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Tese de Doutorado

ANÁLISE DOS MECANISMOS DE AÇÃO ANTINOCICEPTIVA E ANTIINFLAMATÓRIA DO FLAVONÓIDE MIRICITRINA: ESTUDOS *IN VIVO* E *IN VITRO*

Flavia Carla Meotti

Santa Maria, RS, Brasil

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por

Flavia Carla Meotti

Tese apresentada ao Programa de Pós-Graduação em Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

MECANISMOS ENVOLVIDOS NA AÇÃO ANTINOCICEPTIVA E ANTIINFLAMATÓRIA DO FLAVONÓIDE MIRICITRINA: ESTUDOS *IN VIVO* E *IN VITRO*".

AUTORA: Flavia Carla Meotti
ORIENTADOR: Adair Roberto Soares Santos
CO-ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, maio de 2006.

O consumo de flavonóides através da dieta está positivamente relacionado com a diminuição do risco de câncer, ateroesclerose e agravamento de doenças relacionadas com inflamação e estresse oxidativo. Estes compostos são descritos, principlamente, pelas suas ações antiinflamatórias e antioxidantes. A miricitrina é um flavonóide que possui propriedades de inibir proteínas quinases, a síntese de NO entre outras. Desta forma, é de grande interesse a pesquisa destes compostos com finalidade terapêutica. No presente trabalho investigou-se as propriedades antinociceptivas e antiinflamatórias do flavonóide miricitrina, bem como os possíveis mecanismos envolvidos em tal processo. A administração sistêmica (oral ou i.p.) e central (i.c.v. ou i.t.) de miricitrina reduziu de forma dependente da dose o número de contorções abdominais induzidas pelo ácido acético. O tratamento i.p. com a miricitrina também preveniu a nocicepção induzida pela injeção i.pl. de glutamato, capsaicina e PMA, bem como, a nocicepção induzida pela injeção i.t. de glutamato, SP, TNF-α e IL-1β em camundongos. Além disso, o tratamento com miricitrina inibiu a hiperalgesia mecânica induzida pela BK, mas não aquela induzida pela epinefrina e PGE_2 em ratos. Análises de Western blot revelaram que o tratamento com miricitrina inibiu completamente a ativação da PKCα e PKCε induzida pela injeção i.pl. de PMA. A antinocicepção causada pela miricitrina no teste do ácido acético foi significativamente revertida pelo pré-tratamento dos animais com o inativador da proteína $G_{i/o}$ (toxina pertussis); com o bloqueador de canal de K^+ (glibenclamida); com o precursor de NO (L-arginina) e o $CaCl_2$. Entretanto, a antinocicepção provocada pela miricitrina não foi afetada pelo tratamento com antagonista opióide (naloxona); pelo tratamento neonatal com capsaicina (destrói 80% das fibras sensorias não mielinizadas do tipo C) e não está relacionada com uma ação sedativa, relaxante muscular ou hipotérmica do composto. Além disso, ensaios *in vitro* em fatias de córtex cerebral de ratos revelaram que a miricitrina inibe o transporte de cálcio em uma condição despolarizante, embora, quando em alta concentração, miricitrina inibe o transporte de cálcio também em condição não-despolarizante. A miricitrina reduziu a alodínia mecânica induzida pela ligadura parcial de nervo ciático (dor neuropática) e pela injeção i.pl. de FCA (dor inflamatória crônica). Este mesmo tratamento reduziu o edema de pata, as

alterações morfológicas e a atividade da MPO (enzima pró-inflamatória) na pata injetada com FCA, porém não reduziu a migração de neutrófilos ao local da inflamação. A miricitrina mostrou potente ação antioxidante frente à peroxidação lipídica induzida por Fe^{2+}. De acordo com o presente trabalho pode-se concluir que a miricitrina é dotada de atividade antinociceptiva e antiinflamatória quando avaliada em modelos de nocicepção aguda e crônica. Os mecanismos de sua ação antinociceptiva e antiinflamatória incluem a ativação de proteína $G_{i/o}$ e abertura de canais de K^+, a inibição da PKC, da síntese de NO, bloqueio do transporte de Ca^{2+}, inibição da atividade da MPO e neutralização de radicais livres.

Palavras-chave: flavonóides, miricitrina, dor aguda, dor crônica, nocicepção, inflamação, mecanismos de ação, antioxidantes.

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

MECHANISMS INVOLVED IN THE ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITY OF MYRICITRIN: *IN VIVO* AND *IN VITRO* STUDIES.

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DATE AND PLACE OF THE DEFENSE: Santa Maria, 2006

The intake of flavonoids is closely associated with diminished risks of cancer, atherosclerosis and other chronic inflammation disturbance related. These compounds are described, mainly, by their anti-inflammatory and antioxidant activities. Myricitrin is a flavonoid that inhibits protein kinases and NO production. Therefore, the research of the mechanisms by which these compounds exerts their effects is extremely interesting for the therapeutic application. In this study, the antinociceptive and anti-inflammatory activities of myricitrin as well as its mechanisms of action were investigated. The systemic (p.o. or i.p.) and central (i.c.v. or i.t.) administration of myricitrin reduced in a dose-dependent manner the visceral pain induced by acetic acid. The i.p. treatment with myricitrin also prevented nociception induced by i.pl. injection of glutamate, capsaicin, PMA and by i.t. injection of glutamate, SP, TNF-α and IL-1β in mice. In addition, myricitrin treatment inhibited mechanical hyperalgesia induced by BK, but not that induced by epinephrine or PG_{E2} in rats. Western blot analysis revealed that myricitrin treatment fully prevented PKCα and PKCε activation by PMA in mice hindpaw. The antinociception caused by myricitrin in the acetic acid test was significantly reverted by $G_{i/o}$ protein inactivation (pertussis toxin treatment); treatment with ATP-gated K^+ channel blocker (glibenclamide), $CaCl_2$ and L-arginine (NO precursor) administration. However, myricitrin-induced antinociception was modified neither by antagonist opioid (naloxone) nor by neonatal capsaicin treatment (which depletes 80% of unmyelinated C fibers). In addition, myricitrin effects were not associated with sedative and muscle-relaxant action. *In vitro* assays using slices of cerebral cortex of rats revealed that myricitrin inhibited calcium transport in a depolarizing condition; however, at higher concentration, it inhibited calcium transport also in a non-depolarizing condition. Myricitrin increased nociceptive threshold in mechanical allodynia induced by both partial sciatic nerve ligation (neuropathic pain) and FCA i.pl. injection (inflammatory chronic pain). This same treatment decreased paw edema, morphological alterations and MPO activity (proinflammatory enzyme) in FCA hindpaw. On the other hand, it did not reduce neutrophils migration to inflammation site. Myricitrin produced potent antioxidant activity when assessed by Fe^{2+}-induced lipid peroxidation. In conclusion, the present study showed that myricitrin exhibits antinociceptive and anti-inflammatory activity when analyzed in acute and chronic models of nociception. The mechanisms involved in the myricitrin beneficial effects included $G_{i/o}$ protein activation, ATP- gated K^+ channels opening, inhibition of PKC, NO synthesis, wedged of Ca^{2+} transport, inhibition of MPO activity and scavenger action.

Key words: flavonoid, myricitrin, acute pain, chronic pain, nociception, inflammation, mechanisms of action, antioxidants.

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LISTA DE ABREVIATURAS

ATP	trifosfato de adenosina
BK	Bradicinina
$CaCl_2$	cloreto de cálcio
CCK	Colecistocinina
FCA	adjuvante completo de Freund
CGRP	peptídeo relacionado ao gene da calcitonina
CI_{50}	concentração que inibe a resposta em 50%
COX	ciclooxigenase
DI_{50}	dose que inibe a resposta em 50%
EDTA	ácido etilenodiaminotetracético
EPI	Epinefrina
ERK	quinase regulada por sinal extracelular
Eros	espécies reativas de oxigênio
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.pl.	Intraplantar
i.t.	Intratecal
ICAM-1	molécula de adesão intercelular 1
IL-1α	interleucina-1 alfa
IL-1β	interleucina-1 beta
IL-6	interleucina-6
IL-8	interleucina-8
IM	inibição máxima
iNOS	sintase do óxido nítrico induzível
JNK	quinase c-*jun* N-terminal
L-NOARG	Nω-nitro-L-arginina
LOX	Lipoxigenase
MAPKs	proteínas quinases ativada por mitógenos
MIP-1β	proteína inflamatória de macrófago-1β
MPO	Mieloperoxidase
NaCl	cloreto de sódio
NF-κB	fator nuclear-κB
NK	Neurocinina
NMDA	ácido N-metil-D-aspártico

NO	óxido nítrico
NPY	neuropeptideo Y
PBS	solução salina tamponada
PG	Prostaglandina
PGE_2	prostaglandina E2
PI-3K	fosfoinositol 3-quinase
PKA	proteína quinase A
PKC	proteína quinase C
PMA	forbol 12-miristato 3-acetato
s.c.	subcutânea
SNC	sistema nervoso central
SNP	sistema nervoso periférico
SP	substância P
TNF-α	fator de necrose tumoral-alfa
TRPV1	receptor vanilóide transiente do tipo 1
v.o.	via oral

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais encontram-se no item **ARTIGOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA, DISCUSSÃO** e **CONCLUSÕES** desta tese.

1. INTRODUÇÃO

Sendo um dos principais sintomas clínicos para a detecção e avaliação de doenças, a dor exerce seu importante papel, pois atua como um mecanismo de defesa para manter a integridade do organismo. Por outro lado, a dor é um fenômeno de suprema relevância uma vez que diminui drasticamente a qualidade de vida dos portadores deste sintoma, que em alguns casos, pode tornar-se uma doença. A dor causa reações emocionais negativas e, quando persistente, torna-se debilitante e causadora de sofrimento (CHAPMAN & GAVRIN, 1999; JULIUS & BAUSBAM, 2001; GRIFFIS et al., 2006). Enquanto a dor nociceptiva (dor fisiológica) é tipicamente transitória e requer um estímulo intenso para ser desencadeada, a dor patológica (dor clínica) é frequentemente persistente e está em geral, associada à inflamação (WOOLF & MANNION, 1999; MENDELL & SAHENK, 2003).

A recente aplicação da biologia molecular à fisiologia sensorial e pesquisa da dor tem levado ao descobrimento de múltiplos mediadores envolvidos neste processo, além de um extraordinário progresso no entendimento dos mecanismos de ação dos neurotransmissores e drogas envolvidas na modulação central e periférica da dor (LEVINE & TAIWO, 1994; WOOD & DOCHERTY, 1997; MILLAN, 1999). Neste sentido, muitos esforços têm sido dedicados, buscando compreender os mecanismos celulares e moleculares envolvidos na origem da dor, principalmente,

na dor crônica, com o objetivo de encontrar drogas eficazes, com baixos efeitos colaterias e que possam ser empregadas nestas circunstâncias. De fato, atualmente não existe tratamento satisfatório e nem medidas adequadas e específicas para o controle da dor crônica (KINGERY, 1997; WOOLF & MANNION, 1999; MENDELL & SAHENK, 2003).

A manifestação neurofisiológica da dor é denominada nocicepção (TJØLSEN & HOLE, 1997; ALMEIDA et al., 2004). De uma forma geral, o estímulo nociceptivo depende da ação de mediadores que são comuns ao processo inflamatório, principalmente, em casos de dor crônica (FERREIRA & NAKAMURA, 1979; CLATWORTHY et al., 1995; KHASAR et al., 1999; BENNETT et al., 2000; JI & STRICHARTZ, 2004). Neste contexto, substâncias capazes de diminuir a condição inflamatória podem ser empregadas no tratamento contra a dor. Na verdade, a grande maioria dos fármacos presentes no mercado que são utilizados para o controle da dor, possuem um cunho antiinflamatório (MENDELL & SAHENK, 2003).

Neste sentido, vários trabalhos descrevem a potente atividade antiinflamatória dos flavonóides. Os flavonóides possuem ações sobre mediadores celulares como proteínas quinases, enzimas da cascata inflamatória e ação antioxidante, que caracterizam seu potencial antiinflamatório (MIDDLETON et al., 2000; HAVSTEEN, 2002; EDENHARDER & GRÜNHAGE, 2003; CALIXTO et al., 2003; 2004). Estes compostos são metabólitos secundários de plantas que ocorrem de forma natural e amplamente distribuída em vegetais superiores, apresentando-se em altas concentrações em frutas, vegetais, grãos, sementes, chás e vinho (RAMELET, 2000).

Além de suas ações antiinflamatória e antioxidante, os flavonóides são descritos por seus efeitos contra doenças crônicas como câncer, doenças cardiovasculares e coronarianas, diabetes, entre outras (MIDDLETON et al., 2000; BIRT et al., 2001; HAVSTEEN, 2002). As ações biológicas dos flavonóides sobre o organismo animal acontecem, principalmente, pela semelhança entre a estrutura química destes compostos e muitas moléculas inerentes à bioquímica normal de células animais, dentre elas: bases de ácidos nucléicos, coenzimas, hormônios esteróides, neurotransmissores. Esta semelhança explica, em parte, seus efeitos inibitórios sobre enzimas, ligação em receptores, indução de genes. Além disso, a alta mobilidade dos elétrons no núcleo benzóico dos flavonóides contribue para sua ação antioxidante e neutralizadora de radicais livres (RADOUCO-THOMAS et al., 1964; CASLEY-SMITH, 1976; ABATE et al., 1990; HAVSTEEN, 2002). Apesar

das várias atividades biológicas descritas para os flavonóides, pouco se sabe a respeito do efeito destas substâncias no controle da dor.

A miricitrina é um flavonóide pertencente ao subgrupo flavonol e tem sido descrita pela sua ação antioxidante, “scavenger” de radicais livres, antitumoral e anti-hipercolesterolêmica (EDENHARDER & GRÜNHAGE, 2003; YASUKAWA et al., 1990; KIMIRA et al., 1998). É encontrada principalmente em frutas de vegetais do gênero *Pouteria* (marmelo) (MA et al., 2004a) e da espécie *Manilkara zapota* (“sapodilla”) (MA et al., 2004), em folhas de plantas do gênero *Eugenia* (pitanga e jambolão) (SCHMEDA-HIRSCHMANNET et al., 1987) e látex da *Croton draco* (sangue de dragão) (TSACHEVA et al., 2004). Além disso, outros trabalhos demonstram que a miricitrina é capaz de inibir proteínas quinases como a PI-3K e PKC; inibe a ativação do NF-κB; diminui a produção de NO e a super expressão da enzima iNOS (CHEN et al., 2000).

Tendo em vista as atividades biológicas da miricitrina, é surpreendente que, até a atualidade, nenhum estudo farmacológico tenha sido realizado para investigar uma possível ação antinociceptiva e antiinflamatória deste flavonóide. Neste contexto, o presente estudo tem como objetivo investigar os efeitos da miricitrina em modelos de nocicepção aguda e crônica (através de modelos de nocicepção neuropática e inflamatória), bem como esclarecer os mecanismos pelos quais ela esteja exercendo tal efeito.

2. REVISÃO BIBLIOGRÁFICA

2.1. DOR

A sensação de dor tem papel fisiológico e funciona como um sinal de alerta para percebermos que algo está ameaçando a integridade física do organismo (CHAPMAN & GAVRIN, 1999). Neste sentido, a dor é um sintoma clinicamente importante para a detecção e avaliação de doenças, bem como, para induzir um comportamento de precaução e, consequentemente, limitar os possíveis danos (MILLAN, 1999; WOOLF, 2000; ALMEIDA et al., 2004). A dor foi definida pela Associação Internacional para o Estudo da Dor (IASP) como sendo "uma experiência emocional e sensorial desagradável associada com uma lesão tecidual real ou potencial ou descrita em termos de tal lesão".

Já está bem estabelecido que a dor é uma experiência complexa e envolve não apenas a transdução de estímulo nocivo ambiental, mas também o processamento cognitivo e emocional pelo encéfalo (CHAPMAN & GAVRIN, 1999; JULIUS & BAUSBAM, 2001; ALMEIDA et al., 2004). ALMEIDA e colaboradores (2004) descrevem que, enquanto o sistema sensorial permite a localização espaço-temporal, a qualificação física e a intensidade do estímulo nocivo, o componente cognitivo afetivo atribui emoções à experiência, sendo responsável pelas respostas comportamentais à dor.

Em termos de duração, uma sensação dolorosa pode ser transitória, aguda ou crônica. No tipo transitória, a ativação dos nociceptores é feita na ausência de qualquer dano tecidual. Na dor aguda, ocorre geralmente lesão e ativação dos nociceptores no sítio lesionado. Já a dor crônica é causada geralmente por uma lesão ou patologia, sendo que com o passar do tempo, pode ser perpetuada por fatores que não os causadores iniciais da dor (LOESER & MELZACK, 1999; WOOLF & MANNION, 1999; ZIMMERMANN, 2001; MENDELL & SAHENK, 2003).

O componente sensorial da dor denomina-se nocicepção (TJØLSEN & HOLE, 1997). Assim, enquanto a dor envolve a percepção de um estímulo aversivo, a nocicepção refere-se às manifestações neurofisiológicas geradas pelo estímulo nocivo (TJØLSEN & HOLE, 1997; ALMEIDA et al., 2004). A função de alerta da dor reflete a ativação fásica de sensores denominados nociceptores, os quais são sensiblizados quando o estímulo é potencialmente perigoso, ou seja, excedem uma determinada faixa considerada fisiológica (estímulo inócuo) (BURGESS & PERL, 1967; MILLAN, 1999).

Os nociceptores estão localizados na porção distal dos neurônios aferentes sensoriais que estão amplamente distribuídos na pele, vasos, músculos, articulações e vísceras e são sensíveis à estímulos térmicos, mecânicos ou químicos (JULIUS & BASBAUM, 2001). Existem ainda os chamados nociceptores silenciosos ("silent" ou "sleeping"), que são uma pequena proporção das fibras aferentes, os quais normalmente não são responsivos a estímulos.

Entretanto, quando influenciados por mediadores inflamatórios, ou após a administração de agentes flogísticos, apresentam atividade espontânea ou tornam-se sensibilizados e respondem a estímulos sensoriais (JULIUS & BASBAUM, 2001).

A sensibilização dos nociceptores ocorrida, por exemplo, em casos de mudança de temperatura (estímulo nocivo térmico), diferença osmótica ou distensão do tecido (estímulo nocivo mecânico), hipóxia ou lesão tecidual seguida de inflamação (estímulo nocivo químico), resulta na liberação local de mediadores químicos tais como bradicinina, prótons, serotonina, histamina, metabólitos do ácido araquidônico, ATP, adenosina, citocinas, aminoácidos excitatórios, SP, NO, NPY, CCK, neurotrofinas, bombesina, CGRP, opióides, somatostatina, acetilcolina entre outros (JULIUS e BASBAUM, 2001; GRIFFIS et al., 2006). Estes mediadores interagem com receptores específicos, levando a uma propagação do sinal nociceptivo graças a um aumento na permeabilidade da membrana neuronal e consequente geração do potencial de ação (CARLTON & COGGESHALL, 1998; RAJA et al., 1999; PASERO et al., 1999). É importante ressaltar que estes mediadores podem ser liberados não somente pelos neurônios sensoriais, mas também por fibras simpáticas e por células não neuronais como plaquetas, células endoteliais, fibroblastos, células de Schwann e células inflamatórias (BESSON, 1997).

A estimulação dos nociceptores periféricos faz com que a informação nociceptiva seja levada através das fibras aferentes ao SNC. Os longos axônios das fibras nociceptivas que se localizam em nervos periféricos estendem-se de seus corpos celulares contidos em uma estrutura denominada gânglio da raiz dorsal. Após emergir de seu corpo celular, o axônio aferente primário bifurca-se para enviar prolongamentos à medula espinhal e outro para inervar os tecidos corporais (MILLAN, 1999).

As fibras aferentes primárias (neurônios de primeria ordem) estão classificadas conforme sua estrutura, diâmetro e velocidade de condução do estímulo. As fibras Aβ são mielinizadas, com diâmetro maior que 10 μm e velocidade de condução de 30-100 m/s. As fibras Aδ são pouco mielinizadas, variando seu diâmetro entre 2-6 μm, com velocidade de condução de 12-30 m/s. Fibras não-mielinizadas do tipo C possuem diâmetro entre 0,4-1,2 μm e velocidade de condução de 0,5-2 m/s (SHELLEY & CROSS, 1994; MILLAN, 1999; JULIUS e BASBAUM, 2001). Neurônios com corpos celulares de maior diâmetro dão origem à fibras sensoriais do tipo Aβ que detectam estímulos inócuos aplicados à pele, músculos e articulações, não contribuindo para a nocicepção. Entretanto, a estimulação dessas fibras pode aliviar a dor. Isto é o que ocorre, por exemplo, quando elas são ativadas por fricção da pele pela mão após alguma lesão (SHELLEY & CROSS, 1994; MILLAN, 1999; JULIUS & BASBAUM, 2001).

Os corpos celulares de pequeno e médio diâmetro dão origem à maioria dos nociceptores, incluindo fibras C e do tipo Aδ. As fibras do tipo Aδ podem ser classificadas em fibras Aδ do tipo I, respondem a estímulos de calor intenso (até 53°C) e fibras Aδ do tipo II respondem a temperaturas menores que 43°C. A maioria das fibras C são polimodais, sendo ativadas por estímulos nocivos

mecânicos ou térmicos e ainda por estímulos nocivos de origem química como ácidos e capsaicina (SHELLEY & CROSS, 1994; MILLAN, 1999; JULIUS & BASBAUM, 2001).

O corno dorsal da medula espinhal funciona como uma estação relê para a transmissão da dor. Os nociceptores chegam de maneira altamente organizada no corno dorsal. As fibras aferentes primárias C e Aδ têm suas terminações principalmente nas lâminas mais superficiais: lâmina I (zona marginal) e lâmina II (substância gelatinosa) (BESSON & CHAOUCH, 1987). Além disso, a informação nociceptiva se projeta do corno dorsal da medula para o tálamo através de neurônios de segunda ordem, ou neurônios sensoriais secundários, que compreendem as vias ascendentes. Os neurônios sensoriais secundários recebem seus sinais sensoriais pela liberação de glutamato e SP dos neurônios aferentes primários; além disso, este processo excitatório também depende de canais de cálcio e sódio, sendo os canais de cálcio os principais reguladores da liberação de neurotransmissores (HILL, 2001).

Após a interação direta ou indireta com neurônios de projeção no corno dorsal, os axônios de neurônois de segunda ordem formam tratos aferentes que transmitem os impulsos nociceptivos para estruturas do tronco cerebral e diencéfalo, incluindo tálamo, substância cinzenta periaquedutal, região parabraquial, formação reticular da medula, complexo amigdalóide, núcleo septal, hipotálamo, entre outras (ALMEIDA et al., 2004). As vias ascendentes denominadas monossinápticas projetam-se diretamente para centros cerebrais superiores (trato espinomesencefálico, espinoparabraquial, espinotalâmico e espinorreticular). As vias polissinápticas (via pós-sináptica da coluna dorsal e do trato espinocervical) possuem uma estação relê de neurônois de segunda ordem antes de projetarem aos centros cerebrais superiores (MILLAN, 1999). O tálamo desempenha um papel fundamental na integração do impulso de dor. A partir do tálamo, neurônios de terceira ordem transmitem impulsos para o cótex cerebral, onde ocorre o processamento que resulta em consciência da dor (FÜRST, 1999).

Neste sentido, o organismo também possui mecanismos intrínsecos para o controle da dor, pois após a estimulação de diferentes núcleos do tálamo, os sinais são transmitidos para diversas áreas do córtex sensorial somático, substância cinzenta periaquedutal, hipotálamo, amígdala e cerebelo. Um circuito modulador endógeno descendente conectando a substância cinzenta periaquedutal, mais especificamente o núcleo magno da rafe e estruturas adjacentes da medula rostral ventromedial e o corno dorsal da medula é responsável pela ativação de conexões que promovem inibição ou facilitação da nocicepção. Entretanto, o sistema de controle descendente não é simplesmente inibitório ou facilitatório. Cada forma de lesão induz um tipo diferente de plasticidade sobre o sistema, fenômeno que acontece em casos de dores crônicas principalmente. Os sistemas de neurotransmissão mais amplamente estudados nesta conexão é o sistema glutamatérgico, GABAérgico, neuropeptídérgico, serotoninérgico, opioidérgico e noradrenérgico (MILLAN, 2002; REN & DUBNER, 2002; VANEGAS & SCHAIBLE, 2004).

2.1.1. Dor devido a um processo inflamatório

Nos casos de lesão tecidual, como por exemplo, em traumas mecânicos ou invasão por agentes infecciosos, o organismo aciona mecanismos cujo propósito é limitar o dano e auxiliar a regeneração. Esses mecanismos fazem parte da resposta inflamatória, que é caracterizada por quatro sinais cardinais: dor, rubor, calor e tumor; em alguns casos pode acometer o membro/órgão com perda da função (GALLIN et al., 1982). A inflamação é essencial para a manutenção da saúde dos indivíduos, pois é a maneira pela qual o organismo reage contra agentes potencialmente danosos como bactérias, vírus e outros patógenos (HERSH et al., 1998).

A inflamação envolve, entre outros, a ação do sistema complemento, sistema de coagulação, resposta imunológica humoral e celular, citocinas, hormônios, angiogênese e processos de reparo. Um importante mecanismo na inflamação é o recrutamento de macrófagos para atuar contra microorganismos invasores ou suas toxinas (HAVSTEEN, 2002). Estes agentes inflamatórios são reconhecidos por anticorpos específicos presentes na superfície dos macrófagos. Após este reconhecimento, ocorre uma intensa produção de radicais livres, com intuito de matar a célula invasora (HAMPTON et al., 1998; MacMAHON et al., 2005). Além disso, este processo libera citocinas, as quais promovem a propragação da resposta. Dentre outras ações, as citocinas induzem a expressão de genes de enzimas envolvidas no processo inflamatório, como a COX (MASFERRER et al., 1994).

A formação de prostaglandina pela COX é um dos responsáveis pela sensação de dor (MASFERRER et al., 1994). Além deste metabólito do ácido araquidônico, outros mediadores inflamatórios como a histamina, serotonina, cininas, citocinas, neuropeptídeos, aminoácidos excitatórios, prótons, neurotrofinas, ATP, NO, opióides, entre outros (JULIUS e BASBAUM, 2001; MacMAHON et al., 2005; GRIFFIS et al., 2006) oriundos do sangue, ou de células do tecido lesado, células adjacentes ou de células inflamatórias contribuem para a transmissão da nocicepção, bem como, para a inflamação e processo de recuperação.

