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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

EFEITO DOS SESQUITERPENOS POLIGODIAL E DRIMANIAL SOBRE PARÂMETROS GLUTAMATÉRGICOS EM SISTEMA NERVOSO CENTRAL DE RATOS E CAMUNDONGOS.

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À minha família...

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SUMÁRIO

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ABREVIATURAS

SNC	Sistema Nervoso Central
iGluR	Receptores ionotrópicos para Glutamato
mGluR	Receptores metabotrópicos para Glutamato
NMDA	N-Metil-D-Aspartato
AMPA	Ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico
KA	Ácido Caínico
GLAST	Transportador de glutamato e aspartato em ratos
GLT-1	Transportador de glutamato em ratos
EAAC1	Transportador de aminoácido excitatório
EAAT1	Transportador de aminoácido excitatório em humanos
EAAT2	Transportados de aminoácido excitatório em humanos
EAAT3	Transportador de aminoácido excitatório em humanos
EAAT4	Transportador de aminoácido excitatório em humanos
EAAT5	Transportador de aminoácido excitatório em humanos
AMPc	Adenosina 3,5-monofosfato cíclico
NO	Óxido nitrico
GMPc	Guanosina-5'-monofosfato cíclico
1S-3R ACPD	Ácido aminociclopentano-1,3-dicarboxilico
MK-801	(+)-5-metil-10,11-diidro-5-H-dibenzo[a,d]ciclohepten-5,10-imina
LDH	Lactato desidrogenase.
CNQX	6-ciano-7-nitroquinoxalano-2,3-diano

APRESENTAÇÃO

Este tese é constituída por quatro partes:

Parte I. Uma breve Introdução e Objetivos;

Parte II. Os resultados obtidos serão apresentados na forma de artigos científicos, publicado, submetido à publicação e artigo em preparação. Nestes artigos constam Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas;

Parte III. Discussão e Conclusões;

Parte IV. Referências Bibliográficas da Tese.

RESUMO

Produtos naturais derivados de planta têm contribuído enormemente para o desenvolvimento de drogas terapêuticas. Poligodial e drimanial são sesquiterpenos isolados da casca de *Drymis winteri* (Winteraceae), que apresenta propriedades antinociceptivas. O glutamato periférico possui ações nociceptivas; assim, neste estudo investigamos os efeitos destes compostos em vários parâmetros do sistema glutamatérgico de cérebro de ratos e camundongos. O poligodial, *in vitro*, inibiu significativamente a união do glutamato aos seus receptores, a captação de glutamato em céluas de astrocitos, assim como a captação de glutamato por fatias de córtex, hipocampo e estriado de cérebro de ratos, e estimulou a liberação de glutamato em preparações de sinaptossomas. O drimanial, *in vitro*, inibiu significativamente a união do glutamato aos seus receptores, a captação de glutamato em células de astrocitos e em preparações de vesículas sinápticas, assim como a captação de glutamato por fatias de córtex, hipocampo e estriado de cérebro de ratos, e estimulou a liberação de glutamato em preparações de sinaptossomas. Quando efetuamos experimentos, *in vivo*, em camundongos injetados i.p., observamos que a captação de glutamato por fatias de hipocampo e estriado foi inibida por ambos (poligodial e drimanial) enquanto em fatias de córtex não tiveram efeito. Estes resultados demonstram que há uma possibilidade destes dois compostos aumentarem as concentrações extracelulares de glutamato, apontando para um possível efeito neurotóxico, o que sugere cautela com suas utilizações terapêuticas.

ABSTRACT

Natural products including those derived from plants, have over the years greatly contributed to the development of therapeutic drugs. Polygodial and drimanial are sesquiterpenes isolated from the bark of the plant Drymis Winteri (Winteraceae) that exhibit antinociceptive properties. Since peripheral glutamate presents nociceptive actions, in this study it was investigated the effects of these compounds on the glutamatergic system in rat brain and mice. Polygodial *in vitro* inhibited glutamate binding, glutamate uptake by astrocytes, as well as by cortical, hippocampal and striatal slices, and increased synaptosomal glutamate release. The drimanial *in vitro* inhibited glutamate binding, glutamate uptake by astrocytes, synaptic vesicles, as well as by cortical, hippocampal and striatal slices, and increased synaptosomal glutamate release. When injected intraperitoneally in adult male mice, we observed that both polygodial and drimanial inhibited the glutamate uptake in slices from hippocampus and striatum, and did not affects glutamate uptake by cortical slices. These concurrent effects would predispose to an increase in the extracellular glutamate concentrations, leading to possible neurotoxic effects (excitotoxicity) of these natural compounds, which would suggest the need for some caution in their therapeutic application.

I. INTRODUÇÃO

I.1.INTRODUÇÃO

Produtos naturais, incluindo derivados de plantas, têm com o passar dos anos, contribuído largamente para o desenvolvimento de novas drogas terapêuticas (Calixto, et al., 2001). Comunidades primitivas já se utilizavam de plantas para tratar enfermidades, e estas práticas provinham do acúmulo secular do conhecimento (empírico), originando o que se convencionou chamar de medicina popular (Schultz, 1991). Aristóteles dizia que da curiosidade brotaram as ciências, na sua obra também encontramos o mais antigo conjunto botânico. Aristóteles, Teófrasto e Plínio, os mais afamados cientistas do seu tempo, dividiram o reino vegetal em três grandes grupos: árvores, arbustos e ervas. (Simões, et al., 1989). Embora muitas drogas importantes hoje tenham sido obtidas direta ou indiretamente de fontes naturais, como morfina, ácido acetil salicilico entre outras, é estimado que em torno de 40% de todos os medicamentos no mercado são obtidos de recursos naturais, sendo 25% de plantas. (Calixto, et al., 2001) Porém, do ponto de vista de suas atividades farmacológicas, até agora a relevância de produtos naturais, particularmente substâncias derivadas de plantas como uma fonte de novas drogas permanece pouco explorada. Estima-se que existam em torno de 250.000 espécies no mundo mas, somente uma pequena parte tenha sido alvo de investigações científicas. Em torno de 140.000 metabólitos secundários principalmente derivados de plantas superiores, têm sido isolados e identificados quimicamente. (Calixto, et al., 2001).

Mais de 50% das espécies de animais e plantas existentes no planeta (identificadas ou não) ocorrem nos trópicos, mais da metade das plantas tropicais

vive na Amazônia (Moraes, 2003). Juntos os países da América Latina possuem grande parte da biodiversidade mundial. Há inúmeras espécies de plantas e também uma grande riqueza em animais, micro-organismos e em recursos marinhos. Apesar dessa rica fonte natural, esses países nunca usaram corretamente a biodiversidade em benefício de seu próprio desenvolvimento, (Calixto, 2005). O Brasil possui em torno de 20-22% de todas as plantas e micro-organismos existentes. Entretanto, não mais do que 25.000 espécies têm sido objeto de qualquer estudo científico (Calixto, 2005).

De acordo com a Organização Mundial da Saúde em torno de 65-80% da população mundial de países em desenvolvimento, devido à pobreza e à falta de acesso à medicina moderna, depende essencialmente de plantas em suas principais necessidades terapêuticas (Calixto, 2005).

No Brasil, a utilização de plantas medicinais tem origem na cultura dos diversos grupos indígenas que habitavam o País (Simões, et al., 1989).

A pesquisa acadêmica trouxe novos conhecimentos sobre as plantas e suas propriedades terapêuticas. Ela pode ser entendida como uma atividade sistemática de investigação com registro detalhado dos dados, de maneira a permitir a sua verificação, comparação ou reprodução (Simões, et al., 1989).

Popularmente, as plantas medicinais de pequeno porte são conhecidas por ervas e geralmente são utilizadas inteiras. Para plantas maiores (arbustos, árvores) é comum a distinção de uma parte específica a ser utilizada (raízes, folhas, frutos, sementes, flores, etc.). Esta parte é geralmente secada à sombra, podendo ser picada grosseiramente e utilizada em preparações diversas (Simões, et al., 1989). Na medicina popular as preparações usuais de plantas medicinais

são nas formas de chás (Simões, et al., 1989) Além desse emprego, derivados de plantas têm sido muito úteis para o desenvolvimento de novas drogas terapêuticas**.** Um exemplo de planta medicinal é a *Drymis Winteri*, de onde são extraídos os compostos utilizados neste trabalho, Figura I.1

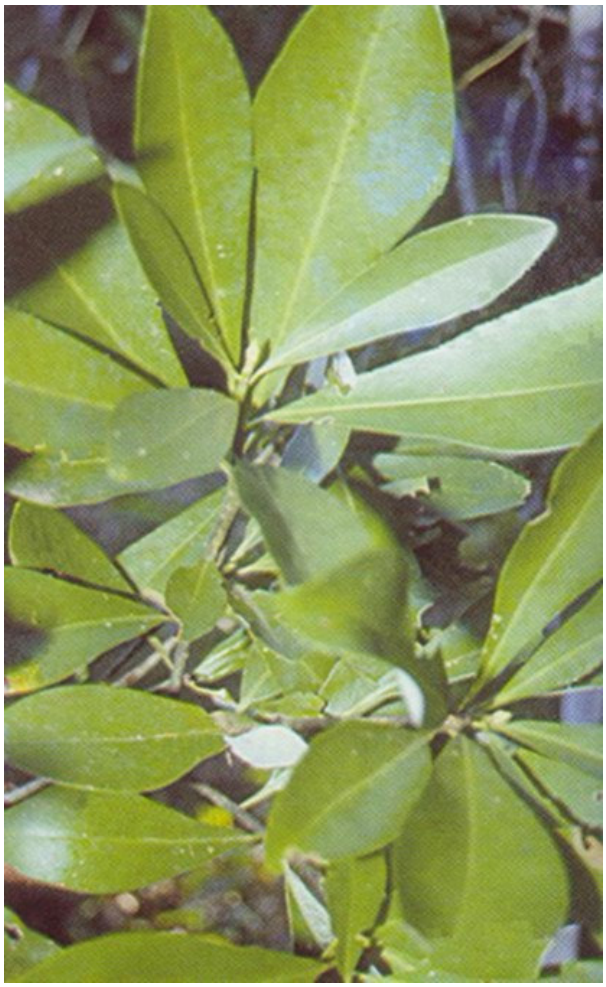
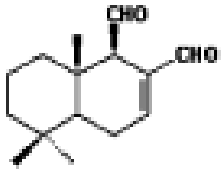


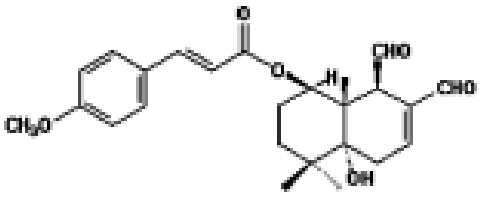
FIGURA I.1. Aspectos gerais das partes aéreas de uma planta de *Drymis Winteri* Fonte: Ervas e Plantas.

I.1.1. POLIGODIAL E DRIMANIAL

Os sesquiterpenos poligodial e drimanial são os principais constituintes isolados da Drymis winteri (Winteraceae) (Cechinel Filho, et al., 1998) (Figura I.2), conhecida popularmente como “Casca de Anta”, esta planta é encontrada no Brasil e em alguns países da América do Sul, cujas cascas e folhas são largamente utilizadas em medicina popular, na forma de chás e infusões, como antiinflamatório, antiespasmódico, antipirético e para o tratamento de asma, alergia e bronquite. (André, et al., 2004; Morton, 1981; Simões, et al., 1989).



Poligodial



Drimanial

FIGURA I.2. Estrutura química do poligodial e do drimanial

Extratos hidroalcoólicos da *Drymis winteri* atuaram em contrações induzidas por mediadores inflamatórios, e o principal constituinte isolado deste extrato, o poligodial, em concentrações micromolares produzem inibição similar de contrações induzidas por neurotransmissores em traquéia isolada de cobaia (El Sayah, et al., 1997 e 1998). Mendes e colaboradores (1998) demonstraram que extratos de *Drymis winteri* produziram inibição dependente de dose, efeito antinociceptivo, este de longa duração quando vários modelos de dor foram avaliados. Assim como o principal constituinte da *Drymis winteri*, o poligodial também teve efeito anti-hiperalgésico quando injetado intra-peritonialmente em camundongos nos modelos de hiperalgesia produzida pelo ácido acético, zymozan, formalina e capsaicina, assim como, contra bradicinina e substância P. Da mesma forma, verificou-se que o poligodial tem efeito antinociceptivo principalmente prevenindo dor neurogênica produzida por formalina e capsaicina (Mendes, et al., 2000).

Quando ratos foram tratados no período neonatal com os sesquiterpenos drimanial e poligodial, observou-se uma longa e pronunciada inibição da nocicepção e da hiperalgesia térmica, nestes animais quando adultos (André, et al., 2004) Estudos farmacológicos "*in vitro"* realizados com o sesquiterpeno poligodial demonstraram que este composto produz relaxamento dependente de sua concentração, quando testado em vasos com endotélio íntegro (artérias pulmonares de coelhos e cobaias e aorta torácica de coelho) e no corpo cavernoso de coelho. Estas ações parecem envolver a liberação de óxido nítrico (NO) ou substância relacionada ao NO do endotélio vascular, por meio de um mecanismo dependente da ativação da guanilato ciclase e do aumento dos níveis

de GMPc (André, et al.,1999 e 2004). Da Cunha e colaboradores (2001) demonstraram em avaliações feitas em ratos e camundongos propriedades antiinflamatórias e antialérgicas do poligodial em diferentes concentrações, inibindo significativamente edema de pata assim como edema de orelha.

