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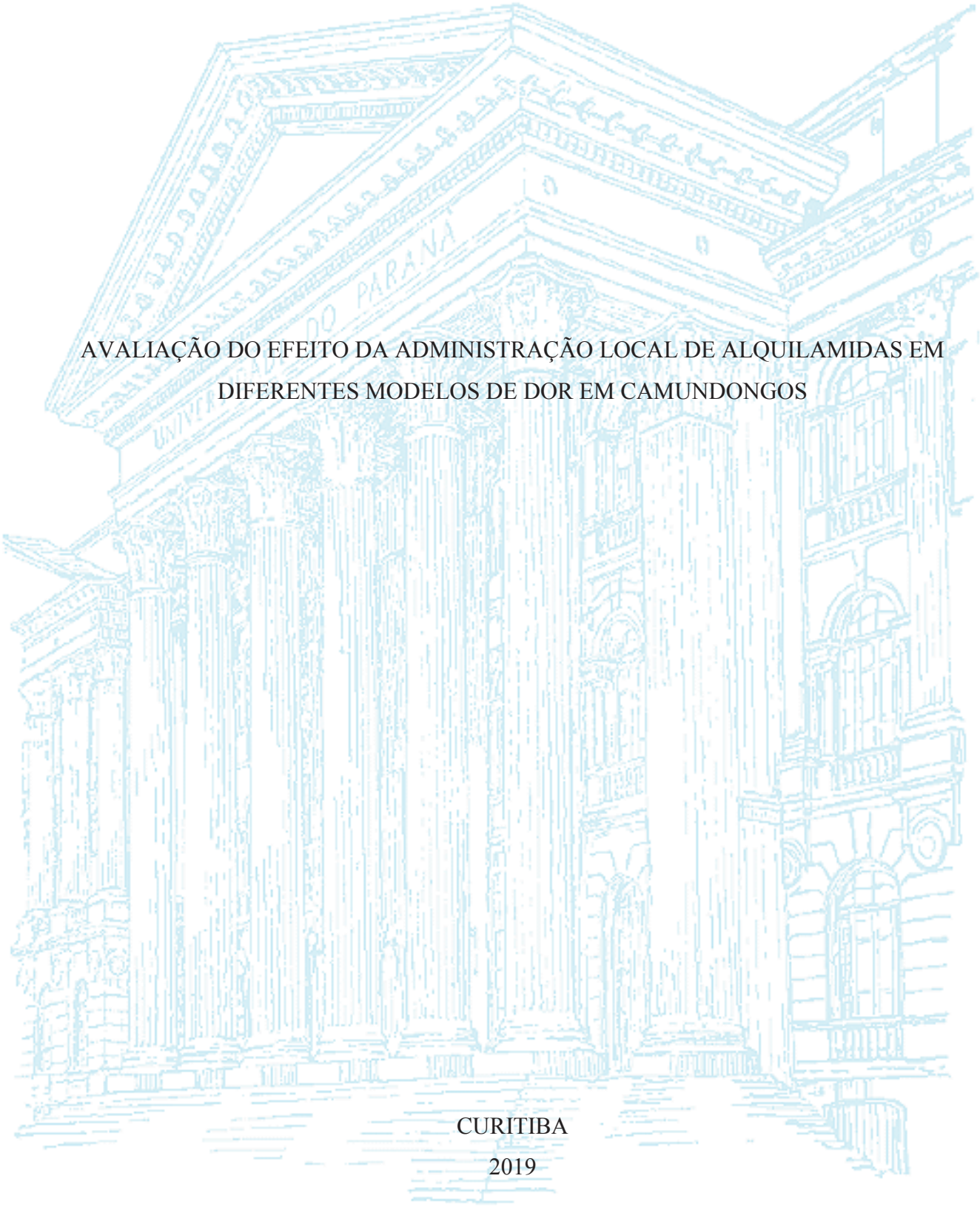

AVALIAÇÃO DO EFEITO DA ADMINISTRAÇÃO LOCAL DE ALQUILAMIDAS EM DIFERENTES MODELOS DE DOR EM CAMUNDONGOS

CURITIBA

2019

JORGE LUIZ DALLAZEN

AVALIAÇÃO DO EFEITO DA ADMINISTRAÇÃO LOCAL DE ALQUILAMIDAS EM DIFERENTES MODELOS DE DOR EM CAMUNDONGOS

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **JORGE LUIZ DALLAZEN** intitulada: **AVALIAÇÃO DO EFEITO DA ADMINISTRAÇÃO LOCAL DE ALQUILAMIDAS EM DIFERENTES MODELOS DE DOR EM CAMUNDONGOS**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

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Novos horizontes. Se não for isso, o que será?

- Humberto Gessinger.

RESUMO

A *Acmella oleracea* (L.) R.K. Jansen (Asteraceae), popularmente conhecida como jambu, é uma planta nativa do norte do Brasil, onde é muito utilizada na culinária devido ao seu sabor acre e pungente. Na medicina popular, é empregada para o alívio de dores de dentes em virtude das sensações de anestesia e formigamento causadas na mucosa oral. Estas propriedades são decorrentes da presença de uma classe de metabólitos secundários denominados de alquilamidas, sendo a principal e majoritária o espilantol. Portanto, tendo em vista as sensações quimestésicas (pungente e anestésica) evocadas por essas moléculas, esse estudo teve como objetivo avaliar o efeito da administração local (intraplantar) da fração hexânica (FH) rica em alquilamidas do jambu, e da isobutilalquil amida sintética (IBA) em diferentes modelos de dor aguda, inflamatória, neuropática e pós-operatória em camundongos. A análise fitoquímica da FH realizada através de cromatografia gasosa acoplada à espectrometria de massas, revelou a presença de diversas alquilamidas, incluindo o espilantol. A administração intraplantar (i.pl.) de FH e IBA em doses crescentes (0,1 – 30 μg/20 μL) induziu efeito *dual*, antinocicepção promovida em dose baixa, e nocicepção em doses elevadas. O efeito antinociceptivo de HF e IBA (0,1 μg/20 μL, i.pl.) foi promovido pelo bloqueio de canais TRPV1 e independente do sistema opioide. Além disso, FH promoveu a estabilização de mastócitos, e ambos FH e IBA sensibilização de canais TRPA1. Por outro lado, a nocicepção induzida por FH e IBA (30 μg/20 μL, i.pl.) é mediada pela ativação/sensibilização de canais TRPA1. Em adição, IBA promoveu a ativação de canais TRPV1, e FH a degranulação de mastócitos, ambos em 30 μg/20 μL (i.pl.). A administração local de FH 0,1 μg/20 μL, i.pl. elevou o limiar mecânico (teste de von Frey, 0,04 – 4 g), e nas doses de 0,1 e 30 μg/20 μL, i.pl., elevou o limiar térmico (placa quente, 52 ± 0,1 °C) da pata dos animais. Entretanto, IBA (30 μg/20 μL, i.pl.) reduziu o limiar mecânico, e em ambas as doses o limiar térmico. No modelo de inflamação aguda induzida por carragenina (300 μg/20 μL, i.pl.), o pré-tratamento com FH e IBA (0,1 μg/20 μL, i.pl.) reduziu a alodinia mecânica e o edema de pata durante 3 e 5 h, respectivamente. No pico inflamatório (3 h), FH e IBA reduziram a migração celular (mieloperoxidase, MPO), os níveis de citocinas pró-inflamatórias (TNF-α e IL-1β), de lipoperóxidos (LOOH), e preveniram a depleção das enzimas antioxidantes (catalase, CAT, e superóxido dismutase, SOD) e de glutationa (GSH) na superfície plantar. Ainda, FH reduziu os níveis de PGE_2 e manteve os níveis basais de IL-10. O efeito do tratamento local com FH ou IBA (0,1 μg/20 μL, i.pl.) também foi avaliado nos modelos de dor neuropática induzida pela ligadura parcial do nervo ciático (LPNC), e no modelo de dor pós-operatória induzida pela cirurgia de incisão plantar (CIP). FH e IBA reverteram a alodinia mecânica e térmica ao frio (acetona) induzidas pela LPNC, bem como parâmetros estáticos (*print* e área da pata) e dinâmico (tempo de pisada) em alterações de marcha dos animais mensuradas pelo aparelho *CatWalk*®. Por fim, ambos os tratamentos com FH e IBA reduziram a alodinia mecânica induzida por CIS, entretanto, apenas FH reduziu a hiperalgesia térmica e escores totais de comportamentos nociceptivos espontâneos. Em conjunto, podemos concluir que as alquilamidas naturais do jambu presentes na FH, bem como a sintética IBA apresentam potencial farmacológico para o tratamento local de dor aguda, inflamatória, neuropática e pós-operatória.

Palavras-chaves: Jambu; Isobutilalquil Amida; Nocicepção; Dor Inflamatória; Dor Neuropática; Dor Pós-operatória.

ABSTRACT

Acmella oleracea (L.) RK Jansen (Asteraceae), popularly known as jambu, is a native plant from northern Brazil, where it is widely used in culinary due to its pungent and acrid taste. In the folk medicine, it is used to relief of toothache due to the anesthesia and tingling sensations caused in the oral mucosa. These properties are due to the presence of a class of secondary metabolites named alkylamides, which the main and majority is the spilanthol. Therefore, in view of the chemesthetic sensations (pungent and anesthetic) evoked by these molecules, the aim of this study was to evaluate the effect of local (intraplantar) administration of hexane (HF) rich in jambu alkylamides, and of synthetic isobutylalkyl amide (IBA) in different models of acute, inflammatory, neuropathic and postoperative pain in mice. The phytochemical analysis of HF performed by gas chromatography coupled to mass spectrometry, revealed the presence of several alkylamides, including the spilanthol. Intraplantar (i.pl.) administration of HF and IBA at increasing doses (0.1 - 30 μg / 20 μl) induced a dual effect, antinociception at low dose, and nociception at high doses. The antinociceptive effect of HF and IBA (0.1 μg/20 μL, i.pl.) was mediated by blocking TRPV1 channels and independent of the opioid system. In addition, HF promotes the stabilization of mast cells, and both HF and IBA sensitization of TRPA channels. On the other hand, HF and IBA promoted nociception (30 μg/20 μL, i.pl.) by the activation/sensitization of TRPA1 channels. In addition, IBA promoted TRPV1 activation, and 30 μg FH the mast cell degranulation, both at 30 μg/20 μL (i.pl.). Local administration of 0.1 μg/20 μL HF promoted mechanical threshold elevation (von Frey test, 0.04 - 4 g), and at 0.1 and 30 μg/20 μL, i.pl., the thermal threshold elevation (hot plate, 52 ± 0.1 ° C) of the animals hindpaw. However, IBA (30 μg/20 μL i.pl.) reduced the mechanical threshold, and at both doses, the thermal threshold. In the carrageenan-induced acute inflammation model (300 μg/20 μl, i.pl.), the pre-treatments with FH and IBA (0.1 μg/20 μl, i.pl.) reduced mechanical allodynia and edema of paw for 3 and 5 h, respectively. At the inflammatory peak (3 h), HF and IBA reduced cell migration (myeloperoxidase activity, MPO), the levels of proinflammatory cytokines (TNF-α and IL-1β), lipoperoxides (LOOH), and prevented the depletion of the antioxidant enzymes (catalase, CAT, and superoxide dismutase, SOD) and glutathione (GSH) on hindpaw surface. Furthermore, HF reduced the levels of PGE2 and promoted the maintenance of basal levels of IL-10. The effect of local treatment with HF or IBA (0.1 μg 20 μL, i.pl.) was also evaluated in the neuropathic pain models induced by partial sciatic nerve ligation (PSNL), and in the postoperative pain model induced by plantar incision surgery (PIS). HF and IBA reversed the mechanical and thermal cold allodynia (acetone) induced by the PSNL, as well as static parameters (print and paw area) and dynamic (treading time) changes in animals' gait mensured in CatWalk® apparatus. Finally, both HF and IBA treatments reduced the mechanical allodynia induced by PIS, however, only HF reduced thermal hyperalgesia and total spontaneous nociception scores. Together, we can conclude that the natural jambu alkylamides present in HF and the synthetic IBA present pharmacological potential for the local treatment of acute, inflammatory, neuropathic and postoperative pain.

Keywords: Jambu; Isobutylalkyl Amide; Nociception; Inflammatory Pain; Neuropathic pain; Post-operative pain.

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LISTA DE ABREVIATURAS

a.C. – antes de Cristo;

m – Metro;

s – Segundo;

ex. – Exemplo;

i.pl. – Via intraplantar.

Artigo Científico 1

g – grams;

i.p. – Intraperitoneal route;

i.pl. – Intraplantar route;

min – Minute;

s – Seconds;

s.c. – Subcutaneous route;

Artigo Científico 2

g – grams;

h – Hour;

i.p. – Intraperitoneal route;

i.pl. – Intraplantar route;

min – Minute;

mm – Millimeter;

O.D. – Optic Density;

Artigo Científico 3

d7 – Seven Days after PSNL Surgery;

g – Grams;

h – Hour;

i.p. – Intraperitoneal route;

i.pl. – Intraplantar route;

min – Minute;

mm – millimeter;

Post – 24 h after PIS;

s.c. – Subcutaneous route;

LISTA DE SIGLAS

5-HT – receptor de serotonina;

AINEs – Anti-Inflamatórios Não Esteroidais;

AMPA – α-amino-3-hidroxi-5-metil-4-isoxazolepropionico;

ANVISA – Agência Nacional de Vigilância Sanitária;

ASICs – Canais Iônicos Sensíveis a Ácido;

ATP – Adenosina Trifosfato;

B1 – receptores de bradicinina tipo 1;

B2 – receptores de bradicinina tipo 2;

BDNF – Fator Neurotrófico Derivado Do Cérebro;

CAT – Enzima Catalase;

Ca_v – Cálcio Voltagem Dependente;

$Ca_v\alpha2\delta$ – Cálcio Voltagem Dependente Subunidade α2δ;

CGRP – Peptídeo Relacionado ao Gene da Calcitonina, *calcitonin gene–related peptide*;

COX-1 – Ciclooxigenase 1;

COX-2 – Ciclooxigenase 2;

DPPH – Radical Livre 2,2-difenil-1-picrilhidrazilo;

DRASIC – Receptores Homólogos do ASIC;

EPs – Receptores de prostaglandina;

ERK – Quinases Extracelulares Fosforiladas Reguladas por Sinal;

EUA – Estados Unidos da América;

FDA – *Food and Drug Administration*;

FH – Fração Hexânica;

GPCRs – Receptores Acoplados a Proteína G;

GRD – Gânglio da Raiz Dorsal;

H_1 – Receptores de histamina;

IASP – Associação Internacional para o Estudo da Dor*, International Association fot the Study of Pain*;

IBA – Isobutilalquil Amida Sintética;

IL-1β – Interleucina 1β;

KCNK ou K_v2P – Canais de Potássio de Dois Poros;

K_v – Canais de Potássio Voltagem Dependente;

LOOH – Lipoperoxidação Lipídica;

MAPK/MKP – 3 – Proteína Quinase Ativada por Mitógeno fosfatase 3;

MDGE – Receptores *mammalian degenerin*;

MPO – Enzima Mieloperoxidase;

MRGPRD – Receptores Mas tipo D Relacionados a Proteína G;

Na_v – Canais de Sódio Voltagem Dependente;

NGF – Fator de Crescimento Nervoso;

NMDA – N-metil D-Aspartato;

NSC – Canais Não Seletivos a Cátions;

P2X – Receptores Catiônicos de Adenosina Trifosfato;

PAR2 – Receptor de Protease 2;

PGE_2 – Prostaglandina E_2;

RDC – Resolução da Diretoria Colegiada;

RENAME – Relação Nacional de Medicamentos Essenciais;

RENISUS – Relação de Plantas Medicinais de Interesse ao SUS;

RTK – Receptor de Tirosina Quinase;

SOD – Enzima Superóxido Dismutase;

SP – Substância P;

SUS – Sistema Único de Saúde;

TNF-α – Fator de Necrose Tumoral α;

TRPA1 – Receptor de Potencial Transitório Anquirina 1;

TRPM8 – Receptor de Potencial Transitório Melastatin 8;

TRPs – Receptores de Potencial Transitório;

TRPV1 – Receptor de Potencial Transitório Vanilóide 1;

TRPV2 – Receptor de Potencial Transitório Vanilóide;

Artigo Científico 1

ALFAC – mixture of ethanol, formaldehyd, and acetic acid;

ANOVA – Analysis of Variance;

B – Basal;

C48/80 – Compound 48/80;

CAPS – Capsaicin;

CAPZ – Capsazepin;

CINA – Cinnamaldehyde;

CNS – Central Nervous System;

DICLO – Diclofenac;

DMSO – Dimethyl Sulfoxide;

EEAO – Ethanolic Extract of *Acmella oleracea*;

FORM – Formalin;

GC–MS – Gas Chromatography–Mass Spectrometry;

GLU – Glutamate;

HC-030031 – 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-N-(4-isopropylphenyl)-acetamide;

HF – Hexanic Fraction;

IBA – Isobutylalkyl Amide;

KCNK – Two-Pore Domain Potassium Channels;

KETO – Ketotifen;

LIDO – Lidocaine;

MOR – Morphine;

NAL – Naloxone;

RTX – Resiniferatoxin;

TRPA1 – Transient Receptor Potential Ankyrin 1;

TRPV1 – Transient Potential Receptor Vanilloid 1;

V – Vehicle;

Artigo Científico 2

AA – Ascorbic Acid;

ANOVA – Analysis of Variance;

ATC – Trichloroacetic Acid;

B – Basal;

BHT – Butylated Hydroxytoluene;

CAT – Catalase;

COX – Cyclo Oxygenase;

DEXA – Dexamethasone;

DMSO – Dimethyl Sulfoxide;

DPPH – 2,2-Diphenyl-1-picrylhydrazyl;

DTNB – 5,5′-dithiobis-(2-nitrobenzoic acid);

EDTA – Ethylenediaminetetraacetic Acid

EEAO – Ethanolic Extract of *Acmella oleracea;*

ELISA – Enzyme-Linked Immunosorbent Assay;

FOX2 – Ferrous Oxidation-Xylenol Orange;

GC-MS – Chromatography-Mass Spectrometry;

GSH – Glutathione;

HF – Hexanic Fraction;

HO-1 – Heme Oxygenase-1;

HTAB – Hexadecyltrimethylammonium Bromide;

IBA – Isobutylalkyl Amide;

ICAM-1 – Intercellular Adhesion Molecule 1;

IL-10 – Interleukin 10;

IL-1β – Interleukin 1 Beta;

LOOH – Lipid Hydroperoxides;

MAPK – Mitogen-Activated Protein Kinase;

MPO – Myeloperoxidase;

N – Naive;

NF-κB – Nuclear Factor Kapa B;

NSAIDs – Non-Steroidal Anti-Inflammatory Drugs;

PGE_2 – Prostaglandin E_2;

ROS – Reactive Oxygen Species;

SOD – Superoxide Dismutase;

TMB – 3,3’,5,5’-tetramethylbenzidine;

TNF-α– Tumor Necrosis Factor Alpha;

TRPA1 – Transient Receptor Potential Ankyrin 1;

TRPV1 – Transient Potential Receptors Vanilloid 1;

U – unit;

V – Vehicle;

Artigo Científico 3

ANOVA – Analysis of Variance;

B – Basal;

$Ca_V\alpha2\delta$ – Calcium Channel alpha-2-delta;

DMSO – Dimethyl Sulfoxide;

GABA – Gabapentin;

GC-MS – Chromatography-Mass Spectrometry;

HF – Hexanic Fraction;

IBA – Isobutylalkyl Amide;

KCNK/K_2P – Two-Pore Domain Potassium Channels;

LH – Left Hind Paw;

MOR – Morphine;

PIS – Plantar Incision Surgery;

PSNL – Partial Sciatic Nerve Ligation;

RH – Right Hind Paw;

V – Vehicle;

TRPA1 – Transient Receptor Potential Ankyrin 1;

TRPV1 – Transient Potential Receptors Vanilloid 1;

LISTA DE SÍMBOLOS

® – Marca Registrada;

μ – Micro;

α – Alfa;

β – Beta;

δ – Delta;

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1 INTRODUÇÃO

Historicamente, a humanidade sempre utilizou recursos naturais para suprir seu sustento, necessidades básicas de alimentação e proteção, bem como para a cura de doenças (VIEGAS et al., 2006). Inevitavelmente, a dor foi uma das primeiras razões que estimulou o homem a buscar nas plantas a fonte de seu alívio. Registros mesopotâmicos em tábuas de argila datados de 2600 anos a.C., já documentavam o uso de *Cupressus sempervirens* (cipreste-italiano) e *Commiphora species* (guggul) para o tratamento da inflamação (DIAS et al., 2012). Além disso, plantas como *Papaver somnniferum* (papoula) e *Cannabis sativa* (maconha) foram utilizadas ao longo de 6000 anos para o tratamento das mais diversas condições dolorosas (NEWMAN et al. 2000; DUTRA et al. 2016).

A dor nos acompanha desde os primórdios, sendo fundamental para o desenvolvimento e manutenção da integridade do nosso organismo. Entretanto, ao perder seu caráter protetivo, a dor torna-se causa de sofrimento, incapacidade, problemas sociais, prejuízos financeiros e um grande fardo aos sistemas de saúde (PATEL, 2009; HENSCHKE et al. 2015). De difícil manejo, muitas vezes o tratamento empregado para o alívio da dor é ineficaz, com baixa adesão do paciente, acompanhado de diversos efeitos adversos e risco de abuso (FINNERUP et al. 2015).

Tendo em vista o potencial das plantas e da grande diversidade de metabólitos produzidos por elas, não podemos descarta-las durante o processo de desenvolvimento de novas alternativas terapêuticas. O Brasil é detentor da maior biodiversidade do planeta, a qual tem grande impacto econômico e estratégico para o avanço científico de nosso país (PALHARES et al. 2015).

Portanto, é de extrema importância a realização de estudos e pesquisas utilizando produtos naturais derivados de plantas, como extratos e princípios ativos isolados, visando compreender melhor seus efeitos biológicos e toxicológicos, bem como seus mecanismos de ação, uma vez que estes podem se tornar alternativas terapêuticas mais eficazes e seguras. Agregado a isso, está a obtenção e repasse de informações corretas e seguras para a população que faz o uso de plantas medicinais apenas embasada no conhecimento popular.

1.1 DOR E NOCICEPÇÃO

Em 1979, a Associação Internacional para o Estudo da Dor (do inglês, *International Association for the Study of Pain*, IASP) conceituou a dor como: "uma experiência sensorial e emocional desagradável associada a uma lesão tecidual real ou potencial, ou descrita em termos de tal dano". O componente emocional torna a dor uma experiência subjetiva, ou seja, cada indivíduo relaciona e percebe esse evento de maneira diferente, e por ser desagradável, reconhecemos que os estímulos gerados podem representar prejuízo ao nosso organismo. Além disso, a dor pode ocorrer na ausência de lesão ou estímulos danosos, mas mesmo assim não podemos desconsiderá-la como "dor".

Recentemente foi proposto uma nova definição: "a dor é uma experiência somática mutuamente reconhecível que reflete a apreensão de uma pessoa à uma ameaça para sua integridade física ou existencial" (COHEN et al. 2018). Este conceito traz as relações inter-indivíduo (paciente e clínicos), ressaltando as dificuldades e limitações de comunicação e interpretação da dor por ambas as partes.

Quando utilizamos modelos animais para o estudo da dor, convencionalmente se utiliza o termo "nocicepção" ao invés de "dor", o qual é definido como o processo neural de codificação do estimulo nocivo (IASP, 2018). Isso se dá ao fato de não conseguirmos avaliar e mensurar o componente subjetivo dos animais. Apesar disso, atualmente muitos estudos vem incluindo em seus protocolos experimentais, testes que avaliam comportamentos de dor não evocada/induzida, espontâneos, relacionados ao bem-estar animal e com o mínimo de interferência do manipulador (KAPPOS et al. 2017; PITZER et al.; 2016; SHEPHERD; MOHAPATRA 2018). Tais modelos estão sendo implementados decorrente aos fracassos e falhas que ocorrem durante a translação de possíveis novas terapias da bancada experimental (estudo não-clínico) para o leito dos pacientes (estudo clínico) (MOGIL 2009; MOGIL et al. 2010; PITZER et al. 2016; WHITESIDE et al. 2013; YEZIERSKI; HANSSON 2018).

A dor pode ser classificada de diferentes maneiras, levando em consideração sua duração (aguda, subcrônica, crônica), fisiopatologia (fisiológica, nociceptiva, neuropática), e contexto clínico (pós-cirúrgica, relacionada a câncer, neuropática, degenerativa) (SINATRA *et al.*, 2009).

1.2 DOR AGUDA E INFLAMATÓRIA

A dor é crucial para a proteção, sobrevivência e interação dos organismos com o mundo físico ao seu redor (BASBAUM et al. 2010). O componente fisiológico da dor, ou seja, a nocicepção, pode ser iniciada na periferia a partir de estímulos nocivos de natureza química, física, mecânica ou térmica (BALIKI; APKARIAN 2015). Estes estímulos ativam neurônios aferentes primários especializados, os nociceptores, que podem ser categorizados em duas classes levando em consideração o calibre de seu axônio, grau de mielinização e propriedades de condução: as fibras Aδ possuem diâmetro médio, são pouco mielinizadas, respondem a temperaturas na faixa de 43 - 53 ºC e sua velocidade de condução é de 5-30 m/s; já as fibras C possuem diâmetro pequeno, são amielinizadas, respondem aproximadamente à estímulos de 43 ºC e possuem velocidade de condução lenta (> 2 m/s) (DUBIN; PATAPOUTIAN 2010; JULIUS 2001). Ambas apresentam limiar elevado de ativação e terminações livres na periferia que inervam pele, tecidos profundos e órgãos. Apesar delas responderem a uma diversa gama de estímulos, sendo denominadas de polimodais, as fibras C respondem a estímulos químicos, mecânicos e térmicos (calor e frio), e as fibras Aδ, a estímulos químicos, mecânicos e/ou térmicos (calor) (DUBIN; PATAPOUTIAN 2010; TRACEY 2017).

Os estímulos nocivos são em sua grande maioria percebidos por diversos receptores expressos ao longo dos nociceptores, que por sua vez geram potencias de ação, despolarizando a membrana desses neurônios e propagando o sinal nociceptivo. Dentre os diversos receptores, podemos citar: os receptor de potencial transitório (TRPs), os quais são responsáveis por detectar agentes químicos e térmicos, sendo o TRPV1 e V2 (vanilóides 1 e 2) responsáveis por detectar o calor nocivo, e o TRPA1 (anquirina 1) e M8 (melastatina 8), pela resposta ao frio nocivo; canais iônicos sensíveis a ácido (ASICs); canais ou receptores catiônicos de adenosina trifosfato (ATP) P2X; receptor de serotonina (5-HT), bradicinina (B1 e B2), histamina (H_1), prostaglandina (EPs) e outros muitos receptores acoplados a proteína G (GPCRs); receptor de tirosina quinase (RTK); e canais não seletivos a cátions (NSC) e voltagem dependentes, receptores *mammalian degenerin* (MDEG), homólogos do ASIC (DRASIC), canais de potássio de dois poros (KCNK), TRPs, e receptores Mas tipo D relacionados a proteína G (MRGPRD), os quais são responsáveis por detectar estímulos mecânicos nocivos (SCHOLZ; WOOLF 2002; DUBIN; PATAPOUTIAN 2010; GEPPETTI et al. 2015; PEIRS; SEAL 2016; TRACEY 2017) (FIGURA 1).

Como outros neurônios somatossensoriais, os nociceptores são pseudounipolares, com os corpos celulares localizados no gânglio da raiz dorsal (GRD), ou do trigêmeo. A partir do

GRD eles bifurcam, enviando um axônio para a periferia, e outro para o corno da raiz dorsal da medula espinhal, ou subnúcleo caudal, respectivamente. No corno dorsal da medula, as fibras aferentes primárias do tipo C adentram em lâminas superficiais I e II (mais responsivas à estimulação nociva), e as do tipo Aδ em lâminas II, III e IV e V (mais sensíveis ao toque), onde realizam a primeira sinapse com interneurônios excitatórios (75%), inibitórios, ou ainda com neurônios da ampla faixa dinâmica (do inglês, *wide-dynamic range neurons*), através da liberação de neurotransmissores como o glutamato, substancia P (SP) e peptídeo relacionado ao gene da calcitonina (do inglês, *calcitonin gene–related peptide*, CGRP) (DUBIN; PATAPOUTIAN 2010; PEIRS; SEAL 2016; WOLLER et al. 2017).

Na sequência, após a sinapse no corno dorsal da medula, as fibras ascendem contralateralmente ao encéfalo principalmente pela via espinotalâmica, passando pelo bulbo, ponte e mesencéfalo sem fazer sinapses, até alcançarem o tálamo, uma espécie de "centro distribuidor", onde realizam a terceira sinapse. No percurso do tronco encefálico, os axônios vão se posicionando ao longo do lemnisco medial, até alcançar o córtex somatossensorial primário, onde ocorre a interpretação do impulso nervoso baseado na discriminação e descrição da dor (intensidade e localização da dor), com projeções para: córtex pré-frontal (planejamento de ações); córtex cingulado anterior (dimensão desagradável); insula (codificação emocional, sensorial e cognitiva); amigdala ("antecipação" e aversão à dor); gânglio da base e substancia cinzenta periaquedutal (vias descendentes da dor) (BALIKI; APKARIAN 2015B; PEIRS; SEAL 2016; KUNER; FLOR 2016) (FIGURA 1).

Apesar da dor aguda ser adaptativa, comumente após um estimulo nocivo e/ou lesão tecidual, pode ocorrer um processo inflamatório caracterizado por calor, rubor, edema, dor e perda de função. Estes eventos são decorrentes da migração de células imunes, dilatação dos vasos e aumento do fluxo sanguíneo, extravasamento plasmático e liberação de mediadores inflamatórios (NOURSHARGH; ALON 2014; JI et al. 2016). SP e CGRP são liberados por nociceptores e contribuem para vasodilatação e extravasamento de plasma e células dos vasos sanguíneos para o tecido lesionado (JULIUS 2001; JI et al. 2016).

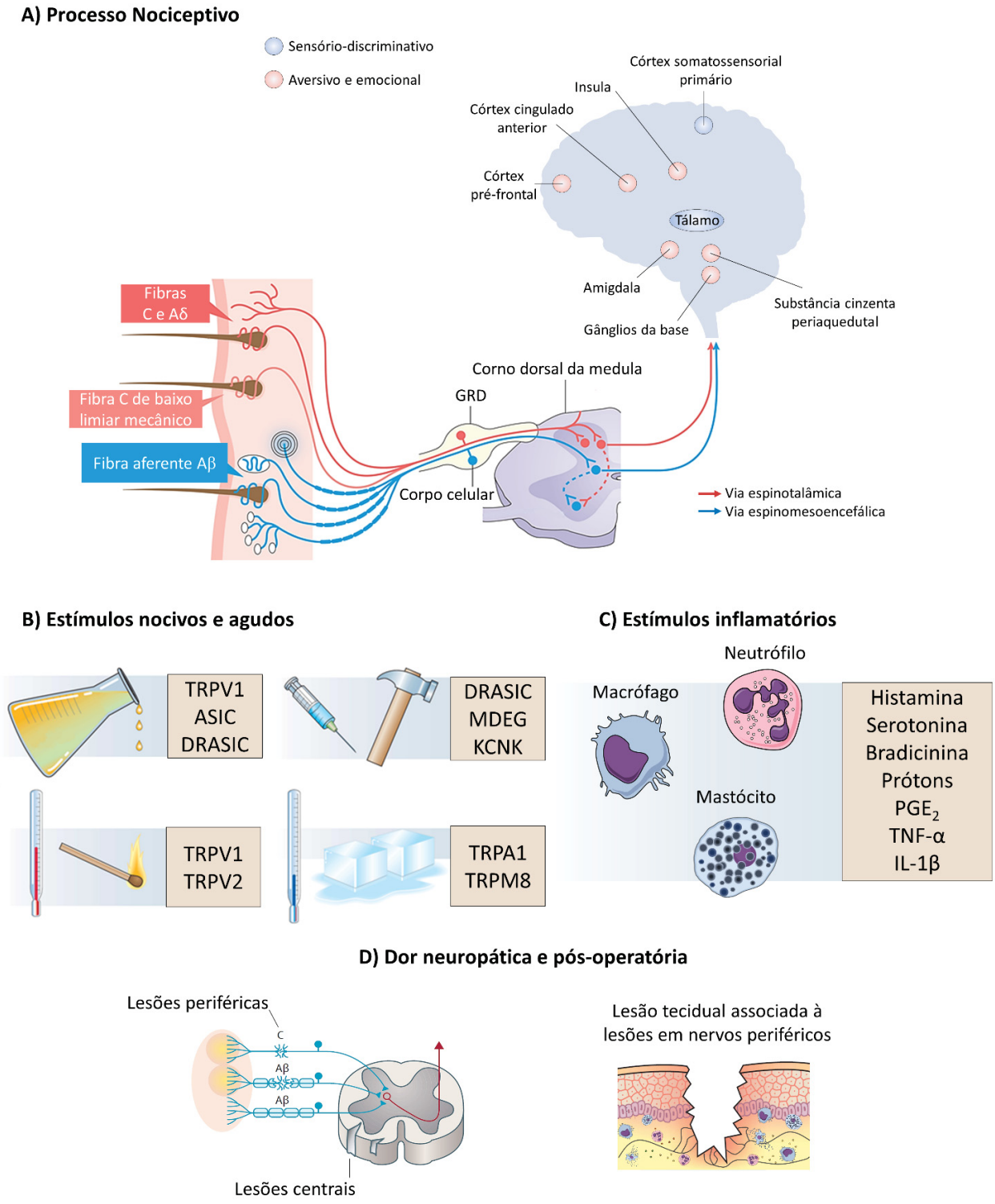
Células imunes, como os macrófagos, migram ao sítio inflamado e liberam citocinas como interleucina-1β (IL-1β) e fator de necrose tumoral-α (TNF-α), que podem ativar e sensibilizar os nociceptores periféricos, causando dor. A sensibilização dos nociceptores ocorre por modulação (fosforilação) de canais TRPV1 e A1, bem como de canais de sódio voltagem dependente (Na_v) 1.7 – 1.9, alterando assim as propriedades de membrana, reduzindo o limiar de ativação e facilitando os potencias de ação (JI et al. 2016; PINHO-RIBEIRO et al. 2017; COOK et al. 2018). Além dessas citocinas, outros mediadores como histamina, serotonina (5-

HT) e fator de crescimento neural liberados por mastócitos, prostaglandina E_2 (PGE_2) sintetizada por macrófagos e neutrófilos, bradicinina, prótons e ATP liberados no meio, também resultam na ativação e sensibilização dos nociceptores (JULIUS 2001; PINHO-RIBEIRO et al. 2017). Em conjunto esses eventos levam a dor provocada por estímulos não nocivos, denominada de alodinia, bem como dor exacerbada frente a estímulos que por si só são dolorosos, denominada de hiperalgesia (COOK et al. 2018) (FIGURA 1).

Enquanto a sensibilização periférica aumenta e facilita os disparos dos nociceptores em direção ao corno dorsal, eventos subsequentes na medula podem gerar a sensibilização central, levando à persistência e cronificação da dor. Decorrente da estimulação prolongada de fibras C e consequente ativação de receptores glutamatérgicos AMPA (α-amino-3-hidroxi-5-metil-4-isoxazolepropionico), os neurônios da ampla faixa dinâmica localizados na lâmina V, passam a receber estímulos mecânicos de fibras Aδ e codifica-los como dor (*wind-up*). Essa facilitação central é decorrente de uma cascata de eventos que podem ser resumidos em: fosforilação do receptor NMDA (N-metil D-Aspartato) e remoção do magnésio que bloqueava sua ativação (voltagem dependente); ativação de canais de cálcio com aumento da síntese e liberação de neurotransmissores peptidérgicos (SP e CGRP) em neurônios glutamatérgicos; participação de astrócitos e micróglia com consequente liberação de citocinas, prostaglandina e quimiocinas; e redução da atividade inibitória gabaérgica e da glicina (BASBAUM et al. 2010; JI et al. 2016; PINHO-RIBEIRO et al. 2017; WOLLER et al. 2017; COOK et al. 2018).

Em adição, decorrente também da migração celular, ocorre o processo de “explosão respiratória”, com consequente e excessiva produção de radicais livres e lipoperoxidação lipídica, gerando um grande desbalanço de todo sistema antioxidante endógeno enzimático (catalase e superóxido dismutase) e não enzimático (glutationa) (REUTER et al. 2010; COSTA et al. 2018).

FIGURA 1 – ILUSTRAÇÃO ESQUEMÁTICA DO PROCESSO NOCICEPTIVO (A), RECEPTORES ATIVADOS POR ESTÍMULOS NOCIVOS (B) E INFLAMATÓRIOS (C), BEM COMO ILUSTRAÇÕES GRÁFICAS DA DOR NEUROPÁTICA E PÓS-OPERATÓRIA (D).



FONTE: adaptado de SCHOLZ, WOOLF (2002); PEIRS, SEAL (2016).

O tratamento para a dor inflamatória envolve principalmente anti-inflamatórios não esteroidais (AINEs, ex.: paracetamol, diclofenaco e ibuprofeno) e esteroidais, como os corticoides (dexametasona, predinisolona e betemetasona). De maneira geral, os AINEs atuam através da inibição da enzima ciclooxigenase 1 e 2 (COX-1 e 2), sendo alguns mais seletivos para a COX-2 (induzida), impedindo assim a conversão do ácido araquidônico em prostaglandinas (DAY; GRAHAM 2013). Já os anti-inflamatórios esteroidais atuam através da ligação com o receptor glicocorticoide no citoplasma das células, que por sua vez atuará na regulação da transcrição gênica, resultando na supressão de genes inflamatórios e ativação de genes anti-inflamatórios (BARNES 2006).

1.3 DOR NEUROPÁTICA

A dor neuropática é definida pela IASP (2018) como uma dor causada por uma lesão (trauma) ou doença (patologias associadas, como tumores, processos infecciosos, diabetes, doenças autoimunes e neurodegenerativas, ou induzida por quimioterápicos) do sistema nervoso somatossensorial, tendo origem em nervos periféricos ou centrais (neuropatia central) (KUNER; FLOR 2016; MEACHAM et al. 2017) (FIGURA 1).

Com foco na dor neuropática periférica, ela pode ser iniciada e sustentada pela hiperexcitabilidade das fibras aferentes (A e C) que promovem disparos ectópicos devido a superexpressão de diversos canais iônicos e receptores ao longo de seus axônios, como por exemplo: canais de sódio voltagem dependente (Na_v 1.3 e 1.6 em neurônios mielinizados, e Na_v 1.7 e 1.8 em amielinizados), canais de cálcio voltagem dependente (Ca_v), TRPs, como TRPV1-2 e TRPA1-M8. Em contrapartida, canais responsáveis por hiperpolarizar, repolarizar ou manter o limiar basal da membrana, como os canais de potássio voltagem dependente (K_v) e canais de potássio de dois poros (K_v2P), passam a ser menos expressos nessas fibras (FINNERUP; JENSEN 2006; MEACHAM et al. 2017; COLLOCA et al. 2017). Em adição, infiltrados de células imunes (neurófilos, macrófagos e mastócitos) contribuem ainda mais para a sensibilização e sustentação da hiperexitabilidade dos neurônios aferentes afetados através da liberação de citocinas inflamatórias (IL-1 β, IL-6, TNF-α, IL-17A e IL-5), ativando seus receptores e sensibilizando outros canais (Na_v) e TRPs (COHEN; MAO 2014; KUNER; FLOR 2016; COLLOCA et al. 2017; MEACHAM et al. 2017; PINHO-RIBEIRO et al. 2017; SOMMER et al. 2018).

