MARCELA ZAMBRIM CAMPANINI E SILVA

FORMULAÇÕES TÓPICAS CONTENDO EXTRATO DE *Pimenta pseudocaryophyllus*: AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E DA EFICÁCIA ANTI-INFLAMATÓRIA E ANTIOXIDANTE *IN VIVO*

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Londrina

Orientadora: Profa. Dra. Rúbia Casagrande
Co-orientadora: Profa. Dra. Sandra R. Georgetti

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Londrina, 22 de Março de 2013.

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“O cientista não é aquele que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas.”

Claude Lévi-Strauss

SILVA, Marcela Zambrim Campanini e. **Formulações tópicas contendo extrato de *Pimenta pseudocaryophyllus*:** avaliação da atividade antioxidante *in vitro* e da eficácia antioxidante e anti-inflamatória *in vivo*. 2013. 129f. Dissertação (Mestrado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina. 2013.

RESUMO

A resposta inflamatória induzida pela exposição à radiação ultravioleta (UV) pode resultar no excesso de espécies reativas de oxigênio (EROs), causando um desequilíbrio oxidante/antioxidante na pele, o que leva a diversos danos foto-oxidativos. Neste contexto, o interesse por extratos vegetais com propriedades antioxidantes, como o extrato etanólico *de P. pseudocaryophyllus* (PPE), tem crescido. Assim, o objetivo do trabalho foi caracterizar quimicamente o PPE por meio de identificação de compostos fenólicos por Cromatografia Líquida de Alta Eficiência (CLAE) e quantificação de polifenois e flavonoides totais, e avaliar sua atividade antioxidante (AO) e de duas formulações tópicas (F1, com alto conteúdo lipídico, e F2, com baixo conteúdo lipídico) adicionadas do mesmo. Realizaram-se também estudos de estabilidade das formulações e estudos de liberação *in vitro* dos antioxidantes presentes em F1 e F2. Em seguida, avaliou-se a eficácia das formulações contra danos causados pela radiação UVB. As formulações foram administradas no dorso dos animais, que receberam uma dose de radiação UVB de 4,14 J/cm2. Após 12 h foi realizada a eutanásia dos animais e as peles foram retiradas para avaliação da eficácia das formulações adicionadas de extrato contra danos oxidativos e inflamatórios. As quantidades de polifenois e de flavonoides encontradas no extrato foram de 199,33 mg/g e 28,32 mg/g, respectivamente. Por CLAE, foram identificados o eugenol, a rutina e o ácido tânico. Os resultados mostram que o PPE e as formulações incorporadas com o mesmo apresentam elevada atividade AO, com IC50 de 4,75 e 3,0 µg/mL, para os testes de DPPH e ABTS, respectivamente. F1 e F2 mantiveram suas características físico-químicas e funcionais durante o estudo; porém, sob condições aceleradas de estabilidade (45º C/75% de umidade relativa (UR)), F1 permaneceu mais estável quando comparada à F2 sob as mesmas condições. Além disso, os resultados de liberação do PPE a partir de F1 e F2 mostraram-se satisfatórios. Em relação aos estudos *in vivo*, F1 e F2 adicionadas de PPE foram capazes de inibir a formação de edema, o aumento da atividade da mieloperoxidase (MPO) e de metaloproteinase-9 (MMP-9), a diminuição dos níveis de glutationa reduzida (GSH) e o aumento de interleucina-1 (IL-1). Porém, as formulações não foram capazes de inibir o aumento dos níveis de fator de necrose tumoral-α (TNF-α). A radiação UVB não influenciou os níveis de interleucina-10 (IL-10), contudo, a F2 adicionada de PPE aumentou os níveis dessa interleucina. Portanto, os resultados deste estudo sugerem o uso de formulações tópicas adicionadas de PPE como fonte AO contra danos causados pelo estresse oxidativo induzido por radiação UV.

Palavras-chave: Antioxidante. *Pimenta pseudocaryophyllus*. Formulação tópica. Radiação UVB. Estresse oxidativo.

SILVA, Marcela Zambrim Campanini e. **Topical formulations containig *Pimenta pseudocaryophyllus* extract:** evaluation of *in vitro* antioxidant activity and *in vivo* antioxidant and antiinflammatory efficacy. 2013. 129p. Dissertation (Master's Degree Dissertation) – State University of Londrina, Londrina. 2013.

ABSTRACT

The inflammatory response induced by exposure to ultraviolet (UV) radiation can result in excessive reactive oxygen species (ROS), causing an oxidant / antioxidant imbalance in the skin, which leads to many fotoxidative damages. In this context, the interest in plant extracts with antioxidant (AO) properties, such as the *P. pseudocaryophyllus* ethanolic extract (PPE), has grown. Thus, the objective of the study was to chemically characterize the PPE through identification of phenolic compounds by High Performance Liquid Chromatography (HPLC), quantification of polyphenols and flavonoids, and to evaluate the AO activity of the extract and of two topical formulations (F1, with high lipid content, and F2, with low lipid content) added with it. We also performed stability studies of the formulations and *in vitro* release studies of the AO substances present in F1 and F2. Then, the efficacy of the formulations against damage caused by UVB radiation was evaluated. Formulations were administered at the back of the animals, which received an UVB radiation dose of 4.14 J/cm2. After 12 h, animals were killed and the skins were removed. The quantities of polyphenols and flavonoids found in the extract were 199.33 mg/g and 28.32 mg/g, respectively. By HPLC, we identified eugenol, rutin and tannic acid on PPE. Results show that PPE and formulations incorporated with it present high AO activity, with IC50 of 4.75 and 3.0 µg/mL, for DPPH and ABTS tests, respectively. F1 and F2 kept their physico-chemical and functional characteristics during the study, but, under accelerated stability conditions (45° C/ 75% of relative humidity (RH)), F1 was more stable when compared to F2 under the same conditions. Furthermore, results of release study of PPE from F1 and F2 were satisfactory. Regarding the *in vivo* studies, F1 and F2 added with PPE were able to inhibit edema formation, increase in myeloperoxidase (MPO) and metalloproteinase-9 (MMP-9) activities, decrease of reduced glutathione (GSH) levels and increase in the interleukin-1 (IL-1). However, the formulations were not able to inhibit the increase in production of tumor necrosis factor-α (TNF-α). UVB radiation did not affect the levels of interleukin-10 (IL-10). However, F2 containing PPE increased this interleukin levels. Therefore, the results of this study suggest the use of topical formulations added with PPE as AO source against UV radiation-induced oxidative stress damage.

Key Words: Antioxidant. *Pimenta pseudocaryophyllus*. Topical formulation. UVB radiation. Oxidative stress.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SÍMBOLOS

ABTS	2,2’ azinobis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
AO	Antioxidante
AP-1	Ativador de proteína-1
$CaCl_2$	Cloreto de cálcio
CLAE	Cromatografia líquida de alta eficiência
DNA	Ácido desoxirribonucleico
DPPH	2,2-difenil-1-picrilidrazil
EGF	Fator de crescimento epidérmico
EGTA	Ácido etileno glicol tetracético
ELISA	Ensaio imunoenzimático
ERKs	Quinases reguladas por sinal extracelular
EROs	Espécies reativas de oxigênio
$FeCl_3.6H_2O$	Cloreto férrico hexahidratado
FRAP	Poder antioxidante férrico
F1	Formulação 1
F2	Formulação 2
GSH	Glutationa reduzida
HCl	Ácido clorídrico
$HO^{\bullet}$	Radical hidroxil
HOCl	Ácido hipocloroso
HOX	Ácidos hipoalosos
HTAB	Brometo de hexadecil trietil amônio
H_2O_2	Peróxido de hidrogênio
H_3PO_4	Ácido ortofosfórico
IFSCC	International Federation of Societies of Cosmetic Chemists
IL	Interleucina
IκB	Fator inibidor de κB
JNKs	Quinases c-Jun N-terminal
KCl	Cloreto de potássio
KH_2PO_4	Fosfato de potássio monobásico

KOH	Hidróxido de potássio
K_2HPO_4	Fosfato de potássio dibásico
LPO	Peroxidação lipídica
MAPKs	Proteínas quinases ativadas por mitógeno
MEC	Matriz extracelular
MMP	Metaloproteinase
MPO	Mieloperoxidase
NaCl	Cloreto de sódio
NADPH	Nicotinamida Adenina Dinucleotídeo Fosfato reduzido
NaOH	Hidróxido de sódio
NaH_2PO_4	Fosfato de sódio monobásico
NF-κB	Fator nuclear- κB
NO	Óxido Nítrico
$ONOO^-$	Peroxinitrito
OPT	O-ftalaldeído
O_2	Oxigênio molecular diatômico
$O_2^{\bullet-}$	Radical ânion superóxido
$^1O_2$	Oxigênio singlete
PBS	Tampão fosfato salino
PGs	Prostaglandinas
PPE	Extrato etanólico de *Pimenta pseudocaryophyllus*
RL	Radicais livres
RNA	Ácido ribonucléico
$RO_2^{\bullet}$	Radical peroxil
SAPKs	Quinases ativadas por estresse
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em gel de poliacrilamida com duodecil sulfato de sódio
SH	Grupamento sulfidrila
SIDA	Síndrome da Imunodeficiência Adquirida
SOD	Superóxido dismutase
TG	Triglicerídeos
TIMPs	Inibidores teciduais de metaloproteinases
TNF-α	Fator de necrose tumoral-α

TPA	12-o-tetradecanoilforbol-acetato
TPTZ	2,4,6 tripiridil-S-triazina
Tris	Hidroximetil aminometano
UR	Umidade relativa
UV	Ultravioleta
X^-	Haletos

SUMÁRIO

1 INTRODUÇÃO

1.1 Estresse oxidativo e os danos ao organismo

Radicais livres (RL) são átomos ou moléculas que contêm um ou mais elétrons desemparelhados em seu orbital de valência (GUTTERIDGE; HALLIWELL, 2000). Com o intuito de estabilizar sua configuração eletrônica, interagem facilmente com outras substâncias por meio de reações de oxi-redução (JENSEN, 2003; HALLIWELL, 2009).

RL derivados de oxigênio são conhecidos como espécies reativas de oxigênio (EROs) e representam a classe mais importante de RL gerada pelo organismo (MILLER; BUETTNER; AUST, 1990). Entre as EROs, destacam-se o radical ânion superóxido ($O_2^{\bullet -}$), o radical hidroxil ($HO^{\bullet}$), o radical peroxil ($RO_2^{\bullet}$) e algumas espécies não radicalares, como o peróxido de hidrogênio (H_2O_2) e o oxigênio singlete ($^1O_2$), que, apesar de não possuírem elétron livre em seu orbital de valência, podem facilmente reagir e formar RL (GULÇIN, 2006).

Em concentrações baixas ou moderadas, as EROs podem ser benéficas para a célula, estando envolvidas em diversos processos fisiológicos, como produção de energia, regulação do crescimento celular, fagocitose e síntese de substâncias importantes, tais como hormônios e enzimas (MARTINDALE; HOLBROOK, 2002; SCHNEIDER; OLIVEIRA, 2004). Na pele, as EROs são constitutivamente produzidas em queratinócitos por processos específicos, tais como oxidações enzimáticas e respiração aeróbia, e podem ser induzidas por diferentes citocinas, fatores de crescimento e outros estímulos fisiológicos. EROs geradas constitutivamente regulam os níveis e a atividade das proteínas fosforiladas e das proteínas-quinases dentro dos queratinócitos (FUCHS et al., 2001).

Com o intuito de controlar a geração das EROs, existem no organismo sistemas antioxidantes, que restabelecem o equilíbrio redox (VASCONCELOS et al., 2007). De forma geral, um antioxidante (AO) pode ser definido como uma substância que, quando presente em baixas concentrações em comparação ao substrato oxidável, retarda ou inibe a oxidação de biomoléculas (SOUSA et al., 2007).

No entanto, há situações em que o equilíbrio entre a produção de EROs e as defesas antioxidantes pode ser prejudicado devido à produção exacerbada dessas espécies reativas ou à deficiência das defesas antioxidantes da célula (HALLIWELL,

2009). Esse desequilíbrio é denominado estresse oxidativo, um processo durante o qual as EROs em excesso podem desregular as vias de transdução redox-sensíveis, de citotoxicidade e de apoptose. Além disso, podem oxidar e danificar lípideos celulares, proteínas e ácido desoxirribonucléico (DNA), levando à modificação e frequentemente à inutilização dessas estruturas, inibindo a sua função normal (VALKO et al., 2007; FUCHS et al., 2001). Os lipídeos são a classe mais suscetível ao ataque pelas EROs, uma vez que estão presentes em grande quantidade nas membranas celulares, as quais estão em contato com fluidos ricos em oxigênio e metal (BUEGE; AUST, 1978).

Assim, o estresse oxidativo está envolvido nas etapas de envelhecimento, transformação e morte celular, com consequências diretas em processos patológicos, como a indução do câncer e a propagação da Síndrome da Imunodeficiência Adquirida (SIDA) em pacientes HIV positivos. Participa também da fisiopatologia de diversas doenças crônicas, como doenças autoimunes, cardiopatias, doenças pulmonares, intoxicação por xenobióticos, entre outras (VASCONCELOS et al., 2007).

Várias são as fontes celulares de RL, como absorção de energia radiante (luz ultravioleta (UV), raio X), metabolismo energético de substâncias químicas exógenas ou drogas, reações de óxido-redução que ocorrem durante processos metabólicos normais e a presença de metais de transição, como ferro e cobre, que doam ou aceitam elétrons livres durante reações intracelulares e que catalisam a formação de RL (FARBER, 1994). Entre essas fontes, a radiação UV desempenha papel de destaque na indução do estresse oxidativo cutâneo (AL SHAAL; SHEGOKAR; MÜLLER, 2011).

1.2 Consequências da exposição cutânea à radiação UV

Nos mamíferos, a pele é basicamente constituída por duas camadas separadas pela membrana basal. A epiderme é a camada superficial, um epitélio queratinizado, estratificado e escamoso que está em contato permanente com o meio ambiente. A derme é um tecido conjuntivo composto por colágeno, fibras elásticas e uma mistura de proteínas de matriz extracelular (FELDMEYER et al., 2010).

A radiação UV da luz solar é o cancerígeno físico mais proeminente e onipresente em nosso ambiente natural. É altamente genotóxica, mas não penetra em outros órgãos além da pele no organismo (GRUIJL, 1999). A radiação UVB (280–320nm) é o espectro da radiação UV com maior capacidade de induzir câncer de pele em animais, podendo causar também danos ao DNA, destacando-se a formação de dímeros ciclobutanopirimidina e de fotoprodutos que induzem mutação nas células epidérmicas. A radiação UVB também aumenta a expressão de genes por meio de vias de sinalização intracelulares de transdução, além de suprimir a resposta imune. Esses três efeitos contribuem cooperativamente para o desenvolvimento do câncer de pele (ICHIHASHI et al., 2003). Na realidade, acredita-se que a radiação UVB seja responsável por 90% da dose carcinogênica da luz solar (AFAQ; MUKHTAR, 2006).

Além disso, a radiação UV é a principal causa de formação exacerbada de EROs na pele ao longo da vida, podendo ser absorvida por ligações conjugadas presentes em diferentes cromóforos na pele, como melanina, DNA, ácido ribonucleico (RNA), proteínas, lipídeos, água, aminoácidos aromáticos como tirosina e triptofano, entre outros. Essas substâncias tornam-se, então, quimicamente reativas, o que possibilita sua participação em diferentes reações fotoquímicas e interações secundárias envolvendo EROs, sendo modificadas ou danificadas (GRUIJL, 1999; GONZÁLEZ; LORENTE; CALZADA, 2008). Dessa forma, considerando que a pele está cada vez mais exposta à radiação UV, o risco de danos foto-oxidativos cutâneos induzidos pelas EROs tem aumentado consideravelmente (PODDA et al., 1998).

Com o intuito de remover RL da pele, estão presentes na epiderme defesas antioxidantes enzimáticas e não enzimáticas, como superóxido dismutase (SOD), glutationa peroxidase, tiorredoxina, catalase, vitaminas C e E, carotenóides e glutationa reduzida (GSH) (AL SHAAL; SHEGOKAR; MÜLLER, 2011). Entre essas substâncias, destacam-se as moléculas que contêm o grupamento tiol, tais como a GSH e a tiorredoxina (FUCHS et al., 2001). Quando existe uma diminuição das defesas antioxidantes induzida, por exemplo, pela exposição à radiação UV, a pele torna-se vulnerável ao ataque das EROs. Neste contexto, a GSH é um marcador epidermal sensível ao estresse oxidativo mediado pela radiação UVB, e sua relevância em estudos relacionados à avaliação dos danos causados pela exposição

a esta radiação tem sido demonstrada (CASAGRANDE et al., 2006a; MARQUELE-OLIVEIRA et al., 2007).

A GSH é um tripeptídeo encontrado intracelularmente em altas concentrações essencialmente em todos os organismos aeróbios. Seu grupamento sulfidrila (SH), altamente polarizável, possibilita a eliminação de RL diretamente por transferência de hidrogênio, o que a torna um ótimo nucleófilo para reações com compostos químicos eletrofílicos. Além disso, atua como cofator para a enzima GSH-peroxidase, que elimina os peróxidos, e regenera as vitaminas E e C. Assim, acredita-se que a GSH surgiu na evolução bioquímica como uma proteção contra EROs geradas por processos oxidativos tanto no organismo quanto no ambiente (HUBER; ALMEIDA, 2008; CARINI et al., 2000).

A pele, por funcionar como uma barreira de proteção contra agentes externos, auxilia na manutenção e na execução de funções imunológicas cruciais. Na epiderme, os queratinócitos, os melanócitos e as células dendríticas de Langerhans agem em conjunto com mastócitos, macrófagos, granulócitos e fibroblastos. Estes subconjuntos de células comunicam-se e interagem por meio de citocinas, liberadas principalmente por queratinócitos epidérmicos (WEISS et al., 2004; CARINI et al., 2000).

Em condições normais, o revestimento endotelial dos microvasos é bastante permeável a moléculas pequenas, como a glicose, e quase impermeável a moléculas grandes, como proteínas plasmáticas (ex. albumina). Durante a inflamação aguda induzida pela radiação UV, sinais químicos como histamina e prostaglandinas (PGs), liberados por células como os macrófagos e os mastócitos, podem ativar vias de sinalização endotelial que têm como alvo elementos estruturais (por exemplo, actina e miosina) que regulam a permeabilidade vascular. O resultado é o alargamento das junções interendoteliais e a separação de células endoteliais, formando verdadeiros espaços entre as mesmas. Isso caracteriza o aumento da permeabilidade vascular, que permite o extravasamento de proteína microvascular e fluido para o interstício, causando o edema (KVIETYS; GRANGER, 2012; WIDYARINI et al., 2001).

Uma das primeiras respostas detectáveis nas células cutâneas expostas à radiação UV é a ativação de múltiplos receptores de superfície celular de citocinas e de fatores de crescimento, destacando-se receptores do fator de crescimento

epidérmico (EGF), do fator de necrose tumoral-α (TNF-α) e da interleucina-1 (IL-1) (RITTIÉ; FISCHER, 2002).

Estudos demonstram que baixas doses de radiação UVB (5-40 mJ/cm^2, bem abaixo daqueles que causam vermelhidão na pele humana), induzem em cultura de queratinócitos humanos a expressão de citocinas como TNF-α e interleucinas 1α, 1β e 6 (IL-1α, IL-1β, IL-6), as quais promovem reações de células efetoras na pele, como leucócitos e células endoteliais (CARINI et al., 2000). A inflamação decorrente da liberação dessas substâncias representa uma tentativa de proteção do organismo para restaurar um novo estado homeostático após perturbação causada por um estímulo nocivo. No entanto, este processo pode também ser destrutivo, resultando em doenças inflamatórias como a psoríase e a dermatite de contato. Nestes processos cutâneos, as citocinas IL-1 e TNF-α liberadas pelos queratinócitos têm demonstrado ser cruciais (FELDMEYER et al., 2010). Na realidade, os níveis dessas duas citocinas muitas vezes podem ser correlacionados com a gravidade das referidas doenças (KADOSHIMA-YAMAOKA et al., 2009).

A IL-1 e o TNF-α induzem respostas sistêmicas e locais. Ambos promovem a adesão de neutrófilos e de outras células estimulando a expressão de moléculas de adesão como as moléculas de adesão intercelular (ICAMs), moléculas de adesão celular-vascular (VCAMs) e selectinas (KADOSHIMA-YAMAOKA et al., 2009; FELDMEYER et al., 2010; BANERJEE; SAXENA, 2012). A IL-1 e o TNF-α também aumentam a produção celular de óxido nítrico (NO), que, além de causar danos teciduais por si só, é um componente essencial para a síntese de peroxinitrito ($ONOO^-$), um oxidante celular extremamente citotóxico (COMBES et al., 2001). Com base nestas atividades, tanto a IL-1 quanto o TNF-α podem representar alvos potenciais para intervenção terapêutica.

