]
Alopecia	A. Rosemary extract showed hair growth	[18]
Anti-aging	A. Rosm1 has a strong antioxidant capacity; B. Rosemary essential oil nanoparticles showed great	[19,20]

	capacity of hydration and improve the elasticity	
Ultraviolet protection	A. Rosemary and citrus extract were able to improve cell protection against UV; B. Rosemary extract reduce skin damage caused by sun	[21,22]
Other studies	A. A secondary metabolite present in rosemary extract showed stabilizer emulsion properties	[23]

Anti-inflammatory Activity

The inflammatory activity of *R. officinalis* extract is attributed to the presence of carnosol and carnosic acid [53] and of ursolic, oleanolic, and micromeric acids [24].

The bioactive compound carnosic acid was reported to be a potent nitric oxide (NO) inhibitor; NO is a pro-inflammatory mediator that induces or enhances the inflammatory process [35]. Low concentrations of this metabolite (6.2 µg/mL) inhibited NO by approximately 72%, whereas complete inhibition was reported at concentrations of >12.5 µg/mL [36]. Using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, the *R. officinalis* extract was shown to possess strong antioxidant activity, supporting its potential as an anti-inflammatory agent. The extract also exhibited antiplatelet activity, which is instrumental for the improvement of microcirculation. The maximum platelet inhibition occurred at a carnosic acid concentration of 31 µg/mL [36].

Mice with atopic dermatitis that were topically treated with carnosol showed a significant reduction in skin lesions compared with the control animals [26]. Atopic dermatitis is a chronic inflammation of the skin characterized by the presence of eczematous and pruritic lesions related to several factors, such as inflammatory cells, cytokines, and enzymes (e.g., STAT3, iNOS, and COX-2) [37]. The lymph node weight and size were significantly increased, which is associated with a high immunoglobulin E production [38]. Carnosol was able to reduce the

amounts of immunoglobulin E, neutrophils, and inflammatory cytokines (TNF-α and IL-1β) in the blood of mice. Histological sections of the ear and dorsum showed that the skin of the animals treated with carnosol was thinner and showed less infiltration of inflammatory cells and fewer mast cells compared with that of mice in the control group. Carnosol was able to reduce the expression of the enzymes iNOS and COX-2. Although it did not affect the expression of STAT3, the metabolite was able to inhibit the activity of this enzyme in *in vitro* assays; different concentrations of carnosol (1.2 and 5 µM) reduced NO production in LPS-treated RAW 264.7 cells [26].

R. officinalis did not show significant anti-inflammatory effects in induced dermatitis. In a study, the effect of extracts of marigold and rosemary on irritant contact dermatitis induced by sodium lauryl sulfate was evaluated in healthy human volunteers. Both extracts were incorporated into the base cream DAC (Deutscher Arzneimittel Codex = German Pharmaceutical Codex) at a 5% concentration. The effect of this formulation on irritant contact dermatitis was evaluated visually using bioengineering methods (e.g., chromametry and tewametry). These extracts were shown to have no anti-inflammatory effect on existing dermatitis; however, when applied simultaneously with the irritant, the inflammatory process was reduced, indicating that they exerted a protective effect against the development of induced dermatitis [54].

Evaluation of the topical anti-inflammatory effects of the extracts of rosemary leaves prepared with solvents of increasing polarity in vivo was performed using the croton oil-induced ear edema test. The extract obtained with n-hexane and chloroform exhibited important dose-dependent anti-inflammatory activity, whereas that obtained with methanol had a weak anti-inflammatory effect.

The extracts obtained with chloroform ((ID50 = 83 µg/cm2) and n-hexane (ID50 = 265 µg/cm2) showed an anti-inflammatory activity similar to that of indomethacin (ID_{50} = 93 $\mu g/cm^2$) (used as reference, with a discrete effect) [24].

Skin Cancer

The effects of *R. officinalis* hydroalcoholic extract were tested on human melanoma A375 cells; theextract resulted in a dose-dependent reduction of human melanoma cell proliferation. Mutations in melanocytes are attributed to the excessive exposure of the skin to sunlight and induce the development of melanomas [55-57]. The cell cycle proliferation was inhibited *in vitro* because of the cytotoxic andcytostatic activity of the hydroalcoholic extract [39]. When

a mouse model of skin cancer inducedby 7,12-dimethylbenz[a]anthracene was treated with a 500 or 1000 mg/kg oral dose of *R. officinalis* hydroalcoholic extract for a period of 15 days, it led to a decrease in the number, diameter, weight, and incidence of tumors and an increase in the latency period [40].

Rosmarinic acid was shown to exhibit chemopreventive activity against 7,12-dimethylbenz[a] anthracene-induced skin cancer; this was attributed to its anti-lipid peroxidation potential andits ability to modulate the detoxification cascade and expression patterns of p53, bcl-2, caspase-3, and caspase-9 [41]. Carnosic acid was shown to have an important protective role against melanoma. This secondary metabolite inhibited the proliferation and adhesion of B16F10 melanoma cells in a dose-dependent manner through the inhibition of the expression of cell migration markers (MMP-9, TIMP-1, uPA, and VCAM-1) and phosphorylation of signaling molecules (Akt, FAK, and Sr) [42].

Wound Healing

Healing is a complex dynamic process that results in the restoration of the anatomical barriers of the skin that may have been compromised by diseases or burns [58]. Diabetes was induced in male BALB/c mice by the intraperitoneal injection of alloxan [1]. After confirmation of hyperglycemia,incisions that were 4 mm in diameter were made on the backs of the mice and the mice were allocatedto one of four treatments: control: vehicle, aqueous extract, and essential oil. The male BALB/c mice in the essential oil group showed healing, angiogenesis, and improvements in granulation tissue at several stages compared with those in the aqueous extract group [1]. Another study using diabetic rats topically treated with oil extracted from *R. officinalis* reported accelerated wound healing in bothdiabetic and nondiabetic animals [43].

Another study that explored the healing potential of rosemary was performed on 63 Wistar rats allocated to one of three treatments: control; 2% *R. officinalis* cream; and 4% *R. officinalis* cream. Wounds were made on each rat, and a solution containing *Candida albicans* was applied onto these wounds. The results showed that wound healing in the 4% *R. officinalis* cream group was faster than that in the other groups [44].

Skin Flap Survival

Skin flaps are used in the reconstruction of soft tissues and large wound defects. This techniquehas been employed in plastic surgery, and its efficacy is dependent on the location of the wound andextent of the defect [59,60].

One study compared animals allocated to three treatment groups: group I (control, the

tissue was cleaned only with saline solution); group II (application of the essential oil on the tissue twice per day for 1 week following skin evaluation); and group III (application of rosemary oil before and after evaluation and at the end of the week, 30 min before the surgery). The survival rates were 29%, 59%, and 67% for groups I, II, and III, respectively. Compared with the control (group I), tissue necrosis was significantly lower and tissue viability was significantly higher in groups II and III. Topical application of the oil in the week before the surgery increased the tissue survival rate (higher in group III than in group II). The study reported antioxidant, anti-inflammatory, and vasodilatory activities of the oil as factors for the increased tissue survival [45].

Transdermal Effects

Transdermal drug delivery means that the drug is able to reach systemic circulation when administered topically. Drugs can penetrate the skin through three different pathways: transappendageal (when the drug permeates through hair follicles and through sweat and sebaceous glands); paracellular (when the drug passes through the intercellular space); and transcellular (when the drug passes through cells) [61]. The antinociceptive effects of the essential oil extracted from R. officinalis were analyzed following the incorporation of three concentrations (0.1%, 0.5%, and 1%) of the essential oil in a 1% diclofenac sodium gel. Two in vivo tests, the tail flick tests and formalin test, were performed to compare the antinociceptive effects of the gel base (control), gel with 1% diclofenac, and gel with 1% diclofenac plus 0.1%, 0.5%, and 1% rosemary oil. In both the in vivo assays, the 0.1% and 0.5% concentrations resulted in no significant antinociceptive effects whereas the essential oil concentration of 1% (in the diclofenac sodium gel) resulted in a reduction in pain. The study demonstrated that the essential oil is rich in monoterpenoids, especially 1,8-cineole, which are capable of promoting cutaneous absorption [46].

Antifungal Treatment

Dermatophytes are the most common agents causing topical mycoses [62]. The World Health Organization estimates that 20% of the global population is affected by dermatomycoses [63]; the prevalence of these diseases tends to increase with age and is dependent on the climate and location [64]. *R. officinalis* was reported to be active against dermatophytes in vivo [22].

The antifungal activity of rosemary essential oil was tested against *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, and *Candida krusei* [47]. Such dermatophytes are

the most common agents causing topical mycoses [62]. It was found that an oil concentration of 8% was capable of inhibiting the growth of *Candida* sp. A similar study evaluated the effect of *R. officinalis* hydroalcoholic extract against two dermatophytes, *Microsporum gypseum* and *Trichophyton rubrum*, and showed that a concentration of 10% *R. officinalis* extract was responsible for 86% inhibition of fungal growth [48].

Cosmetic Properties

Ginoid Lipodystrophy (GLD, Cellulite)

Most postadolescent women have cellulite [65,66]. Cellulite is manifested by topographic disorders of subcutaneous tissue, such as nodules, edema, and abnormal fibrosis [67].

It is believed that gynoid lipodystrophy (GLD) is a chronic inflammatory process in which adipocyte malfunction causes a higher content of altered lipids to be retained. This increases the cellular volume and compromises blood circulation and the normal physiological state of pregnancy, after which the mother retains a higher content of lipids to guarantee caloric reserves [68].