Após a injúria tecidual ou após a invasão do tecido por microorganismos e, consequente reconhecimento do antígeno, mediadores primários específicos são liberados e se ligam aos seus receptores de membrana (FERREIRA & NAKAMURA, 1979; NAKAMURA & FERREIRA, 1987; KHASAR et al., 1999). Em decorrência desta ligação ocorre ativação da cascata de sinalização intracelular, através de segundos mensageiros. A ativação de quinases, lipases, oxigenases, peroxidases, entre outras enzimas, resulta na síntese de mediadores a partir de lipídeos de membrana, na abertura de canais iônicos, ou ainda, na ativação de fatores de transcrição gênica, os quais são translocados ao núcleo e se ligam ao gene promotor, iniciando a síntese de outros mediadores, dentre eles, citocinas e fatores quimiotáticos (KETTLE & WINTERBOURN, 1991; CARTER et al., 1999; LEVINE & REICHLING, 1999; KITAURA et al., 2000; XAGORARI et al., 2002; CALIXTO et al., 2003; 2004). Nas células inflamatórias este mecanismo acontece como um

sistema de amplificação de sinal e recrutamento de células até que o agente causador do dano seja eliminado e então, inicia-se a recuperação do tecido.

Tanto a liberação de mediadores primários quanto a síntese de novos mediadores são responsáveis pela ativação e/ou sensibilização de nociceptores adjacentes à lesão. A sensibilização dos nociceptores diminui o limiar de ativação e aumenta a probabilidade de que estes disparem com estímulos de menor intensidade (FERREIRA & NAKAMURA, 1979; NAKAMURA & FERREIRA, 1987; KHASAR et al., 1999; COUTAUX et al., 2005; HOLDEN & PIZZI, 2003). Desta forma, estes nociceptores são ativados por estímulos que em condições normais seriam inócuos (FINNERUP & JENSEN, 2004; MILLAN, 1999). Isto é bem evidenciado através de modelos experimentais, onde a injeção de um agente pró-inflamatório como o FCA sensibiliza os locais injetados, tornando estes animais responsivos a estímulos térmicos e mecânicos inócuos a um animal não injetado (LARSON et al., 1986). De fato, a dor é uma característica peculiar da inflamação e a dor inflamatória é o maior problema clínico em vários distúrbios inflamatórios, como, por exemplo, a artrite reumatóide (MacMAHON et al., 2005).

Portanto, nestas situações encontra-se um quadro de estimulação constante dos nociceptores, a qual é reponsável por alterações plásticas, não somente no tecido nervoso periférico, mas também em nível central; isto é muito comum em casos de neuropatia periférica, onde a lesão nervosa gera um processo inflamatório crônico, com alterações plásticas no SNP e SNC (MacFARLANE et al., 1997; JI & WOOLF, 2001; WOOLF & SALTER, 2000; CODERRE et al., 1993; JI & STRICHARTZ, 2004). De fato, do ponto de vista clínico, um dos aspectos mais problemáticos da dor de origem inflamatória é a possibilidade da progressão de um estado agudo para um estado prolongado, podendo desta forma, aumentar a susceptibilidade de instalação de um quadro de dor inflamatória crônica (WOOLF & MANNION, 1999; MENDELL & SAHENK, 2003).

Nestas condições, a inflamação perde sua característica de proteção ao organismo e torna-se uma patologia. A grande preocupação na busca dos mecanismos que envolvem a inflamação é devido ao seu envolvimento em doenças crônicas, incluindo câncer, diabetes, doenças neurodegenerativas, cardiovasculares e reumáticas (NAGRA et al., 1997; MIDDLETON et al., 2000; HAVSTEEN, 2002; KAMIYA et al., 2003; MacMAHON et al., 2005; GARCIA, 2005).

2.1.2. Dor neuropática

Diferentemente da dor nociceptiva (dor aguda), a dor neuropática (dor crônica) não possui função biológica protetora. Mais do que um sintoma, a dor neuropática é a própria doença, pois a sensação de dor é interminável, podendo durar anos ou décadas após a injúria inicial. Pode ser refratária a múltiplos tratamentos e, frequentemente, associada à ansiedade, medo, depressão e insônia; na maioria das vezes é debilitante, incapacitando o paciente de realizar ações do cotidiano (CHAPMAN & GAVRIN, 1999; WOOLF & MANNION, 1999; MENDELL & SAHENK, 2003). A dor neuropática foi definida em 1994 pela IASP como a "dor iniciada ou causada por lesão primária ou disfunção do sistema nervoso". A etiologia da dor neuropática é heterogênea e pode ser devida a um insulto primário ao sistema nervoso periférico ou central (ZIMMERMANN, 2001). As neuropatias originam-se quando ocorre uma lesão nos nervos ou nas demais estruturas que transmitem a sensação dolorosa, e podem resultar de trauma mecânico, lesão nervosa (amputação ou compressão), efeitos tóxicos de drogas, doenças como diabetes ou síndrome da imunodeficiência adquirida (HIV/AIDS) (MENDELL & SAHENK, 2003).

A principal sintomatologia clínica das neuropatias periféricas é dor espontânea ou hipersensibilidade da área afetada, causando um fênomeno conhecido como hiperalgesia (reposta aumentada à dor após um estímulo nocivo) e/ou alodínia (sensação de dor frente a um estímulo que seria inócuo) (FINNERUP & JENSEN, 2004); isto se deve, principalmente às alterações ocorridas na medula espinhal (CODERRE et al., 1993; MacFARLANE et al., 1997; JI & WOOLF, 2001; WOOLF & SALTER, 2000). Em algumas situações a dor neuropática pode estender-se além dos limites dos nervos lesados, manifestando-se bilateralmente, o que sugere alterações nos mecanismos centrais (CODERRE et al., 1993).

Os mecanismos exatos da instalação do quadro de dor neuropática ainda não são inteiramente compreendidos. No entanto, MacFARLANE e colaboradores (1997) sugerem que o desenvolvimento de dor crônica após lesão de nervo ocorra através de alterações na medula espinhal, como excitabilidade aumentada, inibição diminuída, restruturação organizacional das células e, eventualmente, mudança no fenótipo. Estas alterações ocorrem principalmente devido a uma estimulação excessiva dos nociceptores, uma vez que estes estão com limiar de ativação mais baixo (hipersensibilidade) (COUTAUX et al., 2004).

A excitabilidade aumentada ocorre em função de uma estimulação repetitiva sobre as fibras não mielinizadas do tipo C, o que resulta em uma prolongada descarga no corno dorsal da medula espinhal. Este fenômeno é conhecido como "Wind up" (DAVIES & LODGE, 1987; HERRERO et al., 2000) e significa um aumento progressivo no número de pontenciais de ação por estímulo que ocorre em neurônios do corno dorsal. Estes episódios repetitivos de "wind up" podem levar à potenciação a longo prazo (long-term potentiation, LTP), a qual envolve um aumento prolongado na transmissão sináptica. Ambos "wind up" e LTP fazem parte do processo de sensibilização envolvido na maioria dos estados de dor crônica (POCKETT, 1995).

Este fenômeno está associado, principalmente, a um aumento da resposta de receptores glutamatérgicos e os receptores das taquicininas (NK-1 e NK-2) (OTSUKA & YOSHIOKA, 1993; LI & SANDKUHLER, 1997; HERRERO et al., 2000). Além disso, a fosforilação da PKC está intimamente relacionada com o fenômeno de sensibilização central, uma vez que é o principal segundo mensageiro acionado pela ativação dos receptores sensíveis às taquicininas (CHEN & HUANG, 1992; OTSUKA & YOSHIOKA, 1993).

Além de um aumento na excitabilidade neuronal, outra alteração na medula espinhal responsável pela dor crônica é a diminuição da neurotransmissão inibitória, principalmente no que se refere à neurotransmissão GABAérgica (IBUKI et al., 1997; MALAN et al., 2002; WOOLF, 2004). Outro mecanismo que contribui para a diminuição da inibição é a eficácia reduzida dos opióides endógenos (ZHANG et al., 1998).

Após a lesão de nervo periférico há uma mudança drástica nos níveis de transcrição de algumas substâncias, incluindo citocinas, enzimas da síntese de prostaglandinas e síntese de NO, outro fenômeno que ocorre é a expansão das fibras Aβ (LEKAN et al., 1996; RAMER et al., 1999; COSTIGAN et al., 2002; XIAO et al., 2002; HOLDEN & PIZZI, 2003). Em modelos experimentais de neuropatia periférica estas fibras invadem a lâmina II do corno dorsal da medula, tornando estas fibras pró-nociceptivas (LEKAN et al., 1996; RAMER et al., 1999).

A lesão de nervos periféricos é frequentemente acompanhada de inflamação local transitória, a qual contribui para o início da sensação de dor. Neste sentido, assim como na dor inflamatória, na dor neuropática estão envolvidos múltiplos mediadores inflamatórios (CLATWORTHY et al., 1995; BENNETT et al., 2000; JI & STRICHARTZ, 2004;). Corroborando com esta afirmação, estudos mostram que a extensão da hiperalgesia está diretamente relacionada com a extensão da resposta inflamatória ao sítio da lesão do nervo (CLATWORTHY et al., 1995; TRACEY & WALKER, 1995). Além disso, drogas antiinflamatórias aliviam a hiperalgesia em modelos animais de injúria do nervo (CLATWORTHY et al., 1995; BENNETT et al., 2000).

Na dor neuropática, assim como acontece no processo inflamatório, a ação dos mediadores sobre seus receptores, tanto em nervos periféricos quanto centrais, inicia uma cascata de sinalização que culmina na manutenção do potencial de ação. Por ser um evento crônico, a principal característica desta patologia são mudanças plásticas causadas por alterações na expressão gênica de receptores, canais iônicos, proteínas intracelulares, neuromoduladores, mediadores de sinalização extracelular, entre outros (WOOLF & COSTIGAN, 1999; XIAO et al., 2002; COSTIGAN et al., 2002; JI & STRACHARTZ, 2004; CALIXTO et al., 2004a; FERREIRA et al., 2005; QUINTÃO et al., 2005).

Assim, os mecanismos patofisiológicos da dor neuropática refletem, em grande parte, aqueles ocorridos na inflamação, embora, alguns autores relatem que a lesão de nervo produz, em neurônios aferentes primários, alterações neuroquímicas ligeiramente distintas daquelas

produzidas na inflamação (HOKFELT et al., 1994; WOOLF & COSTIGAN, 1999; YAJIMA et al., 2003).

2.2. ATIVIDADES BIOLÓGICAS DOS FLAVONÓIDES

Flavonóides são contituintes essenciais das células de vegetais superiores. Devido a sua coloração e odor, são responsáveis pela comunicação do vegetal com o ambiente, atraindo agentes polinizadores como pássaros, insetos e outros animais (HARBORNE, 1986; 1988). Flavonóides também apresentam propriedades regulatórias semelhantes às vitaminas lipossolúveis, interferem no metabolismo celular e atuam juntamente com hormônios regulando o crescimento do vegetal (FRAGNER, 1964; GROTEWELD et al., 1994; JIANG et al., 1999). Estes compostos interferem ainda na transferência de elétrons durante a fosforilação oxidativa que ocorre no cloroplasto e possuem um importante papel na fixação do nitrogênio (CANTLEY & HAMMES, 1976; MUKOHATA et al., 1978; MORTENSON & THORNELEY, 1979).

Em mamíferos, os flavonóides ocorrem somente através da dieta. Estes compostos estão presentes em frutas, vegetais, grãos, sementes, chás e vinho. Em indivíduos saudáveis, estima-se uma média de consumo de 1–2 g de flavonóides por dia. Apesar de o homem há várias décadas associar saúde com dieta de produtos de origem vegetal e usar ervas medicinais para o tratamento de algumas doenças, somente há poucos anos as propriedades bioquímicas e farmacológicas dos flavonóides começaram a ser desvendadas (HAVSTEEN, 2002).

Flavonóides são gamma-benzopironas e constituem uma família de compostos com pequenas variações em sua estrutura química. Alguns deles estão ligados a um monossacarídeo (maior parte encontrada na natureza), aumentando sua solubilidade em água. A porção aglicona (sem o monossacarídeo) constitui-se de difenilpropanos (C6-C3-C6) e está ilustrada na figura 1 (HARBORN, 1989). O grupo compreende antocianidinas, flavonol, isoflavonol, flavona, isoflavona, flavana, isoflavana, flavanona, flavanol, isoflavanol, entre outros (HARBORNE, 1967; CROFT, 1998). Assim, estes compostos compartilham a mesma estrutura primária e, conseqüentemente, compartilham algumas atividades biológicas; embora, devido a certas modificações estruturais, podem apresentar propriedades distintas (HAVSTEEN, 2002).

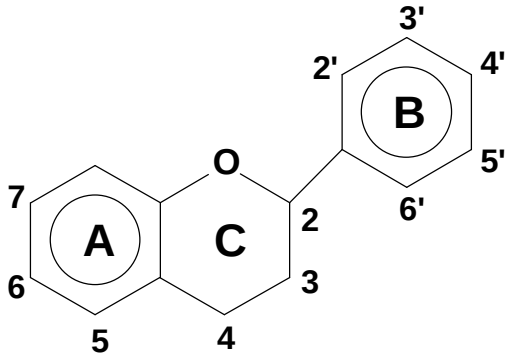

Figura 1: Estrutura química base (difenilpropano) dos flavonóides.

2.2.1. Biodisponibilidade de flavonóides oriundos da dieta

A absorção intestinal e metabolismo de flavonóides da dieta ainda não estão bem estabelecidos, pois uma série de divergências a respeito deste processo é encontrada na literatura. A grande maioria dos estudos encontrados na literatura trata da biodisponibilidade da quercitina, provavelmente, pelo grande número de atividades biológicas deste flavonóide (PODHAJCER et al., 1980; MANACH et al., 1998; HOLLMAN et al., 1999; GRAEFE et al., 1999; YAMAMOTO et al., 1999; AZIZ et al., 1998; WALLE et al., 2001).

A maior parte dos flavonóides encontrada na natureza está sob a forma glicosilada, poucas quantidades são encontradas como aglicona, uma vez que esta é mais instável; sendo assim, a forma glicosilada é a mais comumente ingerida na dieta (WALLE, 2004; YOKOMIZO & MORIWAKI, 2005). Quando ingeridos, os flavonóides chegam ao lúmen intestinal e, parte sofre ação de β-glicosidases de bactérias intestinais, liberando a molécula de monossacarídeo. A porção aglicona pode ser absorvida com as micelas biliares nas células epitelias e então, passar para a linfa (DAI et al., 1997; SPENCER et al., 1999); ou podem atravessar a membrana do enterócito através de difusão passiva (MUROTA & TERAO, 2003). Os flavonóides que não sofrerem hidrólise serão absorvidos para o interior do enterócito através dos transportadores de glicose dependentes de sódio (SGLT1) (MUROTA & TERAO, 2003) e, ao atingirem o enterócito, sofrem ação das β-glicosidases contidas nesta célula (WALLE, 2004). Posteriormente, as agliconas dos flavonóides são conjugadas, ainda no enterócito, pela UDP-glicuroniltransferase, sulfotransferase ou metiladas pela catecol-*O*-metiltransferase (PISKULA & TERAO, 1998; CRESPY et al., 1999; DE SANTI et al., 2000; MUROTA &TERAO, 2003). Quando não conjugados no enterócito, os flavonóides são conjugados no fígado (MUROTA & TERAO, 2003) ou circulam no sangue ligados à albumina (PODHAJCER et al., 1980).

É importante mencionar que para alguns flavonóides a conjugação pode diminuir as propriedades farmacológicas da molécula (MANACH et al., 1998). No caso da quercitina, a glicuronidação no carbono 4', o qual consiste no grupo catecol (quercetina 4'-*O*-β-glicuronídeo) reduz drasticamente a atividade antioxidante da molécula, enquanto a glicuronidação no carbono 3 do grupo propano (quercetina 3-*O*-β-glicuronídeo) não altera esta propriedade (YAMAMOTO et al., 1999; WALLE, 2004).

Os flavonóides conjugados podem atravessar as membranas celulares através dos transportadores associados a proteínas de resistência a multidrogas (MRP) (WALLE, 2004). A conjugação serve como um sistema de transporte na corrente sangüínea, uma vez que os flavonóides são pouco solúveis em água (MUROTA & TERAO, 2003). O'LEARY e colaboradores (2001) demonstraram que células de hepatoma (HepG2), células do intestino, fígado e neutrófilos são capazes de clivar a quercetina e o ácido glicurônico, liberando a quercetina aglicona no tecido

(MUROTA & TERAO, 2003). Além disso, SHIMOI e colaboradores (2001) sugerem que durante a inflamação há um aumento da liberação da aglicona do seu conjugado, através do aumento da atividade da β-glicuronidase, um evento que acompanha a inflamação.

O pico de concentração plasmática para flavonóides como a quercetina ocorre 1,5 a 2 horas após a ingestão (AZIZ et al., 1998) e a meia-vida de eliminação plasmática é cerca de 22 horas (HOLLMAN et al., 1999); embora alguns autores relatem que a meia-vida de eliminação plasmática possa chegar a 72 horas, provavelmente, pela recirculação entero-hepática (WALLE, 2004). Os flavonóides são degradados a ácidos orgânicos (ácido cafeico e cinâmico, entre outros) e então, excretado com a urina (GRAEFE et al., 1999; BOURNE & RICE-EVANS, 1999). Estudos com a quercetina [^{14}C] marcada radiotivamente, mostram que o CO_2 expirado através dos pulmões é o maior metabólito deste flavonóide (WALLE et al., 2001).

A baixa solubilidade dos flavonóides frequentemente apresenta um problema para a aplicação médica destas substâncias. Neste sentido, o desenvolvimento semi-sintético de formas solúveis na água tem sido um importante avanço na medicina. As formas apresentadas são hidroxietilrutosídeos e inositol-2-fosfatoquercetina, os quais são aplicados no tratamento de hipertensão e microsangramentos (CALIAS et al., 1996).

2.2.2. Atividade antioxidante e neutralizadora de radicais livres

Os flavonóides são compostos fenólicos e, desta forma, estão propensos à oxidação formando quinonas. Uma das propriedades mais discutidas dos flavonóides é a sua habilidade de neutralizar radicais livres (AFANAS'EV et al., 1989; HAENEN et al., 1997; PIETTA, 2000; EDENHARDER & GRÜNHAGE, 2003; KOSTYUK et al., 2003; 2004; YOKOMIZO & MORIWAKI, 2005; SAWAI et al., 2005).

Radicais livres são substâncias que carregam um elétron desemparelhado. Estas espécies são altamente reativas e aumentam no curso de muitos processos fisiológicos, especialmente, cadeia respiratória, pela redução parcial do oxigênio, e oxidações catalizadas pelas oxigenases. Estas reações são muito comuns, uma vez que, o oxigênio é um ótimo aceptador de elétrons, formando as denominadas espécies reativas de oxigênio (EROs) (PRATICÒ & DELANTY, 2000).

As EROs incluem radicais livres, como radicais hidroxil ($OH^{\bullet}$) e ânion superóxido ($O_2^{\bullet -}$), dióxido de nitrogênio ($NO_2^{\bullet}$); bem como moléculas não radicalares como o peróxido de hidrogênio (H_2O_2), peroxinitrito ($ONOO^-$); ácido hipocloroso ($HOCl^-$), ânion hidroxila (HO^-), entre outros. Estas moléculas possuem uma grande reatividade e podem levar à lipoperoxidação, oxidação de carboidratos, proteínas e DNA (PRATICÒ & DELANTY, 2000).

A sobrevivência da célula frente à toxicidade dos radicais livres no curso normal do metabolismo celular se dá graças à ação dos antioxidantes endógenos. Os antioxidantes são substâncias que direta ou indiretamente protegem os sistemas celulares dos efeitos tóxicos

produzidos por radicais oxidativos (HALLIWELL, 1995). Compostos com ação biológica e função antioxidante podem ser moléculas protéicas com grupos tiólicos como as metalotioneínas, enzimas glutationa peroxidase (GSH-Px), superóxido dismutase e catalase, moléculas não-protéicas como a glutationa e substâncias exógenas como a vitamina C e os flavonóides () (KRISHNA et al., 1996; EVANS et al., 1997; McKENZIE et al., 1998; HALLIWELL, 1999; EDENHARDER & GRUNHAGE, 2003; HAWSE et al., 2006). Assim, sob condições normais, os sistemas antioxidantes celulares minimizam os danos causados pelas EROs, porém, quando a produção de radicais livres excede a capacidade protetora da célula tem-se o estresse oxidativo.

O estresse oxidativo está relacionado com uma série de patologias, uma vez que, alterações no equilíbrio redox podem ser tóxicas, e determinantes do tempo de vida celular (PARKES et al., 1998). Os metabólitos das EROs estão associados a processos neurodegenerativos e outras desordens como câncer, catarata, isquemia, enfisema pulmonar, diabetes mellitus, envelhecimento precoce e cirrose hepática (FUJITA et al., 1988; COHEN, 1989; HALLIWELL & GUTTERIDGE, 1990; FLOYD, 1990; BOHM et al., 1998; HAVSTEEN, 2002).

Outra situação em que há grande produção de EROs constituem as reações inflamatórias. Nestas condições, macrófagos e neutrófilos produzem EROs como primeira linha de defesa contra bactérias, vírus, metástases e demais partículas estranhas ao organismo (FUJITA et al., 1988; KUJUMGIEV et al., 1999; LIMASSET et al., 1999; HAMPTON et al., 1998). A produção de radicais livres também acelera a formação de eicosanóides e citocinas, exacerbando a resposta inflamatória. Esta reação é importante como mecanismo de defesa, porém, um excesso na formação de EROs pode levar à oxidação e perda da função de moléculas essenciais, como grupos sulfidrílicos de proteínas e enzimas, lípideos de membrana, ácidos nucléicos (HADDAD, 2002).

Nestas condições em que as alterações no *status oxidativo* implicam lesão tecidual e agravamento da patologia, antioxidantes exógenos e substâncias capazes de neutralizar radicais livres, como os flavonóides, podem ser aplicados com sucesso a fim de minizar os danos. Flavonóides são fáceis e irreversivelmente oxidados a ρ-hidroquinona, a qual, em uma reação reversível, é oxidada a ρ-quinona. Este último é facilmente polimerizado e posterirmente excretado. Então, os flavonóides são inativados por oxidação, o que justifica seu alto potencial antioxidante e baixo potencial pró-oxidante; além disso, estes compostos perdem elétrons mais facilmente do que ganham (FUJITA et al., 1988; LAUGHTON et al., 1989; AFANAS'EV et al., 1989; JÖRGENSEN et al., 1998; BOHM et al., 1998; CAI et al., 1999; HAVSTEEN, 2002).

Flavonóides são capazes de proteger ácidos graxos insaturados de membranas celulares e antioxidantes endógenos da oxidação (ZLOCH & SIDLOVA, 1977; ZLOCH & GINTER, 1979; BOHM et al., 1998; PIETTA, 2000; KOSTYUK et al., 2003; YOKOMIZO & MORIWAKI, 2005) e suprimem a produção de $ONOO^-$, o qual é um dos maiores causadores de danos oxidativos em situações de sepse (HAENEN et al., 1997).

Além de suas propriedades antioxidantes e neutralizadoras de radicais livres, os flavonóides também possuem alta capacidade de remover íons de metais pesados com potencial de oxi-redução. Então, esta propriedade também confere proteção à toxicidade das EROs, uma vez que a formação de radicais livres e oxidação de biomoléculas é catalisada por íons de metais pesados (AFANAS'EV et al., 1989; HALLIWELL & GUTTERIDGE, 1990; BROWN et al., 1998). Interessantemente, esta propriedade quelante de metais torna alguns flavonóides aptos a neutralizarem espécies radicalares usando o próprio metal como agente catalítico. Esta habilidade foi descrita há pouco tempo e os autores descrevem-na como uma atividade mimética da enzima superóxido dismutase (KOSTYUK et al., 2004).

2.2.3. Atividade antiinflamatória

Outro exemplo de aplicação terapêutica dos flavonóides é na inflamação. Estes compostos são descritos por suas propriedades antiinflamatórias *in vitro* e *in vivo*, incluindo processos inflamatórios crônicos (VAN CAUWENBERGE & FRANCHIMONT, 1968; SANCHEZ de MEDINA ET AL., 1996; BOHM et al., 1998; MIDDLETON et al., 2000; HAVSTEEN, 2002). Flavonóides são excelentes inibidores da inflamação por exterminar radicais livres e por regular a produção de eicosanóides (FUJITA et al., 1988; BOHM et al., 1998; MIDDLETON et al., 2000; CHEN et al., 2000; RASO et al., 2001; HAVSTEEN, 2002).

Os flavonóides possuem ação imunomodulatória (GAO et al., 2001) e vêm sendo empregados no tratamento de doenças alérgicas do trato respiratório há várias décadas (FROSTAD, 1977; BENNETT et al., 1981). Desde 1930, o flavonóide sintético cromoglicato disódico foi introduzido no mercado com o nome “Intal” na forma de solução nasal para o tratamento profilático de rinite alérgica e em casos de asma crônica (FROSTAD, 1977; Havsteen, 2002). A ação antialérgica dos flavonóides é atribuída à capacidade destes compostos de inibir a síntese de prostaglandinas e o transporte de cálcio em mastócitos, inibindo a secreção de serotonina e histamina (FEWTRELL & GOMPERTS, 1977, 1977a; BENNETT et al., 1981; CHEN et al., 2000; RASO et al., 2001).

Durante a inflamação, os flavonóides evitam a degradação do tecido conjuntivo devido sua ação neutralizadora de radicais livres. Além disso, alguns flavonóides possuem ação sobre a hialuronidase, uma enzima que, durante o processo inflamatório hidrolisa gliconas do tecido conjuntivo. Embora a ação desta enzima seja importante para iniciar o processo de reparo do tecido, ela rompe a integridade do mesmo, tornando-o mais susceptível à invasão pelo infectante (HASATO et al., 1979; LI et al., 1997). Flavonóides também inibem outras metaloproteinases da matriz, como a elastase de neutrófilos, a qual está intimamente relacionada com o processo inflamatório (SARTOR et al., 2002). A inibição das hialuronidases pelos flavonóides acontece porque esses polifenóis se ligam ao sítio ativo da enzima, competindo com o substrato. No caso das colagenases a inibição pode se dar pela complexação com o metal no sítio catalítico da enzima, prevenindo a formação do estado de transição (CETTA et al., 1978; KUTTAN et al., 1981; FINE et al., 1992; DOWD et al., 1995).

Os flavonóides também apresentam influência sobre a atividade de proteases e fosfolipases. Os produtos da hidrólise destas enzimas são segundos mensageiros envolvidos em muitos processos celulares, dentre eles aqueles envolvidos na resposta inflamatória (KYO et al., 1998; MANTLE et al., 1999).

