CAMILLA CAROLINA ARRIERO RODRIGUES

EFEITO PROTETOR DA FORMULAÇÃO TÓPICA CONTENDO ÁCIDO VANÍLICO CONTRA INFLAMAÇÃO E O ESTRESSE OXIDATIVO INDUZIDOS PELA RADIAÇÃO UVB EM CAMUNDONGOS SEM PELO.

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

Orientadora: Profa. Dra. Rúbia Casagrande

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RODRIGUES, Camilla Carolina Arriero. **Efeito protetor da formulação tópica contendo ácido vanílico contra inflamação e estresse oxidativo induzidos pela radiação UVB em camundongos sem pelo.** 120 f. Dissertação (Mestrado em Biomedicina). Universidade Estadual de Londrina, Londrina, Paraná, 2019.

RESUMO

A radiação ultravioleta B (UVB) é um dos principais fatores de risco para doenças dermatológicas e a exposição crônica à radiação UVB tem sido relacionada ao câncer de pele e ao envelhecimento cutâneo prematuro. Estes eventos desencadeiam o excesso de espécies reativas de oxigênio que levam ao desequilíbrio de moléculas pro e antioxidantes, e se instala no organismo o processo denominado estresse oxidativo. Neste caso os antioxidantes endógenos não conseguem manter a homeostase e se faz necessário o uso de terapias exógenos no intuito de melhorar esse desequilíbrio. Neste sentido, o ácido vanílico tem se mostrado um promissor regulador desse desequilíbrio, ele é a forma oxidada da vanilina, é um composto fenólico, presente no extrato da baunilha, um agente aromatizante encontrado em plantas e frutos comestíveis, que possui atividade antioxidante. Desta forma, os objetivos da presente pesquisa foram preparar uma formulação tópica contendo ácido vanílico e avaliar a estabilidade físico-química e funcional da formulação, bem como avaliar seus efeitos terapêuticos tópicos nos danos cutâneos inflamatórios e oxidativos induzidos pela radiação UVB em camundongos sem pelo. As formulações contendo ou não o ácido vanílico foram preparadas e armazenadas em temperatura de 4°C, temperatura ambiente (TA) e 40±2°C/75±5% de umidade relativa (UR) pelo período de seis meses (180 dias) e em tempos pré-determinados alíquotas foram avaliadas quanto ao aspecto visual, pH, separação de fases após centrifugação. Em adição, foi avaliada a estabilidade funcional pelo método do sequestro do radical $ABTS^{+}$ para testar a atividade antioxidante da formulação. Para os experimentos *in vivo*, as formulações contendo ou não o ácido vanílico foram administradas no dorso dos animais uma hora antes, cinco minutos antes e cinco minutos após o período de irradiação que durou cinco horas e meia (4,14 J/cm^2). Após 2, 4 e 12h foi realizada a eutanásia dos animais para os testes de edema, cutâneo, histologias para espessura epidérmica, queratinócitos apoptoticos e colágeno dérmico, metaloproteinase 9 (MMP-9), poder redutor do íon ferro (FRAP), sequestro do radical cátion ABTS, glutationa reduzida (GSH), catalase (CAT), hidroperóxidos lipídicos (LOOH) e produção de ánion superóxido ($O_2^{\bullet-}$) e as peles dos animais foram retiradas e armazenadas no -80°C. Os resultados de estabilidade físico-química e funcional mostraram que a formulação foi estável nas diferentes temperaturas de armazenamento por 6 meses. A formulação contendo ácido vanílico reduziu o edema cutâneo, a espessura epidérmica, o número de queratinócitos apoptóticos e os danos nas fibras de colágeno, além da secreção/atividade da MMP-9. Ainda, foi possível observar que a administração tópica de ácido vanílico incorporado a formulação resultou em uma melhora da capacidade antioxidante da pele pela manutenção do FRAP, sequestro do $ABTS^{+}$, os níveis dos antioxidantes endógenos GSH e atividade da CAT, e pela redução da produção de LOOH e de $O_2^{\bullet-}$. Em suma, estes resultados sugerem o uso do ácido vanílico veiculado em formulação tópica como estratégia relevante para doenças cutâneas causadas pela exposição à radiação UVB.

Palavras-chave: Ácido vanílico. Estresse oxidativo. Composto fenólico. Pele.

RODRIGUES, Camilla Carolina Arriero. **Protective effect of topical formulation containing vanillic acid against UVB-induced inflammation and oxidative stress in hairless mice.** 120 p. Dissertation (Master's degree in Biomedicine). Universidade Estadual de Londrina, Londrina, Paraná, 2019.

ABSTRACT

Ultraviolet B (UVB) radiation is a major risk factor for dermatological diseases and chronic exposure to UVB radiation has been linked to skin cancer and premature skin aging. These events trigger the excess of reactive oxygen species that lead to the imbalance of pro and antioxidant molecules, and the process called oxidative stress is installed in the body. In this case the endogenous antioxidants cannot maintain homeostasis and it is necessary to use exogenous therapies in order to improve this imbalance. In this sense, vanillic acid has been shown to be a promising regulator of this imbalance, it is the oxidized form of vanillin, a phenolic compound, present in vanilla extract, a flavoring agent found in plants and edible fruits, which has antioxidant activity. Thus, the objectives of the present research were to prepare a topical formulation containing vanillic acid and to evaluate the physicochemical and functional stability of the formulation as well as to evaluate its topical therapeutic effects on the inflammatory and oxidative skin damage induced by UVB radiation in hairless mice. Formulations containing or not vanillic acid were prepared and stored at 4°C, room temperature (RT) and 40 ± 2°C / 75±5% relative humidity (RH) for six months (180 days) and at pre-determined aliquots times were assessed for visual appearance, pH, phase separation after centrifugation. In addition, functional stability was assessed by the ABTS + radical sequestration method to test the antioxidant activity of the formulation. For the in vivo experiments, the formulations containing or not vanillic acid were administered on the animals back one hour before, five minutes before and five minutes after the irradiation period which lasted five and a half hours (4.14 J / cm2). After 2, 4 and 12 h the euthanasia of the animals was evaluated for edema, cutaneous, histologies for epidermal thickness, apoptotic keratinocytes and dermal collagen, metaloproteinase 9 (MMP-9), iron ion reducing power (FRAP), radical cation ABTS, reduced glutathione (GSH), catalase (CAT), lipid hydroperoxides (LOOH) and superoxide ion production ($O2^{\bullet -}$) and animal skins were removed and stored at -80°C. The physico-chemical and functional stability results showed that the formulation was stable at different storage temperatures for 6 months. The formulation containing vanillic acid reduced cutaneous edema, epidermal thickness, number of apoptotic keratinocytes and damage to collagen fibers, as well as secretion / activity of MMP-9. Furthermore, it was possible to observe that the topical administration of vanillic acid incorporated into the formulation resulted in an improvement of the antioxidant capacity of the skin by the maintenance of FRAP, $ABTS^{+}$ sequestration, GSH endogenous antioxidant levels and CAT activity, and the reduction of the production of LOOH and $O2^{\bullet -}$. In summary, these results suggest the use of vanillic acid in topical formulation as a relevant strategy for cutaneous diseases caused by exposure to UVB radiation.

Keywords: Vanillic acid. Oxidative stress. Phenolic compound. Skin.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2’ azinobis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
AP-1	Ativador de proteína-1
ARE	Elemento responsivo a antioxidante
$CaCl_2$	Cloreto de cálcio
CAT	Catalase
DNA	Ácido desoxirribonucleico
DPPH•	2,2-difenil-1-picrilhidrazil
EDTA	Ácido etilenodiamino tetra-acético
EPM	Erro padrão da média
EROs	Espécies reativas de oxigênio
Fe_2^+	Ferro
FeCl3	Cloreto férrico
FRAP	Poder antioxidante de redução férrica
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
HCl	Ácido clorídrico
HO-1	Hemeoxigenase-1
H_2O	Água
H_2O_2	Peróxido de hidrogênio
HOCl	Ácido hipocloroso
H_3PO_4	Ácido ortofosfórico
KCl	Cloreto de potássio
K_2HPO_4	Fosfato de potássio dibásico
KH_2PO_4	Fosfato de potássio monobásico
KOH	Hidróxido de potássio
LOOH	Hidroperóxidos lipídicos
LPO	Peroxidação lipídica
MMPs	Metaloproteinases da matriz
NaCl	Cloreto de sódio
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NaOH	Hidróxido de sódio

NBT	*Nitroblue tetrazolium*
NF-κB	Fator nuclear kappa B
NO•	Radical óxido nitrico
NOX_2	NADPH oxidase 2
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
O2	Oxigênio molecular
$O2^{\bullet-}$	Radical superóxido
$^1O_2$	Oxigênio singlete
OH	Hidroxila
•OH	Radical hidroxil
RNAm	Ácido ribonucleico mensageiro
RO^2•	Radical peroxil
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em gel de poliacrilamida com duodecil sulfato de sódio
SOD	Superóxido dismutase
TCA	Ácido tricloro acético
TPTZ	2,4,6 tripiridil-S-triazina
Tris	Hidroximetil aminometano
UV	Ultravioleta
UVB	Ultravioleta B
VA	Ácido Vanílico

SUMÁRIO

1. INTRODUÇÃO

1.1 ESTRUTURA DA PELE HUMANA

Por ser o maior órgão do corpo humano, a pele que contempla 16% do peso corporal, desempenha múltiplas funções, como barreira de proteção contra agentes externos, substâncias químicas, patógenos e a radiação ultravioleta (RUV). Em adição, impede também a perda excessiva de água e eletrólitos do corpo, exercendo importante função termorreguladora (AFAQ; ADHAMI; MUKHTAR, 2005; FONSECA et al., 2011; KHAVKIN; ELLIS, 2011). O pH da pele encontra-se em torno de 4,6 e 5,8 e desempenha um papel importante nas condições de pele. O manto ácido é essencial para a barreira protetora da pele. Neutraliza agressores à base de alcalinos, inibe o crescimento de bactérias, restaura e mantém o ambiente ácido ideal para a flora natural da pele prosperar (ALI; YOSIPOVITCH, 2013).

A pele é composta pelas camadas: epiderme, derme e hipoderme (Figura 1). A epiderme é a camada superior da pele, um epitélio queratinizado, estratificado e escamoso e é dividida em estratos, sendo eles:

Basal que tem células cilíndricas ou cuboides, a qual separa a epiderme da derme, é responsável pela contínua renovação da epiderme (MENON, 2002)

Espinhoso com células em formato poligonal ou achatadas, de núcleo central.

Granuloso tem células achatadas, núcleo central e citoplasma com grânulos basófilos chamados de querato-hialina. Sua função principal é formar barreira contra a penetração de substâncias, inibindo a desidratação do organismo (LAZAR, 2002).

Lúcido presente apenas na epiderme da pele espessa (palma das mãos e planta dos pés).

Córneo é mais externo, tem células achatadas, mortas e sem núcleo, é a principal barreira de permeabilidade para as substâncias externas além de fator limitante da penetração dos ativos através da pele (FOLDVARI, 2000). Sua barreira lipídica é o principal desafio para a entrega tópica de agentes terapêuticos (MENON; CLEARY; LANE, 2012)

As principais células que compõe a epiderme são os queratinócitos, que representam 95% da sua composição (FONSECA et al., 2011; KHAVKIN; ELLIS, 2011). Os queratinócitos produzem uma barreira a partir de sua diferenciação à medida que se movem da camada basal para o estrato córneo. Os queratinócitos são produzidos e renovados na epiderme aproximadamente a cada 28 dias. Essas células levam 14 dias para atingir o estrato córneo e outros 14 dias para descamarem (LOSQUADRO, 2017).

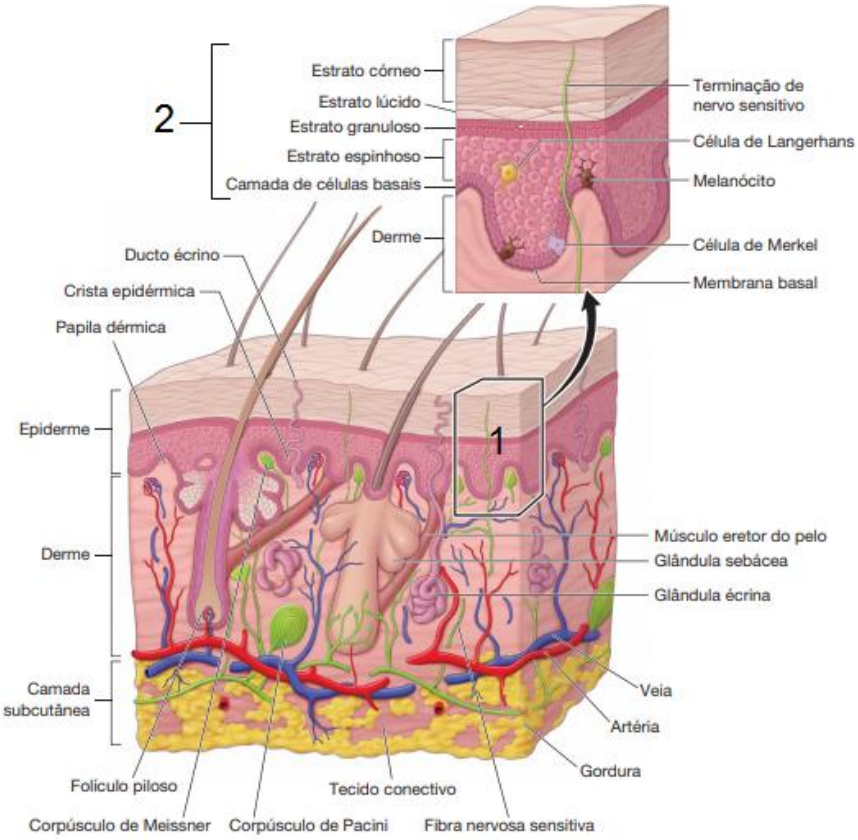

Figura 1: Corte transversal da pele, delimitação das camadas: epiderme, derme e hipoderme, (1) ampliação da área de epiderme e começo de derme (2) e diferenciação dos estratos da epiderme.

A epiderme também é constituída pelo sistema melânico, composto pelas células denominadas melanócitos que produzem melanina; células de Langerhans, de função imunológica, com a função de captar, processar e apresentar os antígenos aos linfócitos T; células de Merkel que são interligadas com o sistema nervoso, responsáveis pela sensibilidade cutânea (BENSON; WATKINSON, 2012; CHANG, 2010).

A melanina é o principal pigmento biológico envolvido na pigmentação cutâneo, sendo determinante nas diferentes colorações da pele, atua como um filtro óptico, absorve a energia e estabiliza os radicais livres originados pela radiação (BENSON; WATKINSON, 2012). A melanina armazenada no citoplasma dos queratinócitos, oferece máxima proteção ao DNA contra os efeitos negativos da radiação ultravioleta (COSTIN; HEARING, 2007).

A derme situa-se abaixo da epiderme, é constituída por tecido conjuntivo, que fornece resistência à pele. Composta por colágeno e fibras elásticas (produzidas por fibroblastos), vasos sanguíneos e linfáticos, nervos e terminações nervosas, folículos pilosos e glândulas sebáceas e sudoríparas. Além de fibroblastos, a derme possui células relevantes imunologicamente como células dendríticas, macrófagos, mastócitos e linfócitos (HEATH; CARBONE, 2013).

A última camada e mais profunda é a hipoderme, composta por células adiposas que reforçam a estrutura do tecido conjuntivo e funciona como barreira protetora isolante. Além disso, apresenta também como função a reserva de energia, e permite a mobilidade sobre estruturas de suporte, moldando os contornos do corpo. Os adipócitos formam a maior parte das células nessa camada e estão organizados em septos de tecido conectivo fibroso, podendo ser encontrado nervos, vasos sanguíneos e linfáticos (WOLFF et al., 2003).

1.2 A PELE COMO VIA DE ADMINISTRAÇÃO

A pele tem se mostrado uma eficiente via de administração de formulações medicamentosas. A aplicação tópica possibilita a administração de medicamentos de forma segura e efetiva, além de evitar efeitos como irritação gastrointestinal, toxicidade sistêmica e metabolismo hepático. Uma das grandes vantagens da administração tópica de fármacos é a ação direta no sítio alvo ou muito próximo, necessitando assim, de uma menor quantidade de substância ativa, e consequentemente, menos efeitos secundários (BENSON; WATKINSON, 2012)

Alguns compostos são aplicados a pele, para permanecerem na superfície como os protetores solares e os cremes barreira, enquanto que outras substâncias destinam-se a atingir a epiderme e derme, como por exemplo os analgésicos locais ou os antifúngicos (UEDA et al., 2010).

O transporte de ativos pela pele pode ocorrer por três vias: Intracelular (diretamente através de células), intercelular (em torno das células) ou por anexos da pele (TROMMER; NEUBERT, 2006). A primeira ocorre com a difusão do ativo que passa diretamente pelos corneócitos e pela matriz lipídica intercelular; a segunda o ativo passa entre as células permitindo a difusão de solutos lipofílicos ou apolares através da matriz lipídica contínua (rota proposta para a maioria dos ativos) e a terceira pelos apêndices cutâneos (glândulas ou folículos pilosos) (ALLEN, L. V. JR.; POPOVICH, N. G.; ANSEL, 2007).

Em média, na superfície cutânea existem 40-70 folículos pilosos e 200-250 ductos sebáceos por metro quadrado de área de pele e por eles a absorção cutânea é muito intensa (CHIEN; LIU, 1986), entretanto, estes apêndices da pele representam apenas 0,1% da superfície cutânea total, e desta maneira, a absorção percutânea pela via transepidérmica se torna a principal via de permeação de absorção de fármacos (ALLEN, L. V. JR.; POPOVICH, N. G.; ANSEL, 2007).

É necessário ter em consideração o tipo de fármaco a veicular, uma vez que cada fármaco está relacionado com o tipo de transporte. Substâncias hidrofílicas ou com carga penetram com mais dificuldade o estrato córneo, devido à sua natureza lipídica e seu teor baixo de água. Por esta razão, o transporte de substâncias lipofílicas é facilitado, devido à

dissolução nos lipídeos intercelulares do estrato córneo (STAHL; WOHLERT; KIETZMANN, 2011).

1.3 RADIAÇÃO ULTAVIOLETA E SUAS CONSEQUÊNCIAS NA PELE

A luz solar é um espectro eletromagnético, e emite radiação, que alcança a terra sob a forma de ondas. O espectro eletromagnético é dividido em três regiões distintas: região elétrica com as ondas de rádio e micro-ondas, radiação óptica que compreende as radiações infravermelha visível e ultravioleta e a terceira que compreende os raios X, raios gama e os raios cósmicos (SHETTY et al., 2015).

As ondas UV (ultravioleta) são as que estão no espectro de 100 a 400nm e têm potencial carcinogênico, são capazes de causar foto envelhecimento e imunossupressão. O espectro da radiação UV subdivide-se em três bandas de comprimento de onda, denominadas UVA (320-400nm), UVB (320-280nm) e UVC (100-280nm). A radiação UV é um dos principais fatores ambientais que afeta a pele de acordo com seus comprimentos de onda (BALUPILLAI et al., 2015; GONZÁLEZ; FERNÁNDEZ-LORENTE; GILABERTE-CALZADA, 2008; SVOBODOVA; WALTEROVA; VOSTALOVA, 2006).