As alterações que podem ocorrer a nível central (medula) em casos de neuropatia periférica são: elevada atividade de enzimas quinases que fosforilam receptores e canais, levando assim ao aumento da excitabilidade pós-sináptica; aumento da expressão de vesículas transportadores de glutamato, de neurotransmissores (aminoácidos e neuropeptídios); aumento da sinalização em receptores AMPA e NMDA; aumento da expressão de Ca_v; aumento da atividade da micróglia, glia e astrócitos com liberação de diversas quimiocinas; redução e prejuízo de mecanismos inibitórios da dor, como interneurônios gabaérgicos, bem como das vias descendentes (VON HEHN et al. 2012; COHEN; MAO 2014; KUNER; FLOR 2016; COLLOCA et al. 2017; MEACHAM et al. 2017; PINHO-RIBEIRO et al. 2017; SOMMER et al. 2018).

Outros fenomenos importantes podem ocorrer e contribuir para as manifestações clínicas da neuropatia, como a alteração de fenótipo das fibras, ou seja, fibras que normalmente não expressavam neuropeptídios (CGRP, SP e fator neurotrófico derivado do cérebro, BDNF), como as Aβ e Aδ, passam a expressar e alteram suas propriedades de transdução e transmissão da dor. Dependendo da lesão, alterações atróficas (denervações) podem diminuir o tamanho do corpo celular no GRD e o diâmetro do axônio, com eventual morte neural e redução do número de fibras. Isso pode causar tanto a diminuição da sensibilidade, quando hiperalgesia, além de privar o órgão de fatores de crescimento e outras neurotrofinas (VON HEHN et al. 2012; COHEN; MAO 2014; KUNER; FLOR 2016; COLLOCA et al. 2017; MEACHAM et al. 2017).

O oposto à perda de fibras pode ocorrer com brotamento contralateral de neurônios aferentes de outras fibras não lesadas em direção às áreas próximas ao trauma (formação de neuromas) e no GRD, ou ainda o brotamento de neurônios autonômicos simpáticos no GRD e expressão de receptores adrenérgicos α em fibras somatossensoriais aferentes. Esse tipo de dor, também chamada de síndrome complexa de dor regional, tem grande participação do sistema simpático, o qual leva à vasoconstrição local, redução da nutrição e oxigenação, bem como alterações de temperatura e/ou cor nas extremidades afetadas, seguidas de inchaço ou atrofia e dor agravada pelo frio ou estresse. Em adição, no GRD pode também ocorrer o brotamento de fibras Aβ em lâminas I e II (SCHMIDT; WILLIS 2007; COHEN; MAO 2014; KUNER; FLOR 2016; COLLOCA et al. 2017).

Em conjunto, essas alterações provocam nos pacientes acometidos dor espontânea em forma de queimação, pontadas, picadas e formigamento devido aos disparos ectópicos das fibras, alodinia mecânica e térmica ao frio, bem como hiperalgesia devido alterações centrais (FINNERUP; JENSEN 2006; JENSEN; FINNERUP 2014; MEACHAM et al. 2017).

O tratamento para a dor neuropática envolve uma ampla gama de medicamentos, sendo os de primeira escolha: inibidores da recaptação de serotonina (duloxetina e venlafaxina), antidepressivos tricíclicos (amitriptilina e imipramina) e anticonvulsivantes ligantes da subunidade α2δ de canais de cálcio (gabapentina e pregabalina). Os de segunda escolha incluem: opioide (tramadol), gel ou *patches* de capsaicina (8%, agonista TRPV1) e anestésico local bloqueador de canal de sódio (lidocaína). Por fim, como terceira linha sugerem-se: opioides fortes (morfina, oxicodona e metadona) e neurotoxina que atua pelo bloqueio neuromuscular e inibição da liberação de acetilcolina (toxina botulínica A) (FINNERUP et al. 2015; ATTAL 2018).

1.4 DOR PÓS-OPERATÓRIA

A dor pós-operatória é caracterizada por ser uma dor aguda e decorrente da sensibilização periférica e central. Dependendo da área e extensão da cirurgia, é comum ocorrer traumas teciduais e consequente ativação e sensibilização de fibras aferentes primárias (C e Aδ). Como consequência destes eventos, muitos pacientes apresentam dor em repouso ou em movimento, bem como alodinia e hiperalgesia no local e em regiões próximas à lesão (POGATZKI-ZAHN et al. 2017).

A sensibilização periférica pode ocorrer em decorrência da: diminuição do pH (~ 6,8) e pressão de oxigênio no sítio cirúrgico, com consequente aumento da concentração de lactato e prótons, contribuindo para a ativação de canais iônicos ASIC presentes nas fibras nociceptivas (KIM et al. 2007; KANG et al. 2013; KIDO et al. 2013); migração de neutrófilos e liberação de citocinas (IL-6, IL-1β e TNF-α), fator de crescimento nervoso (NGF) e síntese de PGE_2 (SAHBAIE et al. 2012); e migração de mastócitos com consequente liberação de histamina, serotonina (5-HT) e triptases (ativação do receptor de protease 2, PAR2) (OLIVEIRA et al. 2011, 2017).

Por sua vez, a sensibilização central decorre principalmente da ativação de receptores AMPA (fosforilação da subunidade GluR1 do receptor via fosfoquinase Cγ), e independentemente de receptores NMDA (ZAHN et al. 2005). Outros mecanismos também vêm sendo propostos, como: ativação de quinases extracelulares fosforiladas reguladas por sinal (ERK) 1/2 em neurônios e micróglia (SHI et al. 2013; VAN DEN HEUVEL et al. 2015), bem como desregulação do sistema endocanabinóide endógeno (inibição de receptores canabinóides espinhais do tipo 1 e 2) e da proteína quinase ativada por mitógeno (do inglês, *mitogen-activated protein kinase*, MAPK) fosfatase (MKP)-3 (SAHA et al. 2013).

Além disso, a dor pós-operatória pode envolver tanto componentes inflamatórios, quanto neuropáticos, uma vez que durante o processo podem ocorrer lesões em nervos, levando a disparos de potenciais de ação espontâneos (POGATZKI-ZAHN et al. 2017) (FIGURA 1).

O tratamento da dor pós-cirúrgica tem como objetivo, além de promover seu alívio, evitar a sua cronificação (LAVAND'HOMME 2017). O arsenal terapêutico para o tratamento da dor pós-operatória envolve anti-inflamatórios não esteroidais e corticosteroides, opioides, antagonista NMDA (quetamina), anestésicos locais e anticonvulsivantes ligantes da subunidade α2δ de canais de cálcio (CHOU et al. 2016).

1.5 POTENCIAL TERAPÊUTICO DOS PRODUTOS NATURAIS

Os produtos naturais obtidos de plantas são resultado de seu metabolismo primário e secundário. O metabolismo primário produz metabólitos que têm importância funcional e vital para a planta, como por exemplo os carboidratos, proteínas, lipídios e ácidos nucleicos. Esses metabólitos primários são encontrados com baixa variabilidade entre outros exemplares da mesma planta e sofrem pouca influência de fatores externos. Por outro lado, os produtos de biossíntese do metabolismo secundário, ou seja, os metabólitos secundários, tem produção extremamente limitada por fatores ambientais e geográficos, sendo encontrados em concentrações variadas entre as plantas. Esses compostos têm papel ecológico e adaptativo ao meio, proporcionando proteção contra herbívoros e microrganismos, proteção contra raios ultra-violeta e defensores agrícolas, atração de polinizadores, atração de animais dispersores de sementes. Geralmente estes metabólitos apresentam estruturas únicas e complexas, bem como atividades biológicas que despertam interesse de pesquisadores e indústrias das áreas farmacêutica, médica, cosmética, alimentícia e agronômica. Dentre os muitos metabólitos secundários, podemos citar: flavonóides, alcalóides, terpenóides, taninos, alquilamidas, etc. (PAUL 2002).

Esses metabólitos podem estar presentes tanto em preparações tradicionais, como as infusões (chás) de plantas medicinais, quanto em produtos industrializados, como nos medicamentos. É importante mencionar que, segundo a Resolução da Diretoria Colegiada (RDC) n° 26/2014, planta medicinal é definida como espécie vegetal utilizada com propósitos terapêuticos, e quando seca e estabilizada, passa a ser denominada de droga vegetal. Ambas podem ser utilizadas para a preparações de chás por meio de infusão, decocção ou maceração em água.

A planta fresca ou droga vegetal pode ser submetida a processos de extração e fracionamento químico e físico, resultando em derivados vegetais que contém as substâncias responsáveis pelas suas ações terapêuticas, os fitocomplexos (conjunto de todos os metabolitos primários ou secundários). Subsequentemente, os fitocomplexos, ou ainda compostos isolados, podem dar origem a medicamentos, os quais são também definidos pela RDC nº 26/2014. Os fitoterápicos são medicamentos obtidos com emprego exclusivo de matérias-primas ativas vegetais, tendo sua eficácia e segurança baseada em evidências clínicas, podendo ser simples (uma espécie vegetal), ou composto (mais de uma espécie vegetal). Por outro lado, os produtos tradicionais fitoterápicos também são aqueles obtidos com emprego exclusivo de matérias-primas ativas vegetais, entretanto sua segurança e efetividade são baseadas em dados publicados na literatura técnico-científica. Ambos têm finalidade profilática, curativa ou paliativa, mas diferem no ato de registro: os fitoterápicos devem apresentar registro, e os produtos tradicionais fitoterápicos, registro ou notificação. Além disso, este último não pode ser indicado para doenças graves, nem administrado pelas vias injetável e oftálmica. Vale ressaltar que substâncias ativas isoladas (sintéticas, semissintéticas ou naturais), ou em associação com extratos (vegetais ou animais), não são considerados como fitoterápico ou produto tradicional fitoterápico.

O uso de plantas medicinais como remédios teve seus primeiros registros documentados a milhares de anos atrás por civilizações mesopotâmicas, egípcias, e gregas (CHEN et al. 2017). Desde então, em países em desenvolvimento da Ásia, África e América latina, o uso desses recursos naturais como tratamento primário de doenças é estimado em 70 – 95%. Já em países desenvolvidos, 70 – 90% das pessoas utilizam produtos naturais como complementação aos tratamentos (WHO 2011).

A história mostra que a dor foi importante para o descobrimento dos primeiros fármacos e medicamentos, os quais tem ligação direta com as plantas medicinais e seus princípios ativos. Na Alemanha, em 1806, Friedrich Serturner deu início ao desenvolvimento de medicamentos a partir de plantas com o isolamento da morfina proveniente da *Papaver somnniferum,* um dos primeiros e mais importantes princípios ativos utilizados para o tratamento de dor. Em adição, outra planta muito tradicional da América do Sul, mais precisamente do Peru, despertou o interesse dos europeus devido sua atividade estimulante. Em 1530, as folhas de *Erythroxylon coca* foram levadas para a Europa e intensamente estudadas. Em 1859 – 1860, os químicos alemães, Albert Niemann e Wilhelm Lossen, isolaram pela primeira vez o alcaloide cocaína. Na sequência, em Viena, o farmacologista Karl Damian Ritter von Schroff percebeu a interessante propriedade anestésica deste composto. Mais tarde, apenas

em 1884, que, Sigmund Freud, propôs o uso da cocaína como anestésico, e seu amigo, Carl Koller realizou pela primeira vez a sua aplicação como tal. Entretanto, a intensa atividade no sistema nervoso central, efeitos tóxicos, e o risco de dependência química, tornaram necessário o descobrimento de outros anestésicos. Sendo assim, em 1900 e 1905, o alemão Alfred Eihorn sintetizou a benzocaína e procaína, respectivamente, dando início a uma grande sequência de descobertas de novos anestésicos locais. Drogas como a lidocaína, a primeira derivada amida utilizada na clínica, foi sintetizada em 1944 e vem sendo utilizada até os dias atuais (RUETSCH et al. 2001).

Entretanto, foi a partir de 1828 que ocorreu o marco do desenvolvimento das indústrias farmacêuticas. O descobrimento da salicilina, princípio ativo da *Salix alba*, pelo alemão Johann Buchner, e as seguidas modificações estruturais em ácido salicílico por Raffaele Piria (1839), e em ácido acetil salicílico por Felix Hoffman (1897), fizeram com que se chegasse a um dos mais utilizados medicamentos da história, a aspirina. Este feito proporcionou uma das primeiras patentes do ramo, além de impulsionar e fortalecer uma das mais poderosas indústrias farmacêuticas, a Bayer (DUTRA et al. 2016).

Desde então, estima-se que 49% dos fármacos aprovados pelo FDA (do inglês *Food and Drug Administration*) são diretamente produzidos ou derivados de produtos naturais (NEWMAN; CRAGG 2016). O mercado de produtos naturais cresce ano após ano e já atinge 83 bilhões de dólares (WHO 2011), sendo 28 bilhões apenas do mercado de fitoterápicos, distribuído principalmente entre Europa, Ásia e Estados Unidos. Apesar do Brasil ser detentor da maior biodiversidade do planeta (20 – 22%) com aproximadamente 45.000 espécies de plantas, o nosso mercado de fitoterápicos movimenta apenas 261 milhões de dólares, menos de 5% do total (DUTRA et al. 2016). Mesmo com cifrões modestos, o número de publicações científicas brasileiras com produtos naturais surpreende. Até 2015, foram publicados 34.614 artigos, sendo aproximadamente um terço publicado entre 2011 e 2013 (DUTRA et al. 2016).

Devido ao crescente interesse popular e institucional, as plantas medicinais e fitoterápicos vem sendo gradativamente incluídos no âmbito do Sistema Único de Saúde (SUS). Em 2006 foi criada a Política Nacional de Plantas Medicinais e Fitoterápicos, pelo Decreto nº 5.813, que tem como principal objetivo "garantir à população brasileira o acesso seguro e o uso racional de plantas medicinais e fitoterápicos, promovendo o uso sustentável da biodiversidade, o desenvolvimento da cadeia produtiva e da indústria nacional". Portanto, com o intuito de atingir este objetivo, as ações necessárias para tal foram detalhadas em 2008 no Programa Nacional de Plantas Medicinais e Fitoterápicos, aprovada pela Portaria Interministerial nº 2.960. Dentre muitas ações, podemos destacar: a inserção de plantas medicinais e fitoterápicos

no SUS, visando sempre segurança, eficácia e qualidade; reconhecimento das práticas populares e tradicionais que se baseiam no uso de plantas medicinais e remédios caseiros, bem como o desenvolvimento de estratégias de comunicação, formação técnico-científica e capacitação no setor de plantas medicinais e fitoterápicos para melhor atender as necessidades da população; desenvolvimento de instrumentos de fomento à pesquisa, tecnologias e inovações em plantas medicinais e fitoterápicos; e promoção do uso sustentável da biodiversidade de nosso país. Estas práticas trabalham em conjunto com a Política Nacional de Práticas Integrativas e Complementares do SUS, aprovada pela portaria nº 971/2006, que se propõe garantir o acesso aos medicamentos fitoterápicos, além do monitoramento da qualidade dos mesmos pelo Sistema Nacional de Vigilância Sanitária, a qual é coordenada pela Agência Nacional de Vigilância Sanitária (ANVISA). Em conjunto, elas controlam e regulam a produção e venda de plantas medicinais e fitoterápicos no Brasil (PALHARES et al. 2015).

A Relação Nacional de Medicamentos Essenciais (RENAME) atualmente inclui 13 medicamentos fitoterápicos para uso ginecológico, tratamento de queimaduras, artrite, osteoartrite e auxiliares terapêuticos de gastrite e úlcera. Como parte dos programas mencionadas anteriormente, em 2009 foi divulgada uma Relação de Plantas Medicinais de Interesse ao SUS (RENISUS). Composta por 71 plantas, ela tem como intuito orientar a cadeia produtiva e o desenvolvimento de pesquisas. Dentre elas: *Lippia alba* (Verbenaceae), *Arnica montana* (Asteraceae), *Calendula officinalis* (Asteraceae), *Harpagophytum procumbens* (Pedaliaceae), *Uncaria tomentosa* (Rubiaceae), *Vernonia condensata* (Asteraceae), *Casearia sylvestri*s (Salicaceae), e *Zingiber officinale* (Zingiberaceae), apresentam indicações para tratamento de condições dolorosas.

Os avanços são revelados pela aceitação da população e de profissionais da saúde. No Brasil, 2.160 Unidades Básicas de Saúde já disponibilizam fitoterápicos ou plantas medicinais, sendo que 260 disponibilizam a planta *in natura*, 188 a droga vegetal, 333 o fitoterápico manipulado e 1.647 o fitoterápico industrializado. Em 2017, foram realizados 66.445 atendimentos de fitoterapia, distribuídos em 1.794 estabelecimentos da Atenção Básica em 1.145 municípios. A dispensação de fitoterápicos em 2017 e 2018 chegaram a 1.888.826 e 2.328.919, respectivamente. Além disso, em 80 municípios é praticada a Farmácia Viva, a qual disponibiliza plantas medicinais como auxiliar no tratamento de doenças de menor gravidade (SISAB – Sistema de Informação em Saúde para a Atenção Básica, Plantas medicinais e fitoterápicos no SUS, 2019).

Coincidentemente, o primeiro fitoterápico genuinamente brasileiro tem indicações analgésicas e anti-inflamatórias. O Acheflan®, produzido com o óleo de *Cordia verbenacea*, está dentre os 15 fitoterápicos mais vendidos no Brasil (DUTRA et al. 2016). Em uma revisão recente, Newman e Cragg (2016) mostraram que de 1981 a 2014 foram aprovados 1562 novos medicamentos pelo FDA, sendo 17 analgésicos, 5 anestésicos, e 51 anti-inflamatórios. Dos analgésicos, 5 deles apresentavam relação com produtos naturais, sendo que apenas 1 foi derivado direto, 3 mimetizavam e 2 apresentavam grupamentos farmacofóricos derivados de um produto natural. Todos os anestésicos foram moléculas sintéticas. E ao que diz respeito dos anti-inflamatórios, 1 foi uma biomacromolécula e 13 derivados direto de produtos naturais.

Apesar da maioria dos medicamentos lançados no mercado ainda terem natureza sintética, a utilização de produtos naturais no processo de desenvolvimento de novos fármacos pode facilitar o descobrimento de potenciais moléculas alvo. Estima-se que em um universo de aproximadamente 20 milhões de químicos sintéticos, menos de 20% apresentarão atividade biológica relevante. Em contra partida, de 160 mil compostos naturais, ao final de uma triagem, 80% apresentarão potencial atividade biológica, representando assim um caminho mais curto na descoberta de novas moléculas alvo (HARVEY et al. 2015).

Novas técnicas e ferramentas, como a bioinformática, ou o refinamento de outras já existentes, vêm fortalecendo e impulsionando a pesquisa com base em produtos naturais. O rastreio e triagem (*screening*) de moléculas alvo tem sido facilitada pela utilização de diversas bibliotecas digitais e banco de dados de produtos naturais (ex.: *Dictionary of Natural Products*). As melhorias nos processos de extração, purificação e caracterização de moléculas, além da possibilidade de prever a interação da molécula com seu alvo farmacológico através de estudos de relação estrutura-atividade quantitativa (do inglês*, quantitative structure−activity relationship*) utilizando *softwares* computacionais e interação proteína-proteína *in vitro*, otimizam a pesquisa pré-clínica e direcionam estudos *in vivo*. O "rastreio de alto rendimento" *in vitro* (do inglês*, high-throughput screening*), combinado às abordagens genômicas e metabolômicas, vem acelerando a descoberta de moléculas alvo e otimizando a biossíntese de metabólitos secundários. Por fim, um conceito e prática recentemente introduzidos sugerem a otimização do processo de descoberta de novos fármacos utilizando produtos naturais como pontos de partida para síntese e modelagem química, tendo seus farmacóforos (parte da molécula essencial à atividade) como inspiração para novas descobertas (HARVEY et al. 2015; CHEN et al. 2017; RODRIGUES et al. 2016). Como resultado de todas essas novas abordagens citadas acima, temos que aproximadamente 15% dos ensaios clínicos realizados atualmente estão relacionados com plantas medicinais (HARVEY et al. 2015).

Sendo assim, para aproveitar toda a biodiversidade que há em nosso país, algumas mudanças e melhorias são necessárias. A implementação de leis de proteção e acesso à biodiversidade, como o aperfeiçoamento do Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, Lei da Biodiversidade nº 13.123/2015 e decreto nº 8.772/2016 que a regulamenta, juntamente com fomentos à pesquisa e parcerias entre universidades e indústrias, são pontos cruciais para o desenvolvimento de científico na área de produtos naturais. É imprescindível a mudança da relutância de pesquisadores em trabalhar com produtos naturais; além de mais estudos toxicológicos e farmacocinéticos dos produtos. Contudo, há empecilhos que são intrínsecos à esta área, como: a complexidade e/ou o baixo rendimento de muitas moléculas, tornando-as de difícil síntese e produção em larga escala (CALIXTO 2003; DUTRA et al. 2016).

1.6 *Acmella oleracea* E ALQUILAMIDAS

A *Acmella oleracea* (L.) R.K. Jansen, Asteraceae (sinônimos *Spilanthes acmella* (L.) Murr., *Spilanthes acmella* var. oleracea (L.) C.B. Clarke, dentre outros), é popularmente conhecida como “jambu” ou agrião-do-pará. Trata-se de uma planta herbácea anual, perene, semi-ereta ou quase rasteira que possui 20 – 40 cm de altura. Apresenta caule cilíndrico, carnoso e de ramos decumbentes. Possui raiz pivotante com inúmeras ramificações laterais com folhas compostas, opostas, membranáceas e pecioladas. As flores são pequenas, amareladas, com áreas púrpuras na pálea do cálice, dispostas em capítulos globosos terminais (FIGURA 2) (FAVORETO; GILBERT 2010).

FIGURA 2 – FOLHAS E FLORES DO JAMBU.

FONTE: disponível e adaptado de: <http://armazemdasespeciarias.com.br/wp-content/uploads/2015/11/shutterstock_147228356.jpg>.

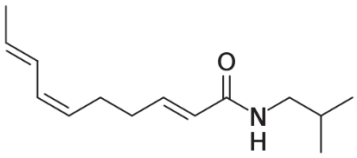
O jambu é uma planta nativa da Amazônia e muito cultivado no estado do Pará. Por crescer facilmente em regiões de clima úmido e quente, ele foi introduzido ao resto do mundo através dos trópicos e subtrópicos, sendo hoje encontrado no norte da Austrália, África, Ásia, Índia e Sri Lanka (LIM 2016).

Devido ao sabor acre e pungente de suas flores e folhas, e à propriedade sialagoga (salivante), o jambu é tradicionalmente utilizado como condimento e tempero na culinária local do norte do Brasil em pratos como o pato no tucupi e tacacá, e para abrir o apetite (FAVORETO; GILBERT 2010).

O jambu é rico em diversos metabólitos secundários, como os óleos essenciais (limoneno e β-cariofileno), fenóis (ácido vinílico e ácido trans-ferulico), fitoesterois (álcool mirístico e estigmasterol), triterpenos (α- e β-amirina), e ésteres (ácido láurico, mirístico, palmítico, linoléico e linolênico) (PRACHAYASITTIKUL et al. 2009; FAVORETO; GILBERT 2010). Entretanto, esta planta apresenta em abundância outra classe de metabólitos ativos, chamados de alquilamidas (isobutilamidas), sendo a principal a (2E,6Z,8E)-N-isobutil-2,6,8-decatrienamida, ou espilantol (CHENG et al. 2015) (FIGURA 3). Diversas outras alquilamidas já foram descritas em flores e folhas do jambu. Boonen et al. (2010) descreveu 11 diferentes alquilamidas (oito delas isobutilamidas, duas metilbutilamidas e uma feniletilamida) em um extrato etanólico de jambu, sendo 88,8% delas o espilantol. Semelhantemente, Cheng et al. (2015), também demonstrou que o espilantol era a alquilamida majoritária em um extrato etanólico das flores (84.5 mg/g).

As alquilamidas são um grupo promissor de metabólitos secundários e são constituídas por uma cadeia carbônica alifática e insaturada (“cauda” apolar, ácido graxo), ligada a um grupo funcional amida (“cabeça” polar), que por sua vez pode conter grupos radicais variados (isobutil, metil, fenil, benzil, etc.) (BOONEN et al. 2012; GREGER 2016).

FIGURA 3 – ESTRUTURA QUÍMICA DO ESPILANTOL.



FONTE: adaptado de BOONEN, et al. (2010).

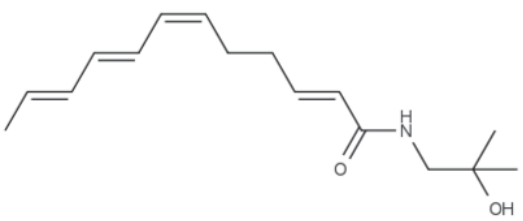
Estas moléculas são responsáveis por causar uma sensação única de formigamento e dormência no local onde é aplicada, principalmente na mucosa oral sugerindo um efeito anestésico. Essas sensações quimiotáticas justificam seu uso na medicina popular para o tratamento de dor de dente, além das diversas aplicações na culinária, em produtos farmacêuticos e cosméticos (PRACHAYASITTIKUL et al. 2013).

Além de ser indicado como anestésico local na medicina popular, o jambu também é indicado para: ferimentos na boca e cicatrizante; como antitussígeno, antiasmático e antigripal; antibacteriano, antifúngico, antimalárico, antituberculose; contra problemas hepáticos, estomacais e digestivos; estimulante sexual; inseticida e desinfetante (FAVORETO; GILBERT; 2010; LIM 2016; PRACHAYASITTIKUL et al. 2013) .

Muitos estudos já demonstraram os efeitos anti-inflamatório e antinociceptivo de diferentes extratos do jambu, administrados de maneira sistêmica, em diferentes modelos de dor (CHAKRABORTY et al. 2004; RATNASOORIYA et al. 2005; NOMURA et al. 2013; RIOS; OLIVO 2014).

As alquilamidas podem ser encontradas em plantas dos gêneros da família Asteraceae, como: *Anacyclus*, *Echinacea* e *Heliopsis*, mas também do gênero *Zanthoxylum* da família Rutaceae (GREGER 2016). As plantas do gênero *Zanthoxylum*, como a *Szechuan pepper* (*Zanthoxylum piperitum*), são nativas da África e Ásia, onde são conhecidas como "árvores da dor-de-dente" (*toothache trees*) e tradicionalmente usadas para tratar dor de dente e artrite (TSUNOZAKI et al. 2013). Da mesma maneira que o jambu, a *Szechuan pepper* é rica em alquilamidas, sendo a principal o sanshool (FIGURA 4). Já foi atribuído a este composto o bloqueio de canais de sódio presentes nos nociceptores Aδ, e assim explicando suas sensações de anestesia, dormência e formigamento (TSUNOZAKI et al. 2013).

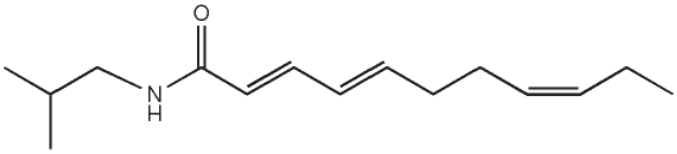
FIGURA 4 – ESTRUTURA QUÍMICA DO SANSHOOL.



FONTE: BAUTISTA et al. (2008).

Entretanto, estudos utilizando sanshool e seu derivado sintético mais estável, a isobutilalquil amida (N-isobutil (2E, 4E, 8Z)-unadeca-2,4,8-trienamida, ou apenas IBA, FIGURA 5), demonstraram que, quando injetados via intraplantar em ratos, estes compostos induziram comportamentos tipo nociceptivos de lambida na pata, bem como aversão quando dados na água de beber (BRYANT; MEZINE 1999; SUGAI et al. 2005; LENNERTZ et al. 2010; KLEIN et al. 2011). Estes comportamentos foram atribuídos principalmente à ativação de canais TRPV1 e TRPA1 em neurônios do gânglio da raiz dorsal e fibras aferentes primárias, bloqueio de canais de potássio de dois poros (KCNK 3, 9 E 18), além da ativação de neurônios da ampla faixa dinâmica e de mecanorreceptores de baixo limiar (LENNERTZ et al. 2010; SUGAI et al. 2005; BAUTISTA et al. 2008;TULLEUDA et al. 2011; KOO et al. 2007; KLEIN et al. 2011; SAWYER et al. 2009).

FIGURA 5 – ESTRUTURA QUÍMICA DA IBA.



FONTE: ALBIN; SIMONS (2010).

Mais de 300 alquilamidas pertencentes a mais de 25 famílias de plantas já foram descritas na literatura. De modo geral, esses compostos apresentam diversas atividades biológicas, como: pronociceptivo, antinociceptivo, analgésico, anti-inflamatório e imunomodulador (BAUTISTA et al. 2008; GULLEDGE et al. 2018; DE LA ROSA-LUGO et al. 2017; NOMURA et al. 2013; RADUNER et al. 2006; GERTSCH 2008). Este potencial farmacológico resulta em diversas pesquisas científicas, patentes e ensaios clínicos. Recentemente uma revisão publicada por Silveira et al. (2018), mostrou que o espilantol esteve envolvido em 1.444 patentes depositadas entre os anos de 1996 e 2016, sendo 30 sobre suas propriedades farmacológicas, 30 com aplicação em cosméticos, 31 de métodos de obtenção, e 406 sobre propriedades sensoriais.

Neste sentido, três patentes no ramo cosmético depositadas pelas empresas Natura Cosmeticos AS (Brasil), Gattefossé SAS (França) e Dr. Belfer William (Estados Unidos da América, EUA), estavam associadas ao efeito "anti-idade" (do inglês, *anti-age*), por provocar relaxamento facial e "anestesia" local. O depósito da empresa francesa foi publicado em 8 territórios (Áustria, Brasil, Canada, Europa, França, Japão, Espanha e EUA), originando os produtos Gatuline® Expression AF e Gatuline® In-tense. Por sua vez, a patente brasileira foi depositada em 4 territórios (Brasil, Canada, Europa, e Estados Unidos), e deu origem ao produto Spilol®. Ainda, o jambu e o espilantol são aplicados em diversos produtos de higiene pessoal e bucal, como os géis Buccaldol® (Alphamega, França) e Indolphar® (ID Phar, Bélgica).

As diversas propriedades sensoriais de extratos ricos em espilantol, ou dele puro, vem sendo agregadas em produtos alimentícios e bebidas por muitas empresas, como a OGAWA & CO (Japão), a qual utiliza a alquilamida para realçar o sabor e dar refrescância a bebidas com gás; a Kraft Foods Global Brands LCC (EUA) utiliza espilantol para estimular a salivação em composições orais que deixam a boca seca; e pela empresa Symrise GMBH & CO.KG (Alemanha), que utiliza o espilantol em comidas e suplementos dietéticos devido à sua pungência. A tendência de utilização dessa alquilamida em diferentes formulações e áreas farmacêuticas, alimentícias e cosméticas tende a aumentar. Uma patente de 2009 da empresa japonesa Takasago Perfumary CO menciona um método de baixo custo para síntese do espilantol em larga escala. Por enquanto, a média de preço do espilantol varia de U$ 150 – 300,00/5 mg.

A partir de uma pesquisa breve em "clinicaltrials.gov", utilizando a palavra "alkylamide" como ferramenta de busca, é possível encontrar três estudos clínicos com caráter de intervenção. O primeiro foi postado em 2013 (EUA, NCT02003651) e está ainda em andamento utilizando um extrato alcoólico de *Echinacea* (Quick Defense®, Gaia Herbs) para gripe em mulheres de 18 a 55 anos. O segundo (NCT02187549), postado em 2014 e ainda em andamento (EUA), avalia a segurança e efetividade de uma alquilamida isolada "HYADD" em hidro-gel administrada via intra-articular em adultos com osteoartrite nos joelhos. E, por fim, o terceiro foi postado em 2017 (Suíça, NCT03070314) e estava em fase 4, mas foi suspenso em 2018 por falta de participantes (crianças de 4 a 12 anos). Ele tinha o intuito de avaliar o efeito de um extrato alcoólico de *Echinacea* (Echinaforce Jurior®) rico em alquilamidas para o tratamento de gripe. Vale ressaltar que já existe no mercado o Echinaforce® (A.vogel, Suiça) com indicações para prevenir e tratar resfriados, gripes e infecções do trato respiratório superior.

1.7 OBJETIVO GERAL

Investigar os efeitos promovidos pela administração local (intraplantar) da fração hexânica (FH) rica em alquilamidas obtidas das flores de *Acmella oleracea,* bem como da isobutilalquil amida sintética (IBA), em modelos experimentais de dor aguda para elucidar seus mecanismos de ação (artigo científico 1), e também em modelos de dor inflamatória aguda (artigo científico 2), neuropática e pós-operatória em camundongos (artigo científico 3).

1.7.1 Objetivos específicos

Artigo Científico 1:

- Caracterização fitoquímica da fração hexânica (FH) obtidas das flores de *Acmella oleracea*;
- Avaliar o comportamento nociceptivo induzido pela administração local (via intraplantar, i.pl.) de doses crescentes de FH e IBA em camundongos;
- Avaliar o efeito antinociceptivo promovido pelo tratamento local com FH e IBA em baixa dose no modelo de nocicepção induzida por formalina em camundongos;
- Avaliar o envolvimento de canais TRPV1 e TRPA1 nos efeitos antinociceptivo e pronociceptivo promovidos pela injeção intraplantar FH e IBA em dose baixa e elevada em camundongos;
- Avaliar o envolvimento do sistema opioidérgico nos efeitos antinociceptivo e pronociceptivo promovidos pela injeção intraplantar FH e IBA em dose baixa e elevada em camundongos;
- Avaliar o envolvimento do sistema histaminérgico, bem como a participação dos mastócitos, nos efeitos antinociceptivo e pronociceptivo promovidos pela injeção intraplantar FH e IBA em dose baixa e elevada em camundongos;
- Avaliar os efeitos locais promovido pela injeção intraplantar FH e IBA no limiar mecânico e térmico dos animais em baixa e elevada dose;

Artigo Científico 2:

- Avaliar os efeitos dos pré-tratamentos locais com FH e IBA na alodinia mecânica e edema de pata induzidos pela injeção intraplantar de carragenina em camundongos;

- Avaliar os efeitos dos pré-tratamentos locais com FH e IBA (intraplantar) sobre os níveis de citocinas pró-inflamatórias (TNF-α e IL-1β), PGE_2, IL-10, migração celular (atividade enzimática da mieloperoxidase, MPO) e alterações no sistema antioxidante endógeno (níveis de lipoperóxidos, LOOH, atividade enzimática da catalase e superóxido dismutase, CAT, SOD, respectivamente; e níveis de glutationa, GSH) promovidas na superfície plantar dos camundongos decorrentes da injeção intraplantar de carragenina;

- Avaliar a atividade antioxidante da FH e IBA pelo método *in vitro* de sequestro do radical livre 2,2-difenil-1-picrilhidrazilo (DPPH);

Artigo Científico 3:

- Avaliar os efeitos dos pré-tratamentos locais com FH e IBA (via intraplantar) na alodinia mecânica e térmica ao frio, bem como alterações nos parâmetros de marcha, induzidas pela ligadura parcial do nervo ciático em camundongos;

- Avaliar os efeitos dos pré-tratamentos locais com FH e IBA (aplicação tópica) na alodinia mecânica e hiperalgesia térmica ao calor, bem como em escores de comportamentos nociceptivos espontâneos, induzidas pela cirurgia de incisão plantar em camundongos;

2 ARTIGO CIENTÍFICO 1

Distinct mechanisms underlying local antinociceptive and pronociceptive effects of alkylamides from *Acmella oleracea* flowers

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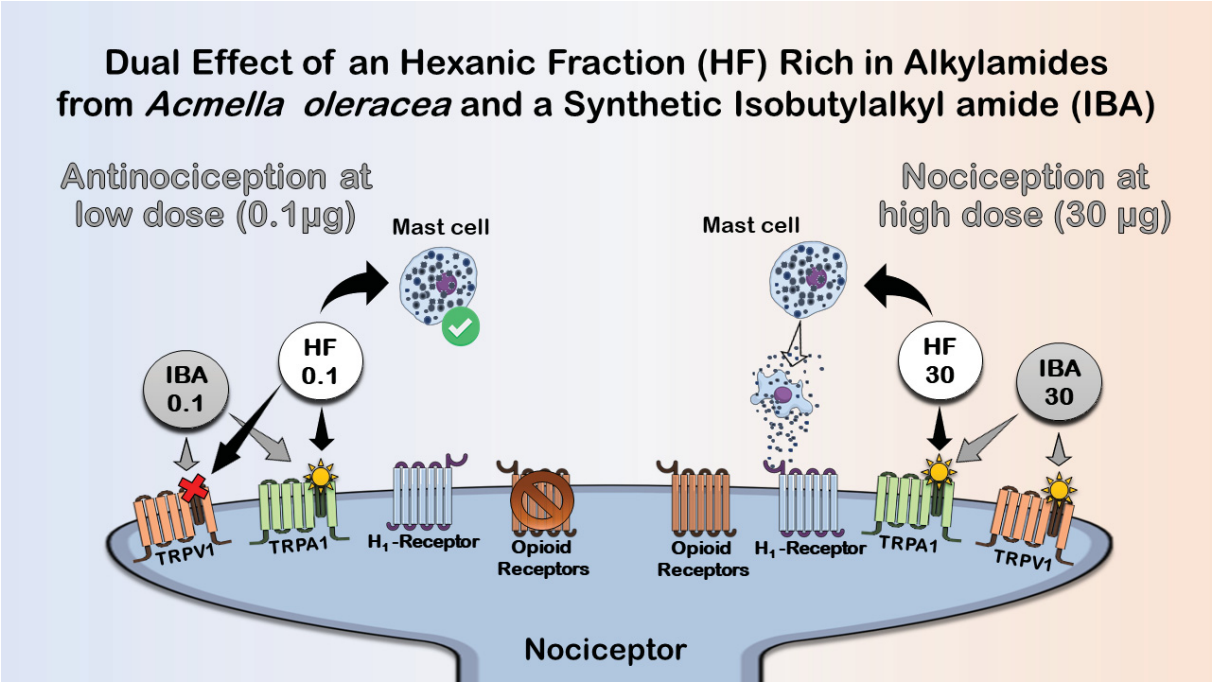
Graphic Abstract 1



Abstract

Acmella oleracea, popularly known as jambu is used in folk medicine to relief toothache. Jambu edible flowers are rich in alkylamides, mainly spilanthol, which are responsible to evoke chemesthetic sensations. This study aimed to investigate the local effects promoted by the intraplantar injection of the hexanic fraction (HF) rich in alkylamides from jambu flowers and compare to the synthetic isobutylamide (IBA). Swiss male mice were intraplantarly administrated with crescents doses of HF and IBA (0.1 – 30 μg/20 μL), and the underlying mechanisms associated to the antinociceptive (0.1 μg) and pronociceptive (30 μg) effects were evaluated in chemical and sensorial tests. HF and IBA (0.1 μg) promoted analgesia in neurogenic and inflammatory phases of formalin test, against glutamate-induced nociception and independent of the activation of endogenous opioidergic system and dependent of TRPV1 modulation, whereas only HF reduced both nociception and mast cell degranulation in hindpaw induced by C48/80. However, both alkylamides potentiated the TRPA1 mediated nociception. In contrast, HF and IBA (30 μg)-evoked nociceptive behaviors were reduced by the activation of endogenous opioidergic system, by TRPA1 antagonist and TRP nociceptive fibers desensitization. In addition, IBA-evoked nociception depends of TRPV1 receptors, and histamine from degranulated mast cells mediates only the HF-induced nociception. Furthermore, on the contrary of IBA, HF elevated both mechanical and thermal paw threshold. Altogether, these results indicate that alkylamides could elicited dual effects, adding new evidences and mechanisms for these opposite actions. Although further research is needed, we confirmed that alkylamides from jambu displays local analgesic and/or anesthetic effects.