Estas moléculas atuam tanto na inibição de algumas enzimas envolvidas na cascata do processo inflamatório, 5-LOX e COX 2 (GUPTA et al., 2000; CHEN et al., 2000; RASO et al., 2001), quanto na migração de leucócitos ao sítio de inflamação (RIEMERSMA et al., 2001). Atuam ainda, sobre moléculas reguladoras da expressão gênica, inibindo a expressão de COX 2, moléculas de

adesão (ICAM-1), citocinas (TNF-α, IL-6, IL-1α e IL-1β); e a ativação de NF-κB, proteína que controla a expressão de citocinas pró-inflamatórias (CHEN et al., 2000; RASO et al., 2001; LIN et al., 2003; CALIXTO et al., 2003). Os flavonóides também inibem a produção de NO em macrófagos, diminuindo a ação vasodilatadora e pró-inflamatória desta molécula (FUSHIYA et al., 1999; LIANG et al., 1999, CHEN et al., 2000).

Os flavonóides podem inibir oxigenases e peroxidases da cascata inflamatória: COX; xantina oxidase, LOX, MPO, por vários mecanismos, (1) capturando seus substratos EROs; (2) quelando metais, Fe^{2+} e Cu^{2+} essenciais para a atividade das enzimas, (3) servindo como substrato para enzima e evitando a oxidação de outras moléculas (4) ou por formar aductos com a enzima, causando alterações covalentes e inativando-a (DIVI & DOERGE, 1996; KOSTYUK et al., 2003; 2004).

A existência de múltiplos alvos intracelulares para os flavonóides pode ser explicada porque estas moléculas competem com o ATP pelo sítio de ligação em quinases intracelulares (MIDDLETON et al., 2000). Então, os flavonóides com grupo catecol são potentes inibidores da PI-3K e PKC (AGULLO et al., 1997; GAMET-PAYRASTRE et al., 1999). Flavonóides inibem a fosforilação de quinases intracelulares e consequentemente, inibem a liberação de TNF-α em macrófagos. XAGORARI e colaboradores (2001; 2002) demonstraram que a inibição da fosforilação das MAPKs (ERK1/2; p38; Akt) é um dos principais mecanismos pelo qual a luteolina inibe a liberação de TNF-α e a ativação do NF-κB. WADSWORTH e colaboradores (2001) demonstraram ainda, que a quercetina também é capaz de inibir a liberação de TNF-α em macrófagos, porém este flavonóide possui uma ação distinta àquela descrita para a luteolina, tendo em vista que sua ação inibitória não é decorrente da inibição da fosforilação de ERK1/2; p38; Akt, mas apenas da JNK. Embora estas proteínas quinases estejam envolvidas na cascata de sinalização intracelular ativada pela PI-3K estes estudos demonstraram que a inibição da MAPKs ocorre de maneira independente da ação dos flavonóides sobre a PI-3K.

A ação de flavonóides sobre quimiocinas não se restringe apenas à inibição da síntese destes peptídeos. LI e colaboradores (2000) demonstraram que os flavonóides são capazes de ligarem-se às quimiocinas (IL-8 e MIP-1β) e impedir a ligação destes peptídeos ao seus respectivos receptores.

Além das ações antiinflamatórias, importantes para evitar o dano tecidual ocorrido nesta situação, os flavonóides também apresentam atividade contra bactérias, fungos, protozoários e vírus. Além de atuarem como germicidas, os flavonóides combatem as toxinas produzidas por estes microorganismos (BRANDAO et al., 1997; LOPES et al., 1998). VECKENSTEDT e colaboradores (1987) observaram os efeitos antivirais dos flavonóides. Evidências mostram que os flavonóides inibem a fusão da membrana do vírus com o lisossoma (MILLER & LENARD, 1981) e inibem a atividade da transcriptase reversa (ONO et al., 1990; WANG et al., 1994). Outro mecanismo pelo qual estes compostos exercem sua ação germicida é através do aumento da

síntese de interferons e ativação de linfócitos T (VECKENSTEDT et al., 1987; WLEKLIK et al., 1987); embora alguns trabalhos mostrem a supressão destas células pelos flavonóides (GAO et al., 2001).

2.2.4. Efeito analgésico dos flavonóides

No leste da Europa, durante séculos os flavonóides vêm sendo indicados na clínica por cirurgiões dentistas para o tratamento da dor e regeneração tecidual (RONZÈRE et al., 1981; SAKAGAMI et al., 1999). Contudo, os mecanismos pelos quais estes compostos regeneram o tecido não são completamente entendidos (RONZÈRE et al., 1981).

Flavonóides aliviam a dor em ferimentos causados por picadas de insetos, cobra, queimaduras ou cortes. Este efeito é atribuído, pelo menos em parte, à inibição de fosfolipases e ciclooxigenases (LIANG et al., 1999; HAVSTEEN, 2002). Embora, outros mecanismos ainda não descritos possam estar envolvidos.

Além de seus efeitos periféricos, flavonóides possuem efeitos sobre o SNC, uma vez que, podem atuar como agonistas dos receptores adenosinérgicos e de receptores $GABA_A$ sensíveis aos benzodiazepínicos (MEDINA et al., 1997; PALADINI et al., 1999; BLARDI et al., 1999).

2.2.5. Outras atividades farmacológicas dos flavonóides

Em função de suas propriedades antioxidantes e antiinflamatórias, os flavonóides podem ser aplicados a muitas outras patologias, as quais estão intimamente ligadas aos processos oxidativos e inflamatórios como diabetes, câncer, doenças vasculares (ateroesclerose), doenças neurodegenerativas, doenças reumáticas, hepatopatias, entre outras (HERTOG et al., 1993; BOHM et al., 1998; MIDDLETON et al., 2000; HAVSTEEN, 2002; YOKOMIZO & MORIWAKI, 2005). Por outro lado, os flavonóides podem apresentar uma atividade específica sobre cada patologia.

Estes compostos são descritos por conterem a progressão do diabetes através da inibição da aldose redutase, uma enzima intrinsicamente envolvida na progressão de catarata e neuropatia diabética (KELLER & LEUENBERGER, 1980; KAMIYA et al., 2003).

Flavonóides inibem a HMG-CoA redutase, uma enzima chave na biossíntese do colesterol; esta propriedade pode auxiliar no tratamento de hipercolesterolemia familiar (KAZAKOV, 1980; AVIRAM & FUHRMAN, 1998). Além disso, eles reduzem a oxidação de lipídeos, o processo inflamatório e agregação plaquetária nas doenças coronarianas (BOURDILLAT et al., 1988; HERTOG et al., 1993; GOKER et al., 1995; AVIRAM & FUHRMAN, 1998; KIMIRA et al., 1998; KOSTYUK et al., 2004; SZEWCZUK et al., 2004; YOKOMIZO & MORIWAKI, 2005).

Estudos epidemiológicos têm mostrado uma relação inversa entre o consumo de vegetais, frutas e sementes ricas em flavonóides e a incidência de câncer em humanos (BLOCK et al., 1992; MESSINA, 1999). BIRT e colaboradores (2001) descrevem as propriedades anticarcinogênicas dos flavonóides. As mais investigadas consistem no aumento da resposta imune contra células tumorais, através da indução de interferons (YASUKAWA et al., 1990; SEN & LENGYEL, 1992). Além disso, os flavonóides interferem em uma série de vias regulatórias da célula, incluindo divisão celular, metabolismo, apoptose, transcrição, reparo de genes nas mutações, transmissão neuronal, inflamação, estresse oxidativo e reposta ao estresse (HARPER et al., 1993; HABTEMARIAM, 1997; OHSHIMA et al., 1998; EDWARDS et al., 1998; BLARDI et al., 1999; CHEN et al., 2000; KO et al., 2005; 2005a).

Os flavonóides são úteis na isquemia/reperfusão não somente por suas ações “scavenger”, mas porque estes compostos possuem ação vasorelaxante (LAW et al., 1999).

2.2.6. Toxicidade dos flavonóides

Salvo em raros casos de alergia (PETERSEN, 1977; HAUSEN & WOLLENWEBER, 1988; HEGUI et al., 1990), pouca relevância toxicológica é dado ao consumo dos flavonóides, uma vez que estes compostos fazem parte da dieta (HAVSTEEN, 2002).

Flavonóides são fracamente antigênicos, por outro lado, reações alérgicas ocorrem em cerca de 3 – 5% da população após ingerir alimentos com alta concentração de flavonóides (HAUSEN & WOLLENWEBER, 1988; HEGUI et al., 1990).

CASLEY-SMITH & CASLEY-SMITH (1986) demonstraram que dose letal 50 (DL_{50}) dos flavonóides, na sua forma aglicona, é igual a 2 g/kg do peso do animal, quando administrados por via intravenosa em ratos. Estudos toxicológicos prévios mostram que a administração constante de doses extremamente altas de flavonóides causa alterações morfológicas na membrana dos hepatócitos, com necrose celular e morte do animal. Além disso, foi observado um efeito mutagênico por alguns flavonóides (CEA et al., 1983; SAHU et al., 1981; CANIVENC-LAVIER et al., 1996). A atividade tóxica e mutagênica dos flavonóides é atribuída, principalmente, à formação de epóxidos durante sua biotransformação (CANIVENC-LAVIER et al., 1996). Porém, em geral, as mutações são reparadas antes de conduzirem a um distúrbio. Desta forma, o risco de conseqüências patológicas ocorridas pela mutação através do consumo de flavonóides é considerado baixo (HABS et al., 1984).

Além disso, os flavonóides inibem enzimas inflamatórias em concentrações muito baixas, semelhantes às encontradas na corrente sanguínea de humanos após a ingestão de alimentos ricos neste composto (KOSTYUK et al., 2003). Assim, a margem de segurança para o uso terapêutico dos flavonóides em humanos é relativamente extensa (HAVSTEEN, 2002). Por outro lado, mais estudos toxicológicos são necessários para assegurar esta afirmação.

2.2.7. Miricitrina

A miricitrina é um flavonóide encontrado principalmente em frutas de vegetais do gênero *Pouteria* (marmelo) (MA et al., 2004a) e da espécie *Manilkara zapota* (“sapodilla”) (MA et al., 2004). Este flavonóide também é encontrado em grandes quantidades em folhas de plantas do gênero *Eugenia* (pitanga e jambolão) (SCHMEDA-HIRSCHMANNET et al., 1987) e látex da *Croton draco* (sangue de dragão) (TSACHEVA et al., 2004).

Estudos farmacológicos prévios, baseados no uso etnofarmacológico destas plantas, demonstraram que elas possuem atividade antidiabética (anti-hiperglicêmica); anti-hemorrágica; antiinflamatória; antiúlcera; imunomodulatória; antimicrobiana; antioxidante; anti-hipertensiva e vasorrelaxante, sendo algumas destas atividades atribuídas à presença de miricitrina no extrato (WAZLAWIK et al., 1997; CASTRO et al., 1999; CONSOLONI et al., 1999; KIM & KIM, 2003; TSACHEVA et al., 2004; SOUZA et al., 2004; SHAH et al., 2004; MANOSROI et al., 2005; SHARMA et al., 2006).

A miricitrina (Figura 2) pertence ao subgrupo flavonol e é formada por dois grupos fenil (A e B) unidos por uma cadeia de três carbonos (C); nesta cadeia propiônica está ligado um monossacarídeo. Esta característica química confere à miricitrina uma série de propridades farmacológicas. A presença do grupo pirrogalol (3 oxidrilas livres no anel B) é muito importante para a atividade antioxidante; neutralizadora de radicais livres, pró-apoptótica, inibidora de proteínas quinases e peroxidases (BROWN et al., 1998; GAMET-PAYRASTRE et al., 1999; PIETTA, 2000; KOSTYUK et al., 2003; SAWAI et al., 2005; KO et al., 2005; 2005a).

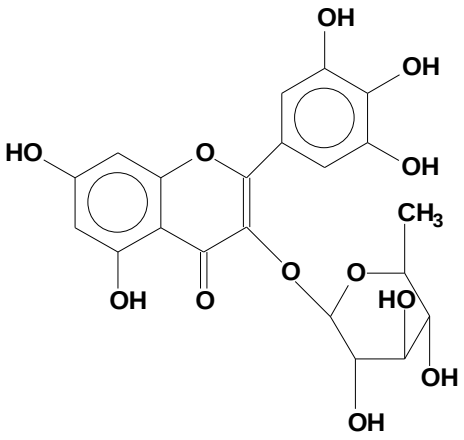

Figura 2: Estrutura química da miricitrina

Estudos em humanos revelam que a ingestão de alimentos contendo miricitrina está positivamente relacionada a um aumento nos níveis de lipoproteínas de alta densidade (HDL) e negativamente relacionada aos níveis de triglicerídeos (KIMIRA et al., 1998). Além disso, este flavonóide reduz a oxidação de lipoproteínas de baixa densidade (LDL). Sob este aspecto, o consumo de alimentos contendo miricitrina pode diminuir potencialmente o risco de ateroesclerose (YOKOMIZO & MORIWAKI, 2005).

Miricitrina tem sido descrita por suas atividades antioxidante e neutralizadora de radicais livres (EDENHARDER & GRÜNHAGE, 2003; YOKOMIZO & MORIWAKI, 2005), propriedade a qual justifica a grande amplitude de benefícios associados ao consumo desta substância. EDENHARDER & GRÜNHAGE, (2003) demonstraram que a capacidade de neutralizar radicais livres confere à miricitrina potente ação antitumoral. Por outro lado, a inibição de crescimento de tumor também é devida à indução de resposta imunológica contra o tumor (YASUKAWA et al., 1990).

Estudos *in vitro* mostraram que a miricitrina é capaz de inibir a produção de NO e a expressão da enzima iNOS, bem como, a ativação do NF-κB induzida por lipopolissacarídeo de *Eschericchia coli* (LPS) (CHEN et al., 2000). Corroborando com estes dados, TSAI e colaboradores (1999) encontraram que a miricetina (aglicona da miricitrina) é capaz de inibir a atividade da IκB quinase, enzima

responsável pela fosforilação da proteína IκB que, quando fosforilada, libera o NF-κB, permitindo sua translocação ao núcleo.

Recentemente, KO e colaboradores (2005a) demonstraram que a miricetina é capaz de inibir metaloproteinases de matriz (MMP) em células de carcinoma coloretal, explicando o efeito anticancerogênico e antiinflamatório deste flavonóide. Neste mesmo estudo, a inibição da ativação da PKCα pela miricetina inibiu o aumento na expressão de MMP, fosforilação da ERK1/2 e a expressão da c-Jun. A presença de três oxidrilas livres no anel B da miricetina torna este flavonóide o mais potente inibidor de ambas proteínas quinases, PKC e PI-3K, esta última fosforila a PKC (AGULLO et al., 1997; GAMET-PAYRASTRE et al., 1999; KO et al., 2005). Além disso, a ação da miricetina sobre a sinalização mediada por PKC confere a esta molécula propriedade pró-apoptótica. A miricetina ativa caspase 3 e caspase 9 em células leucêmicas humanas (HL-60), com decréscimo da proteína Bcl-2 (antiapoptótica) e aumento da Bax (pró-apoptótica) (KO et al., 2005). Interessantemente, estes mesmos efeitos *in vitro* não foram encontrados para a miricitrina; porém, é importante ressaltar, que a administração de ambas as formas miricetina ou miricitrina *in vivo*, resultará em formas conjugadas para serem transportadas pela corrente sanguínea, ou seja, após a ingestão oral de miricitrina ou miricetina, não há distinção entre as duas estruturas (MUROTA &TERAO, 2003). Além disso, ao chegarem no tecido este composto pode ser liberado do seu conjugado, como mencionado na seção 2.2.1 (O'LEARY et al., 2001). Portanto, a justificativa para a escolha de um flavonóide glicosilado em estudos farmacológicos *in vivo,* reside no fato que estes compostos são mais estáveis que seus correspondentes agliconas e, portanto, mais facilmente encontrados na natureza (YOKOMIZO & MORIWAKI, 2005).

3. OBJETIVOS

Levando em consideração: (1) o efeito ambas, miricitrina e sua aglicona, miricetina, em processos de sinalização celular comuns ao processo de dor e inflamação; (2) a atividade antioxidante destes compostos e (3) a atividade antiinflamatória já descrita para os flavonóides, o objetivo do presente estudo foi avaliar o efeito da miricitrina sobre a transmissão nociceptiva aguda e crônica, bem como, desvendar os mecanismos pelos quais este flavonóide exerce tal efeito. As ações da miricitrina sobre a nocicepção aguda foram investigadas utilizando modelos químicos de nocicepção em camundongos e ratos. E as ações sobre a nocicepção crônica foram avaliadas através da administração i.pl. de FCA (modelo de nocicepção inflamatória crônica) e ligadura parcial do nervo ciático (modelo de nocicepção neuropática) em camundongos.

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão dispostos da mesma forma que foram publicados na edição das revistas científicas (**Artigos 1**) ou submetidos, que é o caso dos **Artigos 2** e **3**.

4.1. Efeito antinociceptivo da miricitrina em modelos de dor aguda

Artigo 1

ANALYSIS OF THE ANTINOCICEPTIVE EFFECT OF THE FLAVONOID MYRICITRIN: EVIDENCE FOR A ROLE OF THE L-ARGININE-NITRIC OXIDE AND PROTEIN KINASE C PATHWAYS

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Analysis of the Antinociceptive Effect of the Flavonoid Myricitrin: Evidence for a Role of the L-Arginine-Nitric Oxide and Protein Kinase C Pathways

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ABSTRACT

The present study investigated the antinociceptive effects of the flavonoid myricitrin in chemical behavioral models of pain in mice and rats. Myricitrin given by i.p. or p.o. routes produced dose-related antinociception when assessed on acetic acid-induced visceral pain in mice. In addition, the i.p. administration of myricitrin exhibited significant inhibition of the neurogenic pain induced by intraplantar (i.pl.) injection of capsaicin. Likewise, myricitrin given by i.p. route reduced the nociception produced by i.pl. injection of glutamate and phorbol myristate acetate (PMA). Western blot analysis revealed that myricitrin treatment fully prevented the protein kinase C (PKC) α and PKCϵ activation by PMA in mice hind paws. Myricitrin given i.p. also inhibited the mechanical hyperalgesia induced by bradykinin, without affecting similar responses caused by epinephrine and prostaglandin E_2. The antinociception caused by myricitrin in the acetic acid test was significantly attenuated by i.p. treatment of mice with the nitric oxide precursor, L-arginine. In contrast, myricitrin antinociception was not affected by naloxone (opioid receptor antagonist) or neonatal pretreatment of mice with capsaicin and myricitrin antinociceptive effects is not related to muscle relaxant or sedative action. Together, these results indicate that myricitrin produces pronounced antinociception against chemical and mechanical models of pain in rodents. The mechanisms involved in their actions are not completely understood but seem to involve an interaction with nitric oxide-L-arginine and protein kinase C pathways.

Flavonoids are a γ-benzopyrone family that occur naturally and are widely spread in higher plants (Ramelet, 2000). They are plant secondary metabolites and are chemically defined by their common structure, which is composed of diphenylpropanes (C6-C3-C6) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Harborne, 1989). In mammals, flavonoids occur only through dietary intake. The average daily human intake in the United Kingdom and United States has been present to be 20 mg to 1 g. These compounds are present in fruits, vegetables, grains, nuts, tea, and wine. However, little is reported about quantify flavonoid in food and thus, only a few studies have attempted to assess the relationship between consumption of foods rich in flavonoids and the prevention of certain diseases (Birt et al., 2001).

A variety of biological effects have been ascribed to flavonoids (Birt et al., 2001; Havsteen, 2002; Calixto et al., 2003, 2004). Much attention has been given to their antioxidant (Edenharder and Grünhage, 2003) and anti-inflammatory properties, in vitro and in vivo (Calixto et al., 2003, 2004). In addition, some studies report antitumoral (Clifford et al., 1996) and hepatoprotective (Ferrándiz et al., 1994) action. Flavonoids inhibit cytokine release from RAW264.7 cells (Xagorari et al., 2002) and may modulate the increasing number of cellular processes involving redox reaction, including the regulation of tyrosine phosphatase activity (Gamet-Payrastre et al., 1999). In contrast, little is known about the effects of flavonoids on the modulation of pain transmission.

Myricitrin (Fig. 1) is a flavonoid that belongs to the flavonol subgroup. This flavonoid is found in fruits of genus

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ABBREVIATIONS: NO, nitric oxide; i.pl., intraplantar(ly); PMA, phorbol 12-myristate 13-acetate; L-NOARG, N^{ω}-nitro-L-arginine; PKC, protein kinase C; PBS, phosphate-buffered saline; ANOVA, analysis of variance; PGE_2, prostaglandin E_2; PKA, protein kinase A; TRPV1, transient receptor potential vanilloid type 1.

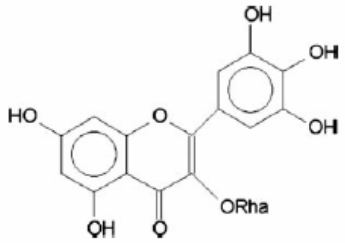

Fig. 1. Molecular structure of myricitrin.

Pouteria (Ma et al., 2004) and *Manilkara zapota* (Ma et al., 2003), leaves of *Eugenia uniflora* (Schmeda-Hirschmann et al., 1987), and latex of *Croton draco* (Tsacheva et al., 2004). In addition, myricitrin has been reported as having an antioxidant action (Edenharder and Grünhage, 2003). It comprises an antimutagenic effect, which is attributed to a free radical scavenger action (Edenharder and Grünhage, 2003). However, the ability of myricitrin to inhibit tumor promotion is also due to the activation of immune responses against tumors (Yasukawa et al., 1990). The intake of myricitrin from food is associated with the level of high-density lipoprotein cholesterol and negatively related to the level of triglycerides (Kimira et al., 1998).

Previous reports demonstrate that myricitrin is able to inhibit nitric oxide (NO) production. In addition, it reduces the overexpression of nitric-oxide synthase and nuclear factor-κB activation induced by lipopolysaccharide on RAW264.7 cells (Chen et al., 2000).

Taking into account the biological activities of myricitrin, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of this flavonoid up to this date. Here, we have therefore attempted to examine the possible antinociceptive action of myricitrin in chemical and mechanical models of nociception in mice and rats. Attempts have been made to further investigate some of the possible mechanisms that underlie the antinociceptive action of myricitrin.

Materials and Methods

Animals

Experiments were conducted using Wistar rats (200–300 g) or Swiss mice (25–35 g) of both sexes, housed at 22 ± 2°C under a 12-h light/dark cycle (lights on at 6:00 AM) and with access to food and water ad libitum. Animals (male and female rat or mice were homogeneously distributed among the groups) were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after protocol approval by the Institutional Ethics Committee and were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified (Zimmermann, 1983). The number of animals and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Abdominal Constriction Induced by Acetic Acid

The abdominal constrictions were induced according to procedures described previously (De Campos et al., 1997) and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of acetic acid (0.6%, 0.45 ml/mouse) at the time of the test. Mice were pretreated with myricitrin by i.p. (0.01–10 mg/kg) or p.o. (1–100 mg/kg) routes, 30 or 60 min before the irritant injection. Control animals received a similar volume of vehicle (10 ml/kg). After the challenge, the mice were individually placed into glass cylinders of 20-cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min.

Algogen-Induced Overt Nociception in Mice

Glutamate-Induced Nociception. In an attempt to provide more direct evidence concerning the possible interaction of myricitrin with the glutamatergic system, we separately investigated whether myricitrin would be able to antagonize glutamate-induced licking in the mouse paw. The procedure used was similar to describe previously (Beirith et al., 2002). A volume of 20 μl of glutamate (10 μmol/paw prepared in saline) was injected intraplantarly (i.pl.) in the ventral surface to the right hind paw. The mice were observed individually for 15 min following glutamate injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. Mice were treated with myricitrin (1–100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before glutamate injection.

Phorbol Myristate Acetate (PMA)-Induced Nociception. The procedure used was similar to that described previously (Siebel et al., 2004). A volume of 20 μl of PMA (50 pmol/paw prepared in saline) was injected i.pl. in the ventral surface of the right hind paw. After the challenge, the mice were individually placed into glass cylinders of 20-cm diameter, serving as observation chambers. The mice were observed individually from 15 to 45 min after PMA injection, and the amount of time spent licking the injected paw timed with a chronometer was considered indicative of nociception. The mice were treated with myricitrin (0.01–10 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before PMA injection.

Capsaicin-Induced Nociception. The procedure used was similar to that described previously (De Campos et al., 1997). After an adaptation period, 20 μl of capsaicin (1.6 μg/paw) was injected i.pl. in the ventral surface of the right hind paw. The mice were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. The mice were treated with myricitrin (1, 10, and 100 mg/kg i.p.) 30 min before capsaicin injection. Control animals received vehicle (10 ml/kg).

Bradykinin-, Epinephrine-, or Prostaglandin E_2-Induced Hyperalgesia. The procedures used were similar to those described previously (De Campos et al., 1997). The rats were pretreated i.p. with myricitrin (30 mg/kg) or vehicle (10 ml/kg) 30 min before injection of 100 μl of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw), prostaglandin E_2 (10 nmol/paw), or only saline solution alone (control group) in the ventral surface of the right hind paw. The nociception threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure to the dorsal site of inflamed or control paws, using a Basile analgesy meter (Ugo Basile, Comerio, Italy) according to the method of Randall and Selitto (1957). The weight on the analgesy meter ranged from 0 to 750 g, and the threshold was expressed as load (grams) tolerated. When bradykinin was used, animals were pretreated with the angiotensin-converting enzyme inhibitor captopril (5 mg/kg s.c.) 60 min before experiments to prevent its degradation (De Campos et al., 1997).

Measurement of Motor Performance, Locomotor Activity, and Corporal Temperature. To evaluate the possible nonspecific muscle-relaxant or sedative effects of myricitrin, mice were submitted to the Rotarod task (Vaz et al., 1996) and open-field test (Rodrigues et al., 2002). Rotarod apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks 25 cm in diameter. The bar rotated at a constant speed of 17 revolutions/min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with myricitrin (30 or 100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before being tested. The results are

expressed as the time(s) for which animals remained on the Rotarod. The cut-off time used was 120 s.

The ambulatory behavior was assessed in an open-field test as described previously (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed with all paws crossing was counted in a 6-min session. Mice were treated with myricitrin (30 and 100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min beforehand.

In addition, some compounds cause antinociception by decreasing basal corporal temperature (hypothermia). To exclude this possibility, we assessed the skin temperature of mice 30 min after they received vehicle (saline) or myricitrin (30 and 100 mg/kg i.p.). A thermosensor (Mallory Ltda., Ceará, Brazil) was placed on the skin in the sacral region, and the procedure was carried out in accordance with the manufacturer's instructions.