Da mesma forma que o principal componente extraído da *Drymis winteri* o poligodial, outro componente extraído desta planta o drimanial possui efeitos antinociceptivos quando testados em modelos de dor induzidos por injeção intraperitonial de ácido acético (Malheiros, et al., 2001). Quando administrado sistematicamente via intraplantar ou rotas espinhal e supraespinhal, o drimanial produziu pronunciada antinocicepção, em modelos de nocicepção causados por formalina, capsaicina e glutamato (Scheidt, et al., 2002). Além disso, drimanial causou marcada inibição da nocicepção induzida por injeção intratecal de um agonista metabotrópico glutamatérgico (1S,3R)-ACPD em camundongos, mas não quando a indução foi realizada pelos agonistas ionotrópicos NMDA, AMPA e kainato ou pela substância P (Scheidt, et al., 2002). O drimanial também inibiu o "binding" de [3H]Glutamato em preparações de membrana de córtex de camundongos. Em experimentos em que nocicepção induzida por formalina, hiperalgesia térmica e edema de pata, hiperalgesia termal e extravasamento plasmático induzido por capsaicina e tratamento neonatal com drimanial também foi verificado o efeito antiinflamatório e antinociceptivo desta droga (André, et al., 2004). Esses resultados sugerem a utilização desses compostos, no desenvolvimento de novas drogas terapêuticas.

I.1.2. GLUTAMATO

O glutamato é o principal neurotransmissor excitatório do sistema nervoso central (SNC) de mamíferos, estando presente na maioria das sinapses centrais (Ozawa, et al., 1998). O glutamato está envolvido nas mais diferentes funções cerebrais, tais como aprendizado e memória, formação de redes neurais e processos relacionados ao desenvolvimento e ao envelhecimento (Castellano et al., 2001; Collingridge e Lester, 1989; Izquierdo e Medina, 1997; Ozawa, et al., 1998; Segovia, et al., 2001) O desequilíbrio deste sistema pode levar a doenças neurodegenerativas agudas como epilepsia (Dingledine et al., 1991; Price, 1999), hipóxia, anóxia e traumatismo craniano; e doenças neurodegenerativas crônicas, como Alzheimer, Huntington entre outras. Por outro lado uma ativação excessiva do sistema glutamatérgico pode provocar dano ou até mesmo a morte neuronal (Lipton e Rosemberg, 1994; Olney, et al., 1978; Price, 1999). A morte neuronal provocada pela estimulação excessiva dos receptores glutamatérgicos é denominada excitotoxicidade (Olney e Ho, 1970; Olney, et al., 1978).

Em cérebro de mamíferos, o glutamato é encontrado em altas concentrações e participa de funções metabólicas idênticas às exercidas em outros tecidos (Meldrum, 2000). No que se refere às suas ações específicas como neurotransmissor, o glutamato é sintetizado nos terminais pré-sinápticos, predominantemente a partir de glutamina, devido à ação da enzima glutaminase. Entretanto, também pode provir do α-cetoglutarato, via glutamato desidrogenase e

α-cetoglutarato aminotransferase (Schousboe et al., 1997). O glutamato sintetizado é armazenado, acoplado a uma H^+-ATPase (V-ATPase), num "pool" vesicular através da atividade de transportadores vesiculares independentes de Na^+, presentes nas membranas das vesículas sinápticas (Fykse e Fonnum, 1996).

Quando um impulso nervoso chega ao terminal pré-sináptico ocorre a despolarização dos terminais, e o glutamato que se encontra nas vesículas é liberado para a fenda sináptica, por exocitose, dependente da concentração de cálcio citosólico (Nicholls e Atwell, 1990). O glutamato uma vez liberado na fenda, vai interagir com seus receptores, localizados nas membranas pós-sinápticas e também nas membranas gliais (Gallo e Ghiani, 2000; Scannevin e Huganir, 2000), (Figura I.3).

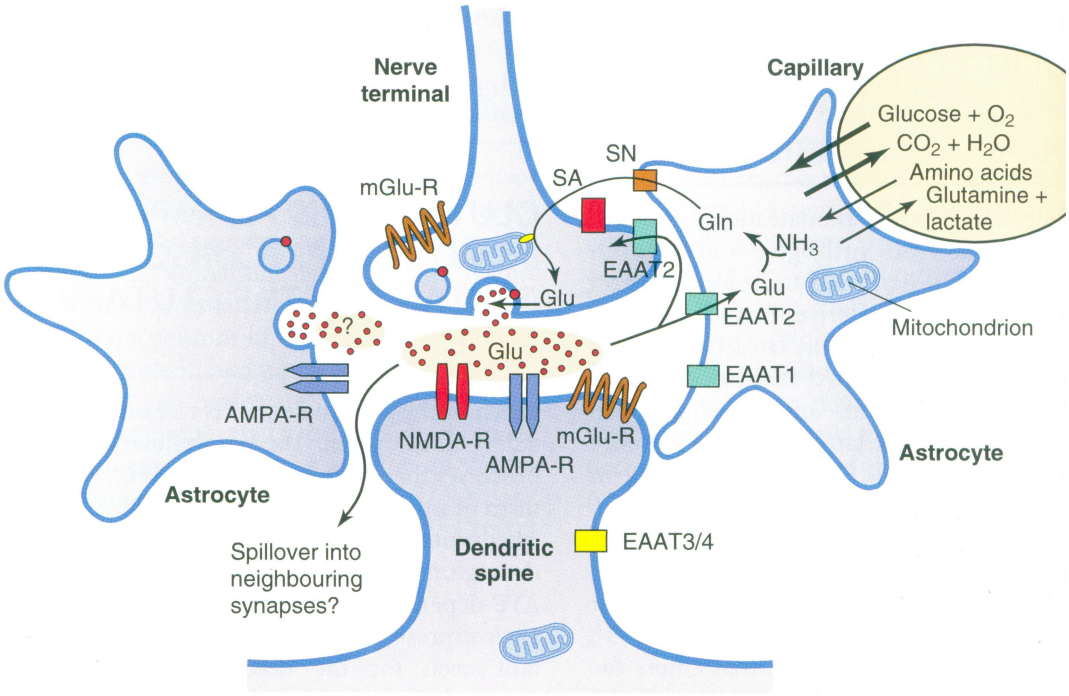


FIGURA I. 3. Representação esquemática de uma sinapse glutamatérgica

Fonte: Basic Neurochemistry

Os receptores glutamatérgicos (GluRs) controlam a maioria das ações do glutamato no SNC, e podem ser classificados de acordo com parâmetros farmacológicos e moleculares em dois grupos: receptores ionotrópicos e metabotrópicos (Figura I.4) (Ozawa, et al., 1998). Os receptores ionotrópicos (iGluR) são canais iônicos específicos para cátions que permitem a passagem de íons quando ativados, promovendo a despolarização da membrana sináptica, desencadeando uma resposta excitatória (Ozawa, et al., 1998). Tendo por base os agonistas específicos, os receptores ionotrópicos podem ser divididos em três grupos: N-Metil-D-Aspartato (NMDA), ácido α-amino-3-hidróxi-5-metil-4-isoxazol propionico (AMPA) e ácido caínico (KA). Todos os subtipos são ativados por glutamato, entretanto cada um deles é ativado seletivamente por um agonista sintético diferente (Ozawa, et al., 1998). Estudos de clonagem têm demonstrado que os receptores AMPA e KA são distintos, embora eles possam ser ativados por alguns mesmos agonistas e antagonistas, sendo frequentemente denominados não-NMDA. Os receptores AMPA e KA estão amplamente distribuídos por todo SNC. Os receptores NMDA são encontrados em todo o SNC, o maior nível é encontrado na região CA1 do hipocampo. (Ozawa, et al., 1998)

Os receptores metabotrópicos (mGluR) pertencem a uma família de receptores que interagem com proteínas ligantes de nucleotídeos da guanina (proteínas G), moduladores dos níveis de efetores intracelulares, através de enzimas como adenilato ciclase e da fosfolipase C, responsáveis pela produção de segundos mensageiros (como AMPc, diacilglicerol e inositol-3-fosfato), que por sua vez ativam e/ou inibem diversos eventos celulares (Conn e Pin, 1997; Ozawa, et al., 1998)

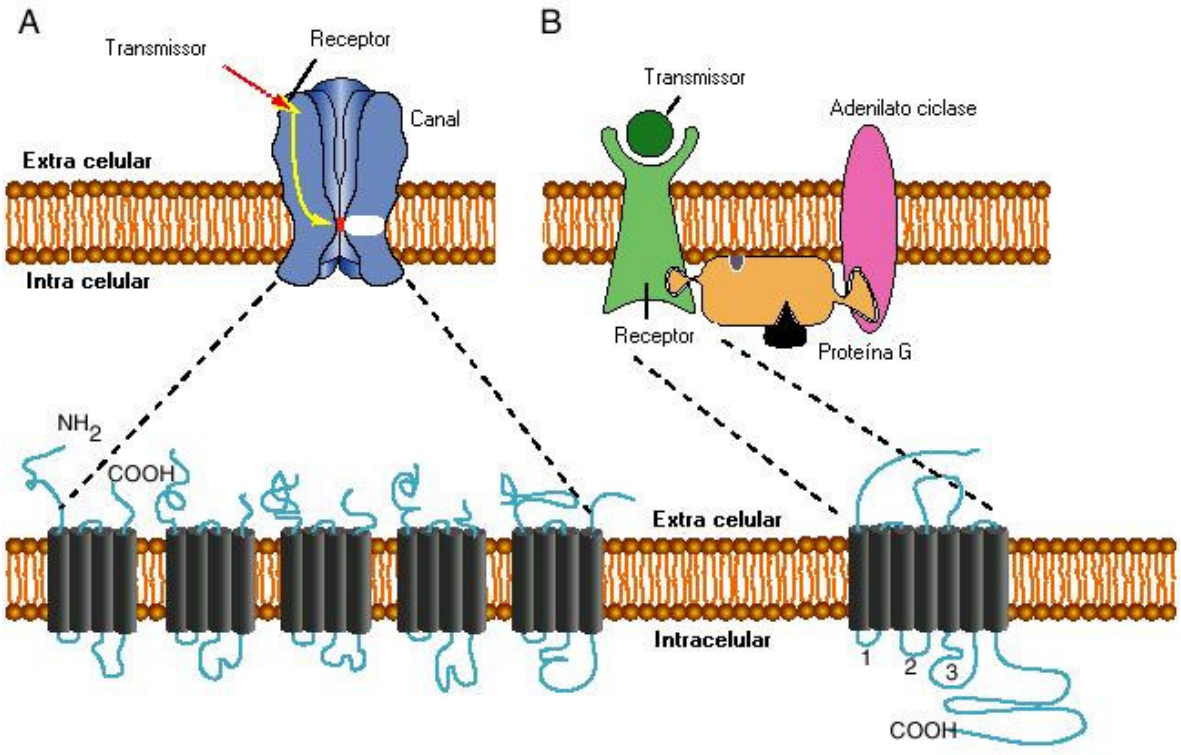


FIGURA I.4. Representação esquemática dos receptores ionotrópicos (A) e metabotrópicos (B). Fonte Zigmond et al, 1999.

I.1.3.ASTRÓCITOS

Os astrócitos são as mais numerosas células não neuronais do SNC e totalizam em torno de 50% do volume do cérebro humano (Tower e Young, 1973). Estas células fornecem suporte metabólico, estrutural e trófico para os neurônios e modulam a atividade sináptica (Chen e Swanson, 2003). Prejuízo às funções dos astrócitos durante isquemia e outros insultos, pode influenciar a sobrevivência dos neurônios, uma vez que estas células são responsáveis pela captação de glutamato, pelo tamponamento de radicais livres, transporte de água e produção de citocinas e NO (Chen e Swanson 2003).

Após a promoção de influxo iônico nas células pós-sinápticas e/ou a modulação da produção de segundos mensageiros, o glutamato precisa ser removido da fenda sináptica por sistemas de transporte dependentes de sódio, localizados principalmente na membrana celular dos astrócitos (Amara e Fontana, 2002; Anderson e Swanson, 2000; Danbolt, 2001; Robinson e Downd, 1997). Devido à ausência de sistemas enzimáticos para metabolizarem o glutamato na fenda sináptica, os sistemas de captação de glutamato são responsáveis pela inativação da ação glutamatérgica. Os transportadores de glutamato são os seguintes: GLAST/EAAT1, GLT-1/EAAT2 (transportadores gliais), EAAC1/EAAT3 (transportador neuronal), EAAT4 (transportador predominante em células de Purkinje no cerebelo) e EAAT5 (transportador encontrado na retina) (Arriza, et al., 1997; Danbolt, 2001; Fairman, et al., 1995; Kanai e Hediger, 1992; Pines, et al., 1992; Storck et al., 1992). Após a captação astrocitária, o glutamato pode ser metabolizado por duas diferentes vias: a formação da glutamina ou ser convertido

a α-cetoglutarato. A transformação em glutamina é realizada pela ação da glutamina sintetase, enquanto que a formação do α-cetoglutarato é feita por desaminação através da glutamato desidrogenase ou ainda pela ação de transaminases. A glutamina é liberada no espaço extracelular e pode ser captada pelos terminais pré-sinápticos neuronais, onde é convertida pela glutaminase em glutamato. Este, por sua vez, é captado pelas vesículas sinápticas e liberado novamente, recomeçando o processo. A transformação do glutamato em glutamina, bem como sua captação pelos neurônios e a sua transformação novamente em glutamato, é conhecida como ciclo glutamato/glutamina (Amara e Fontana, 2002; Anderson e Swanson, 2000; Danbolt, 2001). A remoção do glutamato da fenda sináptica, que ocorre primeiramente por transportadores sódio dependentes de alta afinidade, é o principal mecanismo modulador de ações do glutamato. Neste contexto, os transportadores gliais representam um importante papel na manutenção das concentrações extracelulares de glutamato abaixo dos níveis neurotóxicos (Anderson e Swanson, 2000; Danbolt, 2001).