A cream with extracts of three plants (*Zanthoxylum clava-herculis* (containing magnoflorine and laurifoline), *Annona squamosa* (containing squamosin and kaurenoic acid), and *Rosmarinus officinalis* (containing carnosic acid)) was examined for its effects on cellulite in a single-blind, randomized controlled study of 44 women between 18 and 59 years or age with mild-to-severe cellulite. The formulation led to an improvement in the appearance of the cellulite [36].

Alopecia

Alopecia is characterized by the loss of some or all hair and is classified as a chronic dermatological disorder [69]. The prevalence of alopecia has increased owing to stress and diet-related factors [70]. Excess testosterone in the blood capillaries is significantly associated with this condition; as such, antiandrogenic agents have been reported to reduce hair loss [49].

C57BL/6 mice with testosterone-induced alopecia were treated topically with hydroalcoholic extracts of rosemary (2 mg/day/animal) and showed a significant increase in hair growth after the 16th day of treatment compared with those in the control [49]. A hydroalcoholic extract was tested *in vitro* for the evaluation of 5αR enzyme activity and showed strong inhibition of the binding of dihydrotestosterone (DHT) to its receptor. An *in vitro* assay

in human prostate LNCaP cells indicatedthat 12-methoxy-sarcosalic acid had a key role in the inhibition of the 5αR enzyme and DHT/receptorbinding [49].

Antiaging

Aging is a skin process that occurs owing to intrinsic and extrinsic factors. Intrinsic factors act at the cellular level, whereas extrinsic factors are governed by human behavior, such as chronic exposure to the sun, poor nutrition, smoking, and excessive alcohol consumption [71]. These internal and external agents lead to the production of reactive oxygen species (ROS) [72]; when ROS levels exceed the cell's neutralization capacity, damage to cell constituents occurs, ultimately leading to celldeath [73].

A new compound was isolated from the hydrophilic fraction of an aqueous methanol extract andwas named Rosm1. This biomolecule had a strong antioxidant capacity to neutralize ROS, similar to vitamin E, and was able to inhibit free radical-mediated reactions *in vivo* and *in vitro*, protecting lipids and cell constituents from oxidative stress [8].

Lipid nanoparticles have been used to increase the cutaneous permeation of drugs [74,75]. These nanoparticles were loaded with *R. officinalis* essential oil incorporated into a gel and an in vivotest was performed to assess the moisturizing ability of this formulation and any increase in elasticity. The gel containing nanoparticles loaded with rosemary oil showed a greater capacity to hydrate andincrease of the elasticity of skin compared with the gel containing free essential oil [50].

Rosmarinic acid was encapsulated within chitosan microparticles. The release profile of the loaded particles was studied in two distinct media: water and coconut oil. A slower release profile observed in coconut oil was attributed to the lower solubility of the chitosan particles [76].

R. officinalis extract has strong antioxidant activity, which is mainly attributed to its phenolic compounds. Antioxidant activity is generally attributed to free radical scavenging, but secondary metabolites may play a biological role in the regulation of apoptosis, cell signal transduction,and xenobiotic metabolism in the liver [77].

Ultraviolet (UV) Protection

UVA radiation induces the production of ROS, and UVB is absorbed by molecules such as DNAand proteins, subsequently damaging the cells [78-80].

Rosemary extract was tested alone and in combination with citrus extract obtained from

grapefruit in vivo and *in vitro* for its protective effects against UV irradiation [4]. In the *in vitro* cell viability measurement using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, keratinocytes (HaCaT cells) were treated with the extracts separately and in combination. Then, the cells were exposed to UV radiation at intensities of 800 and 1200 J/m^2. Both extracts increased the viabilityof the keratinocytes. The effects of a 1:1 mixture of citrus extract and rosemary extract was tested on cells exposed to UV radiation; this mixture showed superior effects on the increase of cell viability compared to those of either extract alone, indicating that the polyphenolic compounds of each extracthave different targets for cell protection [4].

When irradiated with UV at a dose of 800 J/m^2, the citrus and rosemary extracts (50 µg/mL each) showed 40% and 13% protection against UV light, respectively. When combined (100 µg/mL), 70% protection was observed. The synergistic effect was even greater when 1200 J/m^2 of radiation was used. The extracts also had the ability to decrease the formation of free radicals during UV exposure. The study also showed that, when used in combination, the extracts were able to protect DNA from damage caused by the formation of free radicals. Oral administration of the combined citrus and *R. officinalis* extracts over the course of 8 weeks increased the UV dose required to induce erythema inthe skin [4].

When the skin is injured by sun exposure, there is a decrease in type I collagen synthesis and excessive degradation by enzymes called metalloproteinases (MMPs). It was demonstrated that rosemary extract inhibits UV-induced metalloproteinase-1, indicating that it may reduce the skin damage caused by sunlight [51].

Other Studies

The impact of rosmarinic, ursolic, and oleanolic acids on the stability of multiple water/oil/water(W/O/W) emulsions has been studied. These acids did not have any effect on the interfacial tension when used as surfactants but were able to improve the stability of these emulsions for a short periodof time. The authors concluded that rosemary extract contained active compounds with cosmetic potential owing to their various biological activities but that they could also be used as stabilizers, and favored the formation of W/O/W emulsions [52].

Conclusions and Future Perspectives

Rosemary extract contains a large variety of bioactive molecules with great therapeutic potential.These include triterpenes (e.g., ursolic and oleanolic acid), tricyclic diterpenes (e.g., carnosic acid andcarnosol), phenolic acids (e.g., caffeic acid and rosmarinic acid), and essential oils. These secondary metabolites have been formulated in topical dosages. Topical administration strategies avoid first-pass metabolism, releasing the drug at the site of action and resulting in a lower risk of side effects.This strategy can be applied to improve the properties of cosmetic products (e.g., those that combat UV exposure, aging, and cellulite), owing to the anti-inflammatory activity and free radical-scavenging effects of *Rosmarinus officinalis*.

The use of nanoparticles in the development of new products for topical administration should be explored further, as they result in the more effective delivery of molecules to their site of action and increase treatment adherence. According to the published literature, rosemary has anti-inflammatory, antimicrobial, and antioxidant properties, which have been extensively reported in oral formulations(e.g., toothpaste or formulations to treat infections). The development of new formulations containing rosemary extracts should be encouraged, as their promise as topical agents is well established.

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4 CAPÍTULO II

Development and evaluation of formulation (oil-in-water) containing *Rosmarinus officinalis* extract for wound healing effect

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ABSTRACT

Rosmarinus officinalis belongs to the *Lamiaceae* family, and its constituents show different properties such as antioxidant, anti-inflammatory, and antimicrobial activity. The aim of this study was to select an extraction method and develop a emulsion with *R. officinalis* extract for wound healing. Maceration, infusion, Soxhlet and ultrasound were used to produce rosemary extracts, which were submitted to antioxidant, compound quantification, cell viability and antibacterial assays. Infusion and Soxhlet showed better results in the DPPH at 2.5 mg/mL and the first extract showed 111.94 ± 4.26 mg GAE/g of ferric reduction potential. In compound quantification infusion showed promising metabolite extraction in phenolic compounds (52.50 ± 2.75 mg GAE/g) and tannins (74.47 ± 5.73 mg TAE/L), although maceration was able to extract a higher amount of flavonoids (72.88 ± 3.84 mg QE/g). The concentration of 6.25-100 µg/mL showed results superior to 80% of cell viability for all extracts, demonstrating its non-toxicity. The infusion and ultrasound extracts affected more strains of skin bacteria in disc diffusion assays. In the minimum inhibitory concentration assay, the infusion extract showed results against *S. aureus* (>1.5 mg/mL), *S. oralis* (>6 mg/mL) and *P. aeruginosa* (>6 mg/mL), while ultrasound showed effects against those three bacteria (>3, >12.5 and 12.5 mg/mL, respectively) and *E. coli* (>25 mg/mL). The infusion extract was chosen to be incorporated into an oil-in-water emulsion, whose stability, texture and sensorial were assessed. It was observed that the blank and extract emulsions showed significant increase in density and viscosity during 90 days, the infusion extract promoted lower spreadability and appropriated texture, and the blank formulation showed great acceptance among the volunteers. According to the results, it is possible to conclude that the emulsion with *Rosmarinus officinalis* extract can be used for wound healing due to the biological activity assessed and its good adhesion.

Key-words: Rosemary; antioxidant; antimicrobial; cell viability; emulsion; wound healing

INTRODUCTION

Wound is a skin process that occurs when tissue is damaged, leading to four stages: vasoconstriction and coagulation, acute inflammation, acellular proliferation and wound remodeling (Teller and White 2011). However, when this process is impaired, it is known as chronic wound, with a higher amount of reactive oxygen species (ROS) released as pro-inflammatory factors that contribute to compromise healing, leading to cell apoptosis and tissue necrosis (Trachootham, Lu et al. 2008, Circu and Aw 2010, Dunnill, Patton et al. 2017).

Plant secondary metabolites delivered into the skin with a pharmacological effect are used in order to develop topical formulations for the treatment of local disorders (Garg, Rath et al. 2015). For example, rosemary extract showed improvement in the healing process of wounds, due to the presence of numerous bioactive compounds with antioxidant capacity (Alizargar, Kuchaki et al. 2012).