Analysis of the Possible Mechanism of Action of Myricitrin

Involvement of the Opioid System. To investigate the possible participation of the opioid system in the antinociceptive effect of myricitrin, mice were pretreated with naloxone (a nonselective opioid receptor antagonist, 5 mg/kg i.p.), and after 20 min, the animals received an injection of myricitrin (1 mg/kg i.p.), morphine (5 mg/kg s.c.), or vehicle (10 ml/kg i.p). Other groups were pretreated with vehicle and after 20 min received myricitrin, morphine, or vehicle, 30 min before acetic acid injection.

Involvement of the L-Arginine-Nitric Oxide Pathway. To investigate the role played by the nitric oxide-L-arginine pathway in the antinociception caused by myricitrin, mice were pretreated with L-arginine (40 mg/kg i.p., a nitric oxide precursor) or D-arginine (40 mg/kg i.p., an inactive isomer of L-arginine), and after 20 min, they received myricitrin (1 mg/kg i.p.), N^{ω}-nitro-L-arginine (L-NOARG; 25 mg/kg i.p., an inhibitor of nitric oxide synthesis), or vehicle (10 ml/kg i.p.). The nociceptive responses to acetic acid were recorded 30 min after the administration of myricitrin, L-NOARG, or vehicle. Other groups were pretreated with vehicle (10 ml/kg i.p.) and after 20 min received myricitrin, L-NOARG, or vehicle 30 min before acetic acid injection (Abacioglu et al., 2001).

Involvement of Capsaicin-Sensitive Fibers. To explore the role of capsaicin-sensitive fibers in the antinociceptive effect of myricitrin, newborn mice were anesthetized with ether and treated s.c. with 50 mg/kg capsaicin on the 2nd day of life with the purpose of inducing the irreversible degeneration of unmyelinated afferent neurons, especially C fibers (Holzer, 1991). Control animals received, by the same route, the same volume of vehicle used to dissolve capsaicin (10% ethanol, 10% Tween 80, and 80% saline). The antinociceptive effect caused by myricitrin (1 and 10 mg/kg i.p.), against the nociceptive response induced by acetic acid, was analyzed at 6 to 7 weeks after the neonatal capsaicin or vehicle treatment. The efficiency of the treatment of mice with capsaicin was confirmed by means of a topical application of 50 μl of capsaicin (0.01%) into the right eye, and the number of wiping motions occurring during the subsequent 1 min was counted as described previously (Ikeda et al., 2001).

Effect of Myricitrin on the PKCα and PKCϵ Activation by PMA

Preparation of Tissue for Western Blot Studies. The mice received myricitrin (1 mg/kg i.p.) or vehicle (10 ml/kg i.p.), 30 min before PMA (50 pmol/paw) injection. The injected paw was isolated 15 min after PMA or phosphate-buffered saline (PBS) injection (Ferreira et al., 2005). The skin and connective tissues of the plantar portion of the hind paws were removed and disrupted using a glass Potter homogenizer in an ice-cold buffer containing protease and phosphatase inhibitors (100 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 μg of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 200 mM NaF, and 2 mM sodium orthovanadate). The homogenate was centrifuged at 1000*g* for 10 min at 4°C; the pellet was discarded, and the supernatant was further centrifuged at 35,000*g* for 30 min at 4°C. The supernatant was collected as a cytoplasm-rich fraction. The resulting pellet was resuspended and considered as a membrane-rich fraction. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). The samples were aliquoted and stored at −80°C until their Western blot analysis.

Western Blot Analysis. To confirm the activation of PKCα and PKCϵ after PMA injection into the mouse paw, Western blot analysis was carried out as previously described (Ferreira et al., 2005). Equivalent amounts of protein (10 and 50 μg for membrane- and cytoplasm-rich fractions, respectively) were mixed in buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromphenol blue) and boiled for 5 min. Proteins were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membranes, according to the manufacturer's instructions (Millipore Corporation, Billerica, MA). The membranes were saturated by incubation overnight with 10% nonfat dry milk solution and then incubated with anti-PKCα and anti-PKCϵ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, the membranes were incubated with adjusted peroxidase-coupled secondary antibodies. The immunocomplexes were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Drugs. The following substances were used: acetic acid, morphine hydrochloride (Merck, Darmstadt, Germany), bradykinin, epinephrine, prostaglandin E_2, capsaicin, naloxone hydrochloride, phorbol 12-myristate 13-acetate, glutamic acid, L-arginine, D-arginine, L-NOARG, and PBS tablets (Sigma-Aldrich, St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Drugs were dissolved in 0.9% NaCl solution, with the exception of myricitrin, which was dissolved in Tween 80 plus saline and capsaicin, dissolved in ethanol plus Tween 80 plus saline. The final concentration of Tween and ethanol did not exceed 5% and did not cause any "per se" effect. The myricitrin, isolated from the plant of genus *Eugenia* by the Department of Chemistry (Federal University of Santa Catarina, Brazil), was characterized by spectral analyses (RMN-^{1}H) and (RMN-^{13}C) and by comparison with the spectrum literature data (Agrawal, 1989) and showed a degree of purity greater than 98%.

Statistical Analysis. The results are presented as mean ± S.E.M., except the ID_{50} values (i.e., the dose of myricitrin reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. When possible, the ID_{50} value was determined using at least three dosages of myricitrin by linear regression from individual experiments, using linear regression software (GraphPad Software Inc., San Diego, CA). Maximal inhibition values were calculated at the most effective dose used. The statistical significance of differences between groups was performed by ANOVA followed by Newman-Keuls test. $p < 0.05$ was considered as indicative of significance.

Results

Abdominal Constriction Induced by Acetic Acid. The results in Fig. 2A show that myricitrin, given i.p. 30 min prior to testing, produced dose-related inhibition of the acetic acid-induced abdominal constrictions in mice, with a mean ID_{50} value (and their respective 95% confidence limits) of 0.33 (0.20–0.54) mg/kg, and the inhibition observed was 84 ± 5% for the dose of 10 mg/kg. Furthermore, given by p.o. route 60 min beforehand, myricitrin (100 mg/kg) caused a partial but significant inhibition (39 ± 4%) of the acetic acid-induced pain (Fig. 2B). Hence, myricitrin was less efficacious and potent in preventing the nociception caused by acetic acid when it was given orally in comparison with when it was

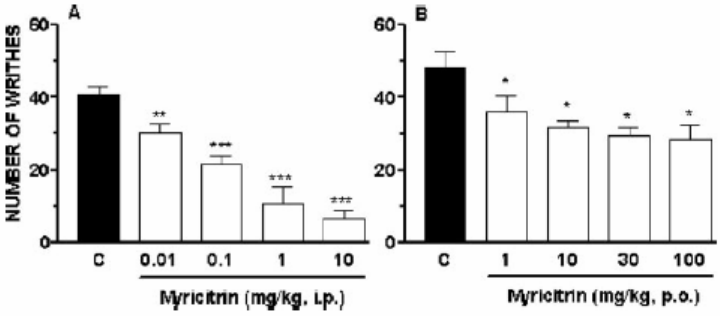

Fig. 2. Effect of myricitrin administered i.p. (A) and orally (B) against acetic acid-induced writhing response in mice. Each column represents the mean of six to eight mice, and the error bar indicates the S.E.M. Control values (C) indicate the mice injected with vehicle, and the asterisks denote the significance levels compared with control groups (one-way ANOVA followed by Newman-Keuls test): *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

given i.p. Thus, the administration of myricitrin by the i.p. route (time point 30 min beforehand) was chosen for all further studies with independent groups of animals.

Glutamate-Induced Nociception. The results presented in Fig. 3A show that myricitrin caused a dose-dependent and significant inhibition of the glutamate-induced nociception, with a mean ID_{50} value of 16.8 (10.3–27.5) mg/kg, and the inhibition observed was 81 ± 10% for the dose of 100 mg/kg.

Capsaicin-Induced Nociception. The i.p. administration of myricitrin produced partial, but significant, inhibition of the capsaicin-induced neurogenic nociception (Fig. 3B). The inhibition observed was 13 ± 8, 42 ± 8, and 37 ± 6% for the doses of 1, 10, and 100 mg/kg, respectively (Fig. 3B).

PMA-Induced Overt Nociception. The i.p. administration of myricitrin also produced a marked and dose-dependent inhibition of PMA-induced licking (Fig. 3C). The mean ID_{50} value from this result was 0.56 (0.33–0.95) mg/kg, and the inhibition was 100% when administrated at 10 mg/kg.

Bradykinin-, Epinephrine-, or Prostaglandin E_2-Induced Hyperalgesia. The results of Fig. 4 show that intraplantar administration of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw), and prostaglandin E_2 (PGE_2; 10 nmol/paw) significantly increased ($p < 0.01$) the sensibility to mechanical stimuli (hyperalgesia) in rats when assessed in the Randall-Selitto model. In addition, bradykinin, epinephrine, and PGE_2 caused a decrease of 40 ± 7, 58 ± 2, and 75 ± 3% on pressure supported in grams compared with control group value. Furthermore, the treatment of rats with myricitrin (30 mg/kg i.p) completely reversed the hyperalgesic effect caused by bradykinin ($p < 0.01$) but did not reduce epinephrine- or PGE_2-induced hyperalgesia (Fig. 4).

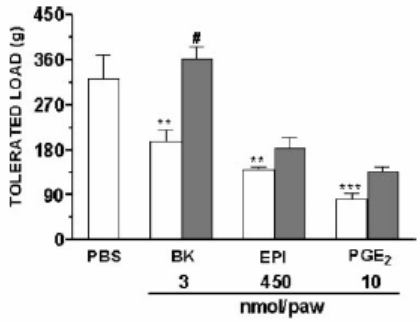

Fig. 4. Effects of i.p. injection of the myricitrin (30 mg/kg) on mechanical hyperalgesia induced by bradykinin (BK; 3 nmol/paw); epinephrine (EPI; 450 nmol/paw) or PGE_2 (10 nmol/paw) in rat paw. The columns indicate the control values (phosphate-buffered solution, injection paw) and the BK-, epinephrine-, or PGE_2-injected paws, in the absence (open) or presence (closed) columns of the myricitrin. Each column represents the mean of six rats, and the error bars indicate the S.E.M. The symbols denote significance levels: **, $p < 0.01$ and ***, $p < 0.001$ compared with saline group; and #, $p < 0.001$ compared with vehicle i.p. plus BK i.pl. group (one-way ANOVA followed by Newman-Keuls test).

Measurement of Motor Performance, Locomotor Activity, and Basal Temperature. The myricitrin treatment (30 and 100 mg/kg i.p.) did not alter response of mice in both tests, motor performance on the Rotarod task and locomotor activity in the open-field test compared with animals that received saline (control group) (Table 1). In addition, the basal temperature of mice was not altered by myricitrin (30 and 100 mg/kg) treatment (Table 1).

Involvement of the Opioid System. The results presented in Fig. 5 show that the pretreatment of mice with

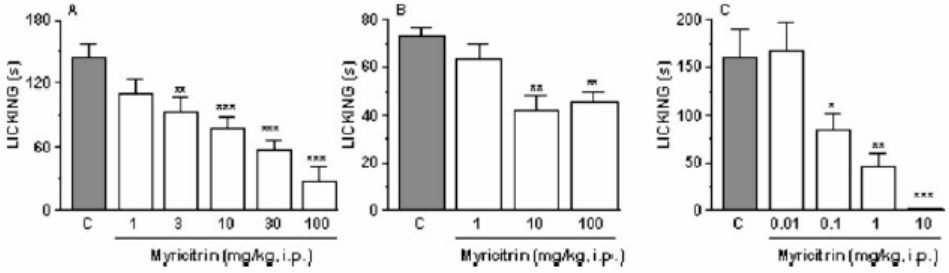

Fig. 3. Effect of myricitrin given i.p. (0.01–100 mg/kg i.p.) against the glutamate-induced (10 μmol/paw; A), capsaicin-induced (5.2 nmol/paw; B), and PMA-induced (50 pmol/paw; C) licking in mice. Each column represents the mean of six to eight mice, and the error bar indicates the S.E.M. Control values (gray bar) indicate the mice injected with vehicle i.p., and the asterisks denote the significance levels compared with control groups (one-way ANOVA followed by Newman-Keuls test): *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

TABLE 1
Effect of myricitrin in the motor performance (Rotarod test), locomotor activity (open-field test), and corporal temperature of mice
Data are expressed as mean ± S.E.M. of six animals.

	Rotarod	Open field	Temperature
	s	*crossing number*	°C
Saline	97.8 ± 14.0	113.2 ± 4.5	29.4 ± 0.6
Myricitrin (30 mg/kg)	97.6 ± 11.4	122.3 ± 29.5	29.0 ± 0.5
Myricitrin (100 mg/kg)	120.0 ± 0	120.4 ± 7.2	28.6 ± 0.5

naloxone (5 mg/kg i.p.), given 20 min beforehand, completely reversed the antinociception caused by morphine (5 mg/kg s.c.) against acetic acid-induced pain, without affecting the antinociception caused by myricitrin (1 mg/kg i.p.).

Involvement of the L-Arginine-Nitric Oxide Pathway. The results presented in Fig. 6 show that mice pretreatment with nitric oxide precursor L-arginine (40 mg/kg i.p.), given 20 min prior to testing, but not with D-arginine (40 mg/kg i.p.) significantly prevented ($p < 0.05$) the antinociception caused by L-NOARG (25 mg/kg i.p.) and by myricitrin (1 mg/kg i.p.) when analyzed against acetic acid-induced abdominal constrictions.

Involvement of Capsaicin-Sensitive Fibers. Finally, the neonatal capsaicin (50 mg/kg s.c.) treatment of mice produced partial, but significant, inhibition (27 ± 7%) of the acetic acid-induced nociception (Fig. 7). In contrast, the same treatment of mice with capsaicin did not significantly change the antinociceptive effect of myricitrin (1 and 10 mg/kg i.p.) compared with the neonatal vehicle treatment group (Fig. 7). Furthermore, successful capsaicin treatment of newborn mice was confirmed by a significant reduction ($p < 0.001$) in the response to topical application of capsaicin to the cornea in capsaicin-treated mice. The mean number of wiping motions was 4.6 ± 1.6 and 18.5 ± 1.7 in capsaicin- and vehicle-treated mice, respectively.

Western Blot Analysis of Protein Kinase C. The possible participation of the PKC pathway on the antinociceptive effect of myricitrin was confirmed through Western blot analysis. Injection of PMA (50 pmol/site) into the mouse paw activated PKCα and, to a lesser extent, PKCϵ isoforms, as indicated by their translocation from cytosol- to membrane-rich homogenates achieved in administered tissues (Fig. 8, A–D). In addition, myricitrin (1 mg/kg) pretreatment significantly prevented the activation of both PKCα and PKCϵ isoforms caused by PMA injection (Fig. 8, A–D).

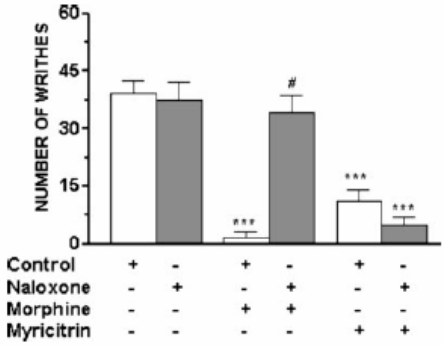

Fig. 5. Effect of pretreatment of mice with naloxone (5 mg/kg i.p.) on the antinociceptive profiles of myricitrin (1 mg/kg i.p.) and morphine (5 mg/kg s.c.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice, and the error bars indicate the S.E.M. The symbols denote significance levels: ***, $p < 0.001$ compared with control group; and #, $p < 0.001$ compared with morphine and myricitrin alone (one-way ANOVA followed by Newman-Keuls test).

Discussion

The present study demonstrates, for the first time, that systemic (i.p. or p.o.) administration of the flavonoid myricitrin, at doses that did not produce any important motor dysfunction, alterations in basal temperature, or any other obvious side effects induced a dose-dependent inhibition of acetic acid-induced visceral nociceptive response in mice. The most relevant additional findings of the present work were that i.p. administration of myricitrin caused significant inhibition of glutamate- and capsaicin-induced nociception and dose-dependent inhibition of the nociceptive response caused by intraplantar injection of PMA; the myricitrin antinociceptive effect in PMA-induced nociception is closely related to inhibition of both PKCα and PKCϵ; myricitrin comprised an antihyperalgesic effect upon the intraplantar injection of bradykinin, but not with epinephrine or PGE_2, in rats; its antinociception against the acetic acid test was significantly reversed by i.p. treatment of mice with L-arginine, but not by naloxone; and the neonatal treatment of mice with capsaicin completely failed to affect myricitrin-induced antinociception in mice.

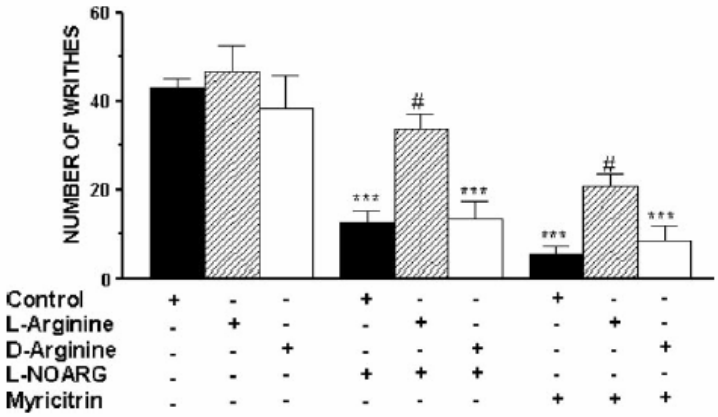

Fig. 6. Effect of pretreatment of mice with L-arginine (50 mg/kg i.p.) or D-arginine (50 mg/kg i.p.) on the antinociceptive profiles of myricitrin (1 mg/kg i.p.) and L-NOARG (25 mg/kg i.p.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice, and the error bars indicate the S.E.M. The symbols denote significance levels: ***, $p < 0.001$ compared with control group; #, $p < 0.05$ compared with L-NOARG and myricitrin alone (one-way ANOVA followed by Newman-Keuls test).

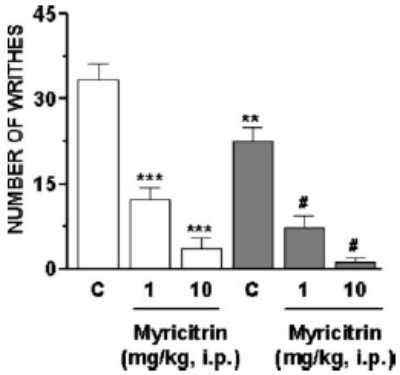

Fig. 7. Effect of neonatal treatment with vehicle (clear bars) and capsaicin (50 mg/kg, dark bars) on the antinociceptive action of myricitrin (1 and 10 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 8 to 10 mice, and the error bars indicate the S.E.M. The symbols denote significance levels: *, $p < 0.05$; ***, $p < 0.001$ compared with control group (vehicle neonatal plus saline i.p.); and #, $p < 0.001$ compared with capsaicin neonatal plus saline i.p. group (one-way ANOVA followed by Newman-Keuls test).

Flavonoids exert important pharmacological actions, such as antioxidant, anti-inflammatory, antiallergic, and anti-ischemic properties, suggesting their potential protective function against cardiovascular and coronary heart diseases and against certain forms of cancer (Gamet-Payrastre et al., 1999; Birt et al., 2001; Havsteen, 2002; Calixto et al., 2003, 2004; Edenharder and Grünhage, 2003). The flavonoid myricitrin produces important antioxidant and antimutagenic effects, which are attributed or not to its free radical scavenger action (Yasukawa et al., 1990; Edenharder and Grünhage, 2003). Although myricitrin is known to possess a strong antioxidant effect, the putative antinociceptive activities of myricitrin, as well as the mechanisms of its action in vivo, have never been reported.

The results reported here indicate that i.p. administration of myricitrin produced consistent and dose-related antinociception when assessed in acetic acid-induced visceral nociception in mice. Compared with standard analgesic drugs (results obtained by our group), myricitrin was about 36.6- to 72.7-fold more potent than acetaminophen, aspirin, and diclofenac in attenuating acetic acid-induced pain (Vaz et al., 1996; Santos et al., 1998). As expected, oral administration of myricitrin was less potent and efficacious than its i.p. administration in preventing the nociception. In fact, it is generally recognized that intact flavonoid glycosides, like myricitrin, are poorly absorbed when given by oral route (Murota and Terao, 2003). Thus, the present result is in agreement with data from the literature demonstrating that the bioavailability (antinociceptive activity) of myricitrin is notably decreased when given orally in comparison with when given i.p.

The acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Collier et al., 1968). This method presents a good sensitivity; however, it shows poor specificity, leaving scope for the misinterpretation of results. This can be avoided by complementing the test with other models of nociception and by a performance motor

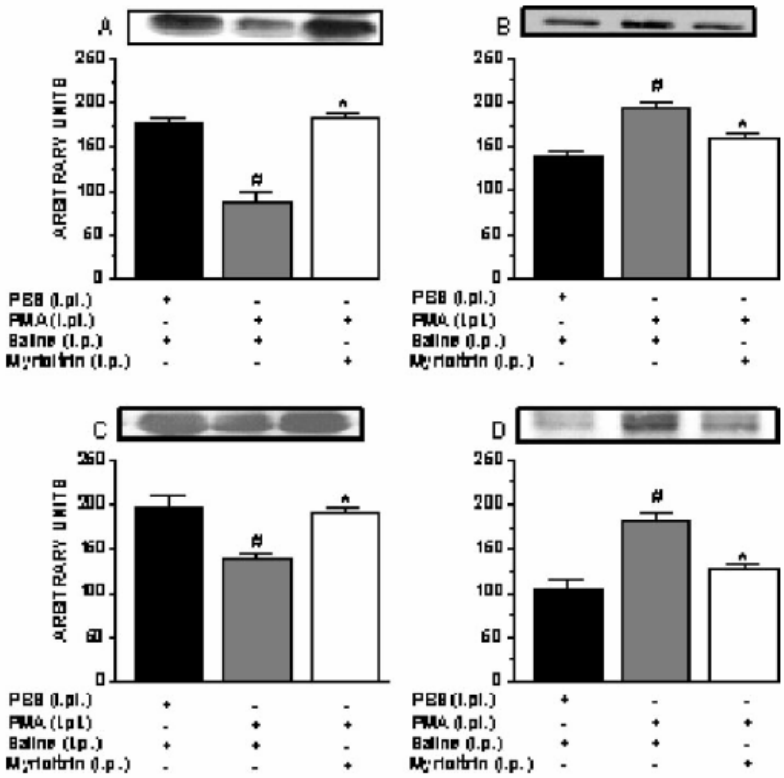

Fig. 8. Western blots showing the inhibitory effect of myricitrin (1 mg/kg i.p.) in the translocation from cytosol (A and C) to membrane (B and D) of PKCα (top panel) and PKCε (bottom panel) in response to i.pl. injection of PMA into mice paw. Mice paw tissues were obtained from basal (PBS) or PMA injected. Membrane and cytosolic levels of PKCα and PKCε were determined using specific antibody. Results were normalized by arbitrarily setting the densitometry of the basal group and are expressed as mean ± S.E.M. ($n = 3$). #, $p < 0.05$ compared with basal values; and *, $p < 0.05$ compared with control groups (saline i.p. plus PMA i.pl.), one-way ANOVA followed by Student-Newman-Keuls test.

test. For this reason, myricitrin was examined for its possible inhibitory action in the Rotarod and open-field tests. In both tests, we could observe that there was no statistically significant interference in performance motor patterns at higher doses than those producing marked suppression of the writhing response.

Another interesting finding of the present study is the demonstration that myricitrin, given i.p., produced a dose-dependent inhibition of the nociceptive response caused by injection of glutamate into the mouse hind paw. Glutamate nociception seems to involve peripheral, spinal, and supraspinal sites of action, and it is greatly mediated by both *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate receptors, as well as by the release of nitric oxide and nitric oxide-related substances (Beirith et al., 2002). Thus, these previous findings and data of the present results may indicate, at least in part, that the antinociceptive action of myricitrin could be associated with its ability to inhibit NO production or through interaction with the glutamatergic system.

Results of the present study also strongly suggest the involvement of protein kinase C, but not protein kinase A, in the antinociception caused by myricitrin. This notion derived from the data showing that i.p. administration of myricitrin dose-dependently inhibited the overt nociception by intraplantar PMA injection (a protein kinase C activator). Another piece of evidence that supports this view was the results demonstrating that myricitrin suppressed the mechanical hyperalgesia induced by bradykinin but not that induced by prostaglandin E_2 in rats. Some studies propose that, in nociceptor, bradykinin binds to the B_2 receptor, causing a direct activation of protein kinase C and the indirect activation of the protein kinase A (Ferreira et al., 2004). Furthermore, the results of the current study show that myricitrin, at a dose that abolished bradykinin-induced mechanical hyperalgesia, was not able to inhibit epinephrine-induced mechanical hyperalgesia. It has been suggested that mechanical hyperalgesia produced by epinephrine depends on the activation of both PKC and PKA (Khasar et al., 1999). Thus, we can speculate that epinephrine produced a powerful hyperalgesic effect by acting in the β-adrenergic receptor via cAMP/PKA independent of PKC second messenger pathways.

Of note, mice treated with myricitrin did not show PKC activation, as indicated by Western blot analyses. PKC activity requires an intracellular translocation from cytosol to cytoskeletal and membrane sites of action. Thus, translocation of PKC from a cytosolic to a membrane-associated location within the cell is a sensitive indicator of activation (Ferreira et al., 2005). In accordance with previous results (Ferreira et al., 2005), we found that i.pl. injection of PMA induced the translocation of a classic (PKCα) and, to a lesser extent, a novel protein kinase C (PKCϵ) isoforms from cytosolic to membrane. The present results show that myricitrin given systemically was able to prevent the activation of both PKC isoforms. Taken together, these results strongly suggest that the antinociceptive effect of myricitrin in PMA-induced nociception is closely related to inhibition of PKC. In fact, experimental evidence now indicates that flavonoids, such as myricitrin, can inhibit phosphoinositide 3-kinases, and, consequently, they inhibit protein kinase C isoenzyme activation (Gamet-Payrastre et al., 1999). However, flavonoids can also inhibit PKC directly (Agullo et al., 1997). The major structural characteristics in potent phosphoinositide 3-kinases and PKC inhibition by flavonoids are the presence of the 3′,4′ OH group on the B ring (Gamet-Payrastre et al., 1999), and myricitrin shares this condition.