A expressão de IL-1 e de TNF-α é regulada em nível transcricional pelo fator nuclear-kB (NF-kB), e, da mesma forma, tanto a IL-1 quanto o TNF-α podem ativar o NF-kB (FELDMEYER et al., 2010). O NF-kB é um fator de transcrição ativado pela radiação UV que atua como importante mediador das reações imunológicas e inflamatórias e da resposta de fase aguda, podendo contribuir para a proliferação celular e/ou morte celular apoptótica (WEI et al., 1999; RENARD et al., 1997). Uma vez ativado, o NF-kB possui a capacidade de ligar-se a uma sequência de 10 pares de bases na região promotora do gene que codifica a cadeia leve k das moléculas de anticorpo das células B (kB) (GLEZER et al., 2000). Na realidade, o NF-kB pode

ser ativado por vários agentes, como TNF-α, IL-1β, lipopolissacarídeos, vírus e DNA dupla-fita. Esses agentes induzem uma cascata de eventos que levam à fosforilação, e consequente degradação, do IkB, uma proteína inibitória que forma um complexo que impede a translocação do NF-kB para o núcleo (RENARD et al., 1997; GLEZER et al., 2000). Como consequência, o NF-kB liga-se a diferentes promotores de genes, tais como os que codificam TNF-α, interleucinas (IL-6 e IL-1) e várias moléculas de adesão, permitindo, assim, sua transcrição (RENARD et al., 1997; CARINI et al., 2000). Independentemente do estímulo, parece haver participação de EROs e do aumento de cálcio intracelular para a ativação do NF-kB (GLEZER et al., 2000).

Percebe-se, na realidade, que, enquanto as citocinas pró-inflamatórias são capazes de estimular o recrutamento de células imunocompetentes e de ativar fatores transcricionais relacionados à inflamação, essas mesmas células e fatores transcricionais também são capazes de estimular a produção daquelas citocinas, caracterizando um ciclo complexo que objetiva reverter alterações causadas por um estímulo externo.

De forma concomitante, existe a atuação de citocinas capazes de inibir os processos inflamatórios, como a interleucina-10 (IL-10), que equilibra os sinais ativadores e inibidores dos mesmos (WEISS et al., 2004; WITKO-SARSAT et al., 2000). A IL-10 é produzida por diferentes tipos de células, como monócitos, linfócitos T e B, queratinócitos e principalmente macrófagos. As propriedades anti-inflamatórias da IL-10 incluem a redução da transcrição e da produção de IL-1β, TNF-α, IL-6 e IL-8 e o aumento da liberação do antagonista do receptor de IL-1. A IL-10 também limita a infiltração de macrófagos e linfócitos e a agregação e proliferação de células B, além de inibir o NF-kB (RIBBONS et al., 1997; WEISS et al., 2004; WITKO-SARSAT et al., 2000).

Outra consequência da exposição à radiação UV é a expressão de genes alterados por meio da ativação de múltiplas vias de sinalização, como as de proteínas quinases ativadas por mitógeno (MAPKs). As MAPKs constituem uma superfamília de proteínas que inclui ERKs (quinases reguladas por sinal extracelular), JNKs (quinases c-Jun N-terminal), SAPKs (quinases ativadas por estresse) e a p38 quinase. A radiação UV pode ativar os quatro subgrupos por meio da ativação de fatores de crescimento e de receptores de citocinas, como o da IL-1β (LI et al., 2010; SACHSENMAIER et al., 1994; JANSSENS; BEYAERT, 2003).

MAPKs ativadas são translocadas para o núcleo, onde fosforilam e ativam vários substratos, incluindo o fator de transcrição ativador de proteína-1 (AP-1), que controla a expressão de diversos genes envolvidos na regulação do crescimento e da diferenciação celular. Estudos demonstram que a inibição do AP-1 prejudica a formação de tumor em modelo de câncer de pele em ratos, o que reforça o conceito de que a ativação de genes-alvo dependentes de AP-1 está intimamente envolvida no desenvolvimento de câncer de pele (RITTIÉ; FISCHER, 2002; LI et al., 2010).

O fator de transcrição redox-sensível AP-1 ainda modula os genes que codificam as metaloproteinases (MMPs) e os seus inibidores teciduais (TIMPs) (FUCHS et al., 2001; LI et al., 2010). As MMPs são uma família de endopeptidases extracelulares conhecidas por facilitar a migração e a metástase por meio de quebra de barreiras formadas por matriz extracelular (MEC), cuja principal constituição é o colágeno (CHEN; PARKS, 2009).

Um fato interessante é que MMPs não são constitutivamente expressas na pele, mas são induzidas em resposta a, por exemplo, citocinas e fatores de crescimento. (FUCHS et al., 2001; LI et al., 2010). Estudos demonstram que a irradiação UV induz a formação de MMPs na epiderme e na derme, e estes resultados suportam a teoria de que MMPs são os principais mediadores do dano no tecido conjuntivo exposto à irradiação UV e no envelhecimento precoce da pele (FONSECA et al., 2011). Sabe-se que o equilíbrio das MMPs secretadas e de seus TIMPs desempenha um papel importante na manutenção da homeostase do tecido conjuntivo. Em processos inflamatórios, incluindo na pele, há um desequilíbrio entre MMPs e TIMPs (FUCHS et al., 2001; EISSA et al., 2007). Em doenças neoplásicas, esse desequilíbrio leva ao excesso de degradação, o que se correlaciona com o caráter invasivo das células tumorais (EISSA et al., 2007).

Uma vez que as MMPs degradam o colágeno presente na MEC, há um aumento dos níveis de colágeno degradado, que, por sua vez, parece regular de forma negativa a síntese de pró-colágeno I (FONSECA et al., 2011). Adicionalmente, estudos demonstram que o principal sinal histopatológico de fotoenvelhecimento é o acúmulo demasiado de componentes da MEC degradados (JENKINS, 2002). Fonseca e colaboradores (2011) demonstraram que uma formulação tópica capaz de inibir o aumento de MMPs decorrente da exposição à radiação UV reduz também a inibição da síntese de pró-colágeno e melhora o processo de colagenização.

Entre os diversos tipos de metaloproteinases, a MMP-2 (gelatinase de 92 kDa), e a MMP-9 (gelatinase de 72 kDa), também chamadas de gelatinase A e B, respectivamente, figuram entre as principais na degradação do colágeno da pele e dos componentes da rede de fibras elásticas (JENKINS, 2002). Quando expressas por células epiteliais, particularmente células cancerosas, a principal função da MMP-2 e da MMP-9 é a ruptura da membrana basal por degradantes de colágeno do tipo IV. Estudos demonstram que epitélios lesados do olho, pele, intestino e pulmão expressam MMP-9, que apresenta elevada atividade elastolítica e fibrilina-degradante, (CHEN; PARKS, 2009; JENKINS, 2002).

As fontes celulares das MMPs envolvidas na lesão aguda do tecido ainda não foram completamente elucidadas, porém, sabe-se que os neutrófilos e macrófagos são fontes de MMP-9. Em neutrófilos, a MMP-9 existe pré-formada em grânulos e é liberada por meio da exocitose. Os macrófagos sintetizam a MMP-9 após o estímulo de mediadores pró-inflamatórios, incluindo complexos imunes pré-formados ou lipopolissacarídeos. Embora os leucócitos sejam contribuintes importantes nos distúrbios teciduais agudos mediados por enzimas, células residentes do parênquima também têm a capacidade de sintetizar o mesmo espectro de MMPs (WARNER et al, 2004; WITKO-SARSAT et al., 2000).

A radiação UV também é capaz de estimular neutrófilos e outras células imunes, aumentando seu consumo de oxigênio. Assim, mais EROs são formadas, iniciando com o $O_2^{\bullet-}$ por meio da ativação da Nicotinamida Adenina Dinucleotídeo Fosfato reduzido (NADPH) oxidase. Também é produzido o $ONOO^-$ a partir de O_2 por reação com NO e o $HO^\bullet$ a partir de H_2O_2 na presença de íons de metais de transição. O potencial de neutrófilos para produzir EROs é importante para a destruição de patógenos, mas pode resultar em danos celulares e teciduais (FRANCK et al., 2009).

Uma enzima importante presente nos neutrófilos e que é ativada após exposição à radiação UV é a hemoenzima mieloperoxidase (MPO), que desempenha papel crucial na primeira linha de defesa da resposta imune inespecífica, sendo responsável pela atividade microbicida dos neutrófilos. Esta enzima permanece armazenada dentro de grânulos citoplasmáticos azurófilos, em concentração relativamente elevada de até 5% do peso seco da célula (JANTSCHKO et al., 2005; HACHIYA; OSAWA; AKASH, 2000). Seu mecanismo bactericida consiste na geração de EROs pelos neutrófilos utilizando peróxido de hidrogênio (H_2O_2) e

haletos (X^-) para produzir ácidos hipoalosos (HOX). O oxidante mais poderoso resultante dessas reações é o ácido hipocloroso (HOCl), que é extremamente citotóxico e reage rapidamente com a maioria das moléculas biológicas, promovendo dano inflamatório tecidual (JANTSCHKO et al., 2005).

Apesar do seu papel indispensável na destruição microbiana, a MPO está presente em um número crescente de doenças, como aterosclerose, artrite reumatoide, câncer de pulmão e outras. Produtos típicos de oxidação da MPO foram detectados também em cérebros de pacientes diagnosticados com a doença de Alzheimer e no sistema nervoso central em pacientes com esclerose múltipla (HOY et al., 2002).

1.3 Pimenta pseudocaryophyllus

Considerando a relevância das consequências da exposição cutânea à radiação UV, novos produtos para o tratamento e para a prevenção dos danos cutâneos mediados pelo estresse oxidativo são necessários. Neste contexto, diversos estudos têm estabelecido e caracterizado antioxidantes de fontes naturais para aplicação tópica (MARQUELE-OLIVEIRA et al., 2007; CASAGRANDE et al., 2006; FONSECA et al., 2011; GEORGETTI et al., 2008). O uso de antioxidantes naturais tem sido associado à diminuição da incidência de doenças relacionadas ao estresse oxidativo (MORAIS et al., 2009), atraindo o interesse medicinal nos potenciais efeitos benéficos dessas substâncias. Entre os compostos naturais com relevante potencial AO, destacam-se os polifenois, uma classe de substâncias com elevada diversidade de estruturas, simples e complexas, amplamente distribuída no reino vegetal (CHUN et al., 2005). Graças às suas propriedades redutoras e à sua estrutura química, são capazes de quelar metais de transição envolvidos na catalisação de reações de peroxidação lipídica (LPO) e de sequestrar EROs e outros RL, agindo tanto na etapa de iniciação como na propagação do processo oxidativo (GEORGETTI et al., 2007; ANDRADE et al., 2007; SOUSA et al., 2007).

Pimenta pseudocaryophyllus, conhecida como “pau-cravo”, “cataia”, “louro-cravo” ou “craveiro”, é uma planta presente nos biomas mata atlântica e cerrado. Popularmente, suas folhas são empregadas no preparo de chás utilizados como calmantes e reguladores da digestão, bem como para o alívio de estados gripais (PAULA et al., 2008; PAULA et al., 2005). A *P. pseudocaryophyllus* faz parte da

família Myrtaceae, uma das mais importantes da flora brasileira (cerca de 30% dos representantes da família podem ser encontrados no Brasil) e que possui espécies de grande interesse medicinal (FARIAS et al., 2009). São 26 gêneros e aproximadamente 1000 espécies, com distribuição predominantemente pantropical e subtropical. Essa família possui dois grandes centros de dispersão, um na América e outro na Austrália (PAULA et al., 2008; PAULA et al., 2005).

A atividade AO *in vitro* e *in vivo* de diferentes espécies pertencentes à família Myrtaceae tem sido demonstrada (ARUN et al., 2011; REYNERTSON et al., 2008). Bellosta e colaboradores (2003) demonstraram, por exemplo, a atividade inibitória da MMP-9 em cultura de células pelo extrato de *Tristaniopsis calobuxus*, uma planta pertencente a esta família. Outro estudo demonstrou também a atividade AO *in vitro* de extrato de *Syzygium cumini*, bem como sua atividade inibitória do estresse oxidativo *in vivo*, inibindo o dano genômico, a LPO e a diminuição de GSH, glutationa-S-transferase, SOD e catalase (ARUN et al., 2011).

O gênero *Pimenta* é um dos que merecem destaque. Possui aproximadamente 15 espécies distribuídas na América, estando sua maioria localizada no Caribe (FARIAS et al., 2009). Os trabalhos relacionados ao gênero *Pimenta* são, predominantemente, de caráter fitoquímico e farmacológico, e demonstram as propriedades antibacteriana, antifúngica, antioxidante, anti-inflamatória, anti-hipertensiva, analgésica e antimutagênica de diferentes espécies do gênero, corroborando diversas utilizações etnofarmacológicas do mesmo (FERNÁNDEZ et al., 2001; RAMOS et al., 2003; SAENZ et al., 2004; JIROVETZ et al., 2007; ZABKA; PAVELA; SLEZAKOVA, 2009; PAULA et al., 2010; GARCIA et al., 2004). Já foi demonstrado, por exemplo, que o extrato de folhas da *Pimenta racemosa* apresenta atividade anti-inflamatória, inibindo a formação de edema de orelha e o aumento da MPO induzidos pelo 12-o-tetradecanoilforbol-acetato (TPA) e o edema de pata induzido pela carragenina (GARCIA et al., 2004; FERNÁNDEZ et al., 2001).

Assim como na maioria das plantas do gênero *Pimenta*, as folhas de *P. pseudocaryophyllus* possuem compostos polifenolicos como taninos e flavonoides, conhecidos por sua elevada atividade AO, em alta concentração (PAULA et al., 2008; PAULA et al., 2005; FAJEMIROYE et al., 2012). Os flavonoides representam um dos grupos fenólicos mais importantes e diversificados, apresentando o núcleo flavina, uma estrutura básica ideal para o sequestro de RL e a quelação de metais

de transição envolvidos na catalisação da formação dos RL (figura 1). O núcleo flavina consiste em 15 átomos de carbono arranjados em 3 anéis fenólicos com baixa massa molecular (BARREIROS; DAVID; DAVID, 2006). Esses compostos possuem diversos efeitos biológicos, incluindo anti-inflamatório, antibacteriano e antialérgico. Também inibem a LPO, a agregação plaquetária, a permeabilidade vascular (e consequente edema) e a atividade de sistemas enzimáticos como ciclooxigenase e lipooxigenase, além de interferirem nos processos de metabolismo do ácido araquidônico e na liberação de histamina por mastócitos e basófilos (COOK; SAMMAN, 1996; BONINA et al.,1996).

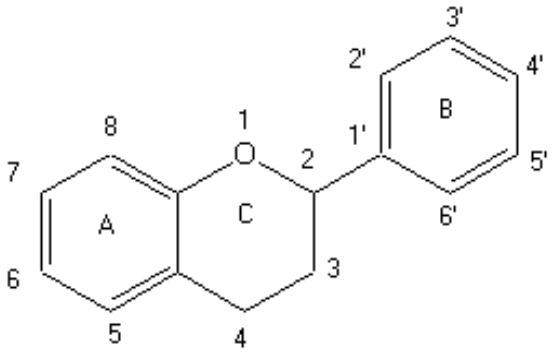

Figura 1: Estrutura básica dos flavonoides.

Taninos são polifenois de massa molecular mais elevada e estrutura mais complexa que os flavonoides. Também apresentam relevante atividade AO e evidências de estudos *in vitro* e *in vivo* apontam que taninos são capazes de inibir a LPO, além de possuírem comprovada atividade anti-inflamatória, antimicrobiana, antiviral, antitumoral e anticâncer (YOSHIDA; HATANO; ITO, 2005; FEDELI et al., 2004). Os taninos são classificados em taninos hidrolisáveis e taninos condensados (figura 2). O primeiro grupo é constituído de misturas de fenóis simples, como pirogalol e ácido elágico, e também de ésteres do ácido gálico ou digálico com açúcares, principalmente glicose. Os taninos condensados são compostos cujas unidades fundamentais são estruturas monoméricas de 2-fenilbenzopiranos com uma estrutura básica de forma C_6-C_3-C_6, como o ácido tânico (VITAL et al., 2004).

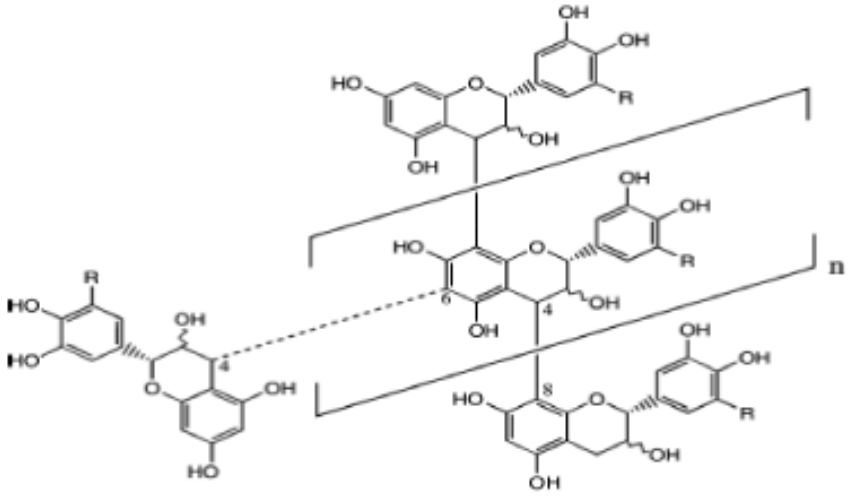

Figura 2: Estrutura básica de um tanino condensado.

Em relação às atividades farmacológicas da *P. pseudocaryophyllus*, recentes estudos *in vivo* demonstraram atividade ansiolítica, antimicrobiana, antinociceptiva e anti-inflamatória desta planta, o que pode ser correlacionado com seu uso popular (FAJEMIROYE et al., 2012; PAULA et al., *in press*).

A *P. pseudocaryophyllus* possui 3 variações: *pseudocaryophyllus*, *fulvescens* e *hoehnei* (PAULA et al., 2010). Apesar de ser a única espécie do gênero nativa do Brasil, (FAJEMIROYE et al., 2012) existem ainda poucos estudos sobre esta planta, tanto *in vitro* quanto *in vivo*. Neste contexto, a avaliação da atividade AO *in vitro* e da possível ação farmacológica da *P. pseudocaryophyllus pod*e representar uma alternativa promissora para a proteção da pele contra danos causados pela radiação UV.

1.4 Desenvolvimento de formulações tópicas

Entre as formas farmacêuticas destinadas à aplicação tópica, destacam-se as emulsões, que são misturas termodinamicamente instáveis que conservam as devidas proporções entre seus componentes e mantêm a superfície interfásica mesmo após serem expostas a tensões decorrentes de fatores como temperatura, agitação e aceleração da gravidade. São formadas por duas fases: a interna (ou dispersa) e a externa (ou dispersante). A estabilidade dessas preparações dá-se pela ação de um agente emulsificante, ou tensoativo, capaz de reduzir a tensão

interfacial entre as duas fases da formulação e de formar membranas protetoras ao redor das partículas, gerando forças repulsivas entre as gotículas (GEORGETTI, 2004; GU; REGNIER; MCCLEMENTS, 2005). Os emulsificantes podem ser aniônicos, catiônicos, não-iônicos ou anfotéricos, dependendo da natureza do grupo da porção hidrofílica da molécula (SCHUELLER; ROMANOWSKI, 1998). Atualmente, bases ou ceras auto-emulsionantes têm sido amplamente utilizadas no preparo de emulsões por possuírem em sua composição uma combinação de tensoativos emulsificantes e componentes graxos (ECCLESTON, 1997).

Apesar da presença dos emulsificantes, devido à natureza instável e dinâmica dessas formulações, as mesmas estão sujeitas à possibilidade de separação de fases, o que depende das condições de temperatura e umidade e do tempo de armazenamento (KALLIOINEN; HELENIUS; YLIRUUSI, 1994; SCHUELLER; ROMANOWSKI, 1998). A desestabilização de emulsões pode ocorrer por diversos mecanismos físico-químicos, como separação gravitacional, floculação, coalescência, hidrólise de tensoativos e destruição microbiana. (GU; REGNIER; MCCLEMENTS, 2005). No caso de preparações que contenham coloides hidrofílicos (géis), por exemplo, os mesmos podem enrijecer e contrair em temperaturas frias e expandir em temperaturas elevadas (KALLIOINEN; HELENIUS; YLIRUUSI, 1994). Entre os processos mais comuns de degradação dos componentes ativos presentes nessas preparações estão as reações de hidrólise, as quais dependem basicamente da temperatura e da quantidade de água disponível no meio (WATERMAN; ADAMI, 2005). Os polifenois, substâncias muito utilizadas como princípio ativo em formulações tópicas, são suscetíveis à ação da temperatura e da umidade, sendo que seu perfil de estabilidade e sua atividade biológica estão fortemente relacionados às condições de processamento e de armazenamento (UNGAR; OLUWATOOYIN; SHIMONI, 2003).