Extracts of *Rosmarinus officinalis*, which belongs to the *Lamiaceae* family and is popularly known as rosemary (al-Sereiti, Abu-Amer et al. 1999), have been used for antioxidant, anti-inflammatory, antidepressant, antinociceptive and antibacterial purposes (Hussain, Anwar et al. 2010, Martínez, González-Trujano et al. 2012, Amaral, de Carvalho et al. 2013, Sasaki, El Omri et al. 2013). The review article published by de Macedo, Santos et al. (2020) revisited a range of its medicinal (anti-inflammatory, skin cancer, wound healing, skin flap survival, transdermal drug delivery and antifungal) and cosmetic (ginoid lipodystrophy, alopecia, antiaging and ultraviolet protection) properties *in vitro* and *in vivo*.

Rosemary secondary metabolites responsible for those therapeutic activities were identified as flavonoids, polyphenols and terpenes (del Baño, Lorente et al. 2004, Borrás-Linares, Stojanovic et al. 2014, Mena, Cirlini et al. 2016, Fernández-Ochoa, Borrás-Linares et al. 2017). Those compounds were identified by chromatographic techniques (Mena, Cirlini et al. 2016, Sharma, Velamuri et al. 2020).

The concentration of bioactive compounds changes according to the extraction method used. Rosmarinic acid concentration varied when submitted to different extraction methods (maceration with stirring, heat reflux and microwave-assisted extraction), temperature and time (Sik, Hanczné et al. 2020). Biological activities also change depending on the type of extraction used. Ultrasound, solid-liquid and supercritical fluid extraction showed different results for phenolic compounds, antioxidant activity, minimum inhibitory concentration and minimum bacterial concentration (Rashidaie Abandansarie, Ariaii et al. 2019).

The aim of this study was to develop a topical emulsion (oil-in-water) with *Rosmarinus*

officinalis extract for wound healing. To this end, four extraction methods were used and the rosemary extracts were evaluated for *in vitro* compound quantification and antioxidant and antibiotic activity. The chosen extract was then incorporated in a topical emulsion, which was characterized and analyzed for stability.

MATERIAL AND METHODS

Materials

2,2-diphenyl-1-picrylhydrazyl (DPPH) and quercetin (95% of purity) were bought from Sigma-Aldrich (São Paulo Brazil), gallic acid and Folin-Ciocalteu were purchased from Dinâmica Química Contemporânea Ltda (São Paulo, Brazil), tannic acid and sodium carbonate by Êxodo Científica (Sumaré, São Paulo, Brazil), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoluim (MTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), Pen-Strep and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ). All other reagents were analytical grade. Rosemary was purchased in popular market in Mogi Mirim (São Paulo, Brazil) and was registered in the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) under register number AE2335F.

Extraction

Four extraction methods were applied in this study, although for purposes of comparability, a proportion of 5 g of rosemary leaves and 100 mL of ethanol (EtOH) 100% were used for all extractions. The maceration method (MAC) was based on extraction made by Cizauskaite, Ivanauskas et al. (2016) with modifications, adding the plant in solvent and rest for 30 minutes. The infusion (INF) method followed Zimmermann, Walch et al. (2011) with some modifications, which consisted of EtOH heating followed by the addition of rosemary leaves and rest for 30 minutes. The methodology employed for Soxhlet (SOX) followed Chaowuttikul, Palanuvej et al. (2020) with modifications, where *Rosmarinus officinalis* leaves were added in an extraction chamber and the ethanol was added in a boiling flask attached to a heating mantle, for 30 minutes. The ultrasound (ULT) extraction used the Oliveira Gde, de Oliveira et al. (2016) methodology with some modifications, keeping the mixture of rosemary and EtOH in an ultrasound bath (Unique USC-800) for 30 minutes. After extraction and cooling (when necessary), the samples were filtered with paper filter and stored in a refrigerator. Prior

to lyophilization, EtOH was removed in an evaporator (Fisotam 802) for 1 hour at 80 °C and 150 rpm, and frozen in a lyophilizer (Lyostar 3, SP Scientific) for 4 h at -40 °C under 100 mTorr of vacuum. Rosemary extracts ranged from -40 °C to 20 °C, in 137 hours (Bellows and King 1972). The lyophilized samples were kept in a refrigerator.

Antioxidant activity

In vitro antioxidant activity was assayed by DPPH (Santos, Torres et al. 2017) and FRAP methods (Urrea-Victoria, Santos et al. 2016) with modifications.

For DPPH, a lyophilized extract solution was prepared using methanol (10 mg/mL) and further dilutions were made (2.5 - 10 mg/mL). In a 96-well microplate DPPH solution, samples and methanol were added in triplicate. After 30 minutes of incubation, sample absorbance was read in a spectrophotometer (Thermo Scientific, Multiskan Sky) at 517 nm of wavelength. In addition, the blank (methanol) and sample blank (extract without reagent) were prepared. The radical DPPH inhibition percentage of the extracts was calculated by:

$$\%=\frac{\text{Control DPPH Abs-Sample Abs}}{\text{Control DPPH Abs}}$$

For FRAP, 10 mg/mL samples in methanol were diluted until 2.5 mg/mL concentration. In a 96-well microplate were added 265 µL FRAP reagent solution, 20 µL of samples and 15 µL of ultrapure water, then the microplate was incubated in the dark for 30 minutes, after which a spectrophotometer measurement was made in 595 nm of wavelength. The values were expressed in milligram of gallic acid equivalent per gram of sample (mg GAE/g), through the calibration curve (Urrea-Victoria, Santos et al. 2016).

For both tests a blank (methanol) and sample blank (extract without reagent) were measured.

Phenol, flavonoids and tannins determination

In vitro compound quantification was assayed by methods for phenolic compounds, flavonoids and tannins. All samples were resuspended in methanol from 10 mg/mL of concentration until 2.5 mg/mL.

For phenolic compounds, the Santos, Torres et al. (2017) methodology was used, with

modifications. In a 96-well microplate, 20 µL of samples were mixed with 180 µL of ultrapure water, 20 µL of Folin-Ciocalteu reagent 1N, 20 µL of methanol and 60 µL of $NaCO_2$ 10%. The measurement was made in a spectrophotometer at 760 nm after 20 minutes of incubation. The values were expressed in milligram of gallic acid equivalent per gram of sample (mg GAE/g), through the calibration curve.

For flavonoids, the Alves and Kubota (2013) protocol was followed. The spectrophotometer measurement was made at 425 nm of wavelength. The results were expressed in milligrams of quercetin equivalent per sample gram (mg QE/L), according to the calibration curve.

For tannins, the Shad, Nawaz et al. (2012) protocol was followed. The spectrophotometer measurement was made at 725 nm of wavelength. The results were expressed in milligrams of tannic acid equivalent per liter (mg TAE/L), according to the calibration curve.

Cell viability assay

The cytotoxicity of rosemary extracts was evaluated according to the Machado, Shishido et al. (2013) methodology. Immortalized human keratinocytes (HaCaT) were cultivated in an RPMI medium with 10 % of bovine fetal serum and incubated at 37 °C and 5% CO_2. After confluence, the cells in the culture bottle were trypsinized with 2.5 g/L trypsin/EDTA 0.2 g/L solution and added to a 96-well microplate. Each well received 1 mL of cell culture and its final concentration was $1x10^6$ cells/mL. *Rosmarinus officinalis* samples were added in different concentrations (6.25 - 400 µg/mL) in microplate wells for 24 hours of incubation. After medium removal, 100 µL of MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution were added to the wells and then incubated for 4 hours at 37 °C and 5% CO_2. Finally, the MTT reagent was removed, 100 µL of ethanol 100% were added and a spectrophotometer (Elx800 Absorbance Microplate Reader) measurement was made at 570 nm. The cell viability values were determined following the proposal by Mosmann (1983).

Microbiological activity

Diffusion discs – screening

The disc diffusion test was performed according to Andrews (2001), Mostafa, Al-Askar et al. (2018), and Pereira, Zulim et al. (2019) with some modifications. Rosemary extracts at 50 mg/mL were filtered with a sterilized Millipore filter (0.22 μm) and added to a sterilized filter paper disc (8mm of diameter). The medium was made starting with the dispersion of 10.5 mL Muller-Hilton agar in a sterilized Petri plate and a mixture with 15 mL of bacterial suspension previously inoculated (100 mL of medium/1 mL of 10^7 CFU) to assure 10^5 CFU/mL of medium concentration. The discs with extracts and a disc with 50 μL of 100,000 UI positive control Pen-Strep (Sigma) solution were added to the agar. The plates were stored at 5 °C for 2 h and then incubated for 24 h at 35 °C. The presence of halos allowed initial screening. Ethanol 100% was tested to evaluate interference in the test.

The bacteria from different biological samples used in the test were collected at the Sorocaba hospital (Table 1).

Table 1. Hospital bacteria samples from different biological material used in screening of maceration, infusion, Soxhlet and ultrasound extracts

Number	Sample	Bacterium
2.1	Tracheal secretion	*Enterobacter cloacae*
2.15	Blood	*Enterobacter sp*
2.32	Urine	*Enterobacter agglomerans*
3.23	Urine	*Escherichia coli*
3.39	Tracheal secretion	*Escherichia coli*
3.57	Urine	*Escherichia coli*
4.11	Blood	*Klebsiella pneumoniae*
4.20	Urine	*Klebsiella pneumoniae*
4.25	Urine	*Klebsiella ozaenae*
5.2	Catheter tip	*Proteus mirabilis*
6.5	Catheter tip	*Acinetobacter*

Evaluation of Minimum Inhibitory Concentration (MIC)

In order to evaluate the inhibitory potential of the Rosemary extracts the classical method of successive dilution (MIC) adapted to 96-well plates was developed. The strains used were determined by the Clinical and Laboratory Standards Institute (CLSI) with modifications

as described below, gram-positive (*Staphylococcus aureus* ATCC 10390 and *Streptococcus oralis* ATCC 9811) and gram negative (*Pseudomonas aeroginosa* ATCC 9721 and *Escherichia coli* ATCC 25922).