I.1.4.GUANOSINA

Um possível papel neuroprotetor para a guanosina tem sido apontado em recentes trabalhos do nosso grupo. Nestas investigações verificou-se que a guanosina foi capaz de prevenir convulsões provocadas por super estimulação do sistema glutamatérgico. Especificamente, pelo ácido quinolínico (um composto que super estimula o sistema glutamatérgico), quando administrado no ventrículo

lateral de cérebro de camundongos e ratos, (Lara, et al., 2001; de Oliveira, et al., 2004; Schmidt, et al., 2000). Quando administrada por via oral crônica ou aguda, (de Oliveira, et al., 2004; Vinadé et al., 2003; 2004) a guo é capaz de atuar como anticonvulsivante. Por outro lado quando testada na tarefa de esquiva inibitória apresentou efeitos amnésicos semelhantes aos antagonistas glutamatérgicos (Roesler, et al., 2000; Vinadé, et al., 2003; 2004; 2005), bem como protegeu as fatias corticais dos efeitos deletérios da privação de glicose e oxigênio, e estimulou a captação de glutamato em culturas de astrócitos (Frizzo, et al., 2001; 2002). Estudos complementares demonstraram que a guanosina parece ser a principal responsável pelos efeitos estimulatórios na captação de glutamato (Frizzo, et al., 2003).

O efeito sobre a captação de glutamato parece ser específico da guanosina, uma vez que os nucleotídeos precisam ser hidrolisados para exercer esse efeito, e não ocorre efeito aditivo quando testados em conjunto com a guanosina (Frizzo, et al., 2003). Também o nível de guanosina no fluido cérebro espinhal encontra-se aumentado no grupo tratado, quando comparado com o controle (Soares, et al., 2004; Vinade, et al., 2005). O aumento provocado pela guanosina na captação de glutamato parece depender da idade, uma vez que os efeitos da guanosina foram observados em culturas de astrócitos com 10 dias *in vitro* e não em 40 dias (Gottifried, et al., 2002). Esse efeito parece se repetir em modelos mais complexos, visto que em fatias de córtex, hipocampo e estriado de cérebros ratos, de diferentes idades, a guanosina só estimulou a captação de glutamato em ratos de 10 dias de vida e ainda seu efeito apresentou-se como dependente da estrutura cerebral utilizada (Thomazi, et al., 2004).

I.1.5.AMINOÁCIDOS EXCITATÓRIOS PERIFÉRICOS

Tem sido demonstrado que o glutamato além de ser o principal neurotransmissor no sistema nervoso central, também possui um importante papel na transdução periférica da dor. Demonstrou-se que terminais sensoriais periféricos expressam receptores glutamatérgicos (GluRs) e que ligandos podem causar comportamentos nociceptivos em vários paradigmas experimentais, sugerindo que o glutamato está envolvido em transdução sensorial periférica e, em particular, transdução nociceptiva. Portanto, a manipulação do sistema glutamatérgico periférico pode conduzir a avanços na terapia da dor sem o risco de sérios efeitos colaterais no SNC. (Carlton, 2001) Existem evidencias de que o sistema glutamatérgico respresenta um importante papel na dor inflamatória. Por exemplo, aplicação periférica de antagonistas de receptores NMDA e não-NMDA atenua ou bloqueia comportamentos nociceptivos em vários modelos de inflamação (Carlton, 2001; Lucifora, et al., 2006; Rustioni, 2005).

O glutamato participa na modulação térmica de hiperalgesia periférica em ratos (Jackson, et al., 1995). Além disso, injeção de antagonista não competitivo de receptor NMDA, MK801, e antagonista competitivo de AMPA/Kainato CNQX reduziu significativamente a resposta hiperalgésica termal em ratos.

I.2. OBJETIVO

Os mecanismos dos efeitos anti-inflamatório e antinociceptivo destes compostos estão bastante descritos na literatura, cuja ação aparece como sendo no sistema nervoso periférico; especificamente, na corda espinal. Como é conhecido que estes efeitos ocorrem, pelo menos parcialmente, via sistema glutamatérgico, e este sistema de transdução tem papel fundamental no sistema nervoso central (fisiológico e patológico), o objetivo deste trabalho foi investigar possíveis efeitos excitotóxicos destas drogas no SNC, via glutamato.

II. RESULTADOS

CAPITULO I

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Naturally occurring compounds affect glutamatergic neurotransmission in rat brain

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Abstract:

Naturally occurring compounds affect glutamatergic neurotransmission in rat brain

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Abstract

Natural products, including those derived from plants, have largely contributed to the development of therapeutic drugs. Glutamate is the main excitatory neurotransmitter in the central nervous system and it is also considered a nociceptive neurotransmitter, by acting on peripheral nervous system. For this reason, in this study we investigated the effects of the hydroalcooholic extracts from *Drymis winteri* (polygodial and drimanial), *Phyllanthus* (rutin and quercetine), *Jathopha elliptica* (jatrophone), *Hedyosmum brasiliense* (3HDS), *Ocotea suaveolens* (Tormentic acid), *Protium kleinii* (αβ-amyrin), *Citrus paradise* (naringin), *soybean* (genistein) and *Crataeva nurvala* (lupeol), described as having antinociceptive effects, on glutamatergic transmission parameters, such as [^{3}H]glutamate binding, [^{3}H]glutamate uptake by synaptic vesicle and astrocyte cultures, and synaptosomal [^{3}H]glutamate release. All the glutamatergic parameters were affected by one or more of these compounds. Specifically, drimanial and polygodial presented more broad and profound effects, requiring more investigation on their mechanisms. The putative central side effects of these compounds, via the glutamatergic system, are discussed.

Keywords: glutamate transmission, antinociception, naturally occurring compounds, CNS, side effects.

1. Introduction

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) participating, via ionotropic (iGLURs) and metabotropic (mGLURs) receptors, in plastic processes, such as those related to memory and learning, neural networks formation, and brain development and ageing (Izquierdo et al, 1997; Ozawa et al., 1998; Segovia et al., 2001). Physiological activation of these receptors is essential for normal brain development and activity, whereas their overstimulation leads to excitotoxicity, as occurs in neurodegenarative disorders and in acute brain injury (Danbolt, 2001; Maragakis and Rothstein, 2001, 2004). Therefore, the maintenance of extracellular glutamate concentrations below neurotoxic levels is critical to brain function (Meldrum, 2000; Danbolt, 2001; Maragakis and Rothstein, 2001; 2004, Chen and Swanson, 2003), which is accomplished by glutamate uptake, mainly by astrocytes through a Ca^{+2}-dependent process (Attwell, 2000; Danbolt, 2001; Chen and Swanson, 2003). Within astrocytes, glutamate is converted to glutamine, released to extracellular space, taken up by presynaptic terminals and accumulated into synaptic vesicles (Robinson and Dowd, 1997), for further releasing to synaptic cleft under suitable stimuli. Therefore, all of these parameters have to work in coordinated way for an adequate functioning of the glutamatergic neurotransmission.

The glutamatergic system is modulated by environmental stimuli (Meldrum, 2000; Ozawa, 1998), including natural products extracted from plants (Martini et al, 2000; Scheidt et al, 2002). As various of these products are used in folk medicine, here we studied the effects of drimanial, polygodial, tormentic acid, jatrophone,

αβAmina,13HDS, genistein, narigin, quercetine, rutin and lupeol on the glutamatergic system of rat brain.

Polygodial and drimanial are dialdehyde unsaturated sesquiterpenes, main constituents isolated from *Drymis winteri (*Winteraceae*)*, a well-known medicinal plant found in Brazil and some South American countries**,** utilized in folk medicine as an anti-inflammatory, antispasmodic, antipyretic, and for the treatment of asthma, allergy and bronchitis (André, 2004; Morton, 1981). Systemic administration of polygodial and drimanial produces marked antinociceptive, anti-inflammatory and anti-allergic effects (Mendes et al., 2000; Da Cunha et al., 2001;Scheidt et al., 2002). Tormentic acid is a naturally occurring pentacyclic triterpene isolated from the stem bark of *Ocotea Suaveloesns* (Lauraceae) that produces dose-related antinociception against the acute visceral, neurogenic and inflammatory pain responses caused by acetic acid and formalin in mice (Beirith et al. 1999). Jatrophone is a diterpene isolated from *Jatropha elliptica* (Euphorbiaceae), a plant used in the folk medicine for the treatment of neoplasia, inflammation, ulcers and diuretic diseases**,** among others (Calixto and Sant'ana 1990). (13-Hydroxy-8,9-dehydroshizukanolide) is a sesquiterpene isolated from stems and leaves of *Hedyosmum brasilliense* (Chloranthaceae)**,** and produces significant inhibition of acetic acid-induced abdominal constriction in mice when given i.p. (Trentin et al. 1999). The genus *Phyllanthus* (Euphorbiaceae) consists of a great number of species widely distributed in most tropical and subtropical countries. The infusion of the leaves, stems and roots of the majority *Phyllanthus* species are largely used to treat hepatitis, disturbances of the kidney and urinary

bladder, intestinal infections, diabetes and exhibit a pronounced antinociception (for review see Calixto et al. 1998, 2001).

Protium kleinii (Burseraceae) is claimed to be useful to treat some inflammatory state (Otuki, 2005) and also exhibit antinociceptive properties (Otuki 2004). Lupeol, a pentacyclic triterpene, isolated from the stem bark of Crataeva nurvala Buch–Ham (Capparidaceae) has been shown to exhibit anti-inflammatory, anti-arthritic, anti-mutagenic and anti-malarial activity in vitro and in vivo systems (Geetha and Varalakshmi, 1998; Sudhahar, 2005).

Flavonoids are a large class of naturally occurring aromatic secondary plants metabolites, as the plant-derived compounds naringin and genistein, which have several effects on human health, mainly related to antioxidant activity (Dugo, 2005) and free radical scavenger activity.

In this paper we examined the effects of these several naturally occurring compounds on various glutamatergic neurotransmission parameters in rat brain. Our aim was to study if the demonstrated therapeutic effects of these compounds involving the peripheral nervous system could be connected with some putative side effects involving the central glutamatergic system in rats (Kruk, 2005).

2. Material and Methods

2.1. Materials

Drimanial, poligodial 13HDS, quercetine, rutin, Lupeol, tormentic acid, jatrophone and αβamyrin were provided from the Federal University of Santa Catarina. Genistein and naringin were obtained from Sigma. The maximum concentrations of compounds used were based on the maximum solubility

achieved. In all experiments the solvents (tween 20, DMSO or ethanol) were added in control groups. L-[^{3}H] Glutamate (48 Ci/mmol) was purchased from Amersham International, UK. Other chemicals were of analytical grade.

2.2. Animals

Adult Wistar rats (200-250 g) were obtained from our local breeding colony and maintained on a 12 hour light-12 hour dark schedule at 25° C, with food and water (ad libitum). The experiments were conducted according to the All experiments were in agreement with Brazilian biodiversity rights and with the Committee on Care and Use of Experimental Animal Resources of UFRGS, Brazil.

2.3. Membranes

2.3.1 Brain membrane preparations

Synaptic membrane preparations were carried out as described by Jones and Matus (1974). Animals were killed by decapitation without anesthesia, and their brains rapidly removed. All subsequent isolation procedures were performed at 4° C, except where otherwise indicated. The brains were homogenized in 0.32M sucrose (10% w/v) and centrifuged at 800 x *g* for 10 min. The supernatant was centrifuged at 27,000 x *g* for 20 min. The pellet was osmotically shocked by resuspension in 5 mM Tris/HCl pH 7.4 fort 30 min. The lysate was made up to 34% (w/w) sucrose by addition of the appropriate volume of 48% (w/W) sucrose. The upper phase of 28,5% (w/w) sucrose was overlaid above the sample phase and a small volume of 10% (w/w) sucrose overlaid onto this upper phase to give a gradients. These density gradients were centrifuged at 60,000 x *g* for 110 min in a

super speed centrifuge swing-out rotor. Three fractions were recovered, being the enriched synaptic membranes recovered from fraction 2 of the gradient.

2.3.2 [^{3}H]glutamate binding assay to neural membranes

The binding assay of [^{3}H]glutamate was performed at 25^{0}C in small polycarbonate tubes (total incubation volume 500 µL) containing 10 mM Tris-HCl pH 7.4, 0.1 mg membrane protein, 40 nM [^{3}H]glutamate and the drugs at specified concentrations. Incubation was started by the addition of membrane preparation. After 30 min of incubation, tubes were centrifuged at 20,800 x *g* for 5 min. The supernatant was discarded, and the walls of the tubes and the pellets surface were quickly and carefully rinsed with cold distilled water. The pellets were solubilized with 0.3 mL of 0.1% sodium dodecyl sulfate (w/v) and NaOH 0,1M overnight. Bound radioactivity was measured by using a Wallac scintillation counter.

2.4. Synaptic vesicles

2.4.1 Synaptic vesicle preparation

Synaptic vesicles were prepared from cerebral cortex as described by Fykse and Fonnum (1988), with some modifications according to Tavares et al. (2000). Briefly, homogenates (10 w/v) from cerebral cortex of rats were prepared in a buffer containing 0.32 M sucrose, 10 mM MOPS/Tris buffer, pH 7.4, and 1 mM EGTA and centrifuged twice for 10 min at 1000 *g*. Both supernatants were pooled and centrifuged for 30 min at 20.000 x *g* to obtain the crude synaptosomal fraction (P2). This fraction was osmotically shocked by resuspension for 30 min in 10 mM MOPS/Tris, pH 7.4, containing 0.1 mM EGTA (approximately 0.8 ml/g of fresh

tissue) and centrifuged at 17,000 x *g* for 30 min. The supernatant containing synaptic vesicles was subjected to 0.4 M and 0.6 M sucrose density gradient centrifugation at 65,000 x *g* for 2 h. The synaptic vesicle fraction was isolated from the 0.4 M sucrose band and stored at -70° C for up to four weeks, with no loss of activity. This fraction does not contain contaminant structures that might interfere with uptake by synaptic vesicles (Naito 1985).

