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Avaliação das atividades anti-inflamatória e cicatrizante de um extrato hidroetanólico de *Plantago australis* e do seu composto verbascosídeo, em modelos *in vitro*

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Sumário

Lista de abreviaturas utilizadas

AKT: Proteína quinase B

ALT: alanina aminotransferase

AP: proteína ativadora

AST: aspartato aminotransferase

CAM: molécula de adesão celular

CAT: catalase

CLAE: Cromatografia Líquida de Alta Eficiência

Fos: proteínas de fosforilação

COX: ciclooxigenase

EGF: fator de crescimento epidermal

G-CSF: fator estimulador de colônias de granulócitos

GM-CSF: fator estimulador de colônias de granulócitos e macrófagos

GPx: glutationa peroxidase

GSH: glutationa

ERO: espécie reativa de oxigênio

ERN: espécie reativa de nitrogênio

IL: interleucina

INF: interferon

iNOS: óxido nítrico sintase

i.p.: intraperitoneal

IRF: fator regulador de interferon

HSV-1: herpes simplex tipo 1

LPS: lipossacarídeo

MAPK: proteína quinase ativada por mitógeno

MCP: quimiocina para monócitos

mg: miligrama

mL: mililitro

mm: milímetro

μM: micro molar

MPO: mieloperoxidase

MIP: proteína inflamatória de macrófago

NF- κB: Fator de transcrição nuclear- kappa B

OMS: Organização Mundial da Saúde

PG: prostaglandina

PL: fosfolipase

SOD: superóxido dismutase

TGF: fator de crescimento transformador

TLR: receptor toll-like, receptor de proteína transmembrâna

TNF-α: fator de necrose tumoral alpha

VSV: vírus da estomatite vesicular

Resumo da Dissertação

Introdução: *Plantago australis* é uma planta da família Plantaginaceae amplamente difundida na América Latina, conhecida popularmente como “tansagem” ou “transagem”. Na medicina popular, suas sementes e folhas são utilizadas principalmente na forma de extrato para o tratamento de diversas patologias. Dentre seus componentes químicos, destaca-se o verbascosídeo, um glicosídeo fenólico derivado do ácido cafeico.

Objetivos: O presente estudo teve como objetivo avaliar as atividades cicatrizante e anti-inflamatória de um extrato hidroetanólico padronizado das folhas de *P. australis* e de seu marcador analítico, verbascosídeo, em modelos *in vitro*.

Materiais e Métodos: A atividade cicatrizante foi avaliada em linhagens celulares de queratinócito humano (HaCat), utilizando teste de migração (*Scratch Test*) para avaliação de migração celular. Como modelo para estudo de inflamação utilizou-se LPS, composto capaz de ativar a resposta inflamatória em células de microglia murina (N9), para posterior quantificação de mediadores inflamatórios (IL-6, IL-10, IL-12p70, INFγ, MCP-1 e TNFα), além da avaliação das atividades de superóxido dismutase (SOD), catalase (CAT) e alteração no potencial de membrana mitocondrial.

Resultados: Os tratamentos realizados com o extrato hidroetanólico padronizado das folhas de *P. australis* e com verbascosídeo induziram um aumento da migração celular de queratinócitos quando comparados ao controle, indicando uma possível atividade cicatrizante. Tanto o verbascosídeo quanto o extrato hidroetanólico padronizado das folhas de *P. australis* induziram aumento das enzimas antioxidantes sugerindo uma possível ativação destas enzimas, por outro lado, os

compostos não induziram alterações na expressão dos mediadores inflamatórios de células de micróglia. Nos experimentos realizados após a indução da inflamação com LPS, pode-se observar que o verbascosídeo induziu redução significativa de TNFα, IL-6, IL-12p70, INFγ e MCP-1. Embora o extrato hidroetanólico padronizado das folhas de *P. australis* também tenha reduzido a inflamação induzida por LPS, tem-se uma redução estatisticamente significativa apenas no mediador TNFα. Interessantemente, os dois compostos foram capazes de reverter os efeitos causados pelo LPS, nos testes de catalase, superóxido dismutase e avaliação do potencial de membrana mitocôndrial.

Conclusão: Considerando os testes realizados neste estudo, o conjunto de resultados leva-nos a sugerir que tanto o extrato padronizado das folhas de *P. australis*, quanto o verbascosídeo, são capazes de aumentar a migração celular da linhagem de queratinócitos, mas não são capazes de induzir qualquer efeito inflamatório.Já em células ativadas por LPS, o verbascosídeo foi capaz de inibir os mediadores inflamatórios, enquanto os dois compostos conseguiram reverter os efeitos oxidativos, reestabelecendo o estado REDOX.

Abstract

Introduction: *Plantago australis* is a plant of Plantaginaceae family widespread in Latin America, popularly known as “tansagem” or “transagem”. In folk medicine seeds and leaves are used mainly in the extract form for the treatment of various diseases. Among its chemical constituents, be noteworthy the verbascoside, a derivative phenolic glycoside of caffeic acid.

Objectives: The present study aimed to evaluate the wound healing and anti-inflammatory activities of *P. australis* hydroethanolic standardized extract and its analytical marker, verbascoside, *in vitro* models.

Materials and methods: The wound healing activity was evaluated in human keratinocyte cells (HaCat) using Scratch Test for cell migration evaluation. For the inflammation study was used LPS model, a compound capable of activating the inflammatory response in microglia murine cells (N9), and subsequent quantification of inflammatory mediators (IL-6, IL-10, IL-12p70, INFγ, MCP-1 and TNFα), apart the evaluation of the activity of superoxide dismutase (SOD, catalase (CAT), changes of mitochondrial membrane potential.

Results: The treatments with the standardized extract from the leaves of *P. australis* and verbascoside, showed an increase in cell migration of keratinocytes when compared to the negative control, indicating a possible healing activity. Both verbascoside and *P. australis* hydroethanolic standardized extract induce increased of antioxidants enzymes suggesting a possible activation of these enzymes, still, not induce changes in the expression of inflammatory mediators in microglia cells. In the experiments after induction of inflammation with LPS, can be seen that verbascoside sharply showed a significant reduction of TNFα, IL-6, IL-12p70, MCP-1 and INFγ,

although the of *P. australis* hydroethanolic standardized extract has also reduced the inflammation induced by LPS, only have a statistically significant reduction in TNF-α. Interestingly, both the compounds were able to reverse the effects caused in N9 cells activated by LPS, in superoxide dismutase, catalase and evaluation of mitochondrial membrane tests.

Conclusion: Considering the test performed in this study, the results set leads us to propose that both standardized extract of the leaves of *P. australis*, as verbascoside alone are capable of increasing cell migration, but are not capable inducing any inflammation or oxidative effects. Already in cells activated by LPS, the verbascoside was able to inhibit inflammatory mediators, while the both compounds were able to reverse the effect of oxidation.

1. INTRODUÇÃO

As plantas medicinais são usadas há milhares de anos. A utilização de espécies vegetais por grande parte da população mundial iniciou-se no processo evolutivo, perdurando até os dias atuais. Os benefícios das plantas foram passados de geração em geração, com aperfeiçoamento da sua utilização para diversas patologias (Rates, 2001). O estudo dos princípios ativos das plantas possibilitou a obtenção de muitos medicamentos na indústria farmacêutica. Atualmente, a decrescente eficácia de drogas sintéticas e as crescentes contraindicações de seus usos têm aumentado a busca por moléculasnaturais alternativas (Petrovska, 2012; Pasquale,1983; Rates, 2001).

A ampla variedade de compostos ativos oriundos de plantas tem valor significativo nas linhas de pesquisas existentes, possibilitando o desenvolvimento de novos fármacosapós a comprovação da eficácia e da avaliação de toxicidade. Embora exista um grande número de estudos utilizando compostos derivados de plantas medicinais, uma ampla gama de plantas e seus derivados permanecem parcialmente explorados ou inexplorados, tanto da perspectiva científica, quanto comercial (Van Wyk, 2015; Foglio *et al.*, 2006). Entre todos os medicamentos comercializados no mundo atualmente, cerca de 40% tiveram origem direta ou indireta de fontes naturais (Rates, 2001). Cerca de 25% dos medicamentos prescritos no mundo são derivados de plantas e 121 utilizando compostos de plantas. Dos 252 medicamentos listados como essenciais pela Organização Mundial da Saúde (OMS), 11% são exclusivamente de origem vegetal (Sahoo *et al.* 2010). No Brasil, embora nenhum esteja incluído na lista de medicamentos essenciais, há mais de 1000 medicamentos registrados como fitoterápicos (OMS, 2005). Estima-se

que o mercado mundial de fitoterápicos movimente cerca de 44 bilhões de dólares por ano (Ministério da Saúde, 2012).

As plantas medicinais desempenham, portanto, um papel muito importante na medicina moderna, fornecendo componentes para fármacos, os quais dificilmente seriam obtidos via síntese. Tem-se como exemplo de drogas importantes obtidas a partir de plantas a digoxina (*Digitalis spp.)*, a vimblastina (*Catharanthus roseus)*, a atropina (*Atropa belladona)*, a morfina e a codeína (*Papaver somniferum*), e o brasileiro Acheflan® (*Cordia verbenacea DC*) (Calixto, 2005; Rates, 2001; Foglio *et al.*, 2006).

O Brasil é reconhecido mundialmente pela sua biodiversidade genética vegetal. Possuindo uma flora exuberante, com cerca de 55 mil espécies catalogadas, correspondendo a aproximadamente 20% da variedade biológica do mundo. Aliado a isso, tem-se outra grande riqueza, o conhecimento etnofarmacológico obtido pelo uso tradicional de plantas como medicamentos pela população (Calixto, 2000; Agra e Barbosa Filho, 1990).

Segundo a OMS cerca de 80% da população mundial utiliza plantas medicinais como principal recurso no atendimento básico de saúde (Wangchuk *et al.*, 2011; Karthshwaran e Mirunalini, 2010). No entanto, mesmo com a grande biodiversidade que há no mundo, muitas espécies são usadas empiricamente, sem comprovação científica quanto à eficácia e segurança, o que demonstra que existe uma enorme lacuna entre a oferta de plantas e as escassas pesquisas (Foglio *et al.*, 2006).

1.1. Plantaginaceae

A família Plantaginaceae da ordem Plantaginales, subclasse Asteridaes, compreende 108 gêneros, com cerca de 2000 espécies (Cronquist 1988; Hefler *et*

al., 2011). Dentre estes, o gênero *Plantago* é um dos maiores, composto por cerca de 250 espécies de plantas ao redor do mundo. As plantas pertencentes a este gênero apresentam um grande número de compostos biologicamente ativos com propriedades farmacológicas. No Brasil foram descritas aproximadamente 16 espécies de *Plantago*, principalmente em áreas tropicais do Sudeste e Sul (Rahn, 1966; Hefler *et al.*, 2011).

As espécies do gênero *Plantago*, popularmente conhecidas como tansagem ou transagem (Lorenzi e Matos, 2008) vêm sendo utilizadas na medicina popular em todo o mundo, principalmente para o tratamento de doenças inflamatórias, de pele, câncer, sistema reprodutivo e doenças nos sistemas respiratório e digestivo (Sahagún *et al.*, 2015; Chiang *et al.*, 2003; Nazarizadeh *et al.*, 2013; Palmeiro *et al.*, 2002).As sementes e folhas são as partes mais usadas das plantas deste gênero, na forma de infusão, emplasto e principalmente na forma de extrato (Hussan *et al.*, 2015; Mahmood e Phipps, 2006; Lopez *et al.*, 2009). As espécies mais utilizadas para fins terapêuticos são a *Plantago australis*, *Plantago lanceolata* e *Plantago major* (Palmeiro *et al.*, 2002).

Como exemplos do uso da família Plantaginaceae, *P. major* tem sido empregada por centenas de anos em Taiwan para o tratamento de resfriado, conjuntivite e hepatite (Chiang *et al.*, 2003). Sementes de *P. ovata* e *P. psyllium* são utilizadas na medicina popular como laxante na Índia e no sul da Europa (Samuelsen *et al.*, 1999). No Brasil, as folhas e sementes de *P. major* são utilizadas como anti-inflamatório, antibacteriano e antisséptico, enquanto no sul do Brasil a *P. australis* é utilizada como diurético, cicatrizante, anti-inflamatório, antibacteriano e laxativo (Palmeiro *et al.*, 2002). Na Áustria, a *P. lanceolata* é indicada nas formas de xarope, chá ou folha fresca, para o tratamento de picadas, infecções virais, de pele

ou do trato respiratório (Volg *et al.*, 2013). As sementes de *P. asiática* são utilizadas no Japão como antiasmático, antitussígeno, anti-inflamatório e no tratamento de diarréia (Samuelsen *et al.*, 1999; Li *et al.*, 2009). Folhas de *P. major* são empregadas na medicina popular da Noruega por suas propriedades cicatrizantes (Samuelsen *et al.*, 1999).

1.1.1. *Plantago australis* (Kunth) Rahn

A *P. australis* (Kunth) Rahn (Figura 1) é descrita como uma erva perene, que mede de 10 a 61 centímetros de altura. Sua raiz principal é inconspícua, e suas raízes secundárias são fibrosas e numerosas, podendo ser espessadas. Possui caule tipo rizoma medindo 0,5 - 2 x 0,4 - 1,7 cm. Suas folhas, 7 - 34,3 x 2,5 - 5,5 cm, podem ser de formas oblanceoladas, elípticas, lanceoladas ou espatuladas, membranáceas, pubescentes, vilosas ou tomentosas. Possui tricomas e suas margens são inteiras ou levemente denteadas, glabras ou ciliadas. O pecíolo não é distinto da lâmina, a nervação é acródoma em direção ao ápice, o qual pode ser agudo ou raramente obtuso de base atenuada. A inflorescência é laxa com 11 - 33,9 cm de comprimento. O escapo, com 4,1 - 30,1 cm de comprimento, cilíndrico, pubescente, tomentoso e com tricomas antrorsos. As sementes medem de 1,2 - 2,8 x 1 - 1,4 milímetros, são de cor castanhas, sendo cerca de três por fruto, são elípticas ou obovais, com face placentar levemente plana (Hefler *et al.*, 2011; Rocha *et al.*, 2002; Rahn, 1996).

Esta espécie de planta é vastamente disseminada na América Latina, sendo no Brasil encontrada com distribuição expressiva nas regiões sul e sudeste. Esta planta habita todos os tipos de solos: argiloso, campos secos ou úmidos, limpos ou roças, terrenos arenosos, campos elevados, locais abertos ou caminhos de matas. A *P. australis*, como todas as espécies do gênero Plantaginaceae, necessita de alta

intensidade de luz, porém suas requisições sobre a água são variáveis (Rahn, 1996). Geralmente floresce de setembro a fevereiro, embora a floração possa ser encontrada em outros meses do ano (Hefler *et al.*, 2011).

a)

b

Figura 1- *Plantago australis* a) Imagem adaptada de Hefler *et al.*, 2011 b) Imagem de arquivo pessoal.

1.2.1. Atividades farmacológicas

A P. australis é uma espécie amplamente utilizada na medicina popular, tendo diferentes usos descritos, tais como antibiótico, anti-inflamatório, diurético, antidiabético, analgésico, antidiarreico, antitussígeno, depurativo, para o tratamento de infecção urinária, cálculos biliares, infecção de garganta, reumatismo, úlceras estomacais, afta, entre outros (Vendruscolo *et al.*, 2005; Vendruscolo e Mentz, 2006; Trojan-Rodrigues *et al.*, 2012; Kajawska e Pieronic, 2015).

Grande parte dos usos descritos popularmente foram estudados e comprovados cientificamente. Palmeiro *et al.* (2002), avaliaram as ações anti-inflamatórias e analgésicas de um tratamento via oral de extrato hidroalcóolico das folhas, sementes e frutos de *P. australis in vivo*. A atividade anti-inflamatória do extrato foi testada utilizado modelo de indução de edema de pata com carragenina. Os ratos machos foram tratados uma hora antes da indução da lesão com até 1000 mg/kg do extrato, e a análise foi realizada de hora em hora por quatro horas. Já para a análise da atividade analgésica foi injetado, via intraperitoneal (i.p.), ácido acético para indução da lesão em camundongos. Os tratamentos foram realizados até a dose de 250 mg/kg de extrato via oral e os animais foram observados por 30 minutos para contorções corporais. Em ambos os testes o extrato de *P. australis* foi capaz de diminuir os parâmetros observados em comparação aos controles positivos, os resultados demonstram que na análise anti-inflamatória o extrato foi capaz de reduzir em quase 42% o edema de pata dos ratos, enquanto na avalição analgésica a redução foi de quase 49%.