As moléculas presentes na pele capazes de absorver a RUV são denominadas cromóforos. Para manifestar os efeitos deletérios nas células da pele, a energia eletromagnética da RUV é absorvida pelos cromóforos celulares (grupos funcionais que absorvem radiação) e convertida em energia química. As moléculas que estão presentes nas células como ácido ribonucléico (RNA), melanina, proteínas, lipídeos, aminoácidos e o ácido desoxirribonucléico (DNA), cromóforo de maior importância na exposição à RUV, são capazes de absorver energia desta radiação, eles ficam em estado excitado e ao retornar ao estado normal liberam luz. Estes cromóforos energizados podem reagir com oxigênio molecular (O_2), gerando as espécies reativas de oxigênio (EROs), que levam a um desequilíbrio do estado oxidativoda pele (XU; FISHER, 2005).

A radiação UVA consegue atravessar a camada de ozônio, apesar de ser a menos eficiente na produção de eritema e subsequente melanogênese, é indutora de processos oxidativos e alcança camadas mais profundas (derme) (TEWARI et al., 2013).

Já a radiação UVB é mais eficiente na produção de danos diretos ao DNA, fotoimunossupressão, eritema, espessamento do estrato córneo e melanogênese, afetando predominantemente a epiderme e ainda, causa danos indiretos na pele principalmente pela produção de EROs, e desta forma é mais relevante para a fotocarcinogênese (EMRI; HORKAY; REMENYIK, 2006). Esta radiação é mais intensa entre as 10 e 16 horas, sendo aconselhável evitar exposição solar durante este período (SBCD, 2005).

Em contrapartida a UVC é a mais energética e lesiva para as células, antes totalmente filtrada pela camada de ozônio, porém devido as rupturas na mesma, em algumas partes do mundo a radiação UVC atinge a terra (SHETTY et al., 2015; TEWARI et al., 2013).

1.4 ESPÉCIES REATIVAS DE OXIGÊNIO E ESTRESSE OXIDATIVO

Um radical livre é qualquer molécula ou átomo que contenha um ou mais elétrons desemparelhados. Os radicais são produzidos por vias metabólicas de forma controlada e executam funções essenciais para homeostase como na respiração aeróbica, na ativação de fagócitos como mecanismo de defesa e podem fornecer controle essencial das vias de sinalização reguladas por redox. No entanto, se produzidos de forma exacerbada estimulados por alguma doença ou xenobióticos podem interagir com vários componentes do tecido, instalando o que chamamos de estresse oxidativo. Tais interações podem causar disfunções agudas e crônicas (SIES; MEHLHORN, 1986).

O mecanismo de ação das EROs é por óxido-redução e são gerados de diferentes maneiras. Uma delas ocorre durante a respiração celular aeróbia, na qual o oxigênio sofre redução, resultando na formação de H_2O e formação de intermediários reativos como o radical superóxido ($O_2^{\bullet -}$), o radical hidroxil ($^{\bullet}OH$), o radical óxido nítrico ($NO^{\bullet}$), o radical peroxil ($RO_2^{\bullet}$) e algumas espécies reativas não radicalares como o peróxido de hidrogênico (H_2O_2), ácido hipocloroso (HOCl) e oxigênio singlete ($^1O_2$), que apesar de não possuírem elétron livre, podem facilmente sofrer reações e formar radicais livre (HALLIWELL, Barry, 2015; KOHEN, Ron; NYSKA, 2002; LIN; BEAL, 2006)

Em condições normais, a concentração das EROs dentro das células é extremamente baixa, pois o organismo apresenta mecanismos endógenos para manter a concentração das espécies reativas dentro dos limites fisiológicos (RIBEIRO et al., 2005). Após formados estas reagem modificando moléculas por oxidação e desempenham um papel crítico nas vias de sinalização celular, como metabolismo, crescimento, diferenciação e sinalização de morte (IDELCHIK et al., 2017).

Além disso, a atividade da xantina oxidase, o citocromo P450- oxidase, enzimas envolvidas na via de produção de prostaglandinas e tromboxanos e a NADPH-oxidase da membrana plasmática de células polimorfonucleares, macrófagos e células endoteliais, que produzem uma grande quantidade de EROs em resposta ao estímulo fagocitário, também constituem importantes fontes endógenas geradoras de EROs (HALLIWELL, B., 2006).

No metabolismo endógeno, existem antioxidantes de defesa que protegem as células contra os efeitos dos radicais livres que podem ser classificados em antioxidantes enzimáticos e não enzimáticos. Entre os enzimáticos destacam-se a superóxido dismutase

(SOD) que decompõe o $O_2^{\cdot-}$ em H_2O_2, catalase (CAT) que converte o H_2O_2 em H_2O e O_2, glutationa peroxidade (GPx) que catalisam a degradação de H_2O_2 em glutationa oxidada (GSSG) e água, glutationa redutase (GR). Entre os não enzimáticos incluem-se o α-tocoferol (vitamina E), β-caroteno, ácido ascórbico (viatamina C) e a glutationa reduzida (GSH) (HALLIWELL, B.; CROSS, 1994; HUBER; ALMEIDA; DE FÁTIMA, 2008; REINIERS et al., 2014).

Quando a produção de EROs ultrapassa a capacidade do sistema antioxidante endógeno em reduzi-las, a concentração de radicais livres aumenta incontrolavelmente, rompendo o equilíbrio oxidante/antioxidante no organismo, instalando o estresse oxidativo (LOBO et al., 2010) que pode ocasionar danos importantes em moléculas como DNA e proteínas, lipídeos bem como causa lesões implicadas na carcinogênese (KANG et al., 1998) e envelhecimento precoce (IVAN et al., 2014; REEVE et al., 2010).

1.5. MECANISMOS FISIOPATOLÓGICOS DA IRRADIAÇÃO UVB

Uma das principais respostas celulares contra essa alteração é o estímulo para produção de maior quantidade de antioxidantes através da indução do fator de transcrição Nrf2 (fator nuclear [derivado eritróide-2] tipo 2), que está presente no citoplasma das células (MALHOTRA et al., 2010). Em condições normais, a proteína Keap1 forma um complexo com Nrf2, regulando sua ação e impedindo sua ida para o núcleo mediada por ubiquitinação e posterior degradação através do sistema proteassomal. Após a exposição à UVB, esse complexo é dissociado e o Nrf2 é deslocado para o núcleo, ligando-se ao elemento de resposta antioxidante (ARE). A interação entre Nrf2 e ARE leva a ativação da transcrição de genes que codificam proteínas com ações antioxidantes/detoxificantes como a hemeoxigenase-1 (HO-1), NADPH quinona oxidoredutase 1 (NQO1), GR e GPx, CAT, SOD. Neste contexto, o Nrf2 tem apresentado efeito protetor por meio da regulação de moléculas com ações benéficas, garantindo a sobrevivência da célula através da eliminação de substâncias oxidantes (KOBAYASHI, Akira et al., 2004; KOBAYASHI, Makoto; YAMAMOTO, 2005; LEE, Tzong-shyuan; CHAU, 2002; MOTOHASHI; YAMAMOTO, 2007).

Acredita-se que a modulação da atividade de Nrf2 por qualquer composto natural ou sintético é uma nova estratégia terapêutica para o tratamento de doenças (ABED et al., 2015; LU et al., 2016; O'CONNELL; HAYES, 2015; YU; KENSLER, 2005; ZHUANG et al., 2014). Com a inibição da atividade de Nrf2, aumenta os danos cutâneos induzidos pela radiação UVB, devido ao aumento da produção de mediadores inflamatórios e metaloproteinase de matriz do tipo 9 (MMP-9) (KUMAR et al., 2011).

A irradiação ultravioleta ativa o fator de crescimento e os receptores na superfície dos queratinócitos e fibroblastos. Os receptores ativados estimulam as cascatas de

transdução de sinal que induzem o fator de transcrição AP-1, que estimula a transcrição dos genes da metaloproteinase de matriz (MMP), a MMP-9 (gelatinase), entre outras (QUAN et al., 2009). As metaloproteinases da matriz são secretadas pelos queratinócitos e fibroblastos e também pelos gránulos terciário dos neutrófilos e quebram o colágeno e outras proteínas que compõem a matriz extracelular dérmica, além de interferirem na transcrição do gene do colágeno (LEE, Jongsung et al., 2008). O reparo imperfeito do dano dérmico prejudica a integridade funcional e estrutural da matriz extracelular. A exposição repetida ao sol provoca acúmulo de danos dérmicos que resultam em enrugamento precoce característico da pele fotodanificada (JOHN; TUSZYNSKI, 2001).

O processo oxidativo induzido pela radiação UVB afeta macromoléculas, incluindo lipídios. Os radicais livres em excesso agem sobre a membrana biológica das células e a disponibilidade de ácidos graxos poli-insaturados regula o processo de peroxidação lipídica (LPO). A LPO leva a uma desorganização da membrana celular com liberação de ácido graxo como o ácido araquidônico a partir de fosfolipídios, o que desencadeia aumento da fosfolipase A2 e da cicloxigenase-2 (COX-2), resultando em níveis aumentados de prostaglandinas, incluindo a prostaglandina E2 (PGE2) e inflamação na pele. Os produtos primários da LPO incluem os LOOH e sua decomposição origina o radical peroxil (ROO$^{\bullet}$) muito reativo e o responsável por propagar a LPO para as outras células (HALLIDAY, 2005).

A radiação UVB leva a uma inflamação aguda, e suas principais características são marcadas pela dilatação vascular e aumento no fluxo sanguíneo causando eritema e calor, extravasamento, deposição extravascular de fluido plasmático e proteínas a chamada exsudação causando edema, além da migração e acúmulo de leucócitos no local da injúria (RITTIÉ; FISHER, 2002). A exsudação indica um aumento na permeabilidade normal dos pequenos vasos sanguíneos em uma área de injúria e, portanto, uma reação inflamatória, este evento é um marco na inflamação aguda (COLEMAN, 2010).

O mecanismo mais comum de extravasamento vascular ocorre pela contração das células endoteliais resultando em espaços interendoteliais aumentados, e é deflagrado por histamina, bradicinina e muitos outros mediadores químicos que chamamos de resposta transitória imediata, pois ocorre rapidamente após a exposição ao mediador. Em algumas formas de injúria, como a radiação UV, o extravasamento vascular se inicia entre 2 a 12 horas após a injuria e pode durar horas ou mesmo dias (COLEMAN, 2010).

Com o excesso de radiação UVB, os queratinócitos entram em apoptose para tentar eliminar o DNA danificado e este processo é proporcional ao tempo de exposição, nesse processo as células alteram sua morfologia apresentando cromatina condensada, citoplasma eosinofílico e são denominadas como células de sunburn. (LIM; HYUN, 2007).

Outro evento ligado a exposição excessiva a radiação UVB é chamada de hiperqueratose, que ocorre devido ao acúmulo de queratinócitos na epiderme. Horas após o

estímulo da radiação UV cessar, os sinais de resposta a danos dos queratinócitos diminuem e ocorre a sua hiperproliferação e subsequente espessamento da epiderme. O aumento da espessura da epiderme tem como objetivo diminuir a penetração da radiação UVB na pele (KUMAR, Vinay et al., 2015).

Considerando os mecanismos fisiopatológicos abordados, a administração de antioxidantes por via tópica tem se mostrado uma alternativa simples e efetiva para a proteção da pele contra os danos oxidativos e inflamatórios causados pela radiação UVB (CAMPANINI et al., 2013; CASAGRANDE et al., 2007).

1.6 ANTIOXIDANTE EXÓGENO: ÁCIDO VANÍLICO

Estudos epidemiológicos indicam que os protetores solares não são totalmente efetivos em prevenir os danos cutâneos induzidos pela RUV (COOPER; RANGER-MOORE; BOWDEN, 2005). Segundo Heurung, Raju e Warshaw (2014) os protetores solares que apresentam em sua formulação benzofenona ou dibenzoilmetano podem causar reações alérgicas e dermatite de contato pela presença de benzofenona-3 (oxibenzona) e a degradação dessas substâncias ativas podem causar uma inflamação local. Desta forma é necessário o uso de protetores ou bloqueadores aliados com outras fontes de antioxidantes com o intuito de prevenir a pele contra a ação da radiação (HEURUNG; RAJU; WARSHAW, 2014).

Substâncias podem atuar como antioxidantes por diferentes mecanismos, por exemplo, neutralização direta de EROs, quelação de metais, aumento de antioxidantes de baixo peso molecular, ativação de genes que codificam enzimas antioxidantes e consequentemente aumento de enzimas antioxidantes e também pela inibição de oxidases (KOHEN, R., 1999; PROCHÁZKOVÁ; BOUŠOVÁ; WILHELMOVÁ, 2011).

Atenção tem sido dada aos antioxidantes de fontes naturais, que de maneira geral são de baixo custo e, portanto, considerados abordagens terapêuticas promissoras em controlar diversas doenças humanas (ZHOU et al., 2009). Os compostos fenólicos são substâncias contendo pelo menos um anel aromático com ao menos um grupo hidroxila ligado a este anel (VERMERRIS; NICHOLSON, 2006). São produtos do metabolismo secundário das plantas, nas quais exibem inúmeras funções fisiológicas dentre elas a ação antimicrobiana, coloração (proteção e atração), aroma em folhas e frutos e também propriedade estrutural como a proporcionada pela lignina. Os compostos fenólicos podem ser classificados de acordo com as unidades de carbono ligadas ao anel aromático de seus membros (DE OLIVEIRA; DE CARVALHO; MELO, 2014). Nos últimos anos houve um interesse crescente da indústria por antioxidantes naturais para inibir os efeitos adversos da radiação UVB e melhorar a saúde da pele radiada. No grande grupo dos compostos fenólicos, os flavonoides e os ácidos fenólicos são os que mais se destacam e, são

considerados os antioxidantes fenólicos os mais comuns de fontes naturais, que em baixas concentrações diminuem os efeitos danosos causados pelos radicais livres (KARAKAYA, 2004). Isso porque esses compostos atuam em diferentes níveis do processo oxidativo, com a capacidade de retardar significativamente ou de prevenir a oxidação de substratos oxidáveis como lipídios, proteínas e DNA (ANDRADE; CUNN; DON, 2007).

A baunilha é reconhecidamente o aromatizante mais empregado na indústria de alimentos e cosméticos no mundo (RAMACHANDRA RAO; RAVISHANKAR, 2000). A produção anual do extrato natural de baunilha chega a 1,2 toneladas em Madagascar, maior produtor mundial (BERGER, 2007). A baunilha é extraída através do processamento das favas de uma orquidácea do gênero *Vanilla*. Existem mais de 110 espécies identificadas, mas apenas três possuem interesse comercial: a *Vanilla pampona*, *Vanilla tahitensis e Vanilla planifólia* (BERGER, 2007). Os componentes majoritários dos extratos de baunilha são: a vanilina (3-metoxi-4-hidroxibenzaldeído) 2 % m/m; 4-hidroxibenzaldeído 0,2 % m/m; 4- hidroxibenzilmetileter 0,02 % m/m; ácido acético 0,02 % m/m; e ácido vanílico com teor aproximado de 0,01 % m/m (BERGER, 2007; RAMACHANDRA RAO; RAVISHANKAR, 2002), entre outros. Embora existam muitos compostos presentes nos extratos de baunilha, a vanilina é a principal responsável pelo aroma característico da baunilha (RAMACHANDRA RAO; RAVISHANKAR, 2000).

O ácido vanílico, um composto fenólico, é a forma oxidada da vanilina, usada como agente aromatizante (LESAGE-MEESSEN et al., 1996). Um derivado do ácido diidroxibenzoico, sua formula molecular é $C_8H_8O_4$ com a presença de anel aromático que entra em ressonância e é responsável ou reduzir moléculas e estabilizar compostos (CIVOLANI et al., 2000). (Figura 2).

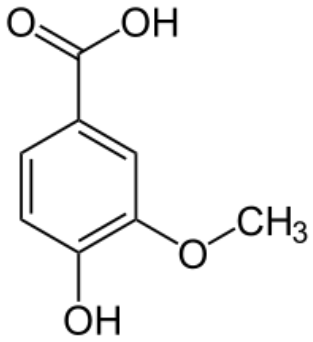

Figura 2: Estrutura molecular do ácido vanílico

Um estudo *in vitro* demonstrou que o ácido vanílico apresentou maior poder antioxidante quando comparado ao poder antioxidante da vanilina (LIBARDI, 2010). Ademais, há também estudos que mostram o ácido vanílico na diminuição do estresse oxidativo e inflamação (SCALBERT et al., 2002). Em plasma de ratos hipertensos, o ácido vanílico foi capaz de reduzir os produtos da peroxidação lipídica e manteve os níveis de antioxidantes enzimáticos e antioxidantes não enzimáticos (KUMAR, Subramanian; PRAHALATHAN; RAJA, 2011). O composto também inibiu a resposta de contorções induzidas por ácido acético em doses via oral nas doses de 3-30 mg/kg, (MORUCCI et al., 2012). Pela via de administração

intraperitonial, inibiu a dor inflamatória por inibição do recrutamento de neutrófilos, estresse oxidativo, produção de citocinas e ativação do fator de transcrição NF-kb em ratos (CALIXTO-CAMPOS et al., 2015). Vinothya et al., 2017 verificou que em ratos hipertensos diabéticos o tratamento com o ácido vanílico diminui significativamente os níveis de glicose, insulina e pressão arterial em comparação com o grupo controle diabético, as atividades antioxidantes foram significativamente aumentadas e os níveis de marcadores de peroxidação lipídica foram significativamente diminuídos (VINOTHIYA; ASHOKKUMAR, 2017). Algumas ações adicionais do ácido vanílico que incluem propriedades antimicrobianas, como demonstrado pela inibição *in vitro* do crescimento de *Listeria spp*. (CAVA-RODA et al., 2012).

Por esta razão, devido as suas propriedades já descritas anteriormente como: antibacterianas (VAZQUEZ-ARMENTA et al., 2018), antimicrobiana (CAVA-RODA et al., 2012), hepatoprotetora (ITOH et al., 2009, 2010), quimioprotetora (ANBALAGAN; RAJU; SHANMUGAM, 2017), neuroprotetora (KHOSHNAM et al., 2017; UL AMIN; SHAH; KIM, 2017), antioxidante e antiinflamatória (CALIXTO-CAMPOS et al., 2015) em outros modelos.

A administração de um antioxidante natural veiculado em emulsões tópicas é uma alternativa simples e eficaz de evitar a depleção de nossas defesas antioxidantes endógenas e ainda prevenir ou reverter os danos do estresse oxidativo na pele, causado principalmente pela exposição à radiação UV (GEORGETTI et al., 2008).

A aplicação tópica possibilita a administração de medicamentos de forma segura e evita efeitos como irritação gastrointestinal, toxicidade sistêmica e o metabolismo de primeira passagem, que reduziria significativamente a concentração do ativo (MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; VIGNOLI; BARACAT; et al., 2015).

Mediante os dados relatados das atividades antioxidantes do ácido vanílico, associados a falta de trabalhos que avaliem sua ação terapêutica vinculado em formulação tópica contra danos oxidativos induzidos pela radiação UVB, o trabalho teve como objetivo avaliar a estabilidade físico-química e funcional da formulação contendo ácido vanílico, bem como estudar sobre o seu efeito terapêutico em modelo de inflamação e estresse oxidativo cutâneo induzidos pela radiação UVB em camundongos sem pelo.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi avaliar a estabilidade físico-química e funcional da formulação contendo ácido vanílico e sua eficácia tópica na inflamação e estresse oxidativo induzidos pela radiação UVB.