Assim, a avaliação da estabilidade de formulações e de seus princípios ativos armazenados sob diferentes condições ambientais durante determinados períodos de tempo constitui uma etapa importante durante o desenvolvimento de novos produtos. Esta avaliação fornece informações sobre o prazo de validade e sobre as condições ideais de armazenamento (CASAGRANDE et al., 2006b). De acordo com a International Federation of Societies of Cosmetic Chemists (IFSCC), o estudo de estabilidade é um procedimento preditivo, baseado em dados obtidos após o armazenamento de produtos em condições que visam a acelerar possíveis

alterações nas formulações. A estabilidade tanto da substância ativa quanto da formulação à qual será incorporada são parâmetros críticos para o preparo de qualquer produto farmacêutico (WATERMAN; ADAMI, 2005). A exposição do produto a temperaturas elevadas permite a aceleração dos processos que influenciam propriedades envolvidas na instabilidade, como viscosidade, partição dos emulsionantes entre as fases, inversão de fases e cristalização lipídica. Um dos testes utilizados em estudos de estabilidade é a centrifugação, na qual a aceleração da gravidade acelera a possível separação de fases da emulsão, proporcionando rápidas informações sobre a estabilidade das preparações. Normalmente avalia-se também o pH das formulações para assegurar-se de que o mesmo encontra-se estável e compatível com os outros componentes utilizados e com o local de aplicação. A verificação da estabilidade funcional do produto, ou seja, da manutenção da atividade pretendida, torna-se relevante, uma vez que as substâncias ativas podem interagir com o veículo, influenciando na solubilidade destes componentes, na sua difusão pelo veículo e na sua partição do veículo para a pele (GEORGETTI, 2004).

Na aplicação tópica de uma substância diversos estágios devem ser considerados para que a mesma atinja o local desejado. Inicialmente, a substância ativa deve ser capaz de difundir a partir da formulação e alcançar a pele (GEORGETTI, 2004). Nesse processo, características como coeficiente de partição, concentração e solubilidade do composto ativo na emulsão desempenham papel fundamental. Assim, os estudos de liberação são bastante utilizados, pois avaliam a capacidade da substância em ser liberada do veículo, tornando-se disponível para penetração no estrato córneo. Esses estudos servem para a avaliação inicial de formulações experimentais na área de desenvolvimento de produtos e podem também servir para avaliar a biodisponibilidade (GETIE et al., 2002; GEORGETTI, 2004). Os estudos de liberação normalmente utilizam membranas sintéticas que funcionam como barreiras porosas que permitem a difusão passiva (a taxa de difusão é determinada pela concentração do soluto) (PAGLIARA et al., 1999).

2 OBJETIVOS

2.1 Objetivos gerais

O presente estudo teve como objetivo avaliar a composição química do PPE e os mecanismos de ação AO do mesmo *in vitro*, bem como avaliar a sua liberação e o seu efeito terapêutico quando veiculado a formulações tópicas em modelo de estresse oxidativo cutâneo/ inflamação induzida por radiação UVB.

2.2 Objetivos específicos

- Caracterizar quimicamente o PPE por meio da determinação do teor de flavonoides e de polifenois totais;
- Identificar os compostos fenólicos majoritários presentes no PPE por Cromatografia líquida de alta eficiência (CLAE);
- Avaliar a atividade AO do extrato utilizando os seguintes métodos *in vitro*: capacidade doadora de átomos de hidrogênio ao radical 2,2-difenil-1-picrilhidrazil (DPPH), capacidade sequestradora do radical 2,2' azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), poder redutor férrico (FRAP);
- Preparar duas formulações tópicas com diferentes conteúdos lipídicos contendo PPE;
- Avaliar as características físicas e físico-químicas das formulações adicionadas do extrato;
- Avaliar a manutenção da atividade AO do extrato após incorporação nas formulações utilizando-se as mesmas metodologias realizadas para o PPE;
- Realizar estudo de estabilidade submetendo-se as formulações a diferentes condições de temperatura e umidade;
- Avaliar a liberação *in vitro* de compostos com atividade AO presentes no PPE a partir das formulações;
- Avaliar o efeito terapêutico tópico das formulações contendo o extrato em modelo de estresse oxidativo/ inflamação cutânea induzida por radiação UVB, com enfoque na diminuição do edema cutâneo; diminuição do infiltrado leucocitário (mensurado pela atividade de MPO); aumento da produção de citocina anti-inflamatória (dosagem de IL-10); inibição da produção de citocinas pró-inflamatórias (dosagem

de TNF-α e IL-1β); diminuição da atividade/secreção de MMP-9; aumento dos níveis do AO endógeno GSH; aumento da capacidade sequestradora do radical ABTS e do poder antioxidante redutor férrico (FRAP).

3 MATERIAL E MÉTODOS

3.1 Material

3.1.1 Reagentes

Ácido tânico, 2,2-difenil-1-picrilhidrazil (DPPH), 2,2' azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), ácido gálico, o-dianisidina, ácido etileno glicol tetracético (EGTA), brometo de hexadecil trietil amônio (HTAB), corante azul brilhante, fenantrolina, N-etilmaleimida, fluoreto de fenilmetilsulfonila, o-ftalaldeído (OPT), 2,4,6 tripiridil-S-triazina (TPTZ) e glutationa reduzida (GSH) foram obtidos da Sigma Chemical Co. (St. Louis, MO, USA). Os solventes de grau HPLC foram obtidos da Panreac (Barcelona, Spain). Kits de ELISA para dosagem das citocinas IL-1β, IL-10 e TNF-α foram obtidos da eBioscience. Eugenol foi obtido da Vetec (Rio de Janeiro, Brazil). Quercetin-3-*O*-rutinoside (rutina) e quercetina foram obtidos da Acros (New Jersey, USA). Folin-Ciocalteau foi obtido da Fluka Chemical Co. (Buchs, Switzerland). Xileno cianol foi obtido da Amresco. Polawax® e Carbopol® foram obtidos da Galena Química e Farmacêutica, enquanto a trietanolamina e os triglicerídeos de ácidos cáprico e caprílico foram obtidos da CQA Química. O propilenoglicol foi obtido da Chemco LTDA. Todos os outros reagentes utilizados foram de grau analítico.

3.1.2 Equipamentos

● Agitador mecânico, Fisatom®; ● Agitador Orbital, modelo 255, Fanem®; ● Balança analítica, modelo HR-120, And®, precisão de 4 casas; ● Banho, modelo 314/2 DN, Nova Ética®; ● Banho de água com temperatura controlada, modelo Banho Maria 100, Fanem®; ● Centrífuga, modelo Baby I 206-BL**,** Fanem®; ● Centrífuga refrigerada, modelo Rotina 46 R, Hettich Zentrifugen®; ● Compartimento de madeira projetado para irradiação; ● Deionizador de água, modelo Purebal Option-Q, Elga®; ● Espectrofotômetro, modelo Evolution 60, Thermo Scientific®; ● Espectrofotômetro de fluorescência, modelo RF-5301PC, Shimadzu®; ● Espectrofotômetro, modelo Helios alfa, Thermo Spectronic®; ● Estufa 0-120 °C, De LEO & Cia®; ● Fonte elétrica para eletroforese, modelo MS

300V, Major Science®; ● Homogeneizador de tecidos Ultra Turrax, modelo T18 basic, IKA®; ● Lâmpada ultravioleta fluorescente, modelo PHILIPS TL/12 40W RS-UVB, MedicalHoland®; ● Leitor de microplaca, modelo Asys Expert Plus, Biochrom®; ● Leitor de microplaca, modelo Victor X3, Perkin Elmer®; ● Pipetas automáticas 20, 100, 200 e 1000 M, modelo LabMate, HTL®; ● pHmetro, modelo Tec-3MP, Tecnal®; ● Radiômetro IL 1700 Research Radiometer. Detectores: SED240 – filtro UVB (290 nm), SED005 – filtro UV (350 nm); SED033 – filtro TRED (633 nm), International Light-USA; ● Sistema de eletroforese Mini Vertical, modelo Mini-Protean® Tetra System, Bio-RAD®; ● Triturador, modelo Tissue-Tearor, Biospec®; ● Ultra-som, modelo TSO, Thornton; ● Sistema de CLAE Shimadzu®. Detector SPD-M10Avp, sistema de controle de forno CTO-10ASvp. Software Classe VP 6.14; ● Coluna de fase reversa analítica Spherisob® (C-18 ODS) (250 x 4.6 mm i.d.; tamanho de particula 5 µm - Waters®); ● Membrana de nitrocellulose 0,45 µm, 47 mm, modelo HAWP04700 - Millipore®.

3.1.3 Material vegetal

O material vegetal foi obtido por coleta realizada em fragmento de vegetação, localizada no Município de São Jerônimo da Serra, Paraná, Brasil. O material testemunho foi depositado no Herbário da Universidade Estadual de Londrina sob número FUEL 43025.

3.2 Métodos

3.2.1 Obtenção do PPE

O PPE foi obtido no Laboratório de Pesquisa de Moléculas Bioativas do Departamento de Química, Centro de Ciências Exatas da Universidade Estadual de Londrina (UEL), localizada em Londrina, Paraná, Brasil. As folhas de *P. pseudocaryophyllus* foram secas em estufa a 40°C e trituradas utilizando-se liquidificador industrial. Em seguida, o PPE (1:10) foi obtido por maceração exaustiva à temperatura ambiente por 12 dias. A mistura resultante foi filtrada e concentrada à metade em evaporador rotatório.

3.2.2 Caracterização química do PPE

3.2.2.1 Determinação do conteúdo de polifenois totais

O conteúdo de polifenois totais foi determinado por método colorimétrico, empregando-se como reagente o Folin-Ciocalteau e o ácido gálico como padrão (curva de 4,0 a 24,0 µg/mL) (KUMATZAWA; HAMASAKA; NAKAYAMA, 2004; GEORGETTI et al., 2006). O PPE foi diluído 1:200 em etanol 80%. Misturou-se 0,5 mL das amostras do extrato com 0,5 mL do reagente Folin-Ciocalteau e 0,5 mL de Na_2CO_3 10%. O branco foi preparado com 0,5 mL de etanol 80%, 0,5 mL do reagente Folin-Ciocalteau e 0,5 mL de Na_2CO_3 10%. Para eliminar a possibilidade da existência de interferentes de cor do extrato, utilizou-se um controle com 0,5 mL da amostra, 0,5 mL de água deionizada e 0,5 mL do reagente Folin-Ciocalteau. Após 1 h de incubação à temperatura ambiente, a absorvância foi determinada a 760 nm. As amostras foram analisadas em triplicata e o conteúdo total de polifenois foi calculado em mg/g de extrato, considerando a quantidade de sólidos totais do extrato (25 mg/mL).

3.2.2.2 Determinação do conteúdo de flavonoides totais

O conteúdo de flavonoides totais presentes no PPE foi determinado por método colorimétrico de complexação utilizando-se o cloreto de alumínio conforme descrito por Georgetti e colaboradores (2006). Para o preparo das amostras realizou-se diluição 1:25 do extrato em etanol 80%. Alíquotas de 0,5 mL de cada amostra do extrato foram misturadas a 0,5 mL de $AlCl_3$ 2%, preparado com etanol 80%. O branco foi constituído de 0,5 mL de etanol 80% e 0,5 mL de $AlCl_3$ a 2%. Para eliminação de interferentes de cor do extrato, foi realizado um controle utilizando-se 0,5 mL de amostra e 0,5 mL de etanol 80%. Após 1 hora de incubação à temperatura ambiente, a absorvância foi determinada a 420 nm. O ensaio foi realizado em triplicata e o conteúdo de flavonoides foi expresso em mg/g de extrato, considerando-se a quantidade de sólidos totais do extrato (25 mg/mL) e utilizando-se uma curva analítica de quercetina como padrão (6,0 – 30,0 µg/mL).

3.2.2.3 Caracterização do PPE por CLAE

Para a identificação dos principais componentes do PPE, utilizou-se coluna C-18 ODS (5 µm), 250 x 4,6 mm, acoplada a uma pré-coluna da mesma fase estacionária. A fase móvel constituiu-se de um gradiente de concentração de acetonitrila (solvente A) e água (solvente B), ambas acidificadas (2% de ácido fórmico) (Tabela 1), na vazão de 1 mL/min e temperatura de 28º C. O volume de injeção foi de 20 µL e a detecção foi realizada em 280 e em 370 nm (RIEGER, 2011).

Preparou-se uma solução do extrato na concentração de 1,25 mg/mL em água e soluções dos padrões eugenol, quercetin-3-*O*-rutinoside (rutina) e ácido tânico foram preparadas na concentração de 2,0 µg/mL em solução contendo 95% de água e 5% de metanol. Anteriormente à injeção, as amostras foram sonicadas e filtradas (0,45 µm).

Tabela 1- Gradiente de concentração da fase móvel utilizada nos ensaios por CLAE

Tempo (min)	% A	% B
0,01	0	100
5	0	100
20	2,5	97,5
30	5	95
50	15	85
60	25	75
65	30	70
70	45	55
75	50	50
80	70	30
85	90	10
90	100	0
95	100	0
110	0	100

3.2.3 Avaliação do mecanismo de atividade antioxidante do PPE

3.2.3.1 Determinação da atividade sequestradora do radical DPPH pelo PPE

O DPPH é um radical livre estável que potencialmente reage com compostos capazes de doar um átomo de hidrogênio. A atividade sequestradora do radical pelo PPE foi determinada pela mudança colorimétrica medida espectrofotometricamente em 517 nm. Essa mudança ocorre devido à redução do radical DPPH (BLOIS, 1958; CASAGRANDE et al., 2007).

Para a medida do sequestro do radical livre, 20 µL do extrato em diferentes concentrações foram adicionados à mistura reacional contendo 1 mL de tampão acetato 0,1M (pH 5,5), 1 mL de etanol e 0,5 mL de solução etanólica de DPPH 250 µM. As concentrações de PPE obtidas no meio reacional foram de 1 a 20 µg/mL (baseando-se no conteúdo de sólidos totais do extrato de 25 mg/mL). A mudança na absorvância foi medida espectrofotometricamente após 15 minutos de incubação à temperatura ambiente. As amostras foram analisadas em triplicata. O branco foi constituído de 1 mL de tampão acetato 0,1M (pH 5,5) e 1,5 mL de etanol. O controle positivo não continha amostra; assim, indica o máximo de elétrons livres do DPPH, o qual é considerado 100% de RL na solução para calcular-se a capacidade doadora de hidrogênio (%) do extrato por meio da seguinte equação:

% Atividade = (1 - absorvância da amostra/absorvância do controle) x 100 (1)

3.2.3.2 Determinação da atividade sequestradora do radical ABTS pelo PPE

A capacidade sequestradora do radical livre ABTS é medida pela supressão da cor do radical devido à diminuição do mesmo no meio e consequente queda de absorvância, quando substâncias antioxidantes são adicionadas (MUNTEANU et al., 2007). O método foi realizado de acordo com Sánchez-Gonzalez e colaboradores (2005), com algumas modificações. A solução ABTS foi preparada em meio aquoso e o cátion ABTS foi obtido após a reação de 7 mM da solução estoque ABTS com 2,45 mM de persulfato de potássio. A mistura foi armazenada em geladeira por 12-

16 h antes do uso. A solução ABTS foi diluída com tampão fosfato (pH 7,4, 0,1 M) até atingir uma absorvância de 0,7 a 0,8 em 730 nm.

Alíquotas de 50 µL das amostras foram adicionadas a 4 mL da solução ABTS diluída, obtendo-se no meio reacional concentrações de PPE de 0,625 a 15,625 µg/mL (considerando-se o conteúdo de sólidos totais do extrato de 25 mg/mL). As leituras foram realizadas espectrofotometricamente a 730 nm após 6 minutos de reação e a capacidade AO foi calculada conforme equação 1. Foi realizado um controle positivo adicionando-se 50 µL de etanol absoluto a 4 mL da solução ABTS diluída. O ensaio foi realizado em triplicata.

3.2.3.3 Determinação do poder antioxidante redutor férrico (FRAP) do PPE

O poder AO de redução férrico do PPE foi avaliado de acordo com Sánchez-Gonzalez e colaboradores (2005), com algumas modificações. O reagente FRAP foi preparado com 2,5 mL de uma solução de 2,4,6 tripiridil–S-triazina (TPTZ) (10 mM) em HCl (40 mM), 2,5 mL de solução de $FeCl_3(6H_2O)$ e 25 mL de tampão acetato (pH 3,6; 0,3 mM). A solução foi incubada a 37°C por 30 minutos.

Para a avaliação da capacidade antioxidante, 900 µL do reagente FRAP preparado previamente foram adicionados de 90 µL de água destilada e de 10 µL do extrato, obtendo-se no meio reacional concentração de PPE de 1,56 µg/mL, baseando-se no conteúdo de sólidos totais do extrato (de 25 mg/mL). As amostras foram incubadas a 37°C por 30 minutos e a leitura foi realizada espectrofotometricamente a 595 nm. Uma curva analítica com diferentes concentrações de trolox (4,0; 8,0; 10,0; 12,5 e 20,0 µM) foi utilizada para posterior cálculo dos resultados em µmol/L equivalente de trolox/µg/mL de extrato. Foi realizado um controle positivo adicionado de etanol. O ensaio foi realizado em triplicata.

3.2.4 Desenvolvimento das diferentes formulações contendo PPE

Uma vez que os princípios ativos possuem características químicas e físicas diferentes e que emulsões fluidas e semi-sólidas possuem propriedades coloidais que podem influenciar a biodisponibilidade do fármaco, ressalta-se a importância da utilização de formulações com diferentes proporções de conteúdo aquoso e lipídico

na avaliação da atividade AO (BARRY, 1983). Assim, foram preparadas formulações tópicas com variação de conteúdo lipídico, adicionadas ou não de PPE. Os componentes utilizados em cada formulação estão expressos na Tabela 2.

A emulsão não-iônica com elevado conteúdo lipídico (formulação 1 – F1) foi preparada com a cera auto-emulsionante Polawax® (álcool cetoestearílico + polioxietileno derivado de éster de ácido graxo de sorbitano 200E). A formulação com baixo conteúdo lipídico (formulação 2 – F2) foi estabilizada com coloide hidrofílico aniônico (carboxipolimetileno, Carbopol®). Triglicerídeos (TG) de ácidos cáprico e caprílico foram adicionados como emolientes, enquanto o propilenoglicol foi usado como agente umectante. O sistema conservante utilizado foi uma mistura de parabenos. Água deionizada foi utilizada no preparo das formulações.

Tabela 2- Componentes das formulações utilizadas nos experimentos

Matérias-primas	%	
	F1	**F2**
A) Polawax®	10,0	2,0
TG de ácidos cáprico e caprílico	5,0	5,0
B) Carbopol® 940 (dispersão 3,0%)	-	6,0
Propilenoglicol	5,0	5,0
Trietanolamina	-	0,2
Água deionizada q.s.p.	100	100
C) Solução de metil (10%) e propilparabeno (2%)	1,0	1,0

q.s.p.: quantidade suficiente para

As fases A e B foram aquecidas a 70°C e misturadas a esta temperatura sob constante agitação em agitador Fisatom 713 D (650 rpm) até resfriamento à temperatura ambiente, quando foi adicionada a fase C. Após 24 h, com auxílio de gral e pistilo, o PPE foi incorporado ou não às formulações na concentração de 5%.

3.2.4.1 Avaliação das características físico-químicas das formulações

Para a avaliação das características físico-químicas, os seguintes testes foram realizados: avaliação visual (cor, consistência e separação de fases) (CASAGRANDE et al., 2009); medição do pH em triplicata em pHmetroTec-3MP TECNAL® (diluição a 10% das formulações em água deionizada); teste de centrifugação em triplicata (2 g de formulação submetidos à centrifugação a 3000 rpm por 30 minutos) (GEORGETTI et al., 2006; MAIA; BADRA, 1999; ANCHISI et al., 2001), que observa a possível separação de fases da formulação.

3.2.4.2 Avaliação da atividade antioxidante do extrato após incorporação nas formulações.

As formulações foram analisadas quanto à ação AO pelos métodos DPPH (item 3.2.3.1), ABTS (item 3.2.3.2) e FRAP (item 3.2.3.3). Assim, as diferentes formulações, adicionadas ou não de 5% de PPE, foram diluídas 1:10 em etanol e agitadas por 15 minutos. Após, a atividade AO das formulações foi comparada à do PPE na mesma concentração final de 5,0 µg/mL, 3,125 µg/mL e 2,5 µg/mL no meio reacional para avaliação da atividade sequestradora de radical livre DPPH, atividade sequestradora do radical ABTS e poder redutor FRAP respectivamente. Foram realizados controles positivos utilizando-se etanol e formulações sem o PPE. Os ensaios foram realizados em triplicata.

3.2.4.3 Estudo da estabilidade do PPE e das formulações tópicas adicionadas de PPE

As formulações e o PPE foram armazenados a 4º C, temperatura ambiente (25ºC) e 40±2ºC/ 75±5% umidade relativa (UR) por 6 meses (CASAGRANDE et al., 2009). Em intervalos de tempo pré-determinados (0, 30, 60, 90 e 180 dias) alíquotas foram coletadas e analisadas. A estabilidade físico-química das formulações foi determinada pelos testes descritos no item 3.2.4.1 e a estabilidade funcional do extrato e das formulações foi verificada pelo método DPPH conforme descrito no item 3.2.4.2.