Outro estudo inferiu que o uso de extrato etanólico bruto das folhas de *P. australis*, foi capaz de reduzir em até 41% a lesão prévia à úlcera, e, em quase 96%, o índice de úlceras estomacais causadas por etanol em ratos, quando comparados ao grupo controle (Burger *et al.*, 2002).

Em 1999, Abad *et al.* publicaram um estudo sobre atividade antiviral de diferentes plantas encontradas na Bolívia. Neste estudo foram analisados a eficácia de vários extratos sobre três tipos de vírus, herpes simplex tipo 1 (HSV-1), vírus da estomatite vesicular (VSV) e poliovírus tipo 1 em diferentes tipos de células. Dos diversos extratos das plantas testadas, o extrato aquoso de *P. australis* foi o que obteve melhores resultados sobre o vírus da estomatite vesicular, em concentrações

variando de 150 a 500 µg/mL, sem apresentar efeitos citotóxicos. Em relação aos vírus de HSV-1 e poliovírus tipo 1, não foi possível observar uma efetividade do extrato.

Palmeiro *et al.* (2003) analisaram a toxicidade oral subcrônica, de um extrato aquoso bruto das folhas de *P. australis*. No estudo, ratos *Wistar* foram tratados com água ou doses de 850 e 1700 mg/kg do extrato aquoso por 60 dias. Ao final, foram realizadas dosagens séricas de ureia, creatinina, glicose, proteínas totais, fosfatase alcalina, albumina, aminotransferases (AST e ALT), exames hematológicos, além de análise histológica de fígado, coração, esôfago, estômago, colón, pulmão e rim. As administrações de ambas as doses do extrato não apresentaram alterações significativas nas análises histológicas. As análises dos parâmetros bioquímicos e hematológicos foram consideradas normais com exceção da dosagem de ALT, que apresentou níveis elevados na dose de 850 mg/kg. Os resultados do estudo sugerem a ausência de alterações de significância clínica nos ratos testados com extrato aquoso de *P. australis*.

1.1.1.2 Composição química

Estudos referentes à composição fitoquímica da *P. australis* têm mostrado uma grande variedade de metabólitos secundários que apresentam atividades biológicas. Rønsted *et al.* (2000) constataram a presença de glicosídeos iridoides, como a aucubina, e de compostos fenólicos, como o verbascosídeo, isoverbascosídeo e salidrosídeo, em um extrato hidroetanólico da planta Gonzales *et al.* (2000) encontraram presença de saponinas, flavonoides e taninos em diferentes tipos de extratos das folhas da planta.

Henn *et al.*, (dados não publicados) realizaram análises de diferentes metabólitos de várias classes de constituintes fitoquímicos de um extrato

hidroetanólico de *P. australis* e encontraram ácidos triterpênicos, flavonoides, glicosídeos iridoides, e derivados do ácido cafeico. O estudo foi realizado por metodologia de cromatografia líquida de alta eficiência CLAE, e no referido extrato, destacou-se o verbascosídeo. De maneira semelhante dados de análise de composição química, realizadas pelo nosso grupo de pesquisa, indicam que o verbascosídeo é adequado para ser utilizado como marcador analítico no desenvolvimento de um extrato padronizado de *P. australis*.

1.1.1.2.1. Verbascosídeo

Verbascosídeo é um composto glicosídico fenólico, também conhecido como acteosídeo, estruturalmente caracterizado pelo ácido cafeico e 4,5-hidroxifeniletanol ligados a um β-(D)-glucopiranosídeo unidos por ligações éster e glicosídicas e uma ramnose ligada na molécula de glicose, representados na Figura 2 (Korkina *et al.*, 2007).

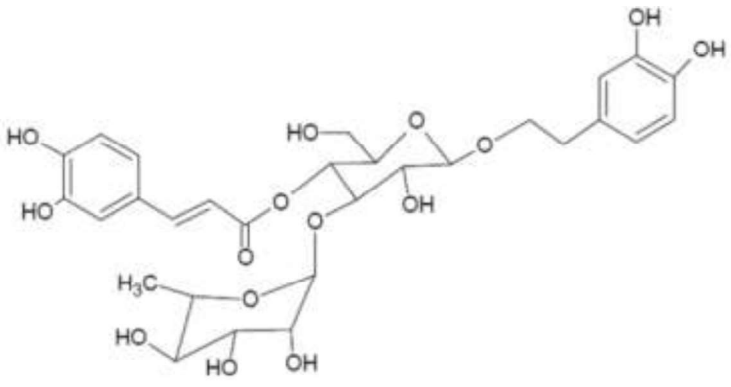

Figura 2. - Representação da estrutura química do composto verbascosídeo.

O verbascosídeo pode ser encontrado em diversas plantas de diferentes famílias (Isacchi *et al.*, 2011). Este composto é conhecido por possuir várias

atividades farmacológicas, como antioxidante, anti-inflamatória, antinociceptiva, anti-hepatotóxico, antiproliferativo, antiulcerogênico, entre outras (Schapoval *et al.*, 1998; Lee *et al.*, 2007; Isacchi *et al.*, 2001; Casamassima *et al.*, 2014; Husmann *et al.*, 2007).

Em um estudo publicado por Lee *et al*. (2013) foram avaliados os efeitos do verbascosídeo sobre a diferenciação de osteoclastos, reabsorção óssea e mecanismos celulares. Neste trabalho foi evidenciado que o verbascosídeo é capaz de reduzir a absorção óssea por osteoclastos maduros, impedir a ativação de MAPKs e de fatores de transcrição como NF-κB e c-Fos, bem como a produção de citocinas inflamatórias TNF-α, IL-1β e IL-6. O verbascosídeo também foi capaz de atenuar os efeitos da indução de osteoporose por ovariectomia.

Speranza *et al.* (2010) avaliaram o efeito anti-inflamatório do verbascosídeo em células THP-1 (leucemia mielomonocítica humana), utilizando um modelo de indução de inflamação celular com lipopolissacarídeo bacteriano (LPS) ou Interferon γ (INF-γ). Os autores demonstram que neste modelo há indução da inflamação, com a produção intensa da atividade de ânion superóxido, conduzindo a estresse oxidativo. Adicionalmente, o tratamento gerou aumento da expressão de NF-κB, e consequentemente indução de óxido nítrico sintase (iNOS). Interessantemente, o tratamento com verbascosídeo na concentração de 100 μM, conseguiu reverter de forma eficiente todos os efeitos causados pelo LPS e/ou INF-γ, diminuindo a expressão e atividade de iNOS, reduzindo a ativação de NF-κB, bem como os níveis de superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx).

Em estudo recente sobre dor neuropática em ratos, pesquisadores do Irã analisaram o possível efeito antinociceptivo do verbascosídeo. Os ratos tiveram dor neuropática induzida por ligação do nervo ciático e receberam aplicação *i.p.*

contendo verbascosídeo nas doses de 50, 100 e 200 mg/mL por 14 dias após a cirurgia. Neste trabalho foram realizados testes de alodinia mecânica, alodinia ao frio e hiperalgesia. A dose de 200 mg/mL demonstrou ser efetiva em todos os testes realizados ($p<0.001$), do terceiro ao décimo quarto dia após a indução da lesão (Amin *et al.*, 2016).

Em extrações de verbascosídeo e teupoliosídeo (56% e 97% p/p) obtidas a partir de flores de *Syringa vulgaris*, Korkina *et al.* (2007), testaram as atividades anti-inflamatória e cicatrizante de seus extratos em ratos *Wistar*. O modelo para ensaio de cicatrização utilizando no estudo consiste no corte de 1,5 cm de espessura e 0,2 cm de profundidade no dorso dos animais. A área da ferida foi analisada nos dias 4 e 8 após a incisão. Os extratos foram aplicados topicamente na concentração de 0,2 mg/mL. Ao final do experimento foi constatado que o extrato contendo 56% de verbascosídeo foi o que apresentou os melhores resultados (dia 0= 459,16 ± 15,68; dia 4= 127,31 ± 28,21; dia 8= 46,29 ± 12,21) em relação ao controle (dia 0= 459,16 ± 15,68; dia 4= 252,96 ± 51,31; dia 8= 150,16 ± 65,46). O extrato contendo 97% de verbascosídeo foi praticamente ineficaz no tratamento da lesão (dia 0= 459,16 ± 15,68; dia 4= 261,25 ± 22,99; dia 8= 124,29 ± 31,23). Nas análises das enzimas da pele dos ratos, o tratamento contendo 56% de verbascosídeo apresentou os melhores resultados nos ensaios de mieloperoxidase (MPO), GPx e glutationa-S-transferase. Já nos experimentos utilizando cultura primária de queratinócito humano ativados por citocinas pró-inflamatórias TNF-α e INF-γ, mostrou que a expressão de quimiocinas para monócitos (MCP-1), para granulócitos e monócitos (IL-8), para células dendríticas (IP-10) e fator de crescimento (G-CSF), foram inibidas de forma eficaz pelo verbascosídeo a 97%, resultado semelhante foi obtido no tratamento com o teupoliosídeo.

1.2. Atividade cicatrizante

Na medicina caseira as plantas são utilizadas no tratamento de diversas doenças. Feridas e queimaduras são exemplos de condições conhecidas pelo fato de serem controladas com medicamentos a base de plantas. Muitos fatores podem influenciar a cicatrização de feridas, como o diabetes, trauma, insuficiência renal e hepática, tabagismo entre outros (Tazima *et al.*, 2008).

A cicatrização de feridas é uma capacidade auto-regenerativa presente nos seres vivos (Balbino *et al.*, 2005). Sejam eles organismos unicelulares, onde se faz por meio de enzimas responsáveis pela recuperação de elementos estruturais e de moléculas de alta complexidade, bem como em organismos superiores onde pode existir de duas formas, seja ela pela cicatrização com restabelecimento da homeostasia com perda da funcionalidade pela formação de cicatriz fibrótica ou pela regeneração total e recomposição da atividade normal do tecido (Balbino *et al.*, 2005; Diegelmann e Evans, 2004).

O ferimento de pele decorrente de lesão por agentes químicos, térmicos, mecânicos ou bacterianos é capaz de induzir uma cascata de reações às quais podem ser divididas em quatro fases: hemostase (1), inflamação (2), proliferação (3) e remodelamento do tecido (4) (Balbino *et al.*, 2005; Harper *et al.* 2014; Alerico *et al.* 2015). O sucesso da cicatrização é dependente da ação sinérgica do sangue, células, plaquetas, citocinas, fatores de crescimento, fibroblastos e queratinócitos (Harper *et al.* 2014; Alerico *et al.* 2015; Diegelmann e Evans, 2004; Flanagan *et al.*, 2000). A seguir são apresentadas em detalhes estas quatro fases da inflamação.

Fase 1 - Hemostase: A resposta normal para a cicatrização se inicia no momento que o tecido é ferido (Diegelmann e Evans, 2004). Com os componentes do sangue derramados pela região da lesão, a resposta imediata do corpo é evitar a

perda de sangue e promover a hemostase (Balbino *et al.*, 2005; Harper *et al.* 2014). Vasos arteriais danificados rapidamente se contraem, acarretando a produção de óxido nítrico, adenosina e outros metabólitos vasoativos capazes de provocar vasodilatação e relaxamento dos vasos arteriais. As plaquetas entram em contato com o colágeno exposto e outros elementos da matriz extracelular, desencadeando a liberação de fatores de coagulação, dando início a cascata de coagulação, bem como fatores de crescimento e citocinas, os quais têm sua passagem vascular facilitada pela liberação de histamina, que aumenta a vasodilatação e permeabilidade vascular (Harper *et al.*, 2014). Além disso, em resposta ao dano às membranas celulares, o ácido araquidônico é utilizado para a síntese das moléculas de sinalização, tais como prostaglandinas, leucotrienos e tromboxanos, os quais têm papeis importantes na estimulação da resposta inflamatória (Harper *et al.*, 2014; Diegelmann e Evans, 2004; Flanagan, 2000).

Fase 2 - Inflamação: A resposta inflamatória pode ser detectada pela presença de calor, inchaço, eritema, dor e perda de função (Flanagan *et al.*, 2000). O objetivo principal desta etapa é impedir a infecção. Os primeiros elementos celulares que se infiltram na ferida são os neutrófilos que interagem diretamente com objetivo de fagocitar partículas antigênicas e corpos estranhos (Flanagan *et al.*, 2000; Harper *et al.*, 2014; Balbino *et al.*, 2005). Logo após, células fagocíticas muito maiores, denominadas macrófagos, são atraídas para a lesão. Os macrófagos são responsáveis por ativar os elementos subsequentes da cicatrização, tais como fibroblastos e células endoteliais (Harper *et al.*, 2014; Diegelmann e Evans, 2004).

Fase 3 - Proliferação: Uma vez que a estimulação da ferida terminou, a hemóstase foi alcançada, a resposta inflamatória equilibrada e o ferimento livre de “entulhos” tem-se início a fase proliferativa. Aqui se inicia a estimulação miogênica e

quimiotática dos queratinócitos pelo TGFα (fator de crescimento transformador) e EGF (fator de crescimento epidermal). Esta fase é composta por três eventos importantes: angiogênese, migração de fibroblastos e epitelização (Tazima *et al. 2008;* Harper *et al.*, 2014). A angiogênese consiste na formação de novos vasos sanguíneos necessários para o ambiente onde foi gerada a lesão. À medida que o processo de angiogênese acontece, é formada uma rede vascular de capilares com ramificações através do ferimento, com isso aumentando a permeabilidade do tecido, formando edema e auxiliando na cura e no aparecimento do tecido de granulação. Além da responsabilidade nutritiva, a angiogênese é responsável pelo aumento da demanda metabólica e do aporte celular, como o aparecimento de fibroblastos para o local da ferida (Harper *et al.*, 2014; Flanagan *et al.*, 2000). A principal função dos fibroblastos é a síntese de colágeno, o qual é o responsável pela sustentação e pela força da cicatriz (Tazima *et al.*, 2008). A epitelização, recobrimento da ferida por novo epitélio, consiste na separação, migração, proliferação, diferenciação e estratificação de queratinócito no local da ferida, estes, provenientes da camada basal da epiderme residual ou da profundidade de apêndices dérmicos (Harper *et al.*, 2014; Mendonça e Coutinho-Netto, 2009).

Fase 4 - Remodelamento de tecido: O estágio final da cicatrização envolve a tentativa de recuperar a estrutura tecidual normal (Harper *et al.*, 2014). Esta fase é marcada pela maturação dos elementos e alterações na matriz extracelular, ocorrendo o depósito de proteinoglicanos e de colágeno (Monsuur *et al.*, 2016; Mendonça e Coutinho-Netto, 2009). Nesta fase, há um equilíbrio entre a síntese e a degradação de colágeno, pela colagenase, e de outras proteínas depositadas na lesão. A perda de equilíbrio dessa relação leva ao aparecimento de cicatrizes

hipertróficas e queloides (Harper *et al.*, 2014; Tazima *et al.*, 2008; Monsuur *et al.* 2016; Mendonça e Coutinho-Netto, 2009).

Numerosos ensaios *in vitro* e *in* vivo têm sido propostos para avaliação de processos de cicatrização de feridas em mamíferos (van den Broek *et al,*. 2014; Schmidt *et al.*, 2009; Ambrosone *et al.*, 2014). O fechamento de feridas que ocorre nos seres humanos são difíceis de estudar em modelos animais, devido às diferenças na fisiologia da pele (Monsuur *et al.*, 2016). Portanto, a busca por novas alternativas para investigar a mobilidade celular e o fechamento da ferida se fazem necessários.

1.2.1. Teste de migração (*Scratch Test*)

O teste de migração (*Scratch Test*) é considerado uma ferramenta conveniente para estimar o potencial de cicatrização de feridas, por exemplo, de diferentes extratos de plantas (Fronza *et al.*, 2009; Zubair *et al.*, 2012; Liang *et al.*, 2007).