2.2 OBJETIVOS ESPECÍFICOS

- Preparar a formulação para veiculação do ácido vanilico;
- Realizar estudo de estabilidade das formulações adicionadas de ácido vanílico;
- Avaliar a eficácia *in vivo* do ácido vanílico adiciona na formulação tópica, na redução da inflamação e do estresse oxidativo induzidos pela radiação UVB com enfoque em:
- Avaliar o edema de pele;
- Avaliar a atividade/secreção de metaloproteinases-9;
- Avaliar os cortes histológicos das peles para os testes de: queratinócitos apoptóticos; espessura epidérmica e colágeno epidérmico;
- Avaliar o poder antioxidante da pele por meio dos ensaios de ABTS (2,2’ azinobis 3-etilbenzotiazolina-6-ácido sulfônico) e FRAP (Poder antioxidante de redução férrica);
- Avaliar a manutenção dos níveis do antioxidante endógeno GSH e atividade da CAT;
- Avaliar a produção de hidroperóxidos lipídicos (LOOH);
- Avaliar da redução de ânion superóxido (SOD);

3. MATERIAIS E MÉTODOS

3.1 MATERIAIS

3.1.1 Materiais de Consumo

Corante azul brilhante R, hematoxilina, eosina, azul de toluidina, tricrômero de masson, glutationa reduzida (GSH), ácido tricloracético (TCA), n-etilmaleimida, fluoreto de fenilmetilsulfonila, fenantrolina, 2,2' azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), 2,4,6 tripiridil-S-triazina (TPTZ), Trolox e ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB), bisacrilamida, *nitroblue tetrazolium* (NBT) e ácido vanílico H36001 (97%) foram obtidos da Sigma-Aldrich (St. Louis, MO, USA). Tert-butil hidroperóxido obtido da Acros (Pittsburgh, PA, USA). Xilene cianol e hidroximetil aminometano (Tris) da Amresco (Solon, OH, USA). Acrilamida e dodecil sulfato de sódio (SDS) da Invitrogem (Carlsbad, CA, USA). Os excipientes usados para o preparo das formulações foram obtidos da Galena (Campinas, SP, Brasil). Todos os outros reagentes utilizados foram de grau analítico.

3.1.2 Materais Permanentes

Agitador mecânico, Fisatom®; Agitador Orbital, 255, Fanem®; Balança analítica, HR-120, A&d®, precisão de 4 casas; Banho-Maria, 314/2 DN, Nova Ética®; Banho-Maria,100, Fanem; Centrífuga refrigerada, Rotina 46 R, Hettich Zentrifugen®; Compartimento de madeira projetado para radiação; Contador β, LS 6000, Beckman® LS 6000; Deionizador de água, Purebal Option-Q, Elga®; Espectrofotômetro, Evolution 60, Thermo Scientific®; Espectrofotômetro, Helios alfa, Thermo Spectronic®; Estufa 0-120 °C, De LEO & Cia®; Fonte elétrica para eletroforese, MS 300V, Major Science®; Homogeneizador de tecidos Ultra Turrax®, T18 basic, IKA; Homogeneizador de tecido, Tissue-Tearor, Biospec®; Lâmpada ultravioleta fluorescente, PHILIPS TL/12 40W RS-UVB, MedicalHoland®; Leitor de microplaca, EnSpire, Perkin Elmer®; Leitor de microplaca, Multiskan 36 GO, Thermo Scientific; pHmetro, Tec-3MP, TECNAL®; Sistema de eletroforese Mini Vertical, Mini-Protean® Tetra System, Bio-RAD; Ultra-som,TSO, Thornton®; Cintilador.

3.2 MÉTODOS

3.2.1 Preparo das formulações tópicas

Foi preparado uma emulsão (gel-creme), utilizando a cera auto emulsionante não íonica Polawax® (álcool cetoestearílico e monoestearato de sorbitol polioxietileno 20 OE) e um colóide hidrofílico, o Aristoflex® (AVC).

A emulsão preparada foi feita pelo método de inversão de fases á 75°C. A fase oleosa foi constituída por Polawax® à 2% (p/p) e a fase aquosa por Aristoflex a 5% (p/p), após foi incorporado o emoliente triglicerídeo de ácido cáprico e caprílico (5%), uma solução conservante (Phenonip®) à 0,4%. A formulação preparada foi dividida em 2 porções: sendo uma reservada para controle (sem a adição de ácido vanílico) e a outra acrescida de 0,5% de ácido vanílico. Na formulação controle foram adicionados 6% de propilenoglicol (umectante e solubilizante) e na formulação contendo o ácido vanílico foi solubilizado os 0,5% do mesmo em 6% de propilenoglicol e adicionado à formulação após 24 horas (Tabela 1).

Tabela 1: Componentes da formulação

Composição % (p/p)	F	F + VA
Polawax®[1] (emulsificante)	2	2
Dispersão de aristoflex®[2] (colóide hidrofílico) a 5%	20	20
Triglicerídeos do ácido cáprico e caprílico (emoliente)	5	5
H_2O deionizada qsp	100	100
Phenonip (conservante)	0,4	0,4
Propilenoglicol (umectante e solubilizante)	6	6
Ácido vanílico (princípio ativo)	-	0,5

[1]Polawax®: Base auto emulsionante não-iônica (álcool cetoestearílico + monoestearato de sorbitol polioxietileno 20 0E).

[2]Aristoflex®: Co-polímero do Ácido Sulfônico acriloildimetiltaurato e vinilpirrolidona neutralizado (formador de gel aniônico).

A concentração utilizada do ácido vanílico foi baseando-se em outros estudos realizados pelo grupo de avaliação terapêutica de formulações tópicas contendo antioxidantes em modelo de inflamação e estresse oxidativo induzidos por radiação UVB (MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; et al., 2016; MARTINEZ; PINHO-RIBEIRO; STEFFEN; SILVA; et al., 2016).

As formulações foram utilizadas apenas 24 horas após o preparo com o intuito de estabilizar os excipientes.

3.2.2 Estudo da Estabilidade das formulações

O estudo de estabilidade tem como objetivo fornecer informações sobre o prazo de validade da formulação, bem como, as condições de armazenamento. Os testes de estabilidade são usados para avaliar a capacidade de um produto em manter seu aspecto original, as características físicas, químicas e microbiológicas (AZZINI, 1999).

Um dos testes de estabilidade realizado nas etapas de pré-formulação é o teste de estabilidade acelerada, que segundo a RE nº.1, 29/07/2005 (ANVISA), tem como objetivo acelerar a degradação química e/ou física de um produto farmacêutico em condições extremas de armazenamento, desta forma, em um curto período de tempo, sob condições

climáticas forçadas, é possível prever os perfis de estabilidade físico-químico dos produtos, de acordo com parâmetros específicos para cada forma farmacêutica.

As formulações adicionadas ou não com o ácido vanílico foram acondicionadas, em frascos de polipropileno do tipo semipermeável (permeável a gases e à umidade), e armazenadas em temperatura de 4°C, TA e de 40±2°C/75±5% de UR pelo período de seis meses (180 dias) (CASAGRANDE; GEORGETTI; VERRI JR.; et al., 2006).

Nos intervalos de tempo determinados (tempo zero, 15, 30, 60, 90 e 180 dias), alíquotas foram coletadas e analisadas quanto à estabilidade físico-química e funcional pelo método de sequestro do radical livre $ABTS^{+}$ (MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; et al., 2016).

3.2.2.1 Avaliação da estabilidade físico-químico e funcional

3.2.2.1.1 Análise visual

Nos intervalos de tempo pré-determinados, as formulações foram analisadas quanto à sua consistência, cor, odor e separação de fases (SHATALEBI; RAFIEI, 2014).

3.2.2.1.2 Determinação do pH

O pH das formulações foi determinado por meio de uma dispersão aquosa 1:10 (p/p). Desta forma, pesou-se 1g de cada formulação e adicionou-se 9g de água deionizada. Após completa a homogeneização em placa com agitação magnética, o pH das amostras foi medido em pHmetro digital. As leituras foram realizadas em duplicatas (ANCHISI et al., 2001; GEORGETTI et al., 2008).

3.2.2.1.3 Teste de Centrifugação

O teste de centrifugação permite informar e comparar as propriedades de estabilidade de diferentes emulsões. Além disso, permite verificar a estabilidade avaliando se houve ou não a separação de fases da formulação, ou seja, permite analisar se houve instabilidade físico-química (AZZINI, 1999).

Cada formulação foi pesada na quantidade de 2g em tubos de ensaios para centrífuga, de formato cônico e graduado. As amostras foram submetidas a 3600 rpm durante 30 minutos, à temperatura ambiente observando-se a possível ocorrência de separação de fases (GEORGETTI et al., 2008).

3.2.2.1.4 Estabilidade funcional

A estabilidade funcional foi avaliada utilizando-se o teste de avaliação da capacidade sequestradora do radical ácido 2,2'-azino-bis-(3-etilbenzotiazolina-sulfônico ($ABTS^+$) determinada pela mudança de cor sob a absorbância em 730nm (CAMPANINI et al., 2013). O cátion $ABTS^+$ foi formado com 7mM de uma solução de ABTS e 2,45mM de persulfato de potássio. A mistura foi armazenada em um frasco âmbar na geladeira por 16 horas antes do uso. Esta solução de radical $ABTS^+$ foi diluída em etanol absoluto para se obter uma absorvância de 0,7 em 730nm.

Para avaliação da possível interferência dos componentes das formulações na atividade antioxidante do ácido vanílico, amostras da matéria-prima foram preparadas diluindo-se 0,04g de ácido vanílico em 10mL de etanol absoluto. Essa solução a 0,004g/mL foi diluída 5 vezes em etanol absoluto e ,posteriormente, mais 5 vezes no mesmo solvente obtendo-se uma solução 160μg/mL de ácido vanílico. As amostras das formulações foram preparadas diluindo-se 1g de cada formulação base e de cada formulação contendo 0,5% do ácido vanílico em 5mL de etanol absoluto. Dessa solução foram diluídas 6,25 vezes em etanol absoluto obtendo-se uma solução de 160μg/mL de ácido vanílico (PEREIRA, 2012).

Para avaliação da atividade sequestradora do radical $ABTS^+$, 25 μL das soluções de ácido vanílico e das formulações acrescidas do mesmo foram adicionadas à 2mL da solução de $ABTS^+$ diluída em etanol absoluto. A reação foi incubada à temperatura ambiente por 6 minutos e a absorvância foi determinada por espectrofotometria a 730nm.

O controle positivo foi preparado com 2mL da solução diluída de $ABTS^+$ em etanol absoluto. O branco era composto da solução de persulfato de potássio 2,45mM diluída em etanol absoluto. Ainda, preparou-se um controle positivo adicionando-se apenas 25μL da formulação sem ácido vanílico, para comparativo da base utilizada com a respectiva formulação adicionada de ácido vanílico. Todas as medidas foram realizadas em triplicata e os resultados foram expressos como % de atividade sequestradora do radical $ABTS^+$ (PEREIRA, 2012; SILVA, 2014).

3.2.3 Determinação da eficácia *in vivo* do ácido vanílico no controle da inflamação e estresse oxidativo induzidos pela radiação UVB.

3.2.3.1 Animais experimentais

Foram utilizados camundongos sem pelo da linhagem HRS/J, de ambos os sexos, adultos pesando entre 20 a 30 g. Os camundongos foram mantidos no Biotério do Centro de Ciências da Saúde da Universidade Estadual de Londrina com temperatura controlada de 22± 2°C, ciclo claro/escuro de 12 horas e com livre acesso a água e ração. Os experimentos

foram realizados conforme as normas da Comissão de Ética no uso de Animais (CEUA) da Universidade Estadual de Londrina (registrado no Ofício Circular CEUA nº70/2017, processo CEUA n° 8225.2017.67).

3.2.3.2 Sistema e fonte de radiação UVB

A fonte de luz utilizada nos experimentos para indução da inflamação/estresse oxidativo foi uma lâmpada 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 313 nm. A fonte de radiação UVB foi instalada em um compartimento de madeira desenvolvido para os experimentos.

A medida da irradiância foi realizada utilizando-se um radiômetro (IL 1700) com detectores para radiação UV (SED 005) e, especialmente, para UVB (SED 240) (CARINI et al., 2000; CASAGRANDE; GEORGETTI; VERRI; et al., 2006).

Os animais foram colocados em caixas plásticas e cobertos com uma tela plástica para que os animais permanecessem dentro das caixas e para garantir que a exposição à luz UVB ocorresse diretamente na região dorsal dos mesmos. Os animais ficaram a 20 cm de distância da fonte de radiação e movimentavam-se livremente na caixa. Foi realizado rodízio com as caixas contendo os animais devido às variações de radiação ao longo da lâmpada. A dose de radiação utilizada para indução da inflamação e do estresse oxidativo foi de 4,14 J/cm^2 (CAMPANINI et al., 2013; IVAN et al., 2014).

3.2.3.3 Protocolo experimental de avaliação da eficácia de formulação tópica contendo ácido vanílico

Os camundongos foram distribuídos em quatro grupos com cinco animais cada:

- Grupo CN: controle não irradiado;
- Grupo CI: controle irradiado e não tratado
- Grupo F: irradiado e tratado com formulação tópica sem o ácido vanílico;
- Grupo F + A: irradiado e tratado com formulação tópica contendo ácido vanílico.

Os camundongos foram tratados topicamente na parte dorsal com 0,5g da formulação controle no grupo F e da formulação contendo o ácido vanílico no grupo F + A (MARTINEZ; PINHO-RIBEIRO; STEFFEN; SILVA; et al., 2016) os tratamentos foram 1h antes, 5 min antes e 5 min após o término da irradiação (CASAGRANDE; GEORGETTI; VERRI; et al., 2006).

Os animais foram eutanásiados com anestesia terminal de 5 % de isoflurano após 12h do final da irradiação para os seguintes ensaios: edema; teste histológico; avaliação de

FRAP; capacidade em reduzir o radical ABTS; níveis de GSH e atividade de metaloproteinase (MMP-9). Para os testes de ânion superóxido (NBT) e catalase (CAT) as peles foram coletadas 2 horas após a irradiação com a eutanásia ocorrendo a partir da anestesia seguido de decapitação e para o teste de hidroperóxidos a eutanásia ocorreu 4 horas após o final da irradiação com a eutanásia por decapitação.

As amostras de pele do dorso dos animais foram limpas com auxílio de algodão e de água deionizada para total retirada das formulações presentes na superfície cutânea. Em seguida, a pele foi retirada e armazenada a -80°C para as análises, para as histologias a pelefoi coletada e armazenadas em tubos falcon contendo formol 10%. O teste de edema cutâneo foi realizado logo após a retirada da pele.

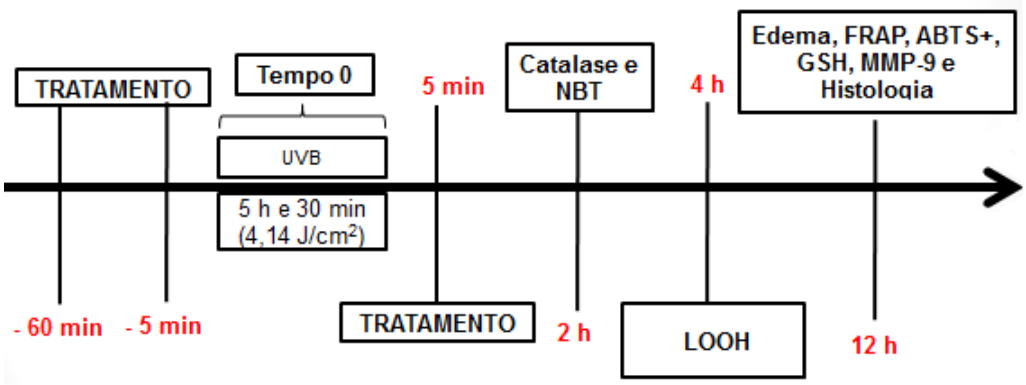

Figura 3 - Esquema geral dos protocolos experimentais para avaliação da eficácia de formulação tópica contendo ácido vanílico. Os animais foram irradiados com radiação UVB durante 5 horas e 30 min (4,14 J/cm2). Uma hora antes, 5 minutos antes e 5 minutos após o final da irradiação os animais foram tratados com as formulações tópicas (com ou sem ácido vanílico). Para os testes de atividade da catalase (CAT) e produção de ânion superóxido (NBT) os animais foram eutanasiados e amostras de pele foram coletadas 2 horas após o final da irradiação, 4 horas (para os testes de produção hidroperóxidos lipídicos [LOOH]) e 12 horas (para os testes de edema, atividade de metaloproteinase-9 [MMP-9], níveis de glutationa reduzida [GSH], avaliação do poder antioxidante redutor de ferro [FRAP] e capacidade em reduzir o radical ABTS).

3.2.4 Avaliação do poder antioxidante redutor de ferro (FRAP)

O ensaio de FRAP mede a redução férrica do 2,4,6 tripiridil-S-triazina (TPTZ) para um produto colorido por meio da ação de antioxidantes que doam elétrons para a reação (KATALINIC et al., 2005). O ensaio foi adaptado e utilizado para avaliar o poder antioxidante de amostras da pele (MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; VIGNOLI; BARBOSA; et al., 2015).

As amostras de pele dos animais dos diferentes grupos foram coletadas e homogeneizadas em 400 µL de KCl (1,15%) com auxílio do Tissue-Tearor (Biospec 985370) e centrifugadas a 1.000 g por 10 min a 4°C. Para a reação foram utilizados 30 µL do sobrenadante do homogenato da amostra e 150 µL de reagente FRAP. O reagente de FRAP foi preparado adicionando 2,5 mL de uma solução 10 mM de TPTZ em HCl 40 mM com 2,5 mL de cloreto de ferro hexahidratado 20 mM e 25 mL de tampão acetato 0,3 mM (pH 3.6), e esta solução foi incubada a 37°C por 30 min antes do uso. Em seguida, foi realizada a leitura em 595 nm (EnSpire, Perkin Elmer). Soluções padrão com diferentes concentrações de trolox (0,5 a 20 nmol) (antioxidante análogo da vitamina E) foram utilizadas para calibração. Os resultados foram expressos como nmol equivalente de Trolox/mg de pele (KATALINIC et al., 2005).

3.2.5 Avaliação da capacidade em reduzir o radical ABTS

A capacidade antioxidante de cada amostra é medida através do decaimento da coloração do cátion $ABTS^{+}$ quando ocorre doação de elétrons da amostra. Para a reação de ABTS, as amostras foram homegeneizadas em 400 µL de KCl a 1,15% com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec 985370) e centrifugadas a 1.000 g por 10 min a 4°C, posteriormente o sobrenadante foi utilizado para análise. A solução de ABTS foi preparada após reagir 7 mM da solução de ABTS com 2,45 mM de persulfato de potássio resultando no cátion $ABTS^{+}$. A mistura foi armazenada em frasco âmbar por no mínimo 16 horas antes do uso. Após as 16 horas, a solução de ABTS foi misturada com tampão fosfato até atingir a absorvância de 0,8 em 730 nm. Foram adicionados em uma placa de 96 poços 7 µL do sobrenadante e 200 µL da solução de ABTS diluída. O branco era composto apenas do tampão. Após 6 minutos de reação, foi realizada a leitura em 730 nm (EnSpire, Perkin Elmer). Uma curva padrão foi preparada com diferentes concentrações de trolox (0,01 a 20 nmol) e os resultados foram expressos em nmol equivalente de trolox/mg de pele (KATALINIC et al., 2005; MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; VIGNOLI; BARBOSA; et al., 2015).