3.2.4.4 Estudo de liberação cutânea in vitro do PPE veiculado às formulações tópicas

Para o estudo, foram utilizadas 6 células de difusão de Franz modificadas, sendo que 4 células foram utilizadas com formulações adicionadas de extrato e 2 delas com formulações controle (sem extrato). As análises foram realizadas em duplicata. As células de difusão são divididas em dois compartimentos, um doador e outro receptor (figura 3). Entre os dois compartimentos (área de 1,77 cm^2), colocou-se uma membrana artificial (nitrocelulose, 0,45 µm). 1 g de cada formulação foi disposto uniformemente sobre a membrana sintética e o compartimento receptor foi preenchido com 16 mL de solução receptora (tampão fosfato (pH 7,4, 0,1 M) com 10% de etanol). O compartimento receptor é composto de uma abertura para amostragem, um tubo coletor e portas para banho circulante que permitem que a temperatura da solução seja mantida a 37°C. As células de difusão permaneceram dispostas em uma placa magnética que promoveu agitação constante do meio a 100 rpm.

Amostras da solução receptora (1 mL) foram coletadas manualmente, com o auxílio de uma seringa graduada, após 12 h. A liberação dos componentes com atividade AO do extrato adicionado às formulações foi avaliada utilizando-se o ensaio de DPPH como descrito no item 3.2.3.1. Os resultados foram expressos na forma de porcentagem de PPE liberado pelas formulações. Todas as medidas foram realizadas em duplicata e formulações sem PPE foram utilizadas como controle.

Figura 3: Célula de difusão de Franz.

3.2.5 Avaliação da eficácia in vivo das formulações contendo PPE na prevenção dos danos induzidos por radiação UVB

3.2.5.1 Animais experimentais

Foram utilizados camundongos sem pelo, machos ou fêmeas, adultos, com peso médio de 30 g, linhagem HRS/J. Os animais foram mantidos no biotério do Hospital Universitário (UEL) em sala com temperatura controlada, ciclo claro/escuro de 12 h e com suprimento de água e ração *ad libitum*. Os experimentos foram conduzidos conforme as normas da Comissão de Ética no Uso de Animais da UEL (protocolo número 34994/209).

3.2.5.2 Sistema e fonte de radiação UVB

A fonte de luz utilizada nos experimentos de indução do estresse oxidativo/ inflamação foi uma lâmpada de luz UVB fluorescente modelo PHILIPS TL/12 40W RS (Medical Holand). A lâmpada emite radiação na faixa de λ de 270 a 400 nm com pico máximo de emissão em torno de 313nm (http://www.ecat.lighting.philips.com.br/l/lampadas/uv/60756/cat/#).

A fonte de luz UVB foi instalada em um compartimento de madeira desenvolvido para os experimentos, localizada a uma distância de 20 cm dos animais. Posicionaram-se 5 caixas plásticas com os diferentes grupos de animais. A parte superior das caixas plásticas foi coberta com uma tela plástica (1 mm de espessura, com rede de malha de 2 mm x 2 mm) para que os animais permanecessem dentro das caixas e para evitar que ficassem sobre duas patas durante a indução, garantindo que a exposição à luz UVB ocorresse diretamente na região dorsal dos mesmos. Os animais movimentaram-se livremente na caixa. Devido às variações de intensidade de radiação ao longo da lâmpada, as posições das caixas foram modificadas periodicamente durante o experimento.

3.2.5.3 Medida da irradiância da lâmpada UVB

A irradiância é a medida da taxa do fluxo de energia em watts (W) (ou J/seg) por unidade de área, ou seja, W/cm^2. A dose de exposição em J/cm^2 é obtida multiplicando-se a irradiância (W/cm^2) pelo tempo de exposição em segundos. Para a medida da irradiância, utilizou-se um radiômetro IL 1700 (Newburyport, MA, USA) equipado com detectores para radiação UV (SED005) e especialmente para UVB (SED240). A medida da irradiância foi realizada em toda a extensão do compartimento, à distância de 20 cm e com a presença da tela plástica utilizada para conter os animais. A dose de exposição (J/cm^2) foi obtida pela multiplicação da irradiância (W/cm^2) pelo tempo de exposição em segundos (CASAGRANDE et al., 2006).

3.2.5.4 Protocolo de avaliação da eficácia das formulações contendo PPE

Após determinação do tempo de irradiação necessário para indução dos danos decorrentes da exposição à radiação UVB (dados não mostrados), foram realizados os tratamentos dos animais com as formulações contendo o PPE.

Os experimentos foram conduzidos com n= 5 animais por grupo e foram repetidos 2 vezes. Os grupos foram os seguintes:

- Grupo 1: controle (não recebeu irradiação)
- Grupo 2: irradiado sem tratamento
- Grupo 3: irradiado tratado com F1 controle (sem PPE)
- Grupo 4: irradiado tratado com F1 contendo PPE
- Grupo 5: irradiado tratado com F2 controle (sem PPE)
- Grupo 6: irradiado tratado com F2 contendo PPE

Os animais foram tratados com 0,5 g de formulação administrada com o auxílio de um pincel no dorso do animal (área de aproximadamente 24,5 cm^2) 1 h antes do início da irradiação, no momento da irradiação e logo após a irradiação. A dose de irradiação utilizada foi de 4,14 J/cm^2 e a eutanásia dos animais foi realizada por meio de anestesia terminal com éter etílico 12 h após o término da irradiação.

As peles dos animais foram limpas com auxílio de algodão e de água deionizada para total retirada das formulações presentes na superfície cutânea. Após, as peles foram retiradas e preparadas conforme as metodologias utilizadas, descritas a seguir.

3.2.5.5 Avaliação de parâmetros inflamatórios cutâneos

3.2.5.5.1 Avaliação do edema cutâneo

Uma das consequências da exposição à radiação UV na pele é a inflamação, caracterizada por, entre outros fatores, aumento da permeabilidade vascular com extravasamento de líquido para o interstício, na epiderme, causando o edema. (KVIETYS; GRANGER, 2012).

A eficácia das formulações contendo PPE no edema induzido por radiação UVB foi verificada observando-se o aumento da espessura da pele, mensurado pela diferença de peso entre os grupos irradiados e o grupo não irradiado. Com o auxílio de um molde, foi coletada uma área constante de pele do dorso de cada animal, que posteriormente foi pesada. O resultado foi expresso em g de pele (AFAQ et al., 2005).

3.2.5.5.2 Avaliação da atividade de MPO

A MPO é uma enzima peróxido de hidrogênio redutase encontrada em grande quantidade em grânulos de monócitos e de polimorfonucleares, como neutrófilos, eosinófilos e basófilos (ARNHOLD; FLEMMIG, 2010). A migração de neutrófilos na pele induzida pela radiação UVB foi verificada pelo método cinético-colorimétrico da avaliação da atividade da MPO. Neste método, a MPO catalisa a oxidação do substrato o-dianisidina, resultando em um composto colorido que é detectado espectrofotometricamente em 540 nm. O ensaio para dosagem de MPO permite inferir indiretamente o número de neutrófilos totais/mg de tecido cutâneo (CASAGRANDE et al., 2006, BRADLEY et al., 1982).

As amostras de pele dos animais foram coletadas em tubos *eppendorf* contendo 400 µL de tampão fosfato 0,05 M (pH 6,0) com 0,5% de brometo de hexadecil trietil amônio (HTAB). Para a realização do ensaio, as amostras foram homogeneizadas com auxílio de triturador Tissue-Tearor (Biospec®) a 16100 *g* durante 2 minutos.

Em microplaca de 96 poços, foi feita uma curva analítica com quantidades crescentes de neutrófilos variando de 97,65 a 100.000 neutrófilos por poço. Essa

curva foi posteriormente utilizada para o cálculo do número de neutrófilos presentes nas amostras. Para a realização da curva analítica, adicionaram-se 100 µL de tampão fosfato 0,05 M (pH 6,0) no 1° poço e 50 µL do mesmo tampão do 2º ao 11° poço. Após, foram adicionados 200.000 neutrófilos no 1° poço e transferiu-se metade da solução presente no poço para o poço seguinte, repetindo este procedimento de diluição até o 11° poço. No 12° foram adicionados 200 µL de água deionizada, sendo utilizado como o branco da reação. Em seguida, foram pipetados 30 µL de amostra em cada poço no restante da microplaca. A reação colorimétrica inicia-se pela adição de 200 µL de uma solução contendo 10% de tampão fosfato 0,05 M (pH 6,0), 0,0167% de o-dianizidina e 0,015% de H_2O_2 em água deionizada. Esta solução não é adicionada no branco. A leitura foi realizada em até 5 minutos depois da reação em 450 nm, utilizando-se espectrofotômetro para placa de poços (Victor X3, Perkin Elmer®).

3.2.5.5.3 Avaliação da produção de TNF-α, IL-1β e IL-10

As amostras de pele dos animais foram coletadas em tubos *eppendorf* contendo 500 µL de solução NaCl 0,9%. Para a realização do ensaio, as amostras foram homogeneizadas com auxílio de triturador Tissue-Tearor (Biospec®). Em seguida, foram centrifugadas a 1455 *g* por 15 minutos a 4º C e o sobrenadante foi retirado para análise.

A quantificação das citocinas TNF-α, IL-1β e IL-10 foi realizada por meio de técnica de enzima imunoensaio (ELISA) com kits comerciais (eBioscience). Microplacas com 96 poços foram incubadas durante a noite a 4° C com anticorpos de captura contra as proteínas de interesse (10 µg/mL). No dia seguinte, as placas foram lavadas com tampão I (tampão fosfato salino (PBS), pH 7,0, com 0,05% de tween 20) e incubadas por 1 h com uma solução a 1% de albumina bovina. Após esse bloqueio e lavagem das placas com tampão I, a curva padrão e as amostras foram adicionadas (100 µL) e incubadas a 4° C por 12 h. Após incubação, lavaram-se as placas com tampão I e os anticorpos policlonais biotinilados (anticorpos de detecção) foram adicionados (100 µL). Após incubação a temperatura ambiente por 1 h, as placas foram lavadas com tampão I e 100 µL de avidina-peroxidase foram adicionados. Após 30 minutos de incubação a placa foi lavada e adicionaram-se 100 µL do reagente contendo 0,04% de ortofenilenodiamina, 0,04% de H_2O_2 em tampão,

a placa foi mantida no escuro em temperatura ambiente por 15 min. A reação enzimática foi interrompida com uma solução de ácido ortofosfórico (H_3PO_4) (1 M) e as absorvâncias determinadas em 450 nm (Victor X3, Perkin Elmer).

A curva padrão foi utilizada para inferir o número de cada citocina presente na amostra e os resultados foram expressos em picogramas (pg) de cada citocina/mg de pele (SAFIEH-GARABEDIAN et al., 1995; VERRI et al., 2007).

3.2.5.5.4 Avaliação da atividade/secreção de MMP-9

A zimografia é um método amplamente utilizado para a detecção de proteases, consistindo em análise qualitativa da atividade por meio da degradação da gelatina adicionada ao gel de eletroforese (KIM et al., 2007). As amostras de pele dos animais foram coletadas em tubos *eppendorf* e, *p*ara a determinação da atividade/ secreção de proteinases, foi realizado um *pool* das amostras de cada grupo de animais. Primeiramente, as peles dos animais foram diluídas 1:4 em tampão tris/HCl 50 mM (pH 7,4) contendo cloreto de cálcio ($CaCl_2$) 10 mM e 1 % de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida) e homogeneizadas com auxílio de Ultra Turrax (T 18 basic, IKA®) durante 2 minutos. Em seguida, as amostras foram duplamente centrifugadas a 12.100 *g* por 10 minutos a 4°C e a fração sobrenadante foi utilizada nos experimentos.

Após, dosou-se a quantidade de proteínas de cada amostra pelo método de Lowry (LOWRY et al., 1951) e então o preparo das amostras foi realizado adicionando-se 50 µL de sobrenadante e 10 µL de tampão Tris/HCl 100 mM (pH 6,8) contendo 4% de SDS, 20% de glicerol e 0,005% de xileno cianol. Esta mistura permaneceu em banho-maria a 37º C durante 8 minutos imediatamente antes de ser aplicada no gel. A placa de gel para a eletroforese foi montada com um gel de concentração e 3 cm de gel de separação. Os constituintes dos géis de separação e de concentração estão expressos na Tabela 3.

Tabela 3 - Constituintes do gel de separação e do gel de concentração.

Substância	Quantidade (µL)	
	Gel de separação	Gel de concentração
Água miliQ	5870	4060
Tampão Tris/HCl 1 M (pH 8,8) com 0,4% SDS	3750	-
Tampão Tris/HCl 0,5 M (pH 6,8) com 0,4% SDS	-	1670
Acrilamida: bis-acrilamida (30:0,8)	5000	860
Gelatina 10%	375	-
Persulfato de amônio 10%	50	33
Temed 20%	10	6,6

O interior da cuba de eletroforese foi preenchido com tampão Tris/glicina 190 mM (pH 8,3) contendo 0,1% de SDS. Antes da aplicação das amostras, o gel foi submetido a uma pré-corrida de 15 minutos a 12 mA. Após, foram aplicados 25 µL de cada amostra. Durante a eletroforese, a corrente aplicada foi de 12 mA no gel de concentração e de 15 mA no gel de separação, sendo esta corrente mantida constante durante 15 minutos após a saída do corante do gel de separação.

Ao término da eletroforese, o gel de poliacrilamida foi lavado em solução de Triton X-100 2% durante 1 h sob constante agitação. Em seguida, o gel foi incubado por 16 h a 37°C em tampão Tris/HCl 50 mM (pH 7,4) contendo 10 mM de $CaCl_2$ e 0,02% de azida sódica (FONSECA et al., 2010).

Para a coloração do gel, após o término da incubação o mesmo permaneceu mergulhado na solução corante contendo 0,25% de azul brilhante, 10% de ácido acético e 50% de metanol em água deionizada. Após, realizou-se a descoloração do gel com solução de ácido acético 20%, quando então a atividade proteolítica pôde ser observada (FONSECA et al., 2010). A análise semi-quantitativa das bandas foi realizada por comparação das diferenças de densidades de cor entre as bandas de cada grupo pelo programa Image J®.

3.2.5.6 Avaliação dos parâmetros antioxidantes cutâneos

3.2.5.6.1 Avaliação dos níveis do antioxidante endógeno GSH

GSH é um importante sistema AO endógeno cuja atividade é sensível ao estresse oxidativo causado por radiação UVB. Assim, estudos confirmam que o estado redox do GSH funciona como um sensor do estresse oxidativo epidermal induzido por radiação UVB, sendo adequado para testar o potencial efeito AO protetor de uma substância de interesse (FONSECA et al., 2011).

As amostras de pele dos animais foram coletadas em tubos *eppendorf* e posteriormente picotadas e diluídas 1:3 em tampão fosfato de sódio monobásico (NaH_2PO_4) 0,1 M (pH 8,0) contendo EGTA 5 mM. Em seguida, foram trituradas em Ultra Turrax (T 18 basic, IKA®) durante 2 minutos e adicionaram-se 200 µL de ácido tricloroacético 30% para cada 1 mL de tampão previamente adicionado. As amostras foram centrifugadas a 1940 *g* por 6 minutos a 4º C e em seguida a 485 *g* por 10 minutos a 4º C, transferindo-se o sobrenadante para tubos *eppendorf*. Ao final, as amostras estavam na concentração de 300 mg de tecido/mL.

Para determinação dos níveis de GSH, 100 µL de amostra foram adicionados a 1 mL de tampão NaH_2PO_4 0,1 M (pH 8,0) contendo EGTA 5 mM. Em seguida adicionaram-se 100 µL de solução metanólica de o-ftalaldeído (OPT) na concentração de 1 mg/mL. A fluorescência foi determinada após 15 minutos em 350 nm e 420 nm, para excitação e emissão, respectivamente, em espectrofotômetro de fluorescência, modelo RF-5301PC, Shimadzu®. A curva analítica foi anteriormente preparada com GSH nas concentrações de 0 a 75 µM (HISSIN; HILF, 1976).

3.2.5.6.2 Capacidade sequestradora do radical ABTS

As amostras de pele dos animais foram coletadas em tubos *eppendorf* contendo 500 µL de solução KCl 1,15%. Para a realização do ensaio, as amostras foram homogeneizadas com auxílio de Ultra Turrax (T 18 basic, IKA®). Em seguida, foram centrifugadas a 1000 *g* por 10 minutos a 4º C e o sobrenadante foi retirado para análise.

Para a determinação da capacidade sequestradora do radical ABTS, a solução de ABTS foi preparada e diluída conforme item 3.2.3.2. A 1 mL desta

solução foram adicionados 40 µL do sobrenadante das amostras. As leituras foram realizadas espectrofotometricamente a 730 nm após 6 minutos de reação. Uma curva analítica com diferentes concentrações de trolox (1 a 25 µM) foi utilizada para posterior cálculo dos resultados em µM equivalente de trolox/mg de pele (KATALINIC et al., 2005). O ensaio foi realizado em duplicata.

3.2.5.6.3 Avaliação do poder antioxidante redutor férrico (FRAP)

As amostras de pele dos animais foram coletadas em tubos *eppendorf* contendo 500 µL de solução KCl 1,15%. Para a realização do ensaio, as amostras foram homogeneizadas com auxílio de triturador Tissue-Tearor (Biospec®). Em seguida, foram centrifugadas a 1000 *g* por 10 minutos a 4º C e o sobrenadante foi retirado para análise.

Para a avaliação do poder redutor férrico, 1 mL do reagente FRAP preparado previamente conforme item 3.2.3.3 foi adicionado de 30 µL de sobrenadante. Após incubação a 37°C por 30 minutos, a leitura foi realizada espectrofotometricamente a 595 nm. Uma curva analítica com diferentes concentrações de trolox (0,5 a 20 µM) foi utilizada para posterior cálculo dos resultados em µM equivalente de trolox/mg de pele (KATALINIC et al., 2005). O ensaio foi realizado em duplicata.

3.2.6 Análises estatísticas

As concentrações de PPE *in vitro* que inibem o processo oxidativo em 50% (IC_{50}) foram estimadas utilizando-se a curva hiperbólica. Os resultados foram analisados pelo teste paramétrico *t* de student para o estudo de liberação e pelo teste paramétrico ANOVA de uma via seguido do pós-teste de comparações múltiplas de Bonferroni para os demais testes *in vitro* e *in vivo.*

Os resultados foram expressos como média ± erro padrão da média. As diferenças foram consideradas estatisticamente significativas para valores correspondentes a $p < 0,05$. Os dados foram analisados pelo software GraphPad Prism®.

4 RESULTADOS E DISCUSSÃO NO FORMATO DE ARTIGOS CIENTÍFICOS

4.1 Topical formulations added with Pimenta pseudocaryophyllus extract: In vitro antioxidant activity and in vivo efficacy against UV-B-induced oxidative stress

Industrial Crops and Products

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Abbreviations

AA: antioxidant activity

ABTS: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

DNA: deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

Formulation 1: F1

Formulation 2: F2

FRAP: Ferric reducing antioxidant power

HPLC: High-performance liquid chromatography

PPE: *P. pseudocaryophyllus* ethanolic extract

RH: Relative humidity

ROS: reactive oxygen species

RT: room temperature

SEM: standard error mean

TPTZ: 2,4,6-tripyridyl-S-triazine

UV: ultraviolet

Abstract

Pimenta pseudocaryophyllus is a plant native from Brazil that presents flavonoids and other polyphenolic compounds in high concentration. In the present study we evaluated: (i) chemical properties of *P. pseudocaryophyllus* ethanolic extract (PPE), (ii) the *in vitro* antioxidant activity (AA) of PPE and of two different topical formulations (F1 and F2) added with PPE, (iii) physico-chemical and functional stability, (iv) *in vitro release studies*, and (iv) *in vivo* capacity of formulations to prevent/treat the UV-B irradiation-induced skin damage. Results show that the polyphenol and flavonoid contents found in PPE were 199.33 mg/g and 28.32 mg/g, respectively, and HPLC results show the presence of eugenol, tannic acid and rutin. Evaluation of the *in vitro* AA demonstrated a dose-dependent effect of PPE and IC_{50} of 4.75 µg/mL in 2,2-difenil-1-picrilidrazil (DPPH) and 3.0 µg/mL in 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. The iron reducing power (FRAP assay) was 0.046 µmol/L trolox equivalent/µg/mL of extract. The capacity to scavenge DPPH radical of PPE was maintained in F1 and F2. However, there was a significant decrease in AA of formulations with PPE compared to extract measured by FRAP and ABTS methods. In addition, both formulations satisfactory released the extract. The evaluation of the functional stability of F1 and F2 did not demonstrate loss of activity by storage at room temperature (RT) and at 4^{o} C/ 6 months. Treatment with F1 and F2 added with PPE significantly increased the capacity to scavenge ABTS radical and the iron reducing antioxidant power (FRAP) of skin in comparison to irradiated animals. In conclusion, the present results suggest for the first time that formulations containing PPE may be a topical source of antioxidant compounds that decrease oxidative damages of the skin.