O *Scratch Test* é um ensaio fácil, de baixo custo, que tem o potencial de avaliar vários fatores envolvidos no processo de cicatrização, incluindo a formação de um novo tecido, migração e proliferação celular *in vitro* (Fronza *et al.*, 2009; Zubair *et al.*, 2012). O teste se baseia na formação de uma fenda em monocamada confluente de células, e após os tratamentos, a realização da captura de imagens em diferentes tempos até o reestabelecimento do contato célula-célula (Liang *et al.*, 2007).

1.3. Inflamação

A inflamação é uma homeostasia corporal em resposta a qualquer tipo de lesão no tecido. É um fenômeno complexo que envolve resposta imune, inata e adaptativa, na área lesada (Hussan *et al.*, 2015). Pode ser de intensidade variável com ações locais, regionais ou sistêmicas, onde há o desencadeamento de uma

cascata de eventos após um estímulo ou agressão. Como consequência, o aumento do calibre e da permeabilidade vascular, recrutamento de leucócitos, estimulação do sistema complemento, ativação de macrófagos e neutrófilos e produção de citocinas (Bak *et al.*, 2013; Hussan *et al.*, 2015;Calixto *et al.*, 2004).

A resposta no início de um processo inflamatório requer a ativação de sinalizadores primários, como macrófagos residentes e mastócitos teciduais. Aos serem ativadas essas células liberam uma grande quantidade de mediadores inflamatórios como, citocinas, quimiocinas, mediadores lipídicos e aminas vasoativas (Medzhitov, 2008; Lawrence e Gilroy, 2007). Quando acionados, esses mediadores irão desencadear a ativação e atração de células inflamatórias iniciando a formação de edema (Lawrence e Gilroy, 2007).

A inflamação aguda é de curta duração no início, podendo persistir por horas ou dias. Este tipo de inflamação é dependente da liberação de mediadores químicos, os quais provocam a formação de edema como um resultado do extravasamento de líquido e de proteínas da microcirculação local e acúmulode leucócitos polimorfonucleares no local da inflamação (Ialenti *et al.*, 1992). Se a resposta à inflamação aguda falhar, ela pode evoluir para uma inflamação crônica (Ialenti *et al.*, 1992; Kumar *et al.*, 2005. Por sua vez, a inflamação crônica, se define como sendo de longa duração e está associada a presença de macrófagos e linfócitos, proliferação de vasos sanguíneos, fibrose e destruição tecidual. Pode estar associada com muitas doenças crônicas, como alergias, aterosclerose, câncer, artrite, doenças autoimunes, entre outros (Gabay, 2006; Pashkow, 2011; Khansari *et al.*, 2008).

A resposta inflamatória aguda envolve a entrega coordenada de componentes do sangue (leucócitos e plasma) para o local da lesão ou infecção (Ialenti *et al.*,

1992). Esta reposta é desencadeada pelos receptores do sistema imune inato, como receptores *Toll-like* (TLRs). Esse reconhecimento inicial da infecção é mediado por macrófagos e mastócitos, os quais conduzem a produção de uma variedade de mediadores inflamatórios, incluindo citocinas e quimiocinas (Medzhitov, 2008; Lawrence e Gilroy, 2007).

As citocinas representam um grupo de substâncias multifuncionais que estão envolvidas em muitas etapas da resposta inflamatória. Estas proteínas são coordenadoras da inflamação, capazes de modular as funções celulares, principalmente do sistema imune (Opal e DePalo, 2000; Dinarello, 2000). As citocinas podem ser classificas como pró- ou anti-inflamatórias, dependendo da maneira que influenciam na inflamação (Calixto *et al.*, 2004). Citocinas pró-inflamatórias, tais como IL-1, TNF-α, IFN-γ e IL-6, estão envolvidas na iniciação e amplificação do processo inflamatório, enquanto citocinas anti-inflamatórias, como a IL-10 e o TGF-β, estão envolvidas modulando negativamente esses eventos (Dinarello, 2000; Opal e DePalo, 2000).

Citocinas podem estar envolvidas, de forma importante, em ambas as inflamações, aguda e crônica (Luheshi *et al.*, 2009; Kumar *et al.*, 2005). De modo geral, as citocinas apresentam controle na ativação, divisão, apoptose e quimiotaxia celular, além de iniciarem e controlarem o processo de cicatrização, com estímulo da reepitelização, angiogênese e deposição de colágeno (Kumar *et al.*, 2005; Harper *et al.*, 2014).

A IL-6 é a principal estimuladora da produção de proteínas de fase aguda, secretada por macrófagos. Está relacionada ao efeito sistêmico da inflamação, podendo atuar como um mecanismo de defesa ou como mecanismo pró-inflamatório na fase crônica da inflamação (Kumar *et al.*, 2005; Gabay, 2006).

A IL-10 é a citocina anti-inflamatória mais importante, sendo secretada por monócitos, macrófagos e células T (Kumar *et al.*, 2005). Esta interleucina é um importante inibidor de citocinas e um potente inibidor da ativação de monócitos/ macrófagos estimulados por citocinas pró-inflamatórias (Opal *et al.*, 1998). É capaz de impedir a formação de óxido nítrico, síntese de prostaglandinas e a atividade de metaloproteinases. A IL-10 é capaz de inibir o efeito de agentes patogênicos intracelulares mediada por LPS por diminuição da produção de TNF-α e IFN-γ (Opal *et al.*, 1998; Opal e DePalo, 2000).

O TNF-α é produto de monócitos/macrófagos, células dendríticas, ou linfócitos, podendo ser estimulado por endotoxinas, produtos microbianos, entre outros estímulos inflamatórios (Kumar *et al.*, 2005). Os efeitos do TNF-α ocorrem após a sua ligação a receptores específicos localizados na membrana celular. Seus efeitos ocorrem sobre a fisiologia celular, onde ocorre a indução de mediadores inflamatórios como eicosanoides e óxido nítrico (Lewis *et al.*, 1991). Juntamente com a IL-1, é a grande responsável pelos efeitos sistêmicos da fase aguda da inflamação (Shen *et al.*, 2009; Kumar *et al.*, 2005; Dinarello, 2000).

A IL-1 é produzida por macrófagos e é controlada por um complexo multiproteico, chamado de inflamossoma, o qual responde a estímulos de microrganismos e decélulas mortas (Kumar *et al.*, 2005; Luheshi *et al.*, 2009)

Ademais, as ativações de TNF e IL-1 (em alguns casos IFN- γ) (Figura 3) são capazes de produzir efeitos locais no endotélio vascular, aumentando a expressão de moléculas de adesão endoteliais, as quais são essenciais para adesão de leucócitos à superfície endotelial antes da migração para o tecido, além de aumentar a atividade pró-coagulante, ativar neutrófilos e aumentar a produção de citocinas (Shen *et al.*, 2009; Luheshi *et al.*, 2009; Dinarello, 2000).

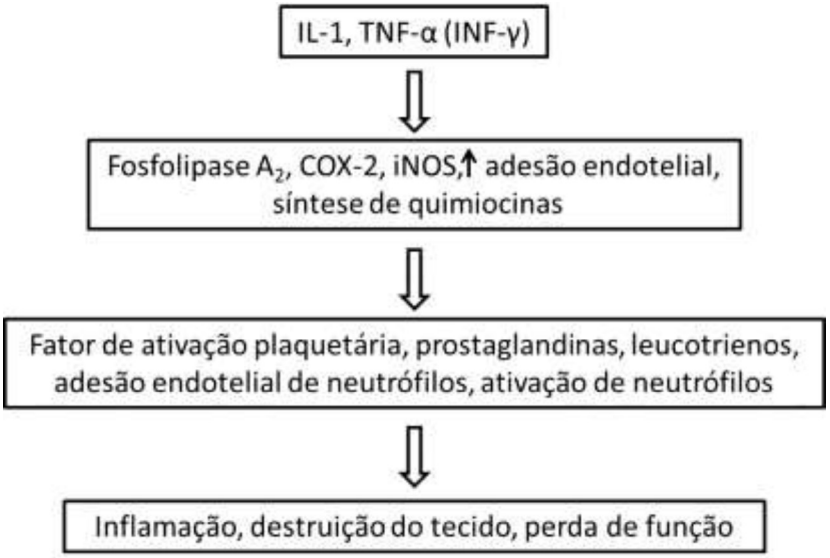

Figura 3- Cascata de inflamação gerada por IL-1 e TNF (Adaptado de Dinarello, 2000).

O INF- γ é secretado por linfócitos e células natural killer. Esta é a citocina responsável pela ativação de macrófagos, capazes de destruir os microrganismos indutores da infecção (Kumar *et al.*, 2005). O INF-γ é capaz de aumentar a atividade do TNF e induzir a produção de óxido nítrico (Calixto *et al.*, 2004).

As quimiocinas são pequenas proteínas que agem como quimioatrativos para tipos específicos de leucócitos (Kumar *et al.*, 2005). São produzidas em resposta ao estímulo inflamatório por macrófagos, células endoteliais, linfócitos T, mastócitos entre outros tipos celulares e são responsáveis pelo recrutamento de leucócitos para o local da inflamação. Também podem ser produzidas em tecidos para controlar a migração normal de células (Sallutto e Makay, 2004; Rossi and Zlotnik, 2000).

Quando ativados, os macrófagos têm como função eliminar o agente causador da inflamação e iniciar o processo de reparo da lesão (Kumar *et al.*, 2005). A ativação dos macrófagos aumenta os níveis de espécies reativas de oxigênio e nitrogênio (H_2O_2, O_2^-, OH^-, NO, $ONOO^-$), citocinas e quimiocinas, proteases,

metabólitos do ácido araquidônico e fatores de coagulação (Federico *et al.*, 2007; Mosser e Edwards, 2008).

O NF-κB é um fator de transcrição central da resposta inflamatória em células imunocompetentes, tanto periférica como em macrófagos, quanto no sistema nervoso central, como em células da glia, por exemplo (Kumar *et al.*, 2005). A atividade do NF-κB é induzida por citocinas pró-inflamatórias, e sua expressão induz a liberação dessas citocinas, formando assim uma retroalimentação positiva (Figura 4) (Bonizzi e Karin, 2004;Carmody and Chen, 2007). O IFN-γ é um importante fator de ativação de macrófagos, mas sozinho é ineficiente. Co-fatores como TNF-α ou LPS também são necessários para proporcionar um segundo sinal para ativação de NF-κB (Kumar *et al.*, 2005).

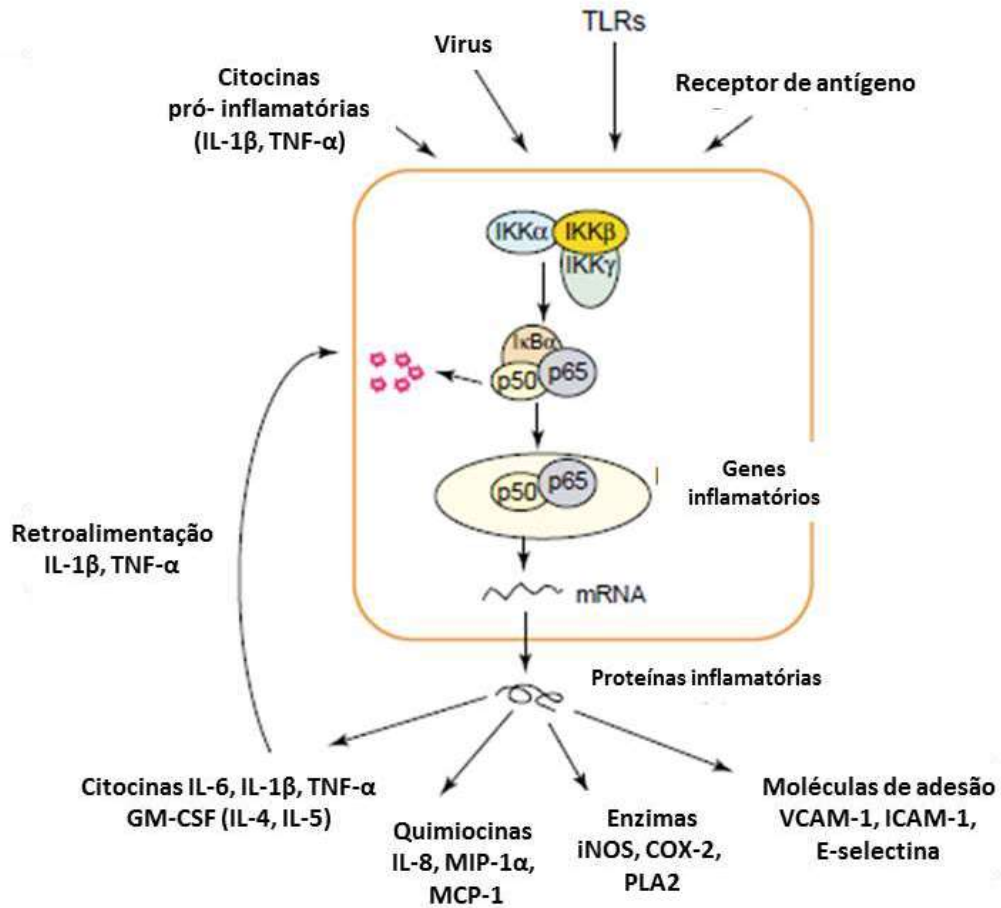

Figura 4- Via clássica de ativação de NF-κB (adaptado de Bonizzi e Karin, 2004).

1.3.1. Estresse oxidativo

A inflamação crônica causa diversos efeitos paralelos celulares, principalmente através da produção excessiva de radicais livres e esgotamento de antioxidantes. Radicais livres produzidos em excesso reagem com ácidos graxos da membrana celular e proteica, prejudicando sua funcionalidade, além disso, podem causar mutações e danos no DNA (Khansari *et al.*, 2009)

Radical livre é definido como qualquer espécie química que contenha elétrons desemparelhados no seu orbital de valência (Khansari *et al.*, 2009). Espécies reativas de oxigênio (EROs) são radicais derivados do oxigênio, que são produzidos a partir de substâncias endógenas e exógenas. As fontes endógenas de EROs incluem mitocôndrias, citocromo P450, peroxissomos e ativação de células inflamatórias (Khansari *et al.*, 2009; Inoue *et al.*, 2003). Durante o processo de transferência de elétrons na mitocôndria pela cadeia respiratória, são produzidos vários intermediários de curta duração, incluindo ânion superóxido ($O_2^{\bullet -}$), radical hidroxil (OH•) e peróxido de hidrogênio (H_2O_2), os quais são tóxicos para a célula (Federico *et al.*, 2007; Guarienti *et al.*,2010; Murray *et al.*, 2011; Roberts *et al.*, 2010).

Além de radicais derivados de oxigênio, existem os radicais derivados de cloro, como o ácido hipocloroso (HOCL) e de nitrogênio, chamados de espécies reativas de nitrogênio (ERN), tais como o peroxinitrito ($ONOO^-$) e óxido nítrico (NO•) (Guarienti *et al.*, 2010; Khansari *et al.*, 2009; Roberts *et al.*, 2010).

Na imunidade inata, os macrófagos desempenham um papel fundamental na eliminação de patógenos através da geração de espécies reativas de oxigênio, nitrogênio e cloro. A reação inflamatória persiste até que os patógenos sejam

eliminados e o processo de reparação do tecido seja concluído (Khansari *et al.*, 2009; Pashkow, 2011).

Na tentativa de manter a integridade dos sistemas biológicos aos danos provocados pelas EROs, os organismos possuem sistemas de defesa antioxidantes. As proteções antioxidantes podem ser enzimáticas como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX), e não enzimáticos, como a glutationa (GSH), vitaminas e compostos fenólicos (Guarienti *et al.*, 2010; Roberts *et al.*, 2010). Além disso, existem situações fisiológicas promotoras de estresse oxidativo, onde a suplementação com antioxidantes exógenos apresentam relevância para manter o equilíbrio pró e antioxidantes (Roberts *et al.*, 2010; Pashkow, 2011).

Dentre as diferentes formas de desencadear um processo inflamatório, que vão desde agentes patogênicos e componentes estruturais de microrganismos a proteínas ou sinais endógenos de danos no tecido, se destaca o modelo utilizando LPS, o qual já está bem caracterizado e elucidado (Hanisch e Ketternmann, 2007).