3.2.6 Avaliação dos níveis do antioxidante endógeno (GSH)

A glutationa (GSH) é considerada o principal marcador para o estresse oxidativo induzido pela radiação UVB, pois é produzida em maior quantidade pelas células epidérmicas (CASAGRANDE; GEORGETTI; VERRI; et al., 2006). O seu grupamento sulfidrila é responsável pela neutralização de substâncias oxidantes, doando elétrons para essas moléculas reativas (D'ORAZIO et al., 2013). Neste ensaio, GSH doa elétrons para o

ácido 5',5'-ditio-bis-(2-nitrobenzóico) (DTNB), convertendo em um produto colorido (SRINIVASAN; SABITHA; SHYAMALADEVI, 2007).

As amostras de pele foram diluídas (1:4) em EDTA 0,02 M e trituradas utilizando o Tissue-Tearor (Biospec 985370). Ao homogenato foi adicionado ácido tricloroacético (TCA) 50% na proporção de 1:0,2 de EDTA e TCA, respectivamente. Em seguida, a mistura foi centrifugada a 2.700 g por 10 minutos a 4°C. O sobrenadante foi retirado e centrifugado novamente a 2.700 g por mais 15 minutos a 4°C. O sobrenadante final foi retirado para análise. Para a quantificação dos níveis de GSH foram adicionados 50 µL do sobrenadante da segunda centrifugação em microplaca juntamente com 100 µL de tampão Tris 0,4 M (pH 8,9) e 5 µL de uma solução de 1,9 mg/mL de DTNB em metanol. Após 5 minutos de incubação, a leitura da microplaca foi realizada no espectrofotômetro (EnSpire, Perkin Elmer) em 405 nm. A curva padrão foi preparada com 5 a 150 µM de GSH. Os resultados foram expressos em µM de GSH/mg de pele (SRINIVASAN; SABITHA; SHYAMALADEVI, 2007).

3.2.7 Determinação da atividade da catalase (CAT)

A enzima CAT tem como função converter o H_2O_2 em água e oxigênio. O princípio da técnica se baseia na decomposição do H_2O_2 seguida da dimininuição de absorvância. A diferença de absorvância por unidade de tempo mede a atividade da enzima (AEBI, 1984).

As amostras de pele dos animais foram homogeneizadas em 500µL de EDTA 0,02M com auxílio do Tissue-Tearor (Biospec 985370). Em seguida, o homogenato foi centrifugado a 2.700g por 10 minutos a 4°C. O sobrenadante foi centrifugado novamente a 2.700 g por 10 minutos a 4°C, e o sobrenadante final foi retirado para análise. Para a reação, foram adicionados 10µL amostra, 160 µL tampão Tris-HCl 1M com EDTA 5mM pH 8,0, 20 µL água deionizada e 20 µL H_2O_2 200mM. Foi incluído um branco para cada amostra preparado com 10 µL do sobrenadante da amostra, 180µL de tampão Tris-HCl 1M com EDTA 5mM pH 8,0 e 20µL de água deionizada. A velocidade com que o H_2O_2 é reduzido pela ação da CAT foi avaliada através da diminuição no valor da absorbância pela diferença entre a leitura inicial e a leitura 30 segundos após a adição do H_2O_2 200mM. A leitura foi realizada em espectrofotômetro de microplacas (EnSpire, Perkin Elmer) a 240nm com temperatura mantida em 25ºC. Os valores de catalase foram expressos como unidade de catalase/mg de pele/minuto (AEBI, 1984).

3.2.8 Determinação de hidroperóxidos lipídicos (LOOH)

A produção de hidroperóxidos lipídicos é baseada no método de quimiluminescência iniciada pelo tert-butil hidroperóxido descrito anteriormente (GONZALEZ FLECHA; LLESUY; BOVERIS, 1991). Esse teste foi adaptado por Martinez et al. (2015). As amostras de pele

foram coletadas e homogeneizadas em 800 µL de tampão fosfato (pH 7.4) com o homogeneizador de tecidos Tissue-Tearor (Biospec 985370) e posteriormente centrifugadas a 700 g por 2 minutos a 4 ºC. Para o ensaio, 250 µL do sobrenadante foram adicionados a 1730 µL de meio de reação (KCl 120mM, tampão fosfato pH 7,4 30 mM) e 20 µL de tert-butil hidroperóxido 3 mM. Este ensaio foi realizado em contador β marca Beckman® LS 6000 (FULLERTON, CA, EUA) em uma faixa de contagem não coincidente com a resposta entre 300 e 620 nm. Todo o experimento foi realizado ao abrigo da luz para evitar a fosforescência dos frascos, a 30 ºC, durante 120 minutos. Os resultados foram medidos em contagem por minuto (cpm) por mg de pele.

3.2.9 Avaliação da produção de ânion superóxido ($O_2^{\bullet-}$)

O ânion superóxido é um íon produzido endogenamente durante o processo de respiração celular aeróbia. No entanto, na exposição acentuada a radiação UVB pode apresentar aumento da sua quantidade, e consequentemente ativa a enzima superóxido dismutase a qual converte o ânion em peróxido de hidrogênio favorecendo a geração de outro radical que é prejudicial para a manutenção da homeostasia da célula (CHA et al., 2014; MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; VIGNOLI; BARBOSA; et al., 2015).

A quantificação do $O_2^{\bullet-}$ foi realizada pelo método baseado na redução do *nitroblue tetrazolium* (NBT). As amostras de pele foram homogeneizadas com o Tissue-Tearor (Biospec 985370) em EDTA 0,02 M e centrifugadas (2000 g por 20 segundos a 4°C). Primeiramente, 50 µL do sobrenadante de cada amostra foi adicionado em microplaca e incubado por 1 hora. Posteriormente, o sobrenadante foi removido e foi adicionado o NBT (1mg/mL) ao meio. Depois de incubar por 15 minutos, o sobrenadante foi retirado e ao precipitado foram adicionados 20 µL de metanol 100% para fixar, 120 µL de KOH 2 M e 140 µL de dimetilsulfóxido (DMSO) para solubilizar o composto formazan (NBT reduzido). A formação desse composto foi medida em espectrofotômetro (EnSpire, Perkin Elmer) a 620 nm e os resultados foram apresentados como densidade óptica (OD)/10 mg de pele (CAMPANINI et al., 2013).

3.2.10 Avaliação do edema de pele

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

As amostras de pele do dorso de cada animal foram coletadas com auxílio de um molde com área fixa de 5 mm de diâmetro e posteriormente foram pesadas (BOLLER et al.,

2010; IVAN et al., 2014; MARTINEZ; PINHO-RIBEIRO; STEFFEN; CAVIGLIONE; VIGNOLI; BARACAT; et al., 2015). A análise foi feita comparando-se o peso de pele entre os diferentes grupos. Os resultados foram expressos em mg de pele.

3.2.11 Avaliação histopatológica por microscopia óptica

Para análise histopatológica, as amostras de pele do dorso dos animais foram coletadas 12 horas após irradiação e armazenadas em formol 10%. Subsequentemente, as amostras de pele foram fixadas em paraformaldeído 4% e desidratadas em banhos de soluções de etanol em concentrações crescentes (70%, 95% e 100%). Em seguida, foram diafanizadas com xilol e incluídas em parafina. Foram preparadas secções de 5 µm que foram coradas com hematoxilina eosina (H & E) e tricrômico de masson. As secções coradas com H & E foram examinadas utilizando microscopia óptica a uma ampliação de 400 vezes (objetiva de 40) para determinação da espessura epidérmica (DENG et al., 2015) e uma amplificação de 1000 vezes (objetiva de 100) para contagem do número de queratinócitos apoptóticos (SCHWARZ et al., 1995). Já as secções coradas com tricrômico de masson foram examinadas utilizando microscopia óptica a uma ampliação de 100 vezes (objetiva de 10) para visualizar alterações nas fibras de colágeno através da análise da intensidade da coloração azul nas áreas dérmicas da pele exposta a radiação UVB com auxílio do software Image J (NIH) (SONG, 2016).

3.2.12 Determinação da atividade/secreção de proteinases por zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS)

As metaloproteinases são importantes na degradação das matrizes extracelulares como o colágeno e podem desenvolver o fotoenvelhecimento cutâneo (BAE et al., 2015; LIMA et al., 2016).

Usou-se para a determinação da atividade da MMP-9 o ensaio de zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) (FONSECA et al., 2011). Este método é amplamente utilizado para a detecção de proteases, consistindo em análise qualitativa da atividade por meio da degradação da gelatina adicionada ao gel de eletroforese (LIM; HYUN, 2007).

Para determinação da atividade da MMP-9 foi utilizado o ensaio de zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) (FONSECA et al., 2011) 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 microtubos e para este teste foi feito um *pool* das amostras de cada grupo de animais. Primeiramente, as peles dos animais foram diluídas (1:4) e trituradas (T18 basic, IKA) em tampão Tris/HCl 50 mM (pH 7,4) contendo cloreto de cálcio (CaCl2) 10 mM e 1% de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida). O homogenato foi duplamente centrifugado a 12.000 g por 10 minutos a 4 °C e o sobrenadante utilizado no ensaio. Também foi realizada dosagem de proteínas das amostras pelo método de Lowry (LOWRY et al., 1951). Alíquota de 50 µL do sobrenadante das amostras foi diluída em 10 µL de tampão Tris/HCl (pH 6,8) contendo 20% de glicerol, 4% de dodecil sulfato de sódio (SDS) e 0,001% de azul de bromofenol. Em seguida estas amostras foram colocadas em banho-maria a 37 ºC durante 8 minutos imediatamente antes de ser aplicada no gel de eletroforese.

A espessura do gel utilizado foi de 1 mm, composto por um gel de separação e um gel de concentração, preparado conforme tabela 2.

Tabela 2. 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 10 mA por 15 minutos. Após, foram aplicados 25 µL de cada amostra. Durante a eletroforese a corrente aplicada foi de 10 mA para o gel de concentração e 13 mA para o gel de separação, sendo que a última corrente foi mantida constante por 15 minutos após a saída do corante do gel de separação.

Ao término da eletroforese o gel de poliacrilamida foi lavado por 1 hora com solução de Triton X-100 2% sob constante agitação, posteriormente o gel foi incubado por 16 horas

a 37 °C em tampão Tris/HCl 50 mM (pH 7,4) contendo CaCl2 10 mM e 0,02% de azida sódica. Ao final da incubação o gel foi corado com uma solução contendo 0,25% de azul brilhante, 10% de ácido acético e 50% de metanol em água deionizada. Para visualização das bandas o gel foi descorado com ácido acético 20% (FONSECA et al., 2011).

3.3 Análise estatística dos resultados

As análises estatísticas foram realizadas utilizando o software GraphPad Prism 6 GraphPad Software Inc., San Diego, EUA). Os resultados foram analisados estatisticamente por análise de variância (ANOVA) com um fator seguido do teste de comparações múltiplas de Tukey e apresentados pela média ± erro padrão da média (EPM) de mensurações feitas com 5 animais em cada grupo por experimento. Os resultados foram representativos de 2 experimentos separados e foram considerados significativamente diferentes para $p<0,05$. Os resultados da estabilidade foram apresentados em triplicata ± EPM.

4. RESULTADOS E DISCUSSÃO – ARTIGO CIENTÍFICO

4.1 PROTECTIVE EFFECT OF TOPICAL FORMULATION CONTAINING VANILLIC ACID AGAINST UVB-INDUCED INFLAMMATION AND OXIDATIVE STRESS IN HAIRLESS MICE

Frontiers in Pharmacology – Ethnopharmacology

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Abstract

Ultraviolet B (UVB) radiation is known to cause skin damage and is associated with oxidative stress, inflammation, DNA damage and apoptosis. Vanillic acid (VA) is a vanillin oxidation derivate, a flavoring agent found in plants and edible fruits, which has antioxidant activity. The objective of this work was to prepare a topical formulation containing VA (TFcVA) to evaluate the physicochemical stability by visual analysis, centrifugation (phase separation) and pH, and functional stability by the ABTS method under different temperature and storage conditions. In addition, the effects of TFcVA on UVB irradiation-induced skin inflammation and oxidative damage in hairless mice was evaluated. TFcVA maintained its physicochemical and functional stability over 180 days. Treatment with TFcVA inhibited the depletion of antioxidant capacity (ferric reduction ability and ABTS, GSH and catalase activity), lipid peroxidation and superoxide anion production induced by UVB radiation. These effects of TFcVA also resulted in reduction of skin edema, epidermal thickness, burned cell count, cutaneous matrix metalloproteinase activity and collagen degradation. In summary, TFcVA was able to reduce the harmful oxidative and inflammatory effects of UVB radiation on the skin.

Keywords: Vanillic acid; Antioxidant; Phenolic compound; Skin;

Highlights

- Topical formulation containing vanillic acid (TFcVA) remained stable during 180 days
- TFcVA inhibited UVB-induced edema.
- TFcVA suppressed the secretion of MMP-9 induced by UVB-irradiation.
- TFcVA inhibited UVB-induced skin oxidative stress.
- TFcVA protected the skin from the deleterious effects of UVB irradiation.

Abbreviations

ABTS	2,2′ -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAT	Catalase
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
FRAP	Ferric reducing antioxidant power
GSH	Reduced glutathione
$HO^{\bullet}$	Hydroxyl radical
HO-1	Heme oxygenase-1
H_2O_2	Hydrogen peroxide
LPO	Lipid peroxidation
LOOH	Lipid hydroperoxides
KC	Keratinocytes
NBT	Nitroblue tetrazolium
Nrf2	Nuclear factor erythroid 2-related factor 2
$O_2^{\bullet-}$	Superoxide anion
ROS	Reactive oxygen species
SBC	Sunburn cells
SEM	Standard error mean
SOD	Superoxide dismutase
TFcVA	Topical formulation containing vanillic acid
TPTZ	2,4,6-tris(2-pyridyl)-*s*-triazine
UV	Ultraviolet
UVB	Ultravioleta tipo B
VA	Vanillic acid

1. Introduction

As the outmost layer of the human body, the skin could be seriously damaged when constantly exposed to chemical pollutants and environmental ultraviolet (UV) radiation. UVB radiation (280 – 320 nm) can affect various skin structures causing edema, erythema, hyperplasia, premature aging, and even lead to skin malignancy. These potential consequences of UVB radiation explains the current understanding that it is the most damaging spectrum of the solar UV emissions, (Tebbe, 2001).

In normal conditions, the concentration of the reactive oxygen species (ROS) within the cells is extremely low, since the organism has endogenous enzymatic and non-enzymatic antioxidant mechanisms that maintain the concentration of the reactive species within the physiological limits (Ribeiro et al., 2005), but studies have shown that skin exposure to UVB radiation increases ROS levels (Yin et al., 2013). When ROS production exceeds the ability of the endogenous antioxidant system to reduce them, the concentration of free radicals increases uncontrollably, disrupting the oxidant / antioxidant balance in the body by installing oxidative stress (Lobo et al., 2010) that can cause damage which are important in molecules such as DNA and proteins, lipids as well as cause lesions implicated in carcinogenesis (Kang et al., 1998) and early aging (Ivan et al., 2014; Reeve et al., 2010).

Due to the depletion of the endogenous antioxidant system and the increase of the ROS, it is necessary to use exogenous therapies that improve this depletion, as well as assist in the elimination of free radicals.

Topical antioxidant supplementation is an effective strategy to neutralize the deleterious effects of ROS generated after UVB irradiation and to reduce the harmful effects of excessive exposure to UVB radiation (Ahmad and Mukhtar, 2001). Therefore, one of the great advantages of topical administration of drugs is the direct action at the destination or in the proximal delivery, requiring less amount of active substance and, as such, resulting in fewer side effects (Benson and Watkinson, 2012; Chang, 2010).

The vanilla aroma is extracted through the processing of the fava beans of an orchid of the genus *Vanilla*, the *vanilla planifolia* is the one that has commercial importance (Berger, 2007). The components of the vanilla extract are various, but vanillin is the most abundant and oxidation of vanillin that originates vanillic acid (VA) or acid (4-hydroxy-3-methoxybenzoic acid). VA is a dihydroxybenzoic acid derivative, a phenolic compound used as a flavoring agent. VA has a hydroxyl group attached to the aromatic ring whose function is to stabilize the compounds through the electron donation, VA can also be found in a very small amount directly in the vanilla extract (Berger, 2007; Ramachandra Rao and Ravishankar, 2002).

In other models, the VA has antibacterial proprieties (Vazquez-Armenta et al., 2018), antimicrobial (Cava-Roda et al., 2012), hepatoprotective (Itoh et al., 2009, 2010), chemopreventive (Anbalagan et al., 2017), neuroprotective (Khoshnam et al., 2018; Ul Amin et al., 2017), besides being a promising antioxidant and anti-inflammatory active principle (Calixto-Campos et al., 2015). In the plasma of hypertensive rats, VA was able to reduce lipid peroxidation products and to increase the levels of non-enzymatic antioxidants and enzymatic antioxidants (Kumar et al., 2011). Treatment of hypertensive diabetic rats with VA significantly reduced glucose, insulin and blood pressure levels, improved antioxidant activities and levels of lipid peroxidation markers were significantly decreased (Vinothiya and Ashokkumar, 2017). Furthermore, it inhibited complete Freund's adjuvant-induced inflammation and pain by inhibiting the recruitment of neutrophils, oxidative stress, cytokine production, and NFκB activation (Calixto-Campos et al., 2015).

These results reinforce the beneficial potential of VA in the improvement of the antioxidant system. It is important to note that despite the establishment of the antioxidant properties *in vitro* and some *in vivo* models of disease models, there are no studies using VA in topical formulations and no evidence on the effect of a formulation containing VA on UVB-induced inflammation and oxidative stress. Thus, we investigated if a topical formulation containing VA (TFcVA) would remain stable in standard stability protocols that would suggest its potential commercialization, and TFcVA in vivo effect in a model of skin oxidative and inflammatory damage triggered by UVB irradiation.

2. Materials and Methods

2.1 Chemicals

Vanillic acid (VA) purity ≥ 97%, brilliant blue R, reduced glutathione (GSH), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ), Trolox, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT) and bisacrylamide were obtained from Sigma-Aldrich (St Louis, MO, USA). Tert-butyl hydroperoxide from Acros (Pittsburgh, PA, USA); Hydroxymethyl aminomethane (Tris) from Amresco (Solon, OH, USA). Acrylamide, sodium dodecyl sulfate (SDS) were obtained from Invitrogen. Isoflurane 5% from Abbott (Abbott Park, IL, USA). Materials for formulation were obtained from Galena (Campinas, SP, Brazil).

2.2 Topical formulation

An emulsion was prepared by the phase inversion method at 75 ° C. The oily phase consisted of 2% (w/w) Polawax® and 5% (w/w) Aristoflex aqueous phase, after incorporating the triglyceride caprylic and caprylic acid emollient (5%), a preservative solution (Phenonip®) at 0.4%. The prepared formulation was divided into 2 portions: one being reserved for control (without the addition of vanillic acid) and the other with 0.5% vanillic acid. In the control formulation 6% of propylene glycol (humectant and solubilizer) was added and in the formulation containing vanillic acid 0.5% of the same in 6% of propylene glycol was solubilized and added to the formulation after 24 hours. Control formulations did not contain VA (Martinez et al., 2016b). The emulsion, and percentage of active principle (Martinez et al., 2016b) were based on previous studies of our group.

2.3 Physicochemical and functional stability of formulations

Regarding stability studies, formulations were packaged in semipermeable polypropylene containers and stored at 4°C, room temperature, and 40 ± 2° C/75 ± 5% of relative humidity (RH) for 6 months. Samples were evaluated 0, 15, 30, 60, 90 and 180 days after preparation (Martinez et al., 2016a). VA raw material was also stored in the same storage conditions to evaluate its functional stability.

