UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Isolamento e avaliação biológica de compostos fenólicos de espécies de *Hypericum* nativas do sul do Brasil

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PORTO ALEGRE, março de 2010.

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Isolamento e avaliação biológica de compostos fenólicos de espécies de *Hypericum* nativas do sul do Brasil

Dissertação apresentada por **Juliana Schulte Haas** para a obtenção do grau de MESTRE em Ciências Farmacêuticas

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“Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A mágica presença das estrelas!”

Das Utopias, Mário Quintana

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SUMÁRIO

LISTA DE ABREVIATURAS

AAPH$^{\bullet}$	2,2'-Azobis (2-amidinopropano) hidrocloreto
CC	Cromatografia em Coluna
CCD	Cromatografia em Camada Delgada
CNS	Central Nervous System
DBH	Dopamina-β-hidroxilase
DPPH$^{\bullet}$	2,2-Difenil-1-picril-hidrazil
EFS CO_2	Extração por fluido supercrítico CO_2
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HP1	Isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano
HP2	7-Hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano
HP3	5-Hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano
HPA	Hipotálamo-pituitária-adrenal
MAO	Enzima monoaminooxidase
RMN	Ressonância Magnética Nuclear
SNC	Sistema Nervoso Central

RESUMO

Espécies de *Hypericum* (Guttiferae) são mundialmente reconhecidas por seus efeitos farmacológicos, destacando-se *H. perforatum* pela atividade antidepressiva. O estudo com espécies brasileiras vem apresentando resultados promissores. *Hypericum polyanthemum* e *H. caprifoliatum* mostraram efeito antinociceptivo, enquanto *H. caprifoliatum* mostrou atividade do tipo antidepressiva. Além disso, verificou-se atividade de *H. polyanthemum* sobre o carrapato *Rhipicephalus (Boophilus) microplus.* Para essas espécies, é descrita a presença de benzopiranos, derivados de floroglucinol e flavonóides. ***Objetivos:*** Isolar compostos fenólicos lipofílicos de *H. polyanthemum* e o flavonóide hiperosídeo de *H. caprifoliatum,* assim como avaliar atividades biológicas de algumas dessas substâncias. ***Materiais e Métodos:*** Realizou-se a análise química através de técnicas cromatográficas (CC, CCD) e espectroscópicas (^{1}H e ^{13}C RMN). Hiperosídeo foi avaliado nos seguintes modelos comportamentais: potenciação do sono barbitúrico, atividade locomotora espontânea, placa quente, contorções induzidas por ácido acético e natação forçada. O efeito antinociceptivo de isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano (HP1), 7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano (HP2) e 5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano (HP3), foi investigado, assim como sua atividade acaricida através do teste de imersão de larvas. ***Resultados e Conclusões***: Além dos benzopiranos HP1, HP2 e HP3, dois compostos não relatados para a espécie *H. polyanthemum,* a xantona 6-desoxijacareubina e um benzopirano de estrutura similar ao ácido eriostemóico, foram isolados de extrato obtido por fluido supercrítico. Hiperosídeo mostrou efeito depressor sobre o SNC, e efeito antiimobilidade, que parece ser mediado pelo sistema dopaminérgico, em ratos. HP1 apresentou efeito antinociceptivo mediado pela neurotransmissão opióide. Nos experimentos para investigação da atividade acaricida, os benzopiranos mostraram ser, pelo menos em parte, responsáveis pela toxicidade dos extratos sobre os ácaros.

Palavras-chave: *Hypericum, Hypericum polyanthemum, Hypericum caprifoliatum,* benzopiranos, flavonóides, atividade no SNC, *Rhipicephalus (Boophilus) microplus*

ABSTRACT

The pharmacological effects of some *Hypericum* species are worldwide recognized, mostly by the comproved efficacy of *H. perforatum* as antidepressant. The south Brazilian species have been showing promissing results. *Hypericum polyanthemum* and *H. caprifoliatum* presented antinociceptive effect, while an antidepressant-like activity of *H. caprifoliatum* was reported. In addition, extracts from *H. polyanthemum* showed to be highly toxic to *Rhipicephalus* (*Boophilus*) *microplus* cattle tick. The chemical characterization reveals that these species are rich in benzopyrans, flavonoids and phloroglucinol derivatives. ***Objectives:*** To isolate lipophilic compounds from *H. polyanthemum* and the flavonoid hyperoside from *H. caprifoliatum*, as well as to carry out a biological evaluation of some of the isolated substances. ***Material and Methods:*** Chromatography and NMR spectroscopy techniques were performed for isolation and structure elucidation. Hyperoside was applied on behavior models, as the open field, pentobarbital sleeping potentiation, hot plate, acetic acid-induced writhing and the forced swimming test. The antinociceptive effect and the acaricide potential of 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) were also investigated. ***Results and conclusions:*** A benzopyran similar to the eriostemoic acid and the xanthone 6-deoxyjacareubin were isolated from the extract obtained by supercritical fluid. Hyperoside showed a central depressor effect and no toxicity signs. The present study provides evidences for the involvement of D2-like dopaminergic neurotransmission on the mechanism of hyperoside antidepressant-like action in rats. HP1 presented opioid-mediated antinociceptive effect. On larvae immersion test, the benzopyrans showed to account for the acaricide properties of *H. polyanthemum* extracts.

Key-words: *Hypericum, Hypericum polyanthemum, Hypericum caprifoliatum*, benzopyrans, flavonoids, CNS activity, *Rhipicephalus* (*Boophilus*) *microplus*

1. INTRODUÇÃO

As plantas sintetizam uma vasta gama de metabólitos primários e secundários, sendo uma importante fonte de novas moléculas com propriedades farmacológicas (QUEIROZ *et al*., 2009). As plantas medicinais vêm sendo usadas para fins farmacológicos há séculos (BALUNAS e KINGHORN, 2005). Nas últimas décadas, o aumento do interesse em investigar novos compostos levou à introdução de vários fármacos importantes, como os antitumorais vimblastina (*Catharanthus roseus*) e taxol (*Taxus* spp), e o antimalárico artemisinina (*Artemisia annua*) (QUEIROZ *et al*., 2009).