2.4.2. [^{3}H]glutamate uptake by synaptic Vesicles

Uptake experiments were performed in a standard medium (final volume of 200 µl) composed of 10 mM MOPS/Tris, pH 7.4, 4 mM KCl, 140 mM potassium gluconate, 0.12 M sucrose, 2 mM $MgCl_2$, and 2 mM ATP (Wolosker et al, 1996), with [^{3}H]glutamate (3 µCi/ml) and 50 µM glutamate, with or without drugs. The uptake was started by the addition of synaptic vesicles (30-40 µg of protein/tube). Incubation was carried out for 10 min at 35° C, and the reaction was stopped by rapid filtration of the assay medium through 0.45 µm Millipore filters, which were quickly flushed three times with 4 ml of 10 mM MOPS/Tris buffer, pH 7.4, at room temperature. Specific uptake was obtained by subtracting the uptake measured in the absence of ATP from the total uptake. Radioactivity was measured by using a liquid scintillation counter (Wallac).

2.5. Astrocytes

2.5.1 Astrocyte cultures

Primary astrocyte cultures were prepared as described previously by Saneto et al. (1987) modified by Frizzo et. al (2001) from cortices of 1-day-old Wistar rats. The plating medium was MEM with 10% FBS plus 10 ng/mL. In each preparation,

cortices from six pups were dissociated with trypsin followed by DNAse I; the cells were plated in three 24-well plates. Astrocyte cultures were maintained in an incubator at 37° C in a humidified atmosphere of 95% air and 5% CO_2. When confluence was achieved (4-5 days *in vitro*, DIV) the medium was replaced by MEM 5% FBS, and 10μM cytosine arabinose (Ara-C) was added for 48h to eliminate mitotic cells. The subsequent medium change was performed in the above medium without Ara-c. EGF, which was described to induce GLT-1 protein expression in astrocyte (Zelenaia et al., 2000), was added to cell plating. Non-sister cultures from the same plating were used for each set of experiments. Cultures were used at 9 DIV, with astrocytes showing a polygonal morphology. The culture media were replaced with fresh media on the evening before glutamate uptake assay.

2.5.2 [^{3}H]glutamate uptake by astrocytes

Uptake was initiated by rinsing and pre-incubating the cultures for 23 min in Hank's balanced salt sodium medium (HBSS) at pH 7.4 containing drugs as indicated. This procedure was carried out in an incubator at 37° C in a humidified atmosphere of 95% air and 5% CO_2. The uptake was performed in HBSS containing 100μM glutamate and 0.01 μCi/mL L-[^{3}H]glutamate for 7 min. Incubation was stopped with two ice-cold washes of 1 mL HBSS, immediately followed by the addition of 0.5 M NaOH. Aliquots of lysates were taken for protein measure. Incorporated radioactivity was measured by using a liquid scintillation counter (Wallac 1409).

2.6. Synaptosomes

2.6.1. Synaptosomal preparation

Animals were killed by decapitation without anesthesia, and the brains were rapidly removed. Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradient (Dunkley et al, 1988), with minor modifications. Homogenates (10 %, w/v) from whole brain were made in 1.28 M Sucrose, 4 mM EDTA and 25 mM DDT (pH 7,4), and centrifuged at 800 x *g* for 10 min. The supernatant containing synaptosomes was submitted to 23, 15, 7 and 3% Percoll solution density gradient centrifugation at 24,000 x *g* for 10 min. The synaptosomal fractions were obtained between 15% and 23% of Percoll gradient and maintained at 4°C. The fraction was suspended **and** gently homogenized in HBSS medium (low K^+, with Ca^{+2}, pH 7.4) containing (in mM): Hepes 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 12, $CaCl_2$ 1.0, and centrifuged at 21,000 x g for 15 min. The supernatant was removed and the pellet gently resuspended in HBSS buffer. The isolated synaptosomal preparation contained 2.8 mg protein/mL. This fraction presented 5% contamination with both inner and outer mitochondrial membranes fragments, microsomes, myelin, as well as neuronal and glial plasma membranes (Nagi et al.,1986).

2.6.2. Synaptosomal [^{3}H]glutamate release

Determination of [^{3}H]glutamate release was accomplished as described by Migues et al. (1999), with minor modifications (Fontella et al., 2004). Prior to the release assay, synaptosomes were loaded with labeled glutamate by incubating the preparation for 15min at 37° C in a non-depolarizing medium (low potassium) consisting of HBSS, in the presence of 2 μM [^{3} H] glutamate. Aliquots of labeled

synaptosomes (1.4 mg protein) were centrifuged at 16,000 x *g* for 1 min. Supernatants were discarded, and the pellets were washed four times in HBSS by centrifugation at 16,000 x g for 1 min (at 4º C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 1 min in the absence (control) or presence of drugs. K^+- stimulated [^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization. Incubation was terminated by immediate centrifugation (16,000 x *g* for 1 min). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. [^{3}H]glutamate release was calculated as a percentage of the total amount of radiolabel present at the start of the incubation period (preloaded synaptosomes). The total amount of glutamate preloaded into synaptosomes was about 9.9 nmoles/mg protein.

2.7. Lactate dehydrogenase assay

In order to evaluate the integrity of astrocytes and synaptosomes, lactate dehydrogenase (LDH E 1.11.27) release was monitored, by incubating astrocytes with drugs during 23 min, and synaptosomes with drugs for 1 min. The LDH activity in the incubation medium and the total LDH content, determined by synaptosomal disruption using 0,1% Triton X-100, were assayed spectrophotometrically using a kit (Doles).

2.8. Protein determination

The protein content was determined according to Lowry *et al*. 1951, using bovine albumin as standard.

2.9. Statistics

All experiments were performed in triplicate and the mean was used for calculations. Statistical significance was assessed by analysis of variance (ANOVA) followed by Duncan's multiple range test when appropriate. A value of $p<0.05$ was considered statistically significant.

3. Results

The effects of hydroalcooholic extracts of plants on [^{3}H]glutamate binding, [^{3}H]glutamate uptake by synaptic vesicles and astrocytes and synaptosomal [^{3}H]glutamate release are exposed in Table 1a and Table1b. The [^{3}H]glutamate binding was inhibited by drimanial, polygodial, 13 HDS, genistein and quercetine. [^{3}H]glutamate uptake by synaptic vesicles was inhibited by drimanial, jatrophone, 13 HDS and quercetine. [^{3}H]glutamate uptake by astrocytes cultures was inhibited by drimanial, polygodial, tormentic acid and 13HDS, while it was enhanced by lupeol. Synaptosomal [^{3}H]glutamate release was stimulated by drimanial, polygodial (both in low and high K^+) and by narigin (low K^+).

The drugs did not affect LDH release, with exception of the highest dose of drimanial on astrocyte cultures (data not shown).

Discussion

Some *in vitro* effects of several naturally occurring compounds on glutamatergic parameters of rat brain are reported in this paper. These compounds were chosen due to their use in folk medicine in Brazil and other countries. Some of them affected significantly the CNS glutamatergic system. Those presenting activity tended to decrease the glutamate uptake by astrocyte cultures and by

synaptic vesicles, as well as increased the glutamate release from synaptosomal preparations. These effects in concert would tend to increase the extracellular glutamate concentrations, which could lead to excitotoxicity. The fact that various compounds also inhibited the glutamate binding could point that they are able to bind to specific sites for glutamate, located in glutamatergic receptors and transporters (which could explain, at least partially, the inhibitory effects of some of them on the uptake).

The most wide-ranging effects were observed with polygodial and drimanial, which are naturally occurring pungent sesquiterpenes containing 1,4-dialdehyde functional groups extracted from the bark of *Drymis winteri*, a Brazilian therapeutic plant used in folk medicine to treat inflammatory diseases, especially those associated to respiratory tract (Pio Corrêa, 1978). Several studies have shown that the two sesquiterpenes exert some important pharmacological actions, such as antinociceptive, anti-inflammatory and anti-allergic properties (Tratsk et al., 1997; Mendes et al., 1998, 2000; Da Cunha et al., 2001). Furthermore, the peripheral spinal and supraspinal antinociceptive action caused by drimanial is greatly mediated by its ability of interacting with metabotropic glutamatergic receptors (Scheidt et al., 2002). Recently, it was also reported that neonatal animals treated with both sesquiterpenes polygodial and drimanial, like the pungent vanilloid receptor agonist capsaicin, produced marked antinociception, as in adult animals, associated with decreased of binding [^{3}H]glutamate sites at the spinal cord. (André et al., 2004)

Some of the results (shown in Table 1, synaptosomal release) could suggest that drimanial and polygodial are acting as depolarizing compounds of neural cell membranes, thus acting as glutamate agonists.

The effects observed in astrocyte preparations were similar to those seen recently by our group in slices (Martini et al., 2006). It could indicate that the effects of both drugs observed in slices, a brain preparation where interactions among neural cells are mostly preserved, probably reflect astrocytic responses in the physiological environment. This gives an additional relevance to the drugs central effects.

The glutamate uptake by astrocytes is the main process involved in patho-physiological neuroprotection against glutamatergic excitotoxicity, by reducing the extra cellular glutamate concentrations below toxic levels. As a result, the effect of the drugs reducing the uptake (and in the instance of drimanial and polygodial also increasing the release) may indicate that these antinociceptive compounds could be harmful to the brain.

Although it is difficult to correlate the *in vitro* concentrations used here with *in vivo* doses used in other studies dealing with therapeutic effects, our results could indicate some putative central side effects, which warn to some care for using these compounds for medical purposes.

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Table Ia. Effect of drugs on glutamate transmission parameters (tween 20 or ethanol as control)

Parameter / Drugs	Glutamate Binding	Vesicle Uptake	Astrocyte Uptake	Glutamate Release Low K^+	High K^+
Control	100.0 ± 20.3(7)	100.0±10.8(5)	100.0±49.6(6)	100.0±11.7(4)	147.4±17.4 #(3)
Drimanial (234 μM)	42.5±4.0*(4)	45.7±10.1*(4)	3.7±1.6*(3)	358.3±45.5*(3)	322.0±45.7*(3)
Polygodial (855μM)	0 (3)	87.9±11.0(4)	2.4+0.4*(3)	374.9+45.0*(3)	385.9+42.7*(3)
Tormentic Acid (400 μM)	94.4±**14.4**(4)	69.8±10.3(4)	5.0±2.0*(3)	160.4±11.7(3)	189.4±21.7(3)
Jatrophone (650 μM)	76.1±5.0(4)	44.0±11.5*(4)	86.2±1.8(3)	124.9±16.0(3)	171.0±45.4(3)
αβAmyrin 20 mg/mL	86.7±13.8(5)	97.4+9.5(4)	115.1±18.9(3)	117.3±12.1(3)	173.4±30.0(3)

Data represent means ± SEM (expressed as percentage of control: 18.8 pmol/ mg prot for binding; 1.2 nmol/mg prot/min for vesicle uptake; 2.7 nmol/mg prot/min for astrocytic uptake). Tween was used as control for binding experiments and ethanol was used for other parameters. The numbers in bracelets indicate independent experiments performed in triplicate. * values significantly different from control group at $p<0.05$, # $p<0.001$ different from control low K^+.

Table Ib. Effect of drugs on glutamate transmission parameters (DMSO as control)

Parameters / Drugs	Glutamate Binding	Vesicle Uptake	Astrocyte Uptake	Glutamate Release Low K^+	High K^+
Control	10.00±17.5 (8)	100.0±20.6(5)	100.0±9.1(6)	100.0±11.3(4)	145.0±15.2(4)#
13 HDS (812 µM)	50.1±2.1*(3)	47.4±12.6*(4)	55.0±10.3*(3)	130.2±10.9(4)	191.3±20.6(4)
Genistein (740 µM)	30.4±5.1*(3)	98.9±11.7(5)	97.9±12.3(3)	143.8±4.6(3)	193.3±11.9(3)
Narigin (345 µM)	91.1±9.5(7)	144.2±22.4(5)	107.7±13.7(3)	152.5±13.7*(3)	198.2±15.1(3)
Quercetine (500 µM)	24.7±6.0*(4)	33.7±10.2**(5)	82.2±4.6(3)	139.4±12.9(3)	196.9±19.1(3)
Rutin (160 µM)	85.7±6.6(4)	118.9±14.7(5)	102.5±4.2(3)	125.3±8.6(3)	152.1±5.2(3)
Lupeol (160 µM)	73.2±0.8(4)	-	132.4±6.3*(3)	161.2±37.3(2)	214.0±8.7(2)

Data represent means ± SEM (expressed as percentage of control: 22,8 pmol/ mg prot for binding; 0,95 nmol/mg prot/min for vesicle uptake; 1,6 nmol/mg prot/min for astrocytic uptake). The numbers in bracelets indicate independent experiments performed in triplicate. * and ** values significantly different from control group at $p<0.05$ and $p<0.01$, respectively. # $p<0.001$ different from control low K^+.

CAPITULO II

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The sesquiterpenes polygodial and drimanial *in vitro* affect glutamatergic transport in rat brain

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ORIGINAL PAPER

The Sesquiterpenes Polygodial and Drimanial in vitro Affect Glutamatergic Transport in Rat Brain

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Abstract Natural products including those derived from plants, have over the years greatly contributed to the development of therapeutic drugs. Polygodial and drimanial are sesquiterpenes isolated from the bark of the plant Drymis Winteri (Winteraceae) that exhibit antinociceptive properties. Since peripheral glutamate presents nociceptive actions, in this study it was investigated the effects of hydroalcooholic extracts from Drymis winteri (polygodial and drimanial) on the glutamatergic system in rat brain. Polygodial and drimanial inhibited glutamate uptake by astrocytes, as well as by cortical, hippocampal and striatal slices, and increased synaptosomal glutamate release. These concurrent effects would predispose to an increase in the extracellular glutamate concentrations, leading to possible neurotoxic effects (excitotoxicity) of these natural compounds, which would suggest the need for some caution in their therapeutic application.