Keywords: Acmella oleracea; jambu; alkylamides; analgesia; pain; TRP receptors

1 Introduction

Acute pain is an important response to protect the body against noxious stimuli. In response to a chemical, mechanical or thermal stimulus, peripheral nociceptors are activated, carrying the nociceptive response to the central nervous system (CNS). However, persistent inflammatory or neuropathic pain, changes the perception of noxious stimuli, due to a shift in the somatosensorial system. Currently, non-steroidal anti-inflammatory drugs, opioids, local anesthetics, anticonvulsants, and antidepressants are employed as analgesic drugs, despite their side effects on health-related quality of life (Woller et al. 2017). Since pain constitutes a public health problem, find new substances that effectively prevent and/or treat pain conditions with minimal adverse effects is burning. Thus, it is remarkable that naturally active principles, mainly from medicinal plants are considered a valuable source for the development of new analgesic drugs (Dutra et al. 2016).

Acmella oleracea (L.) R.K. Jansen (bas. *Spilanthes oleracea*; syn. *Spilanthes acmella* var. *oleracea*; Asteraceae) deserves special attention due to its biological properties. *A. oleracea* is native of South America and distributed worldwide in tropical climate. In Northern Brazil it is popularly known as "jambu", where its fresh leaves are appreciated as a spicy in typical local dishes, due its sialagogue property, pungent and acrid taste. Moreover, in folk medicine jambu flowers are chewing to treat toothache, promoting tingling, numbing and local anesthesia sensations in the mouth (Dubey et al. 2013). This chemesthetic sensations in oral mucosa are strongly related to the presence of alkylamides, which the main and most abundant in jambu flowers is represented by spilanthol. These class of secondary metabolites is basically N-isobutylamides constituted by saturated and unsaturated aliphatic chains linked to an amide group, whose general structures may vary the number of carbons, the position of unsaturated bonds and radical groups (isobutyl, benzyl or methyl group) (Greger 2016).

Szechuan pepper (*Zanthoxylum piperitum*, Rutaceae) or “toothache trees” is widely used in Asian cultures to treat inflammatory pain, like toothache and arthritis (Tsunozaki et al. 2013). Szechuan peppers are rich in sanshool, an active alkylamide that also induces chemesthetic sensations. Interestingly, when sanshool and its synthetic and stable derivative analog named N-isobutyl (2E, 4E, 8Z)-unadeca-2,4,8-trienamida (IBA) were intraplantarly administrated in animals, they evoked nociceptive-like behaviors and aversion when offered in drinking water (Lennertz et al. 2010; Klein et al. 2011).

Pioneer studies with capsaicin and cinnamaldehyde started to elucidate the mechanisms underlying the pungent and paresthetic sensations (Caterina et al. 1997; Bandell et

al. 2004). Nevertheless, the respectively burning and cooling sensations related to the TRPV1 activation by capsaicin and TRPA1 activation by cinnamaldehyde seems to be different to that evoked by sanshool, IBA, and spilanthol (Bryant and Mezine 1999; Sugai et al. 2005; Albin and Simons 2010).

Thus, considering the pungency, paresthesia and analgesia associated to the alkylamides, our aim was to investigate the effects promoted by intraplantar injection of an hexanic fraction (HF) rich in alkylamides from jambu flowers comparing to IBA, in different nociceptive models to better understand the mechanisms of action behind antinociception and nociception promoted by this class of molecules.

2 Material and methods

2.1 Collection, preparation and analysis of hexanic fraction (HF) from *Acmella oleracea* flowers

A. oleracea (L.) R.K. Jansen was collected in Cruzeiro do Sul (Acre, Brazil) and a voucher specimen was deposited in the Herbarium of the Federal University of Acre - Zoobotanical Park (UFAC-PZ: 15099). Initially, jambu fresh flowers (60 g) were subjected to the extraction under reflux (3 h) with absolute ethanol to obtain the ethanolic extract (EEAO, 2.7 g) (Nomura et al, 2013). Subsequently, EEAO was evaporated under reduced pressure, resuspended in water and lyophilized (1.7 g). Part of the lyophilized EEAO was resuspended in ethanol-water (1:1, v/v) and subjected to the liquid-liquid partition in hexane, which provides the hexane fraction (HF, 0.2 g).

The HF was analyzed by gas chromatography-mass spectrometry detection (GC-MS – Varian 4000). The chromatography was developed in a capillary column VF5-MS (Varian) with 30 m x 0.25 mm (i.d.) and 0.25 μm of film thickness. The temperature was: initial 100 ºC, hold 2 min, then heated at 5 ºC/min to 280 ºC, with a total of 40 min. The compounds were detected by Ion Trap MS with electron ionization at 70 eV, which produced characteristic fragmentation profile useful for compound identification, that was developed by comparison of MS profile with those from literature.

2.2 Animals

Swiss (*Mus musculus*) male adult mice (25 - 30 g) were housed in a 12h light/dark cycle, controlled temperature (22 ± 2 ºC), air exhaustion and free access to water and food (Nuvilab CR-1, Quimtia S/A, Brazil). All experiments were approved by the local Ethics Committee of Animal

Experimentation (CEUA/BIO–UFPR, nº 970 and 1107) and conducted in agreement with the "Guide for the Care and Use of Laboratory Animals" (8th edition, National Research Council, 2011).

2.3 Effect of intraplantar injection of HF and IBA

Accordingly, to investigate the effects promoted by alkylamides, and to select the doses for the subsequent experiments, HF and IBA at 0.1; 0.3; 1; 3; 10 and 30 μg/20 μL or their respective vehicles of higher doses (V: 0.6% tween 80 and 6.5% DMSO, both in 0.9% saline) were intraplantarly (20 μl, i.pl.) injected in the plantar surface of the hindpaw. As indicative of nociceptive behavior, the amount of time that animals spent licking the injected paw was recorded from 0-5 min and at 5-10 min, using a stopwatch (Klein et al. 2011).

2.4 Effect of intraplantar injection of HF and IBA on formalin-induced nociception

The dose of 0.1 μg was selected to study the antinociceptive effects of both alkylamides. Mice were pretreated with HF or IBA at 0.1 μg/20 μL (i.pl.), diclofenac (a non-steroidal anti-inflammatory drug, 10 mg/kg, intraperitoneal, i.p.) or their respective vehicles (V: 0.002% tween 80 or 0.02% DMSO, both in 0.9% saline, i.pl. or 0.9% saline, 10 mL/kg, i.p.). After 15 and 30 min from the local and systemic pretreatments, respectively, the animals received the intraplantar injection of 2.5% formalin. As indicative of nociceptive behavior, the amount of time the animals spent licking the injected paw was recorded from 0-5 min and at 15-30 min after formalin injection (Tjølsen et al. 1992).

2.5 Participation of opioid system on dual action of HF and IBA

To investigate the involvement of opioid system in antinociception promoted by intraplantar injection of 0.1 μg HF and IBA, the animals were pretreated with naloxone (a non-selective opioid antagonist, 1 mg/kg, i.p) or vehicle (V: 0.9% saline, 10 mL/kg, i.p.). After 20 min, mice received HF or IBA at 0.1 μg/20 μL (i.pl.) and morphine (a non-selective opioid agonist, 1 mg/kg, subcutaneous, s.c) or their respective vehicles. Following 15 or 30 min from HF and IBA or morphine pretreatments, the nociception was induced by glutamate (20 μmol/20 μL, i.pl.) and the amount of time the animals spent licking the injected paw was recorded for 15 min (da Silva Lopes et al. 2012).

The higher dose of both alkylamides was then selected to study the nociceptive-like behaviors evoked by HF or IBA at 30 μg/20 μL (i.pl.), and the same protocol above described was carried out to evaluate the opioid participation in nociception.

2.6 Participation of TRP receptors on dual action of HF and IBA

To investigate the involvement of TRPV1 and TRPA1 receptors in the antinociception promoted by alkylamides, mice were pretreated with HF or IBA at 0.1 μg/20 μL (i.pl.) or their respective vehicles. After 15 min, animals received an intraplantar injection of capsaicin (TRPV1 agonist, 1 nmol/20 μL) or cinnamaldehyde (TRPA1 agonist, 200 nmol/20 μL). The amount of time the animals spent licking the injected paw was recorded for 5 min (Sakurada et al. 2003; da Costa et al. 2010).

On the other hand, to evaluate the involvement of TRPV1 in the nociception promoted by alkylamides, first animals were pretreated with capsazepine (TRPV1 antagonist, 10 nmol/20 μL, i.pl) or vehicle (1% DMSO, 20 μL, i.pl.). After 15 min, animals received an intraplantar injection of capsaicin (1 nmol/20 μL), HF or IBA at 30 μg/20 μL and the time that mice spent licking the injected paw (s) was recorded for 5 min. In addition, to examine the possible involvement of TRPA1 receptors in the nociceptive-like behavior evoked by alkylamides, mice were pretreated with HC-030031 (TRPA1 antagonist, 100 mg/kg, i.p) or vehicle (1% Tween 80, 5% DMSO, i.p.). Following 30 min, nociception was induced by cinnamaldehyde (200 nmol/20 μL, i.pl.), HF or IBA at 30 μg/20 μL (i.pl.), and the licking behavior was recorded for 5 min.

To confirm whether TRP receptors participate in the nociceptive behavior induced by HF and IBA (30 μg/20 μL, i.pl.), mice were previously desensitized with resiniferatoxin, a potent TRPV1 agonist that causes a high calcium influx, cellular cytotoxicity and consequent necrosis of the nociceptive fibers that co-express TRPV1 and TRPA1 receptors (Pecze et al. 2009). Thus, resiniferatoxin (50 μg/kg, s.c.) or vehicle (V: 5% Tween 80 and 5% ethanol, s.c.) were administered 6 days before 0.1% capsaicin (10 μL) instillation into the eye, to count the wiping movements. The administration of resiniferatoxin causes loss of corneal sensitivity, indicating the nociceptive fibers depletion reveled by reduction of wipe behavior in treted-animals. On the 7th day, the HF and IBA (30 μg/20 μL, i.pl.)-evoked nociceptive behavior was recorded for 5 min.

2.7 Participation of histaminergic system and mast cells on dual action of HF and IBA and hindpaw histochemistry analysis

To investigate the involvement of the histaminergic system in the antinociception promoted by alkylamides, mice were pretreated with HF or IBA (0.1 μg/20 μl, i.pl.), ketotifen (H1 antagonist and mast cell stabilizer, 5 mg/kg, i.p.), and their respective vehicles. Following 15 or 30 min after HF or IBA and ketotifen treatments, nociception was induced by intraplantar injection of compound 48/80 (C48/80, inductor of mast cell degranulation, 10 μg/20 μL) and the licking nociceptive behavior was recorded over the first 10 min.

In another set of experiments, to examine the possible involvement of the histaminergic system in the nociceptive-like behavior evoked by alkylamides, mice were pretreated with ketotifen (5 mg/kg, i.p.) or vehicle (0.9% saline, 10 mL/kg, i.p.), 30 min before intraplantar injection of HF or IBA (30 μg/20 μL, i.pl.), and the hindpaw licking was recorded for 5 min (Vendramini-Costa et al. 2015).

To confirm mast cell infiltration and degranulation in the hindpaw skin, the plantar surface was excised and fixed in ALFAC solution (16:2:1 mixture of ethanol 80%, formaldehyde 40%, and acetic acid P.A.). Samples were embedded in paraffin, sectioned at 5 μm, mounted on gelatinized slides and stained with toluidine blue, that revealed the mast cell degranulation through a metachromatic reaction between proteoglycans into the granules and the cationic dye (Héron and Dubayle 2013). Hindpaw sections were examined under light microscopy and analyzed in a blind fashion. Randomly representative areas were selected for semiquantitative analysis (100x objective), through the total counting of 20 mast cells (10 fields

per section, n=3 per group), which were categorized as intact or degranulated to obtain the percentage of mast cells degranulation (Oliveira et al. 2013; van Diest et al. 2015). Sections were photographed in 63x magnification with a slide scanner (MetaSystems MetaViewer®).

2.8 Local injection of HF and IBA on mechanical and heat paw withdrawal thresholds

To measure the mechanical withdrawal threshold, mice were placed in individual boxes (18×11×20 cm) on an elevated wire mesh platform and tested with 0.004 - 4g von Frey filaments stimulation (North Coast Medical, Inc., Morgan Hill, CA) according to the up-and-down method, to determinate 50% mechanical paw withdrawal threshold (g) (Dixon 1980; Chaplan et al. 1994).

Thermal stimulation was evaluated on a hot-plate apparatus (Ugo Basile, Italy) at a constant temperature (52 ± 0.1 ºC). The time elapsed between the contact of mouse hindpaw into the hot plate and the injected paw withdrawal response (licking, shaking or guarding) was measured automatically and considered as the heat latency (s) (Hunskaar et al. 1986; Klein et al. 2011).

Both measurements were performed before (B: basal) and at 15, 30, 60 and 120 min after the intraplantar injection (20 µL) of HF or IBA (0.1 and 30 µg), their respective vehicles, 1% lidocaine or vehicle (0.9% saline).

2.9 Statistical analysis

All results are presented as the mean ± standard errors of the mean (S.E.M) for each experimental group. Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni's multi-comparison post hoc test. Data concerning the mechanical and heat paw withdrawal were analyzed using two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-hoc test. In all cases, differences were considered significant when $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ and $****P < 0.0001$. All analyzes were performed using the GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA).

3 Results

3.1 Phytochemistry analysis of HF

The HF chromatogram obtained by GC-MS showed several peaks that indicate the presence of alkylamides (Fig. 1). The highest peak **1** was identified as spilanthol, the main alkylamide present in the plant, which presented a characteristic mass spectrum (Leng et al., 2011; Costa et al, 2013). Peak **2** was consistent with 2-methylbutylamide (Boonen et al, 2010; Sharma et al., 2011). Peaks **3** and **4** were not confirmed as alkylamides, but their mass spectra showed similar fragments to the other alkylamides. A similar fragmentation to peak **5** has been described by Leng et al., (2011) in *A. oleracea*, then based on spectral data banks of the equipment, assigned the structure as N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide. Peak **6** showed similar fragments to phenyl-alkylamide (N-phenethyl-2,3-epoxy-nonamide), mainly due to the aromatic rings revealed by tropylium ion (*m/z* 91) (Boonen et al., 2010).

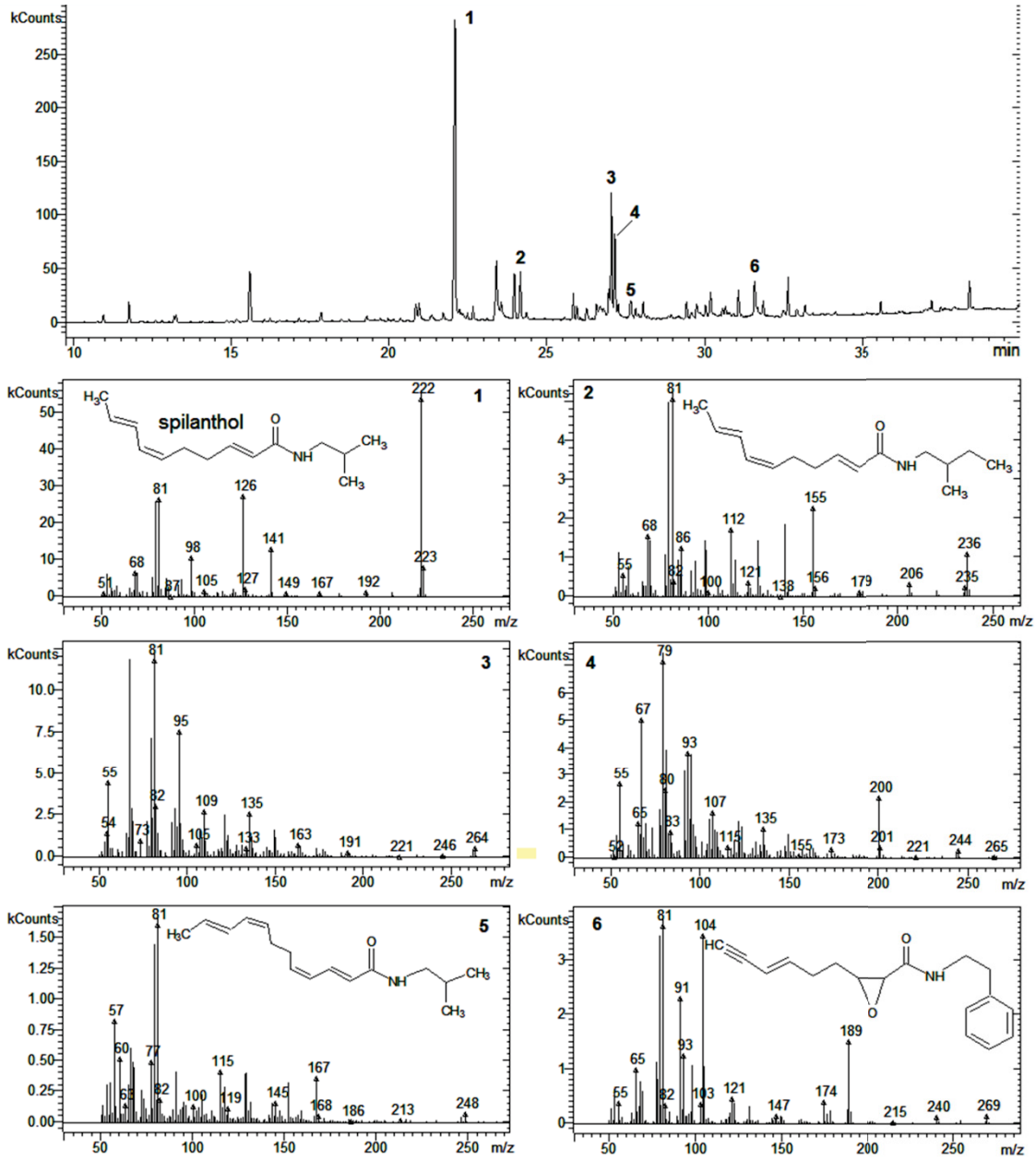


Fig. 1. Chromatogram obtained in the gas phase of the hexanic fraction (HF) from *Acmella oleracea* flowers (GC-MS). Peak 1 was identified as spilanthol based on electron ionization profile. Other peaks observed were identified as different alkylamides described in jambu.

3.2 Effect of crescent doses of HF and IBA injected into the mice hindpaw

Following 10 min of the intraplantar injection of HF and IBA at 0.1, 0.3 and 1 µg and their respective vehicles, any nociceptive-like behaviors were observed. HF at 3, 10 and 30 µg induced significantly licking behavior restricted to the first 5 min (18.7 ± 2.3, 35.7 ± 7.5 and 53.5 ± 6.3s), respectively, when compared to the vehicle group (V: 0.5 ± 0.2s) (Fig. 2A). Similarly, IBA at 3, 10 and 30 µg induced significantly licking behavior just for 5 min in 20.3 ± 2.7, 35.8 ± 9.3 and 57.7 ± 5.2s, respectively, when compared to the vehicle group (V: 0.3 ± 0.2s) (Fig. 2B). From these data, we selected 0.1 µg of HF and IBA to investigate their local antinociceptive effects whereas intraplantar injection of 30 µg was employed to evaluate the nociceptive-like behaviors of alkylamides.

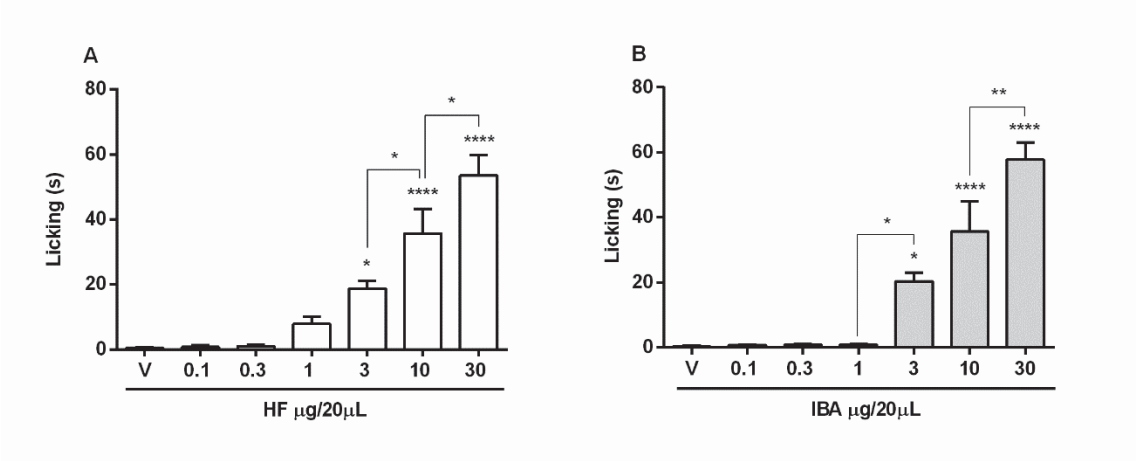


Fig. 2. Effect of crescent doses of HF and IBA injected into the mice hindpaw. The animals received intraplantarly vehicle (V), HF (Panel A) or IBA (Panel B) at 0.1, 0.3, 1, 3, 10 and 30 µg/20 µL. The licking behavior was evaluated for 5 min. Data were expressed as mean ± SEM (n= 6 - 8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ when compared to the vehicle (V) group (ANOVA followed by Bonferroni test).

3.3 Antinociceptive effects of intraplantar HF and IBA in the formalin test

Intraplantar injection of 2.5% formalin into the mouse hindpaw evoked a biphasic licking pain response that was significantly reduced by HF and IBA at 0.1 μg (Fig. 3). In the first phase (neurogenic phase), HF and IBA reduced the formalin-induced nociception in 38.1 and 50.3% respectively, when compared to the vehicle group (V: 101.1 ± 2.7s) (Fig. 3A). In the second phase (inflammatory phase), nociception was reduced in 80.1 and 78.4% by HF and IBA, respectively (V: 189.3 ± 7.1s). The pretreatment with diclofenac (10 mg/kg, i.p.) reduced only the second phase in 56.7% (Fig. 3B).

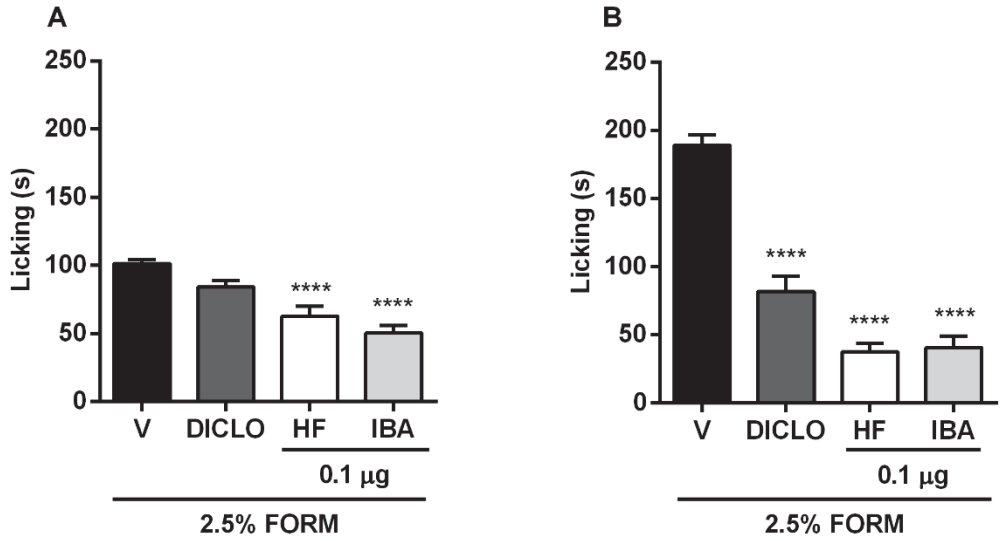


Fig. 3. Antinociceptive effect of intraplantar injection of HF and IBA in the formalin test. Mice were pretreated with vehicle (V), diclofenac (DICLO: 10 mg/kg, i.p.), HF or IBA (0.1 μg/20 μL, i.pl.) before the formalin-induced nociception (2.5% FORM: 20 μL/i.pl.) and licking was evaluated in both first (Panel A) and second (Panel B) phases. Data were expressed as mean ± SEM (n= 6 - 7). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ when compared to the vehicle (V) group (ANOVA followed by Bonferroni test).

3.4 Involvement of the opioid system in the dual effects of intraplantar HF and IBA

Intraplantar injection of glutamate (20 μmol) into the mouse hindpaw evoked a licking pain behavior (Fig. 4A). Pretreatment with morphine (1 mg/kg, s.c.), HF and IBA at 0.1 μg reduced significantly the glutamate-induced nociception in 73.0, 66.9 and 74.4%, respectively (V: 170.5 ± 11.7s) (Fig. 4A). The pretreatment with naloxone (1 mg/kg, i.p.) significantly reversed the antinociception caused by morphine (154.4 ± 13.9s), whereas the antinociception promoted by intraplantar HF and IBA was not antagonized by naloxone (54.6 ± 6.7; 49.4 ± 8.0s, respectively) (Fig. 4A).

On the other hand, as previously observed in Fig. 2, HF and IBA at 30 μg induced pain-like behaviors in mice (Fig. 4B-C). Here, the licking behavior evoked by HF and IBA was significantly reduced by morphine in 81.7 and 91.0%, when compared to the respective vehicle groups (Fig. 4B, V: 48.2 ± 2.4s; Fig. 4C, V: 53.8 ± 4.8s, respectively). Additionally, naloxone pretreatment reverted both HF and IBA-induced nociceptive behavior to 45.2 ± 5.2 and 56.0 ± 5.5s, respectively (Fig. 4B-C).

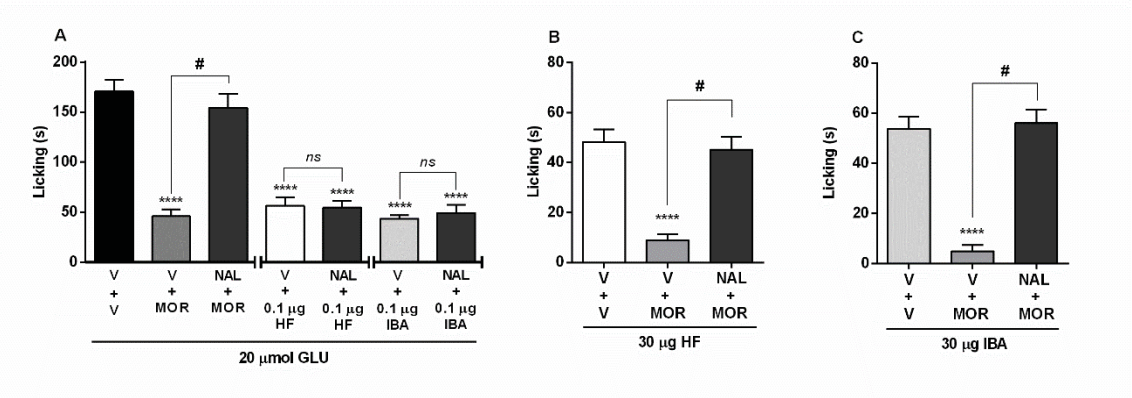


Fig. 4. Participation of the opioid system in the antinociceptive (A) and pronociceptive (B and C) effects of intraplantar injection of HF and IBA. Panel A: Mice were pretreated with vehicle (V) or naloxone (NAL: 1 mg/kg, i.p.), followed by vehicle (V), morphine (MOR: 1 mg/kg, s.c.), HF or IBA at 0.1 μg/20 μL (i.pl.) and nociception was induced by glutamate (GLU: 20 μmol/20 μL, i.pl.). Panel B: Mice were pretreated with vehicle (V) or naloxone (NAL: 1 mg/kg, i.p.), followed by vehicle (V), morphine (MOR: 1 mg/kg, s.c.) and nociception was induced by HF and IBA at 30 μg/20 μL (i.pl.). Data were expressed as mean ± SEM (n= 5 - 6). ****$P < 0.0001$ when compared to the vehicle (V+V) group; $^{\#}P < 0.05$ when compared to V + MOR group (ANOVA followed by Bonferroni test).

3.5 Involvement of TRP receptors in the dual effects of intraplantar HF and IBA

As expected, the nociception induced by the TRPV1 agonist capsaicin (1 nmol, i.pl; 64.5 ± 4.4s) was reversed by TRPV1 antagonist capsazepine (10 nmol, i.pl.) in 45.6%. Similarly, the nociception induced by the TRPA1 agonist cinnamaldehyde (200 nmol, i.pl; 57.1 ± 3.4s) was reduced in 50.4% by the TRPA1 antagonist (HC: 100 mg/kg, i.p.) (data not shown).

Interestingly, pretreatment with HF and IBA at 0.1 μg reduced the capsaicin-evoked licking behavior in 67.0 and 69.0%, respectively (V: 49.4 ± 2.7s, Fig. 5A). Conversely, the nociception induced by cinnamaldehyde was significantly enhanced by 0.1 μg of HF and IBA in 204.1 and 90.6%, respectively (V: 56.0 ± 3.6s, Fig. 5B).

In sharp contrast, the nociception evoked by intraplantar injection of HF at 30 μg was unchanged by capsazepine pretreatment (62.3 ± 5.9s) but was significantly reduced by HC-030031 in 56.3% (V: 61.3 ± 4.1s) (Fig. 6A), whereas the licking pain-behavior evoked by IBA at 30 μg was reduced by both capsazepine and HC-030031 in 32.8 and 87.5%, respectively (V: 54.8 ± 6.4s) (Fig. 6B).

To confirm the involvement of TRP receptors, mice were pretreated with of resiniferatoxin and after 6 days, the desensitization was confirmed through the reduction in 84.9% of wiping events evoked by 0.1% capsaicin instillation into one eye (V: 15.5 ± 0.5). On the following day, the licking behavior induced by 30 μg of HF and IBA was significantly reduced in 80.9% (V: 57.3 ± 2.7s) and 64.9% (V: 47.4 ± 10.6s), respectively (Fig. 6C).

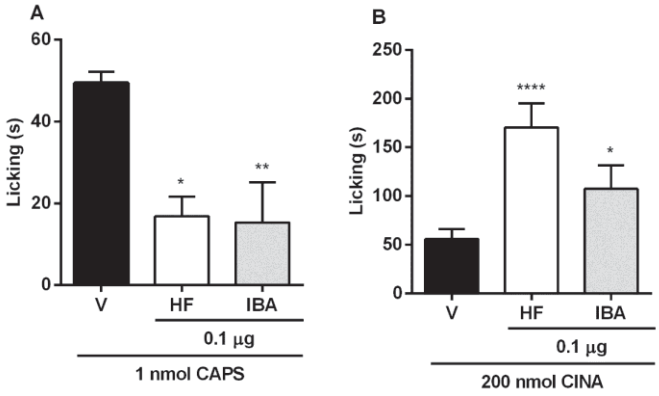


Fig. 5. Effect of intraplantar administration of HF or IBA on nociception induced by capsaicin (A) and cinnamaldehyde (B) in mice. Animals were pretreated with vehicle (V), HF or IBA (0.1 µg/20 µl, i.pl.) before the intraplantar injection of capsaicin (A: CAPS 1 nmol/20 µL) or cinnamaldehyde (B: CINA: 200 nmol/20 µL). Data were expressed as mean ± SEM (n= 6 - 8). **P* <0.05; ***P* < 0.01; *****P* < 0.0001 compared to the vehicle (V) group (ANOVA followed by Bonferroni test).

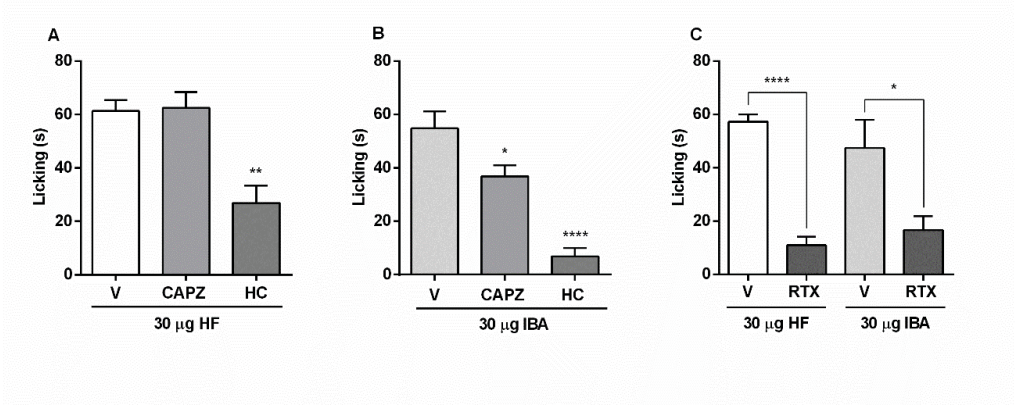


Fig. 6. Participation of the TRPV1 and TRPA1 receptors on HF and IBA-induced nociception. The animals were pretreated with vehicle (V), capsazepine (CAPZ:10 nmol/20 µl, i.pl.) or HC-030031 (HC: 100 mg/kg, i.p.) before the intraplantar injection of HF (A) or IBA (B) at 30 µg/20 µL. Data are expressed as means ± SEM (n= 6 - 8). **P* < 0.05; ***P* < 0.01; *****P* < 0.0001 compared to the vehicle (V) group (ANOVA followed by Bonferroni test). (C) Mice were pretreated with vehicle (V) or resiniferatoxin (RTX: 50 µg/kg, s.c.) and after 7 days, the nociception was induced by intraplantar injection of HF or IBA at 30 µg/20 µL (i.pl.). Data were expressed as mean ± SEM (n= 6 - 8). **P* <0.05; *****P* < 0.0001 compared to the vehicle (V) group (Unpaired T-test).

3.6 Involvement of the histaminergic system and mast cells in the dual effects of intraplantar HF and IBA

The pretreatment with ketotifen and HF at 0.1 μg reduced the nociception induced by C48/80 in 43.2 and 76.9%, respectively, when compared with vehicle group (V: 73.6 ± 6.9s), whereas IBA at 0.1 μg unchanged the C48/80-induced nociception (56.3 ± 6.9s) (Fig. 7A). As can be seen in Fig. 7B, 30 μg of HF-evoked nociceptive licking behavior was reduced by ketotifen in 65.1% (V: 60.2 ± 10.3s), whereas nociception induced by IBA at 30 μg was unchanged (V: 52.2 ± 3.7s).

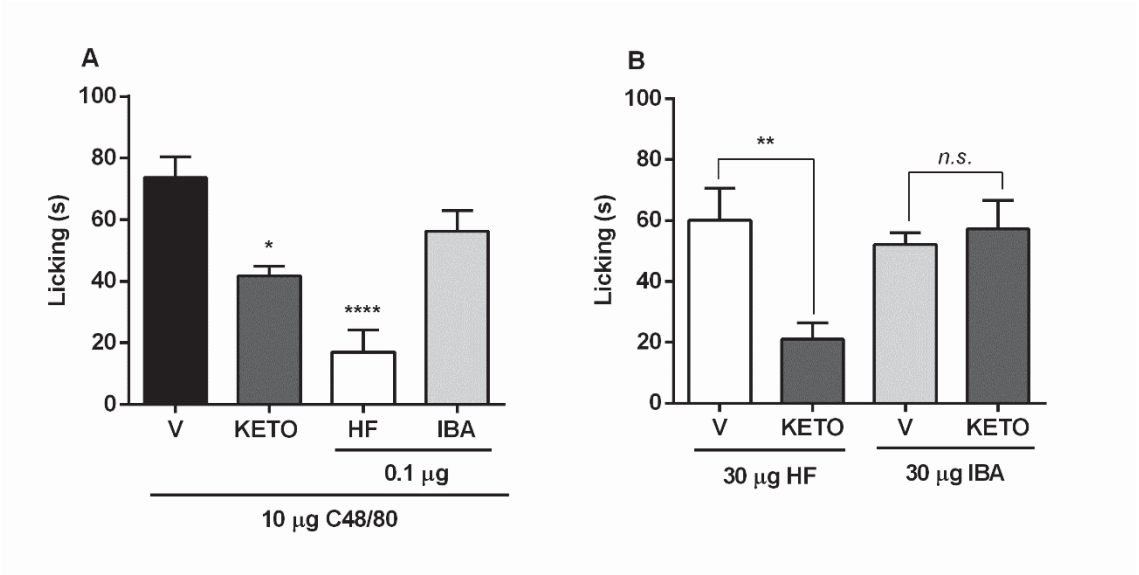


Fig. 7. Evaluation of the histaminergic system in antinociception (A) and nociception (B) promoted by intraplantar injection of HF and IBA. Animals were pretreated with vehicle (V), ketotifen (KETO: 5 mg/kg, i.p.) and HF or IBA (0.1 μg/20 μl, i.pl.). The nociception was induced by C48/80 (10 μg/20 μL, i.pl.) (A). Animals were pretreated with vehicle or ketotifen and the nociception was induced by HF or IBA (30 μg/20 μL, i.pl.) (B). Data were expressed as mean ± SEM (n= 5 - 8). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ when compared to vehicle (V) group (ANOVA followed by Bonferroni test).

Histological analyses of plantar tissues stained with toluidine blue revealed that C48/80 clearly promoted mast cell degranulation. However, following the intraplantar injection of HF or IBA at 0.1 µg or their respective vehicles, we verified the majority presence of intact mast cells, in comparison to the naive group (Table 1). As expected, C48/80 induced-mast cell degranulation was significantly prevented by ketotifen, and interestingly, only 0.1 µg of HF (Fig. 8) but not IBA prevented mast cell degranulation induced by C48/80 (Table 1).

Similarly to the observed with C48/80 (Table 1), the intraplantar injection of HF at 30 µg induced a high mast cell degranulation, which was prevented by ketotifen (Fig. 8). On the other hand, IBA at 30 µg did not promote the hidpaw mast cell degranulation (Table 2).

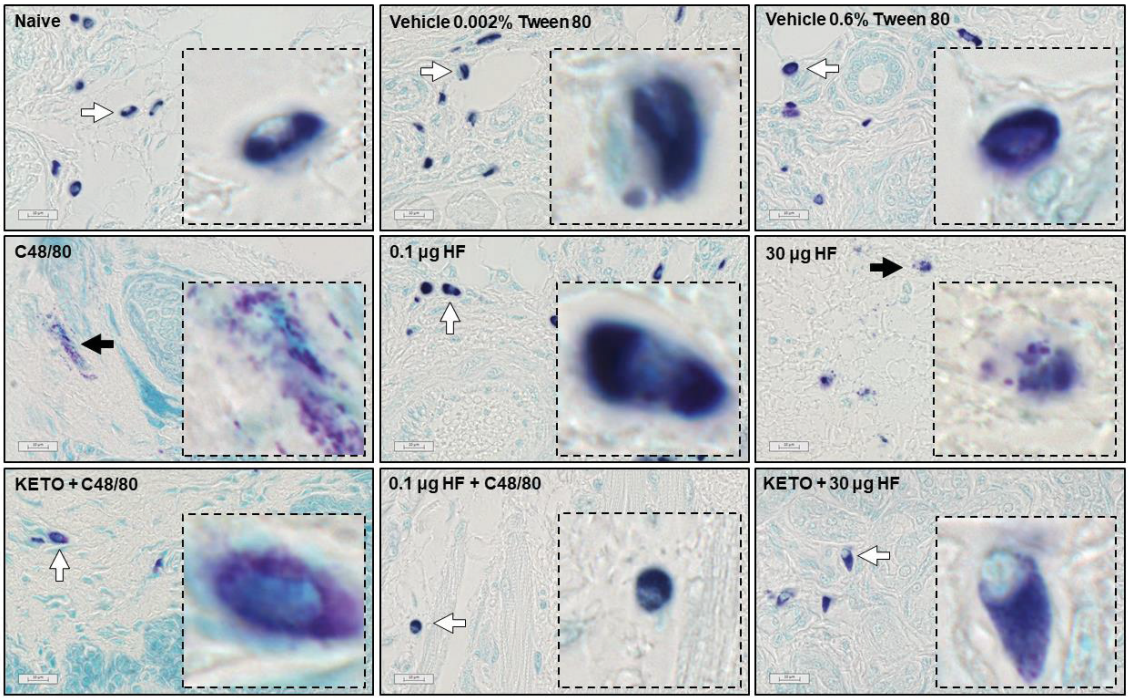


Fig. 8. Representative histochemistry of hindpaw mast cell staining with toluidine blue. Sections were shown in 40x magnification. Arrows in dotted boxes (63x) ⇨ : indicates intact mast cell; ➡ : indicates degranulated mast cell.