The MIC assay followed the method described by Mostafa, Al-Askar et al. (2018) with some modifications. The TSB culture medium was applied by 100 µL and rosemary extracts at 50 mg/mL were applied as an initial sample. Then 100 µL of initial solution were added to a 96-well microplate and serial dilutions were posteriorly made with TSB, starting in the first well. Furthermore, 10 µL of inoculum (0,5 McFarland ± 10^6 CFU/mL) were added to wells containing the extract dilutions and the microplate was incubated for 24 h at 37 °C.

In order to confirm the MIC, a Minimum Bactericidal Concentration (MBC) was applied. The MBC was adapted from the Shin, Ateia et al. (2015) protocol, where 10 µL of MIC samples were removed from the wells and inoculated on agar plates (Aumeeruddy-Elalfi, Gurib-Fakim et al. 2015). The assays were conducted in duplicate.

Formulation development and evaluation

Phytocosmetic development and stability

All raw material was certified by Associação de Certificação Instituto Biodinâmico (IBD) in order to obtain a phytocosmetic.

Oil-in-water (O/A) emulsions (Table 2) were developed following the protocol proposed by Cefali, Ataide et al. (2019). Xanthan gum was previously solubilized, and then benzoic acid, soy lecithin and sorbitol were added in another beaker with hot water. The oil phase contained stearyl alcohol, sunflower oil and sorbic acid, stirred and heated to 70 °C. The aqueous phase was added to the oil phase and, after mixing, xanthan gum was gradually added. After emulsion cooling, the volume was completed with distilled water and pH was adjusted to 5.5-6.5 with sodium hydroxide 0,1 M. Two types of formulation were made, blank (BF) and with 5% of *Rosmarinus officinalis* extract (EF).

Table 2. Blank (BF) and with *Rosmarinus officinalis* extract (EF) formulation components

Components	Concentration %	
	BF	**EF**
Sorbitol	5	5
Benzoic acid	0.3	0.3
Xanthan gum	1.6	1.6
Soy lecithin	1.5	1.5
Stearyl alcohol	4	4
Sunflower oil	5	5
Sorbic acid	0.3	0.3
Sodium hydroxide *u.p.*	pH 5.5 - 6.5	pH 5.5 - 6.5
Rosmarinus officinalis	-	5
Water *u.p.*	100	100

The phytocosmetic with and without extract was assessed following the Cosmetic Products Stability Guide (ANVISA 2004). The emulsions were submitted to preliminary stability in order to evaluate physical properties (color and odor), pH and phase separation for 15 consecutive days. Then, accelerated stability testing was performed at 1, 7, 15, 30, 60 and 90 days, evaluating the aspects previously mentioned as well as density using a pycnometer, and viscosity through a rotational viscosimeter (Brookfield, Mod LV-T, São Paulo, Brasil) with 1.5 rpm at 30 seconds using spindle 4 at 27 ± 2 °C (ANVISA 2004, Cefali, Ataide et al. 2019). Before the stability tests, 5 g of both BF and EF were submitted to phase separation evaluation in a centrifuge with 3000 rpm for 30 minutes. During the stability study, the emulsions were stored at room temperature (with and without light exposure) and hot (climatic chamber, 45 ± 2 °C) and cold (refrigerator, 5 ± 2 °C) conditions. In these evaluations, BF and EF were considered stable if the formulations did not present variation higher than 10% (ANVISA 2004).

Formulation texture analysis

Rheological properties of BF and EF were assessed with a texture analyzer (Stable Micro Systems TAXT plus, United Kingdom). All parameters used in this analysis are shown in table 3. The firmness and work of shear of both formulations were evaluated in a spreadability test, where the emulsion was added in a female cone and pressed down to eliminate air pockets. Firmness, consistency, cohesiveness and work of cohesion were calculated and carried out in a standard size back extrusion container (50 mm diameter) approximately 75% full.

Table 3. Settings and parameters used in textural and spreadability analyses of the blank and extract formulation

Settings and parameters	Textural analyses	Spreadability
Test Mode	Compression	Compression
Pre-Test Speed	1.50 mm/sec	1.00 mm/sec
Test Speed	2.00 mm/sec	3.00 mm/sec
Post-Test Speed	2.00 mm/sec	10.00 mm/sec
T.A. Variable No: 5	0.0 g	-
Target Mode	Distance	Distance
Force	-	100.0 g
Distance	25.000 mm	23.000 mm
Strain	10.0 %	10.0 %
Trigger Type	Auto (Force)	Button
Trigger Force	30.0 g	5.0 g
Probe	A/BE-d35; back extrusion rig 35 mm disc	HDP/SR; spreadability rig

Formulation sensorial analysis

The BF were submitted to sensorial analysis approved by the Ethics Committee (number: 23197519.1.0000.5404) – State University of Campinas.

For this analysis, 0.1 g of emulsion was administered to the forearm of 50 volunteers. The evaluation was made through a questionnaire with the aim of electing the best sensorial parameters (speed absorption, speed drying, stickiness, easy spreading, residual fatty sensorial, dry touch and general evaluation) according to a scale from 1 to 5 (bad, weak, reasonable, good and very good, respectively). In addition, it was evaluated whether the volunteers were interested in using the developed product (Cefali, Ataide et al. 2019).

Statistical analysis

The assays were performed in triplicate and the values were interpreted using ANOVA ($p<0.05$) followed by Tukey test. The results were added to GraphPad Prism 5.0 (Microsoft

Windows) in order to analyze them, make graphs and determine values of effective concentration 50% (EC_{50}) and inhibitory concentration 50% (IC_{50}).

RESULTS AND DISCUSSION

Antioxidant activity

Effective concentration to inhibit 50% (EC_{50}) for all samples in DPPH and FRAP assays is shown in Table 4.

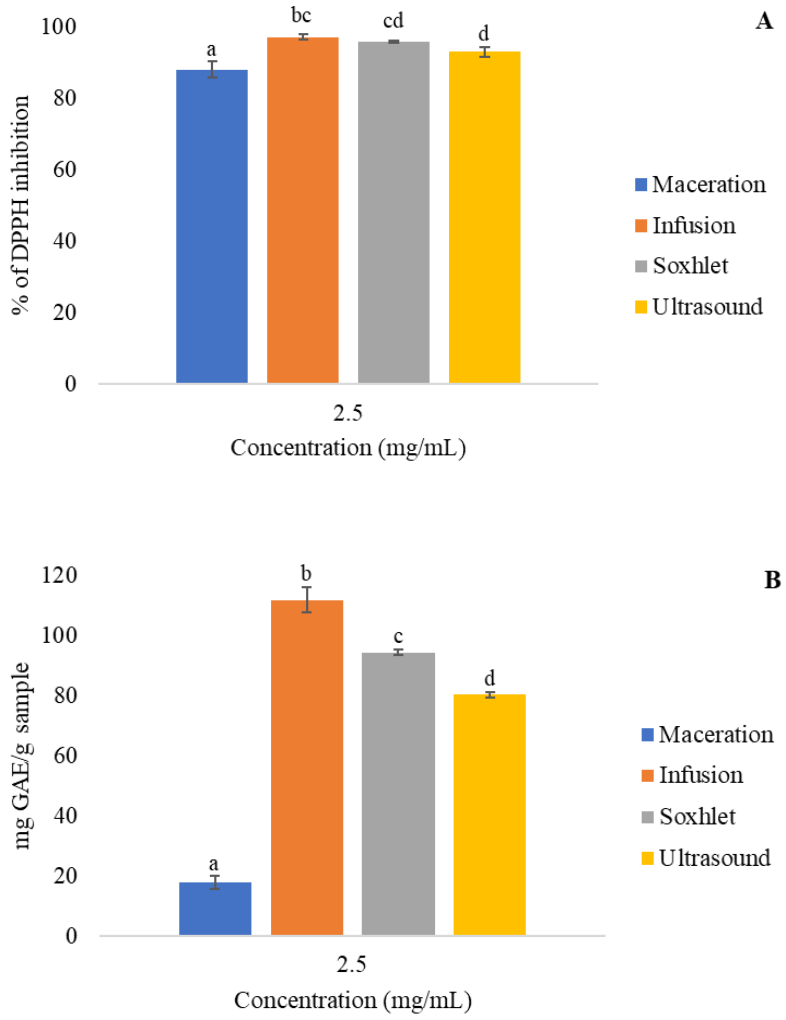
Table 4. Effective concentration (mg/mL) to inhibit 50% of DPPH and FRAP radicals for rosemary extracts obtained by maceration (MAC), infusion (INF), Soxhlet (SOX) and ultrasound (ULT).

Sample	DPPH (mg/mL)	FRAP (mg/mL)
MAC	6.84	7.65
INF	7.73	4.97
SOX	9.38	5.00
ULT	6.83	5.01

Results are presented as average ± standard deviation, n=3.

Antioxidant activities expressed in percentage of inhibition and gallic acid equivalent concentration of DPPH and FRAP assays are shown in Figure 1.

Figure 1. Antioxidant activities in DPPH (A) and FRAP (B) assays of rosemary extracts obtained from maceration, infusion, Soxhlet and ultrasound.



Results are presented as average ± standard deviation, n=3. The letters represent significant difference between samples.

According to Figure 1A *R. officinalis* extracts obtained by the INF and SOX techniques showed higher potential to inhibit DPPH radicals.