Keywords Polygodial · Drimanial · Glutamate · Antinociception · Naturally occurring compounds

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Introduction

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), participating in plastic processes, such as those involved in memory and learning, neural network formation, brain development and ageing [1–3]. However, overstimulation of the glutamatergic system leads to excitotoxicity, as observed in neurodegenerative disorders and acute brain injuries [4–8]. Thus, the maintenance of extracellular glutamate concentrations below toxic levels is essential for normal brain function [4–8]. This is attained by the Na^+-dependent glutamate uptake carried out by transporters located in astrocytic cell membrane [4, 8]. The glutamatergic excitotoxicity may be triggered by increased glutamate release and/or decreased astrocytic glutamate uptake [4, 6, 8]. Accordingly, Rothstein et al. [9], showed that knockout animals in glutamate astrocytic transporters are more sensitive to glutamate toxicity.

The modulation of the glutamatergic system is essential for the physiological functions of CNS, and it is influenced by a number of factors, such as environmental stimuli [2, 7]. Many drugs are targeted at this system [2, 4], including natural products extracted from plants [10, 11]. Polygodial and Drimanial are dialdehyde unsaturated

sesquiterpenes, isolated from *Drymis winteri* (Winteraceae), a well-known medicinal plant found in Brazil and some other South American countries that is used in folk medicine as an anti-inflammatory, antispasmodic, antipyretic, and for the treatment of asthma, allergy and bronchitis [11–13]. Systemic administration of polygodial and drimanial produces marked antinociceptive, anti-inflammatory and anti-allergic effects [11, 14, 15], and they are capable of inducing long-lasting inhibition of the development of nociception and thermal hyperalgesia [13]. Both polygodial and drimanial induced Ca^{+2} uptake in spinal cord of rats and markedly increased intracellular Ca^{+2} in cultured rat trigeminal neurons [16]. Polygodial administered either *i.p.* or *p.o* to mice produces dose-related long-lasting inhibition of abdominal constriction caused by acetic acid, kaolin and zymosan, and the neurogenic and inflammatory pain caused by formalin and capsaicin [14, 17]. Administered either i.p. or i.c.v. to mice, polygodial also produces antinociception against both phases of the formalin response, indicating spinal and supraspinal action sites. Given p.o. to rats, the extract of *D. winteri* reverses the hyperalgesia produced by paw injection of bradykinin and substance P, but not that caused by prostaglandin E_2 or carrageenan [14]. Drimanial exhibited antinociceptive action against pain induced by acetic acid, being about three times less active than polygodial [18].

The mechanisms of the anti-inflammatory and antinociceptive effects of these compounds, which action is basically on peripheral nervous system, specifically on spinal cord [13, 14], are well described in the literature [16, 18]. As it is largely known, these effects occur, at least partially, through the glutamatergic system [11, 19, 20]. The aim of this study was to investigate the effects of these drugs on the glutamatergic system in the CNS. This approach seeks to investigate the involvement of the central glutamatergic system in putative side effects of these drugs, thus neither aiming to investigate the CNS involvement on their anti-inflammatory nor anti-nociceptive actions. Importantly, hyperactivation of the central glutamatergic system is clearly involved in the pathogenesis of various acute and chronic neurodegenarative diseases [4–8].

Material and methods

Animals

Adult Wistar rats (200–250 g) maintained in a 12 h light to 12 h dark schedule at 25°C, with food and water (ad libitum) were obtained from our local breeding colony.

Materials

Drimanial and polygodial (sesquiterpenes) were provided from the Federal University of Santa Catarina (Prof. Calixto). The maximum concentrations of the compounds used were based on the maximum solubility achieved. In all experiments, the solvent (ethanol) was added in control groups.

L-[^{3}H]Glutamate (48 Ci/mmol) was purchased from Amersham International, UK. Other chemicals were of analytical grade.

Astrocytes and brain slices

Astrocyte culture preparations

Primary astrocyte cultures were prepared as described previously by Saneto et al. [21] modified by Frizzo et al. [22] from the cortices of 1-day-old Wistar rats. The planting medium was MEM with 10% FBS plus 10 ng/ml. In each preparation, cortices from six pups were dissociated with trypsin, followed by DNAse I addition and the cells were plated in three 24-well plates. Astrocyte cultures were maintained in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When confluence was achieved (4–5 days in vitro, DIV) the medium was replaced by MEM 5% FBS, and 10 μM cytosine arabinose (Ara-C) was added for 48 h to eliminate mitotic cells. The subsequent medium change was performed in the above medium without Ara-c. EGF, which was shown to induce GLT-1 protein expression in astrocyte [23], was added to cell plating. Non-sister cultures from the same plating were used for each set of experiments. Cultures were used at nine DIV, with astrocytes showing a polygonal morphology. The culture media were replaced with fresh media in the evening before the glutamate uptake assay.

Slice preparations

The animals were decapitated, their brains immediately removed and humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 1.11 glucose, in pH 7.2. The striatum, hippocampus and parietal cortex were dissected into Petri dishes with HBSS and slices (0.4 mm) were obtained using a McIlwain tissue chopper. The slices were separated with the help of a magnifying glass and transferred to 24-well culture plates: one plate was maintained at 35°C and the other on ice. The slices from the first plate were washed once with 1 ml of 35°C HBSS and the second with 1 ml sodium-free HBSS for the analysis of non-specific uptake (see below).

[^{3}H]Glutamate uptake by astrocyte cultures and brain slices

Uptake was initiated by rinsing and pre-incubating the cultures for 23 min or the slices for 23, 25 or 27 min, respectively, for cortex, hippocampus and striatum in Hank's balanced salt sodium (HBSS) at pH 7.4 containing drugs as indicated. For cultures, this procedure was carried out in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The uptake was performed in HBSS containing 100 μM glutamate. The L-[^{3}H]glutamate concentrations and incubation times were: 0.01 μCi/ml and 7 min (astrocytes), 0.33 μCi/ml and 7 min (cortical slices), 0.66 μCi/ml and 5 min (hippocampal slices), and 0.66 μCi/ml and 3 min (striatal slices). Incubation was stopped in all preparations with two ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5 M NaOH. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409).

To measure sodium-independent uptake, the same protocol described above was used, with differences in temperature and the medium used. Sodium-independent uptake was determined on ice (4°C) using N-methyl-D-Glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the specific one. Both the specific and non-specific uptakes were performed in triplicate.

In experiments in slices with guanosine, the drug concentrations used were: drimanial—120 μM (cortex), 230 μM (striatum) or 140 μM (hippocampus); polygodial—140 μM (cortex), 350 μM (striatum) or 560 μM (hippocampus). These concentrations correspond to IC_{50} obtained from the concentration curves.

Synaptosomes

Synaptosomal preparation

Animals were killed by decapitation without anesthesia, and the brains were rapidly removed. Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradients described previously by Dunkley et al. [24], with minor modifications. Homogenates (10%, w/v) from cerebral cortex were made in 1.28 M Sucrose, 4 mM EDTA and 25 mM DDT (pH 7.4), and centrifuged at 800×*g* for 10 min. The supernatant containing synaptosomes was submitted to 23, 15, 7 and 3% Percoll solution density gradient centrifugation at 24,000×*g* for 10 min. The synaptosomal fractions isolated from whole brain were maintained at 4°C. The fraction was suspended, gently homogenized in HBSS (low K^+, with Ca^{+2}) (pH 7.4) medium containing in mM: (Hepes 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2; glucose 12, $CaCl_2$ 1.0) and centrifuged at 21,000×g for 15 min. The supernatant was removed and the pellet gently resuspended in HBSS buffer. The isolated synaptosomal preparation contained 2.8 mg protein/ml. This fraction contained 5% contamination with inner and outer mitochondrial membrane fragments, microsomes, myelin, as well as neuronal and glial plasma membranes [25].

Synaptosomal [^{3}H]glutamate release

Determination of [^{3}H]glutamate release was accomplished as described by Migues et al. [26] with minor modifications by Fontella et al. [27]. Prior to the release assay, synaptosomes were loaded with labeled glutamate by incubating the preparation for 15 min at 37°C in a non-depolarizing medium (low potassium) consisting of HBSS (composition in mM: Hepes 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2; glucose 12, $CaCl_2$ 1.0), in the presence of 2 μM [^{3}H]glutamate. Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 16,000×*g* for 1 min. Supernatants were discarded, and the pellets were washed four times in HBSS by centrifugation at 16,000×g for 1 min (at 4°C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 1 min in the absence (control) or presence of drugs. K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization. Incubation was terminated by immediate centrifugation (16,000×*g* for 1 min). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. [^{3}H]Glutamate release was calculated as a percentage of the total amount of radiolabel present at the start of the incubation period (preloaded synaptosomes).

The total amount of glutamate preloaded into synaptosomes was about 9.9 nmol/mg protein.

Lactate dehydrogenase assay

In order to evaluate the integrity of astrocytes and synaptosomes, lactate dehydrogenase (LDH E 1.11.27) release was monitored, by incubating astrocytes with drugs for 23 min, and synaptosomes with drugs for 1 min. The LDH activity in the incubation medium and the total LDH content, determined by synaptosomal disruption using 1.5% Triton X-100, were assayed spectrophotometrically using a kit (Doles).

Measurement of protein content

The protein content of synaptosomal preparations and astrocyte cultures was determined according to Lowry et al. [28], using bovine albumin as standard. The protein content

of cortical, hippocampal and striatal slices were determined by the method of Peterson [29].

Statistics

All experiments were performed in triplicate and the mean was used for calculations. Statistical significance was assessed by variance analysis (ANOVA) followed by Duncan's multiple range test when appropriate. A value of $P<0.05$ was considered statistically significant.

Results

Results of the synaptosomal release are shown in Figs. 1a and 2a. Drimanial and polygodial at the highest doses used increased the basal and K^+-stimulated glutamate release.

Figures 3a and 4a show dose-response curves of drimanial and polygodial effects, on astrocytic [^{3}H]glutamate uptake, respectively. Both drugs abolished the uptake in a dose-dependent manner.

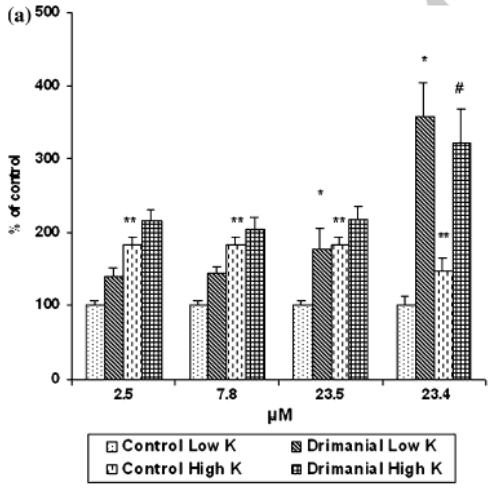
Figures 5a–6a show dose-response curves of drimanial and polygodial effects on [^{3}H]glutamate uptake by cortical, hippocampal and striatal slices. Both drugs inhibited the uptake in all structures studied.

Based upon our previous results [22, 30–34] we investigated whether guanosine (100 μM) could reverse the effects of drimanial and polygodial on [^{3}H]glutamate uptake. Guanosine had no effect, except on the action of drimanial in hippocampus (Figs. 5b–6b).

In order to verify if these drugs affected brain membrane integrity, we measured the effects of polygodial and drimanial on the LDH activity release from synaptosomes (Figs. 1b and 2b), from astrocytic cell cultures (Figs. 3b and 4b) and from brain slices (data not shown). Both drugs did not affect LDH release, with the exception of the highest dose of drimanial on astrocyte cultures.