1. Introduction

Among all the cellular sources of reactive oxygen species (ROS), the ultraviolet (UV) radiation plays a prominent role in the induction of cutaneous oxidative stress. Acute exposure to UV irradiation causes sunburn, DNA damage and connective tissue degradation. Accumulated damage, resulting from chronic sun exposure, causes skin cancer and premature skin aging (photoaging) (Al Shaal et al., 2012; Halliwell, 2009; Vicentini et al., 2011).

Epidemiological studies indicate that the use of sunscreens and sun blockers are not completely effective in preventing UV irradiation-induced skin cancer (Autier et al., 1998; Azizi et al., 2000; Bauer et al., 2005). New targeted chemopreventive approaches need to be identified (Verschooten et al., 2006). Thus, due to the deleterious effects of ROS in the skin, many studies have focused on the establishment and evaluation of antioxidants to enrich the endogenous cutaneous protection system, and thus to prevent and/or treat UV irradiation-induced skin damage. In this context, much attention has been paid to antioxidants from natural sources, especially flavonoids and other phenolic compounds (Atoui et al., 2005; Fonseca et al., 2010).

Pimenta pseudocaryophyllus is present in the Atlantic forest and Brazilian Cerrado. Commonly, teas prepared with its leaves are used as tranquilizers, digestive regulators and for the relief of colds symptoms (Paula et al., 2008). Despite being the only species of the gender native of Brazil (Fajemiroye et al., 2012), there are still few studies about this plant. The leaves of *P. pseudocaryophyllus* present polyphenolic compounds such as tannins and flavonoids in high concentration (Paula et al., 2008; Fajemiroye et al., 2012), which suggests that it might have the ability to act as an antioxidant.

Corroborating, it was reported that the antioxidant activity (AA) could be used to evaluate the functional activity of topical functionalized formulations (Marquele et al., 2005, Georgetti et al., 2006) and even to evaluate the release of antioxidant components from these formulations (Marquele et al., 2006, Vicentini et al., 2009).

Thus, the evaluation of topical formulations added with plant extracts by *in vitro* AA and *in vivo* efficacy is a crucial issue in the study of new pharmaceutical products for skin protection against UV radiation-induced damage. Furthermore, there is no evidence of *in vivo* use of topical formulation containing PPE to prevent

oxidative damages. So, the present study was designed to evaluate the chemical composition, AA of the PPE alone and added in different topical formulations, beyond the *in vitro* release study of antioxidant compounds. In addition, physical-chemical and functional stabilities were also assessed. Finally, the *in vivo* protection of the formulations against oxidative stress caused by UV-B irradiation in hairless mice was evaluated.

2. Material and Methods

2.1. Chemicals

Quercetin dihydrate 99% ($C_{15}H_{10}O_7 \cdot 2H_2O$, Mw = 338.26) and quercetin-3-*O*-rutinoside (rutin) were purchased from Acros Organics (New Jersey, USA). Folin-Ciocalteau was obtained from Fluka Chemical Co. (Buchs, Switzerland) and propylene glycol from Chemco LTDA. 2,2- diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripiridil-s-triazina (TPTZ), tannic acid and galic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Nitrocellulose membrane 0.45 µm, 47 mm, model HAWP04700, white HAWP from Millipore (Sao Paulo, SP, Brazil). Eugenol was obtained from Vetec (Rio de Janeiro, Brazil). Raw materials for formulations were obtained from Galena (Campinas, SP, Brazil). All other reagents used were of pharmaceutical grade.

2.2. Plant material and extract preparation

The leaves of *P. pseudocaryophyllus* were collected in December 2007 at São Jerônimo da Serra (Paraná, Brazil). The plant specimens were identified by A.O.S. Vieira, Departamento de Biologia Animal e Vegetal (Centro de Ciências da Saúde) and a voucher specimen was deposited at the “Herbarium of Universidade Estadual de Londrina (FUEL)” under code no. 43025. The plant material was dried at 40° C, coarsely powdered in industrial blender. The ethanolic extract (1:10) was obtained by exhaustive maceration at room temperature (RT) (25° C) for 12 days. The extract was filtered and concentrated under vaccum.

2.3. Chemical characteristics of PPE

2.3.1. Total flavonoids and polyphenols contents of PPE

Total polyphenol content in PPE was determined by the Folin–Ciocalteau colorimetric method (Georgetti et al., 2006; Kumatzawa et al., 2004). 0.5 mL of PPE solution was mixed with 0.5 mL of the Folin–Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h of incubation at RT. Total polyphenol content was expressed as mg/g (gallic acid equivalents).Total flavonoid content was determined using the aluminium chroride colorimetric method (Georgetti et al., 2007). To 0.5 mL of PPE solution, 0.5 mL of 2% $AlCl_3$ ethanolic solution was added. After 1 h at RT, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin (mg/g) from an analytical curve.

2.3.2. High Performance Liquid Chromatography (HPLC) analysis

The extract was analyzed by high-performance liquid chromatography (HPLC) (Shimadzu) equipped with a photodiode Array detector (SPD-M10Avp), multisolvent delivery system (LC-10Avp), oven control system (CTO-10ASvp) and controlled software Class VP 6.14 software. Chromatography was performed on an analytical reverse phase column Spherisob® (C-18 ODS) (250 x 4.6 mm i.d.; particule size 5 µm) (Waters). The HPLC grade solvents were supplied by Panreac®, and water was purified using Milli-Q-plus filter systems (Millipore). For HPLC runs, a gradient of acidified H_2O (2% Formic Acid) (solvent A) and Acetonitrile (2% formic acid) (solvent B) was used at a flow rate of 1 mL/min, and the volume injected was 20 µL. HPLC gradient: 0 min, 0% B; 5 min, 0% B; 20 min, 2,5% B; 30 min, 5% B; 50 min, 15% B; 60 min, 25% B; 65 min, 30% B; 70 min, 45% B; 75 min, 50% B; 80 min, 70% B; 85 min, 90% B; 90 min, 100% B; 95 min, 100% B; 110 min, 0% B.

UV detection was performed at 200–400 nm (scan) and then set to 280 and 340 nm. UV spectra were recorded for each main peak in the chromatograms. The following compounds were used as references: quercetin-3-*O*-rutinoside (rutin, Sigma), tannic acid and eugenol (Rieger, 2011).

2.4. Determination of in vitro antioxidant efficacy of PPE

2.4.1. Hydrogen-donating ability by DPPH assay

To measure the ability of PPE to scavenge DPPH radical, 20 µL of PPE sample (1 to 20 µg/mL in medium reaction, based on PPE total solids content of 25 mg/mL) were added to the reaction mixture containing 1 mL of 0.1 M acetate buffer (pH 5.5), 1 mL of ethanol and 0.5 mL of ethanolic solution of DPPH 250 µM. The absorbance was measured at 517 nm after 15 minutes of incubation at RT in a Thermo Scientific Evolution® 60 spectrophotometer. The positive control was prepared in the absence of PPE, and it indicates the maximum odd electrons of DPPH, which was considered 100% of free radicals in the solution to calculate the hydrogen-donating ability (%) of PPE. The blank was prepared from the reaction mixture without DPPH solution (Dinis et al., 1994; Casagrande et al., 2007).

2.4.2. Scavenging ability of PPE using ABTS method

The ability to scavenge the ABTS free radical is measured by an absorbance decrease due to suppression of the colored radical (Munteanu et al., 2007). The method was carried out according to Sánchez-Gonzalez et al., 2005, with some modifications. Radical cation ABTS was obtained after the reaction of 7 mM ABTS stock solution with 2.45 mM potassium persulphate. This solution was diluted in phosphate buffer (pH 7.4, 0.1 M) until it reached an absorbance of 0.7 at 730 nm. 50 µL of PPE samples were added to 4 mL of the diluted ABTS solution. The concentrations of PPE obtained in the reaction medium were 0.625 - 15.625 µg/mL (based on PPE total solids content of 25 mg/mL). The absorbance was measured at 730 nm after 6 minutes of incubation at RT in a Thermo Scientific Evolution® 60 spectrophotometer. The positive control was prepared in the absence of PPE and was considered 100% of free radicals in the solution to calculate the scavenging ability of PPE.

2.4.3. Evaluation of the Ferric Reducing Antioxidant Power (FRAP) of PPE

The ferric reduction antioxidant power of PPE was evaluated according to Sánchez-Gonzalez et al., 2005, with some modifications. FRAP reagent was prepared with 2.5 mL of a solution of TPTZ (10 mM) in HCl (40 mM), 2.5 mL $FeCl_3$ ($6H_2O$) solution and 25 mL of acetate buffer (pH 3.6, 0.3 mM). The solution was incubated at 37° C for 30 minutes. For the assay, 900 µL of FRAP reagent were added to 90 µL of water and 10 µL of trolox standard or 10 µL of PPE (1.56 µg/mL in the reaction medium). After incubation at 37° C for 30 minutes, the measurement was performed in spectrophotometer (Thermo Scientific Evolution® 60) at 595 nm. An analytical curve with different concentrations of trolox (4.0 - 20.0 µM) was used for subsequent calculation of results in µmol/L trolox equivalent/µg/mL of extract. A positive control was performed with ethanol.

2.5. Formulations

Formulations were developed varying the content of lipidic and emulsifying agent. Self-emulsifying wax Polawax® (cetostearyl alcohol+ polyoxyethylene derived of a fatty acid ester of sorbitan 20E) was used in both formulations, although in major and minor proportions in formulation 1 (F1) and formulation 2 (F2), respectively. Into F2, anionic hydrophilic colloid (carboxypolymethylene, Carbopol®) was also added as stabilizing agent. Caprylic/ capric triglycerides were used as emollients, and propylene glycol as moisturizer. The preservative used was a mixture of parabens and deionized water was used for the preparation of all formulations (Table A.1). PPE was incorporated (5%) into the formulations at RT. Control formulations did not contain the extract.

2.5.1. Evaluation of physico-chemical characteristics of formulations

In aim to evaluate physico-chemical characteristics, the following tests were performed: visual evaluation (color, consistence and phase separation) (Casagrande et al., 2009); pH measurement in triplicate (10% dilution in deionized water); evaluation of phase separation in triplicate (2 g of formulation submitted to centrifugation at 145 *g* for 30 min) (Georgetti et al., 2006; Anchisi et al., 2001).

2.5.2. Evaluation of the antioxidant activity of F1 and F2 containing PPE

In order to evaluate the AA of PPE after its incorporation to F1 and F2, DPPH (see section 2.4.1), ABTS (see section 2.4.2) and FRAP (see section 2.4.3) methods were performed. Formulations added with PPE were diluted in ethanol to obtain the same concentration used for the analysis of PPE in the reaction medium: 5.0 µg/mL, 3.125 µg/mL and 2.5 µg/mL for DPPH, ABTS and FRAP, respectively. The following controls were included in the test: (i) one positive control was prepared in the absence of sample, and (ii) another by adding the formulations without PPE.

2.5.3. Physico-chemical and functional stability of formulations containing PPE

Formulations containing or not the extract and PPE alone were stored under 3 different conditions: 4° C, RT (25° C) and 40±2° C/ 75±5% of relative humidity (RH) for 6 months (Casagrande et al., 2009). At predetermined time intervals (0, 30, 60, 90 and 180 days), aliquots were collected and analyzed. The physico-chemical stability of the formulations was determined by the tests described in section 2.5.1 and functional stability of PPE, F1 and F2 was measured by DPPH method as described in section 2.5.2. One positive control for each storage condition was added.

2.5.4. In vitro release studies

PPE release rates from the different formulations were measured through 0.45 µm nitrocellulose membranes using modified Franz diffusion cells with a diffusional area of 1,77 cm^2 (Casagrande et al., 2007). The membrane was sandwiched between the upper donor compartment and the lower receptor compartment; 1 g of F1 or F2 containing 5% of PPE was placed on the membrane surface in the donor compartment while the receptor compartment was filled with 16 mL of receptor medium (0.1M phosphate buffer (pH 7.4) with 10% of ethanol), which was in contact with the membrane. During the experiments, the receptor solution was continuously stirred at 100 rpm and kept at 37±1^o C. After 12 h, 1 mL of the receptor medium was aliquoted and the release of PPE antioxidant compounds on F1 and F2 was analyzed by DPPH method (see section 2.4.1). Results are expressed as percentage of PPE released by the formulations. All measurements were performed in duplicate and formulations without PPE were used as control.

2.6. In vivo studies

2.6.1. Animals

Sex matched hairless mice (HRS/J), weighing 20–30 g, were housed in a temperature-controlled room, with access to water and food *ad libitum* until use. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Commitee of State University of Londrina (34994/209).

2.6.2. Formulation administration

Hairless mice were randomly designed to different groups (n=5) and topically treated on the dorsal surface with 0.5 g of F1 or F2 with 5% of PPE or 0.5 g of the respective control formulation without PPE. Formulations were administrated 1 h before, 5 min before and right after the irradiation. Untreated control groups irradiated and non-irradiated were included in the experiments. The results are representative of 2 separated experiments.

2.6.3. Irradiation

The UV-B source of irradiation consisted of a Philips TL40W/12 RS lamp (Medical-Holand) emitting a continuous spectrum between 270 and 400 nm with a peak emission at 313 nm. The lamp was mounted 20 cm above the table where the mice were placed on, resulting in an irradiation of 0.384 mW/cm^2 as measured by an IL 1700 radiometer (Newburyport, MA, USA) with sensor for UV (SED005) and UV-B (SED240). The dose of UV-B used was 4.14 J/cm^2 (Casagrande et al., 2006; Shindo et al., 1994). The mice were killed by ether overdose 12 h after the UV-B exposure, and the full thickness of the dorsal skins were removed and ABTS and FRAP tests were performed on the same day.

2.6.4. Samples preparation

Skin samples of the animals were collected in eppendorf tubes containing 500 µL of 1.15% KCl solution. After, samples were homogenized with Tissue-Tearor (Biospec®). A centrifugation at 1000 *x g* for 10 minutes at 4° C was performed and the supernatant was used on the assays.

2.6.5. Scavenging ability of skin using ABTS method

To determine the ability of ABTS radical scavenging, ABTS solution was prepared and diluted according to item 2.4.2. 40 µL of the supernatant were added to 1 mL of the diluted ABTS solution. Incubation and reading were performed as described in section 2.4.2. An analytical curve with different concentrations of trolox (1- 25 µM) was used for subsequent calculation of results in µM trolox equivalent/mg of skin (Katalinic et al. 2005). The assay was performed in duplicate.

2.6.6. Evaluation of the Ferric Reducing Antioxidant Power (FRAP) of skin

In order to evaluate the FRAP, 30 µL of supernatant were added to 1 mL of FRAP reagent prepared as previously described in item 2.4.3. Incubation and reading were performed as described in section 2.4.3. An analytical curve with different concentrations of trolox (0.5 - 20 µM) was used for subsequent calculation of results in µM trolox equivalent/mg of skin (Katalinic et al. 2005). The assay was performed in duplicate.

2.7. Statistical analysis

The concentration of PPE that caused 50% of DPPH and ABTS scavenging was considered the IC_{50}, which was determined using GraphPad Prism® software, version 6.0. Data were statistically analyzed by *t-student* for release study and one-way ANOVA followed by Bonferroni's t test for *in vivo* tests. Results were expressed as means ± SEM (standard error mean) and considered significantly different when $p < 0.05$ was obtained.

3. Results

3.1. Chemical characteristics of PPE

Results show that PPE presents 199.33 ± 3.79 mg/g and 28.32 ± 1.46 mg/g of polyphenols and flavonoids, respectively. Eugenol, rutin and tannic acid as reference compounds were identified on the ethanolic extract (Fig. B.1). Identification of the individual flavonoids and phenolic compounds was made by HPLC-PDA and comparison of UV data (λmax) with the reported value (Rieger, 2011).

3.2. In vitro antioxidant activity of PPE

The hydrogen donating ability of PPE was evaluated by the use of the stable radical DPPH as presented in Fig. B.2A. The maximum antioxidant activity was 88.17% using the concentration of 20 µg/mL of PPE and the IC_{50} was 4.75 µg/mL. Regarding ABTS method, results showed that PPE exhibited effective and concentration-dependent scavenging activity (Fig. B.2B). The IC_{50} was 3.0 µg/mL and the maximum activity (7.82 µg/mL) was approximately 98.89%, in which a plateau was observed. On FRAP assay, PPE reducing power was 0.046 µmol/L trolox equivalent/µg/mL of extract.

3.3. Formulations

3.3.1. Evaluation of antioxidant activity of the formulations containing PPE

In order to verify if F1 and F2 added with PPE were able to maintain the AA potential of PPE raw material, DPPH, ABTS and FRAP assays were performed with F1 and F2 added with PPE and the results were compared to ethanolic solution of PPE in the same concentration in the reaction medium. Figure B.3A shows that the capacity to scavenge DPPH radical was kept in the formulations with different content of lipidic (45.63%, 44.32% and 45.06% for PPE, F1 and F2, respectively). However, there was a significant decrease in AA of F1 and F2 added PPE measured by FRAP and ABTS methods compared to PPE raw material. The percentages of reduction of ABTS radical were 53.10%, 44.99% and 46.52% for PPE, F1 and F2, respectively (Fig. B.3B). On the FRAP assay, the reducing power was 0.051, 0.039 and 0.041 µmol/L trolox equivalent/µg/mL of extract for PPE, F1, and F2, respectively

(Fig. B.3C). These results demonstrated that there was a reduction of the ABTS scavenging activity and of the iron reducing power for F1 of 15.27% and 22.93%, respectively, and for F2 of 12.39% and 18.83%, respectively.

3.3.2. Stability studies

F1 and F2 maintained their color and consistency characteristics under RT and 4° C. However, a gradual color change of F1 and mainly of F2 under 40±2° C/ 75±5% RH was observed. In general, the pH values of F1 and F2 remained compatible with the skin and both formulations also remained physically stable, showing no phase separation (Table A.2). Regarding functional stability, the hydrogen-donating ability was kept in both formulations at RT and 4° C. However, after 6 months stored at 40±2° C/ 75±5% RH, F1 and F2 lost approximately 7.23% and 21.76% of its AA, respectively (Fig. B.4).

3.3.4. In vitro release studies

PPE release from the formulations was evaluated after 12 h and is presented on Fig. B.5. The percentages of PPE released by F1 and F2 were 14.66% and 13.41%, respectively, without statistical difference between F1 and F2.

3.4. In vivo studies

In this study, UV-B irradiation induced a decrease of approximately 1.58 and 1.84 fold of ABTS scavenging capacity and iron reducing power of the skin, respectively. Both formulations containing PPE inhibited this depletion, maintaining levels similar to control (non irradiated) group in both tests (Fig. B.6).

4. Discussion

Antioxidants from natural sources may provide new possibilities for the treatment and prevention of oxidative stress-mediated diseases, and plant extracts rich in antioxidant compounds have gained special attention (Marquele-Oliveira et al., 2007). As described above, *P. pseudocaryophyllus* has been used as medicinal plant

in folk medication system and is rich in bioactive compounds responsible for that property (Paula et al., 2008; Fajemiroye et al., 2012). In this study, we have found that polyphenols content was approximately 7 times higher than total of flavonoids content of PPE, corroborating other studies that show that *P. pseudocaryophyllus* contains not only flavonoids, but other polyphenolic substances, like tanins (Paula et al., 2008; Fajemiroye et al., 2012). Previously, Paula et al., 2008, and Fajemiroye et al., 2012, have reported flavonoids and phenolic compounds from this plant. Some compounds were isolated as flavonoids and phenolic acids derivatives, and eugenol is reported as main compound of *P. pseudocaryophyllus* in the literature (Paula et al., 2012). In this study, eugenol, rutin and tannic acid were identified on the extract, and these substances may have exerted synergistic effect. Despite eugenol being in greater quantity, it may be suggested that tannic acid and rutin are the main responsible for the antioxidant activity o PPE.

Two or more methods should always be employed in order to evaluate the antioxidant potential of any substance, once oxidative stress depends on the type of generated ROS, how and where it is generated, and the oxidative target evaluated. Furthermore, plant extracts present a diversified phytochemical composition, therefore its AA may be attributed to synergistic action of multiple substances, which belong to different chemical groups and may exert its activity through various mechanisms (Georgetti et al., 2006; Sousa et al., 2007). Thus, the AA of PPE was evaluated by 3 different methods. The ABTS and DPPH methods consist on the verification of the colored radical suppression on the medium, which decreases the absorbance. On FRAP assay, antioxidants in the sample reduce the Fe^{+3}-TPTZ complex to form a blue-colored Fe^{+2}-TPTZ complex, which results in an increase in the absorbance. *P. pseudocaryophyllus* components act as free radical scavengers of negatively and positively charged free radicals, DPPH and ABTS, respectively. Furthermore, *P. pseudocaryophyllus* also demonstrated ferric reduction antioxidant power.