The physical-chemical stability was tested by the following methods: visual evaluation (color, consistency and phase separation) and pH measurement (Georgetti et al., 2006). Functional stability was measured by the ABTS method at 730nm, as previously determined (Martinez et al., 2016a). The TFcVA was diluted in ethanol to obtain a concentration of 160µg/mL, which was also the concentration used for the analysis of the raw material VA in the reaction medium. The following controls were included in the test: (i) a positive control

prepared in absence of sample and (ii) a positive control to which the formulation without VA was added.

After the stability studies, the *in vivo* efficacy of the TFcVA against skin oxidative stress and inflammation caused by UVB irradiation was evaluated.

2.4 *In vivo* efficacy of TFcVA

2.4.1 Animals

The experiments were conducted in female mice hairless (HRS/J), weighing 20-30 g, obtained from the University Hospital of Londrina State University. Mice had free access to water and food at a temperature of 23 ºC ± 2 and a 12 h light and 12 h dark cycles. The Animal Ethics Committee (Of. Circ. CEUA nº70/2017, process CEUA n° 8225.2017.67) of the Londrina State University approved all procedures of this study. All efforts were made to minimize the number of animals used and their suffering.

2.4.2. Experimental protocol

Hairless mice were randomly designed to different groups with 5 mice each: Group 1: control group, non-irradiated; Group 2 control group, irradiated and no treated; Group 3: irradiated group and treated with blank topical formulation (BTF); and Group 4: irradiated group and treated with topic formulation containing VA (TFcVA). Mice received the topical treatment with 0.5g of formulation containing 0.5% of VA or blank control formulation in the dorsum (Martinez et al., 2016b), 1h before, 5 minutes before and 5 minutes after the end of UVB irradiation session (Casagrande et al., 2006b).

2.4.3 Irradiation

The UVB source used in the experiments to induce oxidative stress was a Philips TL/12 RS 40W (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 place the mice were positioned, resulting in an irradiation of 0.209 mW/cm^2 as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with sensor of UV (SED005) and UVB (SED240). The radiation dose for induction of inflammation and oxidative stress was 4.14 J/cm^2 (Ivan et al., 2014; Martinez et al., 2015a).

The animals were terminally anesthetized with 5% isoflurane and dorsal skin samples were collected 12 hours after the exposure to UVB irradiation for edema, matrix metalloproteinase-9 (MMP-9) activity, histology, ferric reducing antioxidant power (FRAP), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and reduced glutathione (GSH)

levels. Samples were collected after 2 hours of exposure to UVB for the tests of catalase (CAT) and superoxide anion production (NBT). Finally, samples collected 4 hours after the end of the irradiation were tested for hydroperoxide production. In these last-mentioned times (2 and 4 hours), the animals were anesthetized with 5% isoflurane followed by decapitation. Skin samples to be analyzed were stored separated for each different test at -80°C, except for the skin edema test, which was performed shortly after collection, and for the histopathological evaluation, for which the samples were stored in 10% formaldehyde solution after collected, until they were processed. The standardization of all conditions of the experiments was published before (Ivan et al., 2014; Martinez et al., 2015a).

2.5 ABTS assay

This assay is based on the inhibition of the absorbance of the radical ABTS. Approximately 30 mg of hairless mice skin was homogenized in 400 µL of KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged at 1000xg for 10 min at 4°C and the supernatant was used measuring the antioxidant capacity of skin. The solution of ABTS was prepared with 7 mM of ABTS and 2.45 mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0,7 – 0,8 in 730 nm was prepared. The supernatant (7 µL) was mixed on ABTS solution and after 6 min the absorbance was determined in 730 nm microplate reader (EnSpire, Perkin Elmer) (Katalinic et al., 2005). Previously, a curve of trolox (0.01 a 20 nmol) was prepared and the results were expressed as nmol equivalent of Trolox/mg skin.

2.6 FRAP assay

This assay was used to evaluate the antioxidant power of ferric reduction of skin (Martinez et al., 2015b). The sample of hairless mice skin was homogenized in 500 µL KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged at 1000xg for 10 min at 4°C and the supernatant was employed for measurement the antioxidant capacity of skin. The reaction consisted in adding the supernatant (30 µL) to the FRAP reagent prepared with 0.3 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloride acid and 20 mM ferric chloride. The FRAP reagent was warmed up to 37°C for 30 min. The absorbance was determined at 595 nm (EnSpire, Perkin Elmer). Previously, a curve of trolox (0.5 – 20 nmol) was prepared and the results were expressed as nmol equivalent of Trolox/mg skin (Katalinic et al., 2005).

2.7 GSH assay

Skin samples were diluted in 0.02M EDTA and triturated using Tissue-Tearor (Biospec 985370). Whole homogenates were treated with 50% trichloroacetic acid. The mixture was then centrifuged at 2,700g for 10 min at 4°C. The supernatant was removed and recentrifuged at 2,700 g for a further 15 min at 4°C. The final supernatant was removed for analysis. For the assay, the reaction mixture contained 50 µL of the sample supernatant, 100 µL of 0.4 M Tris buffer pH 8.9 and 5 µL of a 1.9 mg/mL solution of 5,5'-dithio-bis- (2-nitrobenzoic acid; DTNB) in methanol. The absorbance was determined in a spectrophotometer (EnSpire, Perkin Elmer) after 5 min of incubation at 405 nm. A standard curve of GSH (5–150 µM) allowed the analysis of data and presentation as µMof GSH per mg of skin. The results were expressed in as µM of GSH/skin (Srinivasan et al., 2007).

2.8 Catalase assay

The method is based on the concentration decay of hydrogen peroxide (H_2O_2) which is directly proportional to the absorbance decrease at 240 nm. The difference in absorbance per unit time is the measure of catalase activity (Aebi, 1984).

Skin samples were homogenized in 500 µL of 0.02M EDTA using the Tissue-Tearor homogenizer (Biospec 985370). The homogenate was centrifuged at 2,700 g for 10 min at 4°C twice. The determination of CAT activity on skin was performed on microplate by addition of 10µL sample, 160µL 1M Tris-HCl buffer with 5 mM EDTA pH 8.0, 20 µL deionized water, and 20 µL 200 mM H_2O_2. A white was included for each sample prepared with 10µL of the sample supernatant, 180 µL of 1M Tris-HCl buffer with 5 mM EDTA pH 8.0, and 20 µL of deionized water. The rate at which H_2O_2 is reduced by the action of CAT was evaluated by decreasing the absorbance value by the difference between the initial reading and reading 30 s after the addition of 200 mM H_2O_2. The reading was performed on a microplate spectrophotometer (Enspire, Perkin Elmer) at 240 nm with a temperature maintained at 25°C. The catalase values were expressed as unit of CAT/ mg skin/ minute (Aebi, 1984).

2.9 Evaluation of production lipid hydroperoxides (LOOH)

The production of lipid hydroperoxides was measured by a chemiluminescence method and lipoperoxydation is initiated by addition of tert-butyl hydroperoxide as previously described (GONZALEZ FLECHA et al., 1991) and adapted (Martinez et al., 2015b). Skin samples were collected and homogenized (Tissue-Tearor Biospec 985370) in 800 µL of phosphate buffer (pH 7.4) and then centrifuged at 700 g for 2 minutes at 4 °C. For the assay,

250 µL of the supernatant was added to 1730 µL of reaction medium (120 mM KCl, 30 mM phosphate buffer pH 7.4) and 20 µL of 3 mM tert-butyl hydroperoxide. This assay was performed on a Beckman® LS 6000 β counter (FULLERTON, CA, USA) in a count range not coincident with the response between 300 and 620 nm. The reaction must be carried on in the dark at 30 °C for 120 minutes and the results were measured in counts per minute (cpm) per mg of skin.

2.10 Evaluation of the production of superoxide anion ($O_2^{\bullet-}$)

This method is based on the reduction of nitroblue tetrazolium (NBT) to formazan. Skin samples were homogenized with Tissue-Tearor (Biospec 985370) in 0.02 M EDTA and centrifuged (2000 g for 20 seconds at 4 °C). First, 50 µL of the supernatant from each sample was added and incubated for 1 hour. Subsequently, the supernatant was removed and NBT (1mg/mL) was added to the medium. After 15 minutes of incubation, the supernatant was removed and 20 µL of 100% methanol was added to fix. Then 120 µL of 2 M KOH and 140 µL of dimethylsulfoxide (DMSO) were added to solubilize the formazan compound (reduced NBT). The concentration of formazan formed was measured spectrophotometrically (EnSpire, Perkin Elmer) at 620 nm and the results were presented as optical density (OD)/10 mg of skin (Campanini et al., 2013).

2.11 Skin edema

The effect of topical formulations on UVB-induced skin edema of hairless mice was measured as an increase in the dorsal skin weight. After dorsal skin removal, a constant area (5 mm diameter) was delimited with the aid of a mold, followed by weighing of this constant area (Martinez et al., 2016a). The analysis was obtained by comparing the weight of the skin between groups and the result was expressed in mg of skin.

2.12 Skin histologic evaluation

The dorsal skin samples were collected in formol 10%, fixed in paraformaldehyde 4%, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in paraffin and sectioned to a thickness of 5 µm. The sections were stained with hematoxylin and eosin (H&E) and masson's trichrome stain. The sections stained with H&E were examined using light microscopy at 40x magnification for determination of epidermal thickness (Deng et al., 2015) and a 100x magnification for counting the number of sunburn cells (Schwarz et al., 1995). The sections stained with masson’s trichrome were examined using light microscopy

at a magnification of 10x to visualize changes in collagen fibers by analyzing the intensity of the blue coloration in the dermal areas of the skin exposed to UVB with the aid of the Image J software (NIH) (SONG, 2016).

2.13 Analyses of skin proteinase substrate-embedded enzymography

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate embedded enzymography was used to detect enzymes with gelatinase activity. Assays were carried out as previously described (Saito et al., 2018). The dorsal skin of hairless mice (1:4, w/w dilution) was homogenized (Tissue-Tearor-Biospec 985370) in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.01 M $CaCl_2$ and 1% protease inhibitor cocktail. Whole homogenates were centrifuged twice at 12000xg for 10 min at 4°C. The Lowry method was used to measure protein levels in skin homogenates. Aliquots of 50 µL of samples were mixed with 10 µL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS and 0.005% xylene cyanol. For electrophoresis, 25 µL of the mixture was used were taken for electrophoresis in a gel containing 10% acrylamide and 0.025% gelatin. After destaining in 20% acetic acid, zone of enzyme activity was analyzed by comparing the groups in the Image J software package (NIH, Bethesda, MD, USA).

2.14 Statistical analysis

The bars in the results indicate the mean values ± standard error of the mean (SEM) of 5 mice per group per experiment and are representative of two separate experiments. Data were statistically analyzed by ANOVA followed by Tukey's t test. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different when $p<0.05$.

3. Results

3.1 Physico-chemical and functional stability of TFcVA

After six months under the temperatures of 4°C, RT and 40 ± 2°C to 75% ± 5% RH, the formulations containing or not VA maintained their color, homogeneity and there was no phase separation. The pH value of the TFcVA ranged from 5.4 to 4.9 at different temperatures during the six months, similar to the pH of the skin. The consistency of the base formulation and TFcVA changed at 40°C. The ABTS free radical scavenging ability assay was used to evaluate the functional stability of the TFcVA and the raw material.

Comparing TFcVA with the raw material using the $ABTS^{+}$ methodology, we observed that there was no interference of the formulation excipients in the antioxidant capacity of the TFcVA when compared to the raw material (Figure 1), which made it possible to continue the experiments with this formulation and this methodology.

Figure 2 shows that the ability to scavenger $ABTS^{+}$ radicals at 4°C, RT and 40 ± 2°C at 75% ± 5% RH for the raw material and TFcVA. The TFcVA showed the highest antioxidant activity (%) at the starting day (e.g. day zero) compared to raw material, but in the first 50 days, there was a slight drop, remaining stable up to 180 days, while the raw material VA started with less activity (%), but remained with the same antioxidant activity up to 180 days.

These data on TFcVA physico-chemical and functional stability tests indicate the stability of TFcVA and that it can be used for evaluation of *in vivo* efficacy against damage caused by UVB radiation in the skin.

3.2 TFcVA prevents UVB radiation-induced decrease of skin antioxidants

We performed FRAP and ABTS radical scavenging assays to evaluate the effect of TFcVA in antioxidant capacity of the skin. In both assays, TFcVA inhibited antioxidant depletion keeping their levels close to the non-radiated control group (Figure 3A and 3B). In line with these results, TFcVA also protected mice skin from UVB-induced depletion of GSH (Figure 3C) and CAT. No effect was observed with control formulation (Figure 3D).

3.3 TFcVA reduces UVB radiation-induced lipid hydroperoxides (LOOH) and superoxide anion ($O_2^{\bullet-}$) production

To complete the study of the antioxidant activity of the TFcVA, we evaluated the production of lipid hydroperoxides (LOOH) and superoxide anion ($O_2^{\bullet-}$). Reactive oxygen species (ROS) can oxidize lipids leading to lipid peroxidation (LPO) resulting in products such as LOOH (Martinez et al., 2015b). UVB irradiation increased the LOOH and $O_2^{\bullet-}$ production of the irradiated control groups compared to the non-irradiated control group. In these assays, the TFcVA decreased the production of lipid hydroperoxides (Figure 4A) and reduced $O_2^{\bullet-}$ (Figure 4B). No effect was observed with control formulation.

3.4 TFcVA reduces UVB radiation-induced skin edema

UVB radiation induced significant edema in untreated irradiated mice. On the other hand, mice treated with TFcVA showed a significant inhibition of skin edema after UVB exposure, no effect was observed with control formulation (Figure 5).

3.5 TFcVA reduces UVB irradiation-induced epidermal thickness and apoptosis of keratinocytes

The epidermal thickness of the dorsal skin presented significant increase in the irradiated control groups compared to the non-irradiated control group. By contrast, epidermal hypertrophy was significantly reduced when mice were treated with TFcVA (Figure 6A). We investigated apoptotic keratinocytes after treatment with TFcVA. The results showed that treatment with TFcVA inhibited sunburn cell counts compared with the irradiated control groups, and control formulation induced no effect (Figure 6B).

3.6 TFcVA inhibits UVB radiation-induced skin MMP-9 activity

After exposure to UVB radiation, there is a significant increase in the secretion/activity of metalloproteinases type 9 (MMP-9) that degrade components of the extracellular matrix, such as collagen (Georgetti et al., 2008; Martinez et al., 2016a). In this study, MMP-9 activity/secretion was inhibited by TFcVA and no effect was observed with the control formulation (Figure 7).

3.7 TFcVA reduces UVB irradiation-induced damage of collagen fibers

Tissue sections were subjected to Masson's trichrome staining in order to identify changes of collagen fiber (Figure 8A) formation in the dermal areas of the UVB-exposed dorsal skin (SONG, 2016). Notably, collagen fibers (blue) in the group treated with TFcVA showed lower levels of damage compared to irradiated group. No effect was observed with control formulation (Figure 8B).

4. Discussion

Epidemiological studies indicate that sunscreens are not fully effective in preventing skin damage induced by ultraviolet radiation (UVR) (Cooper et al., 2005). Thus, there is urgent need to develop additional therapeutic approaches to inhibit the deleterious effects of UVR. Considering the essential contribution of oxidative stress to UVR skin damage, the current understanding is that replenishing the skin with antioxidants is a rational and promising approach to prevent radiation damage to the skin (Heurung et al., 2014). Attention has been given to antioxidants from natural sources, which are generally low cost making their use promising for the treatment and prevention of various human diseases (Zhou et al., 2009).

In addition to its flavoring applicability, VA is a phenolic compound capable of donating electrons and stabilizing compounds (Civolani et al., 2000). Phenolic compounds form a substantial part of vegetable foods antioxidants with beneficial biological activities. These compounds received considerable attention because of their important role. Especially, VA has antibacterial proprieties (Vazquez-Armenta et al., 2018), antimicrobial (Cava-Roda et al., 2012), hepatoprotective (Itoh et al., 2009, 2010), chemopreventive (Anbalagan et al., 2017), neuroprotective (Khoshnam et al., 2018; Ul Amin et al., 2017), antioxidant and anti-inflammatory (Calixto-Campos et al., 2015) in other models.

Considering these properties, a TFcVA at 0.5% was prepared followed by physical-functional stability studies under different storage conditions. Stability assessment is a crucial step in acquiring important information about a new product, including its storage conditions (Casagrande et al., 2006a). The TFcVA remained physically stable during six months (without phase separation), except when stored at 40 ± 2 ° C/75 ± 5% RH where it showed a decrease in consistency. The pH values remained within the desired value, close to the skin. The pH measurement is necessary to ensure that the pH value is compatible with the components of the formulation and the application site, avoiding irritation (Casagrande et al., 2006b; Kim et al., 2012). The functional stability performed by the $ABTS^{+}$ assay demonstrated which raw material and TFcVA remained stable in the six months. The promising results on TFcVA stability suggests its potential therapeutic applicability, which we tested in a model with a proeminent physiopathological contribution of oxidative stress making it suitable for testing antioxidants. In this sense, *in vivo* tests were performed to measure the ability of the TFcVA in the improvement of the depletion of endogenous antioxidants and its skin anti-inflammatory efficacy in a mouse model of UVB-triggered skin oxidative stress and inflammation.

UVB radiation produces ROS directly by chromophores - which are molecules responsible for absorbing UV radiation, react and form ROS and indirectly through the inflammatory response (Wei et al., 2002). Several antioxidant systems are present in mammalian tissues to eliminate ROS and protect cells. The balance between antioxidant defenses and ROS production is essential to maintain cell function, whereas a disturbance in favor of the oxidants leads to oxidative stress (Kobayashi and Yamamoto, 2005). Results of the ferric reducing antioxidant power (FRAP) assay correlate well with the levels of antioxidants ascorbic acid, uric acid, and α-tocopherol (Katalinic et al., 2005) while 2,2′-azinobis(3-ethyl- benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity reflects the levels of endogenous antioxidant reduced glutathione (GSH) ((Wei et al., 2002) In this study, UVB irradiation induced a decrease of FRAP and of ABTS scavenging capacity in the skin, reflecting the pro-oxidant effects of this stimulus as described previously (Campanini et al., 2013; Casagrande et al., 2006b; Ivan et al., 2014) The TFcVA improved the antioxidant

capacity of skin after UVB irradiation by increasing ferric and ABTS reducing abilities, which is in good agreement with the direct antioxidant properties of VA.

The enhancement of endogenous antioxidant system occurs via activation of the transcription nuclear factor erythroid 2-related factor 2 (Nrf2) (Malhotra et al., 2010) that interacts with the genes responsible for the regulation of antioxidant enzymes called phase II, such as glutathione peroxidase (GPx), hemeoxygenase 1 (OH-1), quinone oxireductase 1 (Nqo-1), catalase (CAT) and superoxide dismutase (SOD), as well as reduced glutathione (GSH), a non-enzymatic antioxidant (Kobayashi and Yamamoto, 2005; Stępkowski and Kruszewski, 2011). Excessive radiation, high ROS production and activation of inflammatory transcription factors such as NF-kB and AP-1 amplify skin damage. We selected two endogenous antioxidant systems to be tested, the enzymatic CAT and the non-enzymatic GSH. TFcVA inhibited the depletion of these endogenous antioxidants. This antioxidant activity of VA is consistent in varied models of inflammation (Anbalagan et al., 2017; Bhavani et al., 2017; Calixto-Campos et al., 2015; Dianat et al., 2016; Singh et al., 2015; Ul Amin et al., 2017; Vinothiya and Ashokkumar, 2017) and further corroborates that our selection of VA was rational .