Discussion

Polygodial and drimanial are naturally-occurring pungent sesquiterpenes containing 1,4-dialdehyde functional groups, extracted from the bark of *Drymis winteri*, a Brazilian medicinal plant used in folk medicine to treat inflammatory diseases, especially those related with the respiratory system [35]. Several studies have shown that these two sesquiterpenes present antinociceptive, anti-inflammatory and anti-allergic properties [14, 15, 17, 36]. Furthermore, the peripheral spinal and supraspinal antinociceptive action caused by drimanial is greatly mediated by its ability to interact with metabotropic glutamatergic receptors [11]. Recently, it has also been reported that sesquiterpenes polygodial and drimanial produced marked antinociception, as seen with the use of capsaicin, a vanilloid receptor agonist, in neonatal animals as in adult animals. This effect is associated with decreased [^{3}H]glutamate binding in the spinal cord, while only the neonatal capsaicin treatment was capable of inhibiting the expression of TRPV1 in dorsal root ganglia [13]. These findings indicate that, at least in part, the



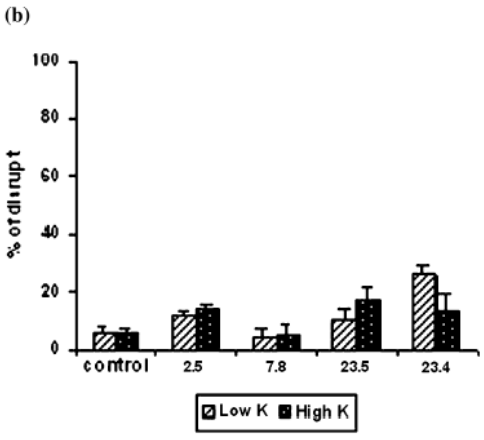


Fig. 1 (**a**) Effect of drimanial (2.5, 7.8, 23.5 and 234 μM) on [^{3}H]glutamate release from synaptosomes ($n=3$). Results are expressed as a percentage of control. Values significantly different from the low potassium control group: $^*P<0.05$; $^{**}P<0.001$. ($P<0.05$ different from the high potassium control. (**b**) Effect of all doses of drimanial LDH activity release from synaptosomes. Data represent the means±SEM

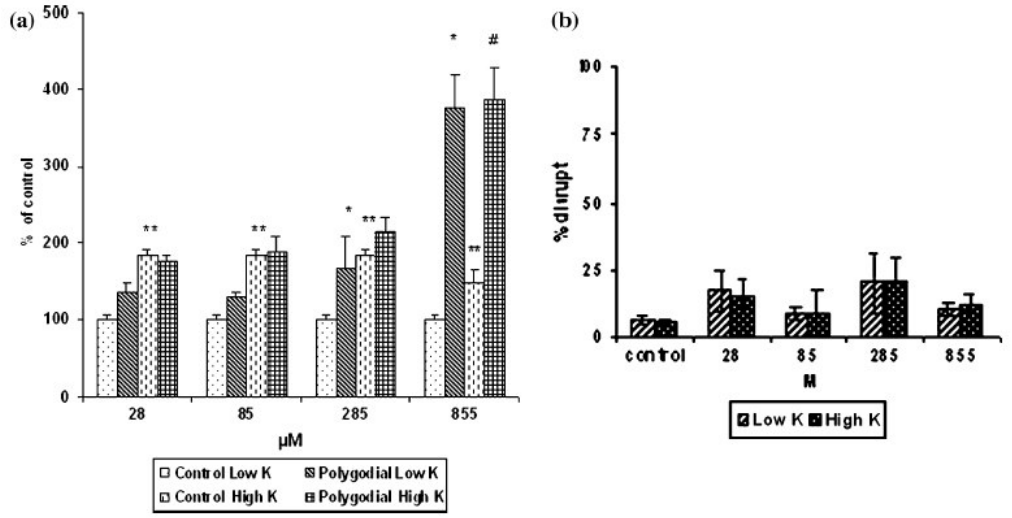


Fig. 2 (**a**) Effect of polygodial (28, 85, 285 and 855 μM) on [^{3}H]glutamate release from synaptosomes ($n = 3$). Results are expressed as a percentage of control. Values significantly different from the low potassium control group: *$P < 0.05$; **$P < 0.001$. ($P < 0.05$ different from the high potassium control. (**b**) Effect of all doses of Polygodial on LDH activity release from synaptosomes. Data represent the means±SEM

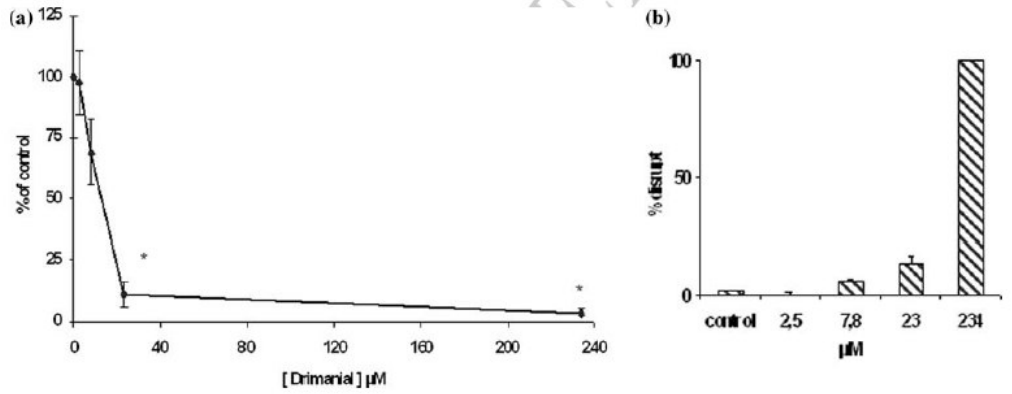


Fig. 3 (**a**) Effect of drimanial (2.5, 7.8, 23.5 and 234 μM) on astrocyte [^{3}H]glutamate uptake ($n = 3$). Results are expressed as a percentage of control. * Indicates values significantly different from the control group at $P < 0.05$. (**b**) Effect of drimanial on LDH activity release from astrocytes ($n = 2$). Data represent means±SEM

antinociceptive actions of polygodial and drimanial, could be associated with their ability of interact with TRPV1.

Owing to the antinociceptive effects of drimanial and polygodial involve the neurotransmitter glutamate at peripheral nervous system [11, 19, 20], we investigated if they could also affect the central glutamatergic system, with putative deleterious actions on the CNS. In fact, they significantly decreased the glutamate uptake by astrocyte cell cultures and slices from brain structures, and increased the glutamate release from synaptosomal preparations.

Since glutamate uptake by astrocytes is the main process involved in patho-physiological neuroprotection against glutamatergic excitotoxicity, by reducing the extra cellular glutamate concentrations below toxic levels, these inhibitory effects suggest putative toxic properties for both sesquiterpenes. Thus, in order to determine if these effects

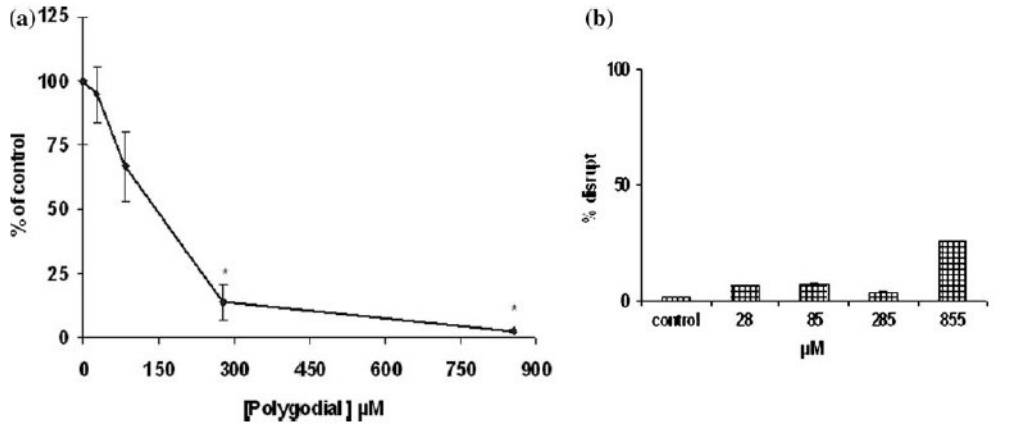


Fig. 4 (**a**) Effect of polygodial (28, 85, 285 and 855 µM) on astrocyte [^{3}H]glutamate uptake (n = 3). * Indicates values significantly different from the control group at $P < 0.05$ (**b**). Effect of polygodial on LDH activity release from astrocytes (n = 2). Data represent the means±SEM

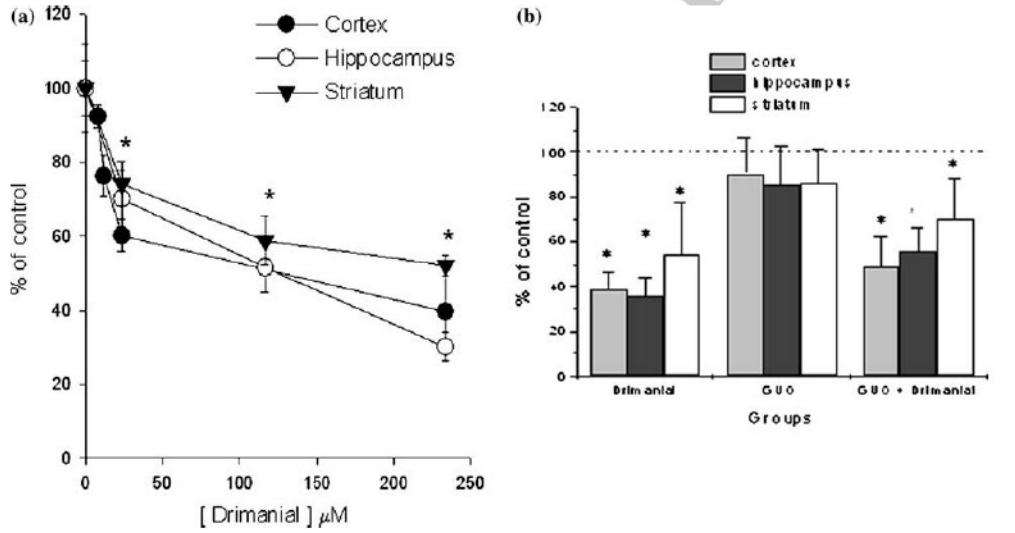


Fig. 5 (**a**) Effect of different concentrations drimanial on L-[^{3}H]Glutamate uptake in cortical (7.8, 11.7, 23.4 and 234 µM), hippocampal and striatal (23.4, 117 and 234 µM) slices (n = 4). Results are expressed as a percentage of control. *Different values from the control ($P < 0.05$). (**b**) Effect of guanosine (100 µM). (Different from drimanial group. Data represent the means±SEM

are also present when astrocytes are in their physiological environment we investigated the effect of both drugs on the glutamate uptake by brain slices, a preparation where the interactions among neural cells are mostly preserved. Slices from some specific brain structures were used in order to elicit information on the regional brain distribution. Both drugs also inhibited glutamate uptake in slices obtained from cerebral cortex, hippocampus and striatum, indicating that astrocytes in their physiological environment were sensitive to the drugs and that their effects were spread throughout the brain.

The effects on glutamate release could suggest that drimanial and polygodial are acting as neural cell membrane depolarizing compounds. Importantly, most of these

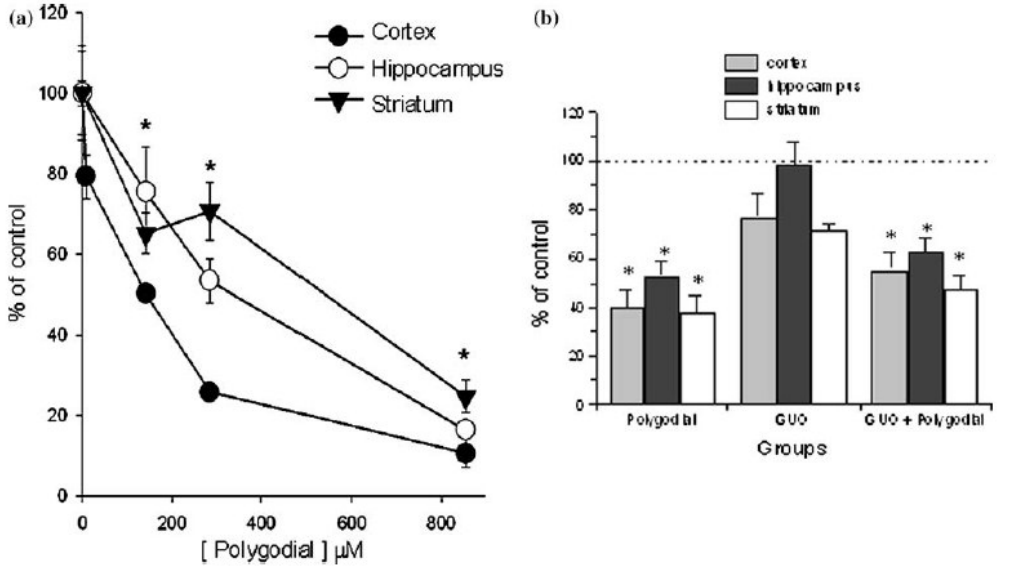


Fig. 6 (**a**) Effect of different concentrations of polygodial on [^{3}H]-glutamate uptake by cortical (85.5, 142.5, 285 and 855 µM) hippocampal and striatal (142.5, 285 and 855 µM) slices. Results are expressed as a percentage of control * indicates values different from the control $P<0.05$ **. (**b**) Guanosine (100 µM). Data represent the means±SEM

effects did not involve cell membrane disruption, as evidenced by the measure of LDH activity release.

Previously, we showed that guanosine in vivo plays neuroprotective roles, preventing seizures induced by overstimulation of the glutamatergic system [30, 31]. Considering that guanosine also enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions [32, 34] and in cultured astrocytes [22, 33] and that this effect may be involved in the neuroprotection exerted by guanosine against glutamatergic excitotoxicity, here we decided to test whether guanosine could reverse the effects of drimanial and polygodial on glutamate uptake. Guanosine had no effect, except partially on the effect of drimanial, in hippocampus. The effects of guanosine are under investigation in our group.

In concert, the effects on release and on uptake, which tend to increase extracellular glutamate concentrations, would lead to injurious effects on brain (excitotoxicity], giving additional relevance to the effects of these drugs on CNS. In fact, our [34, 37] and other [4, 6, 8] groups have results indicating that this decrease in uptake may be implicated in brain injury involving the glutamate system.

Although in vitro concentrations of the drugs used here could be difficult to compare with those of in vivo studies (pharmacological effects), our results point to some caution in their therapeutic use. Whether these substances also exert the same neurochemical effects in vivo is currently under investigation in our group.

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CAPITULO III

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The sesquiterpenes polygodial and drimanial administered intraperitoneally inhibit [3H]glutamate uptake by brain slices from mice

Artigo em preparação

The sesquiterpenes polygodial and drimanial administered intraperitoneally inhibit [3H]glutamate uptake by brain slices from mice

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Abstract

Glutamate is the primary excitatory amino acid neurotransmitter in the central nervous system, and has been shown to have a transduction of sensory input at the periphery. Polygodial and drimanial are sesquiterpenes isolated from the barks of the plant *Drymis winteri* (Winteraceae) and exhibit antinociception properties. As glutamate is considered a nociceptive neurotransmitter, in this study we investigated the *in vivo* effects of the compounds isolated from Drymis winteri (polygodial and drimanial) on central glutamatergic activity of mice. Polygodial and drimanial i.p. administered inhibited the [^{3}H]glutamate uptake by slices from hippocampus and striatum, which could indicate that they could increase extracellular glutamate concentrations, likely causing excitotoxicity. These results point to some caution with the *in vivo* use of these drugs.