The INF and ULT samples showed better EC_{50} results when compared to the others. Bubonja-Sonje, Giacometti et al. (2011) found an EC_{50} of 0.03 mg/mL in a DPPH assay, showing higher capacity to inhibit radicals than infusion and ultrasound extracts. However, their extract was obtained by maceration using 5 g of sample and 50 mL of methanol: water: acetic acid (90: 9: 1 v/v/v) for 24 hours, which could explain this difference. A lyophilized rosemary ethanolic extract DPPH analysis was performed by Pereira, Pinheiro et al. (2017), but with a different method (0.5 mL of sample, 3 mL of ethanol 80% and 0.3 mL of DPPH 0.5 nM). The EC_{50} value was 127.33 µg/mL, which is better than MAC, INF, SOX and ULT.

According to Figure 1B, infusion showed the best result (111.94 ± 4.26 mg GAE/g) for

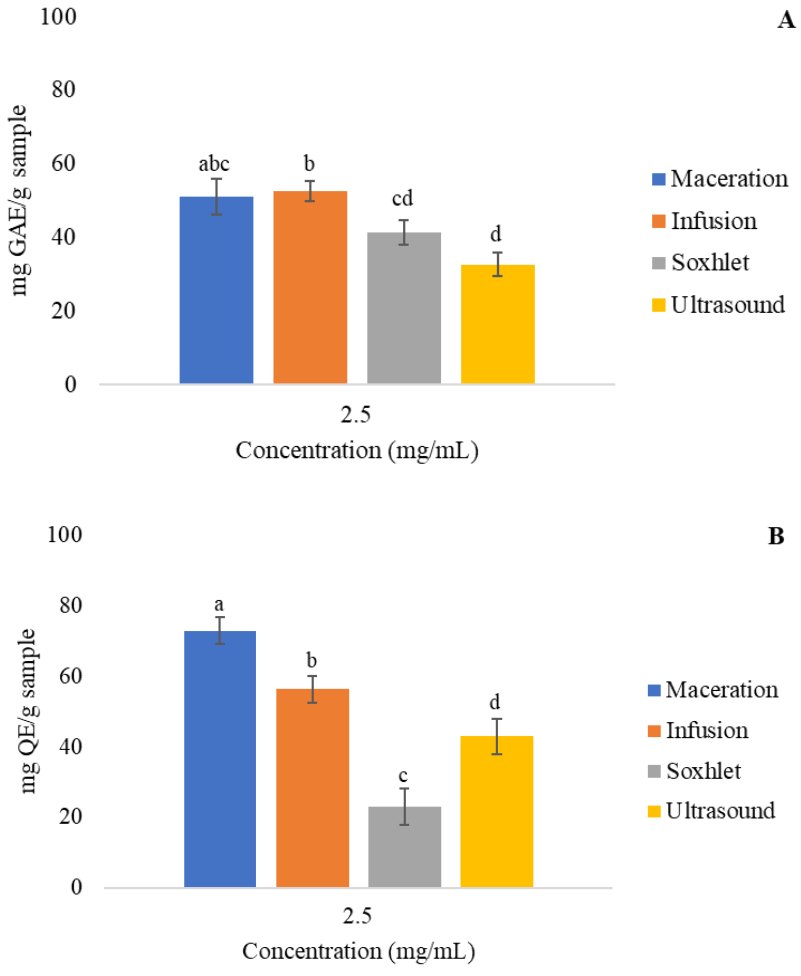
FRAP, with a significant difference between them.

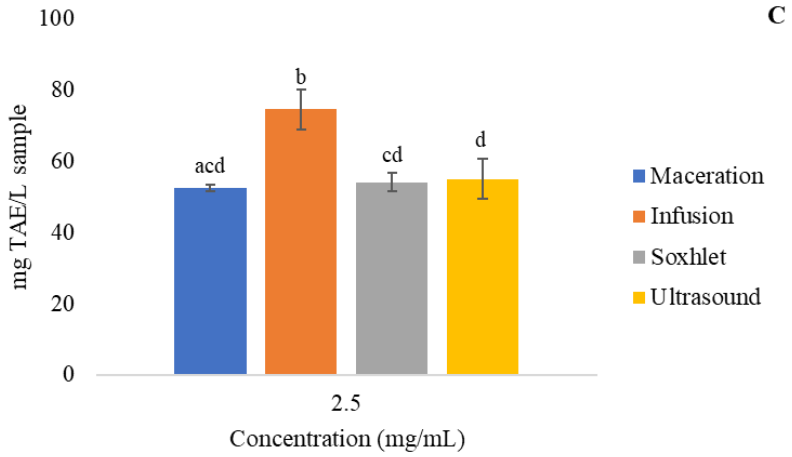
A study by Vital, Guerrero et al. (2016) showed 0.38 mg GAE/g in edible coating with rosemary and oregano essential oils, but all samples obtained from different techniques showed higher values, thus extracts have higher antioxidant activity for FRAP assays. Lagouri and Alexandri (2013) used an aqueous and methanolic extract at 0.1-1.5 g/L, which showed a mean value for EC_{50} of 0.035 mg/mL. The authors employed a different wavelength, namely 593 nm.

Phenol, flavonoids and tannins determination

Rosmarinus officinalis extract (2.5 mg/mL) secondary metabolite quantification results are shown in Figure 2, expressed as standard equivalent.

Figure 2. Determination in mg equivalent/g of sample of total phenolic compounds (A), flavonoids (B) and tannins (C) of rosemary extracts obtained by maceration, infusion, Soxhlet and ultrasound at 2.5 mg/mL of concentration.





Results are presented as average ± standard deviation, n=3. The letters represent significant difference between samples.

In the phenolic compounds assay, extract obtained by infusion showed 52.50 ± 2.75 mg GAE/g, being the largest phenol concentration when compared to other samples. Furthermore, INF and MAC showed no significant difference ($p>0.05$), so both have the same extraction potential.

Afonso, de O Silva et al. (2013) reported 16.67 ± 0.40 mg GAE/g using distilled water as solvent, being an inferior amount when compare to the four extracts.

The maceration extract showed 72.88 ± 3.84 mg QE/g, being considered the best extraction method for flavonoids. None of the samples showed significant differences ($p> 0.05$). Hendel, Larous et al. (2016) found a total flavonoid content of 38.01 ± 0.88 mg QE/g for the Soxhlet method, higher than the value for the same method in this work, which was 22.88 ± 5.09 mg QE/g. The difference in the analytical method was the wavelength, which was 415 nm. The work by Tzanova, Atanasov et al. (2020) listed arguments to be considered when the Soxhlet apparatus was used to extract flavonoids, including the need of this bioactive compound for thermal stability. This information can explain the lower number of flavonoids extracted by the technique mentioned previously, due to degradation in higher temperature.

Infusion showed better results for tannin extraction with 74.47 ± 8.73 mg TAE/g, when compared to other methods. The samples showed significant differences.

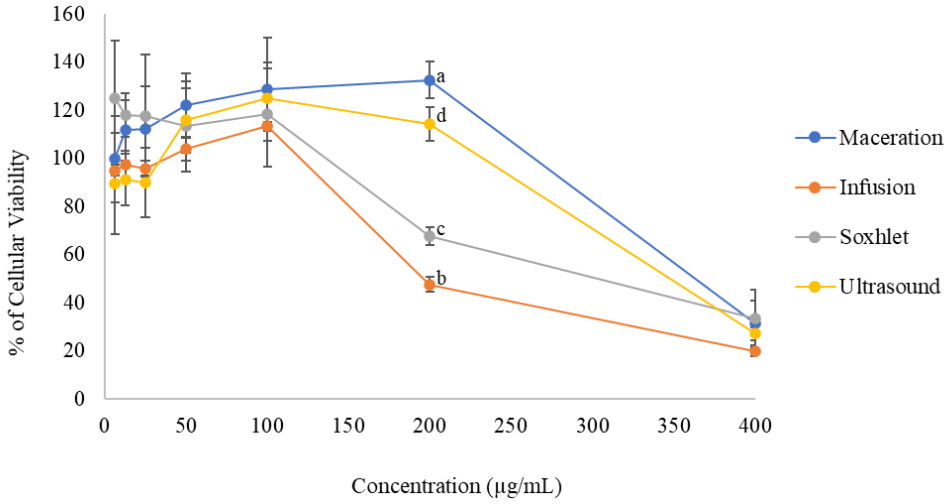
An article published by Kalinda and Naomi Boke (2020) described a qualitative test with ferric chloride for the presence of tannins in dichloromethane, methanolic and aqueous rosemary extract. The results achieved were positive for the secondary metabolite in three samples. However, so far, no studies with tannin quantification for *Rosmarinus officinalis* have

been found.

Cellular viability

Cell viability percentage is shown in Figure 3. The IC_{50} values for INF, SOX and ULT were 193.3, 193.4 and 235.2 µg/mL respectively. The MAC value was inconclusive.

Figure 3. Cell viability percentage in HaCaT of rosemary extract obtained by maceration, infusion, Soxhlet and ultrasound at 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL of concentration.



Results are presented as average ± standard deviation, n=3. The letters represent significant difference between samples.

The concentration of 400 µg/mL showed less cell viability for all samples. Based on the results, it can be considered that the safest extracts were those in concentrations of 6.25-100 µg/mL. In addition, some values exceeded the rate of 100%, which could be an indication that extracts stimulate cell proliferation.

The cell viability profile of HaCaT cells shown by López-García, Kuceková et al. (2013) for *Salvia pratensis* (Lamiaceae) declined with the increase in extract concentration, similar to *Rosmarinus officinalis* in this study. According to ISO10993 (2009) samples that reach at least 70 % or more of viability are considered non-toxic.