UVB skin radiation oxidative end product includes hydroperoxide (LOOH) (Gonzalez Flecha et al., 1991; Shindo et al., 1994). Excessive ROS cause high levels of peroxidation, which are associated with deleterious effects on biological systems, such as inactivation of membrane enzymes and receptors, and increased permeability of ions that may lead to cell membrane rupture. VA maintains the antioxidant levels preventing the oxidation process (Calixto-Campos et al., 2015). In agreement with this notion, TFcVA inhibited LOOH formation (Anbalagan et al., 2017; Bhavani et al., 2017; Vinothiya and Ashokkumar, 2017). During UVB radiation, neutrophil NADPH oxidase produces superoxide anion (F.Y. Yamacita-Borin, A.C. Zarpelon, F.A. Pinho-Ribeiro, V. Fattori and F.Q. Cunha, T.M. Cunha, R. Casagrande, 2015) which contributes to the amplification of oxidative stress and further chemoattraction of neutrophils (G.B. Maru, K. Gandhi, A. Ramchandani, 2014; Shim et al., 2008). This study showed that the TFcVA besides reducing the depletion of the endogenous antioxidants GSH and CAT, also neutralized ROS by electron donation, reducing the amount of $O_2^{\bullet-}$ and lipid hydroperoxides. Therefore, we observed a consistent antioxidant activity of TFcVA in the UVB mouse model of skin damage. Our next question was whether the antioxidant activity would reflect in reduction of disease.

The acute alterations in the skin induced by UVB radiation include marked increased vascular permeability induced by inflammatory mediators resulting in exudation causing edema and vascular dilatation and increased blood flow causing erythema (Coleman, 2010). TFcVA reduced the skin edema caused by UVB nearly reaching basal levels. It is likely that

VA presents an anti-edematogenic effect since it also reduces edema formation in other inflammatory conditions (Calixto-Campos et al., 2015).

UVB radiation is absorbed mainly in the epidermis. Hyperproliferation of epidermal cells, such as keratinocytes, causes hyperkeratosis, which begins hours after exposure to excess UVB, leading to epidermal thickening (Kumar et al., 2015). H&E staining data shows that TFcVA reduced UVB epidermal thickening. UVB also causes other cellular alterations in the skin. UVB induced keratinocytes death by apoptosis, which are called sunburn (SBCs) that display a condensed nuclei and changes in the cellular cytoplasm that becomes eosinophilic (Bayerl et al., 1995; Van Laethem et al., 2005). Reduction in the number of SBCs indicates an increase in keratinocyte photoprotection. Histopathological analysis of H&E stained skin slices demonstrated that TFcVA reduced the number of sunburn cells induced by exposure to UVB.

A cellular inflammatory event that is triggered by UVB is the recruitment of neutrophils. These polymorphonuclear leukocytes produce superoxide anion that will generate hydrogen peroxide. Neutrophils also express the myeloperoxidase enzyme that converts hydrogen peroxide into hypochlorous acid in an environment reach on Cl such as the phagolysosome. However, despite the protective role of this system during infections (Staurengo-Ferrari et al., 2017, 2018) excessive production of hypochlorous acid by recruited neutrophils causes tissue damage by its direct oxidative actions and activation of metalloproteinase type 9 (MMP-9) also secreted by neutrophils (Rosell et al., 2008)]. Keratinocytes and fibroblasts also secrete MMP-9 (Quan et al., 2009). MMP-9 degrades skin collagen and elastic fiber components, and inadequately amplifies the inflammatory response in UVB radiation (Harper et al., 2010). Treatment with TFcVA inhibited MMP-9 activity/secretion after exposure to UVB irradiation. Corroborating this result, the histopathological analysis with trichrome Masson's dye showed that the treatment with TFcVA reduced gelatin degradation. Our data is also supported by evidence that VA decreased MMP-9 activity/secretion in a model of endothelial carcinoma (Bhavani et al., 2017).

In summary, the present study demonstrated to our knowledge, for the first time, that a physical-functional stable TFcVA can be developed using the active principle at a pharmacologically active concentration. TFcVA reduced the skin oxidative stress triggered by exposure to UVB radiation, which explained its therapeutic effect against the deleterious skin damages observed upon UVB radiation. This study opens a new venue of pharmaceutical and pharmacological studies on improving the topical delivery of vanillic acid as active principle that have also the potential to be extended to other inflammatory conditions to be tested.

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Figures

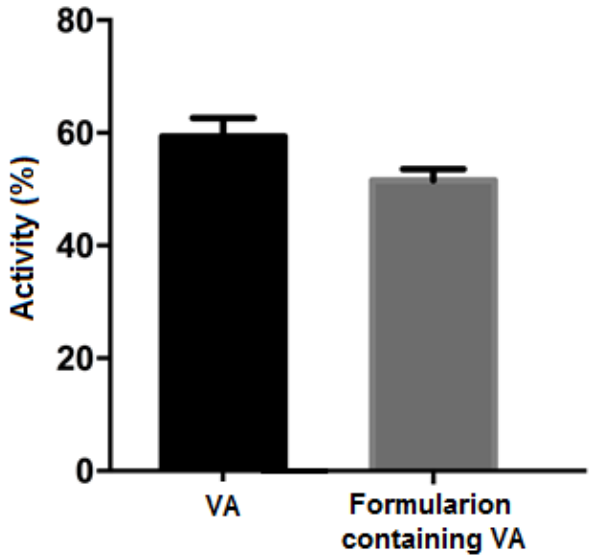


Figure 1 – Interference test of excipients in formulation containing vanillic acid (VA) when compared to vanillic acid (VA) raw material, using the ABTS radical sequestration method.

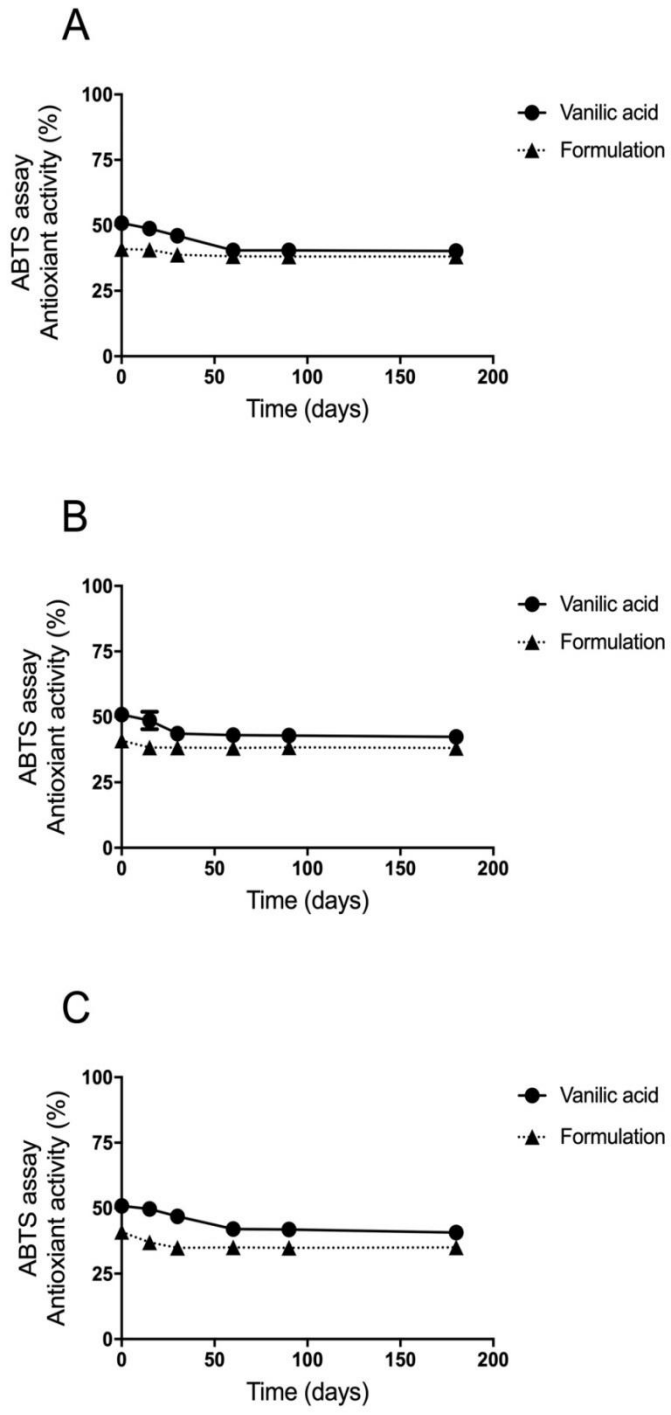


Figure 2 - Stability of ABTS radical reducing ability of vanillic acid (VA) and formulation containing vanillic acid (VA) stored at 4 °C (A), row temperature (RT) (B) and 40°C (C) for 6 months (180 days). Stability of the ABTS radical removal capacity of vanillic and topical acidic raw material formulation containing vanillic acid stored at different temperatures 4 ° C (A), RT (B) and 40 ° C / 75% RH (C) for 6 months.

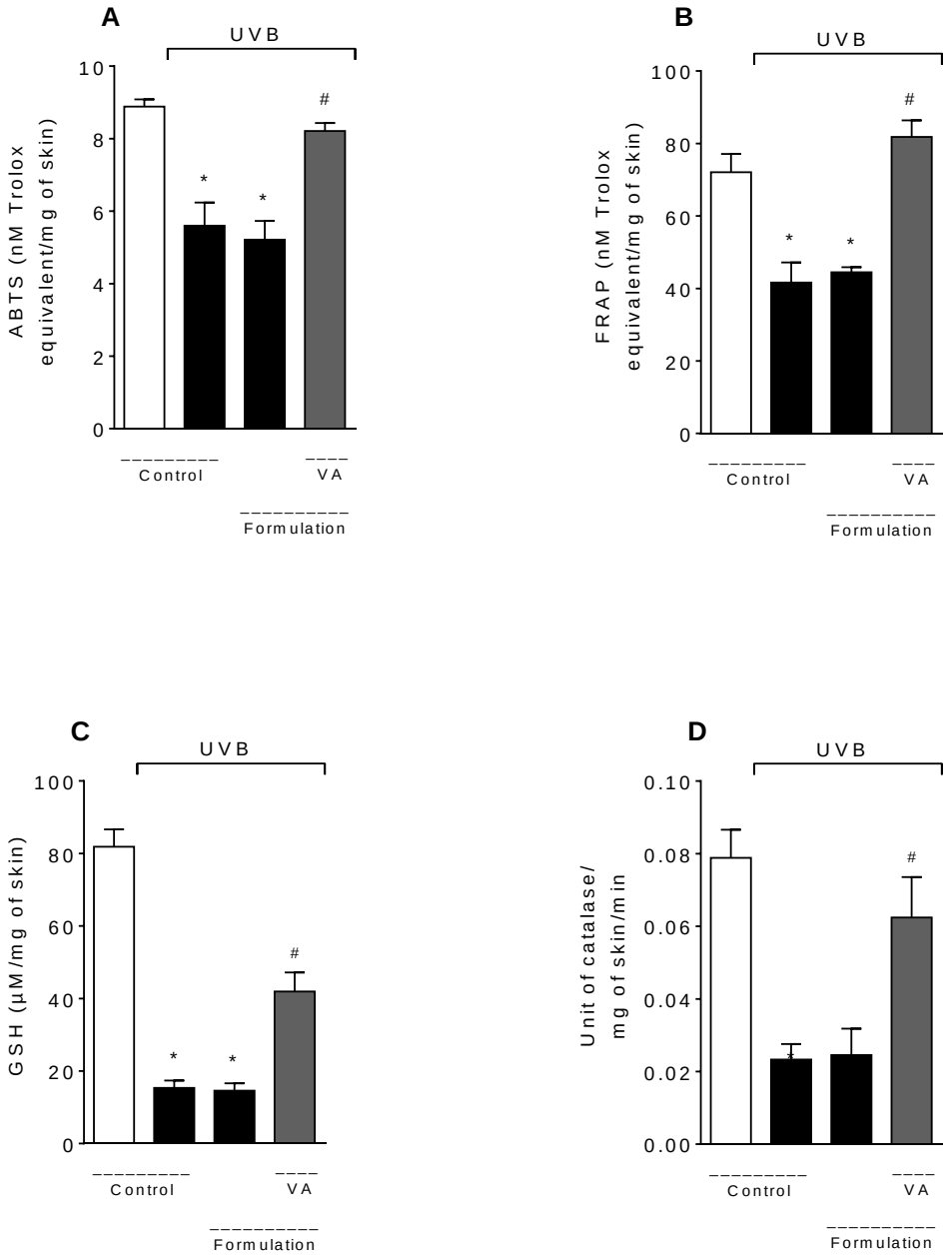


Figure 3 - Topical formulation containing vanillic acid inhibits UVB radiation- induced skin oxidative stress. The antioxidant capacity was determined by ABTS (A), FRAP (B), GSH (C) assays in samples collected 12 h after the end of radiation. The catalase assays (D) was determined in samples collected 2 h after the end of radiation. Bars represent means ± SEM of 5 mice per group per experiment and are representative of two independent experiments. *$p < 0.05$ compared to the non-radiated control group (white bar); #$p < 0.05$ compared to the radiated control groups (black bars).

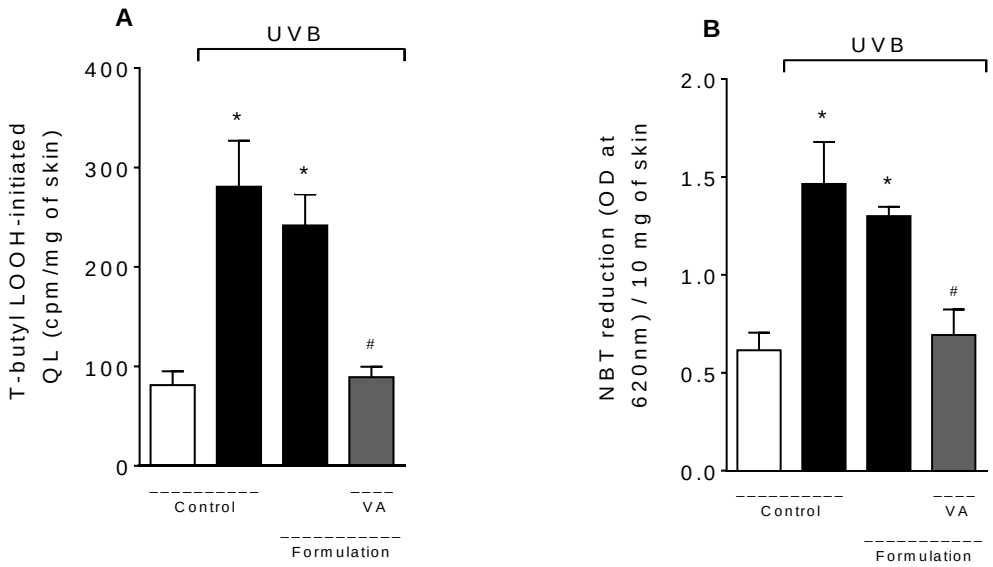


Figure 4 - Topical formulation containing vanillic acid (VA) inhibited UVB-radiation induced lipid peroxidation (LOOH) and superoxide anion production. The t-butyl LOOH initiated chemiluminescence (QL) (A) and nitroblue tetrazolium (NBT) reduction (B) were determined in samples collected 4 h and 2 h after the end of radiation, respectively. Bars represent means ± SEM of 5 mice per group and are representative of two separate experiments. *$p < 0.05$ compared to the non-radiated control group (white bar); #$p < 0.05$ compared to the radiated control groups (black bars).

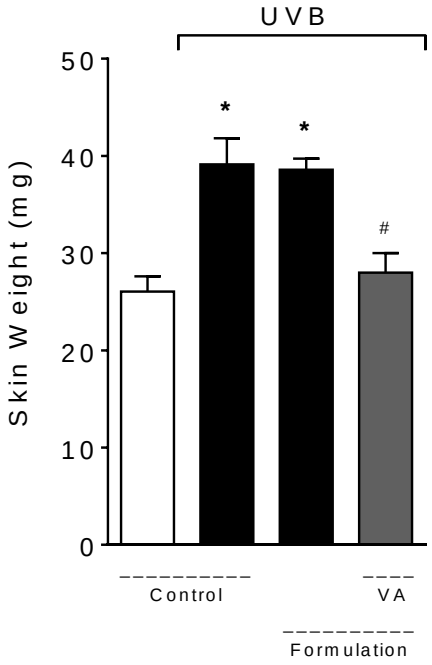


Figure 5 - Topical formulation containing vanillic acid (VA) reduces UVB radiation-induced skin edema. The skin edema were determined in samples collected 12 h after the end of radiation. Bars represent means ± SEM of 5 mice per group and are representative of two separate experiments. *p<0.05 compared to the non-irradiated control group (white bar); #p<0.05 compared to the irradiated control groups (black bars).

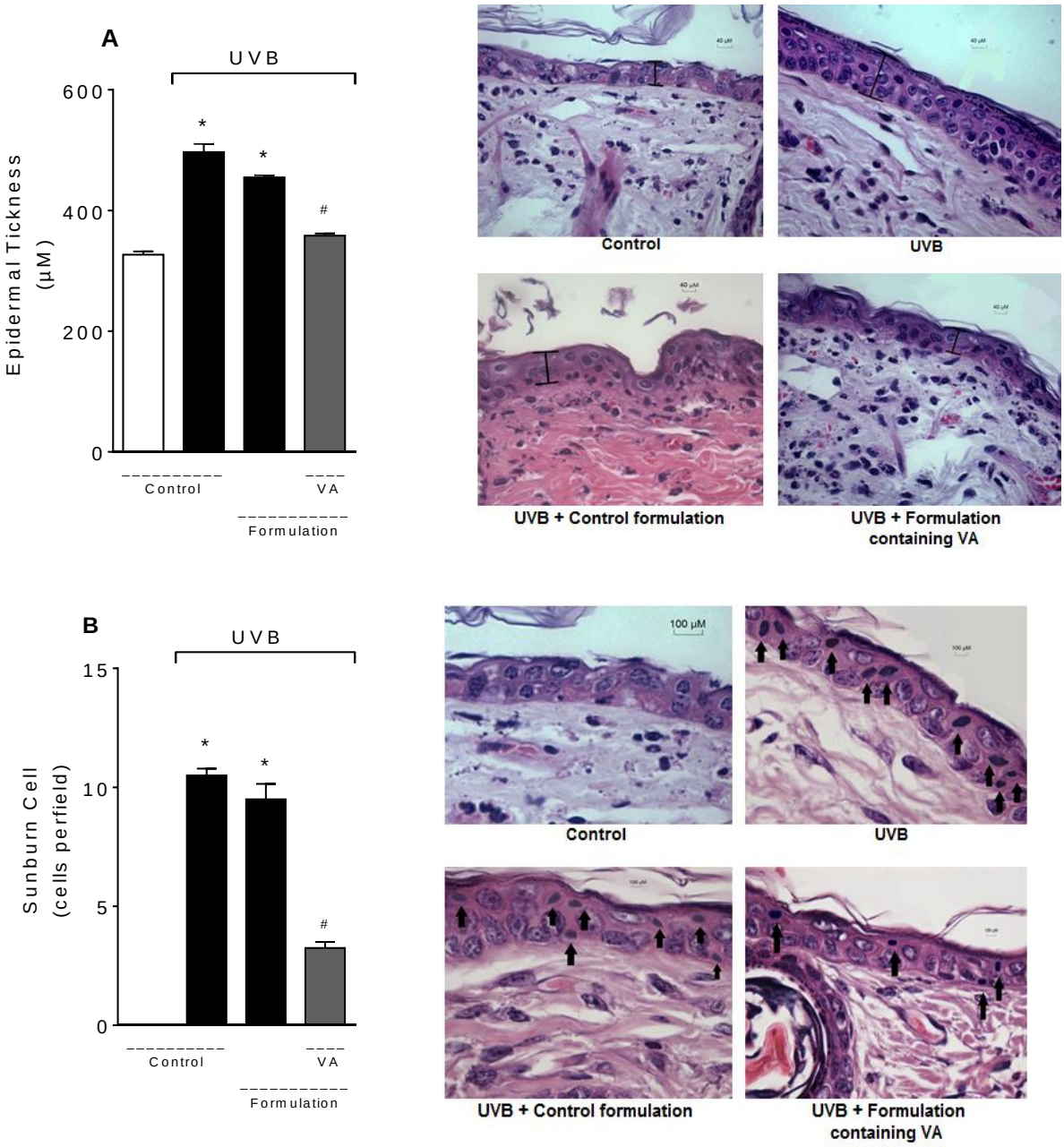


Figure 6 - Formulation containing vanillic acid (VA) reduces UVB radiation-induced epidermal thickness and sunburn cells. Epidermal thickness and sunburn cells were evaluated using hematoxylin and eosin staining (H & E) in skin samples collected 12 h after the end of irradiation. Epidermal thickness (µm) (A) and the number of sunburn cells (B). The sections stained with H & E were examined using light microscopy at 40x (A) magnification and 100x (B). Bars are representative of two separate experiments and represent means ± SEM of 5 mice per group per experiment. *$p < 0.05$ compared to the non-radiated control group (white bar); #$p < 0.05$ compared to the radiated control groups (black bars).