% of mast cell degranulated								
Naive	Vehicle 0.002% Tween 80	Vehicle 0.02% DMSO	0.1 µg HF	0.1 µg IBA	C48/80	KETO + C48/80	0.1 µg HF + C48/80	0.1 µg IBA + C48/80
10.0 ± 2.9	18.3 ± 1,7	13.3 ± 1.6	15.0 ± 2.8	16.6 ± 6.6	71.7 ± 1.7#	38.3 ± 4.4***	36.7 ± 6.7***	78.3 ± 4.4

Table 1. Effect of HF and IBA (0.1 µg/20 µl, i.pl.) on mast cell degranulation. Data were expressed as mean ± SEM (n = 3). One-way ANOVA followed by Bonferroni test. ***P <0.001, when compared to C48/80 group; #P<0.05, when compared to the naive group.

% of mast cell degranulated						
Naive	Vehicle 0.6% Tween 80	Vehicle 6.5% DMSO	30 µg HF	30 µg IBA	KETO + 30 µg HF	KETO + 30 µg IBA
10.0 ± 2.9	23.7 ± 3.3	16.6 ± 3.3	66.7 ± 4.4#	15.0 ± 5.7	23.3 ± 8.8****	23.3 ± 4.4

Table 2. Effect of HF and IBA (30 µg/20 µl, i.pl.) on mast cell degranulation. Data were expressed as mean ± SEM (n = 3). One-way ANOVA followed by Bonferroni test. ****P <0.0001, when compared to HF 30 µg group, #P<0.05, when compared to Vehicle 0.6% Tween 80 group.

3.7 Effect of intraplantar HF and IBA on paw withdrawal thresholds to mechanical and heat stimuli

As shown in Fig. 9A, the intraplantar administration of 1% lidocaine enhanced significantly both mechanical threshold and heat latency of the mice hindpaw. Mechanical thresholds at 30 min were 1.3 ± 0.1g for the vehicle group and 2.8 ± 0.3g for lidocaine group. The heat latency was increased in 34.8 and 73.9%, at 30 and 60 min, respectively, when compared to the respective vehicle groups (V: 13.2 ± 0.5 and 13.8 ± 0.5s, respectively) (Fig. 9B).

At 30 and 60 min following the intraplantar injection of 0.1 µg HF, the mechanical threshold was significantly increased from 1.1 ± 0.1 to 2.6 ± 0.4g and to 2.8 ± 0.4g, respectively. Interestingly, HF at 30 µg did not produce any changes in hindpaw mechanical sensitivity (Fig. 9C). Regarding the heat withdrawal responses evaluated at 15, 30 and 60 min, HF at 0.1 µg increased significantly the hindpaw latency in 22.3, 32.6 and 28.8%, and HF at 30 µg in 26.6, 32.6, 27.3%, when compared to the vehicle group (V: 13.9 ± 0.5; 14.1 ± 0.9, 13.9 ± 0.5s, respectively) (Fig. 9D).

In sharp contrast, the intraplantar administration of IBA at 30 µg significantly decreased the mechanical threshold when compared to vehicle group at 15 and 30 min (1.1 ±

0.1 to 0.1 ± 0.0g and to 0.14 ± 0.1 g, respectively) whereas IBA at 0.1 μg did not change the mechanical threshold of mice (Fig. 9E). Considering the heat withdrawal responses, both intraplantar injections of 0.1 and 30 μg of IBA reduced significantly the thermal latency at 15 and 30 min in 36.8 and 28.6%, and in 47.9 and 40.7%, respectively, when compared to vehicle group (V: 14.4 ± 0.5 and 13.9 ± 0.6 s) (Fig. 9F).

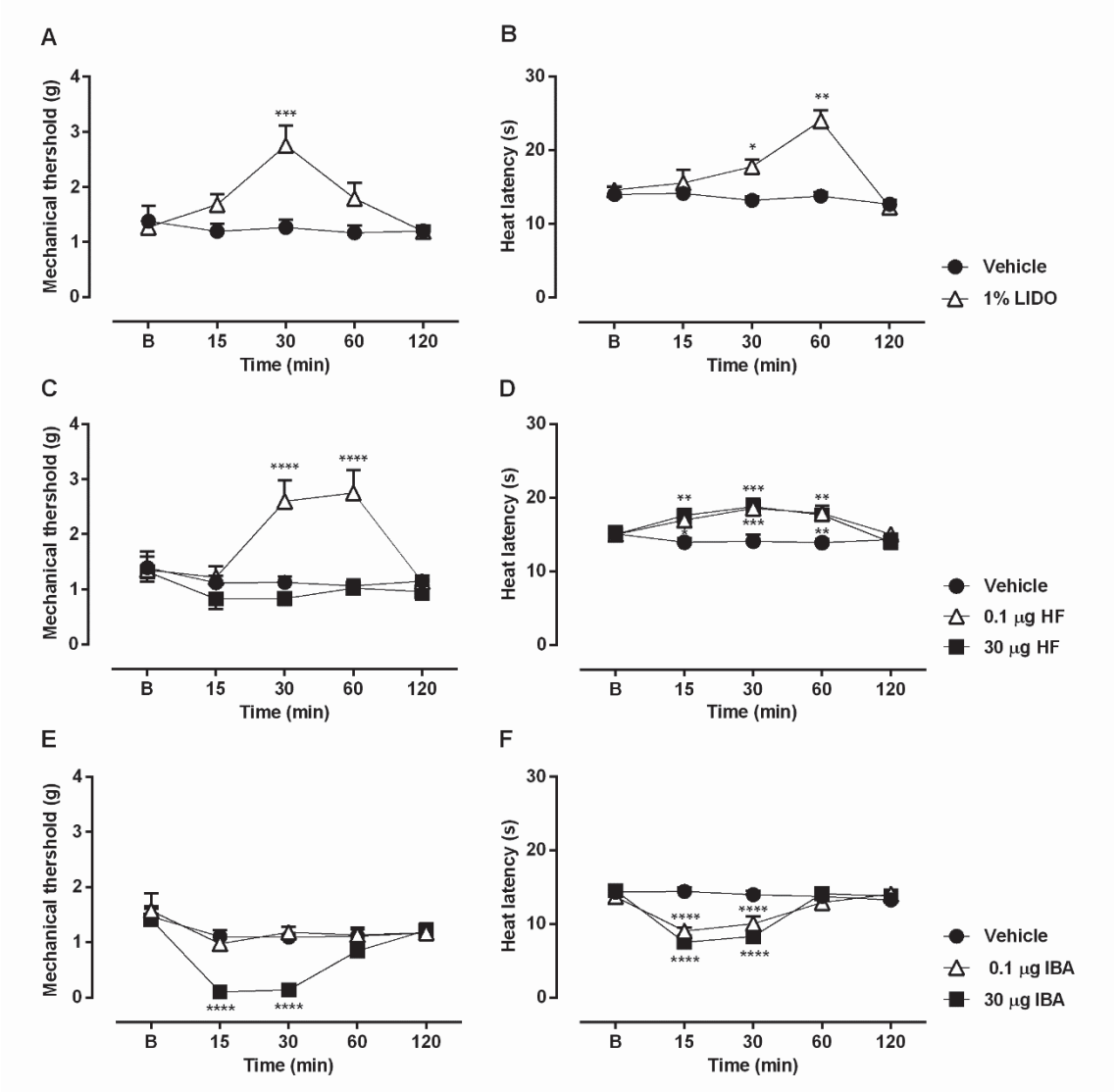


Fig. 9. Effects of lidocaine, HF, and IBA on mechanical and heat withdrawal threshold. Mice received intraplantarly (20 μL) of the vehicle (V), 1% lidocaine (LIDO), HF (0.1 or 30 μg) and IBA (0.1 or 30 μg) for evaluation of the mechanical threshold (g) (Panels A, C, E) and heat latency (s) (Panels B, D, F). Data were expressed as mean ± SEM (n = 6). *P < 0.05; **P < 0.01 ***P < 0.001; ****P < 0.0001, when compared to vehicle (V) group (Two- way ANOVA followed by Bonferroni's multiple comparison post-hoc test).

4 Discussion

The results presented in this study demonstrate for the first time that intraplantar injection of natural alkylamides founded in the HF as well as the IBA, a synthetic analog of sanshool, promoted both antinociceptive and nociceptive behaviors in mice, at lower and higher doses, respectively. Based on the lipophilicity and the apolar property of the hexane solvent, several alkylamides, mainly spilanthol, were concentrated in the HF obtained from jambu flowers. Likewise, Boonen et al. (2010) and Cheng et al. (2015) also found in ethanolic extracts from *A. oleracea* different alkylamides, including spilanthol as the majority. Most of the studies focus on trying to explain the paresthetic sensations promoted by alkylamides, but here we sought to understand some mechanisms of actions underlying these effects, investigating and evaluating at the same time the dual effects of HF and IBA.

Both FH and IBA evoked-licking behaviors elicited in a dose-dependent manner, starting with 3 μg, which could be related to aversive and/or painful sensations. The results reported here match quite closely those previously related to intraplantar IBA-induced nociceptive behaviors in rats (Klein et al. 2011; Tulleuda et al. 2011). Similarly, sanshool (4 mg/20μL, i.pl.) also induced licking behavior in mice (Koo et al. 2007).

The current study found that the intraplantar injection of the lowest dose of HF and IBA (0.1 μg) did not evoke any nociceptive-like behavior, but when administered 30 min prior to the formalin injection, both alkylamides inhibit neurogenic and inflammatory formalin-induced pain behaviors. The neurogenic pain (phase I) is characterized by direct activation of nociceptors, leading to the releasing of glutamate, substance P, calcitonin gene-related peptide and activation of TRP receptors (Mcnamara et al. 2007). In the inflammatory pain (phase II), the nociception is mediated by an intense release of inflammatory mediators, like bradykinin, histamine, and prostaglandins (Abbadie et al. 1997). Spilanthol, also known as affinin, is an abundant constituent of *Heliopsis longipes* (Asteraceae). In agreement with our data, the local administration of affinin (1–600 μg) also reduced both phases of formalin-induced orofacial pain (de la Rosa-Lugo et al. 2017), without any signs of nociceptive-like behaviors. Thus, we suggest that the antinociception observed in the neurogenic phase could be due to the blockade of peripheral ion channels and consequently propagation of action potential, decreasing the release of neurotransmitters in the spinal cord. Concerning the anti-inflammatory effects, different extracts of jambu reduced the inflammatory pain induced by formalin, as well as the prostaglandin synthesis (Ratnasooriya et al. 2005; Nomura et al. 2013). Additionally, spilanthol reduced the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α), COX-2 and iNOS

in murine RAW 264.7 macrophages stimulated with LPS (Chen et al. 2008). According to Tsunozaki et al. (2013), the analgesic effects of sanshool in acute and inflammatory pain models are also related to the blockade of voltage-gated sodium channels in Aδ mechanonociceptors.

Usually, both opioids and local anesthetics reduce the nociceptive behaviors in the first phase of formalin, inhibiting synaptic release of glutamate in spinal cord (Tjølsen et al. 1992; Fischer et al. 2014). Glutamate is the major excitatory neurotransmitter in the CNS and contributes significantly to the nociceptive signaling transmission (Fundytus 2001). Unlike morphine, the local antinociception promoted by HF and IBA at 0.1 µg does not seems to involve opioidergic mechanisms, since naloxone was not able to revert the antinociception on glutamate-induced nociception model. Nevertheless, we observed that the antinociception promoted by the local administration of alkylamides seems to be different of that promoted by systemic treatments, since analgesia induced by both affinin and an ethanolic extract rich in alkylamides from jambu involve opioid system (Déciga-Campos et al. 2010; Nomura et al. 2013). In contrast, we attribute that HF and IBA-evoked licking behavior (30 µg) could be interpreted as nociception, since morphine reduced the nociceptive-like behavior and naloxone reversed morphine induced-analgesia.

Nowadays, the ability of nociceptors to detect chemical, thermal and mechanical noxious stimuli is associated to the expression of TRP receptors, critical molecular transducers (Sousa-Valente et al. 2014). TRPV1 and TRPA1 are commonly co-expressed in nociceptors and could be also activated by capsaicin, cinnamaldehyde, and alkylamides (Patapoutian et al. 2009). The blockade of TRP channels remains as alternative target to pain relief, and in our study, both HF and IBA (0.1 µg) reduced the capsaicin-induced nociception, similarly to the capsazepine. In sharp contrast, HF and IBA (0.1 µg) increased the cinnamaldehyde-induced nociception, suggesting that alkylamides could increase the sensitization of TRPA1 receptors. In humans, IBA-evoked chemestethic sensation, initially described as tingling and pungency, followed by cooling sensation, which were potentiated by simultaneous application of noxious cold stimuli mediated by TRPA1 (Albin and Simons 2010). At least for sanshool and IBA, the mechanisms underlying the aversive tingling and nociceptive behavior were attributed to the activation of TRPV1 and TRPA1 receptors, wide-dynamic-range and low-threshold mechanoreceptors, and inhibition of two-pore domain potassium channels (KCNK) (Sugai et al. 2005; Koo et al. 2007; Bautista et al. 2008; Riera et al. 2009; Sawyer et al. 2009; Lennertz et al. 2010; Tulleuda et al. 2011). At this point, our results suggest differences between the mechanisms underlying the effects of the highest dose (30 µg) of HF and IBA. Notably, the nociception induced by HF seems to be closely related to the activation of TRPA1 receptors,

whereas IBA is able to activate both TRPV1 and TRPA1 receptors. Interestingly, sanshool-induced nociception is reduced by capsazepine (Koo et al., 2007). Similarly, menthol, camphor and cinnamaldehyde presents dual effects on TRPA1 receptors: at lower doses acting as TRPA1 agonists and at higher doses inhibited and/or desensitized TRPA1 receptors (Akopian et al. 2007; Everaerts et al. 2011; Alpizar et al. 2013). Thus, our hypothesis that the antinociception promoted by the HF and IBA could result of TRPV1 modulation are reinforced by molecular docking analysis. By interacting with the TRPV1 THR550 residue, synthetic alkylamides and spilanthol could acting as partial agonist and promote analgesia. However, flexible conformations adopted by alkylamides could interact with TYR511 residue, favoring TRPV1 activation and subsequent pain processing (de la Rosa-Lugo et al. 2017). In addition, instead of inducing nociception, higher doses of capsaicin promotes TRPV1 receptor desensitization, resulting in analgesia (Novakova-Tousova et al. 2007).

It is noteworthy that as previously demonstrated by Pecze et al. (2009), following TRPV1 and TRPA1 systemic desensitization by resiniferatoxin, HF and IBA (30 µg)-induced nociception was found to be markedly reduced. Collectively, our data confirm and reinforce that the dual effects promoted by HF and IBA is associated with TRP receptors expressed in nociceptive fibers.

Notably, nociceptor stimulation and sensitization could also depend of inflammatory mediators released by mast cells, such histamine (Fischer et al. 2017). In this regard, the antihistamine activity of *A. oleracea* has been described wheal test in rats (Ratnasooriya et al. 2005). Moreover, *in vitro* studies showed that natural alkylamides and the synthetic dodeca-2E,4E-dienoic acid isobutylamide from *Echinacea purpurea* inhibited calcium influx, mast cell degranulation and histamine, TNF-α and PGE_2 releasing (Gulledge et al. 2018). Interestingly, our data are also in agreement with these previously published findings, since the local treatment with HF (0.1 µg) and ketotifen prevented both nociception and mast cell degranulation induced by C48/80. Unfortunately, at this moment, we were unable to confirm if the related effects of the lower dose of HF were due to the mast cell stabilization or through interaction with H1 receptor. On the contrary, the local injection of the highest dose of HF (30 µg) induced nociception and an intense mast cell degranulation in mice paws, that were both reduced by ketotifen. At least for the higher dose, we suggest an interaction between mast cells degranulation and released inflammatory mediators in the HF-evoked nociceptive behaviors. However, it is important to point out that the isolated synthetic alkylamide IBA at 0.1 and 30 µg neither prevented C48/80-induced nociception and mast cell degranulation and nor induced

mast cells degranulation, respectively, showing a trend toward suggestion that IBA did not interact with mast cells, but more studies needed to fulfill this claim.

Concerning some pharmacokinetic data from alkylamides, studies have been demonstrated that sanshool display rapid absorption following subcutaneous administration and high bioavailability after intravenous administration (Rong et al. 2016), and that spilanthol also combined an intestinal absorption to a high permeation in blood-brain barrier (Veryser et al. 2016). Despite our study concentrate in evaluating the peripheral effects of alkylamides, the systemic and topical administration of extracts rich in alkylamides from *A. oleracea* and *H. longipes* as well as spilanthol promoted thermal anti-hyperalgesic effects on the hot-plate test (Cilia-López et al. 2010; Nomura et al. 2013; Freitas-Blanco et al. 2016). Indeed, HF (0.1 and 30 μg) *per se* significantly increased the heat paw withdrawal latency, and HF enhanced the paw mechanical threshold, like observed with lidocaine. Our data partially resembles previous studies, which showed that sanshool topically applied into mice hindpaw elevated the mechanical threshold without modify the heat paw latency (Tsunozaki et al. 2013). Thus, it therefore appears that natural alkylamides contained in HF could also promote anesthetic effects, probably due to the blockade of peripheral sodium channels, as lidocaine and sanshool. Confirming previous studies, IBA (30 μg) decreased the paw mechanical threshold (Klein et al., 2011; Tulleuda et al., 2011), effect that has been attributed to the blockade of KCNK channels and consequently hyperexcitability of sensorial neurons (Bautista et al. 2008; Tulleuda et al. 2011). Again, unlikely literature data, IBA (0.1 and 30 μg) *per se* significantly decreased the heat paw withdrawal latency, increasing thermal sensitivity. Besides the present results are in disagreement with the previous reported data (Klein et al., 2011), a recent study demonstrated that KCNK channels are also important to the heat thermal perception (Pereira et al. 2014). In this regard, we speculate that this apparent discrepancy may be attributed to the differences in the doses employed and in chemical structure of alkylamides, which could interact with target pain channels and receptors, resulting in different pharmacological profiles of effects.

In conclusion, we have demonstrated here that intraplantar injection of lower and higher doses of natural and synthetic alkylamides promoted dual antinociceptive and pronociceptive effects, adding new evidences and mechanisms for these opposite actions. Accordingly, the present findings strengthen the possibility that natural alkylamides from jambu flowers, mainly spilanthol, at lower doses, could provide effective pain relief and deserves additional studies to confirm its analgesic and/or anesthetic effects.

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Conflict of interest

The authors declare that there was no conflict of interest.

References

Abbadie C, Taylor BK, Peterson MA, Basbaum AI (1997) Differential contribution of the two phases of the formalin test to the pattern of c-fos expression in the rat spinal cord: Studies with remifentanil and lidocaine. Pain 69:101–110. doi: 10.1016/S0304-3959(96)03285-X

Akopian AN, Ruparel NB, Jeske NA, Hargreaves KM (2007) Transient receptor potential TRPA1 channel desensitization in sensory neurons is agonist dependent and regulated by TRPV1-directed internalization. J Physiol 583:175–193. doi: 10.1113/jphysiol.2007.133231

Albin KC, Simons CT (2010) Psychophysical Evaluation of a Sanshool Derivative (Alkylamide) and the Elucidation of Mechanisms Subserving Tingle. 5:. doi: 10.1371/journal.pone.0009520

Alpizar YA, Gees M, Sanchez A, et al (2013) Bimodal effects of cinnamaldehyde and camphor on mouse TRPA1. Pflugers Arch Eur J Physiol 465:853–864. doi: 10.1007/s00424-012-1204-x

Bandell M, Story GM, Hwang SW, et al (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41:849–857. doi: 10.1016/S0896-6273(04)00150-3

Bautista DM, Sigal YM, Milstein AD, et al (2008) Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels. Nat Neurosci 11:772–779. doi: 10.1038/nn.2143

Boonen J, Baert B, Burvenich C, et al (2010) Journal of Pharmaceutical and Biomedical Analysis Short communication LC – MS profiling of N -alkylamides in Spilanthes acmella extract and the transmucosal behaviour of its main bio-active spilanthol. J Pharm Biomed Anal 53:243–249. doi: 10.1016/j.jpba.2010.02.010

Bryant BP, Mezine I (1999) Alkylamides that produce tingling paresthesia activate tactile and thermal trigeminal neurons. Brain Res 842:452–460. doi: 10.1016/S0006-8993(99)01878-8

Caterina MJ, Schumacher MA, Tominaga M, et al (1997) The capsaicin receptor : a heat-activated ion channel in the pain pathway. 389:

Chaplan SR, Bach FW, Pogrel JW, et al (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63. doi: 10.1016/0165-0270(94)90144-9

Li-Chen W, Nien-Chu F, Ming-Hui L, et al (2008) Anti-inflammatory Effect of Spilanthol from Spilanthes acmella on Murine Macrophage by Mediators. 2:2341–2349

Cheng Y Bin, Liu RH, Ho MC, et al (2015) Alkylamides of Acmella oleracea. Molecules 20:6970–6977. doi: 10.3390/molecules20046970

Cilia-López VG, Juárez-Flores BI, Aguirre-Rivera JR, Reyes-Agüero JA (2010) Analgesic activity of Heliopsis longipes and its effect on the nervous system. Pharm Biol 48:195–200. doi: 10.3109/13880200903078495

da Costa DSM, Meotti FC, Andrade EL, et al (2010) The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. Pain 148:431–437. doi: 10.1016/j.pain.2009.12.002

da Silva Lopes L, Marques RB, Fernandes HB, et al (2012) Mechanisms of the antinociceptive action of (-) Epicatechin obtained from the hydroalcoholic fraction of Combretum leprosum Mart & Eic in rodents. J Biomed Sci 19:68. doi: 10.1186/1423-0127-19-68

de la Rosa-Lugo V, Acevedo-Quiroz M, Déciga-Campos M, Rios MY (2017) Antinociceptive effect of natural and synthetic alkamides involves TRPV1 receptors. J Pharm Pharmacol 69:884–895. doi: 10.1111/jphp.12721

Déciga-Campos M, Rios MY, Aguilar-Guadarrama AB (2010) Antinociceptive effect of *Heliopsis longipes* extract and affinin in mice. Planta Med 76:665–670. doi: 10.1055/s-0029-1240658

Dixon WJ (1980) Experimental Observations. Annu Rev Pharmacol Toxicol 20:441–462

Dubey S, Maity S, Singh M, et al (2013) Phytochemistry, pharmacology and toxicology of spilanthes acmella: A review. Adv Pharmacol Sci 2013:. doi: 10.1155/2013/423750

Dutra RC, Campos MM, Santos ARS, Calixto JB (2016) Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol Res 1–25. doi: 10.1016/j.phrs.2016.01.021

Everaerts W, Gees M, Alpizar YA, et al (2011) The capsaicin receptor TRPV1 is a crucial mediator of the noxious effects of mustard oil. Curr Biol 21:316–321. doi: 10.1016/j.cub.2011.01.031

Fischer L, Lavoranti MI, de Oliveira Borges M, et al (2017) TRPA1, substance P, histamine and 5-hydroxytryptamine interact in an interdependent way to induce nociception. Inflamm Res 66:311–322. doi: 10.1007/s00011-016-1015-1

Fischer M, Carli G, Raboisson P, Reeh P (2014) The interphase of the formalin test. Pain 155:511–521. doi: 10.1016/j.pain.2013.11.015

Freitas-Blanco VS, Franz-Montan M, Groppo FC, et al (2016) Development and evaluation of a novel mucoadhesive film containing acmella oleracea extract for oral mucosa topical anesthesia. PLoS One 11:1–18. doi: 10.1371/journal.pone.0162850

Fundytus ME (2001) Glutamate Receptors and Nociception Implications for the Drug Treatment of Pain. 15:29–58

Greger H (2016) Alkamides: a critical reconsideration of a multifunctional class of unsaturated fatty acid amides. Phytochem Rev 15:729–770. doi: 10.1007/s11101-015-9418-0

Gulledge T V., Collette NM, Mackey E, et al (2018) Mast cell degranulation and calcium influx are inhibited by an Echinacea purpurea extract and the alkylamide dodeca-2E,4E-

dienoic acid isobutylamide. J Ethnopharmacol 212:166–174. doi: 10.1016/j.jep.2017.10.012

Héron A, Dubayle D (2013) A focus on mast cells and pain. J Neuroimmunol 264:1–7. doi: 10.1016/j.jneuroim.2013.09.018

Hunskaar S, Berge OG, Hole K (1986) A modified hot-plate test sensitivie to mild analgesics. Behav Brain Res 21:101–108. doi: 10.1016/0166-4328(86)90088-4

Klein AH, Sawyer CM, Zanotto KL, et al (2011) A tingling sanshool derivative excites primary sensory neurons and elicits nocifensive behavior in rats. J Neurophysiol 105:1701–1710. doi: 10.1152/jn.00922.2010

Koo JY, Jang Y, Cho H, et al (2007) Hydroxy- a -sanshool activates TRPV1 and TRPA1 in sensory neurons. 26:1139–1147. doi: 10.1111/j.1460-9568.2007.05743.x

Lennertz RC, Tsunozaki M, Bautista DM, Stucky CL (2010) Physiological Basis of Tingling Paresthesia Evokedby Hydroxy- -Sanshool. J Neurosci 30:4353–4361. doi: 10.1523/JNEUROSCI.4666-09.2010

Mcnamara CR, Mandel-brehm J, Bautista DM, et al (2007) TRPA1 mediates formalin-induced pain. PNAS, Proc Natl Acad Sci United States Am 104:30. doi: 10.1073/pnas.0705924104

National Research Council (2010). Guide for the care and use of laboratory animals. National Academies Press.

Nomura ECO, Rodrigues MRA, Da Silva CF, et al (2013) Antinociceptive effects of ethanolic extract from the flowers of Acmella oleracea (L.) R.K. Jansen in mice. J Ethnopharmacol 150:583–589. doi: 10.1016/j.jep.2013.09.007

Novakova-Tousova K, Vyklicky L, Susankova K, et al (2007) Functional changes in the vanilloid receptor subtype 1 channel during and after acute desensitization. Neuroscience 149:144–154. doi: 10.1016/j.neuroscience.2007.07.039

Patapoutian A, Tate S, Woolf CJ (2009) Transient receptor potential channels : targeting pain at the source. 8:. doi: 10.1038/nrd2757

Pecze L, Pelsoczi P, Kecskés M, et al (2009) Resiniferatoxin mediated ablation of TRPV1+ neurons removes TRPA1 as well. Can J Neurol Sci 36:234–41. doi: 10.1017/S0317167100006600

Pereira V, Busserolles J, Christin M, et al (2014) Role of the TREK2 potassium channel in cold and warm thermosensation and in pain perception. Pain 155:2534–2544. doi: 10.1016/j.pain.2014.09.013

Ratnasooriya WD, Lanka S, Lanka S (2005) Attenuation of Persistent Pain and Hyperalgesia by Spilanthus acmella Flowers in Rats. 43:614–619. doi: 10.1080/13880200500301944

Riera CE, Menozzi-Smarrito C, Affolter M, et al (2009) Compounds from Sichuan and Melegueta peppers activate, covalently and non-covalently, TRPA1 and TRPV1 channels. Br

J Pharmacol 157:1398–1409. doi: 10.1111/j.1476-5381.2009.00307.x

Rong R, Cui MY, Zhang QL, et al (2016) Anesthetic constituents of Zanthoxylum bungeanum Maxim.: A pharmacokinetic study. J Sep Sci 39:2728–2735. doi: 10.1002/jssc.201600295

Sakurada T, Matsumura T, Moriyama T, et al (2003) Differential effects of intraplantar capsazepine and ruthenium red on capsaicin-induced desensitization in mice. Pharmacol Biochem Behav 75:115–121. doi: 10.1016/S0091-3057(03)00066-2

Sawyer CM, Carstens MI, Simons CT, et al (2009) Activation of lumbar spinal wide-dynamic range neurons by a sanshool derivative. J Neurophysiol 101:1742–1748. doi: 10.1152/jn.91311.2008

Sousa-Valente J, Andreou AP, Urban L, Nagy I (2014) Transient receptor potential ion channels in primary sensory neurons as targets for novel analgesics. Br J Pharmacol 171:2508–2527. doi: 10.1111/bph.12532

Sugai E, Morimitsu Y, Iwasaki Y, et al (2005) Pungent Qualities of Sanshool-Related Compounds Evaluated by a Sensory Test and Activation of Rat TRPV1. Biosci Biotechnol Biochem 69:1951–1957. doi: 10.1271/bbb.69.1951

Tjølsen A, Berge O-G, Hunskaar S, et al (1992) The formalin test: an evaluation of the method. Pain 51:5–17. doi: 10.1016/0304-3959(92)90003-T

Tsunozaki M, Lennertz RC, Vilceanu D, et al (2013) A ‘ toothache tree ’ alkylamide inhibits A δ mechanonociceptors to alleviate mechanical pain. 13:3325–3340. doi: 10.1113/jphysiol.2013.252106x

Tulleuda A, Cokic B, Callejo G, et al (2011) TRESK channel contribution to nociceptive sensory neurons excitability : modulation by nerve injury. 1–17. doi: 10.1186/1744-8069-7-30

van Diest SA, van den Elsen LW, Klok AJ, et al (2015) Dietary Marine n-3 PUFAs Do Not Affect Stress-Induced Visceral Hypersensitivity in a Rat Maternal Separation Model. J Nutr 145:915–922. doi: 10.3945/jn.114.201731

Vendramini-Costa DB, Spindola HM, De Mello GC, et al (2015) Anti-inflammatory and antinociceptive effects of racemic goniothalamin, a styryl lactone. Life Sci 139:83–90. doi: 10.1016/j.lfs.2015.08.010

Veryser L, Taevernier L, Joshi T, et al (2016) Mucosal and blood-brain barrier transport kinetics of the plant N-alkylamide spilanthol using in vitro and in vivo models. BMC Complement Altern Med 16:1–12. doi: 10.1186/s12906-016-1159-0

Woller SA, Eddinger KA, Corr M, Yaksh TL (2017) An overview of pathways encoding nociception. Clin Exp Rheumatol 35:S40–S46

3 ARTIGO CIENTÍFICO 2

Pharmacological Potential of Alkylamides from *Acmella oleracea* Flowers and Synthetic Isobutylalkyl Amide to Treat Inflammatory Pain

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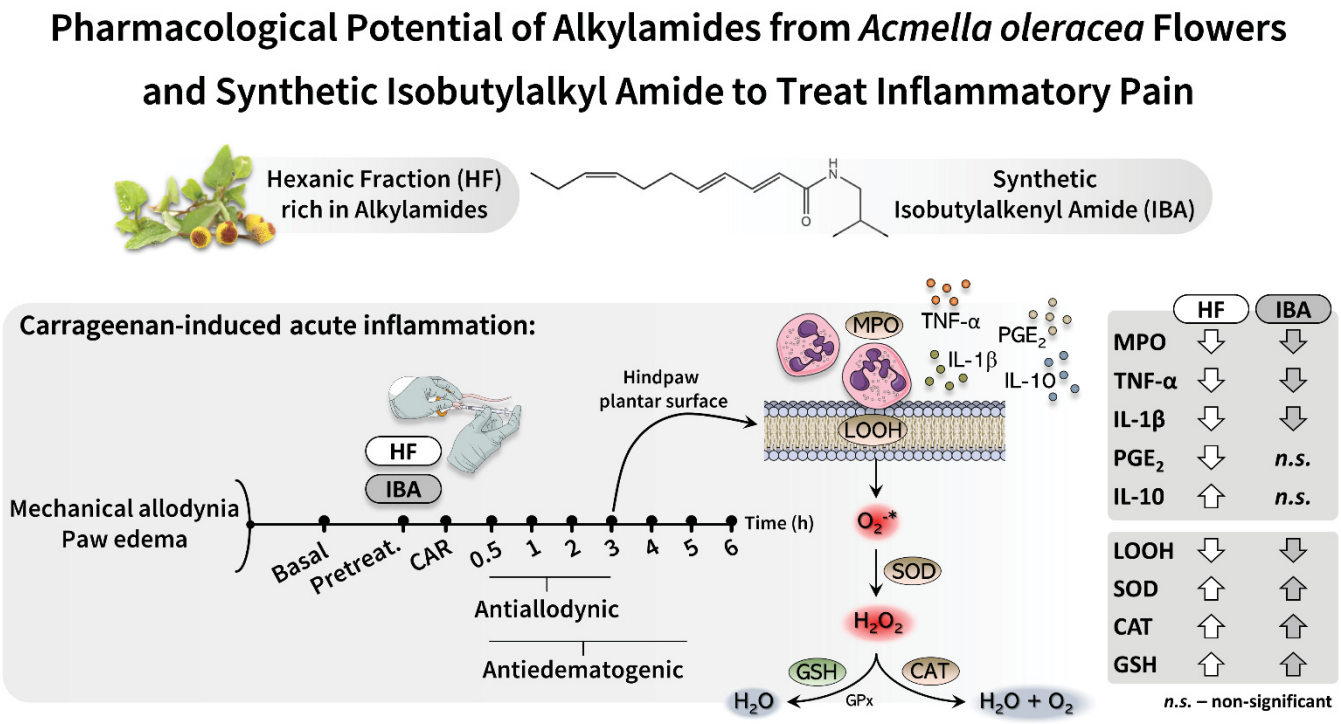
Graphic Abstract 2



Abstract

Acmella oleracea ("jambu") is an Amazonian plant rich in alkylamides. Its flowers are widely used in folk medicine to treat toothache due to tingling, numbness and local anesthesia caused in the mouth. Our group have previously demonstrated the antinociceptive and anesthetic properties of an alkylamide rich-hexanic fraction (HF) obtained from jambu flowers and a synthetic isobutylalkyl amide (IBA) administrated locally at low dose in acute pain models. Thus, here we aimed to evaluate the effectiveness of alkylamides in the acute inflammation induced by carrageenan in mice, and their capacity in scavenging the free radical DPPH *in vitro*. Animals were pretreated with HF and IBA (0.1 μg/20 μL, i.pl.) before 15 min of carrageenan injection (300 μg/20 μL, i.pl.). Mechanical allodynia and paw edema were evaluated previously (basal) and at 0.5 until 6 h. HF and IBA promoted antiallodynic and antiedematogenic effects until 3 and 5 h, respectively. At their maximum effects, the plantar surfaces of injected hindpaws were excised for the measurement of inflammatory and oxidative stress parameters. HF and IBA reduced the myeloperoxidase (MPO) activity, TNF-α and IL-1β levels, prevented the lipid hydroperoxides (LOOH) production, and the depletion of antioxidant agents: superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione (GSH) contents. Furthermore, HF maintained IL-10 levels and decreased PGE_2 synthesis. HF and IBA failed on DPPH assay. Collectively, our results demonstrated the promising anti-inflammatory effects of local pretreatment with alkylamides, supporting the potential of these molecules to treat acute inflammatory pain conditions.

Keywords

Jambu; Alkylamides; Inflammation; Allodynia; Cytokines; Prostaglandin

1 Introduction

Acute inflammation is a physiological process that occur to protect the lesion area and present five cardinal signs: redness, increased heat, edema, pain, and loss of function (Ji et al. 2016). These physical manifestations are characterized by vascular permeability, cell migration, releasing and synthesis of cytokines and prostaglandins, production of free radicals and consequent oxidative stress (Nourshargh and Alon 2014; Costa et al. 2018). Furthermore, peripheral activation and/or sensitization of primary sensory neurons (nociceptors) by pro-inflammatory mediators, as well as central sensitization by non-neural cells, contribute to the appearing of pain induced by normally innocuous mechanical stimuli (allodynia) near to the injured tissue (Pinho-Ribeiro et al. 2017; Cook et al. 2018).

The treatment of inflammatory painful conditions with non-steroidal anti-inflammatory drugs (NSAIDs) and in some cases, using corticosteroid, is well established. However, the adverse effects caused by NSAIDs such as gastric ulcer, renal and hepatic damage, pulmonary and cardiovascular abnormalities (Day and Graham 2013); and by corticosteroid drugs as immunosuppression and edema (Schmidt and Willis 2007) could impair the success of therapy.

In order to develop new effective treatments and with less adverse effects, many research groups are working on medicinal plants, mainly focused on secondary metabolites as a source for drug discovery (Dutra et al. 2016). Alkylamides are a promise group of bioactive compounds present in several genera from Asteraceae family: *Acmella* (*Spilanthes*), *Anacyclus*, *Echinacea* and *Heliopsis*, and also in *Zanthoxylum* from Rutaceae family (Greger 2016). These molecules contain a unsaturated fatty-acid-like portion and an amide group linked to a variable radical (Boonen et al. 2012; Moazami et al. 2015). In oral mucosa, alkylamides are responsible to cause tingle, numbness and local anesthesia sensation (Greger 1984). Preclinical studies have demonstrated the analgesic, anti-inflammatory and immunomodulatory effects promoted by this compounds (Gertsch et al. 2004; Wu et al. 2008; Nomura et al. 2013).

Acmella oleracea (L.) R.K. Jansen, popularly known as “jambu”, is an Amazonian plant very rich in alkylamides, mainly in spilanthol (Cheng et al. 2015; Dallazen et al. submitted data 2018). In Northern Brazil, the flowers of jambu have been used in folk medicine for toothache relief due to paresthesia and chemesthetic sensations evoked in the mouth (Dubey et al. 2013; Prachayasittikul et al. 2013).

Previous data from our research group have demonstrated that the intraplantar treatment with an alkylamide rich-hexanic fraction (HF) obtained from jambu flowers and a synthetic isobutylalkyl amide (IBA, analogue of natural sanshool from *Zanthoxylum piperitum*) promoted antinociceptive effects on acute pain models through Transient Potential Receptors Vanilloid 1 (TRPV1) antagonism and independent of opioid mechanisms. Moreover, natural alkylamides from jambu also prevent the mast cell degranulation and increased both mechanical and thermal paw withdrawal threshold suggesting an anesthetic property (Dallazen et al. submitted data 2018).

Therefore, in the present study we sought to evaluate the pharmacological effect of the local pretreatment with HF and IBA on carrageenan-induced acute hindpaw inflammation model, evaluating inflammatory and oxidative stress parameters.

2 Materials and methods

2.1 Animals

The experiments were conducted using Swiss (*Mus musculus*) male adult mice (25 - 30 g) provided by the local vivarium from Federal University of Paraná (Biological Sciences Sector). The animals were housed in controlled temperature and luminosity (22 ± 2 ºC, 12h light/dark cycle), air exhaustion and free access to water and food (Nuvilab CR-1, Quimtia S/A, Brazil). All experimental protocols were conducted in agreement with the "Guide for the Care and Use of Laboratory Animals" (National Research Council 2011) and previously approved by the local Ethics Committee of Animal Experimentation (CEUA/BIO–UFPR) under approval numbers: 970 and 1107.