Another interesting result of the present study was the demonstration that the L-arginine-nitric oxide pathway is likely to be involved in the antinociception caused by myricitrin. This conclusion derives from the fact that the pretreatment of mice with the substrate of nitric-oxide synthase, L-arginine, at a dose that produced no significant effect on acetic acid-induced pain, significantly reversed the antinociception caused by both myricitrin and L-NOARG (a known nitric oxide inhibitor). In marked contrast, the pretreatment of animals with the inactive isomer of L-arginine, D-arginine, had no significant effect against both myricitrin- and L-NOARG-induced antinociception. In agreement with these findings, it has been reported that myricitrin inhibits NO production and reduces the overexpression of inducible nitric-oxide synthase in RAW264.7 cells (Chen et al., 2000). Our data also demonstrate that the activation of the opioid naloxone-sensitive pathway is probably not involved in the antinociception produced by myricitrin because naloxone significantly reversed morphine, but not myricitrin, antinociception in the acetic acid test.

Previous studies have demonstrated the involvement of vanilloid receptor (TRPV1) in acetic acid-induced writhing (Ikeda et al., 2001). Hence, TRPV1 is stimulated by capsaicin, heat and pH alterations (Julius and Basbaum, 2001). Capsaicin activates TRPV1, which, in turn, induces membrane depolarization and increases cation influx, leading to noxious stimulus (Julius and Basbaum, 2001). Capsaicin administration in newborn mice (48 h old) causes persistent desensitization due to a nonselective loss of small sensory fibers, mostly C fibers (Jancsó et al., 1977). Our results confirm these observations by demonstrating that neonatal treatment of mice with capsaicin significantly blocked the writhing responses induced by acetic acid. However, the capsaicin newborn treatment did not significantly modify myricitrin-induced antinociception. Furthermore, the i.p. administration of myricitrin produces partial, but significant, inhibition of capsaicin-induced neurogenic nociceptive behavior on mouse hind paw. Capsaicin is known to evoke the release of neuropeptides, excitatory amino acids, nitric oxide, and proinflammatory mediators from the peripheral C fibers, and transmits nociceptive information to the spinal cord or causes spinal sensitization through protein kinase C and A activation (Calixto et al., 2005). Thus, the ability of myricitrin to interact with PKC might explain, at least partially, its antinociceptive effect on acetic acid-, glutamate-, capsaicin-, and PMA-induced nociceptive responses. In addition, it has been demonstrated that inhibitors of PKC prevent the phosphorylation of TRPV1, reducing the sensitization of this capsaicin sensitive receptor (Prekumar and Ahern, 2000; Ferreira et al., 2004; Calixto et al., 2005), thus, making it less responsive to agonist action.

In conclusion, the present results provide convincing evidence indicating that myricitrin, a flavonoid occurring naturally and widespread in higher plants, produces systemic antinociception when assessed in chemical models of nociception in mice, as well as producing antihyperalgesic effects in models of painful mechanical hypersensitivity in rats, at

doses that reveal no interference with locomotor activity. The precise mechanisms through which myricitrin exerts its action are currently under investigation, but inhibition of the L-arginine-nitric oxide and blockade of the PKC pathways seems largely to account for myricitrin antinociceptive effect. However, the opioid system, unmyelinated C fibers sensitive to capsaicin and PKA pathway, seem unlikely to participate in the antinociception caused by myricitrin.

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Artigo 2

FURTHER ANALYSIS OF THE ANTINOCICEPTIVE ACTION OF THE FLAVONOID MYRICITRIN: INVOLVEMENT OF THE K^+ AND Ca^{2+} CHANNELS.

Submetido ao Neuropharmacology

Further analysis of the antinociceptive action of the flavonoid myricitrin: involvement of the K^+ and Ca^{2+} channels.

Antinociceptive action of the flavonoid myricitrin

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Abstract

The present study was designed to investigate further the mechanisms involved in the antinociceptive action of myricitrin in chemical behavioral models of pain in mice. Myricitrin given by intrathecal (i.t.) or intracerebroventricular (i.c.v.) route produced dose-related antinociception when assessed on acetic acid-induced visceral pain in mice. In addition, the i.p. administration of

myricitrin caused significant inhibition of the biting behavior induced by i.t. injection of glutamate, substance P (SP), capsaicin, interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α). The antinociception caused by myricitrin in the acetic acid test was fully prevented by i.t. pretreatment with a $G_{i/o}$ protein inactivator, pertussis toxin, and by i.c.v. injection of calcium chloride ($CaCl_2$). In addition, the i.p. pretreatment of mice with the ATP-gated K^+ channel blocker, glibenclamide, prevented partially, but significantly the myricitrin induced-antinociception in the acetic acid test. Calcium uptake analysis (*in vitro*) revealed that myricitrin inhibited $^{45}Ca^{2+}$ influx under a K^+-induced depolarization condition; however, calcium transport was modified in a non-depolarizing condition only at the highest concentration used. In accordance with our previous findings, these results indicate that myricitrin produces consistent antinociception in chemical models of pain in mice. The observations in this study clearly demonstrate the participation of a $G_{i/o}$ protein dependent mechanism, with opening of ATP-gated K^+ channels and reduction of calcium influx in the myricitrin-induced antinociception.

Key words: myricitrin, antinociception, calcium transport, K^+ channels, protein Gi/o.

1. Introduction

The sensation of pain alerts us to real or impending injury and triggers appropriate protective responses. Unfortunately, pain often outlives its usefulness as a warning system and instead becomes chronic and debilitating (Julius and Basbaum, 2001). In this context, the search for drugs that might be useful in the control of pain has become crucial.

Pain control can be achieved at peripheral, spinal and/or supraspinal levels. Pain transmission can begin following a peripheral stimulus where all primary sensory nociceptors make synaptic connections with neurons in the dorsal horn of the spinal cord. Subsets of dorsal horn neurons, in turn, project axons and transmit

pain messages to higher brain centers, including the reticular formation, the thalamus and ultimately the cerebral cortex (Basbaum and Jessel, 2000). The recent application of molecular biology to sensory physiology and pain research has led to the discovery of multiple mediator molecules within the nociceptor. Thus, tissue damage can result in activation of nociceptors through the release of several mediators, including excitatory amino acids, peptides, protons, lipids and cytokines, which bind to receptors and activate signaling pathways, among these protein kinases A and C, calcium/calmodulin-dependent protein kinase, and mitogen-activated protein kinases (MAPKs) (Ji and Strichartz, 2004). Taking this into account, pain can be subject to multiple levels of biochemical and pharmacological controls, involving a diversity of cell types and soluble mediators (Basbaum and Jessel, 2000, Julius and Basbaum, 2001, Ji and Strichartz, 2004).

In this context, we have previously demonstrated several important properties of myricitrin (Fig 1), a naturally occurring flavonoid that is widespread in higher plants, reporting its antioxidant effect (Luo et al., 2002), anti-mutagenic and free radical scavenger action (Edenharder and Grünhage, 2003; Yasukawa et al., 1990), as well as its ability to inhibit the nociceptive response induced by several chemical mediators, mainly by inhibition of protein kinase C (PKC) and nitric oxide (NO) pathways (Meotti et al., 2006). In agreement, previous reports showed that myricitrin is able to inhibit NO production, NO synthase (iNOS) over expression, NF-κB activation, PI 3-kinase and PKC activities (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Chen et al., 2000). In addition, myricetin (aglycone metabolite from myricitrin) inhibits MAPKs phosphorylation (Ko et al., 2005).

Regarding the biological activities of myricitrin and its ability to increase the pain threshold, the aim of the present study was to further investigate the mechanisms involved in the antinociceptive action of this flavonoid. In addition, we

investigated the antinociceptive effects of myricitrin when administered at spinal and supra-spinal levels.

2. Materials and methods

2.1. Animals

Experiments were conducted using adult Swiss mice (25 - 35g) of both sexes and adult male Wistar rats (200 – 250g), housed at 22 $\pm$ 2°C under a 12-h light/12-h dark cycle (lights on at 6:00) and with access to food and water *ad libitum*. Male and female mice were homogeneously distributed among groups, and all animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified by Zimmermann (1983). The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Abdominal constriction induced by acetic acid

The abdominal constrictions were induced according to procedures described previously (Collier et al., 1968) and resulted in the contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal (i.p.) injection of acetic acid (0.6 %, 0.45 ml/mouse) at the time of the test. Mice were lightly anesthetized with ether and a volume of 5 µl of sterile PBS alone or containing myricitrin (0.1 - 10 µg/site) was injected directly into the lateral ventricle (i.c.v.; coordinates from bregma: 1 mm lateral, 1 mm rostral, 3 mm vertical) or between the L5 and L6 vertebrae (i.t.) using a microsyringe connected to polyethylene tubing, as described previously (Laursen and Belknap, 1986; Hylden and Wilcox, 1980). The mice were treated with PBS or myricitrin 10 min before acetic acid injection. After the challenge, the mice were individually placed into glass cylinders of 20 cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min.

2.3. Algogen-induced overt nociception in mice

To test the hypothesis that glutamate, SP, capsaicin and pro-inflammatory cytokines might be involved in the myricitrin antinociception, we examined myricitrin's effects upon these algogens. Animals received an i.p. injection of myricitrin (30 mg/kg) 30 min before i.t. injection of 5 μl of drug. Injections were given to awake animals using the method described by Hylden and Wilcox (1980). Briefly, the animals were restrained manually and a 30-gauge needle, attached to a 50-μl microsyringe, was inserted through the skin and between the vertebrae into the subdural space of the L5-L6 spinal segments. Injections were given over a period of 5 s. The nociceptive response was elicited by glutamate (30 μg/site) (Scheidt et al., 2002); SP (135 ng/site) (Sakurada et al., 1990); capsaicin (30 ng/site) (Sakurada et al., 1996); IL-1β (1 pg/site) and TNF-α (0.1 pg/site) (Choi et al., 2003) with minor modifications. A group of mice received vehicle (PBS) by i.t. route. The amount of time the animal spent biting was evaluated following local post-injections of one of the following agonists: glutamate (3 min after); SP and capsaicin (6 min); IL-1β, TNF-α and PBS (15 min). A bite was defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with the target organ.

2.4. Analysis of the mechanism of action of myricitrin

2.4.1. Participation of $G_{i/o}$ protein

In an attempt to provide more direct evidence concerning the possible participation of $G_{i/o}$ proteins sensitive to pertussis toxin in the antinociceptive action of myricitrin, mice were pretreated with pertussis toxin (0.5 μg/site) by intrathecal route (i.t) or with PBS (5 μl/site). Seven days after the pretreatment mice received myricitrin (1 mg/kg, i.p.), morphine (5 mg/kg, s.c) or vehicle (10 mL/kg); after 30 min, the animals were injected with 0.6% acetic acid. The number of abdominal constrictions was recorded during the 20 min following acetic acid administration.

2.4.2. Involvement of ATP-gated K^+ channels in the antinociceptive action of myricitrin

We next investigated the possible role played by ATP-gated K^+ channels in the antinociceptive action of myricitrin. The animals received, 20 min before myricitrin, an injection of glibenclamide (2 mg/kg, i.p.) or saline (vehicle). The procedure was carried out as described previously (Santos et al., 2005). Thirty minutes after myricitrin (1 mg/kg, i.p.) administration, the animals received 0.45 ml of acetic acid (0.6%) by i.p. route, and the number of abdominal constrictions was recorded over 20 min.

2.4.3. Involvement of calcium channels in the antinociceptive action of myricitrin

To investigate the possible participation of calcium channels in the antinociceptive effects of myricitrin, mice received 5 µl of $CaCl_2$ (200 nmol/site) or PBS (vehicle) by i.c.v. route (Liang et al., 2003). The i.c.v. injection was carried out as described above (section 2.3). After 10 minutes animals were treated with myricitrin (1 mg/kg, i.p.), morphine (2.5 mg/kg, s.c.) or saline (10 ml/kg). Thirty min following drugs administration, the animals received 0.45 ml of acetic acid (0.6%) by i.p. route, and the number of abdominal constrictions was recorded over 20 min.

2.5. Modulation of $^{45}Ca^{2+}$ influx by myricitrin

To investigate the possible myricitrin effects upon calcium transport we assessed the $^{45}Ca^{2+}$ influx into cortical slices of rats. $^{45}Ca^{2+}$ uptake was measured essentially as described by Eason and Aronstam (1984), with some modifications. Two salt solutions were used in these studies: (1) Krebs buffer containing 127mM NaCl, 1.2mM Na_2HPO4, 0.44mM KH_2PO_4, 0.95mM $MgCl_2$, 0.70mM $CaCl_2$, 10mM glucose, and 0.50mM Hepes, pH 7.4 with KCl 5.36mM for baseline or 80mM for K^+-stimulated assay; (2) Lanthanium solution containing 127 mM NaCl, 0.95 mM $MgCl_2$, 10 mM $LaNO_3$, 10 mM glucose, and 0.60 mM Hepes, pH 7.4 with KCl 5.0 mM for baseline or 80 mM for K^+-stimulated assay. To measure $^{45}Ca^{2+}$ uptake, rats were killed by decapitation, the cerebral cortex was dissected, isolated and the parietal cortex was cut into 400 µm slices, which were washed with Krebs buffer (solution 1). The slices (0.8–1.3 mg protein) were preincubated in 96-well

polycarbonate plates for 22 min at 32°C in the absence (control group) or presence of myricitrin (100 and 200 μM). Next, the slices were transferred to medium containing solution 1 plus 21 pmol of $^{45}Ca^{2+}$ and the uptake was monitored for 15 s at 32°C, after which the reaction was stopped by five washes of 2 min each with ice-cold lanthanium solution (solution 2). Immediately after washing, aliquots were lysed with 0.25 mL of a solution containing 0.5M NaOH plus 0.2% SDS and maintained at 60°C for 5 min. An aliquot was taken for determination of the intracellular calcium content by liquid scintillation counting. Nonspecific calcium uptake (20–30% of the total uptake) was determined by carrying out the same experiment using solution 2, which contained the nonspecific voltage-dependent calcium channel blocker lanthanium. Specific uptake was considered as the difference between total uptake and nonspecific uptake.

2.6. Protein measurement

The protein content of the synaptic membrane preparations and of cortical slices was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.7. Drugs

The following substances were used: morphine hydrochloride and acetic acid (Merck, Darmstadt, Germany); capsaicin, glutamic acid, cytokines (tumor necrosis factor-α and interleukin-1β), calcium chloride and phosphate-buffered saline (Sigma, St. Louis, USA); glibenclamide and substance P (Tocris Cookson Inc., Ellisville, USA); $^{45}Ca^{2+}$ was purchased from Amersham International, UK. All other chemicals were of analytical grade and obtained from standard commercial suppliers. Morphine was dissolved in 0.9% NaCl solution and the other drugs were dissolved in phosphate-buffered saline pH 7.0 (PBS), with the exception of myricitrin and glibenclamide, which were dissolved in Tween 80 plus saline, and capsaicin, which was dissolved in alcohol plus saline. The final concentration of Tween and alcohol did not exceed 5% and did not cause any effect “per se”. The myricitrin was isolated from genus *Eugenia* at the Department of Chemistry, Universidade Federal de Santa Catarina, Brazil, it was identified by spectral analyzes

(RMN-1H) and (RMN-13C) and by comparison with the spectrum literature data (Agrawal, 1989), it showed a degree of purity greater than 98%.

2.8. Statistical analysis

The results are presented as mean ± S.E.M., except the ID_{50} values (i.e., the dose of myricitrin reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined using three doses of myricitrin by linear regression from individual experiments using linear regression software (GraphPad software, San Diego, CA). Maximal inhibition values were calculated at the most effective dose used. Data were analyzed by one-way ANOVA followed by Newman-Keuls test, with the exception of the *in vitro* test of calcium uptake, which was analyzed by one-way ANOVA followed by Newman-Keuls test (potassium pretreatment and myricitrin treatment). The statistical differences were considered significant when $P < 0.05$.

3. Results

3.1. Spinal and supraspinal antinociception of myricitrin

The results show that myricitrin, given by i.c.v. or i.t. route 10 min prior to testing, produced dose-related inhibition of the acetic acid-induced abdominal constrictions in mice. The ID_{50} data (and their respective 95% confidence limits) and inhibition values, at a dose of 10 μg/site, are shown in Table 1. It can be seen that myricitrin, administered by either i.c.v or i.t. route, showed similar potency in preventing the nociception caused by acetic acid; however, i.c.v. administration was less efficacious than i.t. in this model of nociception (Table 1). Furthermore, the results demonstrate that myricitrin was very effective in decreasing nociception induced by acetic acid when given by supraspinal (i.c.v.), spinal (i.t.) or systemic (i.p.) routes (Meotti et al., 2006).

3.2. Algogen-induced overt nociception in mice

Intrathecal administration of glutamate, capsaicin, SP, IL-1β and TNF-α caused significant biting behavior in mice when compared to animals injected

intrathecally with PBS ($P<0.001$). The results (seconds) for the biting behavior assessment were: 7.75±3.6 for PBS; 183.1±21.3 for glutamate; 106.2±5.1 for capsaicin; 89.2±6.6 for SP; 116.1±20.8 for IL-1β and 124.1±17 for TNF-α. In all groups, systemic pretreatment with myricitrin (30 mg/kg, i.p.) significantly reduced the biting behavior when compared with mice treated with saline i.p. (control group). The greatest effect of myricitrin appeared against cytokines-induced nociception, for which the inhibition values were: TNF-α (100%); IL-1β (96±2%); SP (84±10%); capsaicin (61±9%) and glutamate (41±13%) (Fig. 2).

3.3. $G_{i/o}$ protein participation

Results presented in Fig. 3 show that inactivation of the $G_{i/o}$ protein, which was caused by an i.t. injection of pertussis toxin seven days before testing, completely abolished the antinociceptive effect of both myricitrin (1 mg/kg) and morphine (5 mg/kg) on the acetic acid-induced visceral pain model. Additionally, the pretreatment with pertussis toxin did not cause any effect “per se” on acetic acid-induced abdominal constriction. In this situation, we can identify an important role of the $G_{i/o}$ protein in the antinociceptive effect of myricitrin, as well as in the antinociceptive effect of morphine.

3.4. Involvement of ATP-gated K^+ channels in the antinociceptive action of myricitrin

The results presented in Fig. 4 show that pretreatment with glibenclamide (2 mg/kg, i.p.) partially, but significantly prevented the antinociceptive effect of myricitrin (1 mg/kg) on the acetic acid-induced visceral pain. Since glibenclamide is a blocker of ATP-gated K^+ channels, this suggests a contribution of this K^+ channel subtype to the myricitrin-induced antinociception. In addition, the same glibenclamide pretreatment did not present any effect “per se”.

3.5. Involvement of Ca^{2+} channels in the antinociceptive action of myricitrin

The results in Fig. 5 show that pretreatment with $CaCl_2$, by i.c.v. route, fully prevented the antinociceptive effect of myricitrin on acetic acid-induced visceral pain. Furthermore, the same pretreatment completely prevented morphine-induced antinociception; however, it did not cause any effect "per se".

3.6. $^{45}Ca^{2+}$ influx

Figure 6 presents myricitrin effects upon $^{45}Ca^{2+}$ uptake into cortical slices from rats. In this assay, the medium containing the higher K^+ concentration (depolarizing condition) led to an increase of 30.0±4.3% ($P<0.05$) in the $^{45}Ca^{2+}$ uptake, when compared to medium with a lower K^+ concentration (non-depolarizing condition). This corroborates *in vivo* antinociceptive results for myricitrin, which at concentrations of both 100 and 200 μM inhibited the $^{45}Ca^{2+}$ uptake in a K^+-induced depolarizing condition ($P<0.001$). The inhibition values were 46±13% and 48±11% for 100 and 200 μM, respectively. On the other hand, in the absence of the depolarizing condition, only the higher concentration of myricitrin was able to reduce $^{45}Ca^{2+}$ influx ($P<0.01$), with an inhibition value of 42±2%. A two-way ANOVA analyses revealed a significant interaction between the presence of myricitrin and the K^+-induced depolarizing condition [$F(2,12) = 4.54$ ($P<0.05$)], suggesting that myricitrin is more effective in an elevated excitability condition.

Discussion

A considerable number of studies have reported that flavonoids exert biological actions, including antioxidant, anti-inflammatory, immunomodulatory, anti-allergic, neuroprotective, anti-mutagenic anti-rheumatic and antinociceptive effects (Havsteen et al., 2002; Calixto et al., 2003; 2004; Meotti et al., 2006). In previous studies, we have demonstrated that systemic administration of myricitrin inhibits chemical models of nociception, including visceral pain induced by acetic acid, licking behavior induced by i.pl. injection of glutamate, capsaicin and phorbol 12-myristate 13-acetate (PMA) in mice. In addition, myricitrin raised the nociceptive threshold in the bradykinin-induced painful mechanical hypersensitivity in rats. Further studies in mice demonstrated that the

antinociceptive effects of myricitrin are due to its ability to inhibit of protein kinase C activationand nitric oxide production. On the other hand, the opioid system, sensitive unmyelinated C fibers and the PKA pathway appear unlikely to participate in the antinociception caused by myricitrin (Meotti et al., 2006).

In the present investigation, we extended the previous studies and demonstrated that spinal or supraspinal administration of myricitrin induces a dose-dependent inhibition of the acetic acid-induced visceral nociceptive response in mice. The acetic acid-induced abdominal constriction is a widely used model of inflammatory pain. The protons from acetic acid can directly activate non-selective cationic channels localized in peripheral afferent fibers (Julius and Basbaum, 2001). In addition, it has been suggested that acetic acid acts by releasing endogenous inflammatory mediators, including bradykinin, substance P, prostanoids, TNF-α, IL-1β, and IL-8, which stimulate the primary sensory neurons (Collier et al., 1968; Vineger et al., 1979; Ribeiro et al., 2000; Ikeda et al., 2001), raising glutamate and aspartate in the cerebrospinal fluid (Feng et al., 2003). Because of its low specificity, acetic acid-induced abdominal constriction is a model of pain that can be prevented by anti-inflammatory, analgesic, muscle relaxant and sedative drugs, which can cause antinociception by working at peripheral or central levels. To identify the location where myricitrin causes antinociception, we administered this flavonoid at systemic, spinal and supraspinal levels. Interestingly, myricitrin was effective in reducing acetic acid-induced pain when administrated by all routes.

To obtain more specific details of the mechanism by which myricitrin causes antinociception, we tested its effects on the biting behavior induced by the excitatory neurotransmitter glutamate; the neuropeptide SP; the TRPV1 agonist capsaicin; and the pro-inflammatory cytokines, IL-1β and TNF-α. Myricitrin systemic treatment potently decreased the biting behavior induced by SP (84%) and to a lesser extent that induced by capsaicin (64%) and glutamate (41%). These data reinforce the opinion on the subject of myricitrin's central effects since systemic administration of this flavonoid was able to reduce biting induced by spinal administration of different mediators.

In this way, a possible interaction between myricitrin and glutamatergic, peptidergic or vanilloid systems could explain, at least in part, myricitrin's antinociceptive effect. In addition, studies have provided evidence that SP, capsaicin and glutamate interact synergistically in the excitation of dorsal-horn nociceptive neurons and in the production of pain, through mechanisms that involve NO production (Liu et al., 1997; Caruso et al., 2005; Sakurada et al., 1996). In line with this, it is noteworthy that the biological effects of myricitrin are, in part, due to its ability in reducing NO levels (Chen et al., 2000; Meotti et al., 2006). As a result, this property could explain the antinociceptive action of myricitrin on the glutamate, SP, capsaicin and acetic acid-induced nocicetive behavior.

Likewise, the present study demonstrated that myricitrin, given intraperitoneally, produces a strong inhibition of the biting response caused by i.t. injection of cytokines, IL-1β and TNF-α. Since cytokines are small regulatory molecules that have well described roles in the inflammatory process (Sommer and Kress, 2004), a powerful inhibition of these polypeptides would elucidate, in part, the anti-inflammatory effects described for the flavonoids (Calixto et al., 2003; 2004). More recently, these polypeptides were reported to induce biting behavior when administered intrathecally (Choi et al., 2003). The binding of IL-1β to its receptor IL-1RI actives tyrosine kinases and calcium-independent PKC (Obreja et al., 2002), which could support, in part, the nociception caused by this peptide. In agreement with this statement, we have reported previously, through *in vivo* and *in vitro* studies, that myricitrin inhibits nociception mainly by its property of PKC inhibition (Meotti et al., 2006). These results together, strongly suggest that myricitrin's effects on IL-1β-induced nociception are due to its property of PKC inhibition.

Furthermore, our results demonstrate that myricitrin completely inhibited TNF-α-induced nociception. Previous reports show that TNF-α-induced nociception involves phosphorylation of p38 (Schäfers et al., 2003). In addition, myricetin has been demonstrated to inhibit MAPKs phosphorylation (Ko et al., 2005). In line with this, myricitrin appears to inhibit TNF-α-induced nociception, at least in part, by decreasing phosphorylation of some intracellular signaling kinases. On the other hand, the antinociceptive action of myricitrin on TNF-α-induced biting behavior could

be strictly related to myricitrin effects upon calcium channels, since recent reports showed that TNF-α activates Ca^{2+} mobilization in cultured sensory neurons (Pollock et al., 2002).

Beside these findings, the present study demonstrates that the inactivation of the $G_{i/o}$ protein, through i.t pertussis toxin treatment, completely prevented the antinociception induced by systemic administration of myricitrin. A similar result was found with the systemic morphine treatment. It is known that $G_{i/o}$ protein activation is responsible for the opening of K^+ channels (Koppen et al., 1996). In this investigation, we observed that pretreatment with glibenclamide, a blocker of ATP-gated K^+ channels, partially, but significantly prevented the myricitrin antinociceptive effect.

Another notable piece of evidence was that the calcium chloride pretreatment fully prevented the antinociception induced by myricitrin and morphine. It is well established that an increase in intracellular Ca^{2+} represents a key step for neurotransmitter release and modulation of cell membrane excitability, as well as activation of some intracellular proteins (Ward, 2004). Evidence has accumulated for the involvement of calcium ions and calcium channels in nociception (Prado, 2001; Cervero and Laird, 2003). Our *in vitro* assays, using rat cortical slices, demonstrated that myricitrin inhibited $^{45}Ca^{2+}$ influx under a K^+-induced depolarizing condition; however, myricitrin modified calcium transport in a non-depolarizing condition only at the highest concentration used. These results may indicate that myricitrin interacts in a distinct way with Ca^{2+} channels, depending on the state of cell depolarization. Considering that myricitrin-induced antinociception was prevented by calcium pretreatment (*in vivo*) and that this flavonoid altered calcium transport (*in vitro*), we can strongly suggest that the block of calcium channels countered the antinociceptive action of this flavonoid.

In conclusion, the present results are in agreement with our previous data and they demonstrate that myricitrin produces antinociception when administered at peripheral or central levels. In addition, this flavonoid inhibits nociception induced by intrathecal administration of glutamate, SP, capsaicin and cytokines. Furthermore, myricitrin antinociception is probably dependent on $G_{i/o}$ protein

activation; opening of ATP-gated K^+ channels; and inhibition of calcium influx. At this time, the myricitrin antinociceptive effects appear to occur by interaction of this flavonoid with calcium channels, ATP-gated K^+ channels, blockade of NO production and inhibition of PKC pathway.