1.3.2. Modelo de indução de inflamação por LPS

O LPS é o principal componente da membrana externa de bactérias gram negativas e um potente ativador da resposta da imunidade inata contra hospedeiros. Sua estrutura é composta por uma camada lipídica e duas camadas de açúcar, um lado hidrofóbico e outro hidrofílico, respectivamente. A porção hidrofóbica é considerada pela maior ação antigênica causada pelo LPS (Raetz e Whitfield, 2008). Após a estimulação com LPS, fagócitos monocíticos, como a microglia, expressam uma variedade de mediadores pró-inflamatórios, incluindo citocinas, quimiocinas e EROs, que orquestram a inflamação e a ativação da imunidade adaptativa (Le *et al.*, 2004; Zhang *et al.*,2012).

A micróglia é um monócito residente no sistema nervoso central, que desempenha um papel crítico na infecção, inflamação, isquemia e neurodegeneração no sistema nervoso central (Le *et al.*, 2004). Essa célula representa a primeira linha de defesa do cérebro, removendo agentes infecciosos e células danificadas (Qin *et al.*, 2007).

A microglia quando ativada por LPS, passando de estado de repouso para estado reativo, induz a liberação de citocinas pró-inflamatórias, como o TNF-α, IL-1β e IL-6, proteína quimiotática de monócito tipo 1 (MCP-1), ERO e ERN (Qin *et al.*, 2007; Lee *et al.*, 2013).

O LPS é reconhecido pelo sistema imune inato em um mecanismo dependente da ativação do receptor do *toll-like* 4 (TLR4). (Guha e Mackman, 2000). A ativação dos receptores TLR4 pode desencadear ativação de diferentes vias de sinalização, como a MAPK (proteina quinase ativada por mitógeno), AP-1 (proteína ativadora 1), IRF3 (fator regulador de Interferon 3) entre outras. Uma das principais vias que são ativadas pelo LPS é a via do NF-κB, que ao se translocar para o núcleo, promove a regulação transcricional de genes promotores de citocinas inflamatórias e de diversas enzimas responsáveis pela geração de espécies reativas (Figura 5) (Bak *et al.*, 2013; Guha e Mackman 2000; Zhang *et al.*, 2012; Lijia *et al.*, 2012).

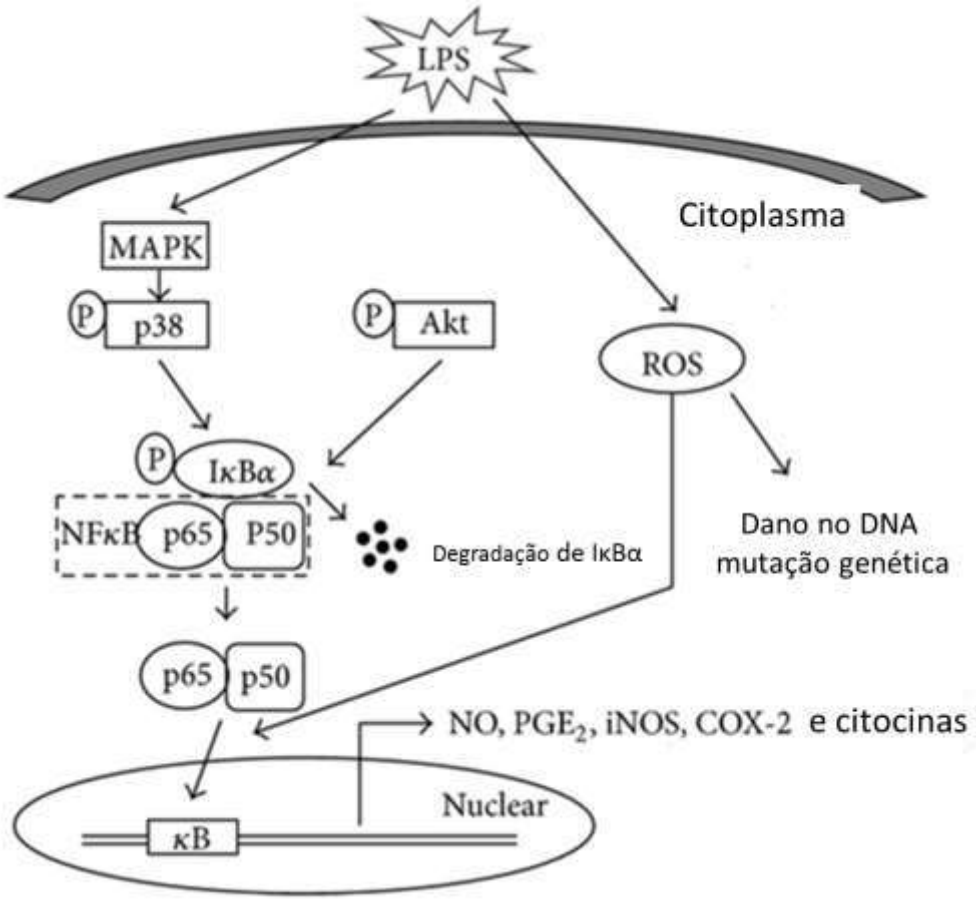

Figura 5- Diagrama esquemático ilustrando as vias de sinalização envolvidas nos efeitos anti-inflamatórios induzidas por LPS (adaptado de Bak *et al.*, 2013).

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2. Objetivos

Avaliar as atividades cicatrizante e anti-inflamatória de um extrato hidroetanólico padronizado das folhas de *Plantago australis* e de seu marcador analítico verbascosídeo em modelos *in vitro*.

a) Produzir um extrato hidroetanólico das folhas de *P. australis* padronizado no composto verbascosídeo.

b) Avaliar a viabilidade celular após tratamento com o extrato hidroetanólico de *P. australis* e de seu composto verbascosídeo em células HaCaT (queratinócitos humanos), usando o ensaio de azul de Tripan.

c) Determinar a atividade cicatrizante do extrato hidroetanólico de *P. australis* e do verbascosídeo em células HaCaT, usando o ensaio de migração celular (*Scratch Test*).

d) Verificar o potencial inflamatório do extrato hidroetanólico de *P. australis* e do verbascosídeo em células de microglia (células N9) pela quantificação dos mediadores inflamatórios IL-6, IL-10, IL-12p70, INFγ, MCP-1 e TNFα.

e) Verificar o efeito do extrato hidroetanólico padronizado de *P. australis* e do verbascosídeo em células N9 de induzir as enzimas antioxidantes superóxido dismutase e catalase.

f) Avaliar a capacidade do extrato hidroetanólico de *P. australis* e do verbascosídeo de induzir alterações no potencial de membrana mitocondrial em células N9.

g) Avaliar os efeitos anti-inflamatórios do extrato hidroetanólico de *P. australis* e do verbascosídeo, em células N9, utilizando o modelo de indução de inflamação com LPS e quantificação de mediadores inflamatórios (IL-6, IL-10, IL-12p70, INFγ, MCP-1 e TNFα).

h) Determinar a atividade antioxidante do extrato hidroetanólico de *P. australis* e do verbascosídeo em células N9, utilizando o modelo de inflamação induzido por LPS, pela quantificação da atividade das enzimas antioxidantes superóxido dismutase e catalase.

i) Determinar a capacidade do extrato hidroetanólico de *P. australis* e do verbascosídeo, utilizando o modelo de inflamação por LPS, de prevenir alterações no potencial de membrana mitocondrial em células N9.

3. Artigo científico redigido em inglês

CAPÍTULO I

Plantago australis (Kunth) Rahn hydroethanolic extract and its constituent verbascoside induce anti-inflammatory and healing activity

Artigo a ser submetido a Journal of Ethnopharmacology

Plantago australis (Kunth) Rahn hydroethanolic extract and its constituent verbascoside induce anti-inflammatory and healing activity

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Abstract

Ethnopharmacological relevance: Plantago australis (Kunth) Rahn is a popular plant widely spread in the Latin America. In folk medicine seeds and leaves are used mainly as extract as anti-inflammatory, diuretic, analgesic, depurative, etc. The verbascoside, a phenolic glycoside derivative of caffeic acid, is a active chemical component described for this specie.

Aims of the study: The aim of the present study was to evaluate the anti-inflammatory and would healing activities of a *Plantago australis* (Kunth) Rahn hydroethanolic extract of leaves (PAHE) and its constituent verbascoside.

Materials and methods: For the wound healing activity we used a Scratch Test, an assay capable to evaluate the ability of migration of keratinocytes cells (HaCat). Inflammation was induced with LPS, a compound of gram negative bacteria membrane, capable of activating the inflammatory response in microglial murine cells (N9). Inflammatory mediators (IL-6, IL-10, IL-12p70, INFγ, MCP-1 and TNFα) were quantificated and the activity of superoxide dismutase (SOD), catalase (CAT), and mitochondrial membrane potential were evaluated.

Results: In the scratch assay, the PAHE and verbascoside, induced a significant increase in migration of keratinocytes, at all concentrations tested when compared to the negative control. The treatments with PAHE and verbascoside induce increased of antioxidants enzymes, suggesting a possible activation of these enzymes, however, did not increased expression of inflammatory mediators in microglial cells. In LPS activated cells the verbascoside showed a significant reduction of TNFα, IL-6, IL-12p70, MCP-1 and INFγ, while the PAHE only present statistically significant reduction in TNFα. Interestingly, both the compounds were able to reduce oxidative parameters in N9 cells activated by LPS.

Conclusion: The results suggest that PAHE and verbascoside have wound healing activity, improving cells migration. In addition, this study suggests that the verbascoside in LPS-activated cells was capable to inhibit inflammation mediators, and both of compounds were able to reverse the oxidation effect.

Key words: *Plantago australis*, verbascoside, inflammation, wound healing, hydroethanolic extract.

1. Introduction

Medicinal plants have been used for thousands of years. The large variety of active compounds from plants it has significant value in several research lines, allowing the development of new drugs (Van Wyk, 2015; Foglio et al., 2006). Brazil is known worldwide for its plant genetic biodiversity, having an exuberant flora possessing about 20-22% of all existing plants (Calixto, 2005). The World Health Organization (WHO) estimates that 80% of the world population uses medicinal plants as the main resource of primary health care (Wangchuk et al., 2011; Karthshwaran and Mirunalini, 2010).

Plantago australis (Kunth) Rahn is a perennial herb that belongs to the Plantaginaceae family, popularly known as “tansagem” ou “transagem” (Hefler et al., 2011). It is widely spread in the Latin America with significant distribution in southern and southeastern regions of Brazil (Rocha et al., 2002; Rahn, 1996). This plant is a specie largely used in folk medicine as anti-inflammatory, diuretic, analgesic, anti-diarrheal, gastric ulcer, depurative, etc (Vendruscolo et al., 2005; Vendruscolo and Mentz, 2006; Trojan-Rodrigues et al., 2012; Kujawska and Pieroni, 2015).

Studies on the phytochemical composition demonstrated that *P. australis* has a wide variety of secondary metabolites that exhibit biological activity. Among such, the presence of iridoid glucosides such as aucubin, and phenolic compounds such as verbascoside, isoverbascoside, and salidroside in a hydroethanolic plant extract (Rønsted et al., 2000).

Verbascoside is a phenolic glycoside compound, also known as acteoside. Structurally this compound is characterized by four chemical groups: a caffeic acid, 4,5-hydroxyphenylethanol linked to a β-(D)-glucopyranoside, rhamnose grouping and one glucose molecule (Korkina et al., 2007). This compound is known to have various pharmacological activities, such as antioxidant, anti-inflammatory, ant nociceptive, and proliferative, anti ulcerogenic, etc (Schapoval et al., 1998; Lee et al., 2007; Hausmann et al., 2007).

Studies on pharmacological activities of *P. australis* are still scarce, so the present study aims to investigate the wound healing and anti-inflammatory activities of *P. australis* hydroethanolic extract (PAHE) and its constituent verbascoside, in two mammalian cell models, keratinocyte and microglial cells.

2. Material and Methods

2.1 Chemical

DMEM (Dulbecco's modified Eagle medium), RPMI (Roswell Park Memorial Institute), FBS (fetal bovine serum), trypsin–EDTA, L-glutamine, penicillin/streptomycin and TB (trypan blue) were obtained from Gibco-BRL (Grand Island, NY, USA). LPS, hydrogen peroxide (H_2O_2) and catalase were purchased from Sigma (St. Louis, MO, USA). Mouse Inflammatory Cytokine was obtained from BD Biosciences (San Diego, CA, USA). Verbascoside used in the analytical and biological assays was also acquired from Sigma (St. Louis, MO, USA).

2.2 Plant material and extraction

The leaves of *P. australis* (Kunth) Rahn were collected Santa Cruz do Sul city, Rio Grande do Sul, Brazil, in a delimited area with coordinates 29°41'46.8"S 52°26'27.4"W, in October of 2014. The plant was identified by the specialist botanic M.Sc. Gustavo Hassemer (Natural History Museum of Denmark, University of Copenhagen, Denmark), and the voucher specimen (ICN: 179648) was deposited in the herbarium of Federal University of Rio Grande do Sul (UFRGS), Brazil. The leaves were dried in a greenhouse (renewal of air) at 40°C, manually triturated and submitted to a sieve analysis (Bertel, Brazil). The hydroethanolic extract were prepared using 395.0 g of leaves and 3950 mL of hydroethanolic solution (30% water and 70% ethanol) and sonicated in an ultrasonic bath (Unique Group, model USC 2880A, Brazil) at 40 KHz for 40 min at 25°C. The extract was paper-filtered and the solvent was evaporated by a rotary evaporator at low temperature (< 40°C) under vacuum, until dried. The mass yield (y%) was calculated and the concentrated extract was stored at – 20°C until further use.

2.3 High performance liquid chromatography analysis

High performance liquid chromatography (HPLC) was developed by Shimadzu Prominence (Japan) equipped with a quaternary, low-pressure mixing pump and inline vacuum degassing, controlled by a CBM-20A interface module, an automatic injector (SIL-20A) and Diode Array Detector (SPD-M20A). HPLC conditions were made according Li et al., 2005, with some modifications. The separation was carried out by a gradient system, using a reverse-phase Phenomenex Luna 5mm C18 (2) (250 x 4.0 mm^2) column maintained at 30°C. The mobile phase consisted of 2% (v/v)

acetic acid solution (solvent A) and acetonitrile (solvent B). The composition gradient used was: 2% (B) to 25% (B) in 40 min, 25% (B) to 100% (B) in 5 min, 100% (B) to 2% (B) in 5 min. The injection volume was 10 µL and the samples were monitored at 320 nm. The verbascoside was identified by comparing the retention times of samples and authentic standard (Sigma-Aldrich, USA). The compound was analyzed qualitatively and whenever possible the quantification was performed and expressed in percentage, correlating the area of the analyte in sample with the area of the verbascoside standard.

2.4 Cells culture and treatments

Immortalized human skin keratinocyte cell (HaCaT) was obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). N9 murine microglial cell line was kindly provided by Dr. Teresa Faria Pais (University of Lisbon, Portugal). The HaCat cell cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. N9 cell was cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in air and were harvested by treatment with 0.15% trypsin–0.08% EDTA in PBS. The HaCaT cells were seeded ($2x10^5$ cells/mL) in complete media and grown for two day prior to treatment with PAHE and verbascoside before evaluations by trypan blue and scratch assay. While, N9 cells were seed ($1x10^3$ cells/mL) in complete media and grow overnight to treatment with the PAHE and verbascoside before evaluations of inflammatory mediators, anti-oxidative enzymes activity and mitochondrial function in a lipopolysaccharide (LPS) stimulation model.

2.5 Trypan blue exclusion assay

The effects of *P. australis* extract and verbascoside on HaCaT cell viability were determined using a trypan blue exclusion assay, as previously described by Uliasz and Hewett (2000). Cells were incubated with PAHE (2.5 to 2000 µg/mL) or verbascoside (2.5 to 100 µg/mL) for 24 h, stained with trypan blue (0.4%), and then viable cells were counted using a Countess® Automated Cell Counter (Invitrogen, USA). The viability was calculated as percentage of negative control.

2.6 Scratch Assay

The cell-based scratch assay has been previously described (Sung et al. 2012). In brief, HaCaT cells were cultured in a 6-well culture plate for 24 h up to 90%-100% confluences of the base was filled. Scratched wound lines on the upside of cultured cells were created by 200 µL yellow micropipette tip. The scratched cells were washed with PBS after removal of culture media, followed by exposure to four concentration (2.5, to 25 µg/mL) of PAHE or verbascoside. The fresh DMEM serum-free medium was used as negative control and fresh medium with SFB 10% as used as positive control. The images were taken immediately (0 h) and after 4, 8, 12, 24 and 48 h. The scratched areas were measured using the software ImageJ® at five different sites from each wound area of gaps. Five different sites of wound area were measured and averaged from five independent experiments. The healing was plotted *versus* time after scratch.