2.2 Pharmacological treatment with Hexanic Fraction from *Acmella oleracea* flowers and Synthetic Isobutylalkyl Amide

The obtention and characterization of the hexanic fraction (HF) from *Acmella oleracea* flowers were previously described by Dallazen et al. (submitted data 2018). Briefly, jambu flowers were extracted using absolute ethanol under reflux, in order to obtain the ethanolic extract (EEAO, Nomura et al. 2013). EEAO was evaporated, resuspended in water, lyophilized, and then resuspended in ethanol-water. Finally, the resuspended part was subjected to a liquid-liquid partition in hexane, providing the hexane fraction (HF), which was analyzed through gas chromatography-mass spectrometry detection (GC-MS).

The dose of 0.1 μg/20 μL administrated via intraplantar (i.pl., in right hindpaw) of both HF and isobutylalkyl amide (IBA) was chosen based on previous study of our group (Dallazen et al. submitted data 2018).

2.3 Carrageenan-induced acute mechanical allodynia and paw edema in mice

The animals were placed in individual boxes (18 cm × 11 cm × 20 cm) on an elevated mesh platform and acclimated for 1 h. Mice were pretreated with vehicle (V: sterile 0.9% saline, 10 ml/kg, intraperitoneally, i.p; 0.002% tween 80 or 0.02% DMSO, both in 0.9% saline, i.pl.), dexamethasone (DEXA: 1 mg/kg, i.p; a synthetic glucocorticoid), HF or IBA (0.1 μg/20 μL, i.pl.). After 15 min from local pretreatment and 30 min after systemic pretreatment the acute inflammatory response was induced by an intraplantar injection of carrageenan in the right hindpaw (300 μg/20 μL, i.pl.) (Carlotto et al. 2016). The mechanical allodynia was accessed with Von Frey filaments (0.004 – 4 g, North Coast Medical, Morgan Hill, CA, USA) based on the Up-and-Down method to determine 50% paw withdrawal threshold (g) (Dixon 1980; Chaplan et al. 1994). Concomitantly, to evaluate the edema, the paw thickness was measured using a digital micrometer (Digimess, São Paulo, SP, BR) and expressed as "variation of millimeter (Δ mm)" between the basal value and the test value at each evaluation (Rossato et al. 2015). Both parameters were evaluated previously, before the treatments (B: basal values), and after 0.5, 1, 2, 3, 4, 5 and 6 h of carrageenan injection.

2.4 Preparation of subcellular fractions of plantar surface of mice hindpaws

At the maximum antiallodynic and antiedematogenic effects of HF and IBA, between 2 and 3 h after intraplantar injection of carrageenan, the animals were euthanized and the plantar surfaces of injected hindpaws were excised and homogenized with 200 mM potassium phosphate buffer (pH 6.5). The homogenate was used to determine the reduced glutathione (GSH) and lipid hydroperoxides (LOOH) levels. Subsequently, the homogenate was centrifuged at 9,000 × g for 20 min at 4 ºC, providing the supernatant, which was used for the determination of superoxide dismutase (SOD) and catalase (CAT) activities, and the pellet, which was used to determine the myeloperoxidase (MPO) levels.

Furthermore, the hindpaw tissue was used for the determination of cytokine (tumor necrosis factor alpha, TNF-α; interleukin 1 beta, IL-1β; and interleukin 10, IL-10) levels through homogenization in RIPA-buffer with protease and phosphatase inhibitors before being centrifuged at 9,000 × g for 20 min (4 ºC). The tissue used for the determination of prostaglandin E_2 (PGE_2) was homogenized in 0.1 M phosphate buffer (pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA) and 10 µM ibuprofen.

2.4.1 Assay for determination of protein concentration

The protein concentration of samples supernatants (5 µL) was determinate by Bradford (1976) method using bovine serum albumin as standard curve (0.062 – 5 mg/mL) to interpolate the values read at 540 nm, according to the recommendation of the manufacturer (Bio-Rad, Hercules, CA, USA),.

2.4.2 Determination of myeloperoxidase activity

To measure the myeloperoxidase (MPO) activity (an indirect marker of neutrophil infiltration), the pellet obtained was re-suspended in 1 mL of 80 mM potassium phosphate buffer (5.4 pH) plus hexadecyltrimethylammonium bromide (HTAB) and centrifuged at 11000 × g for 20 min at 4 ºC. The supernatant (30 µL) was mixed with 0.017% H_2O_2 and 18.4 mM 3,3',5,5'-tetramethylbenzidine (TMB) in phosphate buffer and incubated for 3 min at 37 ºC.

The colorimetric reaction was stopped with sodium acetate (1.46 M, pH 3.0) and the absorbance was determinate at 620 nm. The results are expressed as optic density (O.D.)/mg of protein (Bradley et al. 1982; De Young et al. 1989).

2.4.3 Determination of hindpaw cytokine levels

The cytokine levels were evaluated by enzyme-linked immunosorbent assay (sandwich ELISA). Sample aliquots of supernatant (100 µL, 1:2 dilution) were used to measure tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 10 (IL-10) levels using murine cytokine ELISA kits (Peprotech EC Ltd, London, UK), according to the manufacturer's instructions. The absorbance for IL-1β detection was measured using a microplate reader at 405 nm with wavelength correction to 650 nm, and for TNF-α and IL-10 detections was read at 450 nm with wavelength correction to 620 nm. The values of TNF-α, IL-1β, and IL-10 absorbances were interpolated in a standard curve for each cytokine (0 – 2500; 0 – 4000; 0 – 2000 pg/mL, respectively) and expressed as pg/mg of protein.

2.4.4 Determination of prostaglandin E_2 levels

The prostaglandin E_2 (PGE_2) levels in purified samples from hindpaw tissues was quantified using competitive ELISA kit - monoclonal (Cayman Chemical, Ann Arbor, MI, USA), according to the methodology suggested by the manufacturer. The standard curve of PGE_2 ranged from 7.8 – 1000 pg/mL.

2.4.5 Determination of lipid hydroperoxides content

The content of hydroperoxides (LOOH) in the homogenate was measured based on Ferrous Oxidation-Xylenol orange (FOX2) method (Jiang et al. 1992). Samples (40 µL) were mixed with 90% methanol (1:1), centrifugated at 10,000 × g for 30 min (4 ºC). The supernatants were incubated for 30 min with FOX2 reagent (4 mM butylated hydroxytoluene (BHT) 250 mM, $FeSO_4$, 25 mM H_2SO_4 and xylenol orange at 100 mM). The absorbance of colorimetric reaction was measured at 560 nm, and the results are expressed as mmol/mg of tissue.

2.4.6 Determination of superoxide dismutase activity

The measured of superoxide dismutase (SOD) activity was based in its capacity to inhibit pyrogallol autoxidation. For this, supernatants aliquots (20 µL) were added to 200 mM Tris HCl–EDTA buffer solution (pH 8.5) and vortexed. Then, 25 µL of 1 mM pyrogallol was mixed and incubated for 20 min. The reaction was stopped with 1 N HCl, centrifuged for 4 min at 14000 × g at room temperature, and the absorbance read at 405 nm. One unit (U) of SOD activity was defined as the amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control. The results of SOD activity are expressed as U/mg of protein (Marklund and Marklund 1974; Gao et al. 1998)**.**

2.4.7 Determination of catalase activity

The catalase (CAT) activity was evaluated according to the method of Aebi (1984), based on its capacity to decompose the hydrogen peroxide (H_2O_2) resulting in the decrease of optical density. Supernatants aliquots (5 µL) were mixed to the reaction buffer (1 mM Tris, 5 mM EDTA, and 30 % H_2O_2, pH 8.5), and the absorbance was read at 240 nm for 5 min (minute by minute). The CAT activity was defined as the amount of enzyme required to split 1 nM of H_2O_2 per minute at 25 ºC. The results are expressed as mmol/min/mg of protein.

2.4.8 Determination of reduced glutathione levels

To measure the reduced glutathione (GSH) levels, aliquots from homogenate (50 µL) were mixed with 12.5% trichloroacetic acid (ATC), vortexed vigorously, and centrifugated at 900 × g for 15 min at 4 ºC. Subsequently, the supernatants obtained were mixed with 400 mM TRIS-HCl buffer (pH 8.5) and 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which react with GSH to generate 2-nitro-5-thiobenzoic acid (a yellow compound). The absorbance of the reaction was read at 415 nm, and the values were interpolated into a standard curve of GSH (0.312 – 500 µg/mL) and corrected for the tissue weight (Sedlak and Lindsay 1968). The results are expressed as µg/g of tissue**.**

2.5 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

The antioxidant property of natural products is commonly evaluated by their capacity in scavenging the stable free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH) *in vitro*. The reduction of DPPH produce a colorimetric reaction that can be measured by decreasing of absorbance as demonstrated by Blois (1958). Briefly, 225 µL of ascorbic acid (AA, 50 µg/mL, a positive control), HF or IBA (30 – 1000 µg/mL) were mixed with 75 µL of DPPH (40 µg/mL) and incubated for 5 min. The absorbance was read at 517 nm and the individual values were interpolated in a standard curve of DPPH (0 – 60 µM). The experiment was performed in triplicate for each dilution and the results are expressed as µM DPPH.

2.6 Statistical analyses

The results of inflammatory and oxidative stress parameters were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's multi-comparison post hoc test, and unpaired T-test for comparison between naïve (N) and vehicle (V) groups. The data concern mechanical allodynia and paw edema were analyzed using two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons post-hoc test. All the results are presented as mean ± S.E.M and significant difference was considered when * or $^{\#}P < 0.05$. The

analyses were performed using the GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA).

3 Results

3.1 Effect of local pretreatment with HF and IBA on mechanical allodynia and paw edema induced by carrageenan

The acute inflammatory process induced by intraplantar injection of carrageenan is primarily manifested by increasing of mechanical sensitivity (allodynia) and paw thickness (edema). Carrageenan injection decreased the paw withdrawal threshold from 1.7 ± 0.2 g (B) to 0.4 ± 0.1 g at 0.5 h, and to 0.08 ± 0.0 g at 3 h, maintaining to 0.04 ± 0.0 g until 6 h (Fig. 1A). In the same way, carrageenan-induced edema formation of 1.2 ± 0.1 mm at 0.5 h, 1.7 ± 0.1 mm at 3 h, and 1.8 ± 0.1 mm at 6 h, regarding basal values (B) (Fig. 1B).

The systemic pretreatment with dexamethasone completely abolished the mechanical allodynia and edema induced by carrageenan for 5 and 6 h of evaluation, respectively. Local pretreatment with HF reduced mechanical allodynia in 77.1, 73.5, 76.0, 82.2% at 0.5, 1, 2 and 3 h after carrageenan injection, when compared to its basal value (B: 1.6 ± 0.2 g, Fig 1A). The antiedematogenic effect of HF was observed at 0.5, 1, 2, 3, 4 and 5 h, reducing the paw edema in 56.1, 63.4, 74.3, 78.0, 33.9, and 32.4%, respectively, when compared to the vehicle-pretreated group (Fig. 1B). Similarly, IBA also showed antiallodynic effect at 0.5, 1, 2 and 3 hours, reducing the mechanical allodynia in 76.1, 68.4, 76.1 and 51.0%, respectively, when compared to its basal value (B: 1.5 ± 0.2 g, Fig. 1A). Likewise, IBA inhibited the paw edema formation in 68.4, 58.4, 63.9, 55.7, 21.6, 24.0% at 0.5, 1, 2, 3, 4 and 5 h after carrageenan injection, when compared to the vehicle group (Fig. 1B).

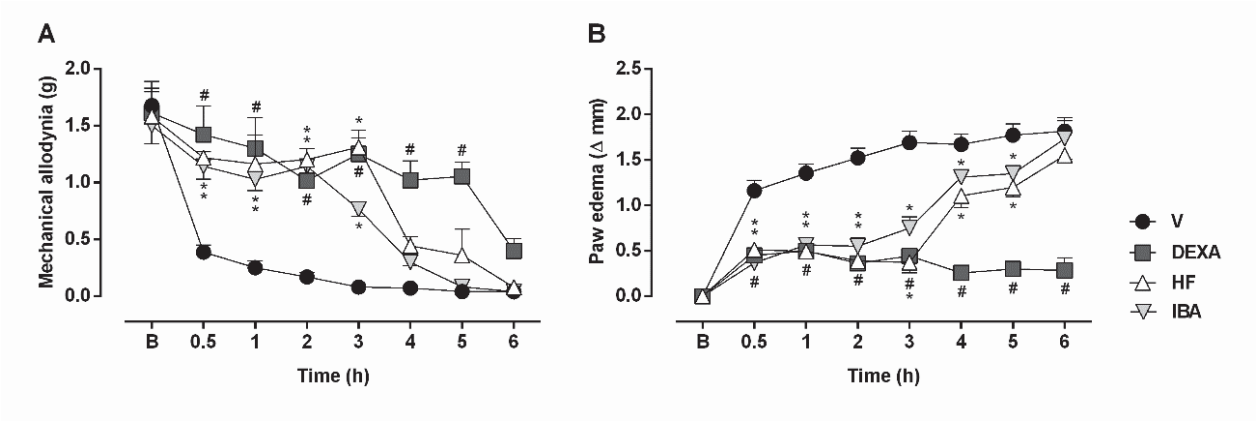


Fig. 1 Effect of HF and IBA on mechanical allodynia (A) and paw edema (B) induced by carrageenan. Mice were pretreated with dexamethasone (DEXA: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.), 30 or 15 min, respectively, before intraplantar injection of carrageenan (300 µg/20 µL). Mechanical allodynia (g) and paw edema (Δ mm) were evaluated before (B: basal value) and after carrageenan injection at 0.5, 1, 2, 3, 4, 5, and 6 h. The results are expressed as mean ± S.E.M (n = 8). $^{*,\#}P < 0.05$ compared to vehicle (V) group (two-way ANOVA followed by Bonferroni test).

3.2 Effect of HF and IBA on neutrophil infiltration and cytokine levels

The intraplantar injection of carrageenan induced a high neutrophil infiltration in hindpaw tissue revealed by high activity of MPO, when compared to naïve group (N: 2.1 ± 0.1 O.D./mg of protein; Fig. 2). The pretreatment with dexamethasone, HF and IBA reduced the MPO activity in 48.7, 68.9 and 49.4%, respectively, when compared to vehicle-treated group (V: 11.1 ± 0.9 O.D./mg of protein, Fig. 2).

The acute inflammatory process induced by carrageenan increased the TNF-α levels (Fig. 3A), which were significantly reduced by dexamethasone, HF and IBA pretreatments in 57.1, 64.4, 57.3%, respectively, when compared to vehicle group (V: 1400.5 ± 243.9 pg/mg of protein). In the same way, the high levels of IL-1β (Fig. 3B) induced by carrageenan also were markedly reduced by dexamethasone, HF and IBA in 85.5, 76.8 and 80.8%, when compared to vehicle group (V: 1192.0 ± 131.5 pg/mg of protein).

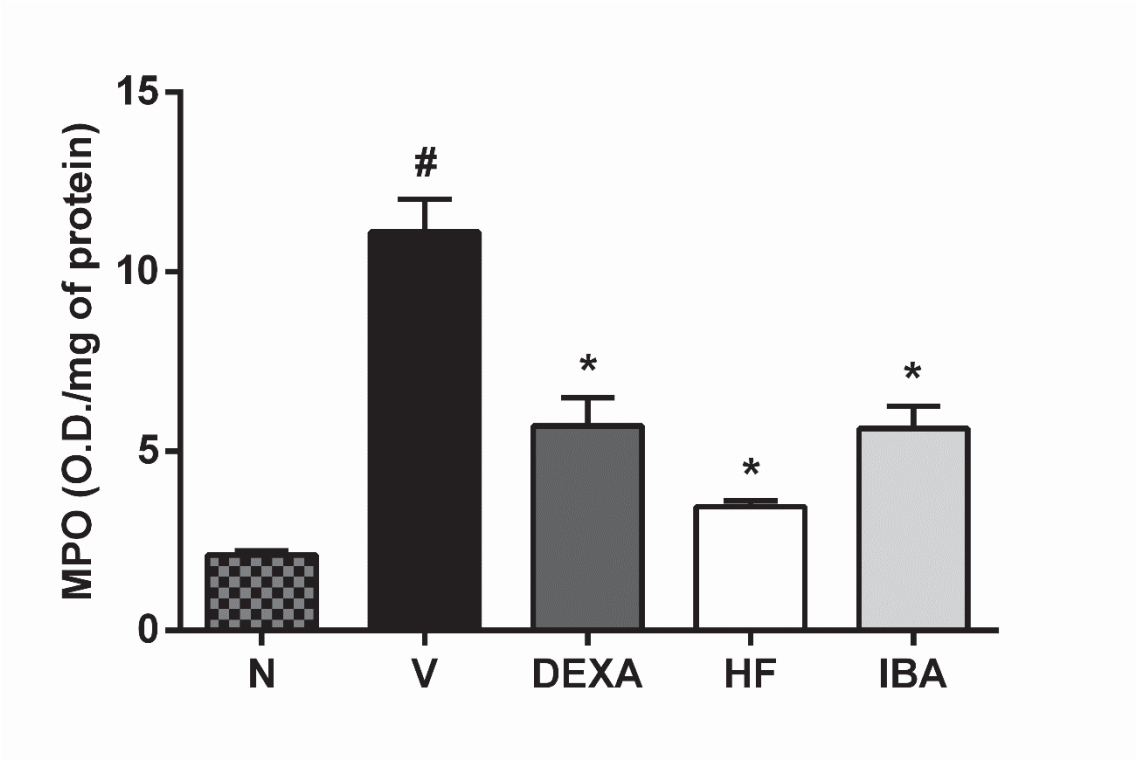


Fig. 2 Effect of HF and IBA on myeloperoxidase (MPO) activity. Mice were pretreated with dexamethasone (DEXA: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.), 30 or 15 min, respectively, before intraplantar injection of carrageenan (300 µg/20 µL). The results are expressed as mean ± S.E.M (n = 7). *$P < 0.05$ compared to vehicle (V) group (one-way ANOVA followed by Bonferroni test); $^{\#}P < 0.05$ compared to naïve (N) group (unpaired *t*-test).

The anti-inflammatory cytokine, IL-10 (Fig. 3C), was reduced by intraplantar injection of carrageenan in 52.7% when compared to naïve group (N: 2221.6 ± 287.4 pg/mg of protein). Dexamethasone and local HF treatments prevented the decreased of IL-10 in 41.6 and 49.3%, respectively, when compared to vehicle group (V: 1052.2 ± 103.8 pg/mg of protein). Intraplantar injection of IBA did not prevent the reduction of IL-10 levels.

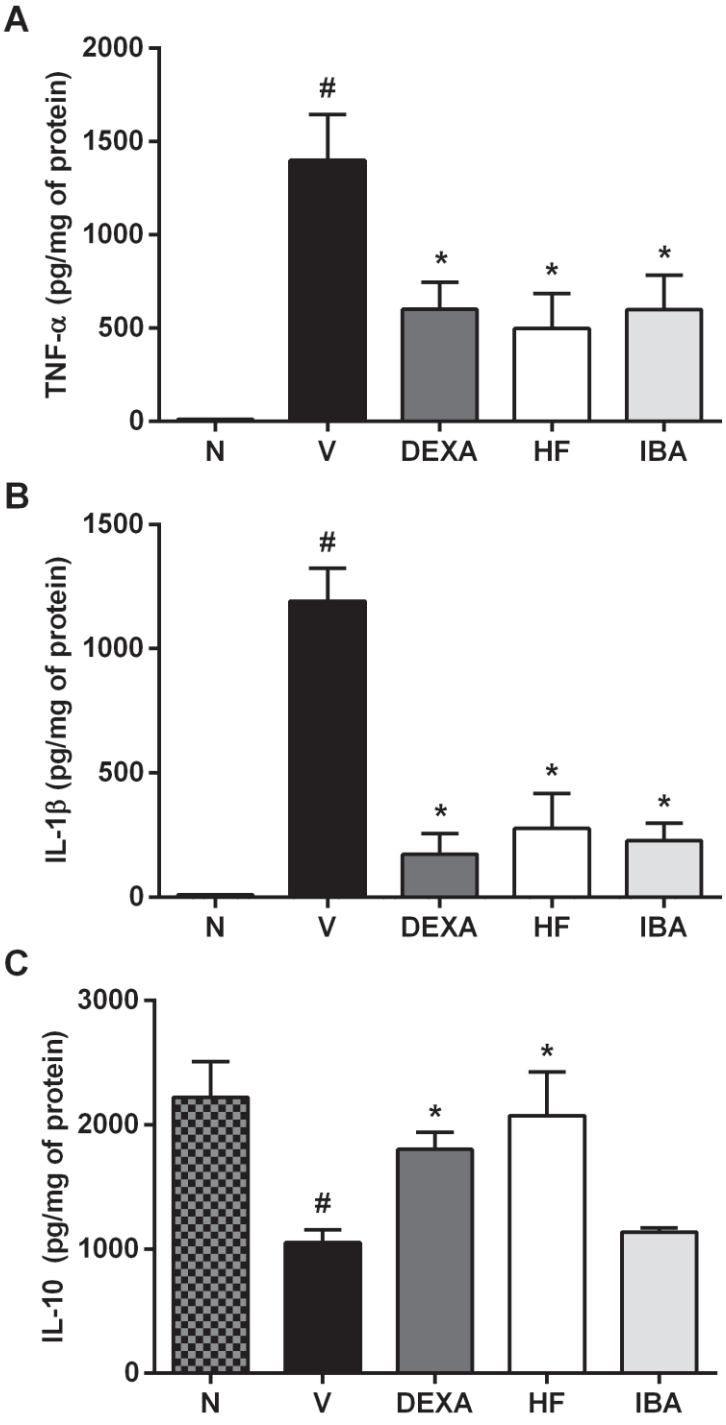


Fig. 3 Effect of HF and IBA on TNF-α (A), IL-1β (B), and IL-10 levels. Mice were pretreated with dexamethasone (DEXA: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.), 30 or 15 min, respectively, before intraplantar injection of carrageenan (300 µg/20 µL). The results are expressed as mean ± S.E.M (n = 5 - 6). *$P < 0.05$ compared to vehicle (V) group (one-way ANOVA followed by Bonferroni test); $^{\#}P < 0.05$ compared to naïve (N) group (unpaired *t*-test).

3.3 Effect of HF and IBA on PGE_2 levels

Intraplantar injection of carrageenan elevates significantly the PGE_2 synthesis (Fig. 4) in inflamed hindpaw tissue when compared to naïve group (N: 224.8 ± 41.5 pg/mL). Dexamethasone and local HF treatments were able to reduce PGE_2 levels in 38.7 and 39.2%, when compared to vehicle-treated group (V: 639.4 ± 23.9 pg/mL). Intraplantar injection of IBA did not prevent the increase of PGE_2 levels.

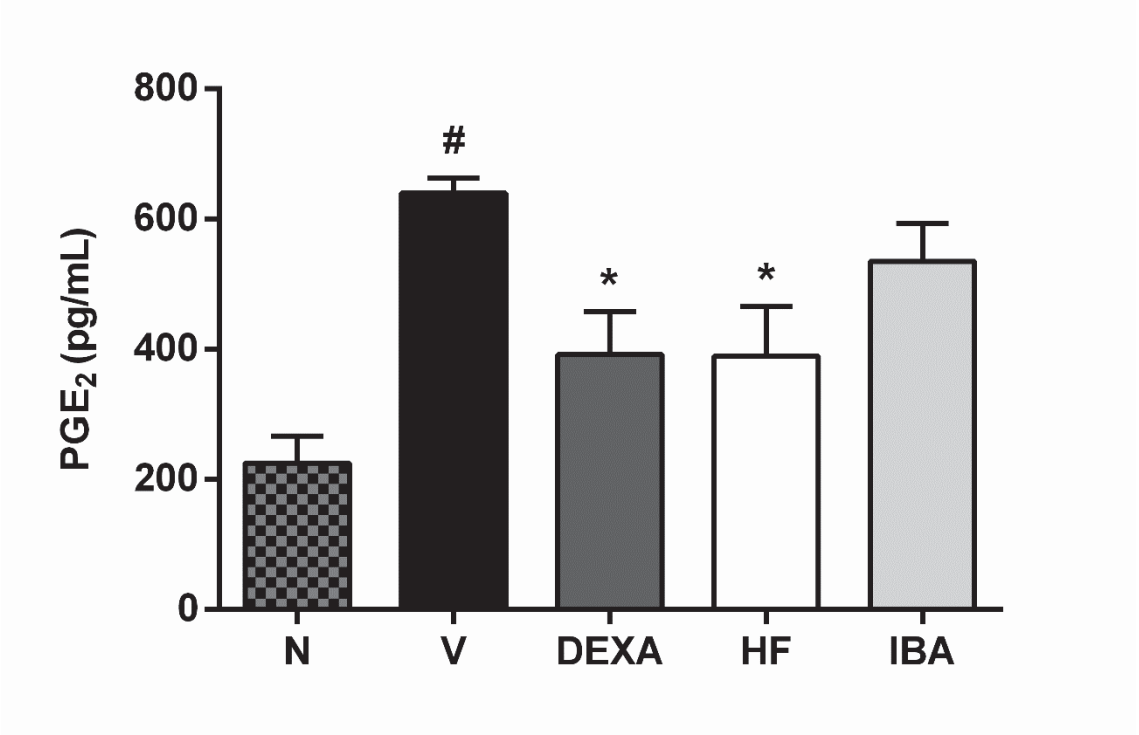


Fig. 4 Effect of HF and IBA on PGE_2 levels. Mice were pretreated with dexamethasone (DEXA: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.), 30 or 15 min, respectively, before intraplantar injection of carrageenan (300 µg/20 µL). The results are expressed as mean ± S.E.M (n = 4). *$P < 0.05$ compared to vehicle (V) group (one-way ANOVA followed by Bonferroni test); $^{\#}P < 0.05$ compared to naïve (N) group (unpaired *t*-test).

3.4 Effect of HF and IBA on antioxidant systems

The intraplantar injection of carrageenan induced an intense oxidative stress response in the hindpaw, which was firstly reveled by high content of LOOH (Fig. 5A) in homogenate tissue of vehicle-pretreated animals, in comparison to the naïve animals (N: 9.9 ± 1.1 mmol/mg of tissue). The systemic pretreatment with dexamethasone, and the local pretreatment with HF and IBA prevented the LOOH production in 75.3, 66.8 and 41,6%, when compared to vehicle group (V: 28.3 ± 1.0 mmol/mg of tissue).

In inflamed tissue the activity of SOD enzyme was quietly decreased in 9.4% (V: 9.5 ± 0.2 U/mg of protein; Fig. 5B), when compared to non-inflamed hindpaw tissue (N: 10.5 ± 0.3 U/mg of protein), and the pretreatments with dexamethasone, HF and IBA restored SOD activity in 13.3, 18.8 and 10.4%, when compared to vehicle group.

CAT activity (Fig. 5C) in vehicle-pretreated animals was significantly reduced in 62.5% when compared to naïve group (N: 0.8 ± 0.1 mmol/min/mg of protein; V: 0.3 ± 0.1 mmol/min/mg of protein). On treated groups, the CAT activity was reestablished in 56.3, 55.2 and 50,7% by dexamethasone, HF and IBA, respectively, when compared to vehicle group.

Levels of non-enzymatic antioxidant agent (GSH) were strongly depleted by the acute inflammatory process induced by carrageenan in 84.8% (2502.2 ± 344.8 µg/g of tissue), when compared to naïve group (N: 16512.6 ± 4121.0 µg/g of tissue) (Fig. 5D). The pretreatments with dexamethasone, HF and IBA prevented the GSH depletion in 64.7, 65.7 and 64.9%, when compared to vehicle group.

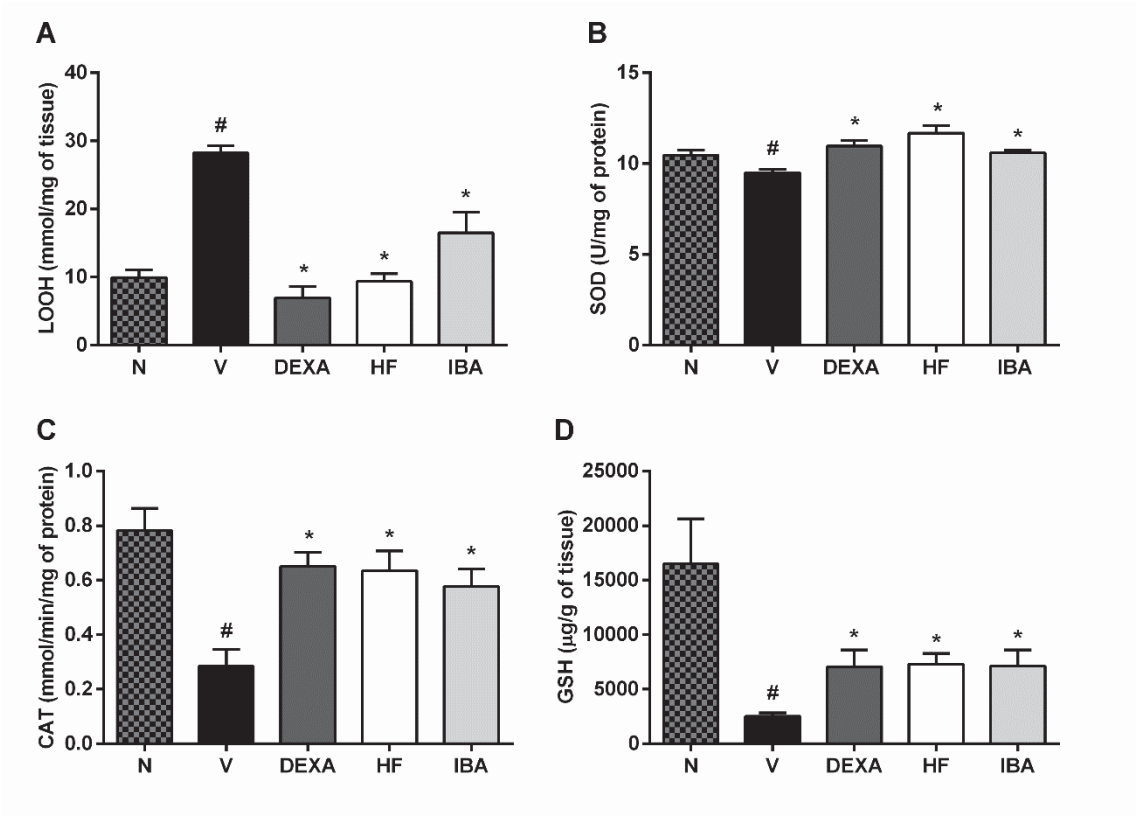


Fig. 5 Effect of HF and IBA on LOOH (A) contents, SOD (B) and CAT (C) activities, and GSH (D) levels. Mice were pretreated with dexamethasone (DEXA: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.), 30 or 15 min, respectively, before intraplantar injection of carrageenan (300 µg/20 µL). The results are expressed as mean ± S.E.M (n = 5 - 7). $^{*}P < 0.05$ compared to vehicle (V) group (one-way ANOVA followed by Bonferroni test); $^{\#}P < 0.05$ compared to naïve (N) group (unpaired *t*-test).

3.5 Effect of HF and IBA on in vitro DPPH free radical-scavenging

Ascorbic acid (AA) and HF at 300 and 1000 µg/mL scavenged the DPPH radicals in 53.1, 43.3, and 100%, respectively, when compared to vehicle group (V). Interestingly, no one tested concentration of IBA was capable to decreasing the DPPH radicals (Table 1).

Sample	µg/mL	DPPH (µM)
V		28.6 ± 0.5
AA	50	13.4 ± 1.6*
HF	30	27.4 ± 0.3
	100	28.2 ± 1.1
	300	16.2 ± 0.4*
	1000	0.0 ± 0.0*
IBA	30	26.7 ± 0.4
	100	27.4 ± 2.0
	300	27.9 ± 1.1
	1000	23.8 ± 1.3

Table 1 Effect of HF and IBA on scavenging the free radical DPPH *in vitro*.

The results are expressed as mean ± S.E.M (triplicate). *$P < 0.05$ compared to vehicle (V) group (one-way ANOVA followed by Bonferroni test).

4 Discussion

The ethnomedicinal knowledge of plants guides and encourage many research groups to investigate new and more efficient therapies to treat pain and inflammation. Alkylamides have been studied for years through pharmacological assays to better elucidate their analgesic and anti-inflammatory properties, and to establish this class of molecules as a target for the development of new drugs. In this sense, this study shows the promising effects of both local pretreatment with natural alkylamides from *Acmella oleracea* and the synthetic isobutylalkyl amide on carrageenan model of inflammation in mice.

The intraplantar administration of carrageenan was responsible to promote intense acute inflammatory response with concomitant and rapid development of mechanical allodynia and edema in just 30 min after its injection, which persisted for 6 hours. The edema occurs mainly as a result of the release of histamine, serotonin, bradykinin, and prostaglandin (Vinegar et al. 1969; Rosa 1972; Della Pasqua et al. 2019). Consequently, these pro-inflammatory mediators lead to activation and sensitization of nociceptors, producing mechanical allodynia (Ji et al. 2016).

We demonstrated that the local pretreatment with HF and IBA promoted remarkable antiallodynic and antiedematogenic effects. Similarly, a cold extract of *Acmella oleracea* aerial parts administrated orally also prevent the edema formation on carrageenan model in rats (Chakraborty et al. 2004). Hernández et al. (2009) showed that an ethanolic extract rich in alkylamides from *Heliopsis longipes* was more potent than the purified spilanthol (mainly alkylamide in the extract) on a model of arachidonic acid-induced ear edema in mice, since the extract present lower ED_{50}. Likewise, synthetic N-pyridyl-(indol-3-yl) alkylamides with 4-fluorobenzyl or benzyl radicals added in indole ring also reduced the paw edema induced by carrageenan in rats, being the alkylamide with 4-fluorobenzyl radical the most potent anti-inflammatory molecule tested (Fouchard et al. 2001).

Furthermore, during the inflammatory process, the sensitization of channels and receptors expressed in the nociceptors, such as TRPV1, have an important role in the allodynia development (Watanabe et al. 2015). In this context, the alkylamides from jambu, IBA, and pure spilanthol have already been described as TRPV1 antagonists (de la Rosa-Lugo et al. 2017; Dallazen et al.submitted data 2018), which could contribute for the antiallodynic effect observed in this study.

The anti-inflammatory effect of natural alkylamides from jambu and synthetic IBA are reinforced by the inhibition of neutrophil infiltration into the inflamed site. The adhesion of this cells on endothelium by interaction with intercellular adhesion molecule 1 (ICAM-1) is a fundamental prior event that leads the neutrophils migration through venular walls to reach the damage tissue (Nourshargh and Alon 2014). Huang et al. (2018), using an *in vitro* model of IL-1β-induced lung inflammation, showed that spilanthol, main alkylamide in HF, suppressed the monocyte adhesion to epithelial cells by decreasing ICAM-1 expression via inhibition of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling pathways. Therefore, at a first moment, we could attribute the inhibition of neutrophil migration, which was revealed by low MPO activity in inflamed paw tissue, to the inhibition of ICAM-1 expression by jambu alkylamides, including spilanthol, and synthetic IBA. Recently, Kim et al. (2018) have shown that an *Acmella oleracea* methanolic extract of whole plant (syn. *Spilanthes acmella* Murray) was capable to suppress MPO activity in lung tissues on a model of acute inflammation induced by LPS intratracheally in mice. The authors suggested that the anti-inflammatory effect is a result of a collective action between spilanthol and others phytochemical constituents founded in the extract (isoquercetin, scopoletin, ferulic and vanillic acids).

During the inflammatory response, the release of immune cytokines, like TNF-α and IL-1β, has a crucial involvement in the sustenance of inflammation through: cell recruitment and migration; development and maintenance of pain and allodynia by directedly activation and sensitization of nociceptors; and edema formation (Cook et al. 2018). Locally, it was observed that HF and IBA prevented the TNF-α and IL-1β releasing. The transcription of these cytokines is regulated by NF-κB pathways activation (Li and Verma 2002). Wu et al. (2008) have shown *in vitro* that spilanthol inactivated NF-κB signaling through inhibition of IκB phosphorylation, reducing the release of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in LPS-treated murine macrophages, corroborating with our *in vivo* results.

In our experimental conditions, the levels of anti-inflammatory IL-10 was reduced after carrageenan injection (Chou 2003), and only the local pretreatment with alkylamides from jambu (HF) maintained basal levels of IL-10, preventing its depletion. IL-10 not only potently inhibits the production of pro-inflammatory cytokines, such as TNF-α, IL-1β (Moore et al. 2001), but also induces the expression of others anti-inflammatory and antioxidant agents, like heme oxygenase-1 (HO-1), a stress-response protein induced during oxidative stress (Lee and Chau 2002). Here, HF, which is rich in spilanthol, maintained IL-10 levels *in vivo*, whereas

Huang et al. (2018) showed that pure spilanthol upregulated heme oxygenase-1 (HO-1) protein expression *in vitro*. Collectively, these data could suggest that spilanthol is somehow involved on IL-10/OH-1 signaling pathway to promote its anti-inflammatory and antioxidant effects. Increased IL-10 levels were observed in mouse splenocyte following *in vitro* incubation with an aqueous extract of *Echinacea purpurea* (Hwang et al. 2004). Interestingly, Chicca et al. (2009) showed that two hydro-alcoholic extracts rich in alkylamides from roots and fresh herb of *Echinacea purpurea* did not change the IL-10 levels in human peripheral blood mononuclear cells stimulated with LPS. However, when the extracts were combined, the authors observed a superstimulation of IL-10 expression, revealing the synergistic immunopharmacological effect of alkylamides. This data could potentially explain the inability of IBA, a single and synthetic alkylamide, in sustaining IL-10 levels in mice hindpaw, different from HF, a jambu fraction rich in alkylamides.

Prostaglandins, mainly PGE_2, are important lipid-derived mediators produced in an inflammatory process and responsible for many of its cardinal signs, such as edema formation and pain sensitization (Kawahara et al. 2015). They are a group of eicosanoids synthetized by constitutive and induced cyclo-oxygenase (COX) 1 and 2, respectively, from arachidonic acid (Ricciotti and Fitzgerald 2011). The potent anti-inflammatory effect of HF observed in our model could be associated with the inhibition of PGE_2 synthesis. In fact, the suppression of COX-2 expression by spilanthol, major alkylamide of jambu (Wu et al. 2008; Huang et al. 2018) reinforce our results. Additionally, the amide carbonyl group present in the spilanthol structure could interact with serine residue (position 516) present in COX-2 active site via nucleophilic attack, resulting in enzyme inhibition (Prachayasittikul et al. 2013). Again, IBA did not prevent the PGE_2 production *in vivo*. We therefore hypothesize that the IBA tested dose (0.1 μg) was not enough to prevent the PGE_2 production, and that the efficacy of HF in reducing PGE_2 levels at low dose could result from the synergism between alkylamides (Prachayasittikul et al. 2013).

Yet other studies using alkylamides from different plants have been conducted to demonstrate the mechanisms of action involved in the anti-inflammatory activity *in vitro*. *Echinacea* extracts and a dodeca-2E,4E-dienoic acid isobutylamide inhibit the mast cell degranulation, TNF-α and PGE_2 releasing (Gulledge et al. 2018). Similarly, *Echinacea* alkylamides inhibited TNF-α expression via cannabinoid CB2 receptor activation (Gertsch et al. 2004), and inhibited COX-2 activity (Raduner et al. 2006; Hinz et al. 2007; Lalone et al. 2010). Guineensine, an alkylamide from *Piper nigrum*, showed analgesic and anti-

inflammatory effects related to inhibition of the endocannabinoid uptake and activation of cannabinoid CB1 receptor (Nicolussi et al. 2014; Reynoso-Moreno et al. 2017). Finally, alkylamides from *Heliopsis helianthoides* and *Lepidium meyenii* also presented potential cannabimimetic action (Hajdu et al. 2014).