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Legends

Figure 1: Chemical structure of myricitrin.

Figure 2: Effects of myricitrin (30 mg/kg, i.p.) on the nociceptive behavior induced by glutamate (GLU 35 μg/site); capsaicin (CAP 30 ng/site); substance P (SP 135 ng/site); IL-1β (1 pg/site) or TNF-α (0.1 pg/site). Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column, control groups) or in the presence (gray column) of myricitrin. The symbols denote significance levels: *$P<0.05$ and ***$P<0.001$ compared to control group, one-way ANOVA followed by Student's t-test.

Figure 3: Effect of pretreatment (seven days) of mice with pertussis toxin (0.5 μg/site, i.t.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and morphine (5 mg/kg, s.c.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of pertussis toxin. The symbols denote significance levels: ***$P<0.001$ compared to control group; #$P<0.001$ compared to group treated with morphine or myricitrin, one-way ANOVA followed by Newman-Keuls test.

Figure 4: Effect of pretreatment of mice with glibenclamide (2 mg/kg, i.p.) on the antinociceptive profile of myricitrin (1 mg/kg, i.p.) against the acetic acid-induced writhing in mice. Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of glibenclamide. The symbols denote significance levels: ***$P<0.001$ compared to control group; #$P<0.001$ compared to myricitrin i.p. group, one-way ANOVA followed by Newman-Keuls test.

Figure 5: Effect of pre-treatment of mice with $CaCl_2$ (200 nmol/site, i.c.v.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and morphine (2.5 mg/kg, s.c.) against the acetic acid-induced

writhing in mice. Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of $CaCl_2$. The symbols denote significance levels: ***P<0.001 compared to control group; #P<0.001 compared to group treated with morphine or myricitrin, one-way ANOVA followed by Newman-Keuls test.

Figure 6: Effect of myricitrin on the calcium influx into rat cerebral cortical slices. Rat cerebral cortical slices were incubated with 21 pmol of $^{45}Ca^{2+}$ in the presence or absence (control groups) of myricitrin (100 and 200 μM). The assays were carried out under non-depolarizing conditions (white column) and under depolarizing conditions (gray column). Values are the means of three experiments with each experimental value being the average of three replicates. Data are expressed as mean ± S.E.M. and the symbols denote significance levels: ##P<0.01 compared to control group (non-depolarizing conditions); ***P<0.001 compared to control group (depolarizing conditions), two-way ANOVA followed by Newman-Keuls test.

Table 1: Effect of supraspinal, spinal or systemic administration of myricitrin on acetic acid-induced visceral pain

	ID_{50}	IM (%)
Myricitrin i.c.v. (mg/site)	5.79 (2.04 – 16.41)	57 ± 5
Myricitrin i.t. (μg/site)	3.32 (1.53 – 7.2)	82 ± 9
Myricitrin i.p. ($mg.kg^{-1}$)[a]	0.33 (0.2 – 0.54)	84 ± 5

The ID_{50} values are reported as geometric means accompanied by their respective 95% confidence limits, using three dosages of myricitrin (n=6 per group). Maximal inhibition values were calculated at 10 μg/site, by i.c.v. and i.t. routes or 10 mg/kg by i.p. route. [a]Data from Meotti et al., 2006

Figure 1

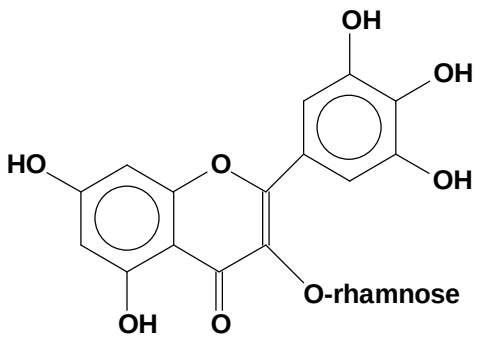


Figure 2

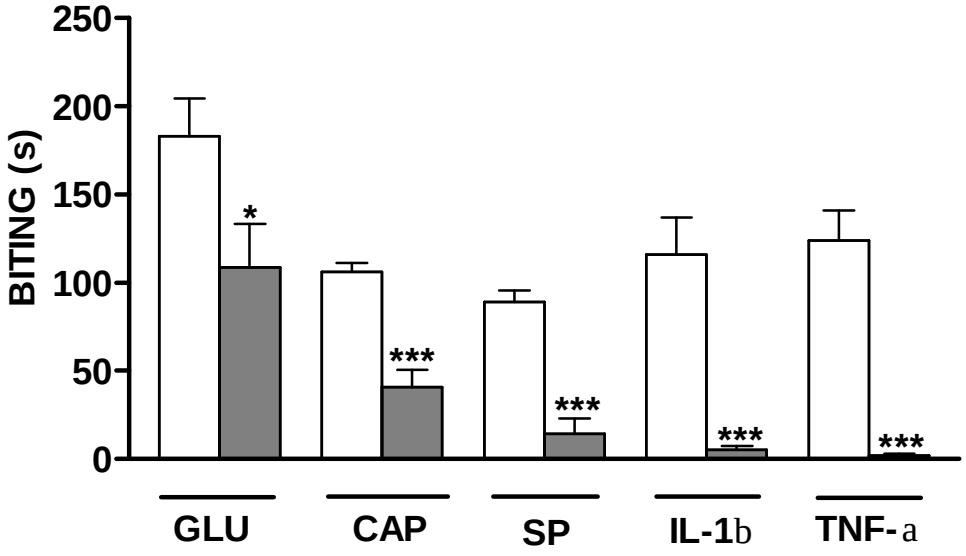


Figure 3

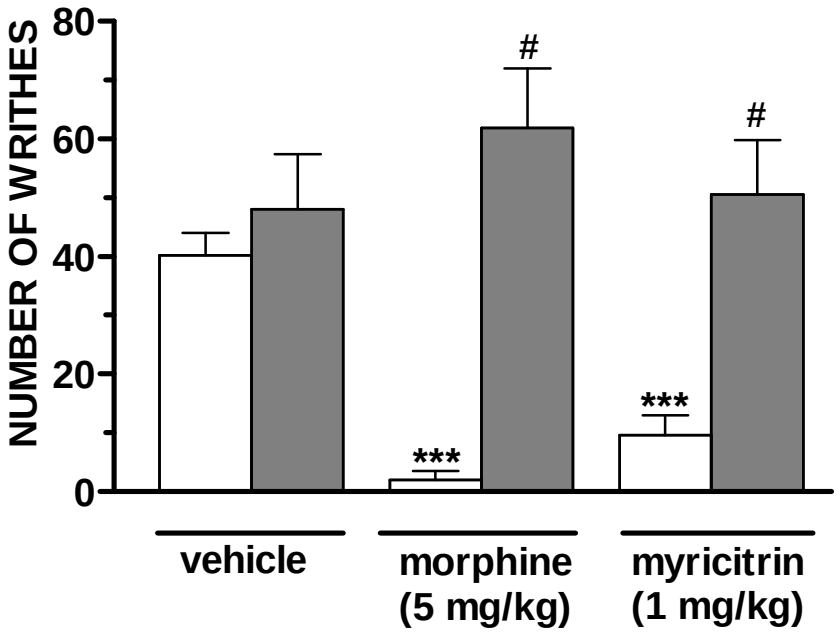


Figure 4

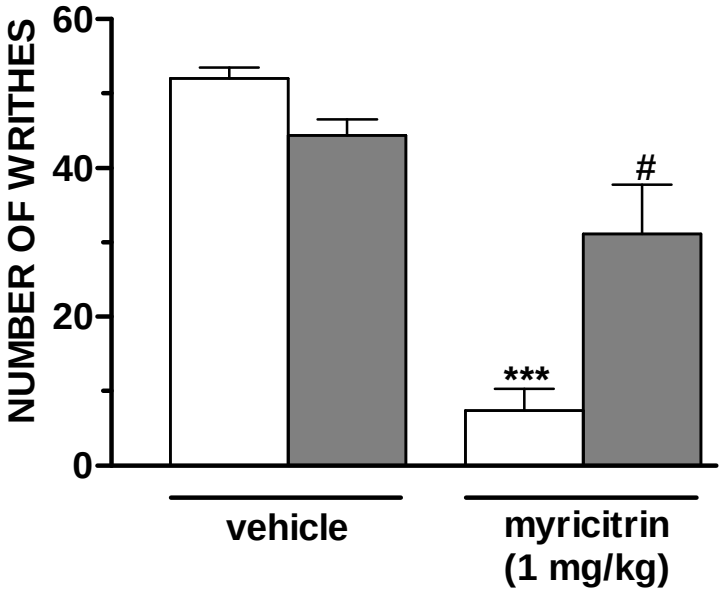


Figure 5

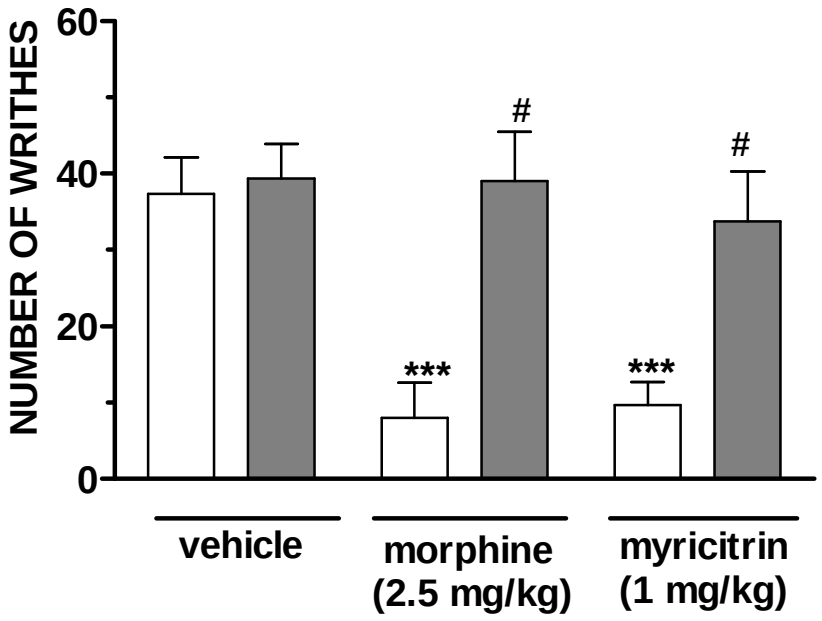
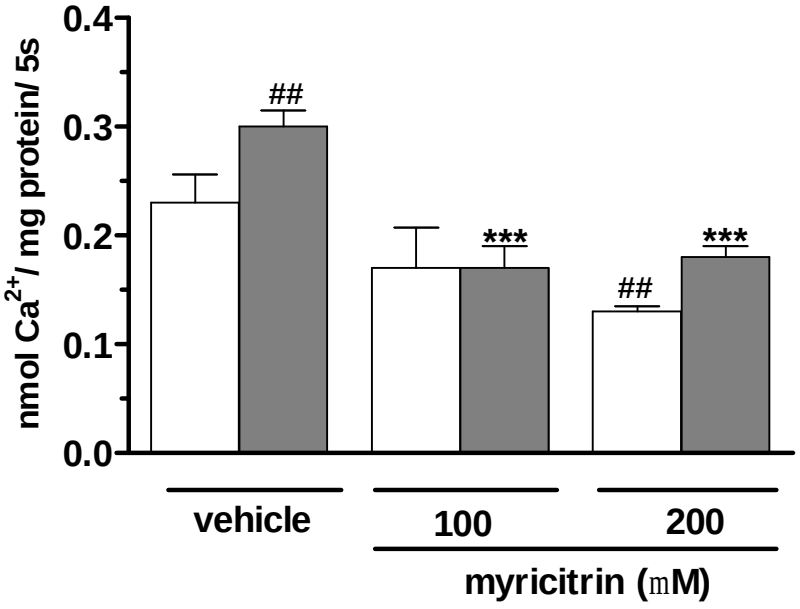


Figure 6



4.2. Efeito da miricitrina sobre a nocicepçao crônica em um modelo de dor neuropática e inflamatória

Artigo 3

ANTINOCICEPTIVE PROPERTIES OF THE FLAVONOID MYRICITRIN IN MODELS OF PERSISTENT INFLAMMATORY AND NEUROPATHIC PAIN

Submetido ao Biochemical Pharmacology

Antinociceptive property of flavonoid myricitrin in models of persistent inflammatory and neuropathic pain in mice

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Abstract

The aim of the present study was to investigate the myricitrin effects upon persistent neuropathic and inflammatory pain. Since, previous studies have ever demonstrated that myricitrin presents antinociceptive effects in several models of acute pain. The neuropathic pain was caused by a partial tying (2/3) of sciatic nerve and the inflammatory pain was induced by an intraplantar (i.pl.) injection of 20 µl of Freund's Complete Adjuvant (FCA) in adult Swiss mice (25-35g). Seven days after sciatic nerve constriction and 24 h after FCA i.pl. injection, mice pain threshold was evaluated through tactile allodynia, using Von Frey Hair (VHF) filaments. Further analyses performed in mice FCA injected were paw oedema measurement, leukocytes infiltration, morphological changes and myeloperoxydase (MPO) enzyme activity. The myricitrin antioxidant property was evaluated by lipid peroxidation assay in liver of rats. The intraperitoneal (i.p.) treatment with myricitrin (30 mg/kg) significantly decreased the paw withdrawal response in persistent neuropathic and inflammatory pain and decreased mice paw oedema. FCA injection increased 4-fold MPO activity and 27-fold the number of neutrophils in to paw of mouse after 24 hours. Myricitrin strongly reduced MPO activity, returning at basal levels, however, it did not reduce neutrophils migration. In addition, myricitrin treatment decreased morphological alterations on epidermis and dermis papilar of mice paw. Myricitrin showed to be a potent antioxidant, decreasing lipid peroxidation induced by $FeCl_2$/EDTA with IC_{50} of 45.0 µM. Together these results indicate that myricitrin produces pronounced anti-allodynic and anti-oedematogenic action in two models of chronic pain in mice. Considering that few drugs are currently available for the treatment of chronic pain, especially of the neuropathic type, the present results indicate that myricitrin might be potentially interesting in the development of new clinically relevant drugs for the management of this disorder.

Key words: myricitrin, allodynia, neuropathic pain, inflammatory pain, antioxidant.

Introduction

Pain is a sensation we have all experienced. For most of us, the pain has been temporary. However, for patients with pathological pain, the hurt experience is unending, with little hope for therapeutic relief. While nociceptive pain (physiological pain) requires intense, high-threshold stimulation and is typically transient, pathological pain (clinical pain), associated with inflammation of peripheral tissue that arises from the initial damage (inflammatory pain) or from lesions to the nervous system (neuropathic pain), is often persistent (Woolf and Mannion, 1999; Zimmermann, 2001; Ji and Strichartz, 2004). In addition, currently available drugs which provide relief of neuropathic and inflammatory pain are effective only in a fraction of such patients. In general, these drugs present low efficacy and a lot of side effects (Woolf and Mannion, 1999; Mendell and Sahenk, 2003).

Neuropathic pain is usually difficult to treat because the etiology is heterogeneous and the underlying pathophysiology is complex. Because no universally efficacious therapy exists for it, neuropathic pain research has been explored with different animal models where intentional damage is done to the sciatic nerve, branches of spinal nerves or in the spinal cord (Seltzer et al., 1990; Malmberg and Basbaum, 1998; Zimmermann, 2001; Ji and Strichartz, 2004). In animal models of neuropathic pain, the extent of hyperalgesia is related to the extent of the inflammatory response at the site of injury (Clatworthy et al., 1995) and anti-inflammatory agents alleviate hyperalgesia in nerve-injured rats (Clatworthy et al., 1995; Bennett, 2000).

In this context, drugs that decrease the inflammatory condition could be successful applied in certain chronic pain state. Taking this into account, several works have described the powerful anti-inflammatory activity of flavonoids (Middleton et al., 2000; Havsteen et al., 2002; Calixto et al., 2003; 2004). Hence, these compounds are broadly distributed in higher plants and known by their antioxidant; anti-inflammatory; immunomodulatory; anti-diabetic; anti-allergic; anti-cancer;

hepatoprotective; neuroprotective; antinociceptive and anti-rheumatic properties (Middleton et al., 2000; Birt et al., 2001; Havsteen et al., 2002; Calixto et al., 2003; 2004; Meotti et al., 2006).

Additionally, we have previously demonstrated that myricitrin, a flavonoid that belongs to flavonol sub-group, was able to inhibit nociceptive response in models of acute pain (Meotti et al., 2006). The myricitrin effects have been attributed, mainly, to the inhibition of PI 3-kinase and PKC activities, NO production, nitric oxide synthase (iNOS) over expression and NF-κB activation, (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Chen et al., 2000; Meotti et al., 2006). More recently, myricitrin showed a potent inhibition of calcium transport, *in vitro*, besides to its *in vivo* effects (data still not published).

Regarding these previous findings, we might suggest that myricitrin is a good candidate for relieve of both, neuropathic and inflammatory, pain. On the other hand, chronic pain differs substantially from acute pain in terms of its persistence and in relation to adaptive changes (Besson, 1999; Woolf and Mannion, 1999; Zimmermann, 2001). In this concern, is noteworthy the rationale research for the myricitrin actions upon chronic pain. Therefore, the present study was designed to investigate the myricitrin antinociceptive effects in models of neuropathic and inflammatory chronic pain. The neuropathic pain was caused by a partial constriction of sciatic nerve and the inflammatory pain was induced by FCA. FCA consists of heat-killed mycobacteria suspended in a mineral oil vehicle, which produces a chronic model of inflammatory condition in rodents (Larson et al., 1985). The mice pain threshold was evaluated through tactile allodynia, using Von Frey Hair (VHF) filaments, and further analyses performed were paw oedema measurement, leukocytes infiltration, morphological changes and MPO enzyme activity into injured paw. In addition, the antioxidant potential of myricitrin was assessed by the *in vitro* lipid peroxidation.

2. Materials and methods

2.1. Animals

Adult female Swiss mice (25 - 35g) and Wistar male rats (200 - 300g) were kept in an automatically-controlled temperature room (23 $\pm$ 2°C) in 12 h light-dark cycles. Food and water were freely available. The experiments reported were carried out in accordance with the current

guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified by Zimmermann (1983), and all experiments were approved by our Ethics Committee for animal use. The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Mechanical allodynia induced by partial sciatic nerve injury

Mice were anesthetized with 7% chloral hydrate (6 ml/kg, i.p.). A partial ligation of the sciatic nerve was performed by tying the distal (2/3) of the sciatic nerve, according to the procedure described in rats (Seltzer et al., 1990) and adapted to mice (Malmberg and Basbaum, 1998). In sham-operated mice, the nerve was exposed using the same procedure, but without ligation. Mice injured nerve did not present paw drooping or autotomy.

The mechanical allodynia was measured as described before (Bortolanza et al., 2002), as the withdrawal response frequency to 10 applications of 0.6 g Von Frey Hair Filaments (VFH, Stoelting, Chicago, USA). Mice were further acclimatized in individual clear Plexiglas boxes (9×7×11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The frequency of withdrawal was determined before nerve injury (baseline), in order to obtain data purely derived from nerve injury-induced allodynia. The operated mice received myricitrin (30 mg/kg; i.p) or vehicle seven days after surgery. The withdrawal response frequency was recorded immediately before (0) and after treatment (0.5, 2, 4, 6 and 24 h).

2.3. FCA-induced inflammation and mechanical allodynia

Mice were lightly anaesthetized with ether and received 20 µL of FCA–Freund's complete adjuvant (1 mg/ml of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate) subcutaneously in the intraplantar surface of the right hind paw (ipsilateral paw).

Twenty four hours after FCA injection the mice were treated with myricitrin (30 mg/kg, i.p) or vehicle. Effects were evaluated against the paw oedema and the mechanical allodynia. Paw oedema was measured by use of a plethysmometer (Ugo Basile) at several time-points (0, 0.5, 2, 4 and 8 h) and was expressed (µL) as the difference between paw volume before (baseline) and subsequent to FCA-injection, the difference indicated the degree of inflammation.

The mechanical allodynia was measured as described before (Bortolanza et al., 2002), as the withdrawal response frequency to 10 applications of 0.6 g von Frey filaments (VFH, Stoelting, Chicago, USA). The frequency of withdrawal was determined before (baseline) FCA injection, in order to obtain data purely derived from the treatments in FCA allodynia. The mechanical allodynia was recorded immediately before (0) and after (0.5, 2, 4 and 8 h) myricitrin treatment.

2.4. Myeloperoxidase assay

The neutrophil infiltration and activation was evaluated indirectly by measuring the myeloperoxidase (MPO) activity, as previously described (Bradley et al., 1982). The experiments were carried out 24 h after FCA i.pl. injection. The animals were subdivided into four groups (n=5/group): **1)** vehicle (saline) i.p. plus PBS i.pl; **2)** vehicle (saline) i.p. plus FCA i.pl.; **3)** and **4)** myricitrin (30 mg/kg) i.p. plus FCA i.pl. Groups 1, 2 and 3 were pre-treated with vehicle or myricitrin i.p., 30 min before PBS or FCA i.pl. injection, whereas, group 4 received myricitrin i.p. 22 h after FCA i.pl. injection.

Following 24 hours from FCA injection, the animals were killed and the subcutaneous tissue of the footpad injected was removed and placed in an eppendorf tube containing 0.75 ml of 80 mM sodium phosphate buffer (pH 5.4) and

0.5% hexadecyltrimethyl ammonium (HTAB). Enzyme assay was carried out as described (Bradley et al., 1982). The reaction product was determined colorimetrically using an ultra microplate reader (absorbance 652 nm), with a molar absorption coefficient of 3.9 x10^4 for 3,3',5,5' tetramethylbenzidine (TMB) salt.

2.5. Histopathological analysis and stereology

In the histopathological analysis animals were subdivided into four groups (n=5/group). The schedule of treatment was the same allows to MPO assay (described above). Twenty four hours after FCA or PBS i.pl. injection, the mice were killed and the injected paw was cut longitudinally into equal halves. The distal proximity was fixed in 4% paraformaldehyde and medial proximity was fixed in a Zenker solution ($HgCl_2$ plus $K_2Cr_2O_7$) by 24 h. The tissues were rinsed, dehydrated and paraffin-embedded. They were sectioned at 5 μm thickness using a rotary microtome. The distal proximity was stained with hematoxylin-eosin and observed by light microscopy using an objective 40x to examine morphological alterations. The medial proximity was stained by May-Grünwald-Giemsa and a representative area of inflammatory cellular response (reticular dermal and hypodermal layers) was selected for quantitative cell counting, using a light microscopy (objective 100x) coupled to camera. The number of neutrophils, eosinophils (polymorphonuclears); lymphocytes, macrophages, and mastocytes was quantified in 20 fields using cycloid test-system, as described by Mandarim-de-Lacerda (1999; 2003). The results are expressed as the mean ± SEM of the number of total cells in an area of 1 mm^2.

2.6. Induction of lipid peroxidation and thiobarbituric acid reactive substances (TBAR'S) determination

$FeCl_2$ and EDTA are used as classical inductors of lipid peroxidation (Gutteridge et al., 1979; Braughler et al., 1988). Animals were decapitated and liver was rapidly homogenized in 50 mM Tris/HCl, pH 7.5 (1/10, w/v) and centrifuged at 4000 x g for 10 min. An aliquot of the liver homogenate (200 μL) was incubated at 37°C in the presence of 100 μM $FeCl_2$, 50 μM EDTA and

myricitrin (0.01 – 1 mM) or vehicle (DMSO) for 1 h. TBAR'S was determined as described by Ohkawa et al. (1979) with minor modifications (Meotti et al., 2004).

2.7. Drugs

The following substances were used: hexadecyltrimethyl ammnonium bromide (HTAB), Freund's complete adjuvant (FCA), 3,3',5,5' tetramethylbenzidine (TMB) (Sigma, St. Louis, USA); chloral hydrate, dimetilformamide and stain reagents May-Grünwald-Giemsa and hematoxylin-eosin were purchased from Vetec (Rio de Janeiro, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Drugs were dissolved in 0.9% of NaCl solution, with the exception of TMB, which was dissolved in dimetilformamide. Myricitrin was dissolved in tween 80 plus saline. In the *in vitro* lipid peroxidation assay myricitrin was dissolved in dimetilsulfoxide (DMSO) plus saline. The final concentration of tween or DMSO did not exceed 10% and did not cause any effect "per se". The myricitrin dose (30 mg/kg) was chosen based on Meotti et al. (2006). Myricitrin was isolated from the plant of genus *Eugenia* in the Department of Chemistry, Federal University of Santa Catarina, Brazil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure (Agrawal, 1989). The chemical purity of myricitrin (more than 98%) was determined by GC/HPLC.

2.8. Statistical analysis

The results are presented as mean ± S.E.M., except the IC_{50} values (i.e., concentration of myricitrin reducing the lipid peroxidation by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The IC_{50} values were calculated using at least three concentrations of myricitrin, determined by linear regression from individual experiments using linear regression software (GraphPad software, San Diego, CA). Maximal inhibition values were calculated at the most effective dose used. The statistical significance of differences between groups was performed by ANOVA followed by Student-Newman-Keuls multiple comparison test. P-values less than 0.05 ($P<0.05$) were considered as indicative of significance.

3. Results

To evaluate myricitrin effects upon neurophatic pain model, we made a partial sciatic nerve ligation in mice. This injury produced a marked development of allodynia on the ipsilateral side seven days after nerve injury procedure (Fig. 1). The acute treatment with myricitrin (30 mg/kg, i.p.) significantly decreased the paw withdrawal response following 30 min from its administration (60 ± 8%), and this effect was kept for 4 h after myricitrin treatment (Fig. 1).

After, we investigated the myricitrin effects on inflammatory pain model, through immunologic reaction induced by i.pl. injection of FCA. The i.pl. injection of FCA produced a profound mechanical allodynia and paw volume enhancement, which were kept during all test. The animals that received myricitrin (30 mg/kg) demonstrated a reduction on mechanical allodynia induced by FCA. This sensitivity reduction started 30 min after myricitrin administration and was maintained for up 4 hours. The more pronounced effect was observed at 30 min, where myricitrin inhibition achieved 50±8%. In addition, at 4 h animal's sensibility was similar to baseline values (Fig. 2A). Furthermore, FCA injection caused an increase on paw volume 24 h after its administration. This effect was significantly reverted by myricitrin (30 mg/kg) beginning 2 hours after treatment. The mainly effect was observed at 2 hours with paw oedema reduction of 25±7% (Fig. 2B).