2.7 Lipopolysaccharide (LPS) inflammation model

For the induction of microglial activation, LPS, a bacterial endotoxin and a generally accepted inducer of pro-inflammatory properties was used. For stimulation experiments the experimental groups were moreover pre-incubated with five differents concentrations of PAHE or verbascoside for 24h (2.5 to 50 µg/mL) and LPS was added for a further 4h (2.5 µg/mL).

2.8 Inflammatory mediator's determination

The BD CBA Mouse Inflammation Kit was used to quantitatively measure Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12p70 (IL-12p70), Interferon-γ (IFN-γ), Monocyte Chemoattractant Protein-1 (MCP-1) and Tumor Necrosis Factor (TNF), and protein levels in tissue suspension. Briefly, 50 µL of samples (cell extract) or known concentrations of standard samples (0–5000 pg/mL) were added to a mixture of 50 µL each of capture antibody bead reagent and phycoerythrin (PE)-conjugated detection antibody. The mixture was then incubated for 2 h at room temperature in the dark and then washed to remove unbound detection antibody. Data were acquired using a FACS Aria III flow cytometer (BD Biosciences) and analyzed using FCAP Array v2.0 software (BD Biosciences).

2.9 Superoxide dismutase (SOD) activity

The SOD activity was determined according to the method of Misra and Fridovich (1972), and the results were expressed as USOD/mg of protein. One unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenochrome formation in 50%.

2.10 Catalase (CAT) activity

The CAT activity was determined by the hydrogen peroxide (H_2O_2) decomposition rate, according to Aebi (1984).

2.11 Mitochondrial membrane potential assay

Mitochondrial membrane potential assay was executed conform manufacturer's recommendations with minor modifications. MitoStatus® TMRE (Tetramethylrhodamine ethyl ester) is a cationic and lipophilic fluorescent dye that is readily sequestered by active mitochondria. When mitochondria membrane is depolarized, the fluorescence shows diminished levels. An aliquot of suspension cell was incubated with MitoStatus® TMRE (57 nM) for 30 min at 37°C, protected from light. Then, samples were analyzed in FACs Calibur Cytometer. The Cell Quest software was used to calculate the median fluorescence. The MitoStatus fluorescence was measured using 488 nm excitation and 585/42 nm band pass emission filters. A total of 10,000 events were measured per sample and the percentage of mitochondrial depolarization was determined.

2.12 Statistical analysis

Data were analyzed with GraphPad Prism 5® and are showed as mean ± SD. The comparison among multiple groups was performed by one-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison test.

3. Results

3.1 Chemical analyses of *P. australis extract*

Analysis by HPLC confirmed the presence of verbascoside in PAHE. The chromatographic profile (fingerprint) obtained by HPLC of PAHE is shown in Fig. 1 by comparing its chromatogram with that obtained from the pure substance. The HPLC

analysis showed that verbascoside was the main constituent of PAHE, corresponding to 6.7467 ± 0.2445 %.

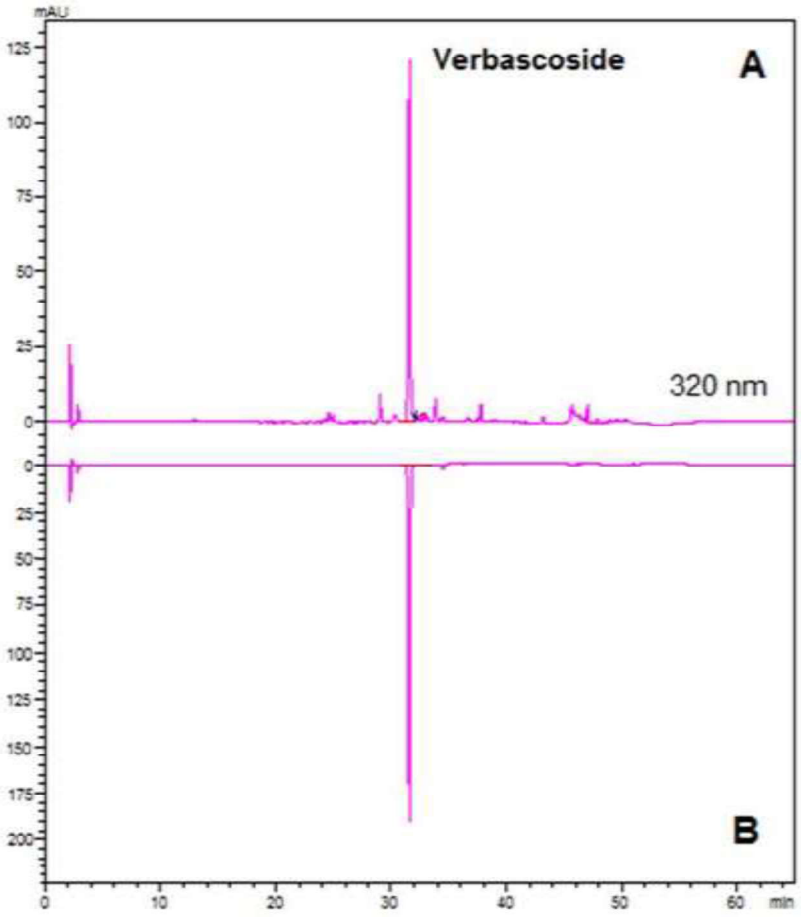

Figure 1. A: HPLC chromatographic of *P. australis* (Kunth) Rahn hydroethanolic extract. B: Chromatogram of verbascoside standard. The average retention time of verbascoside was 31.58 min.

3.2 Viability assay

HaCaT cells were treated with several concentrations of PAHE or verbascoside for 24 h. Our findings showed that PAHE decrease viability but, in a significant way, only in concentrations up to 1000 µg/mL (Figure 2), whereas verbascoside decrease viability in concentrations up to 100 µg/mL (Figure 3). The concentrations used in this preliminary assay were based on our previous results. In this manner, we chose non-cytotoxic PAHE and verbascoside concentrations

(ranging from 2.5 to 50 µg/mL) to follow experiments in order to verify the migration activity in the same cell line.

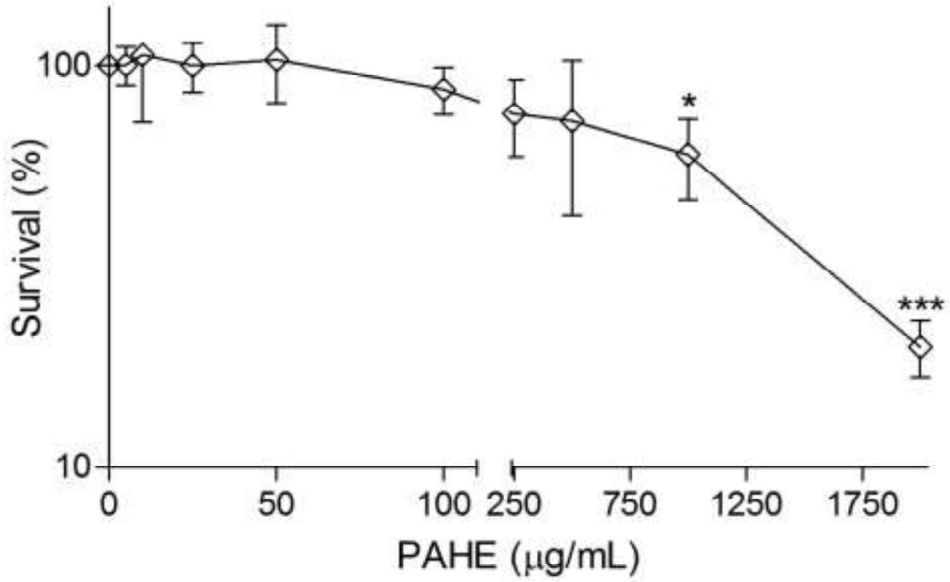

Figure 2. Cytotoxic effects of PAHE in HaCaT cells after 24 h exposure by trypan blue exclusion assay. Results are expressed as mean percentage in treated cells compared to control (solvent) ± standard error median of three independent experiments performed in triplicate. Data significant in relation to negative control group at *$p < 0.05$ and ***$p < 0.001$. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

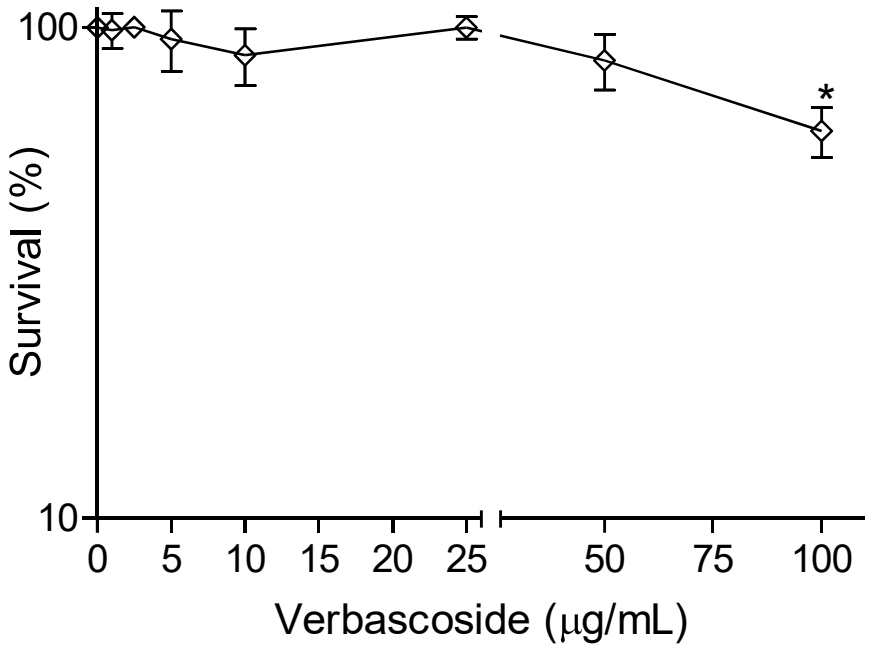

Figure 3. Cytotoxic effects of verbascoside in HaCaT cells after 24 h exposure by trypan blue exclusion assay. Results are expressed as mean percentage in treated cells compared to control (solvent) ± standard error median of three independent experiments performed in triplicate. Data significant in relation to negative control group at *$p < 0.05$. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

3.3 *In vitro* scratch-wound healing assay

We investigated the effect of PAHE and verbascoside concentrations on wound closure in HaCaT cell monolayers. It is evident that the scratch closure occurred at a faster rate in the presence of the different concentrations of PAHE and verbascoside compared to negative control (Figures 4 to 7). We derived the percent wound closure curves by averaging at least five fields from each five independent experiment. Whereas, PAHE induced narrowing of the scratch wound, verbascoside showed less effect. To PAHE, cells showed significantly higher spreading rates compare to negative control in a concentration dependent manner (Figure 4 and 5). At 48 h of culture, wound closure in the negative control was less than 30%, while it had already reached 81.06% in the presence of 25 μg/mL of PAHE (Figure 4). To verbascoside, actions were evident for all the test concentrations, with best results to

accelerates the spreading rate at 5 and 10 µg/mL, with rate of closure reached 58.7 and 57.77%, respectively (Figures 6 and 7).

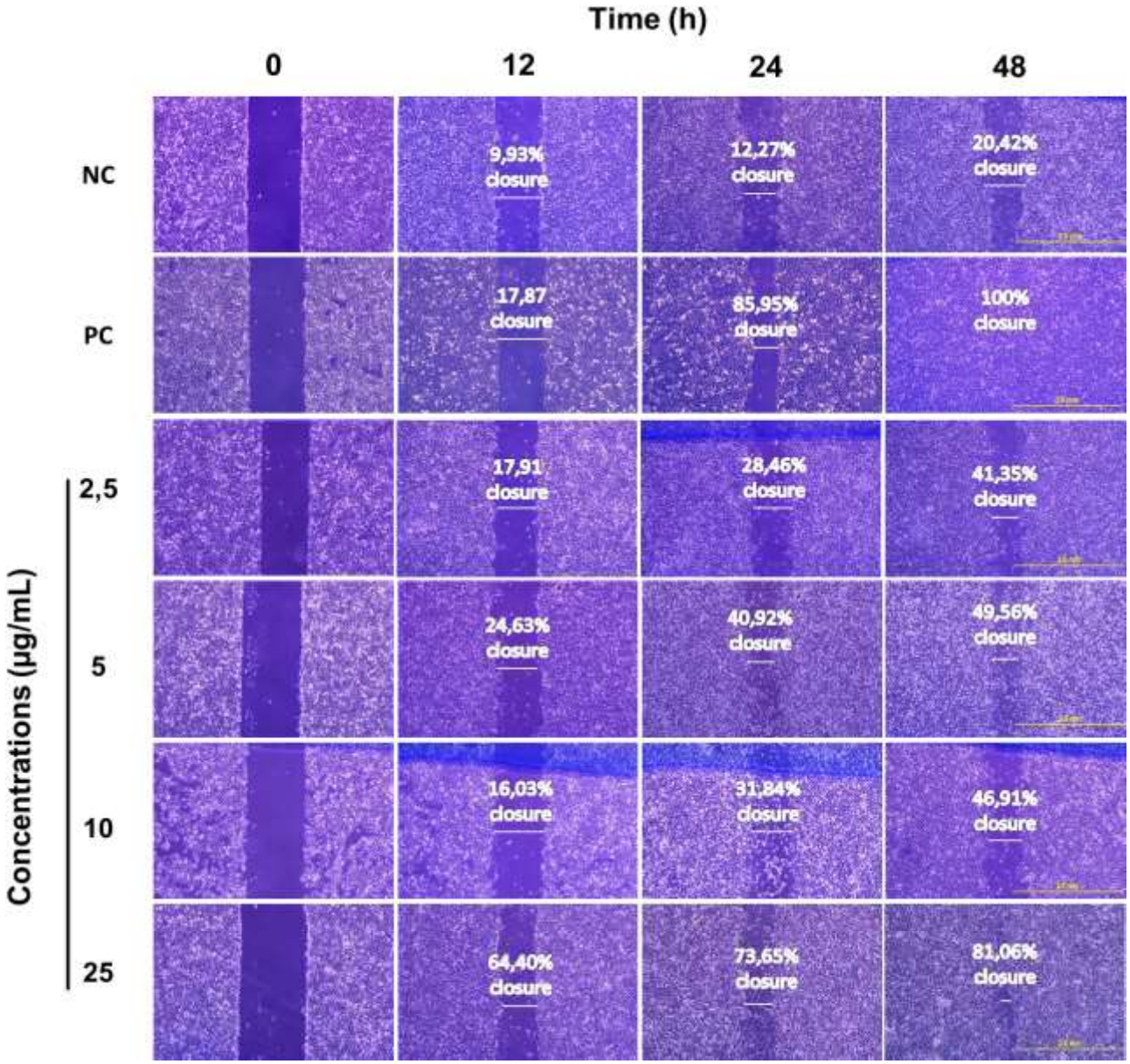

Figure 4. Image of migration test (scratch assay) in HaCaT cells over 48 h of exposure to PAHE. NC: negative control (DMEM FBS-free); PC: positive control (DMEM 10% FBS); PAHE: *Plantago australis* hydroethanolic extract. The measurements were performed considering the average of 5 independent measures per picture, considering time 0 of each treatment with 0%.