In order to verify if F1 and F2 constituents were able to maintain the antioxidant potential of PPE, the same tests used to evaluate the AA of the extract were performed with the formulations added with PPE. The capacity to scavenge DPPH radical was maintained, but FRAP and ABTS assays showed a decrease of AA of PPE after its incorporation in topical formulations. This may be explained by the fact that the antioxidant compounds present in extract may interact with

components of the vehicle and, in the case of emulsions, mainly with the fatty phase (Mambro and Fonseca, 2007). Therefore, it can be suggested that the colloidal properties of self emulsifying base Polawax® used in both formulations could promote interaction with the components of PPE which are responsible for the reducing power and/or with the components able to donate electron to ABTS. So raises the necessity to be concerned about formulations, since one of most challenging tasks in evaluating topical formulations is to deal with the presence of the formulations compounds that may cause interference if using a non specific method (Marquele et al., 2005). However, in stable free radical DPPH assay there was no interference, which means that this method is able to evaluate the AA of the formulations and thus it was used for evaluation of functional stability.

A stable emulsion maintains the proper proportions between its components and maintains the interphase surface even after being exposed to tension resulting from factors such as temperature, agitation and acceleration of gravity (Pather et al., 1995). Thus, two emulsions added with PPE were developed and their physico-chemical and functional stability were evaluated at predetermined times. The formulations developed in this study presented different characteristics, mainly in their lipid content, so several physical instabilities could occur when the complex compounds present in the PPE were added. Therefore, stability testing represents a crucial part of the testing program because the instability of the product modifies essential requisites, i.e. quality, efficacy and safety (Bilia et al., 2001).

During the study, both emulsions remained physically stable and pH values remained satisfactory, which ensures that F1 and F2 are compatible with the application site, avoiding irritation (Casagrande et al., 2009). Regarding functional stability study of DPPH scavenging activity, it was observed that temperature, storage time and type of formulation influenced the AA of PPE. Hydrolysis reactions are one of the most common processes of active components degradation, and depend mainly on the temperature and quantity of available water in the medium (Waterman and Adami, 2005). Since F2 showed a loss of AA approximately 3 times higher than F1, it can be suggested that the higher water content of F2 coupled with drastic conditions of storage destabilized active compounds of PPE. The decrease in the AA of formulations stored at accelerated conditions corroborates the results of visual evaluation, which showed changes in color of formulations, especially of F2. Polyphenols are susceptible to the action of temperature and humidity, and its

stability profile and biological activity are strongly related to the processing conditions and storage (Ungar et al., 2003). Thus, the reduction in AA observed in formulations stored under accelerated conditions may be related to a possible degradation of polyphenols present in the extract.

It is generally assumed that the nature of the delivered pharmaceutical dosage strongly influences the rate and extent of drug release. Release may be improved by selecting the appropriate vehicle. The *in vitro* release studies which measure drug/vehicle interactions are considered to be useful and crucial in pre-formulation step to choose an appropriate vehicle (Ropke et al., 2002; Ozsoy et al., 2004). The release of antioxidant compounds of the PPE from different emulsion systems (F1 and F2) through nitrocellulose membrane was examined and the values found were very close to both formulations, showing that the difference in lipid content did not affect the release of these components of the extract.

Regarding *in vivo* studies, we evaluated the effectiveness of F1 and F2 incorporated with PPE against oxidative damage caused by UV-B irradiation. Once oxidative stress is characterized by the decrease of endogenous antioxidant, several methods have been developed to assess the antioxidant capacity of diverse organs (Halliwell, 2009; Re et al., 1999). The difficulty in measuring each antioxidant component separately and the interactions among them leads to the use of quick, simple and efficient assays, like ABTS and FRAP, which use different technology for measuring antioxidant capacity (Katalinic et al., 2005).

Corroborating the release studies that demonstrated similar results for both formulations, the treatment with these topical formulations containing PPE clearly improved the cutaneous antioxidant capacity to control levels. ABTS assay has been found to correlate well with levels of endogenous glutathione (Kang and Saltveit, 2002), while FRAP assay may reflect levels of ascorbic acid, uric acid and α-tocopherol (Katalinic et al., 2005).

Despite the need of further studies, the prepared formulations added with PPE demonstrate interesting attributes to be explored as potential products to be used against UV-induced damages.

5. Conclusions

It can be inferred from our results that the PPE presented *in vitro* AA and, regarding the development of formulations added with PPE, the most stable formulation was the one prepared with the self-emulsifying wax Polawax® in higher concentration (F1). In addition, both formulations were able to release the antioxidant compounds present in the PPE. Furthermore, formulations containing PPE were able to inhibit UV-induced oxidative damage. Finally, other assays need to be performed in order to achieve a complete understanding of the protective effect of formulations added with PPE.

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Appendice A - Tables

Table A.1: Percent composition (weight/weight) of the formulations 1 and 2

Components	**F1**	**F2**
Polawax®	10.0	2.0
Caprylic/ capric triglyceride	5.0	5.0
Carbopol® 940	-	0.18
Propylene glycol	5.0	5.0
Triethanolamine	-	0.2
Deionized water	74.0	76.0
Solution of methyl (10%) and propylparaben (2%)	1.0	1.0

Table A.2: Physico-chemical characteristic of formulations 1 and 2 containing or not *P. pseudocaryophyllus* ethanolic extract

Formulation	pH	Centrifugation
F1 control	4,67	NS
F1 + PPE	4,65	NS
F2 control	6,65	NS
F2 + PPE	6,46	NS

NS: No separation.

Appendice B - Figures

Figure B.1. Identification of phenolic components of *P. pseudocaryophyllus* ethanolic extract (PPE) using High Performance Liquid Chromatography (HPLC).

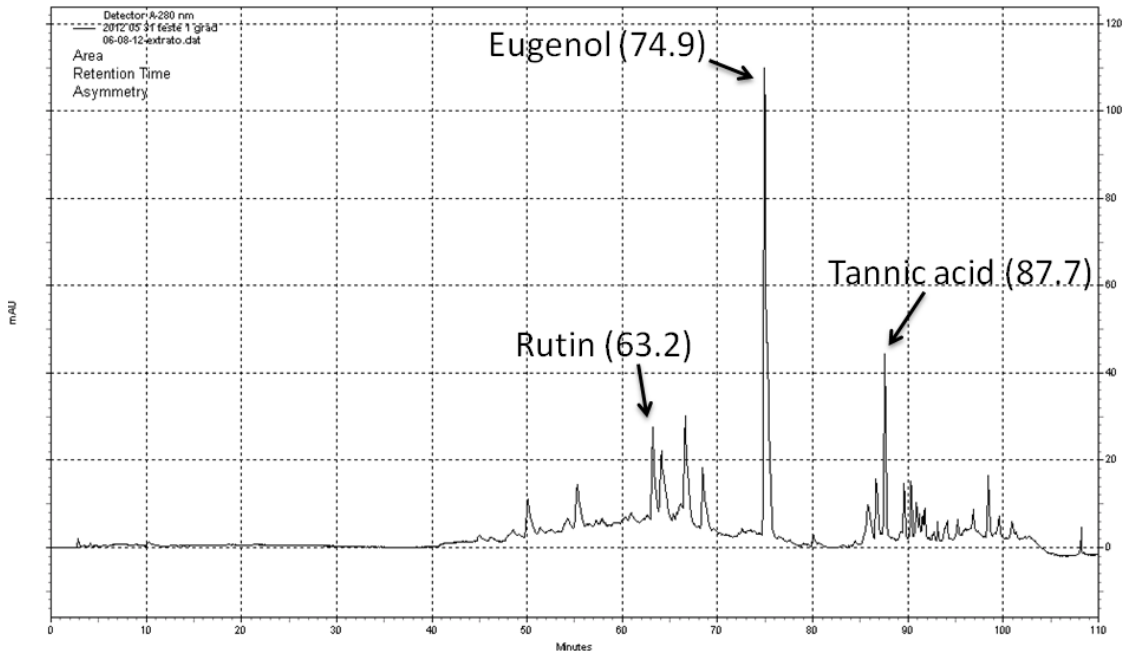


Figure B.2. (A) H-donor ability of *P. pseudocaryophyllus* ethanolic extract (PPE) using stable radical 2,2-difenil-1-picrilidrazil (DPPH) (Concentrations of PPE on the reaction medium: 1 - 20 μg/mL). (B) Scavenging ability of PPE using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (Concentrations of PPE on the reaction medium: 0.625 – 15.625 μg/mL). Results are represented by means ± SEM.

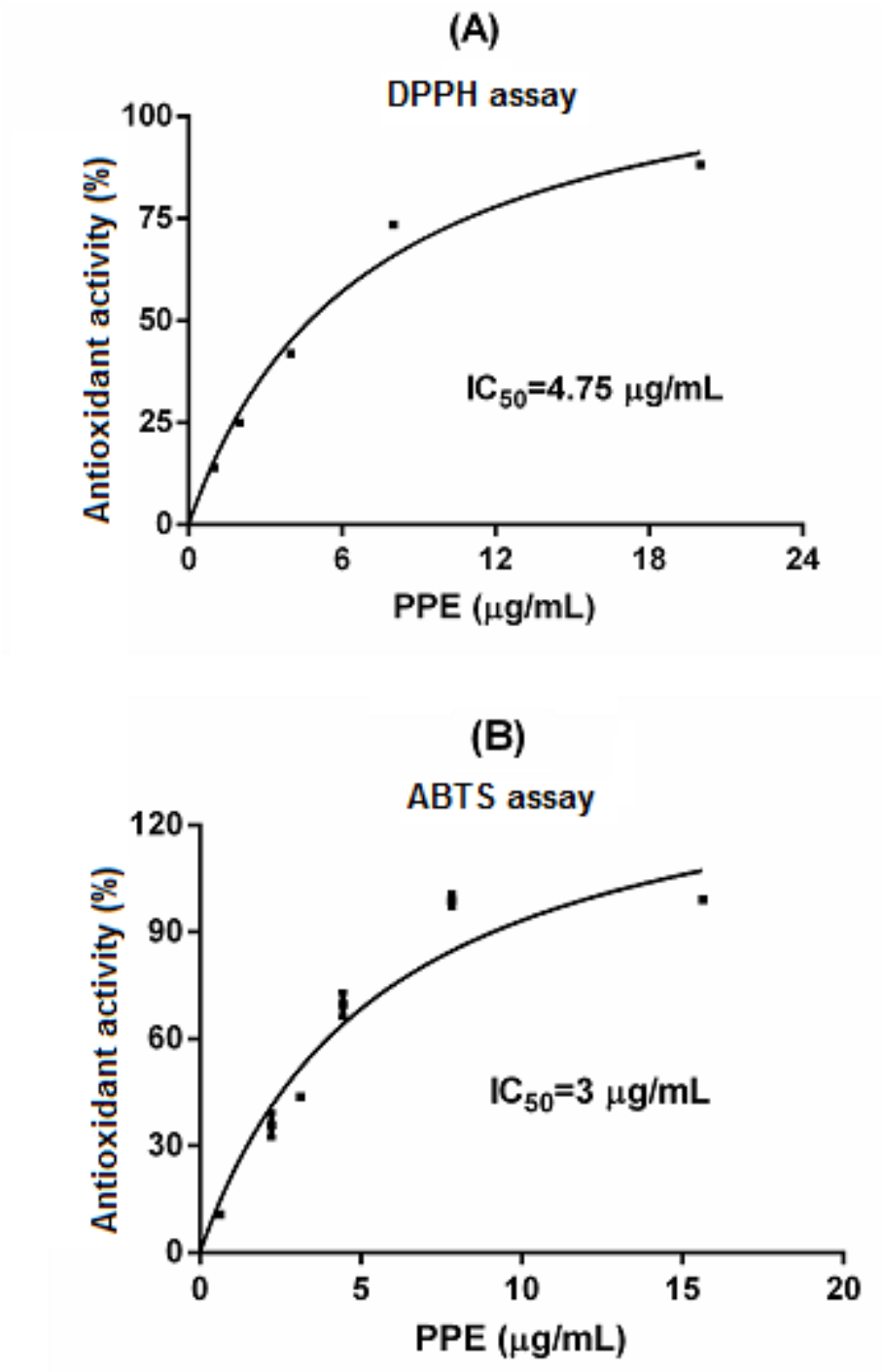


Figure B.3. Evaluation of the antioxidant activity of *P. pseudocaryophyllus* ethanolic extract (PPE) and both formulations (F1 and F2) containing PPE: H-donor ability using stable radical 2,2-difenil-1-picrilidrazil (DPPH) (A), scavenging ability using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (B) and ferric reducing antioxidant power (C).

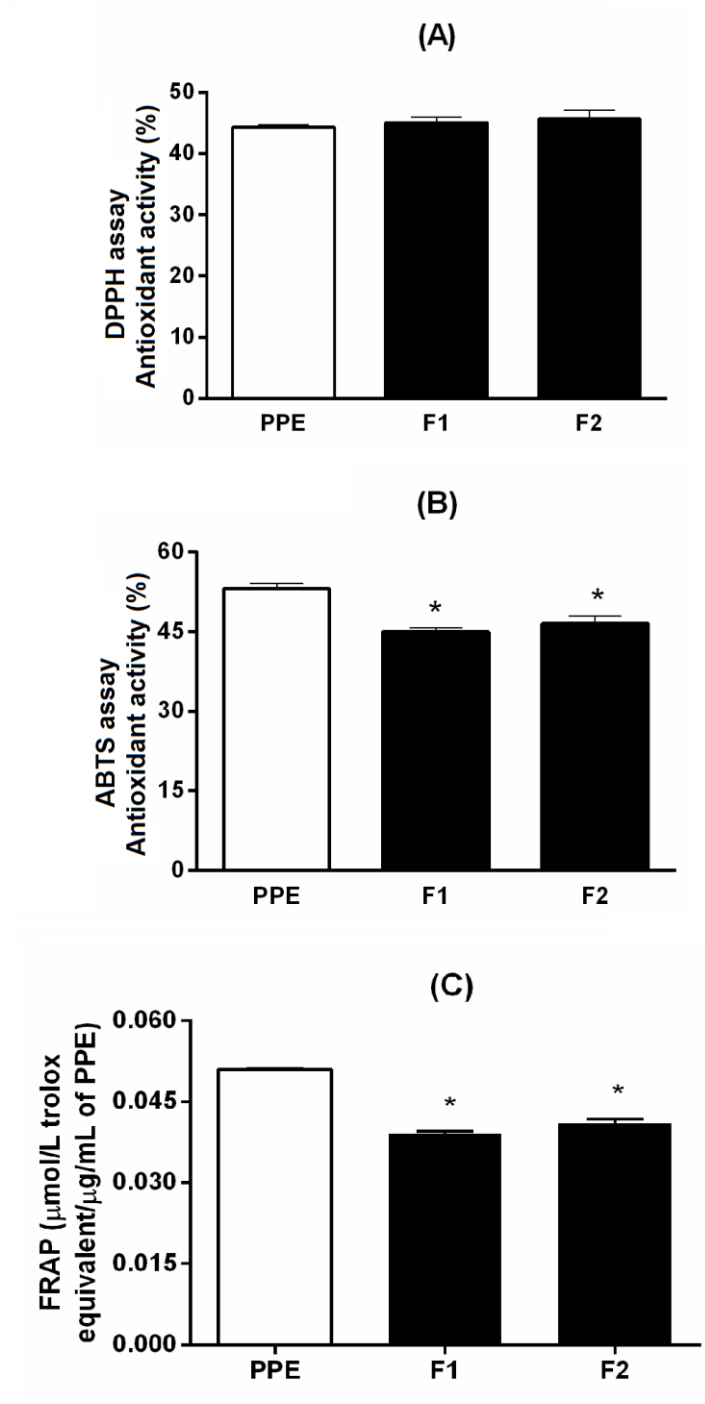


Figure B.4. Stability of hydrogen-donation ability using stable radical 2,2-difenil-1-picrilidrazil (DPPH) of *P. pseudocaryophyllus* ethanolic extract (PPE) (A), formulation 1 (F1) (B) and formulation 2 (F2) (C) containing PPE stored at 4° C, room temperature (RT) and 40° C / 75% relative humid (RH) for 6 months.

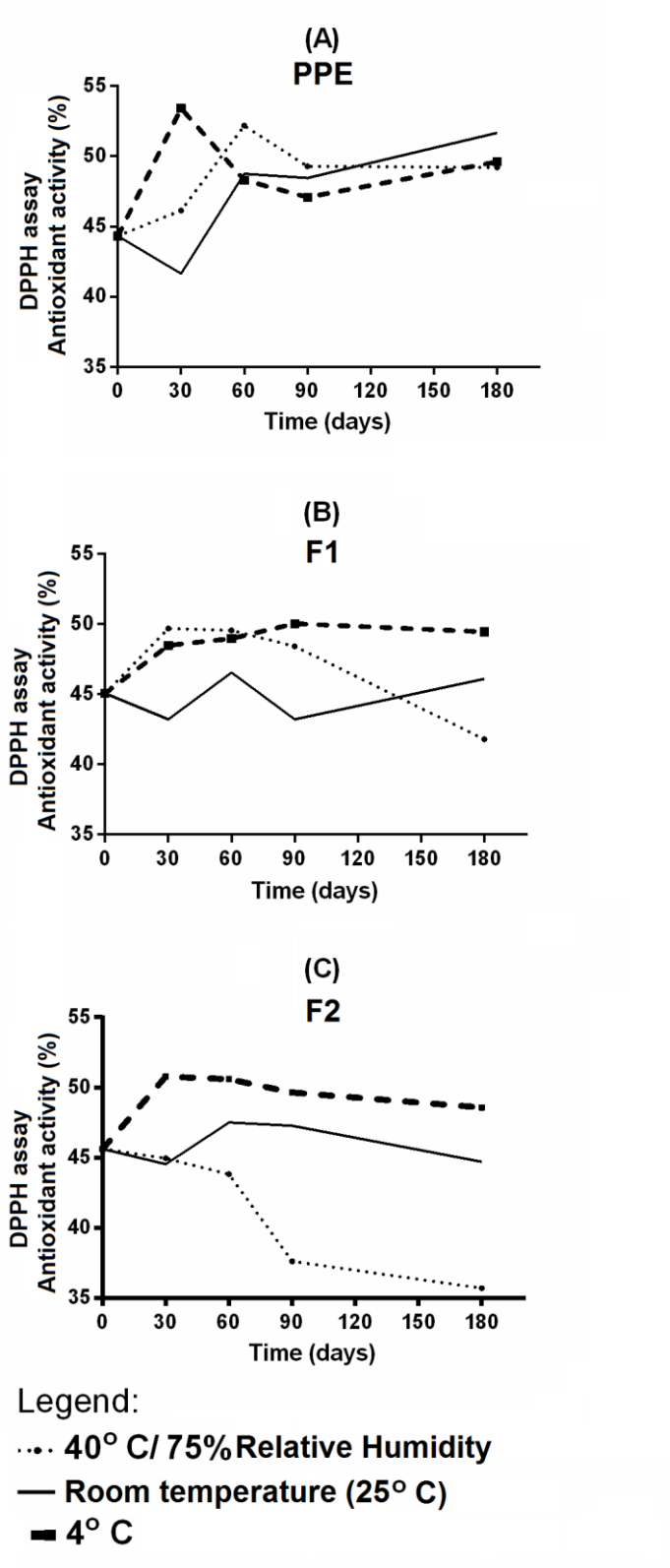


Figure B.5. Percentage of *P. pseudocaryophyllus* ethanolic extract (PPE) release from formulations 1 and 2 measured by hydrogen-donating ability using 2,2-difenil-1-picrilidrazil (DPPH). Results are represented by means ± SEM. No statistical significant difference was detected.