Keywords: Polygodial, drimanial, glutamate, antinociception, naturally-occuring compounds

1 - Introduction

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) participating in plastic processes, as memory and learning, neural networks formation, and brain development and ageing (Ozawa et al, 1998; Segovia et al., 2001, Izquierdo et al, 1997). However, glutamate has been shown to have also a role in transduction of sensory input at the peripheral nervous system (PNS). The fact that the peripheral sensory terminals express glutamate receptors (GluRs) and that glutamatergic agonists can cause nociceptive effects in various experimental paradigms suggests that glutamate is involved in peripheral sensorial transduction, particularly nociceptive transduction (Carlton 2001). The glutamatergic system is modulated by environmental stimuli (Meldrum, 2000; Osawa, et. al., 1998), including natural products extracted from plants (Martini et al, 2000; Scheidt et al, 2002).

Poligodial and drimanial are dialdehyde unsaturated sesquiterpenes, main constituents isolated from *Drymis winteri (*Winteraceae*)*, a well-known medicinal plant found in Brazil and some South America countries that is used in folk medicine as an anti-inflammatory, antispasmodic, antipyretic, and for the treatment of asthma, allergy and bronchitis (André, 2004; Morton, 1981). Systemic administration of polygodial and drimanial produces marked antinociceptive, anti-inflammatory and anti-allergic effects ((Mendes et al., 2000; Da Cunha et al., 2001; Scheidt et al., 2002), and they are capable of inducing a long-lasting inhibition of development of nociception and thermal hyperalgesia (André et al., 2004).

Previous studies with brain rats from our group demonstrated that polygodial and drimanial inhibited L-[^{3}H]glutamate binding in membrane preparations and glutamate uptake by astrocytes, synaptic vesicles (only drimanial) and slices from cortex, hippocampus and striatum, as well as stimulated synaptosomal glutamate release (Martini et. al., 2006).

Considering these effects, here we decided to investigate if these drugs, which exert analgesic effect *in vivo* and affect glutamatergic parameters *in vitro*, can affect L-[^{3}H]glutamate uptake by slices from cerebral cortex, hippocampus and striatum in adults male mice, when injected intraperitoneally.

2 - Material and methods

2.1. Animals

Adults male mice (30-45g), maintained on a 12h light/12h dark cycle and with free access to food and water, were used.

2.2. Materials

L-[^{3}H]glutamate (49 Ci/mmol) was purchased from Amersham International, UK. Other chemicals were of analytical grade.

Polygodial and drimanial (sesquiterpenes) were provided from the Federal University of Santa Catarina, Brazil. The drugs were solubilized in ethanol (12,5 %) and administered at 30 mg/Kg; control animals received a similar volume of 0,9% NaCl solution (ethanol 12,5%).

2.3. L-[^{3}H]glutamate uptake

2.3.1 Slices preparations

Animals were sacrificed 30 min after drugs administration. They were decapitated, their brains were immediately removed and humidified with Hank´s balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0,63 Na_2HPO_4; 4,17 $NaHCO_3$; 5,36 KCl; 0,44 KH_2PO_4; 1,26 $CaCl_2$; 0,41 $MgSO_4$; 0,49 $MgCl_2$ and 1,11 glucose, in pH 7.2. Striatum, hippocampus and parietal cortex were dissected onto Petri dishes with HBSS and slices (0.4 mm) were obtained using a McIlwain tissue chopper. The slices were separated with the help of a magnifying glass and transferred to 24-well culture plates: one plate was maintained at 35° C and the other on ice. The slices from the first plate were washed once with 1 mL of HBSS at 35° C and the second with 1 mL sodium-free HBSS for the analysis of non-specific uptake (see below).

2.3.2 Total uptake

Glutamate uptake was performed according to Thomazi et al (2004). Uptake was assessed by adding 0.33 and 0,66 µCi mL^{-1} L-[^{3}H]glutamate for cortex and hippocampus/striatum respectively, with 100 µM unlabeled glutamate in HBSS at 35°C. Incubation was stopped after 7 min, 5 min or 3 min for cortex, hippocampus or striatum respectively by two ice-cold washes with 1 ml HBSS immediately followed by addition of 0.5 M NaOH, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[^{3}H]glutamate through scintillation counting and for protein measurement.

2.3.3 Sodium-independent uptake

To measure sodium independent uptake, the same protocol described above was used, though with differences in the temperature and the medium used: it was determined on ice 4º C using N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the specific one. Both the specific and non-specific uptakes were performed in triplicate.

2.3.4 Radioactivity quantification

Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409)

2.3.5. Measurement of protein content

Protein content was determined by the method of Peterson (1977), using serum bovine albumin as standard.

2.4. Statistical analysis

All experiments with L-[^{3}H]glutamate were performed in triplicates and the mean was used for the calculations (percentage). Statistical significance was assessed by paired-samples T test.

3. Results

In this study we investigate the effects of the sesquiterpenes polygodial and drimanial i.p. injection on L-[^{3}H]glutamate uptake in cortex, hippocampus and striatum slices from adult mice brain. The uptake was significantly inhibited by polygodial (Fig. 1) and drimanial (Fig. 2) in striatum and hippocampus. No effect was observed in cortical slices

Discussion

Polygodial and drimanial are naturally-occurring pungent sesquiterpenes containing 1,4-dialdehyde functional groups extracted from the bark of *Drymis winteri*, a Brazilian medicinal plant used in folk medicine to treat inflammatory diseases, especially those related with the respiratory system (Pio Corrêa, 1978). Several studies have shown that these two sesquiterpenes present antinociceptive, anti-inflammatory and anti-allergic properties (Tratsk et al., 1997; Mendes et al., 1998; 2000; Da Cunha et al., 2001). Furthermore, the peripheral spinal and supraspinal antinociceptive action caused by drimanial is greatly mediated by its ability to interact with metabotropic glutamatergic receptors (Scheidt et al., 2002). Recently, it has also been reported that the sesquiterpenes polygodial and drimanial produce marked antinociception, as seen with the use of capsaicin, a vanilloid receptor agonist, in neonatal animals as in adult animals. This effect is associated with decreased [^{3}H]glutamate binding in the spinal cord.

Considering that the antinoceptive effects of drimanial and polygodial appear to involve the neurotransmitter glutamate and that the interference of drugs on the glutamatergic system may have beneficial or detrimental action in the CNS, in the present study we investigated the effect of both sesquiterpenos injected intraperitoneally on glutamate uptake by neural cells. This glutamatergic parameter was chosen due their relevance in the maintenance of the physiological glutamatergic tonus, preventing excitotoxicity. We investigated the effect of both drugs on the glutamate uptake by brain slices, a preparation where the interactions among neural (neuronal and astrocytic) cells are mostly preserved. Slices from

some specific brain structures were used, in order to elicit information on the regional brain distribution of their effects. Both drugs inhibited glutamate uptake by slices obtained from cerebral hippocampus and striatum, indicating that astrocytes in their physiological environment were sensitive to the drugs and that their effects were spread throughout the brain. Cortical uptake was not affected by both drugs.

Previously, we showed that these drugs *in vitro* affect the glutamate uptake (Martini et al. 2006). Although it is difficult to compare the *in vitro* drugs concentrations used with those *in vivo* studies (pharmacological effects), these effects on uptake, which tend to increase extracellular glutamate concentrations, would lead to injurious effects on neurons (excitotoxicity), giving additional relevance to the effects of these drugs on CNS. Thus, our results point to some caution in their therapeutic use

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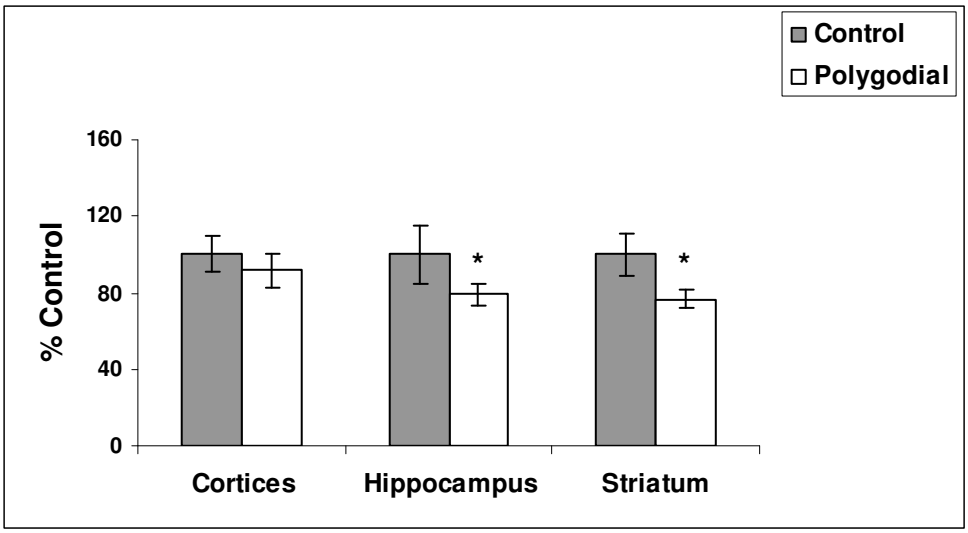

Fig.1. Effects of poligodial (30 mg/Kg) injected intraperitoneally on [^{3}H]glutamate uptake by slices from cortex, hippocampus and striatum mice (n=8). Results are expressed as a percentage of control ± SEM (Cortex 0,496nmol/mgprot/min; hippocampus 0,915nmol/mgprot/min; striatum 0,889nmol/mgprot/min).Values significantly different from the control group: * $p<0,01$

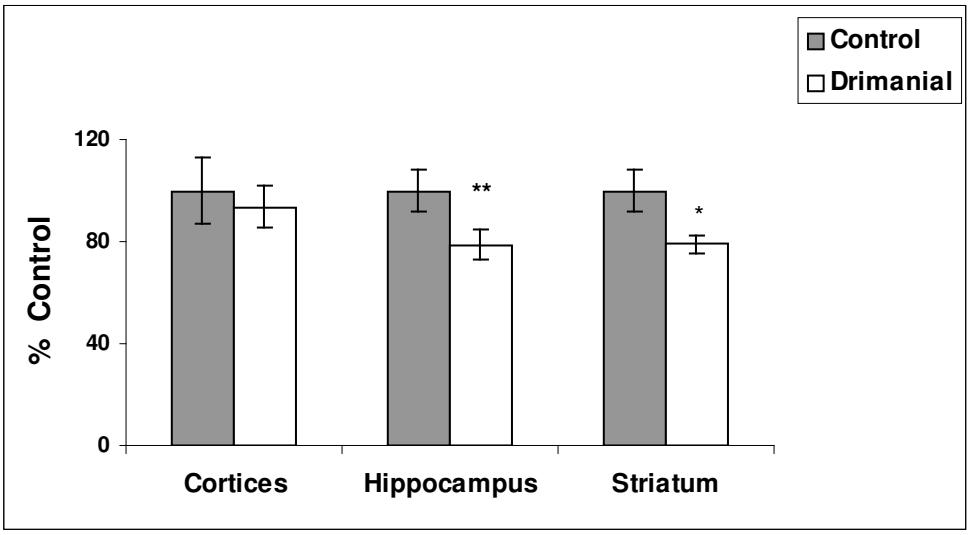

Fig. 2. Effects of drimanial (30 mg/Kg) injected intraperitoneally on [^{3}H]glutamate uptake by slices from cortex, hippocampus and striatum (n=8) mice. Results are expressed as a percentage of control $\pm$ SEM (Cortex 0,605nmol/mgprot/min; hippocampus 1,273nmol/mgprot/min; striatum 1,070nmol/mgprot/min). Values significantly different from the control group: * $p<0,001$ **$p<0,01$

III. DISCUSSÃO E CONCLUSÕES

Produtos naturais e seus derivados constituem uma inesgotável fonte de agentes terapêuticos. Atualmente o uso de plantas medicinais tem sido amplo, em função do fácil acesso e também do baixo custo. Freqüentemente o uso popular de espécies vegetais, na forma de chás e infusões, ocorre sem que haja um estudo que avalie, além dos efeitos farmacológicos, os possíveis efeitos tóxicos, garantindo seu uso de forma segura, principalmente como agentes anti-inflamatórios, antineoplásicos, antiespasmódicos, antidislipidêmcos e analgésicos, entre outros.

Glutamato é o principal neurotransmissor excitatório no SNC de mamíferos, participando em muitos processos, tais como aprendizado e memória, plasticidade, desenvolvimento neural e envelhecimento (Izquierdo e Medina, 1997; Meldrum, 2000; Michaelis, 1998; Ozawa, et al., 1998; Segovia, et al., 2001). Existem evidências de que o glutamato também possui importante papel na transmissão periférica da dor (Beirith, et al., 1998; 2002; 2003). Há demonstrações de que terminais sensoriais periféricos expressam receptores glutamatérgicos (GluRs) e que ligandos glutamatérgicos podem desencadear comportamentos nociceptivos em vários paradigmas experimentais, o que sugere o envolvimento do glutamato na transdução periférica sensorial, em particular na transdução da nocicepção (Carlton, 2001; Jackson, 1995; Lucifora, et al., 2006, Rustioni, 2005).

O uso do glutamato em modelos de nocicepção em camundongos, quando injetado por via intraplantar, produz hiperalgesia e edema de pata. Antagonistas glutamatérgicos ionotrópicos dos tipos NMDA (MK801) e não-NMDA (NBQX ou GAMS), e metabotrópicos (E4CPG) e também o agonista que atua no sítio glicina

do receptor NMDA (felbamato), inibem significantemente a nocicepção induzida por glutamato (Beirith et al., 2002).