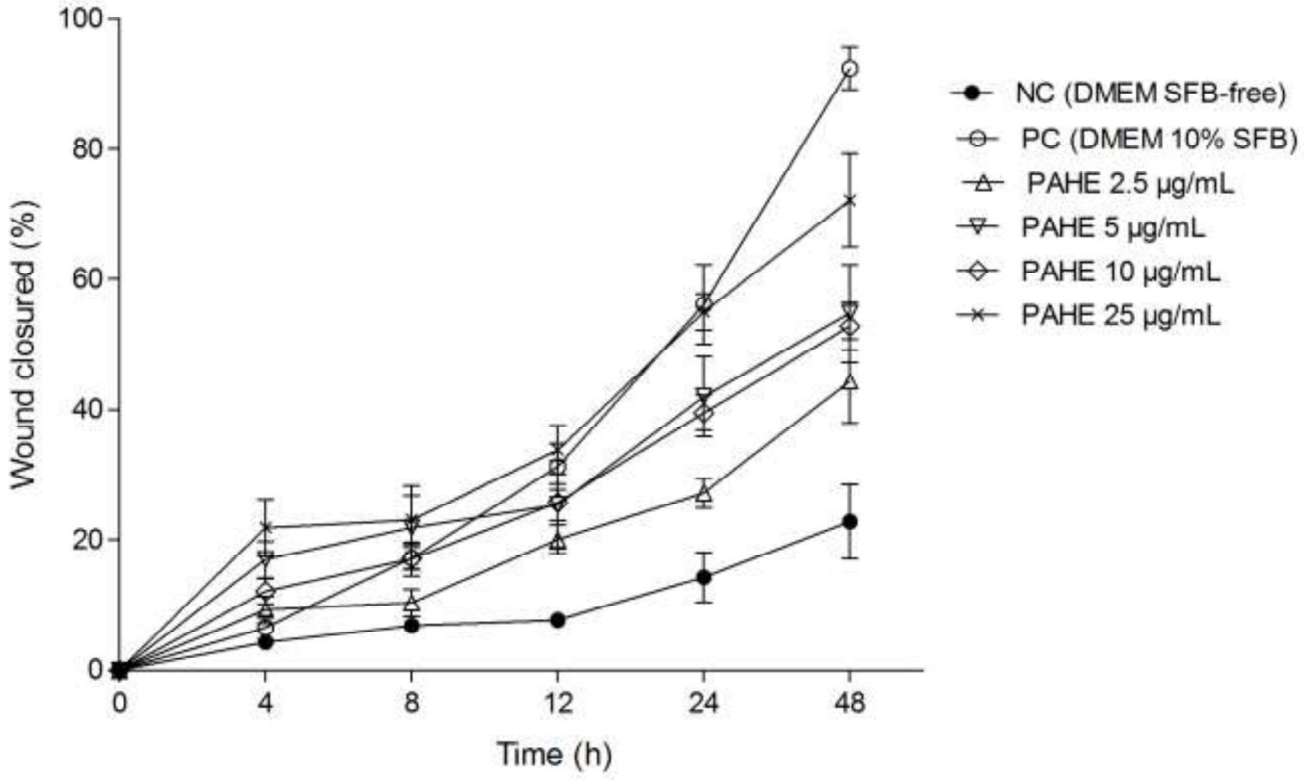

Figure 5. Measurement of HaCaT cell migration after treatment with PAHE Results are presented as mean and standard deviation of five independent experiments. Medium with 10% bovine fetal serum was used as positive control and medium without serum was used as negative control.

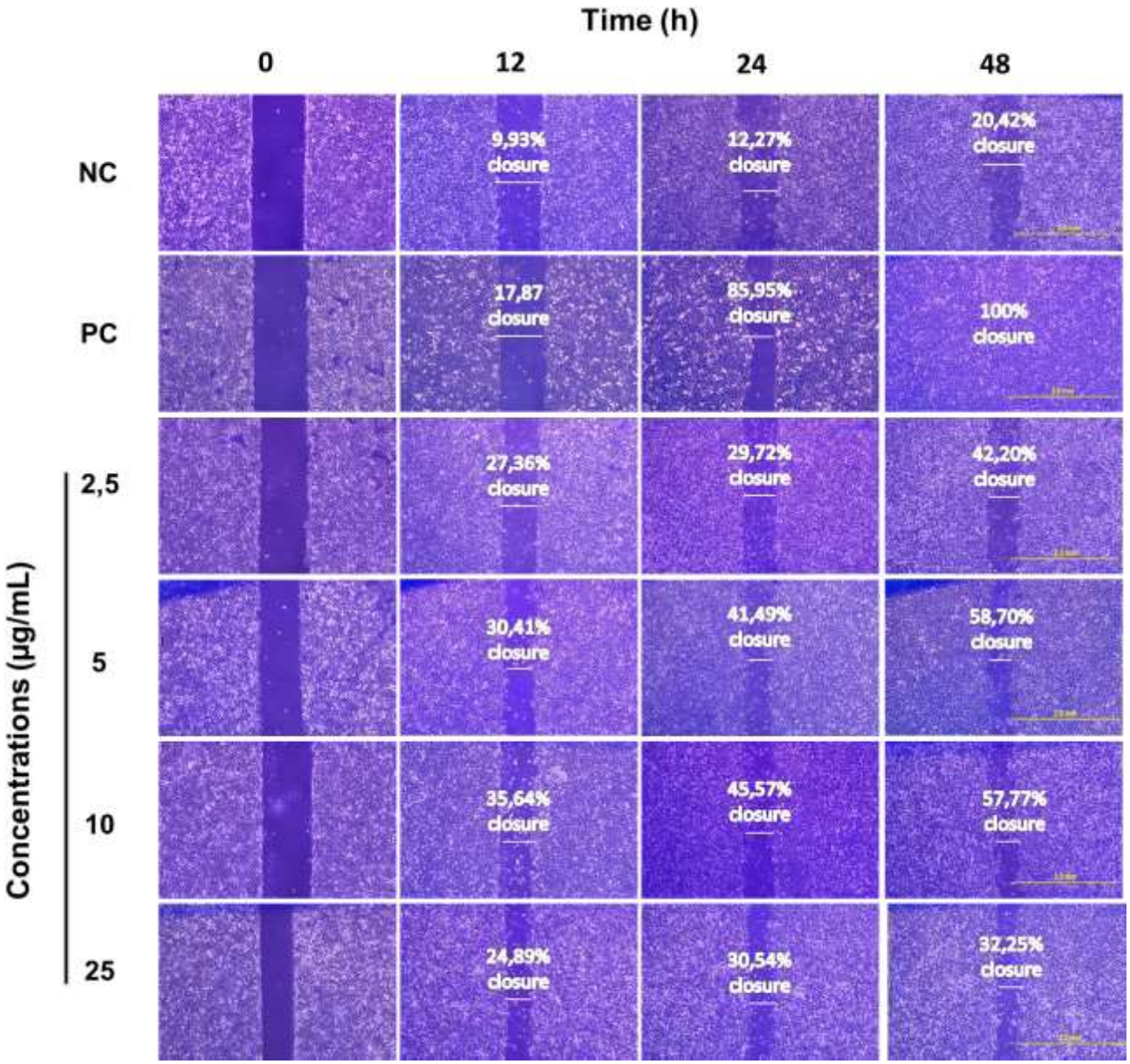

Figure 6. Image of migration test (scratch assay) in HaCaT cells over 48 h of exposure to verbascoside. Medium with 10% bovine fetal serum was used as positive control and medium without serum was used as negative control. The measurements were performed considering the average of 5 independent measures per picture, considering time 0 of each treatment with 0%.

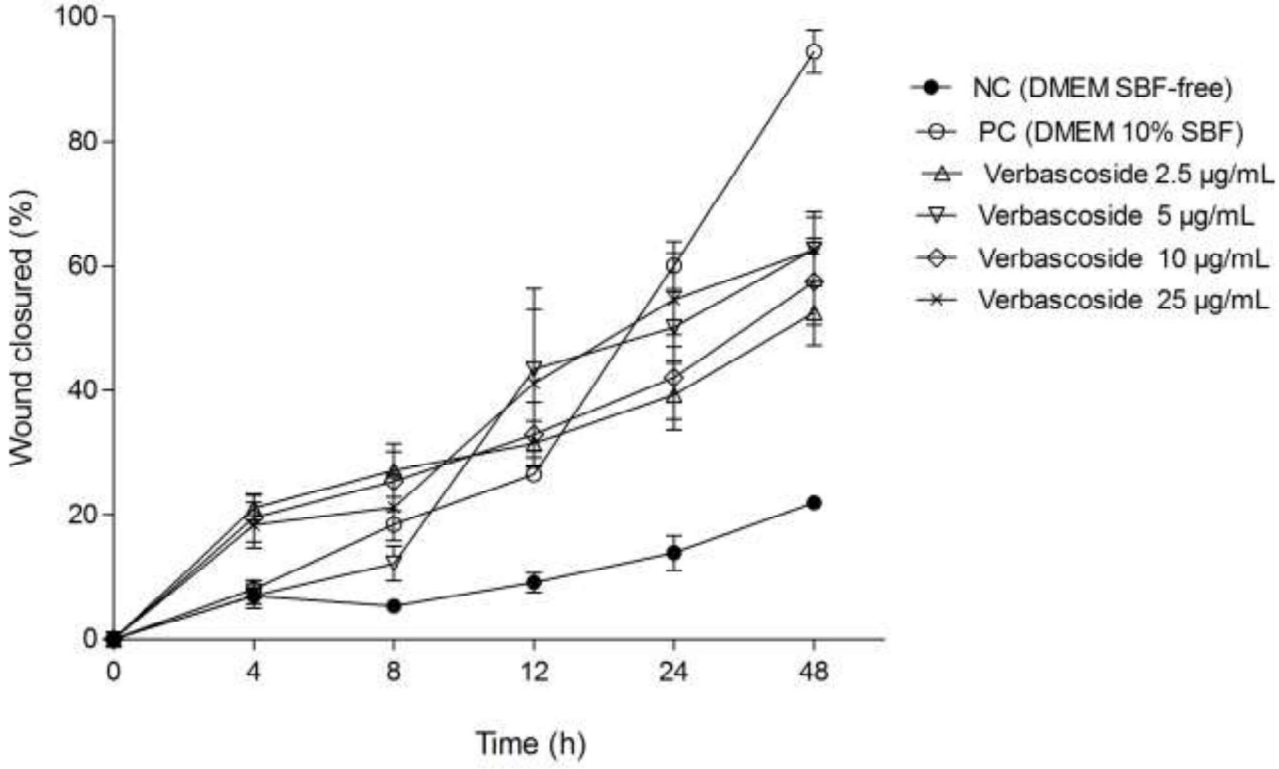

Figure 7. Measurement of HaCaT cell migration after treatment with verbascoside. Results are presented as mean and standard deviation of five independent experiments. Medium with 10% bovine fetal serum was used as positive control and medium without serum was used as negative control

3.4 Effect of HEPA and verbascoside on the production of inflammatory mediadors in LPS-stimulated N9 cells

We measured the production of IL-6, IL-10, IL-12p70, INFγ, MCP1 and TNFα in N9 cell using CBA kit. Treatment of N9 cells with PAHE or verbascoside did not increase the production of any of these inflammation mediators (Figures 8 and 9). LPS alone resulted in a significant increase in cytokine production compared to control group, with the exception of IL-10 (Figures 10 and 11). Interestingly, although the treatment with the PAHE induced a decrease of cytokines LPS-induced in any concentration tested this value was significant (Figure 10). However, treatment with verbascoside at 25 µg/mL considerably inhibited LPS induction of IL-6, IL-12p70, INF-ɣ, MCP-1 and TNFα (Figure 11).

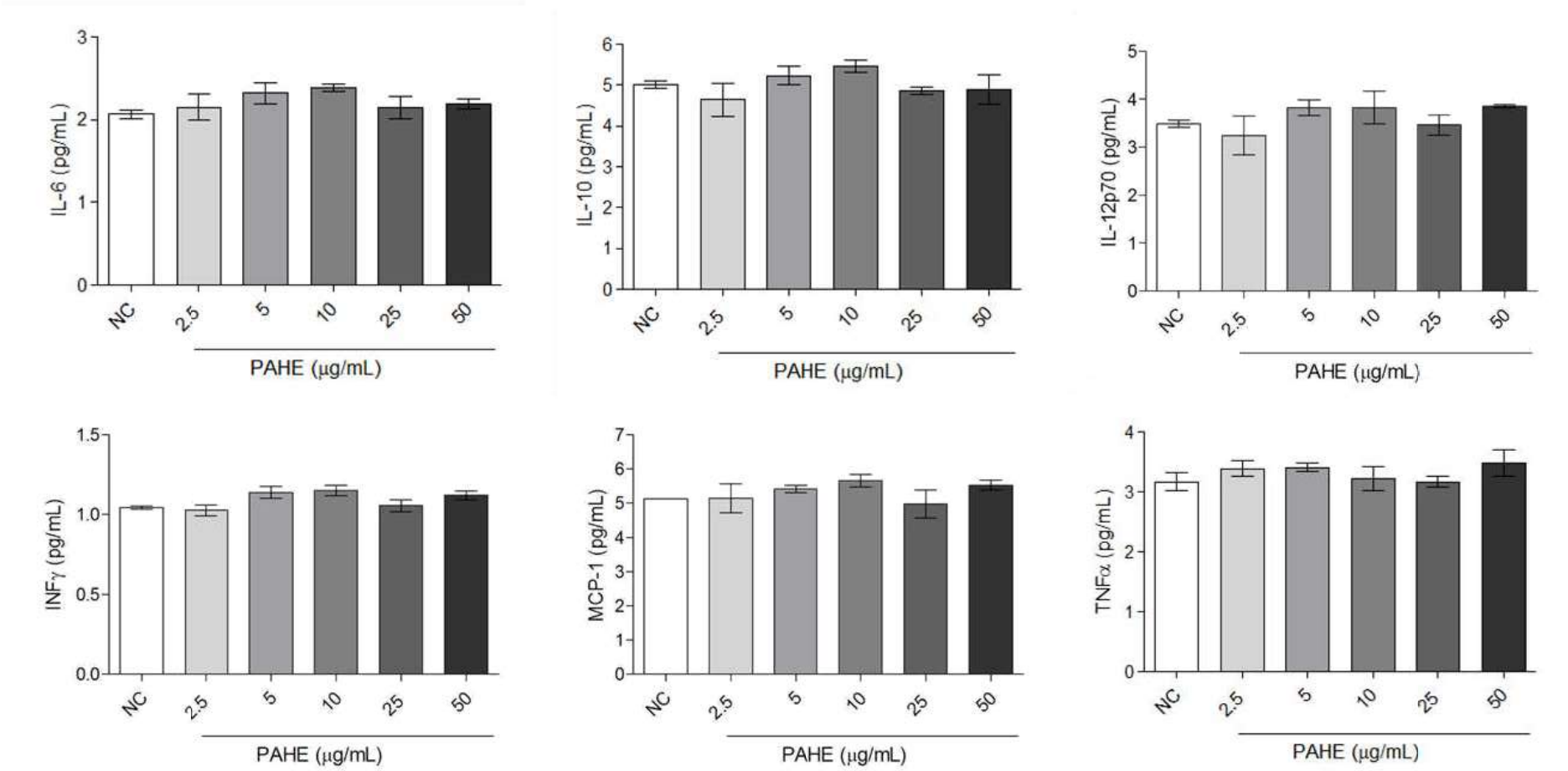

Figure 8. Measured of the inflammatory cytokines in N9 cells after treatment for 24 h with PAHE, using the CBA kit (BD Biosciences), followed by flow cytometry analysis. Results are presented as mean and standard error median of four independent experiments.