An Alamar Blue assay was used to evaluate the cytotoxic potential of *Melissa offinalis* leaf and stem extracts in a study proposed by Moacă, Farcaş et al. (2018). The results for leaves and stems showed an IC_{50} of 301.4 and 109.63 µg/mL, respectively, the former being safer

when compared to rosemary. Thus, *R. officinalis* showed a higher cell viability inhibitory concentration (50%) than *M. officinalis* stems.

Microbiological activity

Diffusion discs – screening

In recent decades, a significant increase in resistance to first-line antibiotics has been observed in different bacterial species. This resistance has affected different hospital situations. By developing systems with antimicrobials of natural origin that have a low probability of developing bacterial resistance and with different sites of action in microorganisms, it is possible to combat antibiotic resistant microorganisms (Gupta and Birdi 2017, Reygaert 2018).

This work evaluated the antimicrobial activity of rosemary extracts against hospital samples. The ethanol 100% extract preparation used does not show interference in antimicrobial activity. On the other hand, Pen-Strep, used as positive control, proved to be more effect against a large number of bacteria when compared to rosemary extracts.

According to the findings, the samples showed different results. The results were dependent on the type of microorganism and the origin of the sample. For *P. aeruginosa* from a catheter sample, all extracts showed antimicrobial activity (halos above 10 mm). However, for *P. aeruginosa* from a urine sample, only SOX was not able to inhibit the microorganism. The same behavior was observed for *Acinetobacter baumanni* and *haemolyticus* from catheter samples.

Even though all samples showed antimicrobial activity, the INF and ULT samples inhibited a greater number of microorganisms than SOX and MAC. The INF sample showed antimicrobial activity (halo above 10 mm) against *Enterobacter agglomerans* from urine and *Escherichia coli* from tracheal secretion. As for the ULT sample, it showed antimicrobial activity (halo above 10 mm) against *E. coli* from urine.

Gazwi, Mahmoud et al. (2020) performed a microbiological screening of *R. officinalis* ethanolic and methanolic extracts in *Escherichia coli* and *Pseudomonas aeruginosa*, the same bacteria of this work. The authors' results showed that only *E. coli* was affected, but MAC, INF and ULT produced an effect against the two strains cited before.

Abramovi, Terpinc et al. (2012) found that a rosemary extract produced an effect against Gram-positive and Gram-negative bacteria. However, due to the presence of carnosic acid the

first class was more susceptible when compared to rosmarinic acid use. INF and ULT showed an effect against gram-negative bacterium, which may be an indication of the absence of carnosic acid. The secondary metabolite cited before according to Gazwi, Mahmoud et al. (2020) induced alterations in bacterial cell leading to nutritional, genetic material and electron transference alternations, and changes in fatty acid production.

An article by Hickl, Argyropoulou et al. (2018) found 0.30, 0.60 and 10 mg/mL of MIC for *S. oralis*, *E. coli* and *S. aureus*, respectively, values which were only surpassed for INF and ULT, with 1.5 and 3 mg/mL of MIC, respectively. A rosemary ethanolic extract was tested in *S. aureus* and *E. coli* for 24 and 48 hours by Weerakkody, Caffin et al. (2010), showing minimum inhibitory concentrations of 5 and >5, and 1.25 and 1.25, respectively, considered better than the INF and ULT samples.

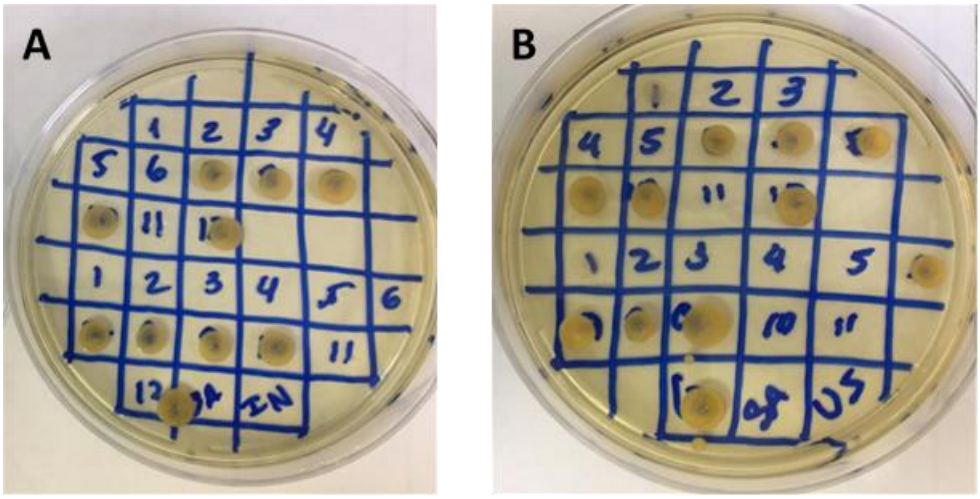
Minimum Inhibitory Concentration determination (MIC)

Our study showed that only the INF and ULT rosemary extracts were able to inhibit the standard microorganisms tested, based on disc diffusion performance. The MIC values are displayed in Table 5 and Figure 4.

Table 5. Minimum Inhibitory Concentration values obtained by microdilution in a 96-well microplate for infusion (INF) and ultrasound (ULT) rosemary extracts at 50 mg/mL of initial concentration.

	MIC (mg/mL)			
Sample	*S. aureus*	*S. oralis*	*E. coli*	*P. aureginosa*
INF	≥1,5	≥6,0	-	≥6,0
ULT	≥ 3,0	≥12,5	≥25,0	≥12,5

Figure 4. MBC assay of infusion (INF) and ultrasound (ULT) extracts in a Petri plate, with different sample concentrations. Microorganism growth in diverse concentrations was observed, shown by the numbers. Number 12 is a positive control of microorganism growth.



Both the INF and ULT extracts showed antimicrobial activity against the microorganisms used in the test. However there are differences in the action. The ULT extract produced an effect against all strains, although INF was two times more effective. These results could be related to the extraction technique, with a higher concentration of phenolic compounds, flavonoids and tannins accounting for greater antioxidant activity.

In a study by Hickl, Argyropoulou et al. (2018), rosemary extracts obtained by the ultrasound assisted method showed MIC values of 0.30, 0.60 and 10 mg/mL for *S. oralis*, *E. coli* and *S. aureus*, respectively. These values were only surpassed for INF and ULT, with 1.5 and 3 mg/mL of MIC, respectively. A rosemary ethanolic extract was tested in *S. aureus* and *E. coli* for 24 and 48 hours by Weerakkody, Caffin et al. (2010), showing minimum inhibitory concentrations of 5 and >5, and 1.25 and 1.25, respectively, being considered better than the INF and ULT samples. The authors mentioned above found a concentration for phenolic content of 56.76 ± 1.05 mg GAE/g in an ethanolic extract, which corroborates higher MIC activity when compared to infusion and ultrasound extracts, which showed 52.50 ± 2.75 and 32.61 ± 3.24 mg GAE/g of phenolics.

The antimicrobial effect of a *Rosmarinus officinalis* extract at 200 mg/mL of concentration on *S. aureus* and *P. aeruginosa* was studied by de Oliveira, de Jesus et al. (2017). This work showed MIC values of 25 and 6.25 mg/mL, using a commercial rosemary extract. In our work MIC values range from 6 to 12.5 mg/mL for *S. aureus* and *P. aeruginosa*, two times lower.

Formulation development and evaluation

Phytocosmetic development and stability

Based on the previous results here reported, the infusion extract (INF) was chosen to be incorporated in the oil-in-water emulsion. Both formulations (BF e EF) showed physical aspects of cream: shiny, creamy, with a white color and a characteristic odor from the base, although the rosemary extract induced a color change from white to yellow. The blank and extract emulsions showed no phase separation in a previous centrifuge test, and showed pH of 5.6 and 5.62, density of 0.82 g/L and 0.84 g/L, and viscosity of 96,666.7 ± 11.015 cP and 106,666.7 ± 8,326.6 cP, respectively.

BF showed no phase separation during 90 days of assay. In all conditions the emulsion's pH did not exceed 10% of variation (ANVISA 2004), but significant differences were reported when compared to the initial sample, at temperatures of 45 ± 2 °C and 5 ± 2 °C. Density and viscosity increased significantly and exceeded the limit of 10% of variation (ANVISA 2004) during the accelerated stability assessment for all conditions.

EF showed no phase separation either. The formulation presented no pH variation higher than 10% (ANVISA 2004) and no significant difference. Density and viscosity were compared to the first day sample. The former parameter showed a significant increase during 90 days. The latter showed a significant increase at room temperature, although when submitted to a higher temperature, only the formulation at day 7 showed a significant increase, and when stored in a refrigerator from day 30, the samples had a considerable increase in this parameter.

During the stability assessment, a darkening of the emulsion's color was observed when stored in a climatic chamber. The same result was reported by Hubinger, Cefali et al. (2010) and Figueiredo, Vilela et al. (2014)

Thus, it can be concluded that BF and EF showed an increase in density and viscosity parameters in the accelerated stability assessment.

Formulation texture analysis

Spreadability is the ability of semisolids to spread over a surface, and is directly linked to viscosity and the type of polymer used in the emulsion composition (Pawar and Pande 2015, Chen, Alexander et al. 2016). According to the results obtained (table 6), the emulsions showed significant differences, with higher values of firmness and work of shear for the formulation with rosemary extract. Considering the definition mentioned above, EF showed higher values for the parameters measured, spreading with more resistance than BF.