Regarding the anti-allodynic and anti-oedematogenic effects of myricitrin on mechanical allodynia and paw edema induced by FCA, we analyzed MPO activity on mice paw. MPO activity, at injured tissue, reflects neutrophils infiltration and degranulation since this enzyme is the most abundant in neutrophils (Bradley et al., 1982). The results on Fig. 3 show that MPO activity enhanced 4-fold 24 hours after FCA administration. Both schedule of treatment, myricitrin 30 min before and 22 h after FCA injection, reduced MPO activity at basal levels (P>0.05 from baseline) (Fig. 3).

To confirm myricitrin effect upon neutrophils migration, we made a histochemical staining and quantify the leucocytes presence on subcutaneous mice paw tissue. The FCA administration caused marked neutrophils migration into paw of mouse. On the other hand, the mastocytes, lymphocytes and macrophages were not altered by FCA 24 h i.pl. administration (Table I). A

massive concentration of inflammatory cells was found on dermis and hypodermis. The neutrophils, mastocyte, macrophage, lymphocyte (plasmocyte) can be seen on Fig 4D. Another cellular alteration was the greater presence of fibroblasts than fibrocytes in the footpad FCA injected, characterizing a pathological condition. The myricitrin treatment neither before nor after FCA administration was able to reduce neutrophils migration (Table I).

In the histological study, the footpad FCA injected showed some morphological alterations on epidermis and connective tissues. In these samples were observed a hyperplasia of epidermal cells, scattering of collagen fibers in papilar and reticular dermis, great presence of inflammatory cells, granulomas, angiogenesis and the presence of active fibroblasts. Both treatments, myricitrin 30 min before or 22 h after FCA, decreased morphological alterations on epidermis and dermis papilar, without changing alterations on dermis reticular. This effect was better observed when myrictrin was administrated 30 min before FCA (Fig 4 - A, B and C).

The antioxidant effects of myricitrin were analyzed by *in vitro* assay using an inductor of lipid peroxidation, the $FeCl_2$/EDTA system (Gutteridge et al., 1979; Braughler et al., 1988), which was incubated with liver homogenate from rat. The lipid peroxidation levels increased 3-fold in the $FeCl_2$/EDTA system when compared to baseline. The myricitrin presence caused a concentration-dependent decrease on lipid peroxidation levels (Fig. 5), with IC_{50} value of 45 (25.0-79.0) µM.

4. Discussion

Pathological (chronic) pain is an unrelenting condition that often become debilitating. At present, few drugs are effective in treating this disorder and great part of them are described by their side effects (Woolf and Mannion, 1999; Mendell and Sahenk, 2003). In this concern, the search for new compounds that could be applied in chronic pain therapy has been essential. In the current study, we demonstrated, for the first time, that systemic (i.p.) administration of the flavonoid myricitrin, produced an inhibition of tactile allodynia induced by both chronic pain models: sciatic nerve partial constriction and chemical FCA-induced inflammation

in mice. It is worth mentioning myricitrin anti-allodynic effect appeared 30 min after treatment (first measurement) and was keeping for up 4h. The additional finds of this study were: myricitrin treatment reduced FCA-induced paw oedema; MPO activity; morphological alterations on epidermis and dermis papilar; without to affect leukocytes infiltration. Furthermore, myricitrin demonstrated a good antioxidant activity when evaluated on lipid peroxidation assay.

Previous studies have demonstrated that myricitrin interacts with certain proteins such as PI-3kinase, PKCα and PKCε decreasing their activities (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Meotti et al., 2006). In addition, this flavonoid has ever been described by the inhibition of inducible nitric oxide synthase (iNOS) over expression, NO production, NF-κB activation (Chen et al., 2000). More recently, a study of our group showed that myricitrin produces a potent inhibition of calcium transport (data still not published). In accordance, further investigations showed that myricitrin exerts an antinociceptive action in models of acute pain, which is closely related to PKC and L-arginina nitric oxide pathways (Meotti et al., 2006), $G_{i/o}$ protein, ATP-gated K^+ and Ca^{2+} channels mechanisms dependent (data still not published).

Taking into account, the purpose of the present study was to investigate the myricitrin effects on the chronic pain models. It is now well recognized that persistent pain, resulting from peripheral injection of FCA or sciatic nerve partial constriction, outcomes in release of multiple inflammatory and nociceptive mediators, resulting in increased long-lasting discharge of primary sensory fibres, that modifies neuronal, neuro-glial and neuro-immune cell phenotype and function in the central nervous system. These alterations can occur at translational or post-translational levels and achieve receptors, ions channels, soluble mediators and molecules played in cell signalling (Woolf and Mannion, 1999; Ji and Strichartz, 2004; Li et al., 2005). In this context, valuable effects of myricitrin counteracting

nerve injury and FCA-induced inflammation are probably associated to its ability to interfere in cell signalling, particularly that related to PKC, NO, Ca^{2+} and K^{+} pathways.

Another interesting result of this work was the number of leucocytes on the FCA injected paw. Previous works have ever reported that FCA i.pl. administration causes massive infiltration of neutrophils (Larson et al., 1985), however, it has never been quantified yet. After 24 h of i.pl. FCA administration, the subcutaneous amount of neutrophils increased around 27-fold, whereas the number of macrophages/lymphocytes and mastocytes was kept similar to saline i.pl. group. Hence, it is extremely relevant to point out that an i.pl. treatment of 24 h with FCA causes intense migration of only neutrophils, preserving the resident number of the other leucocytes. Interestingly, myricitrin, at both schedule of treatments, did not modify leucocytes migration. It was an unexpected fact, once myricitrin treatment inhibited FCA-induced paw oedema, tactile allodynia and MPO activity. It is well described the directly relationship between MPO activity and the presence of neutrophils, since this enzyme is the most abundant in neutrophils (Bradley et al., 1982).

In this work, we found that a decrease on MPO activity did not reflect a direct decrease on neutrophils levels. A reasonable account for it resides because flavonoids, particularly those that comprise cathecol group (like myricitrin), are good substrate for MPO enzyme in concentrations corresponding to circulating plasma flavonoids levels (Kostyuk et al., 2003). In agreement, when incubated *in vitro*, myricitrin was able to inhibit MPO activity from subcutaneous tissue of FCA-injured mice (data not shown). Hence, MPO-catalyzed flavonoids oxidation can prevent the oxidation of other targets, diminishing oxidative damages caused by inflammation. In accordance, it is postulated that anti-inflammatory drugs with oxidizable functional groups can cause inhibition of MPO and it explains, in part, their anti-inflammatory effects (Kettle and Winterbourn, 1991). The ability of

myricitrin to inhibit MPO activity, also would explain its effects against allodynia, oedema and morphological alterations caused by FCA.

Besides, myricitrin showed to be a powerful antioxidant agent, since it inhibited, at low concentrations, the lipid peroxidation in a medium Fe^{2+}-induced free radicals. This antioxidant action can be attributed mainly by the scavenger ability of flavonoids. Given that, flavonoids that contain a cathecol group have ever been described like scavengers and mimics of superoxide dismutase, comprising an important role in the oxidative stress process (Edenharder and Grunhage 2003; Kostyuk et al., 2004).

Oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance in favor of the pro-oxidant, thereby leading to potential damage (Ohkawa et al., 1979; Haddad, 2002; Meotti et al. 2004). The major pro-oxidant agents are the reactive oxygen species (ROS), which play a crucial role in the initiation and progression of pathological conditions, including inflammatory process through induction of mediators such as interleukins (Alder et al., 1999; Haddad, 2002). In addition, ROS can be released in response to TNF-α and LPS (Yoshida et al., 1999) and can serve as intracellular signals for the activation and regulation of redox-sensitive transcription factors (Haddad et al., 2001). Corroborating, antioxidant substances can act as inhibitors of cytokines at both transcriptional and post-transcriptional levels (Hudson, 2001; Haddad et al., 2001; Haddad, 2002). Regarding these mechanisms, antioxidant action and MPO inhibition exerted by myricitrin elucidate its beneficial effects on the inflammatory and neuropathic pain conditions

Although myricitrin was unable to reduce neutrophils migration it was accomplished in reducing paw oedema and morphological alterations on FCA-induced local inflammation. It has been assumed that microvessel permeability can occur independently from leukocyte adhesion and the migration process, however, it happens by ROS-release mechanism dependent (Zhu et al., 2005). These results substantiate the idea that myricitrin, like an antioxidant agent, is a potential candidate for anti-inflammatory drugs research

In summary, the current study provides convincing evidence that myricitrin, a flavonoid occurring naturally and widespread in higher plants, produces systemic anti-allodynic action in two models of persistent inflammatory (FCA-i.pl. injected) and neuropathic (sciatic nerve injured) pain,

when assessed in mechanical stimulus (VHF) in the hindpaw. Besides, myricitrin decreased FCA-induced increased MPO activity, paw oedema and consequently, the subcutaneous morphological footpad alterations. The beneficial effects of myricitrin seem to involve molecular mechanisms such as inhibition of PKC and NO cell signalling, Ca^{2+} and K^{+} transport. Likewise, additional means by which myricitrin has exerted its effects are largely related to its antioxidant activity. Together, the present results indicate that myricitrin might be of potential interest in the development of new clinically relevant drugs for the management of persistent neuropathic and inflammatory conditions.

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Legends

Fig. 1: Effect of myricitrin on mechanical allodynia induced by sciatic nerve injury in response to 10 applications of 0.6 g VFH. The assessment was carried out in mice sham-operated (○), operated and treated with vehicle (●), operated and treated with myricitrin 30mg/kg, i.p. (Δ) seven days after surgery. The baseline (□) was recorded before nerve injury. The results represent the mean ± S.E.M. of eight animals. The symbols denote significant difference *P<0.05 between operated mice plus vehicle and operated mice plus myricitrin by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test.

Fig. 2: Effect of myricitrin on mechanical allodynia **(A)** in response to 10 applications of 0.6 g VFH and paw oedema **(B)** induced by FCA in mice. The animals received vehicle (●) or myricitrin (Δ) 24h after FCA injection. **(A)** The baseline (□) was recorded before FCA injection and in **(B)** baseline paw volume was discounted from total volume for absolutely oedema value. The measure following was 24 hours after FCA-injection (0) and (0.5, 2, 4, 8 h) subsequent to myricitrin (30 mg/kg i.p) treatment. The results represent the mean ± S.E.M. of eight animals. The symbols denote significant difference *P<0.05 between vehicle treated and myricitrin treated mice by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test.

Fig. 3: Effect of myricitrin on MPO activity in mice paw FCA injected. The animals were subdivided into four groups: **1)** saline i.p. 30 min before PBS i.pl.; **2)** saline i.p. 30 min before FCA i.pl.; **3)** myricitrin i.p. 30 min before FCA i.pl.; **4)** myricitrin i.p. 22 h after FCA i.pl. Following 24 hours from FCA-injection the subcutaneous tissue from paws were removed and homogenized in buffer to assessment of MPO activity. The results are expressed as concentration of TMB oxidized with a molar absorption coefficient of 3.9 x10^4 for TMB salt. Each bar represents the mean ± S.E.M. of five animals. The statistical analyzes were performed by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test and the symbols denote significant difference among

groups: $^{\#}P<0.001$ when compared to PBS i.pl. group; ***$P<0.001$ when compared to vehicle i.p. plus FCA i.pl. group.

Fig. 4: Effect of myricitrin on subcutaneous morphological changes and leucocytes infiltration in mice paw FCA injected. The mice were killed and their footpads were removed exactly 24 h after FCA injection, in all groups. The pictures represents: **(A), (B)** and **(C)** footpad section stained by hematoxylin-eosin (objective 40x); **(A)** saline i.p. 30 min before PBS i.pl.; **(B)** saline i.p. 30 min before FCA i.pl.; **(C)** myricitrin i.p. 30 min before FCA i.pl. **(D)** footpad section stained by May-Grünwald-Giemsa (objective 100x) of a representative animal that received saline i.p. and FCA i.pl. Cells migration in an inflammatory condition are showed in **(D):** mastocytes (**mt**); plasmocytes (**p**); neutrophils (**n**); macrophages (**m**) fibroblast active (**f**).

Fig. 5: Effect of myricitrin against lipid peroxidation induced by Fe^{2+}/EDTA on liver of rats *in vitro*. An aliquot of liver was homogenized and incubated at 37°C with 50 μM EDTA; 100 μM $FeCl_2$ and vehicle or myricitrin at different concentrations (0.01-1 mM) during 1h. TBARS were determined as described in Section 2. Each bar represents the mean ± S.E.M. of five animals. The statistical analyzes were performed by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test and the symbols denote: $^{\#}P<0.001$ significantly different from B group (baseline); ***$P<0.001$ significantly different from C group (Fe^{2+}/EDTA plus vehicle - DMSO).

Table I – Leucocytes infiltration on the footpad FCA-injected

Treatment	Neutrophils/ (mm^2)	Mastocytes/ (mm^2)	Macrophages and linfocytes/ (mm^2)
saline i.p. plus saline i.pl.	5.2 ± 3.1	16.9 ± 4.5	15.9 ± 7.0
saline i.p. plus FCA i.pl.	141.6 ± 13.9*	22.8 ± 7.9	15.2 ± 5.9
myricitrin i.p. (before) plus FCA i.pl.	119.8 ± 8.8*	11.2 ± 3.6	12.0 ± 3.2
myricitrin i.p. (after) plus FCA i.pl.	133.0 ± 4.3*	15.9 ± 10.7	15.7 ± 3.5

The number of cells was counted in 20 fields in a representative area of inflammation on the reticular dermal and hypodermal layers. The statistical analyzes were performed by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test and the symbol denotes significant difference when compared to group saline i.p. plus saline i.pl., *P<0.001.

Figure 1

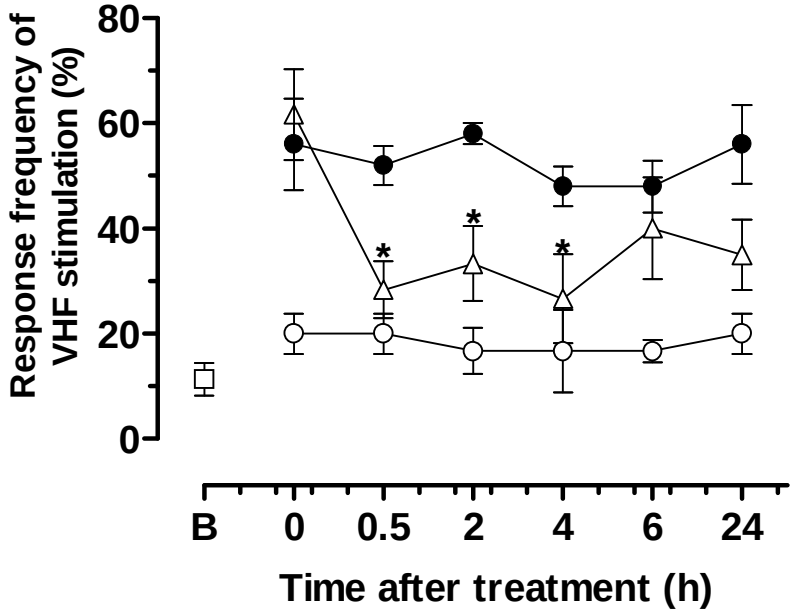


Figure 2A

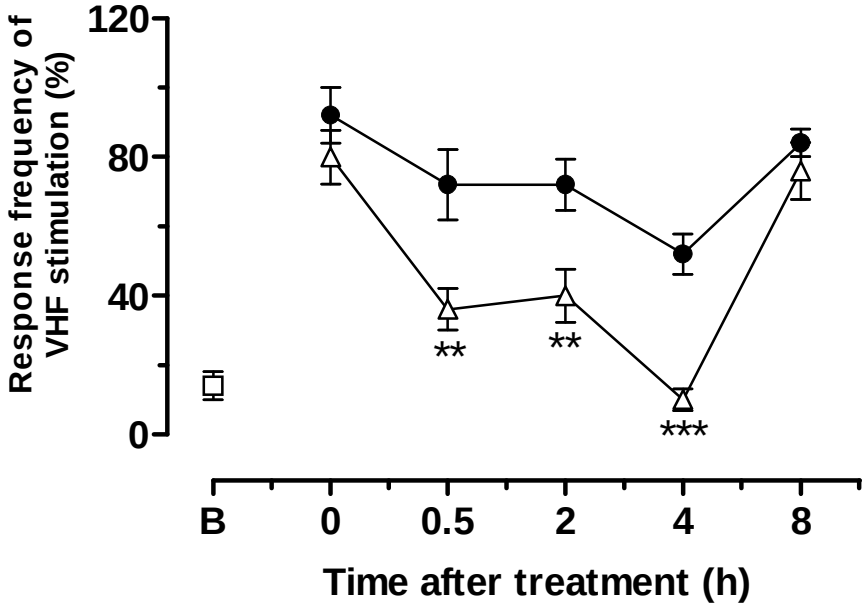


Figure 2B

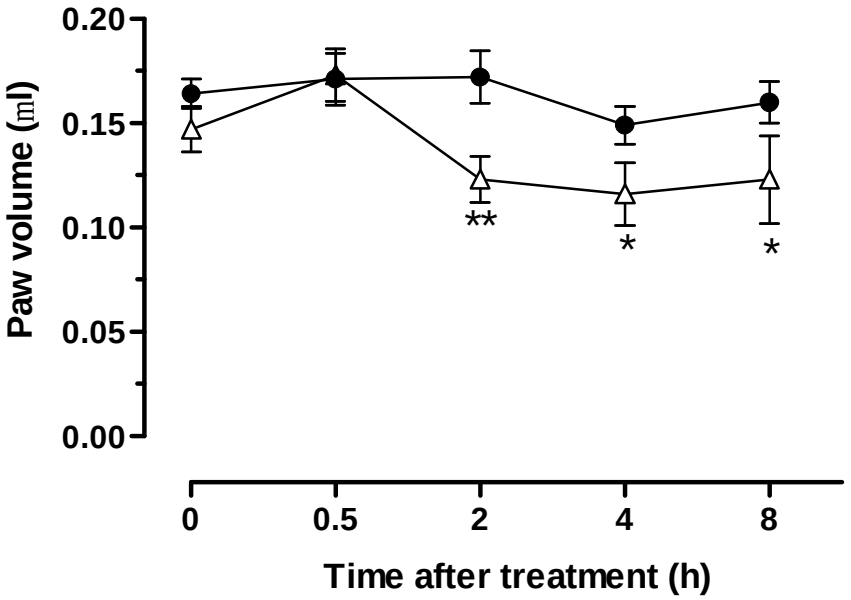


Figure 3

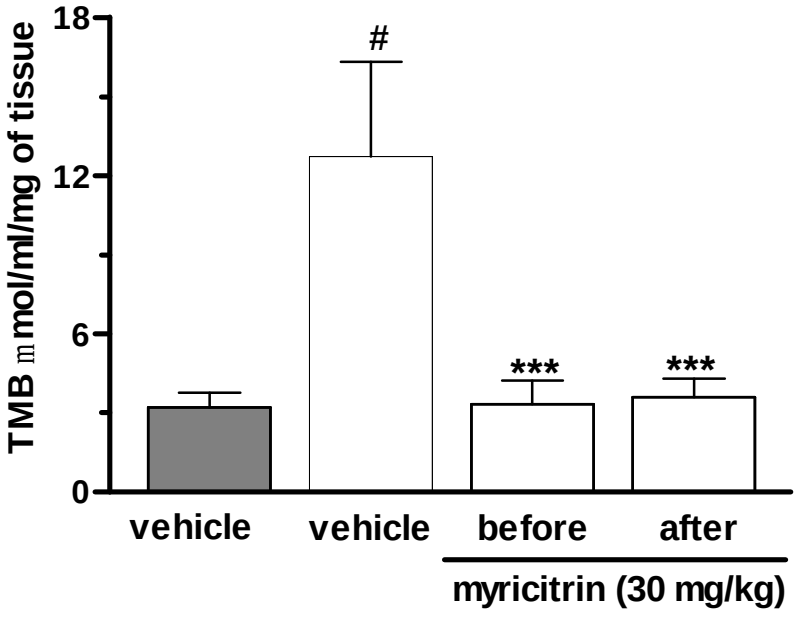


Figure 4

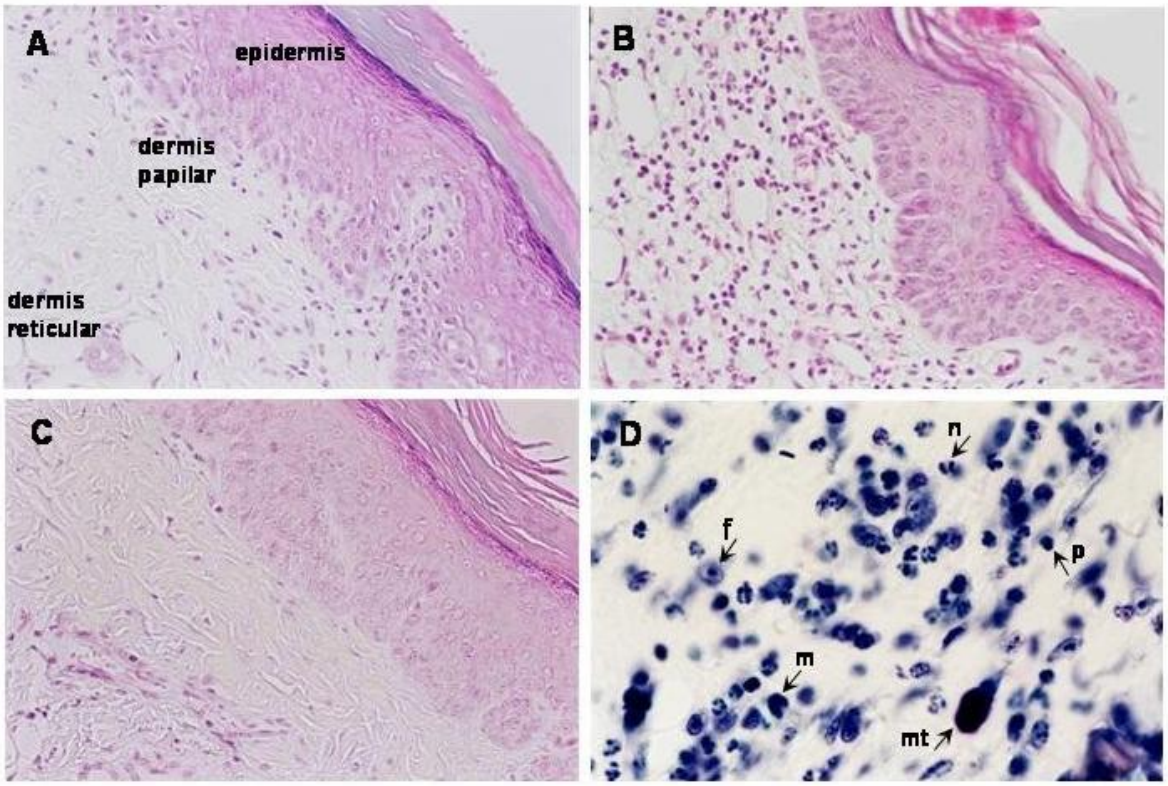
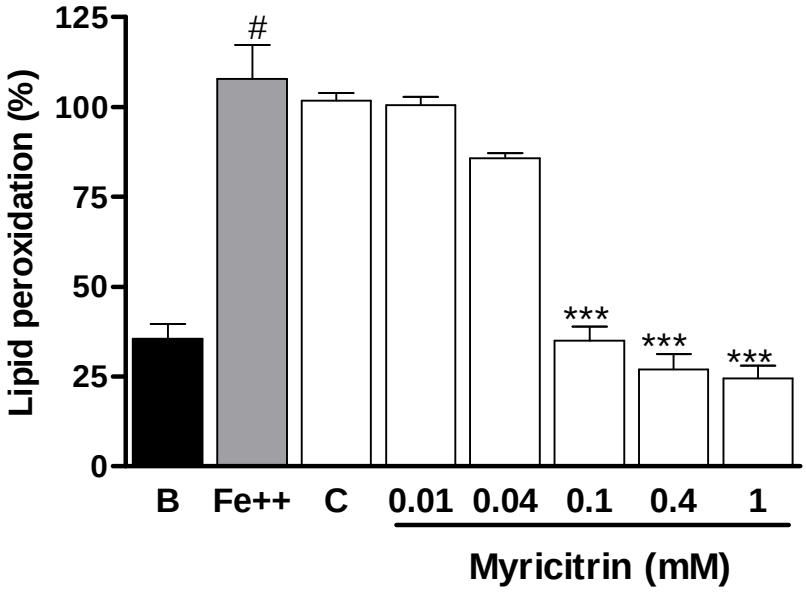


Figure 5



5. DISCUSSÃO

Os flavonóides exercem importantes ações farmacológicas, incluindo ações antioxidante, antiinflamatória, antialérgica, as quais explicam, pelo menos em parte, seu efeito protetor contra doenças cardiovasculares, coronarianas e contra algumas formas de câncer (GAMET-PAYRASTRE et al., 1999; BIRT et al., 2001; HAVSTEEN, 2002; EDENHARDER & GRÜNHAGE, 2003; CALIXTO et al., 2003; 2004). Embora seja um campo pouco explorado, os efeitos analgésicos dos flavonóides também já foram descritos. A analgesia causada pelos flavonóides frente a picadas de insetos, cobra, queimaduras ou corte foi atribuída à inibição de fosfolipases e ciclooxigenases (SAKAGAMI ET AL., 1999; LIANG ET AL., 1999; HAVSTEEN, 2002).

O flavonóide miricitrina tem sido descrito por seus efeitos antioxidante, "scavenger" de radicais livres, antimutagênico, inibidor da síntese de óxido nítrico e da ativação do fator de transcrição NF-κB (YASUKAWA et al., 1990; CHEN et al., 2000; EDENHARDER & GRÜNHAGE, 2003). Estas propriedades sugerem uma possível ação antiinflamatória e analgésica para este flavonóide, embora, seu potencial efeito *in vivo* contra a dor e a inflamação nunca tenha sido investigado antes.

Neste estudo, demonstrou-se que a administração sistêmica (i.p. ou oral) do flavonóide miricitrina causou uma inibição, dependente da dose administrada, das contorções abdominais induzidas pelo ácido acético em camundongos. O efeito antinociceptivo da miricitrina i.p. iniciou 30 minutos após sua administração e permaneceu por quatro horas (dados não mostrados). Além disso, quando administratada pela via oral, a miricitrina foi menos potente e eficaz em prevenir a nocicepção do induzida pelo ácido acético do que quando administrada pela via intraperitoneal. Isto poderia ser explicado por uma menor biodisponibilidade (menor absorção) deste composto quando administrado pela via oral.