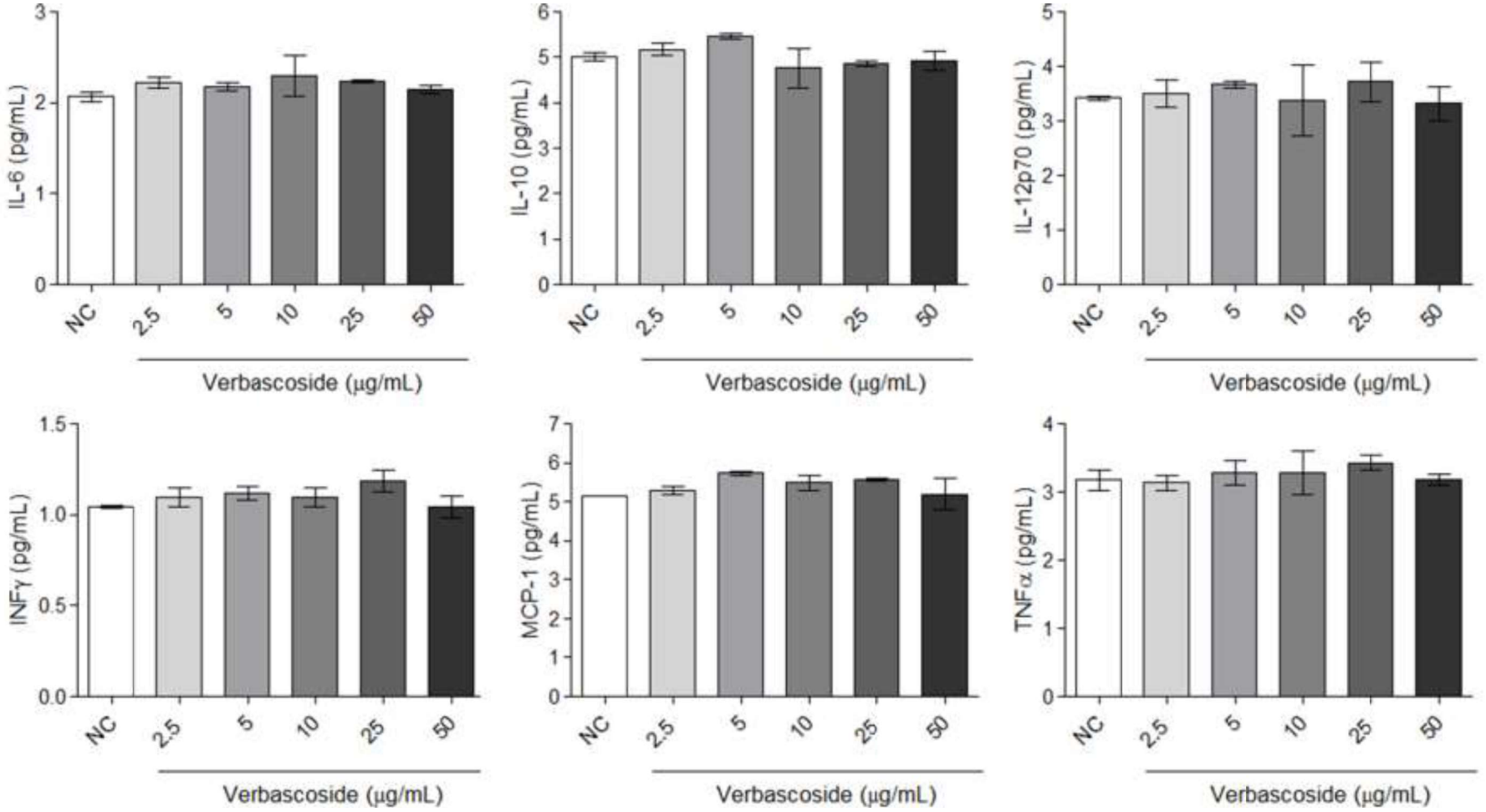

Figure 9. Measured of the inflammatory cytokines in N9 cells after treatment for 24 h with verbascoside, using the CBA kit (BD Biosciences), followed by flow cytometry analysis. Results are presented as mean and standard deviation of four independent experiments.

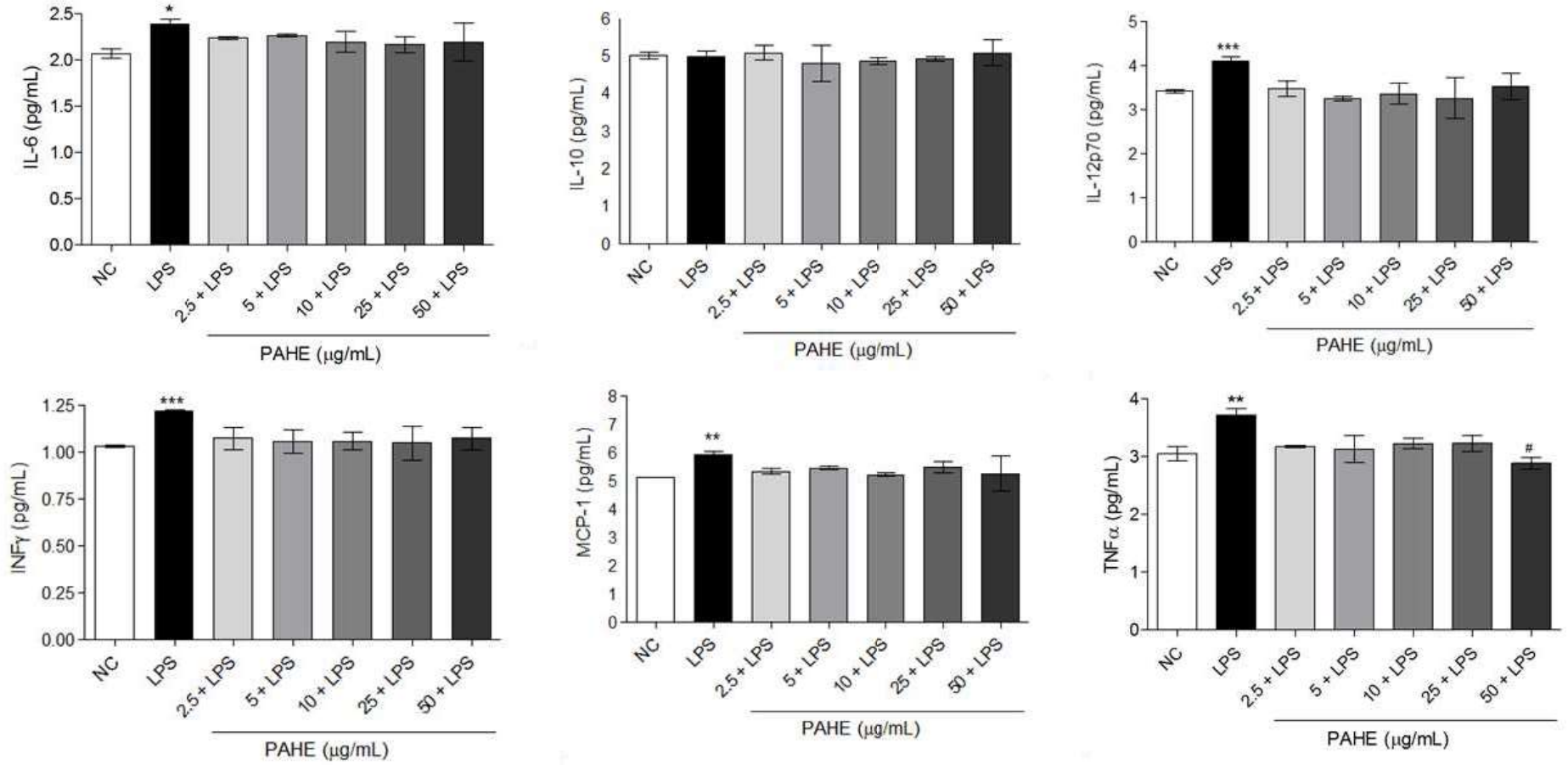

Figure 10. Measured of the inflammatory cytokines in N9 cells using a LPS-inflammation model after treatment for 24 h with PAHE plus 4h of LPS (2.5 µg/mL) , using the CBA kit (BD Biosciences), followed by flow cytometry analysis. Results are presented as mean and standard deviation of four independent experiments. Data significant in relation to negative control group at $*p < 0.05$, $**p$

< 0.01 and ***p < 0.001; $^{\#}p$ < 0.05 when pretreated cells were compared to cells exposed to LPS-challenge. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

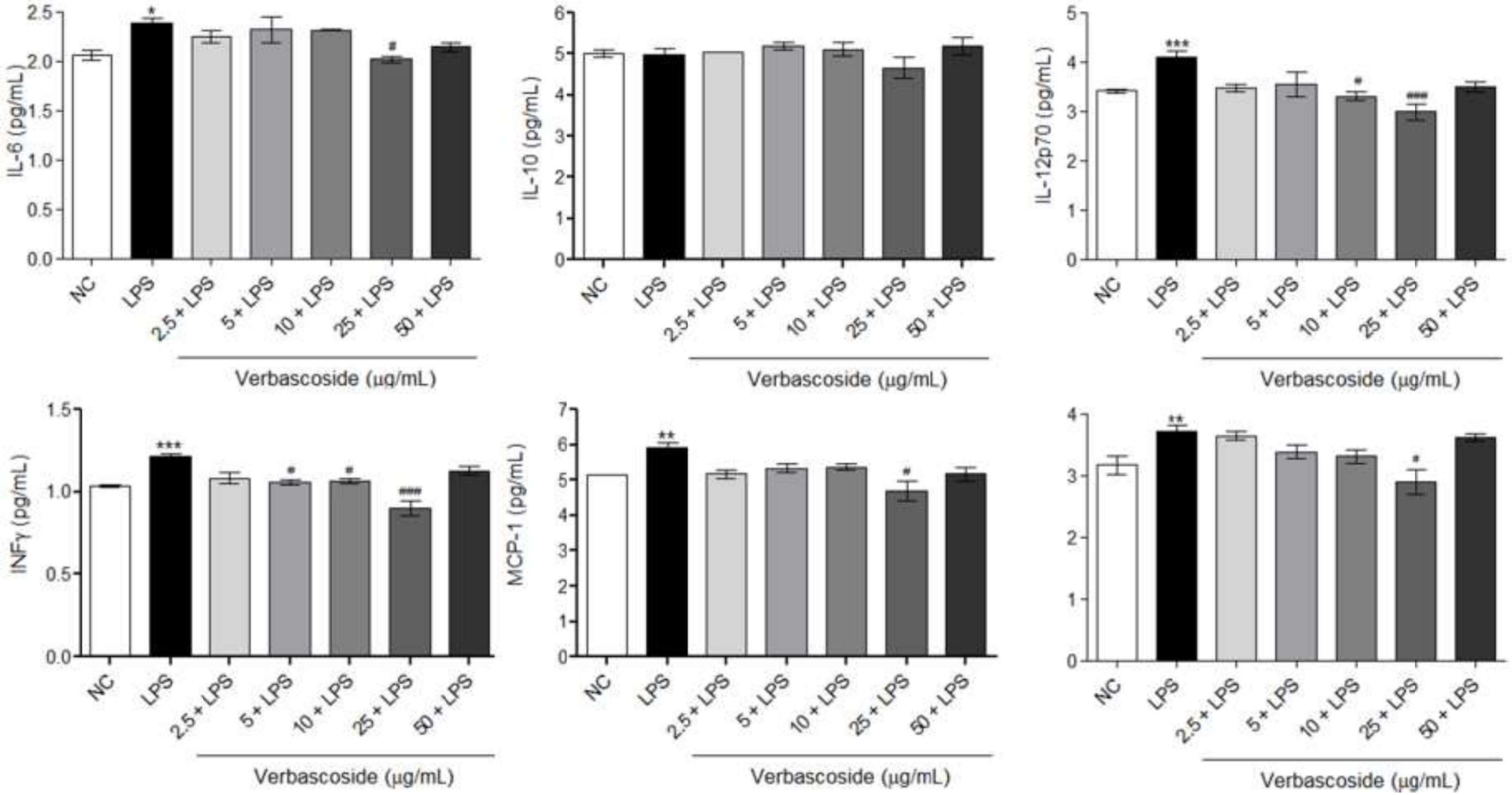

Figure 11. Measured of the inflammatory cytokines in N9 cells using a LPS-inflammation model after treatment for 24 h with verbascoside plus 4h of LPS (2.5 µg/mL), using the CBA kit (BD Biosciences), followed by flow cytometry analysis. Results are

presented as mean and standard error median of four independent experiments. Data significant in relation to negative control group at $^{***}p < 0.001$; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ when pretreated cells were compared to cells exposed to LPS-challenge. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test

3.5 Effect of PAHE and verbascoside on LPS-induced superoxide dismutase and catalase activity increase in N9 cells

The levels of antioxidant enzymes, SOD and CAT were estimated in this study to evaluate the effects of PAHE and verbascoside on modulation of the oxidative stress parameters. The results revealed that SOD and CAT levels significantly increased in PAHE treatment at 10, 25 and 50 µg/mL when compared to negative control, while verbascoside induce only an increase in CAT activity at 25 and 50 µg/mL (Figure 12). The LPS treatment induces a pronounced increase in SOD and CAT activity, probably as result of inflammation process. However, the increase is reversed by PAHE and verbascoside (Figure 13).

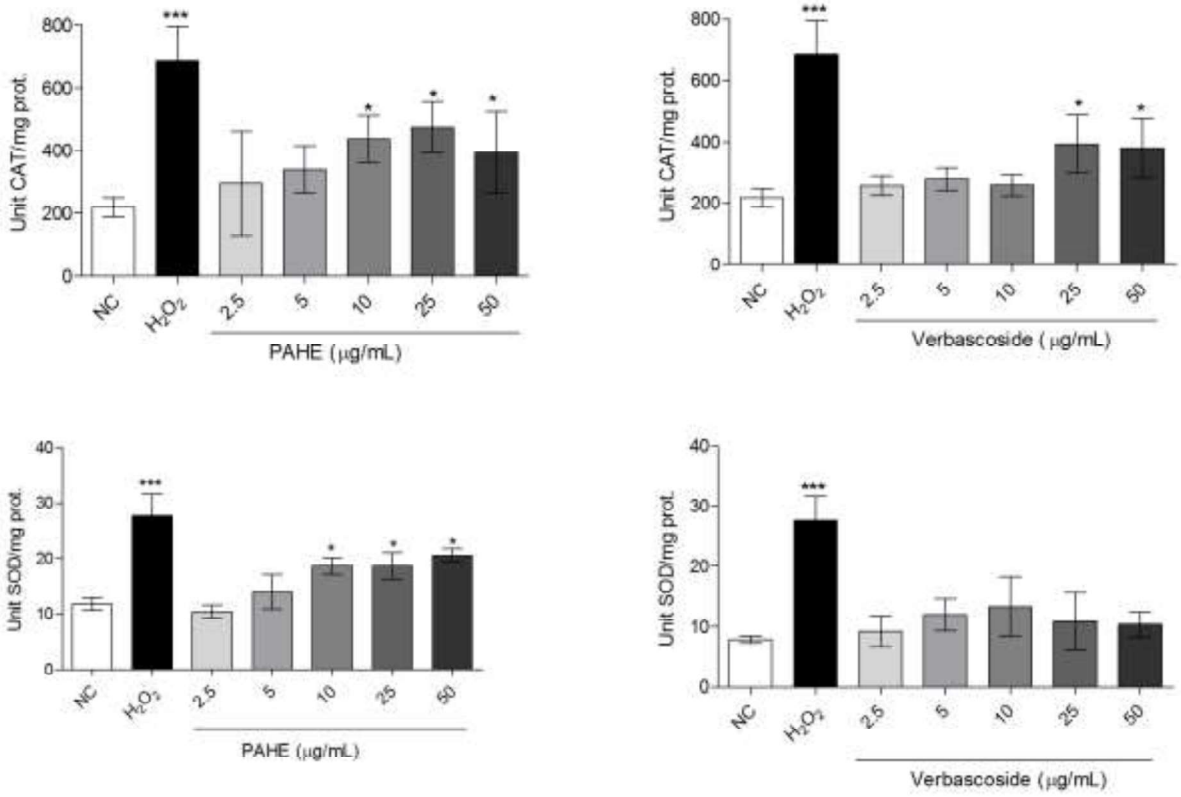

Figure 12. Measurement of the activity of catalase (CAT) and superoxide dismutase (SOD) by spectrophotometry after treatment with PAHE or verbascosídeo. Results are presented as mean and standard error median of three independent experiments. Data significant in relation to negative control group at $*p < 0.05$ and $***p < 0.001$. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