The oxidative stress generated during the inflammation is due to the infiltration of activated neutrophils with high myeloperoxidase (MPO) activity into tissue (Kettle and Winterbourn 1997). Production of reactive oxygen species (ROS) and other free radicals exerts negative influence on endogenous antioxidant system and leads to production of lipid hydroperoxides (LOOH) and cell damage (Pisoschi and Pop 2015). Accumulation of intracellular free radicals is essentially combated by three antioxidant components: superoxide dismutase (SOD) and catalase (CAT) enzymes, and glutathione (GSH). Briefly, SOD convert superoxide radical into H_2O_2, while CAT convert H_2O_2 into water (H_2O) and molecular oxygen (O_2). The non-enzymatic antioxidant, GSH, is cofactor of glutathione peroxidase (GPx), which convert H_2O_2 in H_2O (Weydert and Cullen 2010; Costa et al. 2018).

Natural alkylamides of HF and the synthetic IBA administrated locally prevented the formation of LOOH, the decrease of SOD and CAT activities, and the depletion of GSH levels. However, the restoration of balance between the oxidant and antioxidant system could not be attributed to the scavenging of free radicals, since only HF presented this property at high concentrations (300 and 100 µg/mL), different of IBA. These observations reinforce the notion that the mechanisms whereby HF and IBA promotes the conservation of endogenous antioxidants agents is correlated with the inhibition of immune cell migration, which prevent the respiratory burst and consequent ROS production. Indeed, several extracts from *Acmella oleracea* have been tested on DPPH assay. Ethyl acetate extract of dried flowers and methanolic extracts from aerial parts exhibited high radical scavenging capacity (Wongsawatkul et al. 2008; Wu et al. 2008; Prachayasittikul et al. 2009). However, this property was attributed to the presence of phenolic compounds and coumarin in the extracts obtained using polar solvents, whereas *Acmella oleracea* extracts prepared with nonpolar solvents showed weak antioxidant activity. In this regard, Wongsawatkul et al. (2008) showed that triterpenes and long hydrocarbon chains with esters or alcohols groups identified in hexane extract of aerial parts from *Acmella oleracea* presented low activity on DPPH scavenging. Moreover, hydroalcoholic extracts of *Echinacea purpurea* and *Echinacea angustifolia* (Aarland et al. 2017), and several extracts obtained with polar solvents (water, methanol, and ethanol) from *Zanthoxylum bungeanum* (Chung et al. 2013; Ma et al. 2018) also demonstrated antioxidant effects on DPPH

assay. It is pertinent to note that all these plants are rich in alkylamides, but the antioxidant ability is attributed to the high content of phenols and flavonoids. Recently, Chakthong et al. (2018) showed that the (2E,6E,8E)-N-(2-methylpropyl)-10-oxo-2,6,8-decatrienamide isolated from *Zanthoxylum nitidum* presented low antioxidant activity on DPPH assay. Collectively, we concluded that natural alkylamides from HF and the synthetic IBA displayed a weak free radical scavenging ability.

5 Conclusion

Taken together, we have shown that the local pretreatment with alkylamides from jambu flowers (HF), and with synthetic IBA possess a potent anti-inflammatory property in carrageenan-induced acute inflammation, reducing the allodynia and paw edema in mice. Locally, this is achieved by inhibition of a) neutrophil migration, b) pro-inflammatory cytokines release, and c) oxidative stress. Moreover, HF also reduced PGE_2 synthesis and maintained IL-10 levels. Accordingly, the present findings strengthen the potential of alkylamides to treat inflammatory pain conditions and add promising data to support further studies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Aarland RC, Bañuelos-Hernández AE, Fragoso-Serrano M, et al (2017) Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of Echinacea purpurea and Echinacea angustifolia extracts. Pharm Biol 55:649–656. doi: 10.1080/13880209.2016.1265989

Aebi H (1984) [13] Catalase In Vitro. In: Methods in enzymology. pp 121–126
Blois MS (1958) Antioxidant Determinations by the Use of a Stable Free Radical. Nature 181:1199–1200

Boonen J, Bronselaer A, Nielandt J, et al (2012) Alkamid database: Chemistry, occurrence and functionality of plant N-alkylamides. J. Ethnopharmacol. 142:563–590. doi: 10.1016/j.jep.2012.05.038

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi: 10.1016/0003-2697(76)90527-3

Bradley PP, Priebat DA, Christensen RD, Rothstein G (1982) Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. J Invest Dermatol 78:206–209. doi: 10.1111/1523-1747.ep12506462

Carlotto J, de Souza LM, Baggio CH, et al (2016) Polysaccharides from Arctium lappa L.: Chemical structure and biological activity. Int J Biol Macromol 91:954–960. doi: 10.1016/j.ijbiomac.2016.06.033

Chakraborty A, Devi RKB, Rita S, et al (2004) Preliminary studies on anti-inflammatory and analgesic activities of Securinega virosa (Euphorbiaceae) in experimental animal models. Indian J Pharmacol 36:148–150

Chakthong S, Ampaprom R, Inparn S, et al (2018) New alkylamide from the stems of Zanthoxylum nitidum. Nat. Prod. Res. 1–9. doi: 10.1080/14786419.2018

Chaplan SR, Bach FW, Pogrel JW, et al (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63. doi: 10.1016/0165-0270(94)90144-9

Cheng Y Bin, Liu RH, Ho MC, et al (2015) Alkylamides of Acmella oleracea. Molecules 20:6970–6977. doi: 10.3390/molecules20046970

Chicca A, Raduner S, Pellati F, et al (2009) Synergistic immunomopharmacological effects of N-alkylamides in Echinacea purpurea herbal extracts. Int Immunopharmacol 9:850–858. doi: 10.1016/j.intimp.2009.03.006

Chou TC (2003) Anti-inflammatory and analgesic effects of paeonol in carrageenan-evoked thermal hyperalgesia. Br J Pharmacol 139:1146–1152. doi: 10.1038/sj.bjp.0705360

Chung JW, Oh JU, Lee S, Kim SJ (2013) Inhibition of inducible nitric oxide synthase, cycleooxygenase-2 and lipid peroxidation by methanol extract of Pericarpium Zanthoxyli.

Trop J Pharm Res 12:369–375. doi: 10.4314/tjpr.v12i3.15

Cook AD, Christensen AD, Tewari D, et al (2018) Immune Cytokines and Their Receptors in Inflammatory Pain. Trends Immunol xx:1–16. doi: 10.1016/j.it.2017.12.003

Costa NT, Iriyoda TMV, Alfieri DF, et al (2018) Influence of disease-modifying antirheumatic drugs on oxidative and nitrosative stress in patients with rheumatoid arthritis. Inflammopharmacology 26:1151–1164. doi: 10.1007/s10787-018-0514-9

Dallazen JL, Maria-ferreira D, Barbosa B, et al (2018) Distinct mechanisms underlying local antinociceptive and pronociceptive effects of alkylamides from Acmella oleracea flowers. Submitted data.

Day RO, Graham GG (2013) Therapeutics: Non-steroidal anti-inflammatory drugs (NSAIDs). BMJ 346:34–37. doi: 10.1136/bmj.f3195

de la Rosa-Lugo V, Acevedo-Quiroz M, Déciga-Campos M, Rios MY (2017) Antinociceptive effect of natural and synthetic alkamides involves TRPV1 receptors. J Pharm Pharmacol 69:884–895. doi: 10.1111/jphp.12721

De Young LM, Kheifets JB, Ballaron SJ, Young JM (1989) Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions 26:335–341. doi: 10.1007/BF01967298

Della Pasqua CSP, Iwamoto RD, Antunes E, et al (2019) Pharmacological study of anti-inflammatory activity of aqueous extracts of Mikania glomerata (Spreng.) and Mikania laevigata (Sch. Bip. ex Baker). J Ethnopharmacol 231:50–56. doi: 10.1016/j.jep.2018.11.012

Dixon WJ (1980) Efficient analysis of experimental Observations. Annu Rev Pharmacol Toxicol 20:441–462

Dubey S, Maity S, Singh M, et al (2013) Phytochemistry, pharmacology and toxicology of spilanthes acmella: A review. Adv Pharmacol Sci 2013:. doi: 10.1155/2013/423750

Dutra RC, Campos MM, Santos ARS, Calixto JB (2016) Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol. Res. 112:4–29. doi: 10.1016/j.phrs.2016.01.021

Fouchard F, Marchand P, Le Baut G, et al (2001) Synthesis and pharmacological evaluation of (indol-3-yl) alkylamides as potent analgesic agents. ARZNEIMITTEL-FORSCHUNG-DRUG Res 51:814–824. doi: 10.1055/s-0031-1300120

Gao R, Yuan Z, Zhao Z, Gao X (1998) Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. Bioelectrochemistry Bioenerg 45:41–45. doi: 10.1016/S0302-4598(98)00072-5

Gertsch J, Schoop R, Kuenzle U, Suter A (2004) Echinacea alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS Lett 577:563–569. doi: 10.1016/j.febslet.2004.10.064

Greger H (1984) Alkamides: structural relationships, distribution and biological activity. Planta Med 50:366–375. doi: 10.1055/s-2007-969741

Greger H (2016) Alkamides: a critical reconsideration of a multifunctional class of unsaturated fatty acid amides. Phytochem Rev 15:729–770. doi: 10.1007/s11101-015-9418-0

Gulledge T V., Collette NM, Mackey E, et al (2018) Mast cell degranulation and calcium influx are inhibited by an Echinacea purpurea extract and the alkylamide dodeca-2E,4E-dienoic acid isobutylamide. J Ethnopharmacol 212:166–174. doi: 10.1016/j.jep.2017.10.012

Hajdu Z, Nicolussi S, Rau M, et al (2014) Identification of endocannabinoid system-modulating N-alkylamides from Heliopsis helianthoides var. scabra and Lepidium meyenii. J Nat Prod 77:1663–1669. doi: 10.1021/np500292g

Hernández I, Márquez L, Martínez I, et al (2009) Anti-inflammatory effects of ethanolic extract and alkamides-derived from Heliopsis longipes roots. J Ethnopharmacol 124:649–652. doi: 10.1016/j.jep.2009.04.060

Hinz B, Woelkart K, Bauer R (2007) Alkamides from Echinacea inhibit cyclooxygenase-2 activity in human neuroglioma cells. Biochem Biophys Res Commun 360:441–446. doi: 10.1016/j.bbrc.2007.06.073

Huang WC, Wu LY, Hu S, Wu SJ (2018) Spilanthol Inhibits COX-2 and ICAM-1 Expression via Suppression of NF-κB and MAPK Signaling in Interleukin-1β-Stimulated Human Lung Epithelial Cells. Inflammation 41:1934–1944. doi: 10.1007/s10753-018-0837-0

Hwang SA, Dasgupta A, Actor JK (2004) Cytokine production by non-adherent mouse splenocyte cultures to Echinacea extracts. Clin Chim Acta 343:161–166. doi: 10.1016/j.cccn.2004.01.011

Ji R, Chamessian A, Zhang Y (2016) Pain regulation by non-neuronal cells and inflammation. Science (80-) 354:572–577. doi: 10.1126/science.aaf8924

Jiang ZY, Hunt J V., Wolff SP (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal Biochem 202:384–389. doi: 10.1016/0003-2697(92)90122-N

Kawahara K, Hohjoh H, Inazumi T, et al (2015) Prostaglandin E2-induced inflammation: Relevance of prostaglandin e receptors. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1851:414–421. doi: 10.1016/j.bbalip.2014.07.008

Kettle AJ, Winterbourn CC (1997) Myeloperoxidase: A key regulator of neutrophil oxidant product. Redox Rep. 3:3–15

Kim KH, Kim EJ, Kwun MJ, et al (2018) Suppression of lung inflammation by the methanol extract of Spilanthes acmella Murray is related to differential regulation of NF-κB and Nrf2. J Ethnopharmacol 217:89–97. doi: 10.1016/j.jep.2018.02.011

Lalone CA, Huang N, Rizshsky L, et al (2010) Enrichment of Echinacea angustifolia with Bauer alkylamide 11 and Bauer ketone 23 increased anti-inflammatory potential through interference with COX-2 enzyme activity. J Agric Food Chem 58:8573–8584. doi:

10.1021/jf1014268

Lee TS, Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 8:240–246. doi: 10.1038/nm0302-240

Li Q, Verma IM (2002) NF-κB regulation in the immune system. Nat. Rev. Immunol. 2:725–734. doi: 10.1038/nri910

Ma Y, Wang Y, Li X, et al (2018) Sensory characteristics and antioxidant activity of *Zanthoxylum bungeanum* Maxim. pericarps. Chem Biodivers. doi: 10.1002/cbdv.201800238

Marklund S, Marklund G (1974) Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. Eur J Biochem 47:469–474. doi: 10.1111/j.1432-1033.1974.tb03714.x

Moazami Y, Gulledge T V., Laster SM, Pierce JG (2015) Synthesis and biological evaluation of a series of fatty acid amides from Echinacea. Bioorganic Med Chem Lett 25:3091–3094. doi: 10.1016/j.bmcl.2015.06.024

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19:683–765. doi: 10.1146/annurev.immunol.19.1.683

National Research Council (2011) Guide for the Care and Use of Laboratory Animals. National Academies Press

Nicolussi S, Viveros-Paredes JM, Gachet MS, et al (2014) Guineensine is a novel inhibitor of endocannabinoid uptake showing cannabimimetic behavioral effects in BALB/c mice. Pharmacol Res 80:52–65. doi: 10.1016/j.phrs.2013.12.010

Nomura ECO, Rodrigues MRA, Da Silva CF, et al (2013) Antinociceptive effects of ethanolic extract from the flowers of Acmella oleracea (L.) R.K. Jansen in mice. J Ethnopharmacol 150:583–589. doi: 10.1016/j.jep.2013.09.007

Nourshargh S, Alon R (2014) Leukocyte Migration into Inflamed Tissues. Immunity 41:694–707. doi: 10.1016/j.immuni.2014.10.008

Pinho-Ribeiro FA, Verri WA, Chiu IM (2017) Nociceptor Sensory Neuron–Immune Interactions in Pain and Inflammation. Trends Immunol. 38:5–19. doi: 0.1016/j.it.2016.10.001

Pisoschi AM, Pop A (2015) The role of antioxidants in the chemistry of oxidative stress: A review. Eur J Med Chem 97:55–74. doi: 10.1016/j.ejmech.2015.04.040

Prachayasittikul S, Suphapong S, Worachartcheewan A, et al (2009) Bioactive metabolites from Spilanthes acmella Murr. Molecules 14:850–867. doi: 10.3390/molecules14020850

Prachayasittikul V, Prachayasittikul S, Ruchirawat S, Prachayasittikul V (2013) High therapeutic potential of Spilanthes acmella: A review. EXCLI J. 12:291–312

Raduner S, Majewska A, Chen J, et al (2006) Alkylamides from *Echinacea* Are a New Class of Cannabinomimetics. J Biol Chem 281:14192–14206. doi: 10.1074/jbc.M601074200

Reynoso-Moreno I, Najar-Guerrero I, Escareno N, et al (2017) An Endocannabinoid Uptake Inhibitor from Black Pepper Exerts Pronounced Anti-Inflammatory Effects in Mice. J Agric Food Chem 65:9435–9442. doi: 10.1021/acs.jafc.7b02979

Ricciotti E, Fitzgerald GA (2011) Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol 31:986–1000. doi: 10.1161/ATVBAHA.110.207449

Rosa M Di (1972) Biological properties of carrageenan. J. Pharm. Pharmacol. 24:89–102
Rossato MF, Oliveira SM, Trevisan G, et al (2015) Structural improvement of compounds with analgesic activity: AC-MPF4, a compound with mixed anti-inflammatory and antinociceptive activity via opioid receptor. Pharmacol Biochem Behav 129:72–78. doi: 10.1016/j.pbb.2014.12.001

Schmidt RF, Willis WD (2007) Encyclopedia of Pain. Springer, New York

Sedlak J, Lindsay RH (1968) Estimation Sulfhydryl of Total, Groups Protein-Bound, in Tissue with and Nonprotein Ellman's Reagent. Anal Biochem 25:192–205. doi: 10.1016/0003-2697(68)90092-4

Vinegar R, Schreiber W, Hugo R (1969) Biphasic development of carrageenin edema in rats. J Pharmacol Exp Ther 166:96–103

Watanabe M, Ueda T, Shibata Y, et al (2015) The role of TRPV1 channels in carrageenan-induced mechanical hyperalgesia in mice. Neuroreport 26:173–178. doi: 10.1097/WNR.0000000000000322

Weydert CJ, Cullen JJ (2010) Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc 5:51–66. doi: 10.1038/nprot.2009.197

Wongsawatkul O, Prachayasittikul S, Isarankura-Na-Ayudhya C, et al (2008) Vasorelaxant and antioxidant activities of spilanthes acmella Murr. Int J Mol Sci 9:2724–2744. doi: 10.3390/ijms9122724

Wu LC, Fan NC, Lin MH, et al (2008) Anti-inflammatory effect of spilanthol from Spilanthes acmella on murine macrophage by down-regulating LPS-induced inflammatory mediators. J Agric Food Chem 56:2341–2349. doi: 10.1021/jf073057e

4 ARTIGO CIENTÍFICO 3

Local effects of natural alkylamides from *Acmella oleracea* and synthetic isobutylalkyl amide on neuropathic and postoperative pain models in mice

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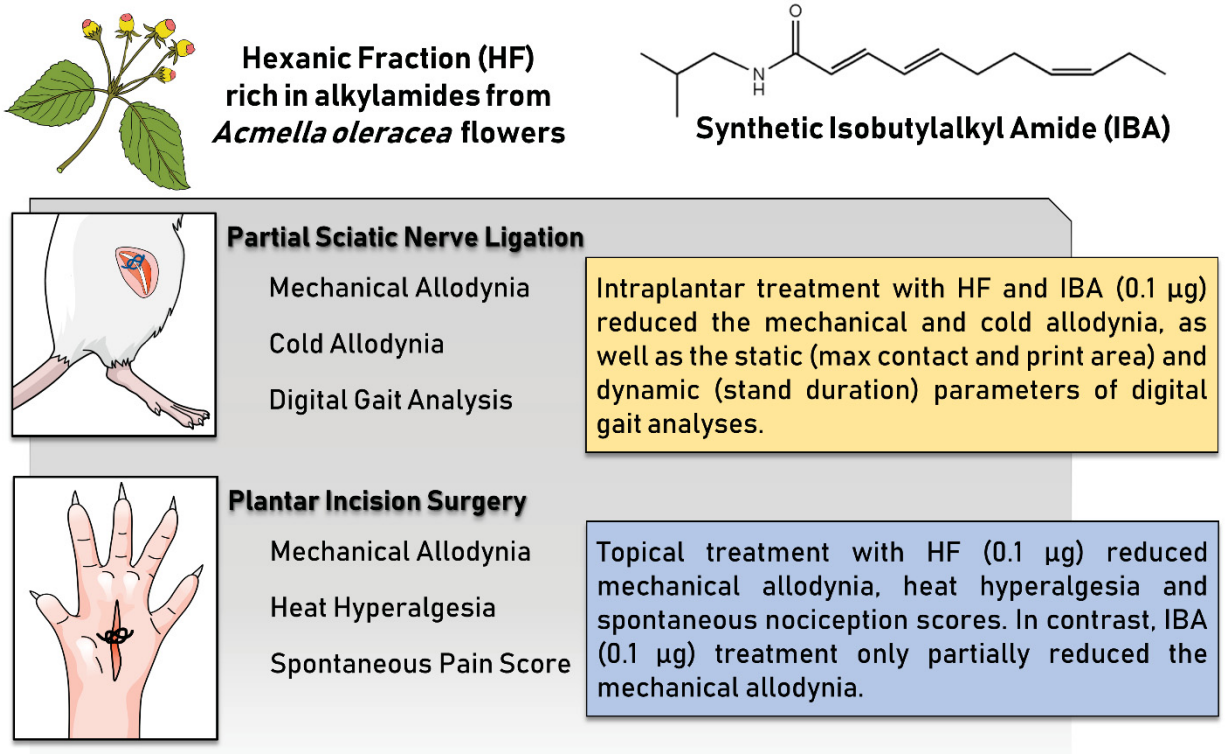
Abstract

Neuropathic and postoperative pain are clinical conditions that impair the patient's quality of life. The current pharmacotherapy of both painful states is ineffective and accompanied by several side effects. In order to develop new therapeutics targets, the secondary metabolites of plants have been extensively studied. *Acmella oleracea* ("jambu") is a native plant from Amazon region and rich in alkylamides, bioactive compounds responsible to induce anesthetic and chemesthetic sensations. We have previously demonstrated that the intraplantar administration of an hexanic fraction (HF) rich in alkylamides from jambu, and the synthetic isobutylalkyl amide (IBA) at 0.1 μg/20 μL promoted antinociceptive and anti-inflammatory effects. Thus, this study aimed to evaluate the local effect of HF and IBA (0.1 μg/20 μL) on neuropathic (partial sciatic nerve ligation, PSNL) and postoperative pain (plantar incision surgery, PIS) models in mice. Seven days after the PSNL, the mechanical (von Frey test) and cold (acetone-evoked evaporative cooling) allodynia, and digital gait parameters were analyzed. The intraplantar HF and IBA treatments attenuated the mechanical and cold allodynia, as well as the static (max contact and print area) and dynamic (stand duration) parameters of digital gait analyses. On the day after PIS, the mechanical allodynia, heat hyperalgesia (hot plate, 52 ± 0.1 °C), and spontaneous nociception scores were evaluated. Topical treatment with HF reduced the mechanical allodynia, heat hyperalgesia and spontaneous nociception scores. In contrast, IBA treatment only partially reduced the mechanical allodynia. Collectively, the local treatment with natural alkylamides from jambu flowers (HF) was effective on both neuropathic and postoperative pain model, instead of IBA, which only had effect on neuropathic pain.

Keywords

Jambu; Isobutylalkyl Amide; Partial Sciatic Nerve Ligation; Plantar Incisional Surgery; Allodynia; Digital Gait Analysis;

Graphic Abstract 3



1 Introduction

Pain is an important physiological reaction against noxious stimuli that occurs to prevent or minimize the tissue damage. However, the sustained or chronic pain result in health problems accompanied by suffering, disability and less quality of life of patients (Hunt and Mantyh 2001; Scholz and Woolf 2002).

Neuropathic pain is a chronic condition caused by a lesion or a disease (diabetic, postherpetic or HIV-related neuropathies, cancer and chemotherapy-induced neuropathy) that affect the structure and function of the somatosensory system, at peripheral and central levels. Despite many possible etiologies, neuropathic pain has common clinical manifestations, such as mechanical and cold allodynia, which means a painful sensation evoked by innocuous stimuli (Cohen and Mao 2014; Jensen and Finnerup 2014; Colloca et al. 2017). The prevalence of neuropathic pain in general population is estimated between 6.9 – 10% (Van Hecke et al. 2014). Unfortunately, the clinical management, including accuracy in diagnostic and prescription of appropriate treatments, is often unsatisfactory and still being a challenge. The first-line of pharmacotherapy includes: tricyclic antidepressants (amitriptyline), serotonin-norepinephrine reuptake inhibitors (duloxetine), and the calcium channel alpha-2-delta (Cavα2δ) ligands (gabapentin) (Finnerup et al. 2015; Cruccu and Truini 2017; Attal 2018). Nevertheless, these available clinical treatments are frequently insufficient and ineffective, accompanied by several side effects, abuse potential, and high economic burden for patients, society and healthcare systems (Scholz and Woolf 2002).

Other distressing condition is the acute postoperative pain. About 80% of patients experienced pain after surgery (75% as moderate to extreme), and less than 50% reported adequate analgesia (Apfelbaum et al. 2003; Gan et al. 2014). The consequence of a surgery is a combination of an intense inflammatory process with nerve injuries, resulting in mechanical allodynia, thermal hyperalgesia and spontaneous pain. The physiopathology of postoperative pain difficults the treatment, which should be started as soon as possible to relief the pain and suffering, and to promote the healing process (Brennan et al. 2005; Uchytilova et al. 2014; Pogatzki-zahn et al. 2017). Inappropriate treatment favors the chronification of pain, delay of rehabilitation, and impaired quality of life (Pak et al. 2018). Even with the well-known adverse effects, the pharmacotherapy of acute postoperative pain basically includes opioids, nonsteroidal anti-inflammatory drugs, and local anesthetics (Chou et al. 2016).

The low efficacy and the high prevalence of side effects of current treatments for neuropathic and postoperative pain become needed and urgent the development of new therapeutic alternatives, improving the patients' quality of life. Accordingly, natural products, such as secondary metabolites from plants, still deserve attention in drug discovery field. It is estimated that 49% of available drugs on market are derived from natural products (Newman and Cragg 2016). In the world, exist around 300,000 plant species, but only 15% had their biological effects investigated (Palhares et al. 2015).

Brazil contemplates the biggest biodiversity of the plant, being 20 to 22% of total (Dutra et al. 2016). One of these is the *Acmella oleracea* (L.) R.K. Jansen (family Asteraceae), a fascinating native plant from Amazon region and introduced in tropical areas around the world (Lim 2016). Popularly known as "jambu", this plant is extensively used in typical culinary dishes from northern Brazil due to its pungent and acrid taste, and also in traditional medicine to treat toothache, throat complaint, cold, stomatitis, among other applications (Dubey et al. 2013; Cheng et al. 2015; Lim 2016). Jambu has a great variety of secondary metabolites, including phenolic compounds (flavonoids, vanillic and trans-ferulic acids), essential oil (limonene and β-caryophyllene), coumarin (scopoletin), phytosterols and triterpenoids (β-sitostenone, stigmasterol, α- and β-amyrins). However, the major and most studied class of bioactivity compounds found in jambu is the alkylamides (Prachayasittikul et al. 2009, 2013; Dubey et al. 2013). These lipophilic compounds basically contain a straight and aliphatic poly-unsaturated carbon chain linked to an amide group with a shorter radical substituted (Boonen et al. 2012; Greger 2016). Numerous alkylamides have been described in jambu, largely in its flowers, but the main and most abundant is the spilanthol (Boonen et al. 2010; Cheng et al. 2015).

Alkylamides are responsible to cause unique chemesthetic sensations (tingling and numbness) in oral mucosa suggesting an anesthetic effect (Boonen et al. 2012). Several biological activities of these molecules have already been demonstrated, like the antinociceptive, analgesic, and anti-inflammatory effects involving many different mechanisms (Gertsch 2008; Nomura et al. 2013; Rios and Olivo 2014; Dallazen et al. submitted data 2018). Our research group have recently shown all these properties promoted by the local treatment (0.1 μg/20 μL, intraplantarly) with an hexanic fraction (HF) rich in alkylamides from *Acmella oleracea* and a synthetic isobutylalkyl amide (IBA) in animal models of acute and inflammatory pain (Dallazen et al. submitted data 2018; Dallazen et al. submitted data 2019).

Although many reports in the literature have already shown the potential of flavonoids, terpenes, alkaloids, coumarin, and phenols to treat chronic pain (Quintans et al. 2014), there are very few studies showing the pharmacological potential of alkylamides to treat painful conditions, such as neuropathic and postoperative pain. Thus, regarding the urgency to develop new therapeutic alternatives to treat these painful conditions, our aim was to investigate the effects of the local treatments with HF and IBA on partial sciatic nerve ligation (PSNL) and plantar incision surgery (PIS) models.

2 Materials and methods

2.1 Animals

The experiments were conducted with Swiss (*Mus musculus*) male adult mice, weighing 25 - 30 g, and provided by the vivarium from Biological Sciences Sector of Federal University of Paraná. The animals were kept in controlled temperature (22 ± 2 ºC) and luminosity (12h light/dark cycle), air exhaustion, water and food (Nuvilab CR-1, Quimtia S/A, Brazil) ad libitum. All experimental protocols of animal handling were conducted in agreement with the "Guide for the Care and Use of Laboratory Animals" (National Research Council 2011) and previously approved by the local Ethics Committee of Animal Experimentation (CEUA/BIO–UFPR: 970 and 1107).

2.2 Experimental design of local treatments

Based on previous publication (Dallazen et al. 2018; Dallazen et al. 2019 submitted data), the following local treatments were performed intraplantarly (i.pl.) or topically in the right hindpaw with the hexanic fraction (HF) from *Acmella oleracea* flowers and isobutylalkyl amide (IBA) at 0.1 μg/20 μL.

For the obtention of HF, initially, jambu flowers were extracted under reflux with absolute ethanol, providing the ethanolic extract (EEAO, Nomura et al. 2013). Then, EEAO was evaporated in reduced pressure. Subsequently, it was resuspended in water, lyophilized, and resuspended again in ethanol-water. The latter was passed through a liquid-liquid partition in hexane, in order to obtain the hexane fraction (HF). The phytochemistry analysis of HF (Dallazen et al. submitted data 2018) was performed using chromatography-mass spectrometry detection (GC-MS).

2.3 Neuropathic pain model

The neuropathic pain was induced via partial sciatic nerve ligation model (PSNL) (Seltzer et al. 1990; Malmberg and Basbaum 1998). For this, mice were anesthetized intraperitonially with xylazine (0.2 mg/kg, i.p.) and ketamine (1 mg/kg, i.p.). The right hindlimb was trichotomized in the high-thigh level, and an incision was made in the skin and in the muscle to expose the sciatic nerve. Then, a partial ligation was made by tying one third to one half of the dorsal portion of the sciatic nerve using 8-0 silk suture. The muscle and skin were closed with 6-0 silk suture. In sham group, the nerve was exposed without ligation. Each behavioral test (mechanical and cold allodynia, and digital gait analysis), described in the sequence, were evaluated previously, before the PSNL procedure (B: basal response), seven days (d7) after the surgery, and after treatments.

2.3.1 Measurement of mechanical allodynia

For the measurement of mechanical paw withdrawal threshold, the animals were placed and in individual clear boxes (18 cm × 11 cm × 20 cm) on an elevated mesh platform and allowed to acclimatize for 1 h before testing. Subsequently, mice were treated with vehicle (V: sterile 0.9% saline, 10 ml/kg, i.p; 0.002% tween 80 or 0.02% DMSO, both in 0.9% saline, i.pl.), gabapentin (GABA: 30 mg/kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.). After 0.5, 1, 2, 3, 4, 5 and 6 h of treatments the mechanical allodynia was accessed with Von Frey filaments stimulation perpendicularly to the ventral surface of hindpaw (0.04 – 4 g, North Coast Medical, Inc., Morgan Hill, CA, USA) to determinate the 50% threshold (g) based on the Up-and-Down paradigm (Dixon 1980; Chaplan et al. 1994).

2.3.2 Measurement of cold allodynia

The cold allodynia was evaluated as previously described by Montrucchio et al. (2013). Mice were placed an elevated mesh platform in individual clear boxes and acclimated for 1 h. Following adaptation, the animals were treated with vehicle (V), gabapentin (GABA: 30 mg/kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.). After 0.5, 1, 2, 3 and 4 h of treatments, the cold allodynia was evaluated by lightly/gently spraying, from a short distance, 20 µL of acetone with an insulin syringe to the ventral surface of hindpaw. The behavioral responses to the acetone-evoked evaporative cooling were observed for 30 s and scored: 0 – no response; 1 –

quick withdrawal, flick or stamp of the paw; 2 – prolonged withdrawal or repeated paw flicking; 3 – repeated paw flicking with licking at the ventral side of the paw (Flatters and Bennett 2004). Acetone was applied alternately three times, with 5 min of interval between them, for each animal. The sum of the scores (total scores) was used for data analysis, being 0 the minimal and 9 maximum score.

2.3.3 Digital gait analysis

Analysis of footprint and gait patterns in mice was made using the Catwalk® XT 10.5 system (Noldus Information Technology, Wagening, the Netherlands) and carried out according Kappos et al. (2017), and Shepherd and Mohapatra (2018). In brief, the CatWalk apparatus consists of an enclosed 130 x 7 cm walkway on a glass platform, where mice were located and allowed to walk freely. A green light illuminates the glass by entering at the edge of the platform and reflecting internally. Upon the glass plate, a red backlight is responsible to create the animal shape silhouette. The run is recorded by a high-speed video camera positioned underneath the green platform, which detected the areas where mice paws touch the illuminated glass, resulting in light scatter and reflection (Illuminated Footprints™ technology). The camera converts the runs into digital images composed by pixels of brightness, and transfers to a computer. The computer software automatically classifies the footprints, indicates errors, and archives the data.

The acquisition settings were: four runs per mice, resulting in one trial; 20 dB of camera gain; green intensity threshold was 0.15 (arbitrary units). Runs were empirically excluded when mice starting/stopping to walk, standing up on two hind foot, or crossed the platform in an atypical manner. Mice where habituated to the experimental room and CatWalk apparatus on the day of the test. All tests were performed at the same period of the day (11:00 AM – 13:00 PM), and all trials were done in the darkness.

The footprint and gait parameters analyzed in this study were:

1) Static parameters:

- Max contact area (cm^2): the surface area of the print at maximum contact;
- Print Area (cm^2): the surface area of the complete paw print;

2) Dynamic parameters:

- Stand duration (s): the duration of contact with the glass plate of the print;
- Swing duration (s): the duration in seconds of no contact of a paw with the glass plate;
- Swing speed (cm/s): is the speed of the paw during Swing.

The data analyzes were mane using the ratio of the ipsilateral (right) to contralateral (left) hind limbs (RH : LH) values obtained in: basal (B: prior to PSNL); seven days after surgery (d7); 0.5, 3 and 24 h after treatments with (V), gabapentin (GABA: 30 mg/kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.).

2.4 Postoperative pain model

The postoperative pain model was carried out according to the plantar incision surgery (PIS) model described by Brennan et al. (1996) and adapted to mice by Pogatzki and Raja (2003). The animals were anesthetized with 2% halothane via nose cone, and the antiseptic preparation on plantar surface of right hindpaw was made with 10 % povidone–iodine solution. Subsequently, a 5 mm longitudinal incision, starting 2 mm from the proximal edge of the heel and extending toward the toes, was made with a number 11 blade (Advantive, SteriLance Medical, Suzhou, CN) through the skin and fascia. With the aid of a curved forceps, the underlying muscle and tendons were carefully elevated, and then replaced to the normal anatomical position. After controlling bleeding and homeostasis, the skin was closed with a single suture of 6-0 nylon in the middle of the incision. Lastly, the wound was covered with 10 % povidone–iodine solution and animals were allowed to recover in their cages until the next day. Sham mice underwent the anesthesia and antiseptic procedures, without an incision.

The behavioral tests: mechanical allodynia, thermal hyperalgesia and spontaneous nociception scores (see below), were evaluated previously, before the PIS procedure (B: basal response), on the next day of surgery (Post: 24 h post-incision), and after the treatments in

different times. Due to incision procedure made on hindpaw, the intraplantar injection used for the treatments could not be performed. Consequently, HF and IBA were diluted in acetone and topically applied on the plantar surface (0.1 µg/20 µL). It is important to mention that mice did not evoke any nociceptive behavior after acetone application in incised paw.

2.4.1 Measurement of mechanical allodynia

The mechanical allodynia was measured as described above. Mice were treated 0.5 h before testing with vehicle (V: sterile 0.9% saline, 10 ml/kg, s.c.; topical 20 µL acetone), morphine (MOR: 1 mg/kg, s.c.), HF or IBA (0.1 µg/20 µL, topically). After 0.5, 1, 2 and 3 h the mechanical allodynia was also accessed with Von Frey filaments (0.04 – 4 g) based on the Up-and-Down method (Dixon 1980; Chaplan et al. 1994).

2.4.2 Measurement of thermal hyperalgesia to heat

The thermal hyperalgesia to heat was evaluated on hot-plate apparatus (Ugo Basile, Italy) at a constant temperature (52 ± 0.1 °C). The contact time of the incised hindpaw on the hot plate was recorded until withdrawal response (licking, shaking or guarding), and measured as heat latency (s). A cut-off time of 30 s was used to avoid tissue damage (Hunskaar et al. 1986; Dallazen et al. submitted data 2018).

Mice were treated before testing with vehicle (V: sterile 0.9% saline, 10 ml/kg, s.c.; topical 20 µL acetone), morphine (MOR: 1 mg/kg, s.c.), HF or IBA (0.1 µg/20 µL, topically), and the heat latency (s) was assessed after 1, 2 and 3 h.

2.4.3 Measurement of spontaneous nociception scores

The measurement of spontaneous nociception scores was based on paw guarding behavior parameters described by Xu and Brennan (2010), adapted to mice. For this, mice were placed and acclimatized in individual boxes on an elevated mesh platform (already mentioned) that allow us to observe clearly the paw position. Both hindpaws were closely observed for 1 min, every 5 min, during 1 h blocks, and scored according to their position and borne weight upon them: 0 – mice place equal weight upon both paws, with the wound blanched; 1 – mice light or partially touch the incised paw on mesh, without blanching the wound; 2 – mice completely removed the incised paw off the mesh, without bearing weight on it. During 1 min

period, the majority score observed was assigned, and the sum of scores (0 to 24 scores) obtained in 12 periods of each paw was subtracted (right – left) to achieve the final spontaneous nociception scores.

The animals were treated with vehicle (V: sterile 0.9% saline, 10 ml/kg, s.c.; topical 20 µL acetone), morphine (MOR: 1 mg/kg, s.c.), HF or IBA (0.1 µg/20 µL, topically), and the spontaneous nociception scores measured after 0.5 h during three blocks of 1 h (0 – 1; 1 – 2; 2 – 3).

2.5 Statistical analyses

The results of mechanical allodynia, digital gait analysis, and thermal hyperalgesia to heat were analyzed using two-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni's multiple comparisons post-hoc test, and are presented as mean ± S.E.M. The data concern total score of cold allodynia and spontaneous nociception scores of postoperative pain model were evaluated with Kruskal-Wallis test, followed by Dunn's multiple comparisons test repeated by each time of evaluation, and are expressed as median and interquartile ranges. The analyzes were performed using the GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA), considering significant difference when $^{*,\#,\$}P < 0.05$.

3 Results

3.1 Local effect of HF and IBA treatments on mechanical allodynia induced by PSNL

The PSNL produced an intense and prolonged mechanical allodynia observed by decreasing of paw withdrawal threshold from 1.6 ± 0.3 g (B), to 0.05 ± 0.0 g seven days after surgery (d7) in vehicle treated group (V) (Fig. 1). The sham procedure did not modify the mechanical threshold in mice. The systemic treatment with gabapentin completely abolished the mechanical allodynia from 0.5 h after its administration, until 5 h of evaluation. Locally, HF reduced mechanical allodynia in 78.0, 72.3, 70.7, and 68.9% at 0.5, 1, 2 and 3 h, respectively, after is injection, when compared to vehicle treated group. The intraplantar treatment with IBA, also reverted the mechanical allodynia in 79.9, 66.7, 64.7, and 76.5% at 0.5, 1, 2 and 3 h, respectively, when compared to vehicle group.