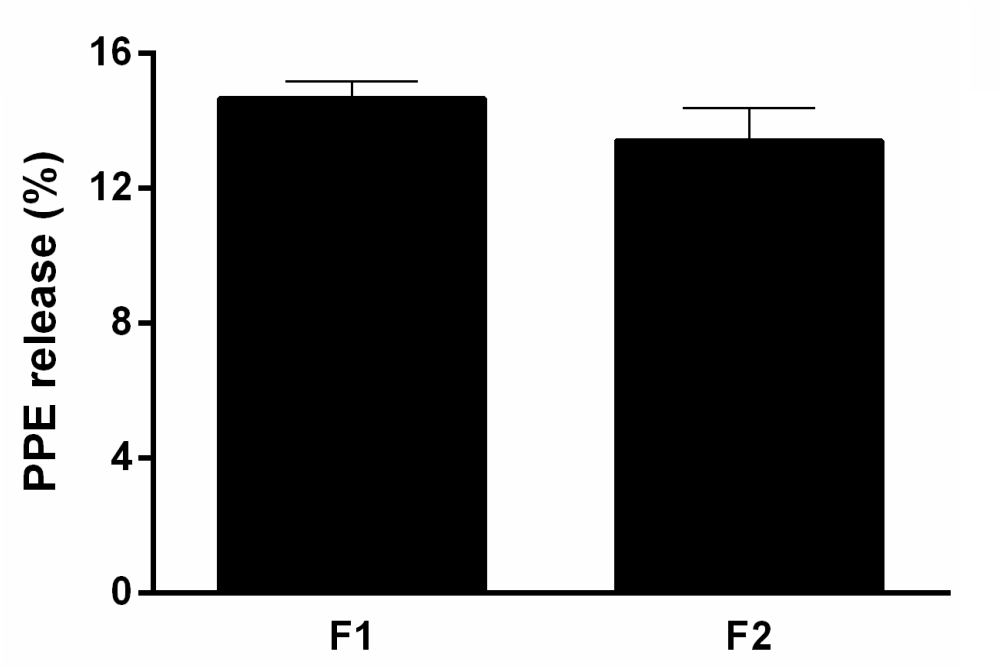
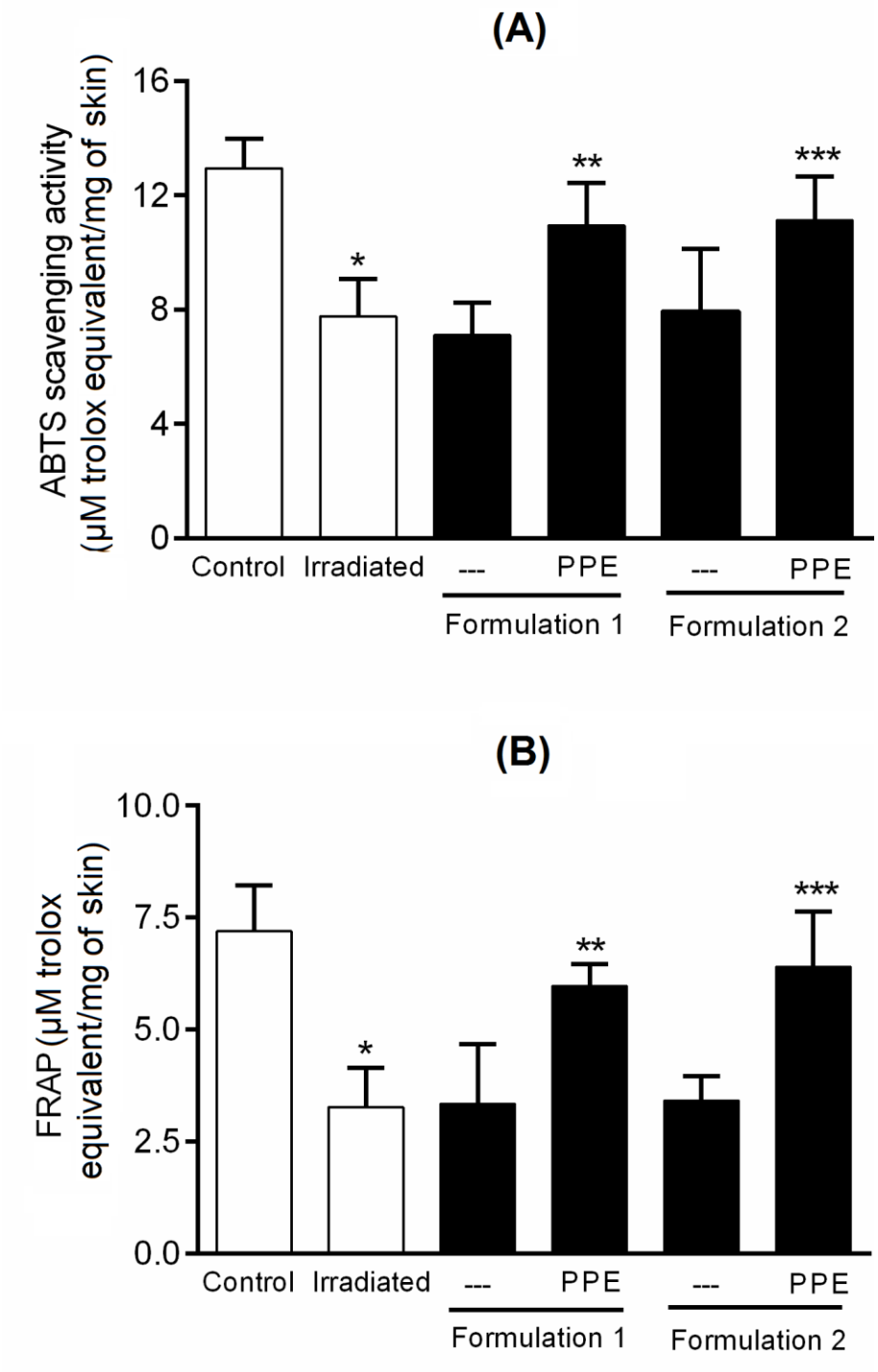


Figure B.6. Formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) increase the antioxidant capacity of skin after UV-B irradiation in 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay (A) and in ferric reducing antioxidant power (B). Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *p < 0.001 compared to the control (non-irradiated) group, **p < 0.001 compared to irradiated group and F1 control group, and ***p < 0.001 compared to irradiated group and F2 control group.



4.2 Efficacy of topical formulations containing Pimenta pseudocaryophyllus extract against UVB-induced oxidative stress and inflammation in hairless mice

Photochemistry and Photobiology B: Biology

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Highlights

- Topical formulations with *Pimenta pseudocaryophyllus* ethanolic extract (PPE) were developed
- PPE formulations prevented the UV-B-induced depletion of reduced gluthathione
- PPE formulations reduced UV-B-induced metalloprotease and myeloperoxidase activity
- PPE formulations modulated IL-1β and IL-10 levels in UV-B-irradiation model
- PPE formulations reduced UV-B-induced oxidative and inflammatory skin damages

Abbreviations

AO: antioxidant
AP-1: activation protein-1
DNA: deoxyribonucleic acid
EGTA: ethylene glycol bis (-aminoethyl ether)-N,N, N0,N0-tetraacetic acid
Formulation 1: F1
Formulation 2: F2
GSH: reduced glutathione
HTAB: hexadecyltrimethylammonium bromide
HOCl: hypochlorous acid
ICAMs: intercellular adhesion molecules
IL: interleukin
MMP: matrix metalloprotease
MPO: myeloperoxidase
NF-kB: nuclear factor kB
OPT: o-phthalaldehyde
PPE: *P. pseudocaryophyllus* ethanolic extract
ROS: reactive oxygen species
SDS: sodium dodecyl sulphate
SEM: standard error mean
SH: sulfhydryl grouping
TNF-α tumour necrosis factor-α
TPA: 12-O-tetradecanoylphorbol-13-acetate
UV: ultraviolet

Abstract

Ultraviolet (UV) irradiation-induced damage of the skin involves an increase in reactive oxygen species (ROS) levels, imbalance of the endogenous antioxidant (AO) system and inflammation. Therefore, plants rich in antioxidant substances may be a promising strategy for treating or preventing UV-induced oxidative and inflammatory damage of the skin. *Pimenta pseudocaryophyllus* is native from Brazil and presents flavonoids and other polyphenolic compounds in high concentration. Thus, the present study evaluated the possible beneficial effects of topical formulations containing *P. pseudocaryophyllus* ethanolic extract (PPE) to inhibit UV-B irradiation-induced oxidative stress and inflammation. PPE was administered on the dorsal skin of hairless mice using two formulations: F1 (non-ionic emulsion with high lipid content) and F2 (anionic emulsion with low lipid content). The treatment was 1 h before, right before and right after the UV-B irradiation (4.14 J/cm^2) session. Skins samples were removed after 12h and the following parameters were evaluated: edema, myeloperoxidase (MPO) activity, cytokines levels (IL-1β, TNF-α and IL-10), matrix metalloprotease-9 (MMP-9) secretion/activity, reduced glutathione (GSH) levels. The UV-B irradiation increased all parameters, except that IL-10 levels were not altered and GSH levels were reduced by exposure to UV-B light. On the other hand, F1 and F2 containing PPE were able to inhibit edema formation, MPO activity, IL-1β production, MMP-9 activity and GSH depletion all to control levels. However, none of the formulations was able to decrease TNF-α levels. F2 containing PPE was also able to increase IL-10 levels. To our knowledge, this is the first study to demonstrate the effectiveness of topical formulations containing PPE to inhibit the UV-B radiation-induced oxidative stress of the skin. Thus, these data suggest the possible usefulness of topical formulations containing this extract to prevent UV-B radiation-induced skin damages.

Keywords: Free radicals; *Pimenta pseudocaryophyllus*; Antioxidant; Oxidative stress; UV-B radiation; Hairless mice.

1. Introduction

As a protective barrier of the body, skin is highly exposed to oxidative stress resulting of, among other sources, ultraviolet (UV) radiation [1, 2]. Reactive oxygen species (ROS) formed as a consequence of UV irradiation may oxidize and damage cellular lipids, proteins and deoxyribonucleic acid (DNA), leading to changes and often to destruction of skin structures, resulting in inhibition of its regular function [3]. The exposure of the skin to UV induces an imbalance between ROS and endogenous antioxidant (AO) systems such as reduced glutathione (GSH) [4, 5, 6]. Furthermore, the release of a network of cytokines, which participate in the onset of cutaneous inflammation, among them TNF-α and IL-1β, is certainly important [7, 8]. These substances may cause widening of interendothelial junctions and separation of endothelial cells, increasing microvascular protein and fluid leakage into interstitium, which results in edema [9, 10]. Neutrophils are also activated, stimulating the activity of myeloperoxidase (MPO), a ROS-generator enzyme [11]. UV irradiation also induces the activity of matrix metalloproteases (MMPs), which can be considered the primary mediators of connective-tissue damage in skin exposed to UV radiation and in the premature aging [12].

Therefore, various efforts have been made to prevent these events caused by sun exposure. In this context, antioxidants from natural sources may provide new possibilities for treatment and prevention of oxidative stress-mediated diseases. In recent years, a lot of researches trying to establish and to characterize natural antioxidants, including both isolated compounds and natural extracts to be topically applied have been performed [5, 12-14]. Peppers and aromatic herbs have been subject of study due to their highly AO properties, generally attributed to the presence of polyphenolic compounds, including flavonoids [15, 16].

Regarding *Pimenta* pseudocaryophyllus, its anxiolytic, antimicrobial, antinociceptive and anti-inflammatory activities have been demonstrated [17, 18]. Studies also show the presence of flavonoids, tanins and other phenolic compounds with AO activity in high concentrations in its leaves [18, 19]. Thus, the development of topical formulations with *P. pseudocaryophyllus* may represent a promising strategy for skin protection against damages caused by UV irradiation.

The present study aimed to evaluate the *in vivo* efficacy of formulations containing *P. pseudocaryophyllus* ethanolic extract (PPE) in the prevention and / or treatment of oxidative damage caused by UV irradiation in the skin of hairless mice.

2. Material and Methods

2.1. Chemicals

Brilliant blue dye, hexadecyltrimethylammonium bromide (HTAB), o-dianisidine dihydrochloride, ethylene glycol bis (-aminoethyl ether)-N,N,N0,N0-tetraacetic acid (EGTA), o-phthalaldehyde (OPT), GSH, sodium dodecyl sulphate (SDS) and acrylamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Raw materials for formulations were obtained from Galena (Campinas, SP, Brazil) and are presented in the formulation section. All other reagents used were of pharmaceutical grade.

2.2. Formulations

Formulations were developed varying the content of lipidic and emulsifying agent. Self-emulsifying wax Polawax® (cetostearyl alcohol+ polyoxyethylene derived of a fatty acid ester of sorbitan 20E) was used in both formulations, although in major (10%) and minor (2%) proportions in formulation 1 (F1) and formulation 2 (F2), respectively. Into F2, 0.18% of anionic hydrophilic colloid (carboxypolymethylene, Carbopol®) was also added as stabilizing agent. Caprylic/capric triglycerides (5%) were added as emollients, and propylene glycol (5%) as moisturizer. The preservative used was a mixture of parabens (1%). Deionized water was used for the preparation of all formulations to complete 100%. PPE was incorporated (5%) into the formulations at room temperature. All concentrations of the formulations raw materials were expressed as percentages weight/weight. Control formulations did not contain PPE.

2.3. Animals

Sex matched hairless mice (HRS/J), weighing 20–30 g, were housed in a temperature-controlled room, with access to water and food *ad libitum* until use. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of State University of Londrina (34994/209).

2.4. Formulation administration

Hairless mice were randomly designed to different groups with 5 mice in each group and topically treated on the dorsal surface with 0.5 g of F1 or F2 with 5% of PPE or 0.5 g of the respective control formulation without PPE. Formulations were administrated 1 h before, 5 min before and right after the irradiation. Untreated control groups irradiated and non-irradiated were included in the experiments. Results are representative of 2–3 separated experiments.

2.5. Irradiation

The UV-B source of irradiation consisted of a Philips TL40W/12 RS lamp (Medical-Holand) emitting a continuous spectrum between 270 and 400 nm with a peak emission at 313 nm. The lamp was mounted 20 cm above the table where the mice were placed on, resulting in an irradiation of 0.384 mW/cm^2, as measured by an IL 1700 radiometer (Newburyport, MA, USA) with sensor for UV (SED005) and UV-B (SED240). The dose of UV-B used was 4.14 J/cm^2 [20, 21]. The mice were killed by ether overdose 12 h after the UV-B exposure, and the full thickness of the dorsal skins were removed and stored at -70^{o} C for further analysis.

2.6. Edema evaluation

The effect of F1 and F2 on UV-B-induced skin edema was measured as an increase in dorsal skin weight. After dorsal skin removal, a constant area (6 mm diameter) was delimitated with the aid of a punch, followed by weighing of this constant area [22, 23]. The result was obtained comparing the weight of the skin between groups and the result was express in g of skin.

2.7. MPO activity

The UV-B-induced leukocyte migration to the skin was evaluated using the MPO kinetic-colorimetric assay as previously described [20, 24]. Skins were collected in 400 µL of 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% of HTAB, and homogenized using Tissue-Tearor (Biospec®). After that, homogenates were centrifuged at 16100 *g* for 2 min at 4 °C. The supernatant was removed to assay. Briefly, 30 µL of sample were mixed with 200 µL of 0.05 M K_2HPO_4 buffer (pH 6.0), containing 0.0167% *o*-dianisidine dihydrochloride and 0.05% hydrogen peroxide. The absorvance was determined after 5 min in 450 nm (Asys Expert Plus, Biochrom). The MPO activity of samples was compared to a standard curve of neutrophils. The results are presented as MPO activity (number of total neutrophils per mg of skin).

2.8. TNF-α, IL-1B and IL-10 assays

Skin samples were removed and then were subsequently homogenized in 500 µL of saline solution (NaCl 0.09%) using Tissue-Tearor (Biospec®). The homogenates were centrifuged at 2000 *g* for 15 min at 4° C and stored at -70° C until further use. Supernatants were used to measure the cytokines. TNF-α, IL-1β and IL-10 contents were determined as described previously by Verri et al. [25] by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience). Absorbances were determined at 450 nm (Victor X3, Perkin Elmer®) and results were expressed as picograms (pg) of each cytocine/ mg of skin [26].

2.9. Analysis of Skin MMP-9 by Substrate-Embedded Enzymography

SDS polyacrylamide gel electrophoresis substrate-embedded enzymography was used to detect MMP-9, an enzyme with gelatinase activity. Assays were carried out as previously described [12, 27]. The total skins taken from each group (1:4, w/w dilution) were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ and 1% of protease inhibitor cocktail in Ultra Turrax (T 18 Basic, IKA®). The entire homogenates were centrifuged twice at 12000 *g* for 10 min at 4°C and the Lowry et al. [28] method was used to measure protein levels on the supernatants.

Aliquots measuring 50 µL were mixed with 10 µL of 100mM Tris–HCl buffer (pH 7.4) containing 4% SDS, 20% glycerol, and 0.005% of xilene cyanol. 25 µL of the mixture (40 µg of protein) were taken for electrophoresis in a gel containing acrylamide 10% and gelatin 0.25%. After electrophoresis, the gels were incubated for 1 h with 2.5% Triton X-100 under constant shaking, incubated overnight in 0.05 M Tris-HCl (pH 7.4), 0.01 M $CaCl_2$ and 0.02% sodium azide at 37 °C, and stained the following day with brilliant blue R. After destaining in 20% acetic acid, zone of enzyme activity were analyzed by comparing the groups in the Image J® program.

2.10. GSH assay

Cutaneous GSH levels were determined using a fluorescence assay as previously described [20]. Firstly, the skin (1:3, w/w dilution) was homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA (buffer 1) using Ultra Turrax (IKA®). After that, homogenates were treated with 30% trichloroacetic acid and then were centrifuged twice (at 1940 *g* for 6 min and at 485 *g* for 10 min) and the fluorescence of the resulting supernatant was measured in a RF-5301PC, Shimadzu® fluorescence spectrophotometer. Briefly, 100 µL of sample were mixed with 1 mL of buffer 1 and 100 µL of OPT (1 mg/mL in methanol). The fluorescence was determined after 15 min (k_{exc} = 350 nm; k_{em} = 420 nm). The standard curve was prepared with GSH in concentrations of 0.0 to 75.0 µM. Results are presented as µM of GSH / mg of skin.

2.11. Statistical analysis

The bars in the figures indicate the mean values ± standard error of the mean (SEM) of 2 separated experiments with n = 5 animals per group. Data were statistically analyzed by one-way ANOVA followed by Bonferroni's t test. Results were considered significantly different when $p < 0.05$ was obtained.

3. Results

3.1. Formulations containing PPE prevent the UV-B-induced edema in the skin

Several studies show that exposure to UV-B light leads to skin edema, which can be considered a marker of skin inflammation [22, 23, 29]. Standard-sized punches of skin were weighted in order to evaluate the capacity of formulations to inhibit UV-B irradiation-induced edema. As shown in Fig. 1, UV-B exposure increased approximately 2.46 fold punch weight of untreated irradiated animals and irradiated animals treated with control formulations. F1 and F2 containing PPE were able to decrease edema formation to control levels.

3.2. Formulations containing PPE prevent the UV-B-induced MPO activity increase in the skin

MPO plays an important role on the defense of the innate immune system and can be used as a marker of the presence of neutrophils or inflammation [11]. Results show that UV-B irradiation induced an increase of approximately 7.63 fold in the MPO activity of untreated irradiated animals and irradiated animals treated with control formulations. F1 and F2 containing PPE inhibited the MPO activity to non-irradiated control levels (Fig. 2).

3.3. Influence of formulations containing PPE on cytokines after UV-B exposure

UV-B light significantly increased TNF-α and IL-1β levels (approximately 3.15 and 2.21 fold, respectively) of untreated irradiated animals and irradiated animals treated with control formulations. F1 and F2 added with PPE decreased IL-1β to control levels (Fig. 3A). However, F1 and F2 added with PPE were not able to decrease TNF-α level (Fig. 3B). UV-B irradiation had no influence in IL-10 levels. Nevertheless, F2 added with PPE increased IL-10 levels by approximately 2.03 fold comparing to control values (Fig. 3C).

3.4. Formulations containing PPE prevent the UV-B-induced MMP-9 activity increase

Results showed a significant increase in the expression/activity of gelatinase in hairless mice skin after UV-B irradiation. The semi-quantitative analysis of MMP-9 in the skin demonstrated that UV-B irradiation induced an increase of approximately 1.63 fold in MMP-9 activity of untreated irradiated animals and irradiated animals

treated with control formulations. F1 and F2 added with PPE were again able to inhibit MMP-9 activity to control levels (Fig. 4).

3.5. Formulations containing PPE prevent the UV-B-induced GSH depletion

In this study, UV-B irradiation induced a decrease of approximately 2.14 fold of GSH levels in untreated irradiated animals and irradiated animals treated with control formulations. Both formulations containing PPE inhibited this depletion, maintaining levels similar to control (non-irradiated) group (Fig. 5).

4. Discussion

In the present study, it was observed that topical formulations containing PPE inhibited the inflammatory and oxidative phenomena in the skin of hairless mice irradiated with UV-B irradiation. The formulations with different lipid contents were able to inhibit UV-B irradiation-induced increase of skin edema, MPO activity, MMP-9 activity and IL-1β production, as well as GSH depletion. F2 was also able to increase the levels of the anti-inflammatory cytokine IL-10. Therefore, these results show in a consistent manner that *P. pseudocaryophyllus* extract can be delivered using topical formulations to reduce UV-B-induced inflammation and oxidative stress of the skin, and a formulation with less lipid content might be more suitable to deliver the extract.

Sunlight coupled with living in an oxygen-rich atmosphere causes unwanted and deleterious consequences to the skin, such as cancer, wrinkling, scaling, dryness, and mottled pigment abnormalities (hyper or hypopigmentation) [30]. Inflammatory chemical mediators including cyclooxygenase derived metabolites of arachidonic acid increase vascular permeability and blood flow [22, 31, 32]. The spectrum of UV light that induces edema in hairless mice and erythema in humans is the same. Because of this similarity, a change in sensitivity to UV radiation in mice as measured by skin edema should reliably predict a changed in the sensitivity in humans [29]. Our results clearly show that PPE incorporated to both formulations was able to inhibit skin edema in animals exposed to UV irradiation. In addition to the present data using an UV irradiation model of inflammation, a fraction of the *P. pseudocaryophyllus* ethanolic extract inhibited the croton oil-induced ear edema [18]. Therefore, the extracts of *P. pseudocaryophyllus* have wider applicability and in

models with different triggering mechanisms. For comparison purposes, it is noteworthy to mention that extracts of a different species of *Pimenta*, *Pimenta racemosa,* inhibits paw and ear edema induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) and carragenin in mice [33, 34].