Table 6. Textural evaluation of firmness (g) and work of shear (g.sec) of both blank (BF) and extract (EF) formulations

Formulation	Firmness (g)	Work of shear (g.sec)
BF	149.70 ± 7.51^{a}	134.61 ± 17.59^{a}
EF	185.30 ± 2.30^{b}	176.87 ± 8.08^{b}

Results are presented as average ± standard deviation, n=3. The letters represent significant difference between samples.

The texture analysis results are shown in table 7. Work of cohesion showed no significant difference. Due to this finding, the *Rosmarinus officinalis* extract was responsible for increasing emulsion firmness, consistency and cohesiveness.

Table 7. Textural evaluation of firmness (g), consistency (g.sec), cohesiveness (g) and work of cohesion (g.sec) for both blank (BF) and extract (EF) formulations

Formulation	Firmness (g)	Consistency (g.sec)	Cohesiveness (g)	Work of Cohesion (g.sec)
BF	172.88 ± 2.56^{a}	1817.18 ± 33.37^{a}	-108.36 ± 2.25^{a}	-886.22 ± 52.97^{a}
EF	183.03 ± 5.83^{b}	1912.78 ± 18.49^{b}	-114.92 ± 2.65^{b}	-999.13 ± 14.26^{a}

Results are presented as average ± standard deviation, n=3. The letters represent significant difference between samples.

Firmness defines whether the spreadability of a formulation tends to be higher or lower, according to its viscosity. The more viscous a cream, the greater its firmness and the lower its spreadability (Mousazadeh, Mousavi et al. 2014). Mousazadeh, Mousavi et al. (2014) showed that an increase in xanthan gum concentration (0-0.3% w/w) led to an increase in spreadability. This finding can prove that BF and EF showed higher firmness due to a higher concentration of gum (1.6 % w/w).

Cohesiveness is related to interaction strength between a formulation's components (Pawar and Pande 2015). Mousazadeh, Mousavi et al. (2014) found greater cohesiveness due to a higher concentration of components in the emulsion. This result may be in accordance with

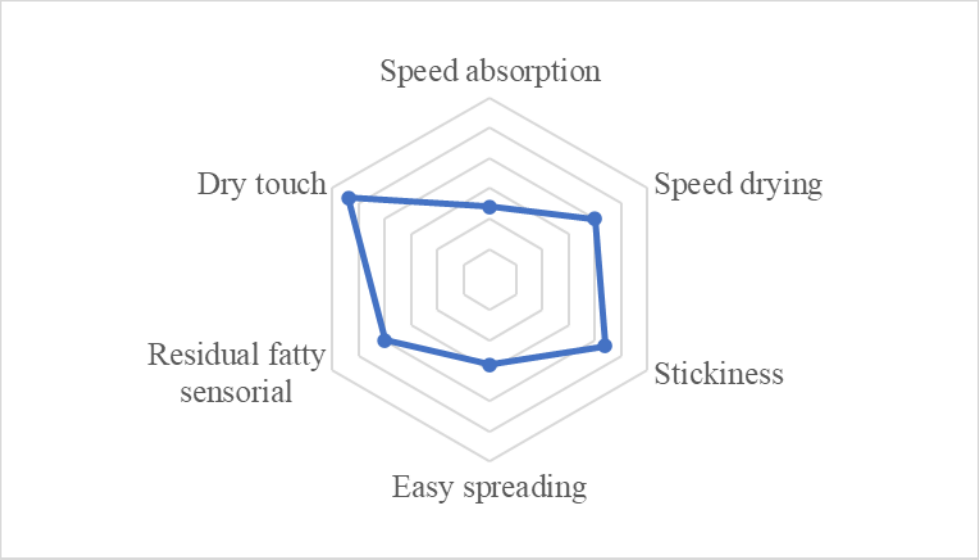
EF since its value was higher than BF, due to the presence of the extract.

Formulation sensorial analysis

The results obtained are shown in figure 5. After the sensorial analysis with 50 volunteers (19 men and 31 women), it was observed that the mean values of each characteristic evaluated indicated that the product showed great speed drying and dry touch, proper to a topic emulsion. Conversely, it showed poor speed absorption, spreadability, stickiness, and residual fatty sensorial. These factors may cause difficulties in product use, since depending on the treatment for which the emulsion is prescribed, it may cause poor adhesion. It was noticed during the test that the previous parameters improved when a volunteer increased the contact surface.

The emulsion had an average overall ratio of 4.08, and 42 participants answered that they would use the product, indicating good acceptance.

Figure 5. Sensorial evaluation of speed absorption, speed drying, stickiness, easy spreading, residual fatty sensorial and dry touch of the O/W emulsion.



CONCLUSION

Bioactive compounds from plants have been studied as a source of new drugs with medicinal and cosmetic properties. These secondary metabolites have shown a biological effect

against free radicals and microorganisms resistant to conventional antibiotics. This study showed that different techniques used to obtain extracts from rosemary had higher antioxidant and antimicrobial activity, due to higher amounts of extracted phenolic compounds, flavonoids and tannins, and extracts in low concentration (6.25-100 µg/mL) showed no toxicity. However, the incorporation of rosemary extracts showed an increase in the density and viscosity of the emulsion during the 90 days of testing. Also, in the sensorial test, the blank formulation had a good acceptance. Therefore, the *Rosmarinus officinalis* topic emulsion can be used to promote wound healing.

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5 DISCUSSÃO GERAL

Os metabólitos secundários são agentes químicos produzidos pelas plantas como forma de sobrevivência (Wink 2003). Essas moléculas têm a capacidade de exercer atividades antioxidante, antimicrobianas e anti-inflamatória (Vauzour, Rodriguez-Mateos et al. 2010, Hussain, Tan et al. 2016, Xie, Chen et al. 2017).

Os ensaios antioxidantes apontaram infusão e soxhlet com maior potencial de inibição de DPPH e infusão com maior ação redutora de ferro. Os resultados de EC50 para ambas as técnicas tiveram resultados inferiores quando comparados aos achados de Bubonja-Sonje, Giacometti et al. (2011), Pereira, Pinheiro et al. (2017) para DPPH, e Lagouri and Alexandri (2013), Vital, Guerrero et al. (2016) para FRAP. Esses valores inferiores podem ser devido ao tipo de solvente utilizado, que pode apresentar maior afinidade por metabólitos antioxidantes, a concentração da amostra para o preparado dos extratos, e ao método extrator.

As técnicas de maceração e infusão apresentaram maior eficiência extrativa de compostos fenólicos de 51,11 ± 4,86 e 52,50 ± 2,75 mg EAG/g. A pesquisa realizada por Bianchin, Pereira et al. (2020) utilizou o extrato de alecrim liofilizado, 10 g de amostra em 100 mL de etanol em banho de água a 70 °C por 30 minutos sob agitação, e como resultado obteve resultado de 46,48 ± 0,08 mg de EAG/g sendo esse valor inferior quando comparado aos dois métodos supracitados. O mesmo autor também relatou quantidades de 11.89 ± 0.58 mg EQ/g de flavonoides totais sendo inferior ao que foi encontrado para as quatro técnicas empregas no presente trabalho, onde a maceração obteve a maior quantidade extraída que foi de 72,88 ± 3,84 mg EQ/g.

Os taninos totais foram avaliados e a técnica de infusão apresentou a maior quantidade extraída. Em contrapartida, não há estudos na literatura que quantifiquem taninos no extrato de *Rosmarinus officinalis*.

Os extratos de MAC, INF, SOX e ULT em uma concentração de 6,25-100 µg/mL apresentaram viabilidade celular superiores a concentração mínima para ser considerado não tóxico de acordo com a ISSO 10993 (ISO10993 2009). Em contrapartida, ensaios de viabilidade em células HaCaT para o extrato de alecrim não foram reportados na literatura, dessa forma a comparação foi feita através da família Lamiaceae. O estudo de Moacă, Farcaş et al. (2018) apresentou um IC_{50} das folhas de *Melissa officinalis* superior, sendo considerado mais seguro quando comparado aos extratos de alecrim obtidos pelas quatro diferentes técnicas anteriormente mencionadas.

A atividade antimicrobiana do extrato de *R. officinalis* foi investigada sozinha ou em associação com extrato de menta e tocoferol por Azizkhani and Tooryan (2015). Os resultados apontaram que o alecrim foi responsável por inibir o crescimento bacteriano e diminuir o número de bactérias. O mesmo efeito antimicrobiano foi visto para os extratos de INF e ULT que apresentaram efeito contra bactéria**s**, no teste de difusão de discos, e bactérias com padrão ATCC, no teste de Concentração Inibitória Mínima.

As diferentes técnicas de maceração, infusão, soxhlet e ultrassom, como visto anteriormente, mostraram diferentes perfis nas atividades antioxidante e microbiológica, quantificação de compostos bioativos e também na viabilidade celular. Esse achado vai de acordo com o que Megateli and Krea (2018), Rashidaie Abandansarie, Ariaii et al. (2019), Sik, Hanczné et al. (2020) demonstraram sobre o impacto que diferentes métodos podem causar nas atividades biológicas e na concentração de metabólitos secundários.

Com base nos resultados apresentados anteriormente, foi desenvolvida uma formulação, óleo em água, de aplicação tópica com incorporação do extrato de infusão.

As características físicas que as emulsões apresentaram foram aparência cremosa, brilhante e odor característico da base, porém o extrato de alecrim foi responsável por causar escurecimento na cor branca para uma amarela.