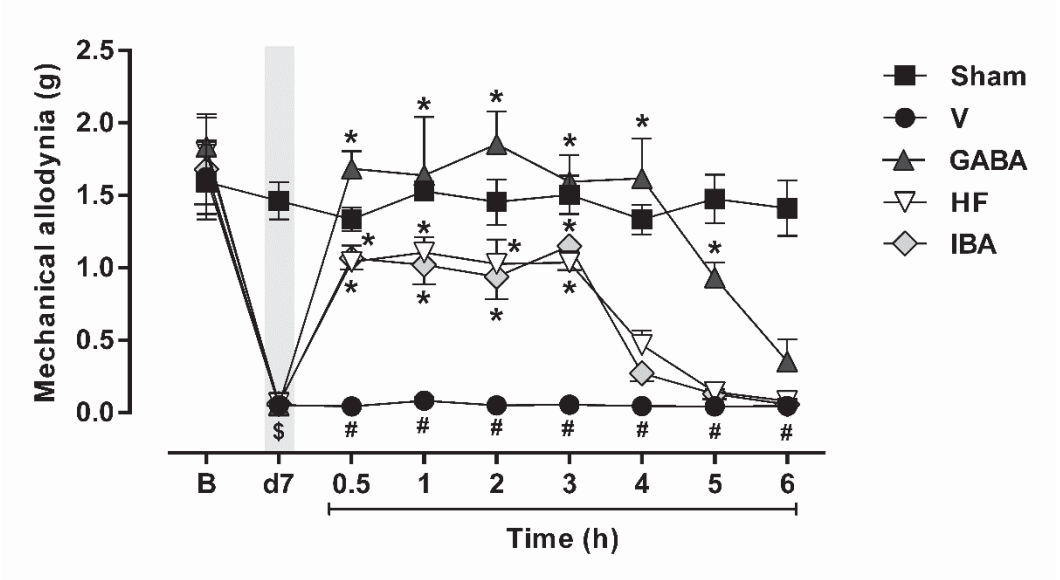


Fig. 1 Effect of HF and IBA on mechanical allodynia induced by PSNL. Mechanical allodynia (g) was assessed using von Frey filaments (0.04 – 4 g). Mice were evaluated before PSNL procedure (B: basal response), seven days (d7) after the surgery, 0.5, 1, 2, 3, 4, 5, and 6 h after treatments with: vehicle (V), gabapentin (GABA: 30 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.). Gray box on day 7 denotes the time point of vehicle or drug administration. The results are expressed as mean ± S.E.M (n = 8). $^{\$}P < 0.05$ compared to basal (B) values, $^{*}P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (two-way ANOVA followed by Bonferroni test).

3.2 Local effect of HF and IBA treatments on cold allodynia induced by PSNL

The PSNL induced cold allodynia reveled by increasing of total scores in acetone-evoked evaporative cooling in all groups seven days after surgery (d7) from 0 ± 0 to 7.7 ± 0.6 scores in vehicle (V) treated group, 6.6 ± 1.5 scores (GABA group), 6.6 ± 2.0 scores (HF group), and 7.0 ± 1.3 scores (IBA group) (Fig. 2). The sham procedure did not induce significant cold allodynia in mice. The systemic treatment with gabapentin reduces the cold allodynia in 76.4, 80.8, 60.7, and 47.9%, at 0.5, 1, 2 and 3 h after its administration, respectively, when compared to vehicle group. The intraplantar treatment with HF attenuated the cold allodynia at 0.5, 1, 2, and 3 h in 69.1, 78.8, 57.1, and 54.2%, respectively, when compared to vehicle group. IBA also decreased the cold allodynia in 69.1, 78.8, and 57.1%, at 0.5, 1, and 2 h, respectively, after its intraplantar administration, comparing to vehicle group.

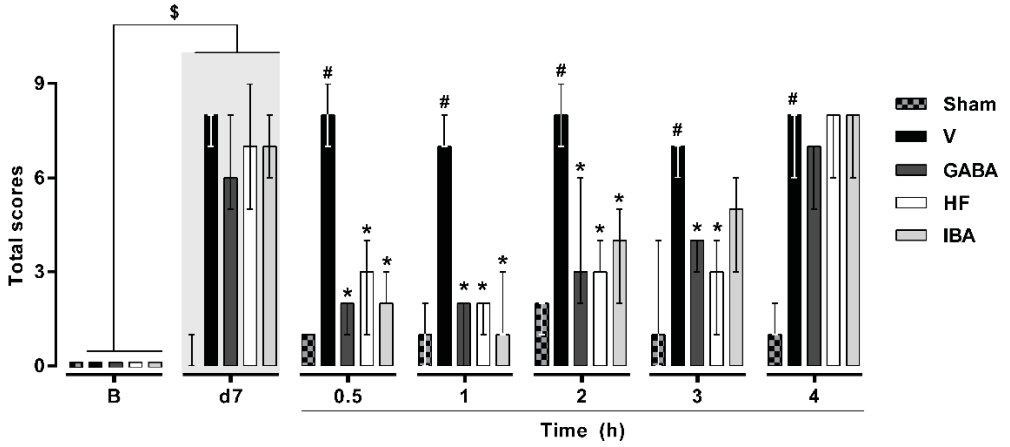


Fig. 2 Effect of HF and IBA on cold allodynia induced by PSNL. Cold allodynia (total scores) was evaluated before PSNL procedure (B: basal response), seven days (d7) after the surgery, 0.5, 1, 2, 3, and 4 h after treatments with: vehicle (V), gabapentin (GABA: 30 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.). Gray box on day 7 denotes the time point of vehicle or drug administration. The results are expressed as median and interquartile ranges (n = 7). $^{\$}P < 0.05$ compared to basal (B) values, $^{*}P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (Kruskal-Wallis test, followed by Dunn's multiple comparisons test).

3.3 Local effect of HF and IBA treatments on gait parameters changes induced by PSNL

The PSNL induced several changes in mice footprint and gait parameters. On static parameters, PSNL significantly reduced the ratio (RH : LH) of max contact and print area of paws in 55.8 and 52.2% in vehicle treated group (Fig. 3 A and B, respectively) seven days after surgery (d7), comparing to its basal value (B). In the same way, paw dynamic parameters of vehicle treated group also presented alterations, like reduction of paw stand duration in 43.9%, decreasing of swing duration in 40.7%, and increasing of swing speed in 52.2% (Fig 3 C, D, and E, respectively). Sham-operated mice did not show changes on static and dynamic paw parameters. The treatment with gabapentin, HF and IBA, increased the ratio of max contact area in 34.1, 33.2, and 32.2%, respectively (Fig. 3 A), and the paw print area in 35.7, 40.4, and 36.5%, respectively (Fig. 3 B), both parameters comparing to the vehicle treated group after 0.5 h of treatments. The paw stand duration was the only dynamic parameter significantly modified by the treatments, which was increased in 28.7, 32.5, and 35.1%, after 0.5 h of gabapentin, HF and IBA administration, respectively, when compared to the vehicle group.

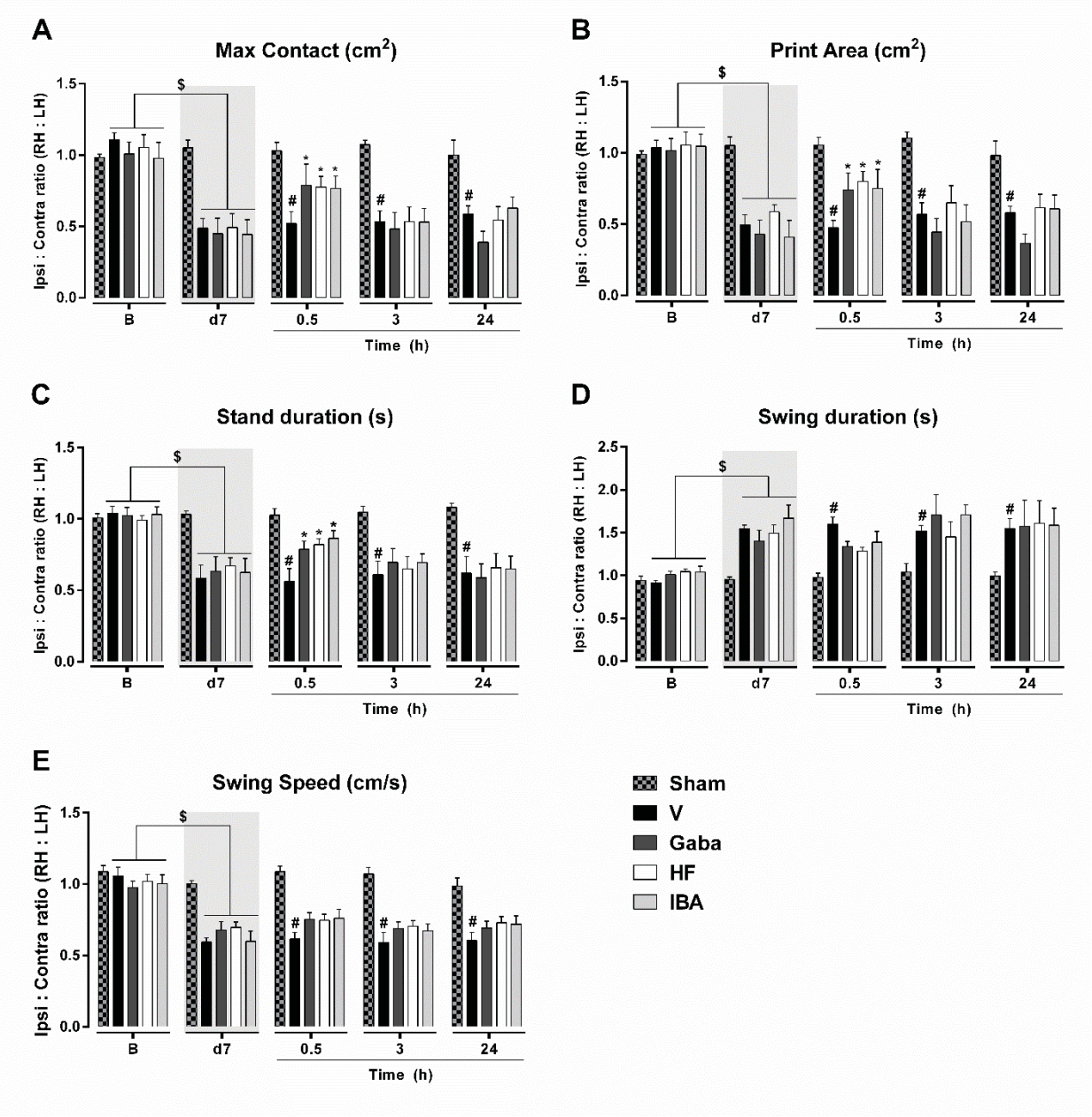


Fig. 3 Effect of HF and IBA on gait parameters changes induced by PSNL. Digital gait was performed on CatWalk® apparatus, and the parameters analyzed were: paw max contact area (A), paw print area (B), paw stand duration (C), paw swing duration (D), and paw swing speed (E). Animals were evaluated before PSNL procedure (B: basal response), seven days (d7) after the surgery, 0.5, 3, and 24 h after treatments with: vehicle (V), gabapentin (GABA: 30 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, i.pl.). Gray box on day 7 denotes the time point of vehicle or drug administration. The results represent the ratio of ipsilateral to contralateral hind limb values (RH : LH), and are expressed as mean ± S.E.M (n = 6). $^{\$}P < 0.05$ compared to basal (B) values, $^{*}P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (two-way ANOVA followed by Bonferroni test).

3.4 Local effect of HF and IBA treatments on mechanical allodynia induced by PIS

On the day after PIS (24 h), the mechanical paw withdrawal threshold markedly decreased from 1.4 ± 0.1 g in basal response (B), to 0.04 ± 0.0 g (Post), as observed in vehicle group (V) (Fig. 4). In sham animals, the mechanical threshold was not altered. As expected, morphine treatment reversed mechanical allodynia in 73.0, 71.4, and 42.1% at 0.5, 1, and 2 h, respectively, when compared to the vehicle group. Interestingly, topical application of HF also reduced the mechanical allodynia at 0.5, 1, and 2 h in 84.1, 84.5, and 77.3%, respectively, when compared to the vehicle group. Locally, IBA just partially reverted mechanical allodynia in 45.1 and 30.2% at 0.5 and 1 h, respectively, after its topical application, when compared to the vehicle group.

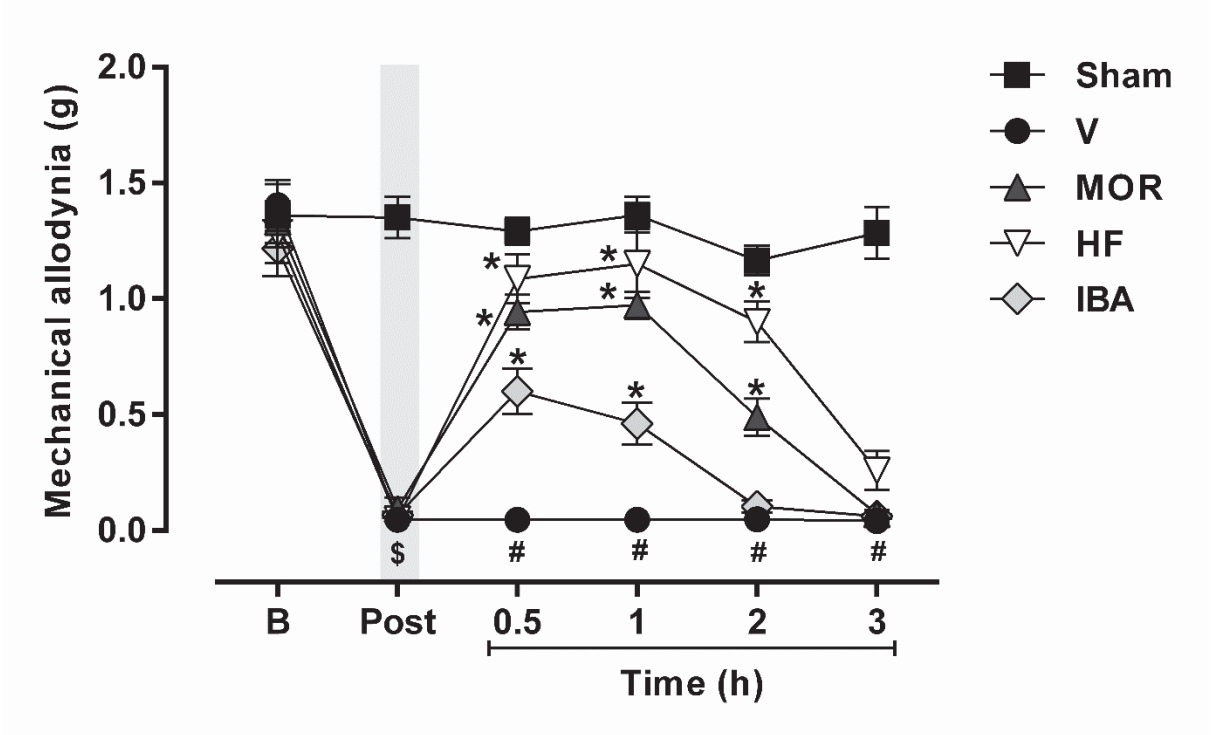


Fig. 4 Effect of HF and IBA on mechanical allodynia induced by PIS. Mechanical allodynia (g) was assessed using von Frey filaments (0.04 – 4 g). Mice were tested before PIS (B: basal response), on the day after (Post) the incision, and 0.5, 1, 2, and 3 h after treatments with: vehicle (V), morphine (MOR: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, topical application). Gray box on the post-incision day denotes the time point of vehicle or drug administration. The results are expressed as mean ± S.E.M (n = 8). $^{\$}P < 0.05$ compared to basal (B) values, $*P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (two-way ANOVA followed by Bonferroni test).

3.5 Local effect of HF and IBA treatments on thermal hyperalgesia to heat induced by PIS

After PIS, mice developed thermal hyperalgesia to heat (Fig. 5), demonstrated by decreasing of heat latency (s) on hot-plate apparatus (Fig. 4) from 12.1 ± 0.4 s (B), to 4.5 ± 0.3 s (Post) in vehicle treat group (V). In sham mice, no changes were observed on heat latency during testing. The subcutaneous treatment with morphine increased the heat latency in 64.0 and 35.0% after 1 and 2 h, respectively, when compared to vehicle group. Topical application of HF on incised paw elevated its heat latency in 62.8 and 41.0%, at 1 and 2 h, respectively, when compared to vehicle group. The local treatment with IBA did not ameliorated the thermal hyperalgesia.

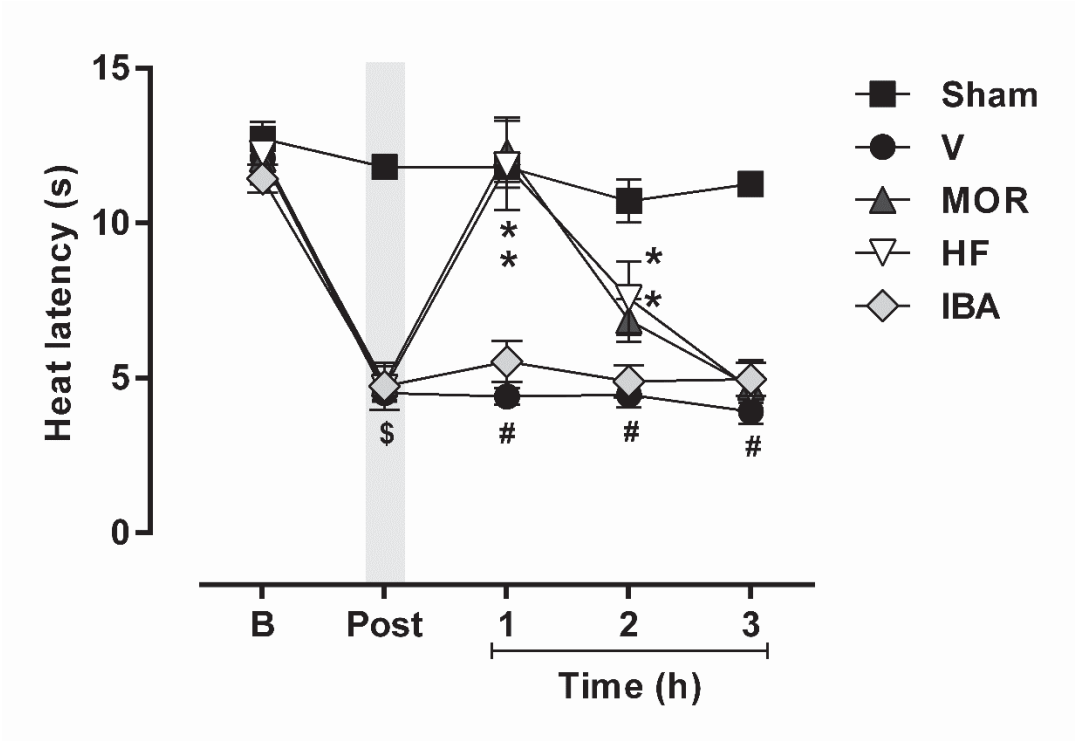


Fig. 5 Effect of HF and IBA on thermal hyperalgesia to heat induced by PIS. Thermal hyperalgesia was performed on hot-plate at 52 ± 0.1 °C. The heat latency (s) of mice was measured before PIS (B: basal response), on the day after (Post) the incision, and 1, 2, and 3 h after treatments with: vehicle (V), morphine (MOR: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, topical application). Gray box on the post-incision day denotes the time point of vehicle or drug administration. The results are expressed as mean ± S.E.M (n = 6). $^{\$}P < 0.05$ compared to basal (B) values, $^{*}P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (two-way ANOVA followed by Bonferroni test).

3.6 Local effect of HF and IBA treatments on spontaneous nociception scores induced by PIS

As expected, PIS induced alteration on position and borne weight upon incised paw 24h post-incision (Post), increasing the spontaneous nociception scores in vehicle treated group (V: 17.0 ± 1.6 scores), and no one alteration was observed in sham animals (Fig. 6). The morphine treatment reduced the scores in the two firsts blocks, 0 – 1 and 1 – 2 h, of assessment in 62.5 and 60.3%, when compared to vehicle group. Topically, HF also decreased the PIS-evoked spontaneous nociception scores in 71.2 and 64.7%, respectively at first (0 – 1 h) and second (1 – 2 h) periods of observation, comparing to vehicle treated group. In contrast, IBA treatment did not modified the paw position and borne weight on the operated paw during the time course of evaluation.

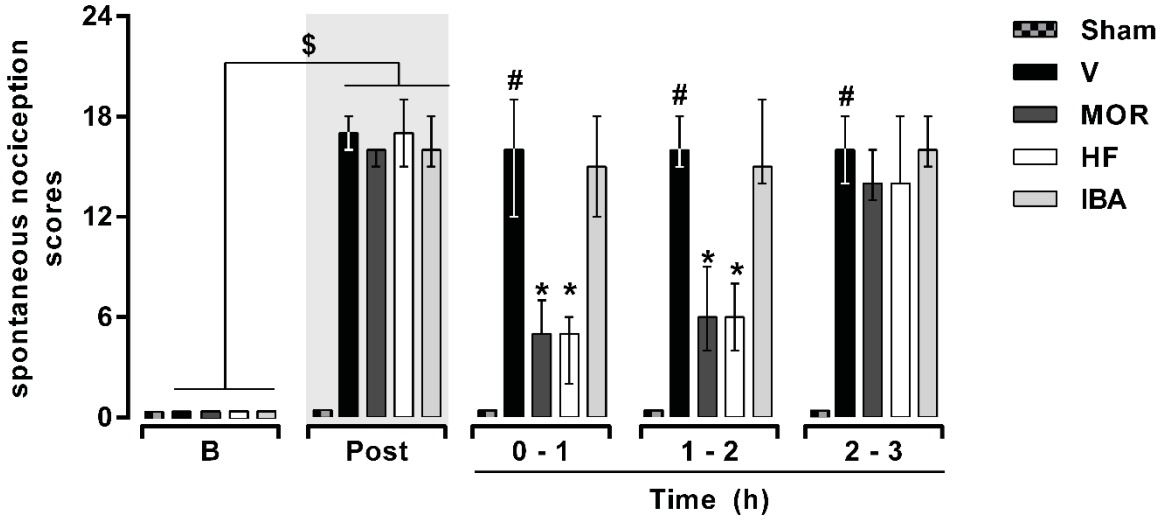


Fig. 6 Effect of HF and IBA on spontaneous nociception scores induced by PIS. The spontaneous nociception scores were measured before PIS (B: basal response), on the day after (Post) the incision, and during blocks of 1 h (0 – 1; 1 – 2; and 2 – 3 h) after treatments with: vehicle (V), morphine (MOR: 1 mg/Kg, i.p.), HF or IBA (0.1 µg/20 µL, topical application). Gray box on the post-incision day denotes the time point of vehicle or drug administration. The results are expressed as mean ± S.E.M (n = 7). The results are expressed as median and interquartile ranges (n = 7). $^{\$}P < 0.05$ compared to basal (B) values, $^{*}P < 0.05$ compared to vehicle (V) group, and $^{\#}P < 0.05$ compared to sham group (Kruskal-Wallis test, followed by Dunn's multiple comparisons test).

4 Discussion

Pain is one of the most social, clinical, and economic problems in the world (Henschke et al. 2015). The pharmacotherapy of neuropathic and postoperative pain is oftentimes ineffective and with serious adverse effects (Finnerup et al. 2015; Chou et al. 2016). Because of this, the biological activities of secondary metabolites of plants have been extensively investigated over the years on pain models. Thus, the current study sought to evaluate the effect of local treatment with natural alkylamides from *Acmella oleracea* and the synthetic alkylamide IBA on neuropathic and postoperative pain models.

Peripheral neuropathic pain is featured by mechanical and cold allodynia, as shown seven days (d7) after PSNL procedure. Gabapentin, a Cavα2δ ligand (Alles et al. 2017), it is a first-line drug for the treatment of neuropathic pain and, as expected, revert for 5 h and 3 h the mechanical and cold allodynia, respectively. Here we demonstrated for the first time that the local administration (i.pl.) of natural and synthetic alkylamides presented antiallodynic effect against mechanical and cold stimulus in neuropathic mice. HF and IBA reverted mechanical allodynia induced by PSNL for 3 h after treatments at 0.1 µg. Similarly, the systemic administration of 100 mg/kg EEAO also reduced the mechanical allodynia for 3 h (Nomura et al. 2013). Considering that local treatments usually have few adverse effects when comparing to systemic drug administration, HF and IBA showed advantages comparing to mentioned drugs.

Under neuropathic conditions the TRPV1 channels are overexpressed and sensitized in peripheral fiber, contributing to arouse pain and mechanical allodynia (Jensen and Finnerup 2014; Marwaha et al. 2016; Basso and Altier 2017; Meacham et al. 2017). Capsaicin, the most studied alkylamide, is a TRPV1 agonist that has been used topically to treat peripheral neuropathic pain as second-line drug (Finnerup et al. 2015). Capsaicinoids are found in plants from Solanaceae family (chili peppers) and are characterized by the presence of a vanillin amine in their molecular structure (Boonen et al. 2012; Greger 2016). High-concentrations patches of capsaicin are responsible to cause desensitization and defunctionalization of TRPV1-expressing neurons (Haanpää and Treede 2012). The intense and continuous activation or blockade of TRPV1 have been reported as an alternative to relief neuropathic pain (Moran et al. 2011; Dai 2016; Marwaha et al. 2016). Natural alkylamides from jambu, spilanthol, and IBA have been described as TRPV1 antagonists, reducing acute nociception (de la Rosa-Lugo et al. 2017; Dallazen et al. submitted data 2018). This mechanism could contribute to reduce the mechanical pain observed in our findings.

Moreover, the local anesthetic lidocaine, a voltage-gated sodium channels (Na_v) blocker, is a second-line drug in the treat of neuropathic pain (Finnerup et al. 2015; Meacham et al. 2017). The Na_v channels (i.e.: Na_v 1.7, 1.8, and 1.9) are also overexpressed and presented alteration in their functionality in peripheral neuropathies, displaying an important role in mechanical allodynia, due to ectopic firing and hyperexcitation of peripheral fibers (Persson et al. 2016; Colloca et al. 2017; Meacham et al. 2017). Sanshool, the natural analogue of IBA, was described as a potent inhibitor of Aδ mechanonociceptors by blocking of several Na_v channels, especially Na_v 1.7 (Tsunozaki et al. 2013). The authors also demonstrated that the sanshool topical application in the hindpaw decreased the mechanical sensitivity and unchanged the heat latency. Likewise, we have recently demonstrated that HF decreased both mechanical and thermal paw sensitivity at 0.1µg (Dallazen et al. submitted data 2018). Thus, the inhibition of action potential in primary afferent fibers showed *in vitro*, in addition to the reduced responsiveness observed *in vivo*, may contribute to the anesthetic property of alkylamides, like HF and sanshool. Moreover, Hoyt et al. (2007) have synthetized numerous benzazepinone substituted with secondary alkylamides that also display a potent anesthetic property by Na_v 1.7 blocking.

However, it is necessary caution to generalize this effect for all alkylamides. The mechanism of action of alkylamides is very distinct between them, and the dose administrated is crucial to promote and determine their effects. For example, IBA, which increased the sensitivity to mechanical and heat stimulus in the paw of naïve animals at high dose (Klein et al. 2011; Tulleuda et al. 2011; Dallazen et al. submitted data 2018), also reverted the mechanical allodynia in neuropathic mice post-PSNL at low dose.

The local injection of HF and IBA surprisingly reduced the cold allodynia for 3 and 2 h, respectively. This positive result was unexpected since HF and IBA could activate/sensitize TRPA1 channels, which is one of the molecular mechanisms involved in cold allodynia (Jensen and Finnerup 2014). There are other pathways responsible to evoke cold allodynia in neuropathic conditions, such as changes in functionality and expression of TRM8, as well as in sodium channels (Na_v) (Jensen and Finnerup 2014; Meacham et al. 2017). Thus, more studies are needed to clarify the mechanisms of HF and IBA on this effect.

Recently, the analysis of gait parameters has been applied as a complementary approach to other stimulus-evoked pain-related behaviors in order to improve the translatability from basic to clinical research (Kappos et al. 2017). The CatWalk® apparatus measures at same time and automatically both static and dynamic parameters of the gait, reflecting the weight distribution between the affected and unaffected hindlimbs of mice. As expected, seven days

after peripheral neuropathic pain induction (PSNL), mice presented changes in gait parameters. The concomitant decrease of both area and duration of paw contact with the platform showed that animals reduced the weight bearing on operated right hindpaw (RH) in relation to non-operated left hindpaw (LH). In addition, the increase of swing duration with reciprocal decrease in swing speed, indicated that the operated limb remained for a longer time without touch the platform in relation to non-operated limb.

In the current PSNL model, the treatment with gabapentin, HF and IBA promoted a partial and shorter effect in weight-bearing indices, when compared to mechanical allodynia assessment. Additionally, gabapentin, HF and IBA treatment did not reverted the duration and speed of swing.

Nevertheless, there are many controversial data in the literature reporting the ineffectiveness of well-established analgesics in reversing the gait abnormalities produced by nerve injury, as well as the lack or not of correlation between von Frey test and gait parameters (Vrinten and Hamers 2003; Gabriel et al. 2009; Mogil et al. 2010). Gabapentin, morphine and EMLA (lidocaine and prilocaine mixture) were not effective on chronic constriction injury-induced gait changes (Mogil et al. 2010); as well as gabapentin and buprenorphine did not alter static and dynamic indices after spared nerve injury (Shepherd and Mohapatra 2018). However, on paclitaxel induced polyneuropathy, the treatment with gabapentin reverted dynamic parameters on gait alterations (Huehnchen et al. 2013).

Despite many authors do not consider the alterations in digital gait analysis as spontaneous/voluntary pain-related behavioral, we have shown that systemic administration of gabapentin, and local injection of HF and IBA, promoted some improvement concerning the weight bearing indices on PSNL model. Furthermore, our results add new evidences that the efficacy of analgesic drugs depends on neuropathic-inducing procedure, and that those alterations can be interpreted as spontaneous and non-evoked/reflexive pain, increased contact sensitivity, and pain-avoidance behavior (i.e.: guardian behavior) (Pitzer et al. 2016).

Likewise, postoperative pain also is a clinical condition that needs careful attention and demand for new therapies. Thus, for the first time, we evaluated the local effect of alkylamides from jambu and synthetic IBA on this painful condition performing the animal model of plantar incision surgery (PIS). On the day after PIS (24 h), mice have developed intense mechanical allodynia, which was reverted by morphine treatment for 2 h, an opioid drug currently used to treat postoperative pain (Zahn et al. 1997; Chou et al. 2016; Rawal 2016). Local treatment with HF and IBA reduced the mechanical allodynia for 2 and 1 h, respectively. Unlike morphine, HF and IBA do not display opioid mechanism (Dallazen et al. submitted data

2018). The mechanical allodynia of postoperative pain is overall induced by an acute inflammatory process in the injured site due to immune cell migration (neutrophil and mast cell), release of pro-inflammatory mediators (cytokines, prostaglandin, histamine), and sensitization of receptors and channels on primary afferent fiber (C and Aδ) (Pogatzki-zahn et al. 2017). The anti-inflammatory effect of HF and IBA has recently demonstrate on acute model of inflammation, which reduced the cell migration, cytokine release and prostaglandin syntheses (Dallazen et al. submitted data 2019). Moreover, the TRPV1 antagonism, anesthetic property, and mast cell stabilization promoted by jambu alkylamides also may contribute to its antiallodynic effect on PIS model (Dallazen et al. submitted data 2018).

Unlike neuropathic pain, which induced cold allodynia, postoperative pain is characterized by hyperalgesia to heat stimuli (Uchytilova et al. 2014; Pogatzki-zahn et al. 2017). Post PIS, the heat latency was strongly decreased, and the treatment with morphine and HF reverted the heat hyperalgesia for 2 h. As previously demonstrated, HF, EEAO, and pure spilanthol increased the heat latency in naïve animals (Cilia-López et al. 2010; Nomura et al. 2013; Dallazen et al. submitted data 2018). Similarly, other extracts rich in alkylamides, like a cold extract from jambu flowers (Ratnasooriya et al. 2005) and a *Heliopsis longipes* ethanolic extract (Acosta-Madrid et al. 2009), also demonstrated an antihyperalgesic effect on carrageenan-induced plantar acute inflammation.

Surgery processes very often induce spontaneous pain in patients, which means pain in rest. This clinical condition is like guarding behavior after PIS in mice (Xu and Brennan 2010). In addition to inflammation process, the nerve injury produced by plantar incision, contribute to peripheral and central sensitization, which lead to ectopic discharges in afferent fibers (Brennan 2011; Pogatzki-zahn et al. 2017). The spontaneous nociception was similar decreased by both morphine (Zahn et al. 1997) and HF treatments during 2 h period. The reduction of guarding behavior may be a collective effect of anesthetic and anti-inflammatory properties of HF.

Two-Pore Domain Potassium Channels (KCNK or K_2P) display an important role in resting potential of neuron membranes and on its excitability through potassium background ("leak") currents (Mathie et al. 2010). In painful conditions, the lower expression and functionally of KNCK channels result in hyperexcitability and spontaneous firing, leading to spontaneous pain, hyperalgesia and allodynia (Marsh et al. 2012; Li and Toyoda 2015). The lack of effect of IBA on heat latency and spontaneous pain score could be explained by the fact that it blocks KNCK 3, 9 and 18 channels (K_2P 3.1, TREK; K_2P 9.1, TASK; K_2P 18.1, TRESK,

respectively) (Bautista et al. 2008; Tulleuda et al. 2011), but the local antiallodynic effect observed in both neuropathic and postoperative pain models remains partially ununderstood.

5 Conclusion

This study reports for the first time the effectiveness of alkylamides to treat neuropathic and postoperative pain. Local administration of natural alkylamides from *Acmella oleracea* flowers (HF) and the synthetic IBA successfully attenuated mechanical and cold allodynia, as well as the static (max contact and print area) and dynamic (stand duration) parameters of digital gait analyses on neuropathic pain model (PSNL). Furthermore, on postoperative pain model, HF was more effective in reducing mechanical allodynia, hyperalgesia to heat and spontaneous nociception, when comparing to IBA, since it only partially reduced the mechanical allodynia induced by PIS. Taken together, our findings showed that alkylamides from jambu and IBA are suitable target for drug development and treatment of pain and its pathological states, such as neuropathic and postoperative pain. Although more studies are necessary to elucidate the HF and IBA mechanisms of action, alkylamides deserve attention and further evaluations to support and fortify their pharmacological effects.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Acosta-Madrid II, Castañeda-Hernández G, Cilia-López VG, et al (2009) Interaction between Heliopsis longipes extract and diclofenac on the thermal hyperalgesia test. Phytomedicine 16:336–341. doi: 10.1016/j.phymed.2008.12.014

Alles SRA, Bandet M V., Eppler K, et al (2017) Acute anti-allodynic action of gabapentin in dorsal horn and primary somatosensory cortex: Correlation of behavioural and physiological data. Neuropharmacology 113:576–590. doi: 10.1016/j.neuropharm.2016.11.011

Apfelbaum JL, Chen C, Mehta SS, Gan TJ (2003) Postoperative pain experience: Results from a national survey suggest postoperative pain continues to be undermanaged. Anesth Analg 97:534–540. doi: 10.1213/01.ANE.0000068822.10113.9E

Attal N (2018) Pharmacological treatments of neuropathic pain: The latest recommendations. Rev. Neurol. (Paris).

Basso L, Altier C (2017) Transient Receptor Potential Channels in neuropathic pain. Curr. Opin. Pharmacol. 32:9–15

Bautista DM, Sigal YM, Milstein AD, et al (2008) Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels. Nat Neurosci 11:772–779. doi: 10.1038/nn.2143

Boonen J, Baert B, Burvenich C, et al (2010) LC-MS profiling of N-alkylamides in Spilanthes acmella extract and the transmucosal behaviour of its main bio-active spilanthol. J Pharm Biomed Anal 53:243–249. doi: 10.1016/j.jpba.2010.02.010

Boonen J, Bronselaer A, Nielandt J, et al (2012) Alkamid database: Chemistry, occurrence and functionality of plant N-alkylamides. J. Ethnopharmacol. 142:563–590

Brennan TJ (2011) Pathophysiology of postoperative pain. Pain 152:S33–S40. doi: 10.1016/j.pain.2010.11.005

Brennan TJ, Vandermeulen EP, Gebhart GF (1996) Characterization of a rat model of incisional pain. Pain 64:493–501. doi: 10.1016/0304-3959(95)01441-1

Brennan TJ, Zahn PK, Pogatzki-Zahn EM (2005) Mechanisms of incisional pain. Anesthesiol Clin North America 23:1–20. doi: 10.1016/j.atc.2004.11.009

Chaplan SR, Bach FW, Pogrel JW, et al (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63. doi: 10.1016/0165-0270(94)90144-9

Cheng Y Bin, Liu RH, Ho MC, et al (2015) Alkylamides of *Acmella oleracea*. Molecules 20:6970–6977. doi: 10.3390/molecules20046970

Chou R, Gordon D, de Leon-Casasola O, et al (2016) Management of Postoperative Pain: A Clinical Practice Guideline. J Pain 17:131–57. doi: 10.1016/j.jpain.2015.12.008.