Este trabalho iniciou investigando vários compostos extraídos de plantas amplamente utilizadas na medicina popular na forma de chás e infusões, com ações terapêuticas relatadas: analgésica, anti-inflamatória, antineoplásica, antinociceptiva, anti-infecciosa e outras.

Inicialmente este estudo realizou um *screening* da atividade de compostos presentes em plantas com atividade analgésica popularmente citada, sobre parâmetros do sistema glutamatérgico em SNC. Esses compostos são de diferentes classes químicas e foram isolados de extratos das seguintes plantas, distribuídas em países tropicais e subtropicais: *Hedyosmum brasiliense* (13-hidroxi-8,9-dehidroshizukanolide - 13 HDS - sesquiterpeno), *Glynice max* (genisteína - flavonóide), *Citrus paradise* (naringina – flavonóide), *Phyllanthus niruri* (rutina e quercetina – flavonóides), *Crataeva nurvala* (lupeol - triterpeno pentacíclico), *Ocotea suaveolens* (ácido tormêntico - triterpeno), *Jatropha elliptica* (jatrofona - diterpeno), *Protium kleini* ($\alpha\beta$-amirina - mistura de dois triterpenos pentacíclicos) e *Drymis winteri* (poligodial e drimanial - sesquiterpenos). A triagem incluiu os seguintes parâmetros do sistema glutamatérgico: união específica do glutamato a receptores, captação astrocitária e vesicular de glutamato, e liberação sinaptossomal de glutamato. Os resultados obtidos foram:

a) 13-hidroxi-8,9-dehidroshizukanolide (13HDS): inibiu a união do glutamato aos seus receptores, a captação vesicular e astrocitária, porém

não apresentou efeito na liberação de glutamato em preparações sinaptossomais;

b) genisteína: inibiu a união do glutamato aos seus receptores e não teve efeito nos demais parâmetros testados;

c) naringina: estimulou a liberação de glutamato por preparações de sinaptossomas e não mostrou efeito nos demais parâmetros testados;

d) quercetina: inibiu a união do glutamato aos seus receptores, bem como a captação vesicular de glutamato e não teve efeito nos demais parâmetros testados;

e) lupeol: somente estimulou a captação astrocitária de glutamato

f) ácido tormentico: somente apresentou efeito inibitório sobre a captação do glutamato pelos astrócitos;

g) jatrofona: inibiu a captação vesicular de glutamato e não teve efeito nos demais parâmetros testados;

h) rutina e αβ-amyrina não apresentaram efeitos sobre os parâmetros testados.

i) poligodial inibiu a união de glutamato aos seus receptores, a captação de glutamato por astrócitos estimulou a liberação de glutamato por preparações de sinaptossoma, não teve efeito sobre a captação de glutamato por preparações de vesículas sinapticas.

j) Drimanial inibiu a união de glutamato aos seus receptores, a captação de glutamato por astrócitos e preparações de vesículas

sinápticas, estimulou a liberação de glutamato por preparações de sinaptossoma.

A partir destes resultados decidiu-se continuar investigando mais detalhadamente somente os compostos poligodial e drimanial, com efeitos mais pronunciados e amplos e estes constituíram o tema central dos artigos científicos e desta tese.

Os sesquiterpenos poligodial e drimanial foram extraídos da *Drymis winteri* (Winteraceae), vulgarmente denominada "casca de anta". Esta planta é encontrada no Sul do Brasil e em países da América Latina. Suas folhas e cascas são muito utilizadas na medicina popular, na forma de chás e infusões como estimulante, antiinflamatório, antiespasmódico, antidiarréico, cicatrizante, no tratamento de asma e alergia, no tratamento de hemorragia uterina e em certas afecções do trato respiratório (Morton, 1981; Simões et al., 1986). Estes compostos têm sido utilizados em várias modelos de dor, tendo sido eficazes na sua prevenção e na anti-inflamação, conforme dados da literatura. (André, et al., 2004; Mendes, et al., 2000; Scheidt et al., 2002; Tratsk, et al., 1997).

Em modelos de dor induzidos pela administração intra-tecal de glutamato, o poligodial administrado por via intra-peritonial não foi capaz de reverter a hiperalgesia (Mendes, et al., 2000), enquanto que o drimanial foi capaz de reverter a hiperalgesia provocada pela injeção intraplantar de glutamato (Scheidt, et al., 2002). Portanto, a ação analgésica do drimanial parece ser, pelo menos em parte, via sistema glutamatérgico.

Ambas as ações fisiológicas e patológicas do glutamato em SNC de mamíferos são mediadas por receptores metabotrópicos e/ou ionotrópicos (Meldrum, 2000; Ozawa, et al., 1998; Segovia, et al., 2001). Esses receptores glutamatérgicos estão envolvidos em respostas celulares fisiológicas ao glutamato (Danbolt, 2001; Meldrum, 2000; Ozawa, et al., 1998).

No primeiro artigo deste trabalho, observou-se, *in vitro*, que o poligodial inibiu completamente a união do glutamato a seus receptores em preparações de membranas sinápticas, de cérebro total de ratos adultos. Nas mesmas preparações, o drimanial reduziu à metade a união específica sódio-independente do glutamato aos seus receptores.

Além de seu papel fisiológico, o glutamato pode ter potentes ações neurotóxicas (excitotoxicidade), envolvidas em vários disturbios agudas (hipóxia, isquemia, traumatismo cerebral) e neurodegenerativas crônicas. Portanto, a manutenção de níveis extracelulares adequados de glutamato é essencial para a modulação dos efeitos fisiológicos e patológicos do glutamato (Danbolt, 2001, Meldrum, 2000; Ozawa, et al., 1998;). A captação de glutamato pelos astrócitos é importante na finalização da sua ação, para que o glutamato não permaneça na fenda sináptica de forma exagerada, induzindo ao dano neuronal. Devido à falta de enzimas capazes de metabolizarem o glutamato na fenda sináptica, um sistema de transportadores presentes principalmente nas membranas gliais é o responsável por esta retirada (Danbolt, 2001). A remoção do glutamato a partir da fenda sináptica, principalmente por transportadores de membrana de alta-afinidade sódio-dependentes, é o principal mecanismo para a modulação das ações do glutamato e para a manutenção de concentrações abaixo dos níveis

tóxicos. Este mecanismo participa da modulação do ciclo glutamato-glutamina. Neste trabalho observou-se que o drimanial e o poligodial inibiram a captação do glutamato em culturas primárias de astrócitos. Na maior concentração testada (855μM, 234μM), tanto o poligodial como o drimanial apresentaram efeito lesivo, rompendo as membranas dos astrócitos, conforme mostram as figuras 3B e 4B do segundo trabalho (efeito sobre a liberação da lactato desidrogenase).

O glutamato pré-sináptico, derivado da glutamina astrocitária, é estocado em vesículas sinápticas através de transportadores sódio-independentes, por um processo gerado por um gradiente eletroquímico de prótons, através de uma H^+-ATPase (V-ATPase) (Danbolt, 2001; Fleck, et al, 2001; Naito e Ueda, 1985). A ação coordenada de ambos os transportadores estoca glutamato no interior das vesículas sinápticas, decrescendo de forma importante a concentração de glutamato na fenda sináptica (Danbolt, 2001; Fykse e Fonnum, 1996), evitando a excitotoxicidade. Como tem sido mostrado que a captação de glutamato por vesículas sinápticas pode ser modulada por compostos exógenos ou endógenos (Amano, et al., 2002; Gasnier, 2000; Rotta, et al., 2003; Tavares, et al., 2000; Wolosker, et al., 1996), neste trabalho investigou-se o efeito dos compostos drimanial e poligodial sobre a captação vesicular de glutamato. Observou-se que o poligodial não alterou este parâmetro, enquanto o drimanial inibiu a captação de glutamato pelas vesículas sinápticas.

O uso de preparações de sinaptossomas para estudos de armazenamento e liberação de neurotransmissores nos terminais pré-sinapticos é de grande utilidade, pelo fato de que as mesmas mantêm as características do terminal neural intacto (Rotta, et al., 2003). Este estudo avaliou os efeitos dos compostos

poligodial e drimanial sobre a liberação de glutamato em condições basais e estimuladas por alto K^+ externo, em preparações sinaptossomais de cérebro total de ratos adultos. Observou-se que o poligodial promoveu um aumento na liberação basal do glutamato. Na liberação de glutamato estimulada por K^+, houve efeito significativo somente em doses altas. Um perfil semelhante foi observado com o composto drimanial. Entretanto, a maior liberação sinaptossomal de glutamato, induzida pela ação destes compostos, em condições basais, poderia ser atribuída a um aumento secundário no glutamato citosólico, como resultado do efeito inibitório sobre a captação vesicular. De acordo com esta proposta, importante ressaltar que tem-se identificado um fator protéico inibitório (IPF), capaz de reduzir a captação vesicular (Lobur, et al., 1990; Özkan, et al., 1997), e então reduzir a quantidade de glutamato vesicular liberado (Tamura, et al., 2001; Amano et al., 2002).

Concentrações extracelulares de glutamato são usualmente mantidas em níveis não-tóxicos por transportadores presentes principalmente nas células gliais (Anderson e Swanson, 2000; Danbolt, 2001). Com o intuito de estudar com mais profundidade a relevância da ação desses compostos, em um sistema mais próximo ao modelo *in vivo*, utilizou-se fatias de cérebro de ratos, preparação em que a arquitetura tecidual é mantida e as células neurais mantêm integridade e interações entre elas. Trabalhou-se com fatias de hipocampo, córtex e estriado, pois essas estruturas estão bastante vinculadas ao papel do glutamato como neurotransmissor e são alvos relevantes para os seus efeitos neurotóxicos. Ambos, poligodial e drimanial *in vitro* inibiram significativamente a captação do glutamato pelas fatias nas três estruturas testadas, sem causar danos às fatias

(conforme medida da atividade da LDH nos meios de incubação - dados não mostrados).

Estudos recentes, realizados por nosso grupo de pesquisa, têm apontado para um possível papel neuroprotetor da guanosina contra a excitotoxicidade do glutamato. A guanosina foi capaz de prevenir convulsões provocadas pelo ácido quinolínico, um composto que hiper estimula o sistema glutamatérgico (Lara, et al., 2001; de Oliveira, et al., 2004; Schmidt, et al., 2000; Vinadé, et al., 2003, 2004). Estudos complementares demonstraram que a guanosina, dentre os derivados da guanina, parece ser a principal responsável pelos efeitos estimulatórios na captação de glutamato exercido pelos astrócitos (Frizzo, et al., 2003). O efeito sobre a captação de glutamato parece ser específico da guanosina, uma vez que os nucleotídeos da guanina precisam ser hidrolisados para exercer esse efeito, e que não ocorre efeito aditivo quando testados em conjunto com a guanosina (Frizzo et al, 2003). Quando as fatias foram incubadas com a metade da dose dos compostos poligodial e drimanial, que causou efeito inibitório máximo (IC50) e na presença de 100 μM de guanosina, não houve reversão da inibição, exceto em hipocampo, onde a guanosina reverteu o efeito inibitório do drimanial.

A modulação do sistema glutamatérgico é essencial para a manutenção das funções do sistema nervoso central. Pelos resultados dos estudos efetuados *in vitro*, até aqui apresentados, observou-se que o composto drimanial teve os seguintes efeitos:

- inibição da união específica do glutamato aos seus receptores;

- inibição da captação do glutamato pelos astrócitos, por preparações de vesículas e por fatias de estruturas cerebrais (hipocampo, córtex e estriado);

- aumento da liberação do glutamato em preparações de sinaptossomas.

Quanto ao poligodial, pode-se dizer que os efeitos encontrados nos estudos *in vitro* foram semelhantes aos acima citados, com exceção da captação vesicular (não houve efeito).

Podemos sugerir que os compostos drimanial e poligodial, utilizados como antiinflamatórios e analgésicos, com efeitos antinociceptivos, podem estar apresentando possíveis efeitos neurotóxicos, uma vez que tendem a aumentar os níveis de glutamato na fenda sináptica, o que é danoso aos neurônios (excitotoxicidade).

Iniciamos uma nova abordagem neste trabalho, tentando mimetizar os ensaios utilizados em modelos experimentais de analgesia. Quando testados *in vivo,* por injeção intraperitoneal em camundongos, na concentração de 30 mg/Kg de peso corporal, verificou-se uma inibição da captação de glutamato pelas fatias de estriado e hipocampo; porém, não houve inibição da captação em córtex cerebral de camundongos adultos.

A ação excitotóxica do glutamato tem sido relacionada à alteração na capacidade de captação deste neurotransmissor pelas células gliais, de forma a manter adequados seu tempo de permanência e sua concentração na fenda sináptica abaixo dos níveis tóxicos. Esta elevada concentração de glutamato pode ser devido à sua liberação aumentada e/ou a diminuição da sua captação astrocitária. Como perspectiva de contribuição a esta ação, será realizada a determinação da concentração liquórica de glutamato, purinas e dos próprios compostos, em animais tratados *in vivo* com os compostos poligodial e drimanial.

Desta forma, podemos inferir que estes compostos podem induzir a uma situação de excitotoxicidade glutamatérgica, devido ao fato destas drogas aumentarem a liberação sinaptossomal de glutamato, aliado a uma inibição da sua captação.

Pelos resultados apresentados neste trabalho, podemos concluir que os compostos derivados da planta *Drymis* winteri, drimanial e poligodial, com efeitos analgésicos e antiinflamatórios, bastante descritos na literatura, e possíveis matérias-primas para o desenvolvimento de novas substâncias com potencial terapêutico, tiveram um comportamento potencialmente tóxico, sugerindo muita cautela no uso para o fim destinado e/ou desenvolvimento de novos fármacos.

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