Durante as análises de estabilidade FB e FE apresentaram ausência de separação de fases, pH de 5.6 e 5.62, densidade de 0.82 g/L e 0.84 g/L, e viscosidade de 96,666.7 ± 11.015 cP e 106,666.7 ± 8,326.6 cP, respectivamente. Durante o decorrer dos ensaios o pH, de ambas formulações, mantiveram-se dentro da variação proposta pelo Guia de Estabilidade de Produtos Cosméticos (ANVISA 2004), porém diferenças significativas foram relatadas quando houve comparação da amostra no dia 0 com os demais dias para FB quando armazenada na câmara climática e geladeira. A densidade e viscosidade apresentaram aumento significativo no decorrer dos ensaios tanto para a emulsão branca quanto para a que continha o extrato.

Foi avaliada a aceitabilidade sensorial do produto com ausência do extrato. FB teve um grau de aceitação por 84% dos voluntários e recebeu uma nota 4,08 em uma análise geral. Esse ensaio é importante para o desenvolvimento de novas formas farmacêuticas e cosméticas, visto que a aceitação da população é um importante fator na adesão do produto seja para fins medicamentosos quanto para fins cosméticos (Isaac, Chiari et al. 2012).

Levando em consideração os testes realizados pode-se dizer a formulação acrescida do extrato de *Rosmarinus officinalis* apresenta potencial de cicatrização de feridas devido ao elevado potencial antioxidante e antimicrobiano, e pela elevada aceitação.

6 CONCLUSÃO

Os extratos de *Rosmarinus officinalis* obtidos por diferentes métodos apresentaram atividade antioxidante elevada, principalmente INF, devido à presença de compostos fenólicos, flavonoides e taninos que são metabólitos secundários responsáveis por esse efeito. Os quatro extratos apresentaram viabilidade celular considerável maior que 80% em concentrações de 6,25-100 μg/ mL. Ademais, os testes de difusão em discos e de Concentração Inibitória Mínima apontaram que os extratos de INF e ULT foram eficazes contra um maior número de cepas e também contra bactérias preconizadas em ensaios farmacopeicos.

O extrato de infusão incorporado a formulação não causou instabilidades durante os 90 dias de estabilidade, porém ambas as formulações estudadas apresentaram aumento significativo na densidade e viscosidade. Ademais INF promoveu uma diminuição da espalhabilidade devido ao aumento na firmeza, consistência e tensão de cisalhamento. Por último o fitocosmético teve uma boa aceitabilidade por 84% dos voluntários e uma nota geral de 4,08 em uma escala de 5.

De acordo com o que foi apresentado, a formulação com extrato de alecrim pode ser aplicada para a cicatrização de feridas cutâneas. Contudo ainda há a necessidade da quantificação dos metabólitos presentes em R. officinalis, ensaio scratch e também um ajuste nos componentes da formulação para manter a densidade e viscosidade dentre dos limites preconizados.

7 REFERÊNCIAS

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8 ANEXOS

8.1 Aceite do Conselho de Gestão do Patrimônio Genético

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AE2335F

A atividade de acesso ao Patrimônio Genético/CTA, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AE2335F**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético/CTA**
Finalidade do Acesso:	**Pesquisa**

Espécie
Rosmarinus officinalis
Rosmarinus officinalis L.
Fonte do CTA
CTA de origem não identificável

Título da Atividade:	**Extração e avaliação da atividade antioxidante de compostos do alecrim visando sua aplicação tópica**

Equipe	
PRISCILA GAVA MAZZOLA	**UNICAMP**
Janaina Artem Ataide	**Unicamp**
Leticia Caramori Cefali	**UNICAMP**
Lucas Miltão	**UNICAMP**
Gabriela Mizael Cavarretto	**UNICAMP**

Luiza Aparecida Luna Silvério	**Unicamp**
Lucas Malvezzi de Macedo	**Unicamp**

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

Identificação do meio onde foi divulgado:	**Trabalho de conclusão de curso apresentado à**

Data do Cadastro:	**26/09/2018 14:44:32**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em **16:32** de **11/08/2020**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - **SISGEN**

8.2 Aceite do Conselho de Ética em Pesquisa

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise sensorial de formulações (bases farmacêuticas) para posterior incorporação de extratos vegetais

Pesquisador: Priscila Gava Mazzola

Área Temática:

Versão: 3

CAAE: 23197519.1.0000.5404

Instituição Proponente: Faculdade de Ciências Farmacêuticas

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.803.645

Apresentação do Projeto:

As informações contidas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram obtidas dos documentos apresentados para apreciação ética e das informações inseridas pelo Pesquisador Responsável do estudo na Plataforma Brasil.

Introdução: Rosmarinus officinalis L., popularmente conhecida como alecrim, é uma planta da família das Lamiaceae. O alecrim possui propriedades terapêuticas e tem sido usado na medicina popular em preparações orais para aliviar cólicas renais, dismenorreia e como antiespasmódico.Ao alecrim, são atribuídas diversas propriedades como: antifúngica, antiviral, antibacteriana, anti-inflamatória, antitumoral, antitrombótica, antinociceptiva, antidepressiva, antiulcerogênica e antioxidante.Os compostos presentes no extrato, agem como antioxidantes, através do mecanismo de doação de hidrogênio para radicais livres formando radicais estáveis e sequestrando radicais superóxidos.O café é uma das bebidas mais consumidas no mundo, com sabor e aroma característicos. O Brasil é o maior produtor e exportador do mundo, com uma produção estimada de mais de 51 mil sacas de 60 kg.O café pertence ao gênero Coffea e a família Rubiaceae que engloba duas das mais importantes espécies vegetais do comércio internacional de café: Coffea arábica L. e Coffea canephora Pierree, conhecidas popularmente como arábica e robusta, respectivamente. Entre todas as diferentes formas e variedades cultivadas, essas duas espécies são responsáveis pela produção de 65% a 70% da produção mundial de café. O café é uma

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Declaração de Pesquisadores	CARTA_RESPOSTA_CEP_Erica_Lucas.pdf	14/01/2020 11:38:58	Priscila Gava Mazzola	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TERMO_DE_CONSENTIMENTO_Erica_Lucas.pdf	14/01/2020 10:46:19	Priscila Gava Mazzola	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_DE_PESQUISA_Erica_Lucas.pdf	14/01/2020 10:39:56	Priscila Gava Mazzola	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_DE_PESQUISA.pdf	28/12/2019 11:12:03	Priscila Gava Mazzola	Aceito
Folha de Rosto	Folha_de_rosto.pdf	09/10/2019 23:31:37	Priscila Gava Mazzola	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 20 de Janeiro de 2020

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador(a))

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8.3 Permissão de uso de artigo científico

Re: [Plants] Manuscript ID: plants-730566; doi: 10.3390/plants9050651. Paper has been published.

P **Plants <plants@mdpi.com>**
27/01/2021 03:28

Para: janaina.a.ataide@gmail.com Cc:plants@mdpi.com; Lucas Malvezzi de Macedo

Dear Janaína,

Thank you very much for your email. Yes, it is allowed.

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Please feel free to let me know in case of any questions.

Looking forward to hearing from you.

Best regards,
Sylvia
Managing Editor
Plants (http://www.mdpi.com/journal/plants)

8.4 Artigos científicos

Artigos publicados:

- **de Macedo, L.M**.; Santos, É.M.; Militão, L.; Tundisi, L.L.; Ataide, J.A.; *Souto, E.B.*; Mazzola, P.G. Rosemary (*Rosmarinus officinalis* L., syn *Salvia rosmarinus* Spenn.) and its topical applications: A review. Plants 2020, 9, 651. (DOI: 10.3390/plants9050651).
- dos Santos, Érica Mendes; **de Macedo, Lucas Malvezzi**; Tundisi, Louise Lacalendola; Ataide, Janaína Artem; Camargo, Gisele Anne; **Alves, Rita C.*; **Oliveira, Maria Beatriz P.P.*; Mazzola, Priscila Gava. Coffee by-products in topical formulations: A review. Trends in Food Science & Technology, v. 2, p. 1, 2021. (DOI: 10.1016/j.tifs.2021.02.064).
- Lourenço, Carolina Botelho; Fava, Ana Laura Masquetti; dos Santos, Érica Mendes; **de Macedo, Lucas Malvezzi**; Tundisi, Louise Lacalendola; Ataide, Janaína Artem; Mazzola, Priscila Gava. Brief descriptions of the principles of prominent methods used to study the penetration of materials into human hair and a review of examples of their use. International Journal of Cosmetic Science, v. 1, p. 1-25, 2020. (DOI: 10.1111/ics.12683).
- Romanhole, Rodrigo Collina; Fava, Ana Laura Masquetti; Tundisi, Louise Lacalendola; **Macedo, Lucas Malvezzi de**; Santos, Érica Mendes dos; Ataide, Janaína Artem; Mazzola, Priscila Gava. Unplanned absorption of sunscreen ingredients: impact of formulation and evaluation methods. International Journal of Pharmaceutics, v. 591, p. 120013, 2020. (DOI: 10.1016/j.ijpharm.2020.120013).

Artigos em redação:

Lucas Malvezzi de Macedo, Érica Mendes dos Santos, Janaína Artem Ataide, Marcelo Lancellotti, Angela Faustino Jozala, Paulo César Pires Rosa e Priscila Gava Mazzola. Development and evaluation of formulation (oil-in-water) containing *Rosmarinus officinalis* extract for wound healing effect.

- Luíza Aparecida Luna Silvério, Érica Mendes Santos, Janaina Artem Ataide, **Lucas Malvezzi de Macedo** e Priscila Gava Mazzola. Natural products applied as excipients in formulations.