O modelo de contorções abdominais induzidas pelo ácido acético em camundongos é descrito como um modelo típico de nocicepção inflamatória visceral, sendo amplamente utilizado como ferramenta para detecção e avaliação de novos agentes com propriedades analgésicas e antiinflamatórias (COLLIER et al., 1968). Os prótons oriundos da dissociação do ácido acético podem ativar diretamente canais de cátion não seletivos localizados nas fibras aferentes primárias (JULIUS & BASBAUM, 2001). Além disso, a injeção de ácido acético na cavidade peritoneal de camundongos promove a liberação de diversos mediadores inflamatórios como PG, BK, SP, TNF-α, IL-1β e IL-8 entre outros (COLLIER et al., 1968; VINEGAR et al., 1979; RIBEIRO et al., 2000; IKEDA et al., 2001), os quais estimulam neurônios aferentes primários, aumentando a liberação de aspartato e glutamato no fluido cerebroespinhal (FENG et al., 2003). Em função disto, este modelo

apresenta uma boa sensibilidade, embora pouca especificidade. Neste contexto, a nocicepção induzida pelo ácido acético pode ser prevenida por agentes antiinflamatórios, analgésicos, relaxantes musculares e sedativos, o que poderia resultar em uma interpretação equivocada de resultados. Com a finalidade de excluir um possível efeito sedativo, distúrbio motor, ou ainda uma hipotermia induzida pela miricitrina, os camundongos foram tratados com doses superiores àquelas usadas no teste das contorções abdominais e foram submetidos ao teste do campo aberto, "rota-rod" e medida da temperatura da pele. Em nenhuma das doses testadas (30 ou 100 mg/kg) a miricitrina foi capaz de interferir na permanência do animal no "rota-rod", no número de cruzamentos no campo aberto e na temperatura do animal.

Como descrito anteriormente, a administração i.p. de ácido acético libera neurotransmissores no corno dorsal e conduz a transmissão nociceptiva em nível central (FENG et al., 2003). Neste sentido, o efeito antinociceptivo causado pela administração sistêmica da miricitrina pode acontecer através de uma modulação tanto periférica quanto central da nocicepção. Para identificar se a miricitrina estaria tendo um efeito central, ela foi administrada por duas outras vias, a via intracerebroventricular (supraespinhal) e a via intratecal (espinhal). Em ambas as vias, a miricitrina inibiu de forma dependente da dose as contorções abdominais induzidas pelo ácido acético. Estes resultados sugerem fortemente que a miricitrina exerce um efeito central na modulação da nocicepção.

Além do envolvimento dos canais sensíveis a ácidos (ASICS), da liberação de citocinas da cavidade peritoneal e de neurotransmissores em nível central, a administração i.p. de ácido acético pode ativar receptores vanilóides. IKEDA e colaboradores (2001) demonstraram o envolvimento do receptor vanilóide nas contorções abdominais induzidas pelo ácido acético. O receptor vanilóide TRPV1 é sensível ao calor, alterações de pH e à capsaicina. A ativação deste receptor induz a despolarização da membrana com aumento do influxo de cátions, levando ao estímulo nocivo (JULIUS & BASBAUM, 2001). Por outro lado, a administração de capsaicina em camundongos neonatos (48 h de vida) causa uma dessensibilização persistente devido a uma diminuição das fibras sensoriais, principalmente, as do tipo C (JANCSÓ et al., 1977). Nossos resultados confirmam estas observações e demonstram que o tratamento neonatal com capsaicina reduziu significantivamente o número de contorções induzidas pelo ácido acético. Entretanto, o tratamento neonatal com capsaicina não interferiu na atividade antinociceptiva da miricitrina.

Embora, as fibras aferentes primárias do tipo C pareçam não contribuir para a ação antinociceptiva da miricitrina, a administração sistêmica deste flavonóide produziu parcial, porém significante inibição da nocicepção neurogênica induzida pela administração i.pl. de capsaicina. Este efeito pode ser explicado pelas ações intracelulares da miricitrina sobre a PKC. De fato, uma série de evidências indica que os flavonóides, tais como a miricitrina podem inibir proteínas quinases como a PI-3K e PKC (AGULLO et al., 1997; GAMET-PAYRASTRE et al., 1999). Sabe-se que a capsaicina libera neuropeptídeos, aminoácidos excitatórios, NO e outros mediadores pró-inflamatórios a partir das fibras C, transmitindo a informação nociceptiva aos neurônios do corno

dorsal, sendo que a sensilização central, ocorre, entre outros meios, através da ativação de proteínas quinase A e C (CALIXTO et al., 2005). Além disso, inibidores de PKC previnem a fosforilação do TRPV1, reduzindo a sensibilização deste receptor, tornando-o menos responsivo à ação do agonista (PREKUMAR & AHERN, 2000, FERREIRA et al., 2004, CALIXTO et al., 2005).

Corroborando com o acima mencionado, de que a ação antinociceptiva da miricitrina se deva uma ação inibitória deste flavonóide sobre a PKC, a administração sistêmica de miricitrina inibiu de forma completa e potente a nocicepção induzida pela administração i.pl. de PMA (ativador de PKC). A principal característica química requerida para inibição da PKC pelos flavonóides consiste na presença das oxidrilas nos carbonos 3' e 4' no anel B da molécula. Além disso, a miricetina, a qual possui um grupo pirrogalol (oxidrila no carbono 3', 4' e 5' do anel B) é descrita, dentre os flavonóides, como o inibidor mais potente de PKC (GAMET-PAYRASTRE et al.,1999). A confirmação do efeito inibitório da miricitrina sobre a PKC foi evidenciada através de análises realizadas por western blot; onde animais tratados com miricitrina (1 mg/kg; i.p.) demonstraram uma significativa redução da ativação desta proteína pelo PMA. De acordo com estas evidências, FERREIRA e colaboradores (2005a) encontraram que a administração i.pl. de PMA induz a ativação de duas isoformas de PKC, a PKCα e PKCε. No presente estudo, observou-se que a administração sistêmica de miricitrina foi capaz de inibir a ativação de ambas isoformas.

Os resultados do presente estudo sugerem fortemente o envolvimento da PKC, mas não da PKA na ação antinociceptiva da miricitrina. Isto se deve ao fato que a administração sistêmica da miricitrina inibiu a hiperalgesia mecânica induzida pela BK em ratos, embora não tenha sido capaz de reduzir a hiperalgesia induzida pela PGE_2 neste mesmo modelo. Estudos prévios sugerem que a BK, ao se ligar no seu receptor B_2 causa uma ativação direta da PKC, porém, indireta da PKA, sendo esta última, ativada após a síntese de prostaglandinas, a qual acontece em decorrência da ativação de PKC (FERREIRA et al., 2004). Além disso, os resultados do presente estudo mostraram que a miricitrina, na mesma dose que reduziu a hiperalgesia induzida pela BK, não foi capaz de diminuir aquela induzida pela epinefrina. A hiperalgesia produzida pela epinefrina depende da ativação tanto de PKC como de PKA (KHASAR et al., 1999). Porém, os resultados obtidos neste estudo sugerem que a ligação da epinefrina em seu receptor β-adrenérgico ativa a via cAMP/PKA, causando uma hipersensibilização que se sobrepõe à ativação da PKC.

Outro resultado interessante deste trabalho foi a constatação que a administração sistêmica da miricitrina inibiu de forma significativa e dependente da dose a nocicepção induzida pela injeção i.pl. de glutamato. A nocicepção induzida pelo glutamato parece envolver sítios de ação periférica, espinhal e supraespinhal. Além disso, a ação do glutamato sobre seus receptores aumenta a produção de NO e substâncias relacionadas à ativação deste mediador (BEIRITH et al., 2002). Dados publicados por CHEN e colaboradores (2000) descrevem que a miricitrina, quando incubada com macrófagos, reduz a produção de NO e a expressão da iNOS induzida por LPS nestas células. Assim, estes dados, juntamente com os resultados do presente estudo, indicam

que a ação antinociceptiva da miricitrina pode estar associada com sua capacidade de inibir a produção de NO. De fato, o efeito antinociceptivo da miricitrina sobre as contorções abdominais induzidas pelo ácido acético foi significativamente revertido pela administração prévia do aminoácido L-arginina (precursor de NO), sugerindo o envolvimento da via da L-arginina-óxido nítrico na atividade antinociceptiva da miricitrina.

Somando-se ao descrito acima, o tratamento prévio dos animais com miricitrina (30 mg/kg; i.p.) preveniu de forma eficaz a nocicepção induzida pela injeção intratecal da SP (84%) e em menor grau aquela induzida pela capsaicina (64%) e glutamato (41%). A SP, capsacina e glutamato interagem sinergicamente durante a ativação de neurônios sensorias no corno dorsal da medula para efetuar a sensação de dor, a qual acontece, dentre outros mecanismos através da produção de NO (SAKURADA et al., 1996; LIU et al., 1997; CARUSO et al., 2005). Neste sentido, a inibição da produção de NO, paralelo à inibição de PKC contribui grandemente para a antinocicepção provocada pela miricitrina.

A administração i.p. de miricitrina (30 mg/kg) produziu forte inibição sobre o comportamento de “biting” causado pela injeção intratecal de citocinas pró-inflamatórias (IL-1β e TNF-α). Já está bem estabelecido que a ligação da IL-1β ao seu receptor IL-1RI ativa PKC independentemente de cálcio (OBREJA et al., 2002), efeito o qual justifica a ação inibitória da miricitrina sobre a nocicepção induzida pela IL-1β. A miricitrina inibiu completamente a nocicepção induzida pela injeção intratecal de TNF-α. SCHÄFERS e colaboradores (2003) descrevem que a nocicepção induzida pelo TNF-α envolve a fosforilação de MAPKs como a p38. Além disso, estudos *in vitro* verificaram uma ação inibitória da miricetina sobre a fosforilação das MAPKs (KO et al., 2005a). Embora, estes mesmos autores descrevem que a inibição da fosforilação das MAPKs pela miricetina é dependente de sua ação sobre a PKC, outros autores demonstram que flavonóides como a luteolina e quercitina podem inibir a fosforilação destas proteínas por uma via independete de PKC (WADSWORTH et al., 2001; XAGORARI et al., 2001; 2002; KO et al., 2005a). De acordo com estes resultados, os efeitos exercidos pela miricitrina podem estar associados a um decréscimo na fosforilação das MAPKs não necessariamente dependente da PKC.

Além de seus efeitos sobre quinases intracelulares, a ação da miricitrina na nocicepção induzida pelo TNF-α pode também ser atribuída a uma inibição dos canais de cálcio. De acordo com esta hipótese, POLLOCK e colaboradores (2002)

verificaram que TNF-α causa mobilização de Ca^{2+} em cultura de neurônios sensoriais. Fortalecendo esta hipótese, o pré-tratamento dos animais com cloreto de cálcio, via i.c.v., preveniu completamente a antinocicepção induzida pela miricitrina.

Já está bem estabelecido que um aumento na concentração de cálcio intracelular representa um passo chave na liberação de neurotransmissores, na modulação da excitabilidade da membrana e ativação de proteínas intracelulares (WARD, 2004). Desta forma, este íon é um dos principais envolvidos na transmissão nociceptiva (PRADO, 2001; CERVERO & LAIRD, 2003). Usando ensaios *in vitro* em fatias de córtex de rato, foi possível evidenciar um efeito inibitório da miricitrina sobre o transporte de ^{45}Ca quando em um meio rico em K^+ (condição despolarizante). Embora, quando em alta concentração, este flavonóide também inibiu a captação em um meio não-despolarizante (baixa concentração de K^+). Estes resultados podem indicar que a miricitrina interage de uma forma distinta com os canais de cálcio, dependendo do estado de despolarização da célula, mas pode perder sua especificidade quando em concentrações mais altas.

Outro interessante resultado aqui apresentado foi que a inativação da proteína $G_{i/o}$, pela administração intratecal de toxina pertussis, preveniu completamente a antinocicepção da miricitrina sobre as contorções abdominais induzidas pelo ácido acético. A ativação de receptores acoplados a proteína $G_{i/o}$, como por exemplo, subtipos de receptores para os opióides, induz, entre outros eventos, a abertura de canais de K^+ (KOPPEN et al., 1996), levando a uma hiperpolarização e consequente inibição do impulso excitatório. Corroborando com esta afirmação, o pré-tratamento dos animais com toxina pertussis preveniu completamente o efeito antinociceptivo da morfina. Apesar de existir uma correlação entre receptores opióides e ativação de proteína $G_{i/o}$, o uso de naloxona (antagonista opióide), em doses que reverteram a ação antinociceptiva da morfina, foi ineficaz em prevenir a antinocicepção causada pela miricitrina, excluindo o envolvimento do sistema opióide na ação antinociceptiva da miricitrina. Por outro lado, o tratamento dos animais com glibenclamida

(bloqueador de canais de K^+ dependentes de ATP) preveniu parcial, embora significativamente, a antinocicepção induzida pela miricitrina.

Desta forma, os efeitos da miricitrina, nos modelos de nocicepção aguda aqui testados parecem envolver, basicamente, a inibição de quinases intracelulares, principalmente PKC, inibição da produção de NO, abertura de canais de potássio e bloqueio dos canais de cálcio.

Considerando as atividades até aqui descritas para a miricitrina, pode-se sugerir uma ação deste flavonóide sobre a nocicepção crônica, embora esta última difira substancialmente da nociepção aguda em termos de persistência e alterações adaptativas (BESSON, 1997; WOOLF & MANNION, 1999; ZIMMERMANN, 2001). Esta característica torna o tratamento da dor crônica muito difícil. De fato, as drogas disponíveis até o momento com o intuito de aliviar as dores crônicas são efetivas somente em uma pequena parcela dos pacientes. Além disso, grande parte delas apresenta baixa eficácia e muitos efeitos colaterais (WOOLF & MANNION, 1999; MENDELL & SAHENK, 2003).

O estudo da dor crônica tem sido amplamente investigado em animais de laboratório, os quais podem ser submetidos a lesões de nervo, tanto periférico, quanto central; estes modelos mimetizam a sensibilização e alterações plásticas ocorridas na dor neuropática (ZIMMERMANN, 2001). Outro modelo de dor crônica consiste, por exemplo, em um modelo de dor inflamatória induzida pela administração periférica de agentes indutores de reposta imunológica como o FCA (*Mycobacterium tuberculosis* atenuada e ressuspendida em óleo mineral) (LARSON et al., 1986).

Neste trabalho, a ligadura parcial de nervo ciático e a injeção i.pl. de FCA tornaram os animais hipersensíveis e responsivos frente a estímulos inócuos aos animais falso-operados ou não injetados (alodínia) (SELTZER et al., 1990). O tratamento com miricitrina (30 mg/kg, i.p.), sete dias após a ligadura do nervo ou 24 horas após a administração de FCA inibiu a alodínia tátil em ambos os modelos; um efeito que iniciou 30 minutos após a administração da substância e perdurou por 4 horas.

A administração de FCA também causou edema na pata injetada e aumentou o número de neutrófilos, embora não alterou o número de macrófafos/linfócitos e mastócitos. O tratamento com miricitrina reverteu o edema, sendo que o efeito do flavonóide iniciou 2 horas após a sua administração e durou por mais de oito horas. Interessantemente, a miricitrina não preveniu a migração de neutrófilos, porém preveniu as alterações morfológicas no tecido subcutâneo da pata e reduziu a atividade da MPO, uma enzima abundante em neutrófilos que está intimamente associada à migração de neutrófilos ao local da inflamação (BRADLEY et al., 1982).

Neste estudo evidenciou-se que o decréscimo na atividade da MPO não refletiu diretamente em um decréscimo no número de neutrófilos. Esta afirmação está de acordo com o fato que flavonóides, particularmente aqueles com o grupo catecol, são bons substratos para a MPO em concentrações correspondentes às circulantes no plasma após a ingestão de alimentos ricos nestas substâncias (KOSTYUK et al., 2003). Além disso, resultados *in vitro* utilizando tecido subcutâneo da pata de animais injetados com FCA mostraram que a miricitrina (100 μM) é capaz de inibir a atividade da MPO (dados não mostrados).

A oxidação dos flavonóides catalisada pela MPO pode prevenir a oxidação de outros alvos, diminuindo os danos oxidativos causados pela inflamação. De acordo com isso, KETTLE & WINTERBOURN (1991) preconizaram que drogas antiinflamatórias com grupos funcionais oxidáveis podem inibir a MPO, explicando seus efeitos antiinflamatórios. Desta forma, a inibição da MPO pela miricitrina, com consequente diminuição dos danos causados pelo estresse oxidativo e pela inflamação, elucidaria, em parte, seus efeitos contra alodínia, edema e alterações morfológicas causadas pelo FCA.

Paralelo a isto, a miricitrina mostrou ser um potente agente antioxidante contra a peroxidação lipídica induzida por Fe^{2+}. Esta ação antioxidante pode ser atribuída, principalmente pela ação “scavenger” deste flavonóide, uma vez que, flavonóides que apresentam o grupo catecol são descritos como potentes agentes “scavenger” e miméticos da superóxido dismutase (EDENHARDER & GRUNHAGE 2003; KOSTYUK et al., 2004).

O estresse oxidativo é definido como um distúrbio no balanço oxidativo da célula em favor dos fatores pró-oxidantes, podendo levar ao dano tecidual (OHKAWA et al., 1979; HADDAD, 2002; MEOTTI et al., 2004). Os maiores agentes pró-oxidantes são as EROs, as quais tem um papel crucial na iniciação e progressão de condições patológicas, incluindo o processo inflamatório através da indução de mediadores tais como interleucinas (ALDER et al., 1999; HADDAD, 2002). Além disso, as EROs podem ser liberadas em reposta ao TNF-α e LPS (YOSHIDA et al., 1999) e podem servir como sinalizadores intracelulares para a ativação e regulação de fatores de transcrição sensíveis à modulação redox (HADDAD et al., 2001). Substâncias antioxidantes podem atuar como inibidores de citocinas em nível transcricional ou pós transcripcional (HUDSON, 2001; HADDAD et al., 2001; HADDAD, 2002). De acordo com estes mecanismos, a ação antioxidante da miricitrina somado ao seu efeito inibitório sobre a MPO esclarece os efeitos benéficos deste flavonóide sobre condições de dor neuropática e inflamatória.

Embora a miricitrina não tenha reduzido a migração de neutrófilos, ela foi capaz de diminuir o edema e as alterções morfológicas induzidas pelo FCA. Já está bem estabelecido que alterações na permeabilidade dos vasos podem acontecer através de processos independentes da adesão e migração de neutrófilos; entretanto, por mecanismos dependentes da liberação de EROs (ZHU et al., 2005). Estes resultados sustentam a idéia de que a miricitrina, como um agente antioxidante, é um potencial candidato para a pesquisa de drogas antiinflamatórias.

A dor persistente, resultante da injeção periférica de FCA ou da constrição parcial do nervo ciático, resulta em liberação de múltiplos mediadores inflamatórios e nociceptivos, causando um aumento na duração e intensidade do potencial de ação em fibras sensoriais primárias. Esta atividade leva a alterações no fenótipo de células neuronais, neurogliais e neurimunes no SNC. Estas alterações podem ocorrer em nível transcripcional ou pós transcripcional e atingem receptores, canais iônicos, mediadores solúveis e outras moléculas envolvidas na sinalização celular (WOOLF & MANNION, 1999; JI & STRICHARTZ,

2004; LI et al., 2005). Neste contexto, os efeitos da miricitrina contra a lesão do nervo e a inflamação induzida pelo FCA estão, provavelmente associados à capacidade deste flavonóide em interferir com a sinalização celular, particularmente, as vias relacionadas com PKC, NO, Ca^{2+} e K^{+}.

Em conclusão, o presente trabalho mostra que a miricitrina é dotada de atividade antinociceptiva e antiinflamatória quando avaliada em modelos de nocicepção aguda e crônica. Os mecanismos de sua ação antinociceptiva e antiinflamatória são mostrados na figura 3.

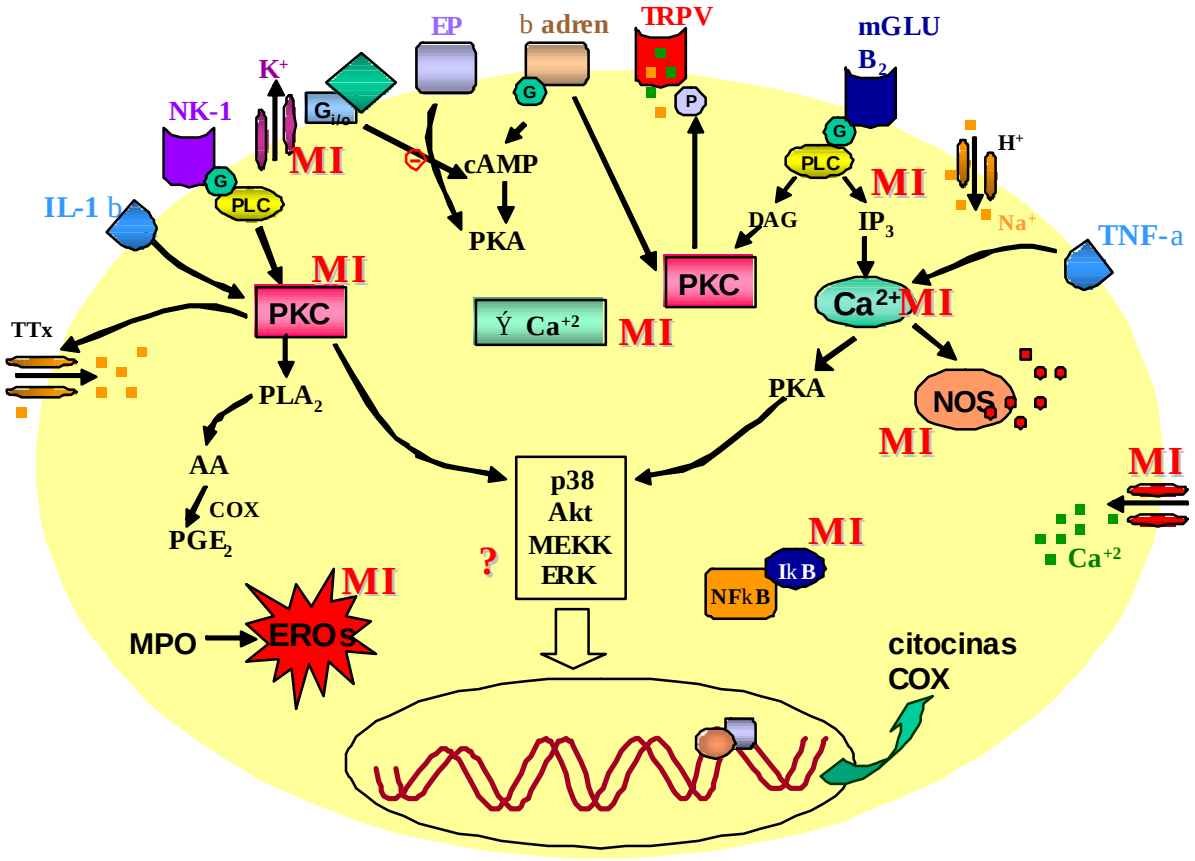


Figura 3: Possíveis sítios de ação da miricitrina com consequente redução na nocicepção e inflamação. MI (miricitrina); TTx (canal de sódio resistente à tetrodoxina); IL-1β (re ceptor para interleucina 1β); TNF-α (receptor para o fator de necrose tumoral α); NK-1 (receptor para neurocinina 1); K^{+} (canal de potássio); $G_{i/o}$ (proteína $G_{i/o}$); EP (receptor para prostaglandina E_2); β adren (receptor β adrenérgico para a epinefrina); TRPV (receptor vanilóide); B_2 e mGLU (receptor metabotrópico para a bradicinina - B_2 ou para o glutamato – mGLU); H^{+} (canal

catiônico sensível a ácidos); PLC (fosfolipase C); PLA_2 (fosfolipase A_2); AA (ácido araquidônico); COX (ciclooxigenase); PGE_2 (prostaglandina E_2); PKC (proteína quinase C); PKA (proteína quinase A); cAMP (adenosina monofosfato cíclico); DAG (diacilglicerol); NOS (óxido nítrico sintase); NF-κB (fator nuclear–κB); MPO (mieloperoxidase); EROs (espécies reativas de oxigênio) Akt, MEKK (quinase quinase de proteína quinase ativada por mitógeno), ERK (quinase regulada por sinal extracelular).

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir que:

- A administração sistêmica (oral e i.p.) e central (i.c.v. e i.t.) da miricitrina causou antinocicepção no modelo das contorções abdominais induzidas pelo ácido acético; o efeito da miricitrina não foi relacionado com uma possível ação sedativa deste composto.
- A administração sistêmica da miricitrina foi capaz de prevenir a nocicepção induzida pela injeção i.pl. de capsaicina, glutamato e PMA; e a nocicepção induzida pela injeção i.t. de glutamato, SP, capsaicina e citocinas pró-inflamatórias em camundongos.
- A administração sistêmica da miricitrina foi capaz de diminuir o efeito hiperalgésico induzido pela BK, mas não aquele induzido pela PGE_2 e epinefrina.
- A administração sistêmica da miricitrina reduziu a alodínia causada pela ligadura parcial do nervo ciático e pela injeção i.pl. de FCA. Os efeitos antiinflamatórios deste flavonóide foram evidenciados através da redução do edema, da lesão tecidual e da atividade da MPO, embora não pela diminuição da migração de neutrófilos.
- Os mecanismos de ação envolvidos na ação antinociceptiva e antiinflamatória da miricitrina envolvem: inibição da PKC; inibição da produção de NO; ativação de mecanismos dependentes da proteína $G_{i/o}$; aumento do influxo de K^+; alterações no transporte de Ca^{2+}; atividade antioxidante e inibição de enzimas do processo inflamatório como a MPO.
- Os mecanismos de ação que parecem não estarem relacionados com a atividade antinociceptiva da miricitrina incluem a PKA; sistema opióide e a neurotransmissão dependente de fibras sensoriais não mielinizadas do tipo C.

7. PERSPECTIVAS

Os resultados obtidos no presente estudo abrem novas perspectivas para a investigação do potencial efeito da miricitrina, dentre eles: suas ações sobre proteínas quinases ativadas por mitógenos e a relação deste evento com o processo de transmissão nociceptiva e inflamação.

Além disso, a miricitrina pode ser aplicada em modelos experimentais que reflitam condições patológicas relacionadas à inflamação e ao estresse oxidativo, como modelos de poliartrite em ratos, modelos de doenças neurodegenerativas e danos oxidativos causados pela intoxicação com metais, desta forma, avaliando o potencial efeito deste flavonóide sobre estas situações, procurando intensificar a aplicabilidade terapêutica e científica desta substância.

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9. OUTROS TRABALHOS PUBLICADOS DURANTE O PERÍODO DE DOUTORAMENTO

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