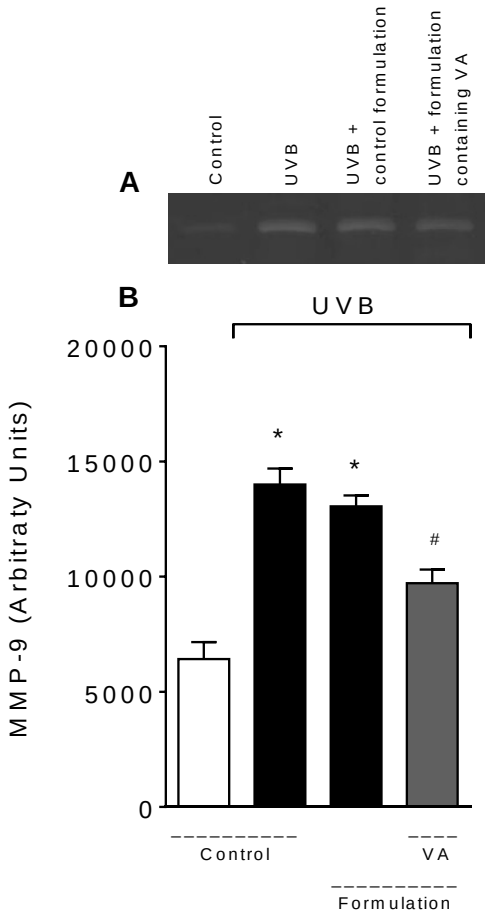
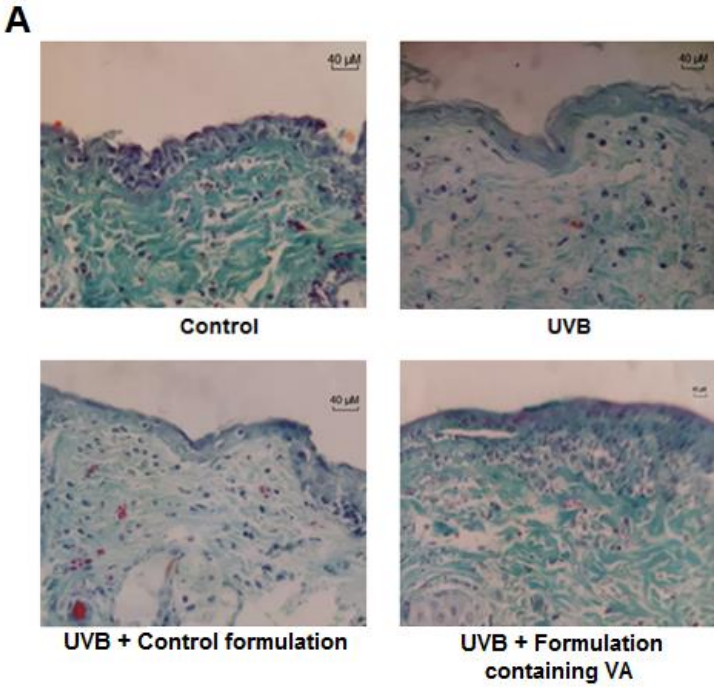


Figure 7 - The topical formulation containing vanillic acid (VA) reduces UVB radiation-induced skin matrix metalloproteinase-9 (MMP-9) activity. Gelatin zymography image. (A) and the activity of MMP-9 was determined in samples collected 12 hours after radiation termination (B). The bars represent means ± SEM of 5 mice per group and are representative of two separate experiments. *p<0.05 compared to the non-irradiated control group (white bar); #p<0.05 compared to the irradiated control groups (black bars)



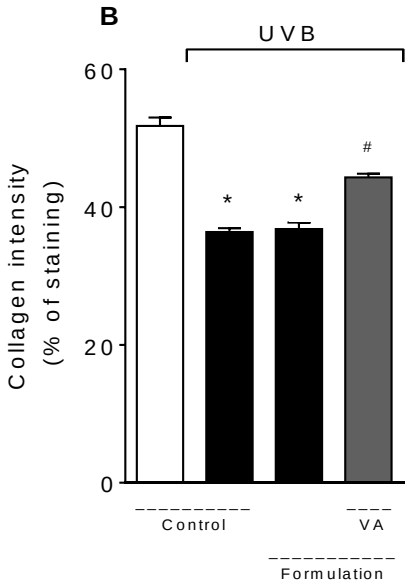


Figure 8 - Formulation containing vanillic acid (VA) inhibits UVB irradiation-induced collagen fibers damage. The collagen fiber formation was determined in samples collected 12 h after the end of irradiation and was evaluated using Masson's trichrome staining. Collagen fiber intensity and bundles shown in blue were analyzed by Image J Program (10x magnification) (A). Collagen intensity (B). Bars are representative of two separate experiments and represent means ± SEM of 5 mice per group per experiment. *$p < 0.05$ compared to the non-radiated control group (white bar); #$p < 0.05$ compared to the radiated control groups (black bars).

5. CONSIDERAÇÕES FINAIS

O ácido vanílico é um antioxidante extraído de fontes naturais, no entanto até o momento não há estudos a respeito do efeito terapêutico da formulação tópica adicionada de ácido vanílico para proteger a pele de danos inflamatórios e oxidativos induzidos pela radiação UVB.

Nos resultados da estabilidade físico-química e funcional da formulação contendo ácido vanílico demonstraram que a formulação se manteve inalterada nos testes visuais e de centrifugação, nas diferentes condições de armazenamento por 180 dias, apenas a consistência que se alterou, o pH permaneceu próximo ao pH da pele durante este tempo nas diferentes temperaturas. Os ensaios *in vivo* mostraram que a formulação tópica contendo ácido vanílico protegeu a pele contra os danos inflamatórios e oxidativos induzidos pela radiação UVB. Com estes resultados, a formulação tópica contendo ácido vanílico se mostrou uma estratégia promissora para controlar e/ou prevenir os danos causados à pele e evitar os efeitos de primeira passagem, além de ser um produto natural e de baixo custo.

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7. ANEXOS

Anexo I – Informações sobre submissão do artigo a revista Frotiers Pharmacology;

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If working with Word please use Frontiers Word templates.

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If you wish to submit your article as LaTeX, we recommend our Frontiers LaTeX templates. These templates are meant as a guide, you are of course welcome to use any style or formatting and Frontiers journal style will be applied during typesetting.

2.3.1.1. Article Type

Frontiers requires authors to carefully select the appropriate article type for their manuscript, and to comply with the article-type descriptions defined in the journal’s "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page. Please note that not

all articles types are available for all journals/specialties. Please contact us if you have any questions. **Please pay close attention to the word count limits.**

Focused Reviews, Frontiers Commentaries and Grand Challenge articles are invited by the chief editor and cannot be part of any Frontiers Research Topic. Unless you were contacted by the chief editor or the editorial office regarding the submission of a paper selected for tier 2 promotion, do not submit a Focused Review or a Frontiers Commentary - instead, submit a Review or a General Commentary.

Please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Protocols and Data Reports.

2.3.1.2. Manuscript Length

Frontiers encourages its authors to closely follow the article word count lengths given in the Summary Table. The manuscript length includes only the main body of the text, footnotes and all citations within it, and excludes abstract, section titles, figure and table captions, funding statements, acknowledgments and references in the bibliography. Please indicate the number of words and the number of figures included in your manuscript on the first page.

2.3.1.3. Language Editing

Frontiers requires manuscripts submitted to meet international standards for English language to be considered for publication.

For authors who would like their manuscript to receive language editing or proofing to improve the clarity of the manuscript and help highlight their research, Frontiers recommends the language-editing services provided by the following external partners:

Editage

Frontiers is pleased to recommend language-editing service provided by our external partner Editage to authors who believe their manuscripts would benefit from professional editing. These services may be particularly useful for researchers for whom English is not the primary language. They can help to improve the grammar, syntax and flow of your manuscripts prior to submission. Frontiers authors will receive a 10% discount by visiting the following link: http://editage.com/frontiers/

The Charlesworth Group

Frontiers recommends the Charlesworth Group Author Services, who has a long standing track record in language editing and proofing. This is a third-party service for which Frontiers

authors will receive a discount by visiting the following link: http://www.charlesworthauthorservices.com/~Frontiers.

Note that sending your manuscript for language editing does not imply or guarantee that it will be accepted for publication by a Frontiers journal. Editorial decisions on the scientific content of a manuscript are independent of whether it has received language editing or proofing by the partner services, or other services.

2.3.1.4. Language Style

The default language style at Frontiers is American English. If you prefer your article to be formatted in British English, please specify this on your manuscript first page. For any questions regarding style Frontiers recommends authors to consult the Chicago Manual of Style.

2.3.1.5. Search Engine Optimization (SEO)

There are a few simple ways to maximize your article’s discoverability. Follow the steps below to improve search results of your article:

- Include a few of your article's keywords in the title of the article;
- Do not use long article titles;
- Pick 5 to 8 keywords using a mix of generic and more specific terms on the article subject(s);
- Use the maximum amount of keywords in the first 2 sentences of the abstract;
- Use some of the keywords in level 1 headings.

2.3.1.6. Title

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.The title should be concise, omitting terms that are implicit and, where possible, be a statement of the main result or conclusion presented in the manuscript. Abbreviations should be avoided within the title.

Witty or creative titles are welcome, but only if relevant and within measure. Consider if a title meant to be thought-provoking might be misinterpreted as offensive or alarming. In extreme cases, the editorial office may veto a title and propose an alternative.

Authors should try to avoid, if possible:

- Titles that are a mere question without giving the answer.
- Unambitious titles, for example starting with "Towards", "A description of", "A characterization of", "Preliminary study on".

- Vague titles, for example starting with "Role of...", "Link between...", "Effect of..." that do not specify the role, link, or effect.
- Include terms that are out of place, for example the taxonomic affiliation apart from species name.

For Corrigenda, Book Reviews, General Commentaries and Editorials, the title of your manuscript should have the following format:

- "Corrigendum: Title of original article"
- "Book Review: Title of book"
- General Commentaries
    - "Commentary: Title of original article" (This does not apply to Frontiers Commentaries)
    - "Response: Commentary: Title of original article"
- "Editorial: Title of Research Topic"

For article types requiring it, the running title should be a maximum of 5 words in length. (see Summary Table)

2.3.1.7. Authors and Affiliations

All names are listed together and separated by commas. Provide exact and correct author names as these will be indexed in official archives. Affiliations should be keyed to the author's name with superscript numbers and be listed as follows: Laboratory, Institute, Department, Organization, City, State abbreviation (USA, Canada, Australia), and Country (without detailed address information such as city zip codes or street names).

Example: Max Maximus, Department of Excellence, International University of Science, New York, NY, USA.

The Corresponding Author(s) should be marked with an asterisk. Provide the exact contact email address of the corresponding author(s) in a separate section.

Correspondence:

Dr. Max Maximus
maximus@gmail.com

If any authors wish to include a change of address, list the present address(es) below the correspondence details using a unique superscript symbol keyed to the author(s) in the author list.

2.3.1.8. Consortium/Group and Collaborative Authors

Consortium/group authorship should be listed in the manuscript with the other author(s). In cases where authorship is retained by the consortium/group, the consortium/group should be listed as an author separated by "," or "and". Consortium/group members can be listed in a separate section at the end of the manuscript.

Example: John Smith, Barbara Smith and The Collaborative Working Group.

In cases where work is presented by the author(s) on behalf of a consortium/group, it should be included in the manuscript author list separated with the wording "for" or "on behalf of". The consortium/group will not retain authorship.

Example: John Smith and Barbara Smith on behalf of The Collaborative Working Group.

2.3.1.9. Headings and Sub-headings

Except for special names (e.g. GABAergic), capitalize only the first letter of headings and subheadings. Headings and subheadings need to be defined in Times New Roman, 12, bold. You may insert up to 5 heading levels into your manuscript (not more than for example: 3.2.2.1.2 **Heading title**).

2.3.1.10. Abstract

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references. The text of the abstract section should be in 12 point normal Times New Roman. See Summary Table for abstract requirement and length according to article type.

For Clinical Trial article types, please include the Unique Identifier and the URL of the publicly accessible website on which the trial is registered.

2.3.1.11. Keywords

All article types: you may provide up to 8 keywords; at least 5 are mandatory.

2.3.1.12. Text

The entire document should be single-spaced and must contain page and line numbers in order to facilitate the review process. Your manuscript should be written using either LaTeX or MS-Word.

Templates are available (see above)

2.3.1.13. Nomenclature

- The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the Acknowledgments.
- Equations should be inserted in editable format from the equation editor.
- Italicize Gene symbols and use the approved gene nomenclature where it is available. For human genes, please refer to the HUGO Gene Nomenclature Committee (HGNC). New gene symbols should be submitted here. Common Alternative gene aliases may also be reported, but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here. Protein products are not italicized.
- We encourage the use of Standard International Units in all manuscripts.
- Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the recommendations by IUPAC.
- Astronomical objects should be referred to using the nomenclature given by the International Astronomical Union provided here.
- Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format:
  urn:lsid:::[:]

  For more information on LSIDs please see Inclusion of Zoological Nomenclature section.

2.3.1.14. Sections

Your manuscript is organized by headings and subheadings. For Original Research Articles, Clinical Trial Articles, and Technology Reports the section headings should be those appropriate for your field and the research itself.

For Original Research Articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field:

Introduction

Succinct, with no subheadings.

Materials and Methods

This section may be divided by subheadings. This section should contain sufficient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval

statement should be included in this section (for further information, see section Materials and Data Policies)

Results

This section may be divided by subheadings. Footnotes should not be used and have to be transferred into the main text.

Discussion

This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior art related to the subject so to place the novelty of the discovery in the appropriate context; discuss the potential short-comings and limitations on their interpretations; discuss their integration into the current understanding of the problem and how this advances the current views; speculate on the future direction of the research and freely postulate theories that could be tested in the future.

For further information, please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Case Reports and Data Reports amongst others or you can check the descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page.

2.3.1.15. Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

2.3.1.16. Author Contributions Statement

The Author Contributions Statement is mandatory and should represent all the authors. It can be up to several sentences long and should briefly describe the tasks of individual authors. Please list only 2 initials for each author, without full stops, but separated by commas (e.g. JC, JS). In the case of two authors with the same initials, please use their middle initial to differentiate between them (e.g. REW, RSW). The Author Contributions Statement should be included at the end of the manuscript before the References.

2.3.1.17. Conflict of Interest Statement

A Conflict of Interest Statement needs to be included at the end of the manuscript before the references. Here, the authors need to declare whether or not the submitted work was carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest. For more information on conflicts of interest, see our Editorial Policies.

2.3.1.18. Contribution to the Field Statement

When you submit your manuscript, you will be required to briefly summarize in 200 words your manuscript's contribution to, and position in, the existing literature of your field. This should be written avoiding any technical language or non-standard acronyms. The aim should be to convey the meaning and importance of this research to a non-expert. While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your statement should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings - whether positive or negative - contribute to progress in your research discipline. This will assist the Chief Editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed statement will also facilitate the identification of the Editors and Reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

Example Statement on: Markram K and Markram H (2010) The Intense World Theory – a unifying theory of the neurobiology of autism. Front. Hum. Neurosci. 4:224. doi: 10.3389/fnhum.2010.00224

Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

2.3.2. References

All citations in the text, figures or tables must be in the reference list and vice-versa. The references should only include articles that are published or accepted. Data sets that have been deposited to an online repository should be included in the reference list, include the version and unique identifier when available. For accepted but unpublished works use "in

press" instead of page numbers. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for the article types that allow such inclusions. Personal communications should be documented by a letter of permission. Website urls should be included as footnotes. Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source. Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal publication for the same preprint exists, the official journal publication is the preferred source.

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

- **SCIENCE, ENGINEERING, and HUMANITIES: For articles submitted in the domains of SCIENCE, ENGINEERING and HUMANITIES please apply Author-Year system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be called according to the surname of the first author, followed by the year. For works by 2 authors include both surnames, followed by the year. For works by more than 2 authors include only the surname of the first author, followed by et al., followed by the year. For Humanities and Social Sciences articles please include page numbers in the in-text citations.

Article in a print journal:

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal:

Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book:

Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract:

Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent:

Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style.

Frontiers Science Endnote Style

Frontiers Science, Engineering and Humanities Bibstyle

- **HEALTH, PHYSICS AND MATHEMATICS: For articles submitted in the domain of HEALTH or the journal Frontiers in Physics and Frontiers in Applied Mathematics and Statistics please apply the Vancouver system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis for Health articles, and in square brackets for Physics and Mathematics articles.

Reference examples

Article in a print journal:

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal:

Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book:

Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract:

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Patent:

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository.
(2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to Citing Medicine.

Frontiers Health Endnote Style

Frontiers Health and Physics Bibstyle

2.3.3. Disclaimer

Any necessary disclaimers which must be included in the published article should be clearly indicated in the manuscript.

2.3.4. Supplementary Material

Frontiers journals do not support pushing important results and information into supplementary sections. However, data that are not of primary importance to the text, or which cannot be included in the article because it is too large or the current format does not permit it (such as movies, raw data traces, power point presentations, etc.) can be uploaded during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to FigShare for permanent storage, during the publication stage of the article, and receive a DOI.

The Supplementary Material can be uploaded as Data Sheet (word, excel, csv, cdx, fasta, pdf or zip files), Presentation (power point, pdf or zip files), Supplementary Image (cdx, eps, jpeg, pdf, png or tif), Supplementary Table (word, excel, csv or pdf), Audio (mp3, wav or wma) or Video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv).

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files. For Supplementary Material templates (LaTex and Word) see Supplementary Material for Frontiers.

Suggested Fonts

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

Headings and subheadings need to be defined in Times New Roman, 12, bold.

The text of the abstract section should be in 12 point normal Times New Roman.

The body text is in 12 point normal Times New Roman.