In general, neutrophils are the first cells recruited from peripheral blood to inflammatory sites. ROS such as superoxide anion are essential for neutrophil recruitment to the inflammatory loci and ROS production is triggered by UV-B irradiation [11, 31, 35]. One of the special neutrophils products is the heme enzyme MPO, which is stored in large amounts in azurophilic granules of these cells [36]. MPO-derived hypochlorous acid (HOCl) reacts with proteins, DNA and lipids to form long-lived oxidants, which have been implicated in processes like carcinogenesis, atherosclerosis and chronic renal failure [31]. F1 and F2 containing PPE were able to decrease UV-induced MPO activity to control levels, demonstrating its anti-inflammatory and protective activity. Corroborating, Garcia et al. [33] and Fernandez et al. [34] also demonstrated the effectiveness of *P. racemosa* against TPA-induced MPO increase in ear tissue.

In ROS-induced inflammation, nuclear factor kB (NF-kB) plays a crucial role. It binds to distinct promoter genes, which encode TNF-α, interleukins (IL-6 and IL-1) and several adhesion molecules, thus allowing their transcription [4, 37]. In this context, UV-B stimulation of cultured human keratinocytes induces the expression of cytokines, such as TNF-α, IL-1α, IL-1β and IL-6 [4]. IL-1 and TNF-α stimulate neutrophils and other cells, increasing expression of adhesion molecules, such as intercellular adhesion molecules (ICAMs) and L-selectins [38-40]. In this study, UV-B irradiation increased both IL-1β and TNF-α levels. Again, F1 and F2 containing PPE decreased IL-1β to control levels, demonstrating the potential anti-inflammatory effect of the extract. However, F1 and F2 with PPE were not able to decrease TNF-α production. This selective effect on IL-1β production, but not TNF-α, suggests that the extract might act at specific intracellular targets related to IL-1β production. The inhibition of IL-1β production by the formulations containing PPE lines up well with the inhibition of MPO activity, since IL-1β is chemotatic for neutrophils [41], which indicates that inhibition of IL-1β production could lead to reduced recruitment of neutrophils and, therefore, reduced MPO activity. Regarding IL-10, UV-B irradiation had no influence on its levels in the present experimental conditions. Nevertheless, F2 containing PPE increased the production of IL-10. IL-10 is an anti-inflammatory

cytokine, which inhibits NF-kB and balances the activators and inhibitors signals of the inflammatory process by reducing the transcription and the production of pro-inflammatory cytokines [42, 43]. Therefore, an additional anti-inflammatory mechanism of F2 compared to F1 was the induction of IL-10 production. It is possible that F2 presents better lipid concentration for the release of active compounds of PPE than F1.

Besides NF-kB, another transcription factor induced by UV irradiation is the activation protein-1 (AP-1), activated by a series of mitogen-activated protein kinases. As a consequence, occurs the induction of MMPs, which degrade the collagen framework of skin [12, 30]. MMP-9 (gelatinase B) is one of the primary enzymes related to degradation of skin collagen and components of the elastic fibers network. It displays the greatest elastolytic and fibrillin-degrading activity [44]. MMPs are produced by fibroblasts, keratinocytes, mast cells, endothelial cells and leukocytes, such as neutrophils, and, like MPO, are released from cytoplasmatic granules [45]. In the present study, UV-B irradiation clearly induced an increase of MMP-9 activity. The capacity of formulations containing PPE to prevent the increase of MMP-9 activity was presented in qualitative and semi-quantitative manners. Similarly, Bellosta et al. [46] demonstrated the activity of the extract of *Tristaniopsis calobuxus*, which, as *P. pseudocaryophyllus*, also belongs to Myrtaceae family, to inhibit MMP-9 activity in mouse macrophages. The activation of MMPs results in elevated levels of degraded collagen, which appear to downregulate type I procollagen synthesis. Thereby, Fonseca et al. [12] suggests that a formulation able to inhibit the decrease of procollagen synthesis might also be able to improve the skin collagenization.

Importantly, UV-B irradiation also leads to an imbalance between ROS and endogenous antioxidants, causing depletion of endogenous antioxidants such as GSH, an epidermal marker that is sensitive to oxidative stress caused by UV-B irradiation [4, 47]. Its sulfhydryl grouping (SH), highly polarizable, allows the removal of radicals directly by hydrogen transfer, which makes it a great nucleophile for reactions with electrophilic chemicals [6]. Besides, it acts as cofactor for glutathione peroxidase and glutathione reductase, which reduce hydrogen peroxide and lipid hydroperoxides [4, 30]. In the present study, the levels of GSH in non irradiated control and in groups treated with F1 and F2 containing PPE were significantly higher

than in the other three groups ($p < 0.01$), suggesting that formulations added with PPE played a strong AO activity by maintaining GSH levels despite UV-B exposure.

To our knowledge, this is the first study to demonstrate the beneficial effectiveness of *Pimenta pseudocaryophyllus* on UV-B-induced skin oxidative stress and inflammation. Thus, these data suggest the possible usefulness of PPE to prevent skin damages caused by UV-B radiation and show the importance to perform further studies with this extract.

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Figures

Figure 1. Formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) inhibit UV-B irradiation-induced edema. Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *p < 0.001 compared to the control (non-irradiated) group, **p < 0.001 compared to irradiated group and F1 control group, and ***p < 0.001 compared to irradiated group and F2 control group.

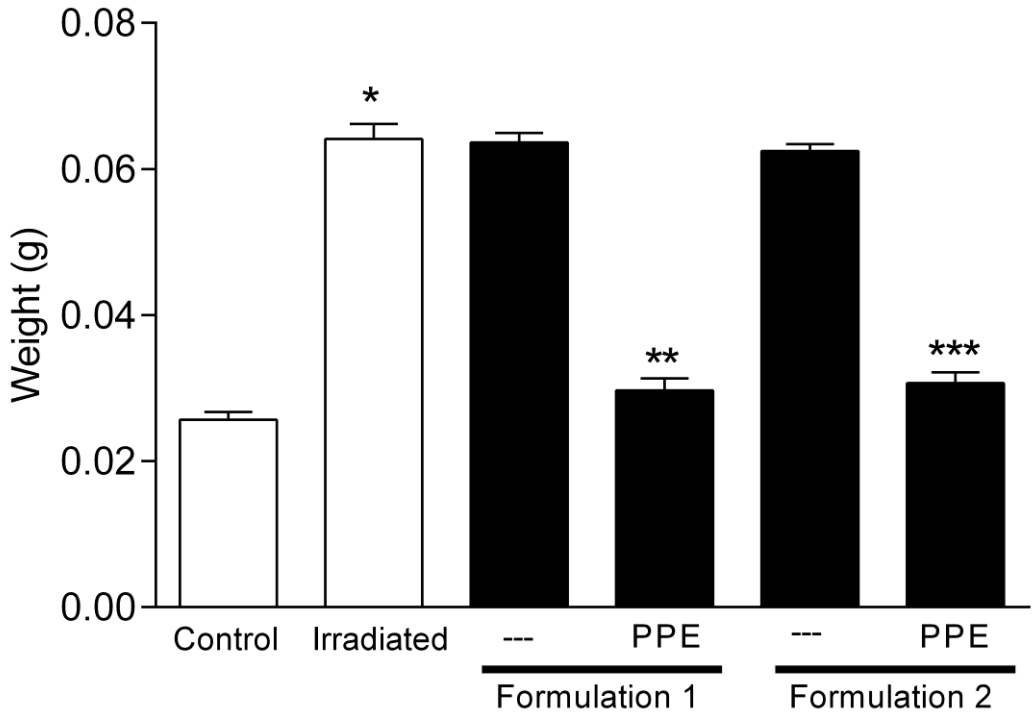


Figure 2. Formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) inhibit the UV-B irradiation-induced increase of myeloperoxidase (MPO) activity. Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *$p < 0.001$ compared to the control (non-irradiated) group, **$p < 0.001$ compared to irradiated group and F1 control group, and ***$p < 0.001$ compared to irradiated group and F2 control group.

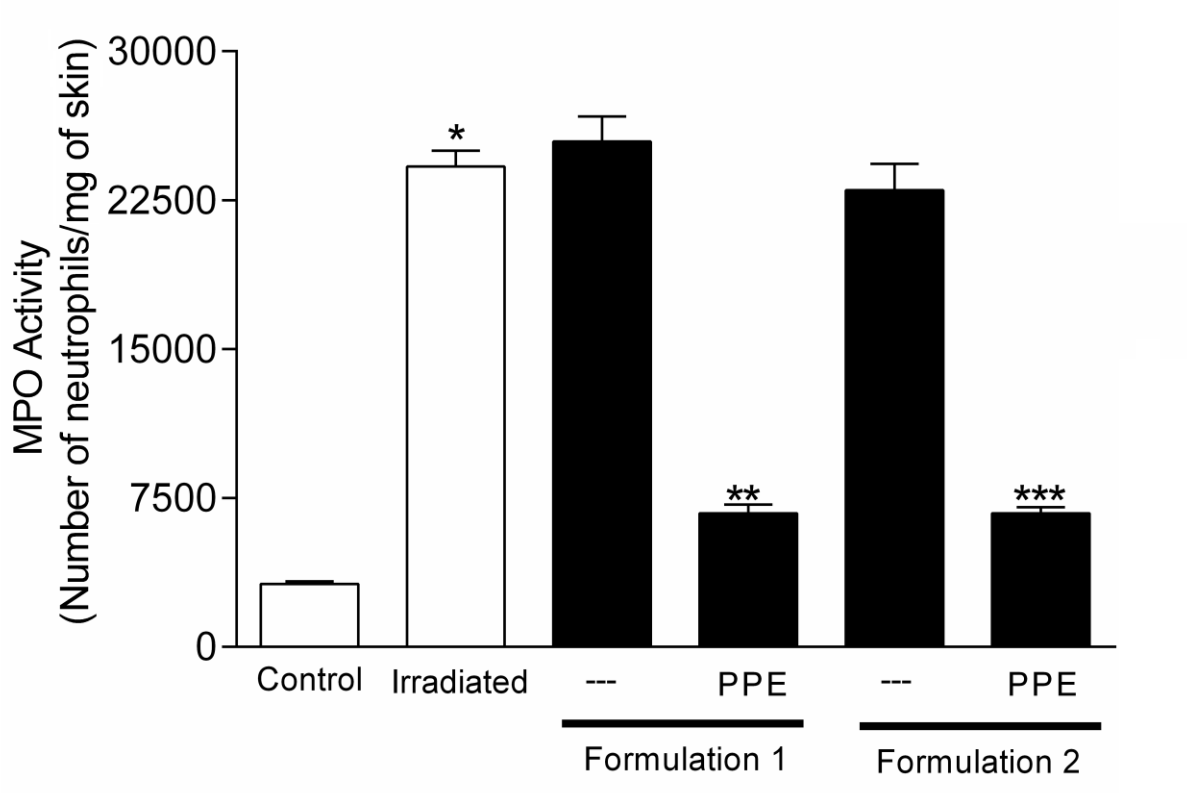


Figure 3. Effect of formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) on cytokine production induced by UV-B irradiation. Mice were treated with F1 or F2 containing PPE or controls and were challenged with UV-B irradiation. The levels of IL-1β (A), TNF-α (B) and IL-10 (C) were determined at the 12 hours after the irradiation. Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *p < 0.001 compared to the control (non-irradiated) group, **p < 0.001 compared to irradiated group and F1 control group, and ***p < 0.001 compared to irradiated group and F2 control group.

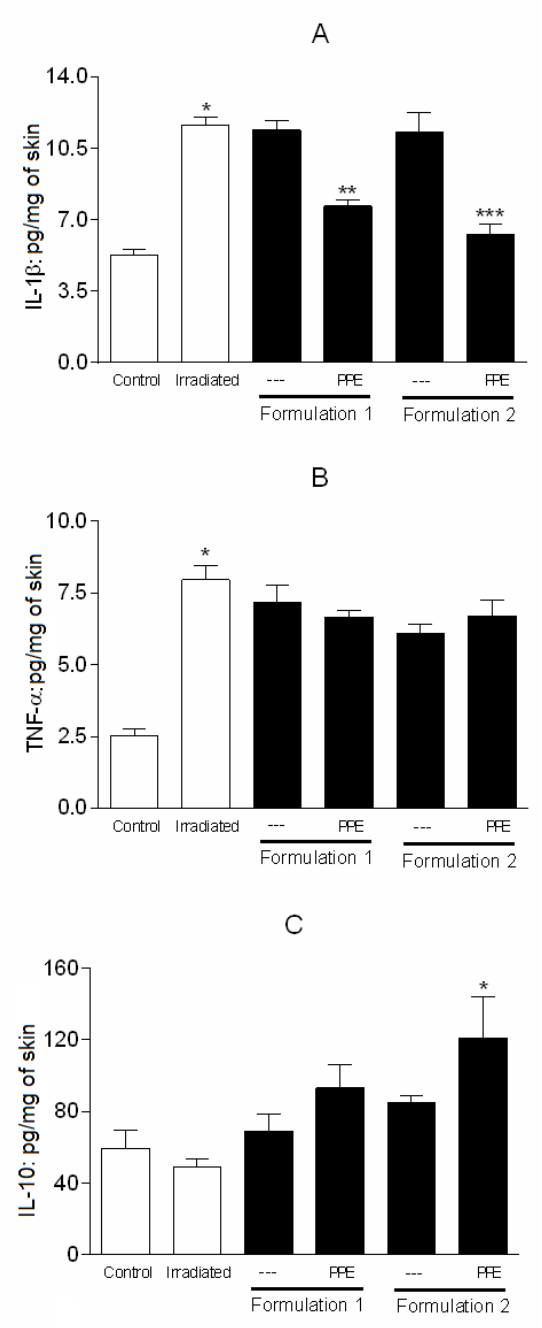


Figure 4. Formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) inhibit UV-B irradiation-induced increase of metalloproteinase-9 (MMP-9) activity. Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *$p < 0.001$ compared to the control (non-irradiated) group, **$p < 0.001$ compared to irradiated group and F1 control group, and ***$p < 0.001$ compared to irradiated group and F2 control group.

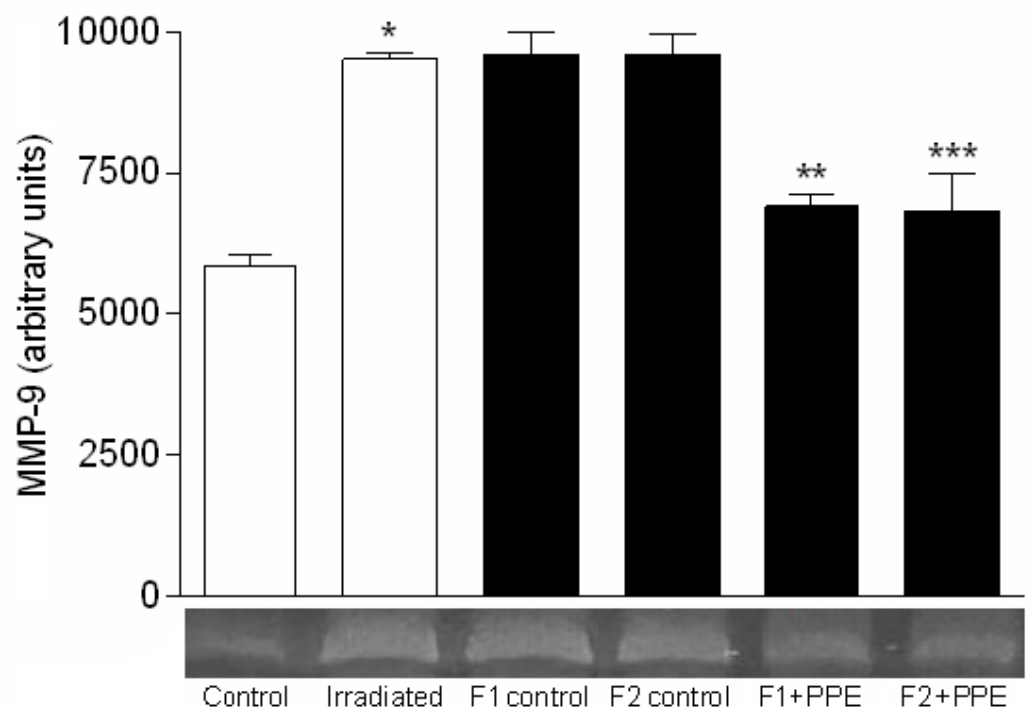
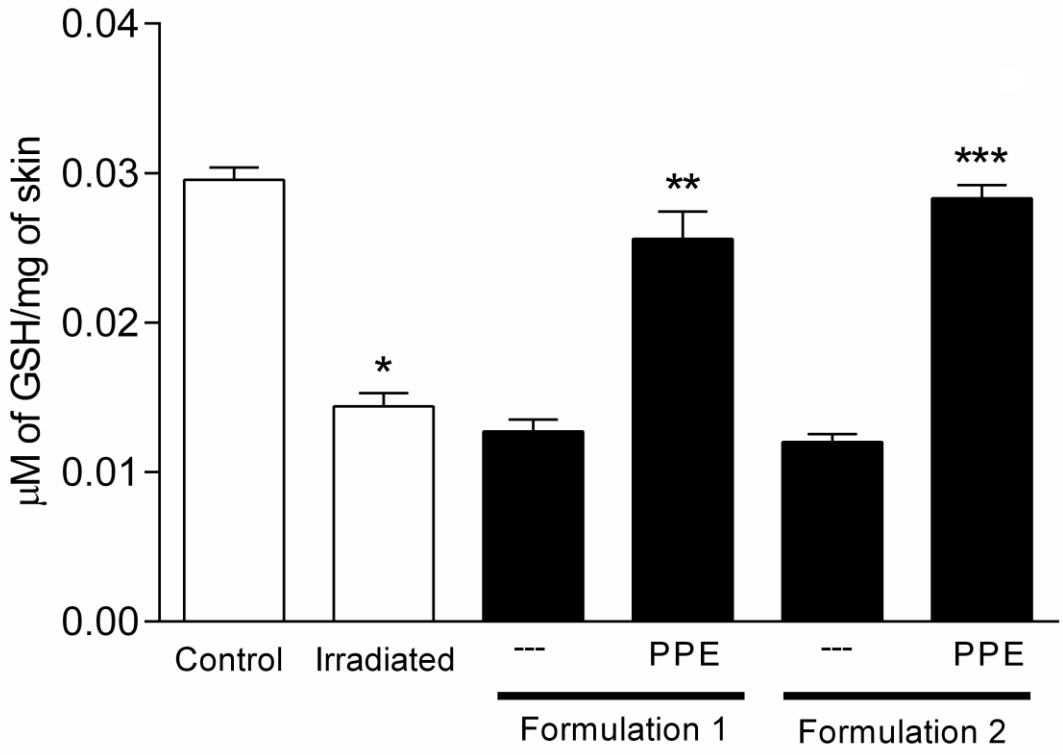


Figure 5. Formulations 1 and 2 containing *P. pseudocaryophyllus* ethanolic extract (PPE) inhibit the reduced glutathione (GSH) depletion induced by UVB irradiation. Bars represent means ± SEM of 2 separated experiments, 5 mice per group. *p < 0.001 compared to the control (non-irradiated) group, **p < 0.001 compared to irradiated group and F1 control group, and ***p < 0.001 compared to irradiated group and F2 control group.



5 CONCLUSÕES

Os resultados dos estudos *in vitro* mostraram que o extrato etanólico de *Pimenta pseudocaryophyllus* (PPE) é rico em polifenois e flavonoides, indicando a presença de eugenol, rutina e ácido tânico. Corroborando a caracterização química, verificou-se elevada atividade antioxidante do mesmo e das formulações adicionadas de PPE. Ainda, verificou-se que ambas as formulações testadas são estáveis, sendo necessário armazená-las à 4º.C e liberam o extrato eficientemente.

Os resultados *in vivo* demonstraram que o tratamento com as formulações contendo o extrato foi eficaz na melhora dos parâmetros inflamatórios e oxidativos da pele induzidos por irradiação UVB.

Assim, esses dados sugerem a possível utilização de formulações tópicas contendo PPE para prevenir e tratar danos foto-oxidativos cutâneos causados pela radiação UVB.

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ANEXO A: Guia para Autores – Industrial Crops and Products

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[1] B.K. Armstrong, A. Kricker, The epidemiology of UV induced skin cancer, J. Photochem. Photobiol.B 63 (2001) 8-18.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, third ed., Macmillan, New York, 1979.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing, Inc., New York, 1994, pp. 281-304.

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