Cilia-López VG, Juárez-Flores BI, Aguirre-Rivera JR, Reyes-Agüero JA (2010) Analgesic activity of Heliopsis longipes and its effect on the nervous system. Pharm Biol 48:195–200. doi: 10.3109/13880200903078495

Cohen SP, Mao J (2014) Neuropathic pain: Mechanisms and their clinical implications. BMJ 348

Colloca L, Ludman T, Bouhassira D, et al (2017) Neuropathic pain. Nat Rev Dis Prim 3:1–20. doi: 10.1038/nrdp.2017.2

Cruccu G, Truini A (2017) A review of Neuropathic Pain: From Guidelines to Clinical Practice. Pain Ther 6:35–42. doi: 10.1007/s40122-017-0087-0

Dai Y (2016) TRPs and pain. Semin Immunopathol 38:277–291. doi: 10.1007/s00281-015-0526-0

Dallazen JL, Maria-ferreira D, Barbosa B, et al (2018) Distinct mechanisms underlying local antinociceptive and pronociceptive effects of alkylamides from Acmella oleracea flowers. Submitted data.

de la Rosa-Lugo V, Acevedo-Quiroz M, Déciga-Campos M, Rios MY (2017) Antinociceptive effect of natural and synthetic alkamides involves TRPV1 receptors. J Pharm Pharmacol 69:884–895. doi: 10.1111/jphp.12721

Dixon WJ (1980) Experimental Observations. Annu Rev Pharmacol Toxicol 20:441–462

Dubey S, Maity S, Singh M, et al (2013) Phytochemistry, pharmacology and toxicology of spilanthes acmella: A review. Adv Pharmacol Sci 2013:. doi: 10.1155/2013/423750

Dutra RC, Campos MM, Santos ARS, Calixto JB (2016) Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol Res 1–25. doi: 10.1016/j.phrs.2016.01.021

Finnerup NB, Attal N, Haroutounian S, et al (2015) Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. Lancet Neurol 14:162–173. doi: 10.1016/S1474-4422(14)70251-0

Flatters SJL, Bennett GJ (2004) Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. Pain 109:150–161. doi: 10.1016/j.pain.2004.01.029

Gabriel AF, Marcus MAE, Walenkamp GHIM, Joosten EAJ (2009) The CatWalk method: Assessment of mechanical allodynia in experimental chronic pain. Behav Brain Res 198:477–480. doi: 10.1016/j.bbr.2008.12.018

Gan TJ, Habib AS, Miller TE, et al (2014) Incidence, patient satisfaction, and perceptions of post-surgical pain: results from a US national survey. Curr Med Res Opin 30:149–160. doi: 10.1185/03007995.2013.860019

Gertsch J (2008) Immunomodulatory Lipids in Plants : Plant Fatty Acid Amides and the Human Endocannabinoid System. Planta Med 74:638–650. doi: 10.1055/s-2008-1034302

Greger H (2016) Alkamides: a critical reconsideration of a multifunctional class of unsaturated fatty acid amides. Phytochem Rev 15:729–770. doi: 10.1007/s11101-015-9418-0

Haanpää M, Treede RD (2012) Capsaicin for neuropathic pain: Linking traditional medicine and molecular biology. Eur Neurol 68:264–275. doi: 10.1159/000339944

Henschke N, Hons BP, Kamper SJ, et al (2015) The Epidemiology and Economic Consequences of Pain. Mayo Clin Proc 90:139–147. doi: 10.1016/j.mayocp.2014.09.010

Hoyt SB, London C, Ok H, et al (2007) Benzazepinone Nav1.7 blockers: Potential treatments for neuropathic pain. Bioorganic Med Chem Lett 17:6172–6177. doi: 10.1016/j.bmcl.2007.09.032

Huehnchen P, Boehmerle W, Endres M (2013) Assessment of Paclitaxel Induced Sensory Polyneuropathy with “Catwalk” Automated Gait Analysis in Mice. PLoS One 8:. doi: 10.1371/journal.pone.0076772

Hunskaar S, Berge OG, Hole K (1986) A modified hot-plate test sensitivie to mild analgesics. Behav Brain Res 21:101–108. doi: 10.1016/0166-4328(86)90088-4

Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2:83–91. doi: 10.1038/35053509

Jensen TS, Finnerup NB (2014) Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. Lancet Neurol 13:924–935. doi: 10.1016/S1474-4422(14)70102-4

Kappos EA, Sieber PK, Engels PE, et al (2017) Validity and reliability of the CatWalk system as a static and dynamic gait analysis tool for the assessment of functional nerve recovery in small animal models. Brain Behav 7:. doi: 10.1002/brb3.723

Klein AH, Sawyer CM, Zanotto KL, et al (2011) A tingling sanshool derivative excites primary sensory neurons and elicits nocifensive behavior in rats. J Neurophysiol 105:1701–1710. doi: 10.1152/jn.00922.2010

Li XY, Toyoda H (2015) Role of leak potassium channels in pain signaling. Brain Res Bull 119:73–79. doi: 10.1016/j.brainresbull.2015.08.007

Lim TK (2016) Edible Medicinal and Non-Medicinal Plants

Malmberg a B, Basbaum a I (1998) Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain 76:215–222. doi: S0304-3959(98)00045-1 [pii]

Marsh B, Acosta C, Djouhri L, Lawson SN (2012) Leak K + channel mRNAs in dorsal root ganglia: Relation to inflammation and spontaneous pain behaviour. Mol Cell Neurosci 49:375–386. doi: 10.1016/j.mcn.2012.01.002

Marwaha L, Bansal Y, Singh R, et al (2016) TRP channels: potential drug target for neuropathic pain. Inflammopharmacology 24:305–317. doi: 10.1007/s10787-016-0288-x

Mathie A, Rees K a, El Hachmane MF, Veale EL (2010) Trafficking of neuronal two pore domain potassium channels. Curr Neuropharmacol 8:276–286. doi: 10.2174/157015910792246146

Meacham K, Shepherd A, Mohapatra DP, Haroutounian S (2017) Neuropathic Pain: Central vs. Peripheral Mechanisms. Curr. Pain Headache Rep. 21

Mogil JS, Graham AC, Ritchie J, et al (2010) Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. Mol Pain 6:. doi: 10.1186/1744-8069-6-34

Montrucchio DP, Córdova MM, Soares Santos AR (2013) Plant Derived Aporphinic Alkaloid S-(+)-Dicentrine Induces Antinociceptive Effect in Both Acute and Chronic Inflammatory Pain Models: Evidence for a Role of TRPA1 Channels. PLoS One 8:2–10. doi: 10.1371/journal.pone.0067730

Moran MM, McAlexander MA, Bíró T, Szallasi A (2011) Transient receptor potential channels as therapeutic targets. Nat. Rev. Drug Discov. 10:601–620

National Research Council (2011) Guide for the Care and Use of Laboratory Animals. National Academies Press

Newman DJ, Cragg GM (2016) Natural Products as Sources of New Drugs from 1981 to 2014. J. Nat. Prod. 79:629–661

Nomura ECO, Rodrigues MRA, Da Silva CF, et al (2013) Antinociceptive effects of ethanolic extract from the flowers of *Acmella oleracea* (L.) R.K. Jansen in mice. J Ethnopharmacol 150:583–589. doi: 10.1016/j.jep.2013.09.007

Pak DJ, Yong RJ, Kaye AD, Urman RD (2018) Chronification of Pain: Mechanisms, Current Understanding, and Clinical Implications. Curr. Pain Headache Rep. 22

Palhares RM, Drummond MG, Dos Santos Alves Figueiredo Brasil B, et al (2015) Medicinal plants recommended by the world health organization: DNA barcode identification associated with chemical analyses guarantees their quality. PLoS One 10:1–29. doi: 10.1371/journal.pone.0127866

Persson AK, Hoeijmakers JGJ, Estacion M, et al (2016) Sodium Channels, Mitochondria, and Axonal Degeneration in Peripheral Neuropathy. Trends Mol Med 22:377–390. doi: 10.1016/j.molmed.2016.03.008

Pitzer C, Kuner R, Tappe-Theodor A (2016) Voluntary and evoked behavioral correlates in neuropathic pain states under different social housing conditions. Mol Pain 12:1–15. doi: 10.1177/1744806916656635

Pogatzki-zahn EM, Segelcke D, Schug SA (2017) Postoperative pain — from mechanisms to treatment. PAIN Reports 2:e588. doi: 10.1097/PR9.0000000000000588

Pogatzki EM, Raja SN (2003) A mouse model of incisional pain. Anesthesiology 99:1023–1027. doi: 10.1097/00000542-200310000-00041

Prachayasittikul S, Suphapong S, Worachartcheewan A, et al (2009) Bioactive metabolites from Spilanthes acmella Murr. Molecules 14:850–867. doi: 10.3390/molecules14020850

Prachayasittikul V, Prachayasittikul S, Ruchirawat S, Prachayasittikul V (2013) High therapeutic potential of Spilanthes acmella: A review. EXCLI J. 12:291–312

Quintans JSS, Antoniolli ÂR, Almeida JRGS, et al (2014) Natural products evaluated in neuropathic pain models - a systematic review. Basic Clin Pharmacol Toxicol 114:442–450. doi: 10.1111/bcpt.12178

Ratnasooriya WD, Lanka S, Lanka S (2005) Attenuation of Persistent Pain and Hyperalgesia by Spilanthus acmella Flowers in Rats. 43:614–619. doi: 10.1080/13880200500301944

Rawal N (2016) Current issues in postoperative pain management. Eur J Anaesthesiol 33:160–171. doi: 10.1097/EJA.0000000000000366

Rios MY, Olivo HF (2014) Natural and synthetic alkamides: Applications in pain therapy. Stud Nat Prod Chem 43:79–121. doi: 10.1016/B978-0-444-63430-6.00003-5

Scholz J, Woolf CJ (2002) Can we conquer pain? Nat Neurosci 5:1062–1067. doi: 10.1038/nn942

Seltzer Z, Dubner R, Shir Y (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 43:205–218. doi: 10.1016/0304-3959(90)91074-S

Shepherd AJ, Mohapatra DP (2018) Pharmacological validation of voluntary gait and mechanical sensitivity assays associated with inflammatory and neuropathic pain in mice. Neuropharmacology 130:18–29. doi: 10.1016/j.neuropharm.2017.11.036

Tsunozaki M, Lennertz RC, Vilceanu D, et al (2013) A ‘ toothache tree ’ alkylamide inhibits A δ mechanonociceptors to alleviate mechanical pain. 13:3325–3340. doi: 10.1113/jphysiol.2013.252106x

Tulleuda A, Cokic B, Callejo G, et al (2011) TRESK channel contribution to nociceptive sensory neurons excitability : modulation by nerve injury. 1–17. doi: 10.1186/1744-8069-7-30

Uchytilova E, Spicarova D, Palecek J (2014) TRPV1 antagonist attenuates postoperative hypersensitivity by central and peripheral mechanisms. Mol Pain 10:67. doi: 10.1186/1744-8069-10-67

Van Hecke O, Austin SK, Khan RA, et al (2014) Neuropathic pain in the general population: A systematic review of epidemiological studies. Pain 155:654–662

Vrinten DH, Hamers FFT (2003) “CatWalk” automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. Pain 102:203–209. doi: 10.1016/s0304-3959(02)00382-2

Xu J, Brennan TJ (2010) Guarding pain and spontaneous activity of nociceptors after skin versus skin plus deep tissue incision. Anesthesiology 112:153–164. doi: 10.1097/ALN.0b013e3181c2952e

Zahn PK, Gysbers D, Brennan TJ (1997) Effect of systemic and intrathecal morphine in a rat model of postoperative pain. Anesthesiology 86:1066–1077. doi: 10.1097/00000542-199705000-00010

5 CONSIDERAÇÕES FINAIS E CONCLUSÃO

A dor é considerada um grande problema clínico, econômico e social. Apesar de diferentes etiologias (inflamação ou lesão em nervos periféricos), a dor causa, na maioria dos casos, limitações, prejuízos financeiros e redução da qualidade de vida dos pacientes. Atualmente, as terapias medicamentosas disponíveis apresentam baixa eficácia, severos efeitos adversos e baixa adesão do paciente, tornando assim necessária a busca de novas alternativas terapêuticas.

O uso de plantas medicinais como fonte de novas moléculas e complexos fitoquímicos é uma ferramenta estratégica para o desenvolvimento de novos fármacos, e o uso etnofarmacológico dessas plantas pode nos guiar nesse processo. Como visto, a *Acmella oleracea* (jambu) apresenta diversos usos populares, dentre eles o uso das suas flores para o tratamento de dores de dente devido a sensação anestésica causada na boca. Esse efeito é atribuído às alquilamidas, uma classe de metabólitos secundários muito abundante no jambu e com diversas propriedades farmacológicas promissoras aqui investigadas.

Primeiramente, nossos resultados revelaram o efeito *dual* e diferentes mecanismos de ação promovidos localmente pela injeção intraplantar de FH (fração hexânica rica em alquilamidas obtidas das flores do jambu), bem como de IBA (isobutilalquil amida sintética), em baixas e elevadas doses:

- A injeção intraplantar de doses crescentes de FH e IBA promoveram a indução de comportamento de lambida da pata em doses mais elevadas;

- FH e IBA em dose baixa promoveram efeito antinociceptivo em ambas as fases, neurogênica e inflamatória, da formalina. Este efeito antinociceptivo ocorreu através do bloqueio ou modulação de canais TRPV1 e independente de receptores opioides. Além disso, também em dose baixa, FH e IBA promoveram a sensibilização de canais TRPA1, e FH a estabilização de mastócitos.

- O comportamento nociceptivo de lambida da pata induzido por FH e IBA em dose elevada foi revertido pelo pré-tratamento com morfina.

- FH em dose elevada promoveu efeito nociceptivo através da ativação de canais TRPA1 e pela indução da degranulação de mastócitos, enquanto que IBA em dose elevada (promoveu efeito nociceptivo através da ativação de ambos os canais TRPV1 e TRPA1;

- Adicionalmente, FH em dose baixa e elevada promoveu o aumento do limiar térmico da pata dos animais, sendo que a menor dose também elevou o limiar mecânico, sugerindo assim um efeito anestésico das alquilamidas do jambu. Ao contrário da FH, IBA em dose baixa e elevada reduziu o limiar térmico da pata dos animais, sendo que a maior dose também reduziu o limiar mecânico.

Na sequência, nossos resultados mostraram os efeitos farmacológicos de FH e IBA no modelo de dor inflamatória aguda induzida por carragenina, demonstrando que:

- Os pré-tratamentos locais com FH e IBA (i.pl.) apresentaram efeitos antialodínico e antiedematogênico no modelo de inflamação induzida pela injeção intraplantar de carragenina;

- Os pré-tratamentos locais com FH e IBA (i.pl) promoveram a redução da migração celular e redução dos níveis de citocinas pró-inflamatórias (IL-1β e TNF-α). Ainda, FH reduziu a síntese de PGE_2 e manteve a concentração de IL-10 em níveis basais;

- Os pré-tratamentos locais com FH e IBA (i.pl) preveniram a formação de LOOH, bem como a depleção dos componentes do sistema antioxidante endógeno (CAT, SOD, GSH);

- FH e IBA não apresentaram efeito antioxidante no modelo de sequestro de radicas livres de DPPH.

E por fim, pela primeira vez na literatura científica, demonstramos os efeitos promovidos pelo tratamento com alquilamidas nos modelos de dor neuropática e pós-operatória:

- Os tratamentos locais com FH e IBA (i.pl.) promoveram efeito antialodínico mecânico e térmico, além da melhora dos parâmetros de marcha dos animais, como área, *print* e tempo de contato da pata, após a indução da neuropatia;

- Os tratamentos locais com FH e IBA (tópico) promoveram efeito antialodínico mecânico no modelo de dor pós-operatória. Entretanto, apenas o tratamento com FH reduziu a hiperalgesia térmica ao calor e comportamentos de dor espontânea.

Coletivamente, concluímos que as alquilamidas naturais do jambu e a sintética IBA apresentaram efeitos biológicos promissores com potencial farmacológico para o tratamento local de dor inflamatória, neuropática e pós-operatória.

REFERÊNCIAS

ATTAL, N. Pharmacological treatments of neuropathic pain: The latest recommendations. **Revue Neurologique**, v. 175, n 1, p. 46-50, 2018.

BALIKI, M. N.; APKARIAN, A. V. Nociception, Pain, Negative Moods, and Behavior Selection. **Neuron**, v. 87, n. 3, p. 474–491, 2015.

BARNES, P. J. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. **British Journal of Pharmacology**, v. 148, n. 3, p. 245-254, 2006.

BASBAUM, A. I.; BAUTISTA, D. M.; SCHERRER, G.; JULIUS, D. Cellular and Molecular Mechanisms of Pain. **NIH Public Access**, v. 139, n. 2, p. 267–284, 2010.

BAUTISTA, D. M.; SIGAL, Y. M.; MILSTEIN, A. D.; et al. Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels. **Nature Neuroscience**, v. 11, n. 7, p. 772–779, 2008.

BOONEN, J.; BAERT, B.; BURVENICH, C.; et al. LC-MS profiling of N-alkylamides in Spilanthes acmella extract and the transmucosal behaviour of its main bio-active spilanthol. **Journal of Pharmaceutical and Biomedical Analysis**, v. 53, n. 3, p. 243–249, 2010.

BOONEN, J.; BRONSELAER, A.; NIELANDT, J.; et al. Alkamid database: Chemistry, occurrence and functionality of plant N-alkylamides. **Journal of Ethnopharmacology**, v. 142, n. 3, p. 563-590, 2012.

BRASIL. Ministério da Saúde. **Agência Nacional de Vigilância Sanitária (Anvisa). Resolução - RDC nº 26, de 14 de maio de 2014**. Dispõe sobre o registro de medicamentos fitoterápicos e o registro e a notificação de produtos tradicionais fitoterápicos. Diário Oficial da União. Disponível em: <http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2014/rdc0026_13_05_2014.pdf>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Política Nacional de Plantas Medicinais e Fitoterápicos**. Disponível em: < http://bvsms.saude.gov.br/bvs/publicacoes/politica_nacional_fitoterapicos.pdf>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Política Nacional de Práticas Integrativas e Complementares do SUS**. Disponível em: < http://bvsms.saude.gov.br/bvs/publicacoes/pnpic.pdf>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Portaria Interministerial nº 2.960, de 9 de Dezembro de 2008.** Aprova o Programa Nacional de Plantas Medicinais e Fitoterápicos e cria o Comitê Nacional de Plantas Medicinais e Fitoterápicos. Diário Oficial da União. Disponível em: <http://bvsms.saude.gov.br/bvs/saudelegis/gm/2008/pri2960_09_12_2008.html>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Portaria nº 971, de 03 de Maio de 2006**. Aprova a Política Nacional de Práticas Integrativas e Complementares no Sistema Único de Saúde. Diário Oficial da União. Disponível em: < http://bvsms.saude.gov.br/bvs/saudelegis/gm/2006/prt0971_03_05_2006.html>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Programa Nacional de Plantas Medicinais e Fitoterápicos**. Disponível em: >http://bvsms.saude.gov.br/bvs/publicacoes/programa_nacional_plantas_medicinais_fitoterapicos.pdf>. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Relação Nacional de Medicamentos Essenciais**. Disponível em: < http://www.conass.org.br/wp-content/uploads/2018/11/RENAME-2018.pdf >. Acesso em: 18/01/2019.

BRASIL. Ministério da Saúde. **Relação Nacional de Plantas Medicinais de Interesse ao SUS**. Disponível em: < http://portalarquivos2.saude.gov.br/images/pdf/2017/junho/06/renisus.pdf>. Acesso em: 18/01/2019.

BRASIL. Presidência da República. **Decreto nº 5.813, de 22 de Junho de 2006**. Aprova a Política Nacional de Plantas Medicinais e Fitoterápicos e dá outras providências. Diário Oficial da União. Disponível em: < http://www.planalto.gov.br/ccivil_03/_Ato2004-2006/2006/Decreto/D5813.htm>. Acesso em: 18/01/2019.

BRASIL. Presidência da República. **Decreto nº 8.772, de 11 de maio de 2016**. Dispõe sobre o acesso ao patrimônio genético, sobre a proteção e o acesso ao conhecimento tradicional associado e sobre a repartição de benefícios para conservação e uso sustentável da biodiversidade. Diário Oficial da União. Disponível em: < http://www.planalto.gov.br/ccivil_03/_ato2015-2018/2016/decreto/D8772.htm>. Acesso em: 18/01/2019.

Brasil. Presidência da República. **Lei n° 13.123, de 20 de maio de 2015**. Dispõe sobre o acesso ao patrimônio genético, sobre a proteção e o acesso ao conhecimento tradicional associado e sobre a repartição de benefícios para conservação e uso sustentável da biodiversidade. Diário Oficial da União. Disponível em: < http://www.planalto.gov.br/ccivil_03/_Ato2015-2018/2015/Lei/L13123.htm>. Acesso em: 18/01/2019.

BRYANT, B. P.; MEZINE, I. Alkylamides that produce tingling paresthesia activate tactile and thermal trigeminal neurons. **Brain Research**, v. 842, n. 2, p. 452–460, 1999.

CALIXTO, J. Biodiversidade como fonte de medicamentos. **Ciência e cultura**, v. 200, p. 37–39, 2003.

CHAKRABORTY, A.; DEVI, R. K. B.; RITA, S.; SHARATCHANDRA, K.; SINGH, T. . Preliminary studies on anti-inflammatory and analgesic activities of Securinega virosa (Euphorbiaceae) in experimental animal models. **Indian Journal of Pharmacology**, v. 36, n. 3, p. 148–150, 2004.

CHEN, Y.; DE BRUYN KOPS, C.; KIRCHMAIR, J. Data Resources for the Computer-Guided Discovery of Bioactive Natural Products. **Journal of Chemical Information and Modeling**, v. 57, n. 9, p. 2099–2111, 2017.

CHENG, Y. BIN; LIU, R. H.; HO, M. C.; et al. Alkylamides of *Acmella oleracea*. **Molecules**, v. 20, n. 4, p. 6970–6977, 2015.

CHOU, R.; GORDON, D.; DE LEON-CASASOLA, O.; et al. Management of Postoperative Pain: A Clinical Practice Guideline. **J Pain**, v. 17, n. 2, p. 131–57, 2016.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2014 July 11. **Identifier NCT02187549, Safety and Effectiveness Study of a Non-Crosslinked HA Alkylamide HYADD(TM) 4 Hydrogel for Osteoarthritis of the Knee;** 2015, October 5. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT02187549?term=alkylamide&rank=1>. Acesso em: 31/01/2019.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2013 December 6. **Identifier NCT02003651, Echinacea and Acute Respiratory Illness;** 2016, December 13. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT02003651?term=alkylamide&rank=2>. Acesso em: 31/01/2019.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2017 March 3. **Identifier - NCT03070314, Echinaforce Junior Bioavailability Trial;** 2018, July 18. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT03070314?term=alkylamide&rank=3 >. Acesso em: 31/01/2019.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2014 July 11. **Identifier NCT02187549, Safety and Effectiveness Study of a Non-Crosslinked HA Alkylamide HYADD(TM) 4 Hydrogel for Osteoarthritis of the Knee;** 2015, October 5. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT02187549?term=alkylamide&rank=1>. Acesso em: 31/01/2019.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2013 December 6. **Identifier NCT02003651, Echinacea and Acute Respiratory Illness;** 2016, December 13. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT02003651?term=alkylamide&rank=2>. Acesso em: 31/01/2019.

ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2017 March 3. **Identifier - NCT03070314, Echinaforce Junior Bioavailability Trial;** 2018, July 18. Disponível em: < https://clinicaltrials.gov/ct2/show/NCT03070314?term=alkylamide&rank=3 >. Acesso em: 31/01/2019.

COHEN, M.; QUINTNER, J.; RYSEWYK, S. VAN. Reconsidering the International Association for the Study of Pain definition of pain. **Pain reports** , v. 3, p. 1–7, 2018.

COHEN, S. P.; MAO, J. Neuropathic pain: Mechanisms and their clinical implications. **BMJ (Online)**, v. 348, p. f7656, 2014.

COLLOCA, L.; LUDMAN, T.; BOUHASSIRA, D.; et al. Neuropathic pain. **Nature Reviews Disease Primers**, v. 3, p. 1–20, 2017.

COOK, A. D.; CHRISTENSEN, A. D.; TEWARI, D.; MCMAHON, S. B.; HAMILTON, J. A. Immune Cytokines and Their Receptors in Inflammatory Pain. **Trends in Immunology**, v. xx, p. 1–16, 2018.

COSTA, N. T.; IRIYODA, T. M. V.; ALFIERI, D. F.; SIMÃO, A. N. C.; DICHI, I. Influence of disease-modifying antirheumatic drugs on oxidative and nitrosative stress in patients with rheumatoid arthritis. **Inflammopharmacology**, v. 26, n. 5, p. 1151–1164, 2018.

DAY, R. O.; GRAHAM, G. G. Therapeutics: Non-steroidal anti-inflammatory drugs (NSAIDs). **BMJ**, v. 346, n. 7917, p. 34–37, 2013.

DIAS, D. A.; URBAN, S.; ROESSNER, U. A Historical overview of natural products in drug discovery. **Metabolites**, v. 2, n. 2, p. 303–336, 2012.

DUBIN, A. E.; PATAPOUTIAN, A. Nociceptors: The sensors of the pain pathway. **Journal of Clinical Investigation**, v. 120, n. 11, p. 3760–3772, 2010.

DUTRA, R. C.; CAMPOS, M. M.; SANTOS, A. R. S.; CALIXTO, J. B. Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. **Pharmacological Research**, p. 1–25, 2016.

FAVORETO, R.; GILBERT, B. Estado da Arte/State of the Art - *Acmella oleracea* (L.) R. K. Jansen (Asteraceae) – Jambu. **Revista Fitos**, v. 5, n. 1, p. 83–91, 2010.

FINNERUP, N. B.; ATTAL, N.; HAROUTOUNIAN, S.; et al. Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. **The Lancet Neurology**, v. 14, n. 2, p. 162–173, 2015.

FINNERUP, N. B.; JENSEN, T. S. Mechanisms of disease: Mechanism-based classification of neuropathic pain - A critical analysis. **Nature Clinical Practice Neurology**, v. 2, n. 2, p. 107, 2006.

FOLHAS E FLORES DO JAMBU. Disponível em: <http://armazemdasespeciarias.com.br/wp-content/uploads/2015/11/shutterstock_147228356.jpg>. Acesso em: 18/01/2019.

GEPPETTI, P.; VELDHUIS, N. A.; LIEU, T. M.; BUNNETT, N. W. G Protein-Coupled Receptors: Dynamic Machines for Signaling Pain and Itch. **Neuron**, v. 88, n. 4, p. 635-649, 2015.

GERTSCH, J. Immunomodulatory Lipids in Plants : Plant Fatty Acid Amides and the Human Endocannabinoid System. **Planta Medica**, v. 74, n. 74, p. 638–650, 2008.

GREGER, H. Alkamides: a critical reconsideration of a multifunctional class of unsaturated fatty acid amides. **Phytochemistry Reviews**, v. 15, n. 5, p. 729–770, 2016.

GULLEDGE, T. V.; COLLETTE, N. M.; MACKEY, E.; et al. Mast cell degranulation and calcium influx are inhibited by an Echinacea purpurea extract and the alkylamide dodeca-2E,4E-dienoic acid isobutylamide. **Journal of Ethnopharmacology**, v. 212, p. 166–174, 2018.

HARVEY, A. L.; EDRADA-EBEL, R.; QUINN, R. J. The re-emergence of natural products for drug discovery in the genomics era. **Nature Reviews Drug Discovery**, v. 14, n. 2, p. 111–129, 2015.

VON HEHN, C. A.; BARON, R.; WOOLF, C. J. Deconstructing the Neuropathic Pain Phenotype to Reveal Neural Mechanisms. **Neuron**, v. 73, n. 4, p. 638-652, 2012.

HENSCHKE, N.; HONS, B. P.; KAMPER, S. J.; et al. The Epidemiology and Economic Consequences of Pain. **Mayo Clinic Proceedings**, v. 90, n. 1, p. 139–147, 2015.

VAN DEN HEUVEL, I.; REICHL, S.; SEGELCKE, D.; ZAHN, P. K.; POGATZKI-ZAHN, E. M. Selective prevention of mechanical hyperalgesia after incision by spinal ERK1/2 inhibition. **European Journal of Pain (United Kingdom)**, v. 19, n. 2, p. 225–235, 2015.

IASP. **Terminology**. Disponível em: <https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698>. Acesso em: 22/01/2019.

JENSEN, T. S.; FINNERUP, N. B. Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. **The Lancet Neurology**, v. 13, n. 9, p. 924–935, 2014.

JI, R.; CHAMESSIAN, A.; ZHANG, Y. Pain regulation by non-neuronal cells and inflammation. **Science**, v. 354, n. 6312, p. 572–577, 2016.

JULIUS, D. Molecular mechanisms of nociception . **Nature**, v. 413, n. September, p. 203–210, 2001.

KANG, S.; LEE, D.; THEUSCH, B. E.; ARPEY, C. J.; BRENNAN, T. J. Wound hypoxia in deep tissue after incision in rats. **Wound Repair and Regeneration**, v. 21, n. 5, p. 730–739, 2013.

KAPPOS, E. A.; SIEBER, P. K.; ENGELS, P. E.; et al. Validity and reliability of the CatWalk system as a static and dynamic gait analysis tool for the assessment of functional nerve recovery in small animal models. **Brain and Behavior**, v. 7, n. 7, 2017.

KIDO, K.; GAUTAM, M.; BENSON, C. J.; GU, H.; BRENNAN, T. J. Effect of deep tissue incision on ph responses of afferent fibers and dorsal root ganglia innervating muscle. **Anesthesiology**, v. 119, n. 5, p. 1186–1197, 2013.

KIM, T. J.; FREML, L.; PARK, S. S.; BRENNAN, T. J. Lactate Concentrations in Incisions Indicate Ischemic-like Conditions May Contribute to Postoperative Pain. **Journal of Pain**, v. 8, n. 1, p. 59–66, 2007.

KLEIN, A. H.; SAWYER, C. M.; ZANOTTO, K. L.; et al. A tingling sanshool derivative excites primary sensory neurons and elicits nocifensive behavior in rats. **Journal of neurophysiology**, v. 105, n. 4, p. 1701–1710, 2011.

KOO, J. Y.; JANG, Y.; CHO, H.; et al. Hydroxy- a -sanshool activates TRPV1 and TRPA1 in sensory neurons. **European Journal of Neuroscience,** v. 26, n. 5, p. 1139-1147, 2007.

KUNER, R.; FLOR, H. Structural plasticity and reorganisation in chronic pain. **Nature Reviews Neuroscience**, v. 18, n. 1, p. 20–30, 2016.

DE LA ROSA-LUGO, V.; ACEVEDO-QUIROZ, M.; DÉCIGA-CAMPOS, M.; RIOS, M. Y. Antinociceptive effect of natural and synthetic alkamides involves TRPV1 receptors. **Journal of Pharmacy and Pharmacology**, v. 69, n. 7, p. 884–895, 2017.

LAVAND'HOMME, P. Transition from acute to chronic pain after surgery. **Pain**, v. 158, n. 4, p. S50–S54, 2017.

LENNERTZ, R. C.; TSUNOZAKI, M.; BAUTISTA, D. M.; STUCKY, C. L. Physiological Basis of Tingling Paresthesia Evokedby Hydroxy- -Sanshool. **Journal of Neuroscience**, v. 30, n. 12, p. 4353–4361, 2010.

LIM, T. K. **Edible Medicinal and Non-Medicinal Plants**. Dordrecht, The Netherlands: Springer, 2016.

MEACHAM, K.; SHEPHERD, A.; MOHAPATRA, D. P.; HAROUTOUNIAN, S. Neuropathic Pain: Central vs. Peripheral Mechanisms. **Current Pain and Headache Reports**, v. 21, n. 6, p. 28, 2017.

MOGIL, J. S. Animal models of pain: Progress and challenges. **Nature Reviews**

Neuroscience, v. 10, n. 4, p. 283–294, 2009.

MOGIL, J. S.; GRAHAM, A. C.; RITCHIE, J.; et al. Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. **Molecular Pain**, v. 6, p. 1–15, 2010.

NEWMAN, D. J.; CRAGG, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. **Journal of Natural Products**, v. 79, n. 3, p. 629-661, 2016.

NEWMAN, D. J.; CRAGG, G. M.; SNADER, K. M. The influence of natural products upon drug discovery. **Natural product reports**, v. 17, n. 3, p. 215–234, 2000.

NOMURA, E. C. O.; RODRIGUES, M. R. A.; DA SILVA, C. F.; et al. Antinociceptive effects of ethanolic extract from the flowers of *Acmella oleracea* (L.) R.K. Jansen in mice. **Journal of Ethnopharmacology**, v. 150, n. 2, p. 583–589, 2013.

NOURSHARGH, S.; ALON, R. Leukocyte Migration into Inflamed Tissues. **Immunity**, v. 41, n. 5, p. 694–707, 2014.

OLIVEIRA, S. M.; DREWES, C. C.; SILVA, C. R.; et al. Involvement of mast cells in a mouse model of postoperative pain. **European Journal of Pharmacology**, v. 672, n. 1–3, p. 88–95, 2011.

OLIVEIRA, S. M.; PH, D.; SILVA, C. R.; et al. Activation by Mast Cell Tryptase in the Development of Postoperative Pain., **Anesthesiology: The Journal of the American Society of Anesthesiologists**, v. 118, n. 3, p. 679–690-679–690, 2013.

DEWICK, P. M. Medicinal natural products: a biosynthetic approach. **John Wiley & Sons**, 2002.

PALHARES, R.M; DRUMMOND, M.G; DOS SANTOS ALVES FIGUEIREDO BRASIL, B, et al. Medicinal plants recommended by the world health organization: DNA barcode identification associated with chemical analyses guarantees their quality. **PLoS One** 10:1–29, 2015.

PEIRS, C.; SEAL, R. P. Neural circuits for pain: Recent advances and current views. **Science**, v. 354, n. 6312, p. 578–584, 2016.

PINHO-RIBEIRO, F. A.; VERRI, W. A.; CHIU, I. M. Nociceptor Sensory Neuron–Immune Interactions in Pain and Inflammation. **Trends in Immunology**, v. 38, n. 1, p. 5-19, 2017.

PITZER, C.; KUNER, R.; TAPPE-THEODOR, A. Voluntary and evoked behavioral correlates in neuropathic pain states under different social housing conditions. **Molecular Pain**, v. 12, p. 1–15, 2016a.

POGATZKI-ZAHN, E. M.; SEGELCKE, D.; SCHUG, S. A. Postoperative pain — from mechanisms to treatment. **PAIN Reports**, v. 2, n. 2, p. e588, 2017.

PRACHAYASITTIKUL, S.; SUPHAPONG, S.; WORACHARTCHEEWAN, A.; et al. Bioactive metabolites from Spilanthes acmella Murr. **Molecules**, v. 14, n. 2, p. 850–867, 2009.

PRACHAYASITTIKUL, V.; PRACHAYASITTIKUL, S.; RUCHIRAWAT, S.; PRACHAYASITTIKUL, V. High therapeutic potential of Spilanthes acmella: A review. **EXCLI Journal**, v. 12, p. 291, 2013.

RADUNER, S.; MAJEWSKA, A.; CHEN, J.; et al. Alkylamides from *Echinacea* Are a New Class of Cannabinomimetics. **Journal of Biological Chemistry**, v. 281, n. 20, p. 14192–14206, 2006.

RATNASOORIYA, W. D.; LANKA, S.; LANKA, S. Attenuation of Persistent Pain and Hyperalgesia by Spilanthus acmella Flowers in Rats. **Pharmaceutical biology,** v. 43, n. 7, p. 614–619, 2005.

REUTER, S.; GUPTA, S. C.; CHATURVEDI, M. M.; AGGARWAL, B. B. Oxidative stress, inflammation, and cancer: How are they linked? **Free Radical Biology and Medicine**, v. 49, n. 11, p. 1603-1616, 2010.

REYNOSO-MORENO, I.; NAJAR-GUERRERO, I.; ESCARENO, N.; et al. An Endocannabinoid Uptake Inhibitor from Black Pepper Exerts Pronounced Anti-Inflammatory Effects in Mice. **Journal of Agricultural and Food Chemistry**, v. 65, n. 43, p. 9435–9442, 2017.

RIOS, M. Y.; OLIVO, H. F. Natural and synthetic alkamides: Applications in pain therapy. **Studies in Natural Products Chemistry**, v. 43, p. 79–121, 2014.

RODRIGUES, T.; REKER, D.; SCHNEIDER, P.; SCHNEIDER, G. Counting on natural products for drug design. **Nature Chemistry**, v. 8, n. 6, p. 531–541, 2016.

RUETSCH, Y.; BONI, T.; BORGEAT, A. From Cocaine to Ropivacaine: The History of Local Anesthetic Drugs. **Current Topics in Medicinal Chemistry**, v. 1, n. 3, p. 175–182, 2001.

SAHA, M.; SKOPELJA, S.; MARTINEZ, E.; et al. Spinal Mitogen-Activated Protein Kinase Phosphatase-3 (MKP-3) is Necessary for the Normal Resolution of Mechanical Allodynia in a Mouse Model of Acute Postoperative Pain. **Journal of Neuroscience**, v. 33, n. 43, p. 17182–17187, 2013.

SAHBAIE, P.; LI, X.; SHI, X.; CLARK, J. D. Roles of Gr-1 + leukocytes in postincisional nociceptive sensitization and inflammation. **Anesthesiology**, v. 117, n. 3, p. 602–612, 2012.

SAWYER, C. M.; CARSTENS, M. I.; SIMONS, C. T.; et al. Activation of lumbar spinal wide-dynamic range neurons by a sanshool derivative. **J Neurophysiol**, v. 101, n. 4, p. 1742–1748, 2009.

SCHMIDT, R. F.; WILLIS, W. D. **Encyclopedia of Pain. Springer, New York**. 2007.

SCHOLZ, J.; WOOLF, C. J. Can we conquer pain? **Nature Neuroscience**, v. 5, n. 11s, p. 1062–1067, 2002.

SHEPHERD, A. J.; MOHAPATRA, D. P. Pharmacological validation of voluntary gait and mechanical sensitivity assays associated with inflammatory and neuropathic pain in mice. **Neuropharmacology**, v. 130, p. 18–29, 2018.

SHI, X. D.; FU, D.; XU, J. M.; ZHANG, Y. L.; DAI, R. P. Activation of spinal ERK1/2 contributes to mechanical allodynia in a rat model of postoperative pain. **Molecular Medicine Reports**, v. 7, n. 5, p. 1661–1665, 2013.

SILVEIRA, N.; SANDJO, L. P.; BIAVATTI, M. W. Spilanthol-containing products: A patent review (1996–2016). **Trends in Food Science and Technology**, v. 74, p. 107-111, 2018.

SINATRA, R. S.; NALINI, V.; WHITNEY, C. J. Pain Physiology and Pharmacology. **Acute Pain Management**, v. 1, p. 4–5, 2009.

Sistema de Informação em Saúde para a Atenção Básica. **Plantas medicinais e fitoterápicos no SUS.** Disponível em: < http://portalms.saude.gov.br/acoes-e-programas/programa-nacional-de-plantas-medicinais-e-fitoterapicos-ppnpmf/plantas-medicinais-e-fitoterapicos-no-sus>. Acesso em: 18/01/2019.

SOMMER, C.; LEINDERS, M.; ÜÇEYLER, N. Inflammation in the pathophysiology of neuropathic pain. **Pain**, v. 159, n. 3, p. 595–602, 2018.

SUGAI, E.; MORIMITSU, Y.; IWASAKI, Y.; et al. Pungent Qualities of Sanshool-Related Compounds Evaluated by a Sensory Test and Activation of Rat TRPV1. **Bioscience, Biotechnology, and Biochemistry**, v. 69, n. 10, p. 1951–1957, 2005.

TRACEY, W. D. Nociception. **Current Biology**, v. 27, n. 4, p. R129–R133, 2017.

TSUNOZAKI, M.; LENNERTZ, R. C.; VILCEANU, D.; et al. A ' toothache tree ' alkylamide inhibits A δ mechanonociceptors to alleviate mechanical pain. **The Journal of physiology**, v. 591, n. 13, p. 3325-3340, 2013.

TULLEUDA, A.; COKIC, B.; CALLEJO, G.; et al. TRESK channel contribution to nociceptive sensory neurons excitability : modulation by nerve injury. **Molecular pain**, v. 7, n. 1, p. 30, 2011.

VIEGAS, C.; DA SILVA BOLZANI, V.; BARREIRO, E. J. OS produtos naturais e a química medicinal moderna. **Quimica Nova**, v. 29, n. 2, p. 326–337, 2006.

WHITESIDE, G. T.; POMONIS, J. D.; KENNEDY, J. D. An industry perspective on the role and utility of animal models of pain in drug discovery. **Neuroscience Letters**, v. 557, p. 65–72, 2013.

WHO. The World Medicines Situation 2011. **The World Medicines Situation**, p. 32, 2011. Disponível em: <http://www.who.int/medicines/areas/policy/world_medicines_situation/WMS_ch6_wPricing_v6.pdf>. Acesso em: 18/01/2019.

YEZIERSKI, R. P.; HANSSON, P. Inflammatory and Neuropathic Pain From Bench to Bedside: What Went Wrong? **Journal of Pain**, v. 19, n. 6, p. 571-588, 2018.

ZAHN, P. K.; POGATZKI-ZAHN, E. M.; BRENNAN, T. J. Spinal administration of MK-801 and NBQX demonstrates NMDA-independent dorsal horn sensitization in incisional pain. **Pain**, v. 114, n. 3, p. 499–510, 2005.

PATEL, N. B. Fisiologia da Dor. In: KOPF, A. Associaçãoo Internacional Para O Estudo da Dor. **Guia para o Tratamento da Dor em Contextos de Poucos Recursos.** Seattle: Iasp Press, 2009.

ANEXO 1 – Certificado da Comissão de ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR: nº 970)

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

UFPR
UNIVERSIDADE FEDERAL DO PARANÁ

Nº 970

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.132887/2016-00

APROVADO/APPROVAL: 10/05/2016 – R.O. 04/2016

TÍTULO: Investigação dos mecanismos envolvidos no efeito nociceptivo da fração rica em alquilamidas isoladas da flores da *Acmella oleracea* (l.) R.K. Jansen, em camundongos

TITLE: Investigation of mechanisms involved in the nociceptive effects of the fraction rich in alkylamides isolated from flowers of *Acmella oleracea* (l.) RK Jansen in mice

AUTORES/AUTHORS: Maria Fernanda de Paula Werner, Jorge Luiz Dallazen, Daniele Maria Ferreira, Fernando Tonholi Dal Lin

DEPARTAMENTO/DEPARTMENT: Farmacologia

Profa. Dra. Ana Vitória Fischer da Silva
Coordenadora da CEUA

ANEXO 2 – Certificado da Comissão de ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR: nº 1107)

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 1107

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.192841/2017-12

APROVADO/APPROVAL: 19/09/2017 – R.O. 08/2017

TÍTULO: Avaliação do efeito da administração local de alquilamidas em modelos experimentais de dor.

TITLE: Evaluation of the effect of local administration of alkylamides in experimental pain models.

AUTORES/AUTHORS: Maria Fernanda de Paula Werner, Jorge Luiz Dallazen, Bruna Barbosa da Luz, Daniele Maria Ferreira.

DEPARTAMENTO/DEPARTMENT: Farmacologia

Profa. Dra. Katya Naliwaiko
Coordenadora da CEUA