2.3.5. File Requirements

For Latex Files, when submitting your article please ensure to upload all relevant manuscript files including:

- tex file
- PDF
- .bib file (if the bibliography is not already included in the .tex file)

Figures should be included in the provided pdf. In case of acceptance, our Production Office might require high resolution filesof the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file, and upload them as 'Supplementary Material Presentation'.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using 'Track Changes'. Editors and Reviewers can only download the PDF file of the submitted manuscript .

2.3.6. Additional Requirements per article types

2.3.6.1. CrossMark Policy

CrossMark is a multi-publisher initiative to provide a standard way for readers to locate the current version of a piece of content. By applying the CrossMark logo Frontiers is committing to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the complete citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response: Commentary: Title of original article".

2.3.6.3. Book Reviews

The title of a book review needs to follow the format "Book Review: Title of book". For book Reviews, you must also provide the full book details at the beginning of the article in this format: "Book Review: Full book reference"

2.3.6.4. Focused Reviews

For Tier 2 invited **Focused Reviews**, to shape the paper on the importance of the research to the field, we recommend structuring the Review to discuss the paper's Introduction, Materials and Methods, Results and Discussion. In addition the authors must submit a short biography of the corresponding author(s). This short biography has a maximum of 600 characters, including spaces

A picture (5 x 5 cm, in *.tif or *.jpg, min 300 dpi) must be submitted along with the biography in the manuscript and separately during figure upload.

Focused Reviews highlight and explain key concepts of your work. Please highlight a minimum of four and a maximum of ten key concepts in bold in your manuscript and provide the definitions/explanations at the end of your manuscript under “Key Concepts”. Each definition has a maximum of 400 characters, including spaces.

2.3.6.5 Systematic Reviews

For Systematic Reviews, the following article structure applies.

- Title: include systematic review/meta-synthesis/meta-analysis as appropriate in the title

Each of the sections should include specific sub-sections as follows

- Abstract

        - Background
        - Methods
        - Results
        - Conclusions
    - Introduction
        - Rationale
        - Objectives
        - Research question
    - Methods
        - Study design
        - Participants, interventions, comparators
        - Systematic review protocol
        - Search strategy
        - Data sources, studies sections and data extraction
        - Data analysis
    - Results
        - Provide a flow diagram of the studies retrieved for the review
        - Study selection and characteristics
        - Synthesized findings
        - Risk of bias
    - Discussion
        - Summary of main findings
        - Limitations
        - Conclusions

2.3.6.6. Data Reports

For Data Reports, please make sure to follow these additional specific guidelines.

1. The data sets (defined as a collection of data that contains individual data units organized in a standardized reusable format, including pre-processed or raw data) must be deposited in a public repository for long-term data preservation prior to submission of the Data Report. The data set(s) is to be fixed and made publicly available upon publication of the Data Report.

2. Our data sharing policy also requires that the dataset be made available to the Frontiers editors and reviewers during the review process of the manuscript. Prior to submission of

your Data Report manuscript, please ensure that the repository you have selected supports confidential peer-review. If it does not, we recommend that the authors deposit the datasets to figshare or Dryad Digital Repository for the peer-review process. The data set(s) can then be transferred to another relevant repository before final publication, should the article be accepted for publication at Frontiers.

Note that it is the authors' responsibility to maintain the data sets after publication of the Data Report. Any published Frontiers Data Report article will be considered for retraction should the data be removed from the final selected repository after publication or the access become restricted.

3. The submitted manuscript must include the following details:

- Detailed statement of contribution of the data report to the field
- Name of the data set
- Name of the database/repository where the data set has been submitted
- Link to the data set for confidential peer-review (which can be updated after acceptance, prior to publication once the data is made public)
- Description of how the data was acquired, data collection period
- Filters applied to the data
- Overview of the data files and their formats
- Reference to and/or description of the protocols or methods used to collect the data
- Information on how readers may interpret the data set and reuse the data

All these elements will be peer-reviewed and are required for the publication of the Data Report.

Any future updates to the data set(s) should be deposited as independent versions in a repository and the relevant information may be published as General Commentaries linked on the Frontiers website to the initial Data Report.

Any detailed analyses or new scientific insights relating to the Data Report can be submitted as independent research articles which can also be linked on the Frontiers website to the Data Report article. The protocols and methodology used to collect the data can also be submitted as Methods articles.

2.3.6.7. Case Reports

Case Reports should include the following:

- Background

- Case Presentation

  For human patients: age, sex and occupation of the patient, presenting symptoms, the patient’s history and any relevant family or social history, and relevant clinical findings

- For animal patients: age, sex, and breed of the animal, presenting problems, the animal's history, and relevant clinical findings.

  Description of laboratory investigations and diagnostic tests.

- Discussion of the underlying pathophysiology and the novelty or significance of the case. Authors are required to obtain written informed consent from the patients (or their legal representatives) for the publication.

2.3.6.8. Policy & Practice Reviews

For Policy and Practice Reviews, the following article structure applies:

- Abstract
- Introduction
- Sections on assessment of policy/guidelines options and implications
- Actionable Recommendations and Conclusions

2.3.6.9. Policy Briefs

For Policy Briefs, the following article structure applies:

- Abstract (bullet point format)
- Introduction
- Sections on Policy Options and Implications
- Section on Actionable Recommendations
- Conclusions

2.3.6.10. Protocols

For Protocols articles, please make sure to follow these additional specific guidelines.

1. The submitted manuscript must include the following sections:

   - An Abstract.

   - An Introduction outlining the protocol and summarizing its possible applications.

- A Materials and Equipment section providing a list of reagents or other materials and/or equipment required to carry out the protocol. For basic-science protocols, the formulation of any solutions, e.g. buffers, should be clearly indicated in the Materials and Equipment section.

- A Stepwise Procedures section listing, stepwise, the stages of the protocol. The timing of each step or related series of steps should be indicated, as should points at which it is possible to pause or halt the procedure without adversely influencing the outcome. For steps requiring repeated measurements, details of precision and accuracy should be presented. Limits of detection or quantification should also be stipulated where appropriate.

- An Anticipated Results section describing, and illustrating with figures, where possible, the expected outcome of the protocol. Any analytical software or methods should be presented in detail in this section, as should possible pitfalls and artifacts of the procedure and any troubleshooting measures to counteract them. These last may also be described in an optional Notes section.

- Code or training data sets referenced by the protocol and useful in its execution should be hosted in an online repository; their accession numbers or other stable identifiers should be referenced in the Anticipated Results.

2. The significance of the protocol and any advance represented by the method compared with other, similar methods should be presented in the contribution to the field statement accompanying your manuscript.

2.3.6.11. Code

The code should be novel and presented in human-readable format, adhere to the standard conventions of the language used (variable names, indentation, style and grammar), be well documented (comments in source), be provided with an example data set to show efficacy, be compilable or executable free of errors (stating configuration of system used).

The code should only call standard (freely accessible) libraries or include required libraries, and include a detailed description of the use-scenarios, expected outcomes from the code and known limitations of the code.

Please therefore make sure to provide access to the following upon submission:

1. Abstract explicitly including the language of code

2. Keywords including the language of the code in the following format:"code:language"” e.g.: "code:matlab"

3. Contribution to the field statement including the utility of the code and its language

4. Main Text including:

    - code description
    - application and utility of the code
    - link to an accessible online code repository where the most recent source code version is stored and curated (with an associated DOI for retrieval after review)
    - access to test data and readme files
    - methods used
    - example of use
    - known issues
    - licensing information (Open Source licenses recommended)

5. Compressed Archive (.zip) of the reviewed version of the code as supplementary material (.zip archives are currently available under the “Presentation” dropdown menu).

2.3.6.12. Registered Report

Registered Reports are empirical research articles outlining a proposed methodology and analyses which are peer-reviewed and pre-registered before data collection. Registered Reports should include an Introduction, Methods and preliminary results from any pilot experiments (if applicable). If the Registered Report is endorsed following peer-review and the research is conducted according to the approved methodology, the manuscript will be given In Principle Acceptance. Following data collection, the authors should submit a complete manuscript containing the peer-reviewed sections included in the Registered Report, as well as the Results and Discussion sections. If the Results include unregistered analysis, these should be indicated separately as ‘Exploratory Analysis’. Authors have 1 year after their registered report is accepted to submit a full manuscript. The format is appropriate for any hypothesis-driven research, including both original studies and replications.

Registered Reports have a maximum word count of 3,000 and may include 2 Figures/Tables. Following data collection, the completed version of the manuscript should follow the guidelines for an Original Research article with a maximum word count of 12,000. Registered Reports incur a A-type article fee, charged after the acceptance of the completed manuscript.

2.4. Figure and Table Guidelines

2.4.1. CC-BY Licence

All figures, tables, and images will be published under a Creative Commons CC-BY licence and permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.2. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

Permissions may be necessary in the following scenarios:

- Republishing
- Modifying/adapting
- Partial Figures

It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.3. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.4. Figure and Table Requirements

Legends

Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

- **All articles are prepared using the 2 column layout:** 2 column articles can contain images 85 mm or 180 mm wide.

2.4.5. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.

JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of **300 dpi at final size**. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use Frontiers ChemDraw Template, if working with another program please follow the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility

Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
- Image areas are not pixilated or stair stepped.
- Text is legible and of high quality.
- Any lines in the graphic are no smaller than 2 points width.

2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies, including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

2.6. Materials and Data Policies

Frontiers is committed to open science and open data, and we strongly encourage authors to maximize the availability of data included in their articles by making generated data publicly available where possible, and ensuring that published data sets are cited in accordance with our data citation guidelines. We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published articles.

Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the Transparency and Openness (TOP) guidelines, and the joint declaration of data citation principles produced by FORCE 11.

2.6.1. Availability of Materials

Authors are strongly encouraged to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

Resource Identification Initiative

Authors wishing to participate in the Resource Identification Initiative should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here.

2.6.2. Availability of Data

Frontiers requires that authors make all data relevant to the conclusions of the manuscript available to editors and reviewers during peer-review to enable complete and objective evaluation of the work described.

We strongly encourage authors to make the raw data supporting the conclusions of the manuscript available in publicly accessible repositories. To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability office at datapolicy@frontiersin.org prior to submission with any queries concerning data reporting.

2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the references list to maximize future usability. The following format should be used:

[Dataset] Author names. (year) Data Title. Repository name. Version. Persistant identifier

2.6.4. Data Availability Statements

Data availability statements are required for all manuscripts published with Frontiers. During the submission process, authors will be asked to detail the location of the raw data underlying the conclusions made in the manuscript, and whether it will be made available to other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analysed in the manuscript. These datasets should be cited in accordance with our data citation guidelines.

A statement will be automatically generated using the information provided in the submission form; however, manuscripts containing incomplete or incorrect statements will be prevented from entering the review process.

Examples of acceptable statements

1. **Datasets are in a publicly accessible repository:**

   The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

2. **Datasets are available on request:**

   The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

3. **All relevant data is contained within the manuscript:**

   All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

4. **Restrictions apply to the datasets:**

   The datasets for this manuscript are not publicly available because: [VALID REASON]. Requests to access the datasets should be directed to [NAME, EMAIL].

5. **Data has been obtained from a third party:**

   The data analyzed in this study was obtained from [SOURCE], the following licenses/restrictions apply [RESTRICTIONS]. Requests to access these datasets should be directed to [NAME, EMAIL].

6. **No datasets were generated for this study**

2.6.5. Recommended and Required Repositories

Authors are required to deposit the following data-types in public, community-supported repositories, such as those listed below, prior to publication of an associated Frontiers manuscript:

^ Genetic sequence variants should be annotated according to the guidelines established by the Human Variome Project.

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

2.6.6. Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code, allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

2.6.7. Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) or European Nucleotide Archive (ENA). Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare or dryad.

2.6.8 Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyse data, false discovery rates (FDR) for large-scale studies and threshold or cut-off rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence

database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification, miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

2.7. Statistics

Frontiers requires that all statements concerning quantitative differences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be sufficiently detailed to be reproduced.

3. Editorial Policies and Publication Ethics

Frontiers' ethical policies are a fundamental element of our commitment to the scholarly community. These policies apply to all the Frontiers in journal series. Frontiers has been a member of the Committee of Publication Ethics since January 2015 and follows COPE guidelines where applicable.

3.1. Authorship and Author Responsibilities

Frontiers follows the International Committee of Medical Journal Editors guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Provide approval for publication of the content;
- Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:

AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

The corresponding author takes primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The

corresponding author should also be available post-publication to respond to any queries or critiques.

Requests to modify the authors list after submission should be made to the editorial office using the authorship changes form.

3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

i. *Original content and duplicate publication:* Frontiers only publishes original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.
ii. *Redundant publication:* Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.
iii. *Fabrication and falsification:* Frontiers opposes both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).
iv. *Plagiarism:* Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see here.

We reserve the right to contact the affiliated institutions of authors, who have not acted according to good research and publication practices.

3.3. Translations

Frontiers accepts manuscript submissions that are exact translations of previously published work. This should be clearly stated in the manuscript upon submission. Permission from the original publisher and authors needs to be sought and also stated in the manuscript, and the relevant documents should be provided as supplementary data for verification by the Editor and the editorial office. The original work from which the manuscript has been translated should be clearly referenced.

- *"This is a (‘language’) language translation/reprint of (‘insert title here’) originally published in (‘insert name here’). (‘Insert name here’) prepared this translation with support from (insert name of funding source, if any). Permission was granted by (‘Insert name here’)."*

Please note that Frontiers may request copies of related publications if there are any concerns about overlap or possible redundancy.

3.4. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with COPE guidelines, we expect that “original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations.” This condition also applies to an author’s own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author’s thesis so long as this is the only form in which it has appeared, is in line with the author’s university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list. For some examples, please check our in Manuscript Requirements and Style Guide at 2.3.1

Conferences, Proceedings and Abstracts

Manuscripts that first appeared as conference papers must be expanded upon if they are to be considered as original work. You are required to add a substantial amount of original content in the form of new raw material (experiments, data) or new treatment of old data sets which lead to original discussion and/or conclusions, providing value that significantly exceeds the original conference version. As a rule of thumb, at least 30% of content must be original. Authors submitting such work are required to:

- Seek permission for reuse of the published conference paper if the author does not hold the copyright (proof of permission should be submitted as supplementary material or sent to editorial.office@frontiersin.org with the manuscript ID upon submission).

- Cite the conference in the Acknowledgements section, or the references section if applicable.

Blogs

Although permissible, extended manuscript content which previously appeared online in non-academic media, e.g. blogs, should be declared at the time of submission in the acknowledgements section of the manuscript.

3.5. Image Manipulation

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (eg. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique must be declared in the methods section of the manuscript. Image grouping and splicing must be clearly stated in the manuscript and the figure text. Any concerns raised over undeclared image modifications will be investigated and the authors will be asked to provide the original images.

3.6. Conflicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional affiliations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment.

The Frontiers review system is designed to guarantee the most transparent and objective editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent. Failure to

declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

What Should I Disclose?

As an author, disclosure of any potential conflicts of interest should be done during the submission process. Consider the following questions and make sure you disclose any positive answers:

1. Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work?
2. Do you have financial relationships with entities that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work?
3. Do you have any patents and copyrights, whether pending, issued, licensed and/or receiving royalties related to the research?
4. Do you have other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

If you failed to disclose any of the potential conflicts of interest above during submission, or in case of doubt, please contact as soon as possible the Frontiers Editorial Office at editorial.office@frontiersin.org with the details of the potential conflicts.

Example statement: “Author xxx was employed by company xxxx. All other authors declare no competing interests.”

The handling editors and reviewers will be asked to consider the following potential conflicts of interest before accepting any editing or review assignment:

3.7. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

3.7.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations. Frontiers follows International Association of Veterinary Editors guidelines for publication of studies including animal research. Approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the study. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research.

Humane Endpoints

All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

3.7.2. Studies involving human subjects

Research involving human subjects is expected to have been conducted in accordance with the World Medical Association's Declaration of Helsinki. Studies involving human participants must be performed in accordance with relevant institutional and national guidelines, with the appropriate institutional ethics committee's prior approval and informed written consent from all human subjects involved in the study including for publication of the results. Conformation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work and confirm that study subjects (or when appropriate, parent or guardian) have given written informed consent. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee]. All subjects gave **written informed consent** *in accordance with the Declaration of Helsinki.*

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. In order to protect subject anonymity, identifying information should not be included in the manuscript unless such information is absolutely necessary for scientific purposes AND explicit approval has been granted by the subjects.

3.7.3. Inclusion of identifiable human data

Frontiers follows the ICMJE recommendations on the protection of research participants, which state that patients have a right to privacy that should not be violated without informed consent. We require non-essential identifiable details to be omitted from all manuscripts, and written informed consent will be required if there is any doubt that anonymity can be maintained.

It is the responsibility of the researchers and authors to ensure that these principles are complied with, including the obtaining of written, informed consent.

Written informed consent can be documented on a form provided by an institution or ethics committee, and it must clearly state how the identifiable data will be used. Frontiers also makes available its own form , which may be used for this purpose, but use of the Frontiers form is not required if a suitable alternative form of consent, meeting the ICMJE recommendations, is used. We consider it to be the author' duty to encourage participants or patients whose consent for publication is required to read and understand the ICMJE guidelines, for their information prior to completing the consent form. Participants should also be encouraged to ask any questions and to ensure they are comfortable before they sign the consent form.

The completed consent forms should be stored by authors or their respective institutions, in accordance with institutional policies. Frontiers does not need to view the completed form, and this should not be included with the submission. The completed form should be made available on request from the editor or editorial office, both during the review process and post-publication.

The determination of what constitutes identifiable data lies with our editors and editorial office staff, and manuscripts may be rejected if the required consent documents cannot be

provided. Please note that written informed consent for publication is required for all case report articles where the patient or subject is identified or identifiable.

3.7.4. Clinical Trials

The World Health Organization defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." In accordance with the Clinical Trial Registration Statement from the International Committee of Medical Journal Editors (ICMEJ), all clinical trials must be registered in a public trials registry at or before the onset of participant enrolment. This requirement applies to all clinical trials that begin enrolment after July 1, 2005. To meet the requirements of the ICMJE, and Frontiers', clinical trials can be registered with any Primary Registry in the WHO Registry Network or an ICMJE approved registry.

Clinical trial reports should be compliant with the Consolidated Standards of Reporting Trials (CONSORT) both in terms of including a flow diagram presenting the enrolment, intervention allocation, follow-up, and data analysis with number of subjects for each and taking into account the CONSORT Checklist of items to include when reporting a randomized clinical trial.

The information on the clinical trial registration (Unique Identifier and URL) must be included in the abstract.

3.8. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed using the below template. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabeled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet authorship criteria has been included. The contribution to the field statement should be used to clearly state the reason for the Correction. Please note, a correction is not intended to replace the original manuscript.

The title of the submission should have the following format: "Corrigendum: Title of original article". It is advised to use the corrigendum Word and LaTeX templates.

If the error was introduced during the publishing process, the Frontiers Production Office should be contacted.

3.9. Retractions

As a member of the Committee on Publication Ethics (COPE), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

- Retractions are not about punishing authors.
- Retraction statements should be public and linked to the original, retracted article.

While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

- Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)
- Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)
- Major plagiarism
- The reporting of unethical research, the publication of an article that did not have the required ethics committee approval
- Legal issues pertaining to the content of the article e.g. libellous content
- Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold (‘gift’) authorship
- Politically-motivated articles where objectivity is a serious concern
- The singling out of individuals or organizations for attack
- Faith issues (e.g. intelligent design)
- Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)

Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy here

3.10. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at support@frontiersin.org.

For any ethical concerns, please contact us at editorial.office@frontiersin.org.