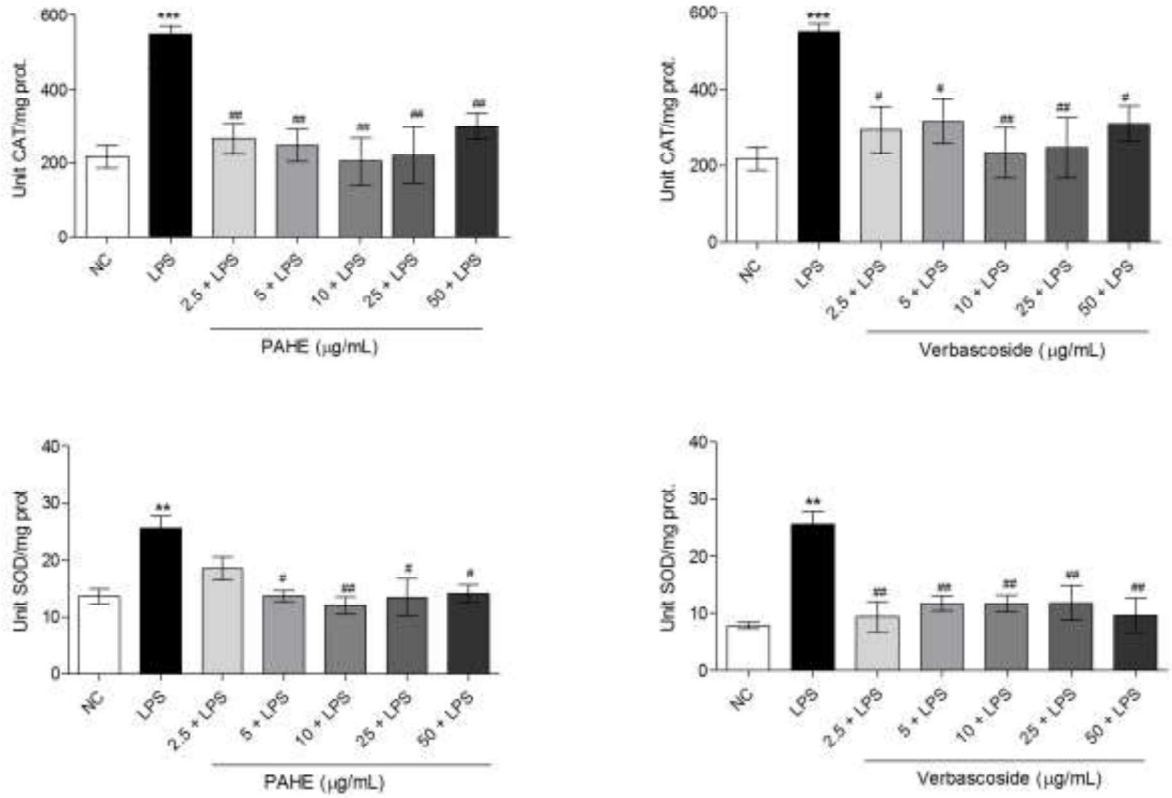

Figure 13. Measurement of the activity of catalase (CAT) and superoxide dismutase (SOD) in N9 cells using a LPS-inflammation model by spectrophotometry, after treatment with PAHE or verbascosídeo plus 4h of LPS. Results are presented as mean and standard error median of three independent experiments. Data significant in relation to negative control group at $^{**}p < 0.01$ and $^{***}p < 0.001$; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when pretreated cells were compared to cells exposed to LPS-challenge. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

3.6 Effects of HEPA and verbascoside on LPS-induced mitochondrial dysfunction in N9 cells

The PAHE and verbascoside do not alter mitochondrial function by Mitostatus staining (Figure 14). We also evaluated the effects of *P. australis* extract and verbascoside on LPS-induced mitochondrial dysfunction and observed that both decrease the mitochondrial dysfunction induced by LPS (Figure 14).

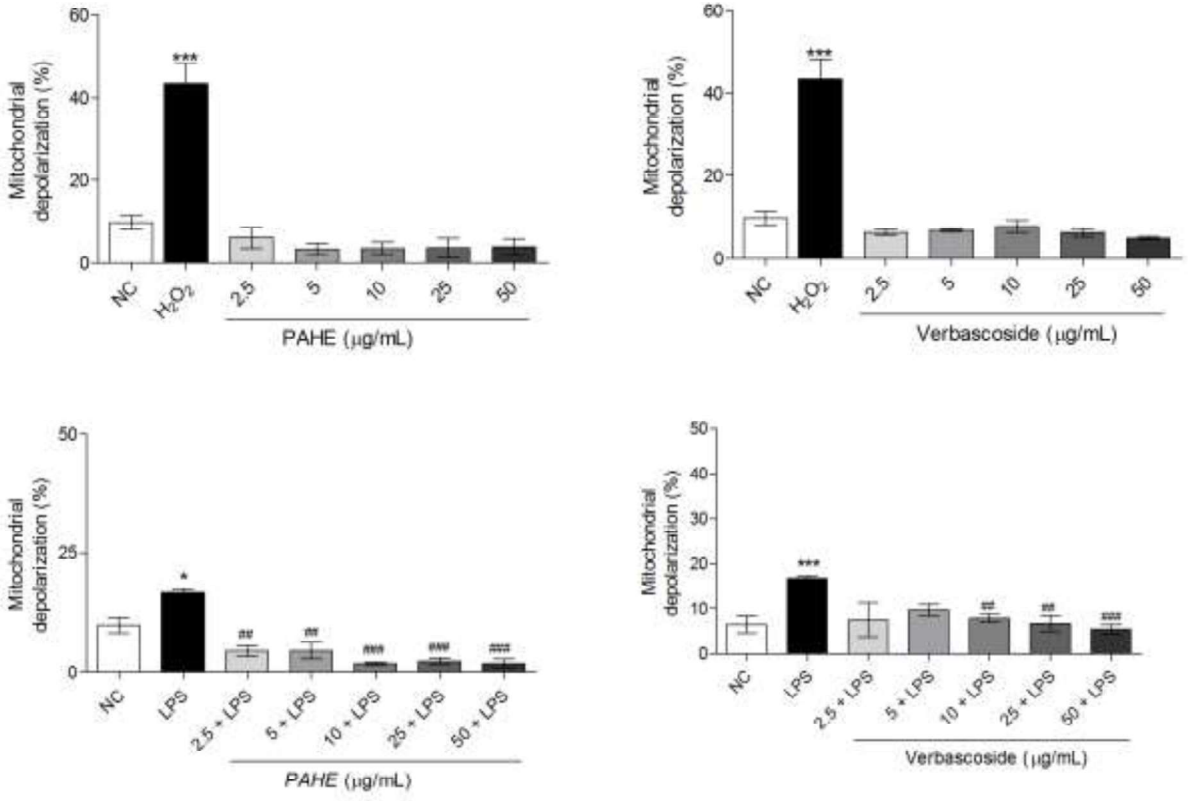

Figure 14. Measured of mitochondrial despolarization in N9 cells using a LPS-inflammation model by Mitostatus stain followed by flow cytometry analysis, after treatment with PAHE or verbascosídeo plus 4h of LPS. Results are presented as mean and standard error median of three independent experiments. Data significant in relation to negative control group at $^{***}p < 0.001$; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ when pretreated cells were compared to cells exposed to LPS-challenge. Statistical analyses were carried out using one-way ANOVA and Tukey's multiple comparison test.

4. Discussion

Several species of the genus *Plantago* are used in folk medicine as anti-inflammatory, cicatrizing and other medicinal treatments (Palmeiro et al., 2002). One of the species of this genus is the *P. australis*, which remains understudied. The *P. australis* is widely disseminated in Latin America with significant distribution in southern and southeastern regions in Brazil. In this study we produce a *P. australis* hydroethanolic extract to verbascoside (6%), a flavonoid glycoside which has been shown to be present in this plant (Andary et al., 1988), and evaluate the healing and

anti-inflammatory potential of this extract, as well as it's analytical marker verbascoside.

We first PAHE to verbascoside cytotoxicity to determine nontoxic concentrations usable *in vitro*. Then, we investigated PAHE to verbascoside wound closure ability on keratinocyte cells. Our results showed that both PAHE to verbascoside have effect on wound healing stimulating activity on HaCaT cells using the scratch assay.

The wound healing is a complex process involves control homeostasis, inflammation, proliferation and formation and remodeling of the new tissue (Harper et al. 2014; Monsuur et al., 2016). Upon injury of the skin occurs cellular response that involves action of fibroblasts, keratinocytes, endothelial cells, and macrophages (Alerico et al. 2015; Flanagan et al., 2000). The migration of keratinocytes is essential for wound re-epithelialization and re-establishment of skin remodeling (Sinvamani et al., 2007). For this reason, many studies have used systems that simplify the complex mechanism of repair, such as the scratch-wound in vitro assay (Slevin et al., 2002; Oberringer et al., 2007; D'Agostino et al., 2015). The cure of chronic wound involves the antibiotics, anti-inflammatory therapy, or combination of both. Some of these drugs are associated to unwanted adverse effects, for that the search for new alternatives is necessary (Nayak et al., 2010). Scratch Test assay is considered a valuable tool to estimate the wound healing potential of different plants-based extract (Fronza et al., 2009; Zubair et al., 2012). In this sense, this is the first work to show that the PAHE is effective in wound healing process. On the other hand, is well documented that verbascoside promotes skin repair and ameliorates skin inflammation (Vertuani et al., 2011), so probably the keratinocyte migration increasing can be associated with high concentrations of verbascosideo in *P. australis* hydroethanolic extract. In this sense, Korkina et al., (2007) showed that verbascoside promotes skin repair and ameliorates skin inflammation due to its ROS scavenging, antioxidant, iron chelating, and glutathione transferase (GST) activity inducing properties. Furthermore, the anti-inflammatory activity of verbascoside has been confirmed by an *in vitro* test performed on cell cultures of primary human keratinocytes, in which verbascoside was able to significantly reduce, in a dose-dependent manner, the release of pro-inflammatory chemokines (Kostyuka et al., 2011; Speranza et al., 2010).

In according to this, using a LPS-inflammation model in N9 microglial cells we showed that both PAHE and verbascoside, in greater or lesser degree, were able to reduce the inflammatory mediators (IL-6, IL-12p70, INFɣ, MCP-1 and TNFα), ameliorate oxidative stress parameters (SOD and CAT activity), restoring mitochondrial function.

Inflammation is a body homeostasis in response to any injury to the tissue. It is a complex phenomenon that involves immune, innate and adaptive responses in the injured area (Hussan et al., 2015). The initial response of this process requires the activation of primary flags, as resident macrophages and mast cells. To be activated, these cells have function to eliminate the causative agent of inflammation and start the damage repair process (Federico et al., 2007). Lipopolysaccharide (LPS), an endotoxin from gram-negative bacteria, is a pathogen capable to activate phagocytic monocytes, such as microglia (Le et al., 2004; Raetz and Whitfield, 2002). Once activated, this cells are capable to express a variety of inflammatory mediators, including cytokines and chemokines, reactive oxygen species (ROS) and reactive nitrogen species (NOS) (Qin et al., 2007;Lee et al., 2013).

Palmeiro et al. (2002) suggested that the anti-inflammatory mechanisms of *P. australis* extract might be related to prostaglandin synthesis inhibition. Recently, it was reported that verbascoside attenuated mast cell-derived inflammatory responses by inhibiting mouse double minute 2 and exhibits an anti-inflammatory activity by suppressing IL-32 signaling pathway (Nam *et al.*, 2015; Yoou et al., 2015). Now, we showed that this effect could be associated with the decrease of other inflammation mediators such as IL-6, Il-12p70, INF-γ, MCP-1 and TNF-α.

The CAT and SOD enzymes represent the first line of endogenous defense which has great importance in limiting damage to the phospholipid membrane and to biological macromolecules produced by ROS (Droge, 2002). Many studies have demonstrated how inflammation causes an oxidative stress with increases in the production of ROS (Valko, 2007). As mentioned before, some works have been pointed the antioxidant effect of verbascoside (Korkina et al., 2007; Vertuani et al. 2011), as we have demonstrated in this work. Probably the rescue of mitochondrial function induced by PAHE and verbascoside is related to this antioxidant activity.

5. Conclusion

In this work we showed that *P. australis* hydroethanolic extract has wound healing and anti-inflammatory activity and that the verbascoside can be the compound partly responsible for these activities in functions of antioxidant property and decrease of pro-inflammatory chemokines release. Besides, these results strengthened the wound healing potential and anti-inflammatory activity of traditionally used *P. australis*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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4. Considerações finais

Considerando a grande utilização de plantas medicinais por diferentes populações e a falta de conhecimento sobre as plantas e seus devidos usos, é necessária a pesquisa sólida e consistente. Ainda que bem difundido e bem aceito pela população, o uso de plantas medicinais não apresenta credibilidade para o tratamento de diversas doenças, impulsionando assim, a busca pelo entendimento de como agem, sua composição química, e qual a melhor forma de uso. Atrelado a isso, a facilidade de obtenção e o baixo custo da utilização de plantas medicinais resulta na possibilidade de utilização como prática alternativa e/ou complementar à medicina convencional impulsionando essa busca pelo conhecimento.

A presente dissertação de mestrado intitulada “Avaliação das atividades anti-inflamatória e cicatrizante de um extrato de *Plantago australis* (Kunth) Rahn, e do seu marcador analítico, verbascosídeo” teve por objetivo a realização de experimentos *in vitro* para elucidação das possíveis atividades farmacológicas de um extrato hidroetanólico padronizado das folhas de *P. australis* e do verbascosídeo. Com isso, considera-se que estes objetivos tenham sido atingidos adequadamente, implicando em uma melhor compreensão dos efeitos dos compostos frente as atividades farmacológicas testadas.

Os resultados deste trabalho nos levam a idealizar uma futura aplicação clinica desses compostos, na forma de formulação fitoterápica, tendo em vista a ampla utilização da planta *P. australis* na medicina popular.

5. Conclusão

Os resultados obtidos nesse trabalho indicam que o extrato hidroetanólico das folhas de *P. australis* e seu marcador analítico, verbascosídeo, apresentaram efeitos cicatrizantes e anti-inflamatórios em modelos *in vitro*.

- Tanto o extrato de *P. australis* quanto o verbascosídeo não apresentaram citotoxicidade em baixas concentrações quando testados em células de queratinócito.

- O extrato de *P. australis* e o verbascosídeo demonstraram possuir acentuado poder de indução de migração celular *in vitro* nas diferentes concentrações testadas, sendo esse feito mais acentuado nos tratamentos realizados com o extrato.

- Nas diferentes concentrações do extrato de *P. australis* e do verbascosídeo não foram observadas alterações dos mediadores inflamatórios, indicando ausência de potencial inflamatório em células de microglia murina.

- Diferentes concentrações do extrato de *P. australis* e do verbascosídeo foram capazes de alterar os parâmetros oxidativos analisados quando comparados ao controle negativo, pelos testes de catalase e superóxido dismutase, indicando uma possível proteção via ativação de enzimas antioxidantes.

- O extrato de *P. australis* e o verbascosídeo não foram capazes de induzir alterações no potencial de membrana mitocondrial em células de microglia murina.

- O verbascosídeo foi capaz de reduzir significativamente a liberação de todos os mediadores inflamatórios dosados em células de microglia quando ativada a inflamação, já o extrato de *P. australis* foi capaz de reduzir apenas um.

- Os compostos testados conseguiram reverter os efeitos causados pelo LPS em células de microglia murina, nos testes de medida das atividades de catalase e superóxido dismutase, assim como na avaliação do potencial de membrana mitocondrial, demonstrando a restauração do estado redox.

6. Perspectivas

O conjunto de dados obtidos nesse trabalho possibilitou determinar as atividades cicatrizante e anti-inflamatória do extrato de *P. australis* e do seu marcador analítico verbascosídeo *in vitro*. Desta maneira, as perspectivas de continuidade deste estudo incluem:

- Realizar dosagem do mediador inflamatório NF-κB após tratamentos com extrato hidroetanólico de *P. australis* e do verbascosídeo em células de microglia (células N9), utilizando modelo de indução de inflamação com LPS.
- Quantificar óxido nítrico pela reação de Griess, em células N9, utilizando o modelo de inflamação induzido por LPS, e tratamentos com extrato hidroetanólico de *P. australis* e do verbascosídeo.
- Avaliar a atividade cicatrizante, do extrato hidroetanólico de *P. major* e do seu composto verbascosídeo, através de um corte histológico na região mediana dorso-caudal da pele em ratos *Wistar*:
    - Analisar a presença de complicações pós-operatórias e gerais de pele.
    - Realizar medidas de tensão da ruptura de pele integra e da cicatriz.
    - Realizar avaliações histológicas do tecido cicatricial.
- Determinar o potencial anti-inflamatório do extrato hidroetanólico de *P. australis* e do seu composto verbascosídeo *in vivo*, utilizando modelo de indução de lesão de pata com carragenina em ratos *Wistar* machos:
    - Realizar dosagem de mediadores inflamatórios no sangue dos ratos utilizados no experimento.

- Elaborar uma formulação fitoterápica utilizando extrato hidroetanólico de *P. australis* para uso clínico.

7. Anexos

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