Muitos fármacos listados nas Farmacopéias modernas são originados de plantas, como a quinina, fármaco antimalárico extraído das cascas de *Cinchona* spp. A pesquisa bem sucedida com produtos naturais é condicionada à forma como a planta em estudo é selecionada (TAGBOTO e TOWNSON, 2001). Dados quimiotaxonômicos, informações sobre o uso tradicional de espécies vegetais em diferentes culturas (etnofarmacologia ou etnobotânica), observação a campo, ou seleção randômica são critérios que podem ser usados para essa seleção. Uma estratégia para o isolamento de compostos bioativos pode ser o fracionamento bio-guiado, onde são extraídas moléculas de frações biológica ou farmacologicamente ativas (RATES, 2001; QUEIROZ *et al*., 2009).

A dificuldade de grande parte da população em acessar preparações farmacêuticas implica no uso de remédios tradicionais, sendo estimado que cerca de 20 mil plantas sejam usadas medicinalmente em todo o mundo (TAGBOTO e TOWNSON, 2001). Nos últimos anos, a utilização de formulações naturais para o tratamento de enfermidades vem crescendo. Segundo a Organização Mundial de Saúde, 70% das pessoas utilizam alguma terapia considerada não convencional. Nos Estados Unidos, esse número chega a um terço da população. Apesar do aumento da popularidade, os indivíduos que fazem uso não conhecem os efeitos adversos provocados por essas preparações, já que são limitadas as informações sobre a eficácia, mecanismo de ação, farmacocinética e segurança, fazendo-se necessários estudos químicos e farmacológicos (MESSINA, 2006; MISCHOULON,

2007; KINRYS *et al*., 2009; MISCHOULON, 2009). No entanto, muitas vezes as formulações vegetais apresentam melhor perfil em relação aos efeitos adversos que os fármacos sintéticos, os quais apresentam estudos sobre eficácia e segurança, além de apresentarem melhor aceitação e aderência ao tratamento. É o caso de algumas plantas usadas para o tratamento de depressão e ansiedade (ERNST, 2006; KINRYS *et al*., 2009).

Distúrbios de humor e ansiedade são os distúrbios psiquiátricos mais freqüentes na prática clínica. Freqüentemente esses sintomas ocorrem ao mesmo tempo, causando problemas socioeconômicos e dificuldades pessoais (SARRIS e KAVANAGH, 2009). A Organização Mundial da Saúde estima que a depressão afete cerca de 121 milhões de pessoas em todo o mundo, sendo a principal causa de morbidade, incapacidade e mortalidade prematura. Os antidepressivos são eficazes em cerca de 60% dos casos, e menos de 25% das pessoas têm acesso ao tratamento. O índice de efeitos adversos causados pelos antidepressivos é alto, além de serem necessárias semanas para que os medicamentos alcancem o efeito esperado (MACHADO *et al.,* 2008; WHO, 2009). Embora existam remédios naturais para o tratamento da maioria dos problemas médicos e fisiológicos, para o tratamento de desordens psiquiátricas o número de exemplares é reduzido (MISCHOULON, 2009).

Hypericum perforatum é usado tradicionalmente no tratamento de depressão leve a moderada, sendo o agente antidepressivo mais prescrito na Alemanha. É uma das dez espécies vegetais mais pesquisadas em todo o mundo, sendo que preparações à base da planta são responsáveis por uma grande parcela da venda de fitoterápicos (BILIA *et al*., 2002; MENNINI e GOBBI, 2004; MISCHOULON, 2007).

Meta-análises e estudos clínicos mostram que extratos da planta apresentam melhor atividade que o placebo, assim como efeito semelhante ao de antidepressivos clássicos, como inibidores da recaptação de serotonina, com

menor manifestação de efeitos adversos (KASPER *et al*., 2006; 2008; LINDE *et al*., 2008; RAHIMI *et al*., 2009; SARRIS e KAVANAGH, 2009).

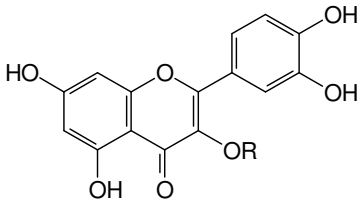
O gênero *Hypericum* é pertencente à família Guttiferae, constituída de 1200 espécies. A subfamília Hypericoideae é formada pelas tribos Vismiae, Cratoxyleae e Hypericeae. A última, a qual pertence o gênero *Hypericum*, apresenta cerca de 450 espécies distribuídas em todo o mundo, principalmente em regiões temperadas e subtropicais montanhosas. Essas espécies foram classificadas em 31 seções por ROBSON (1981). As seções foram definidas pela distribuição geográfica das espécies, mecanismo de polinização, presença de glândulas negras e/ou glândulas pardas. Na região sul do Brasil concentram-se espécies nativas pertencentes a duas seções: *Brathys* e *Trigynobrathys* (ROBSON, 1981; 1990).

Diferentes espécies de *Hypericum* são extensamente utilizadas desde a antigüidade na medicina popular pelo seu efeito sobre diversas patologias, como ação antidepressiva, anti-helmíntica, diurética, antibiótica, antiviral, anti-herpes, no tratamento de ferimentos e queimaduras (TROVATO *et al.,* 2001; MENDES *et al.,* 2002; MENNINI e GOBBI, 2004; DUGOUA *et al.,* 2006; SÁNCHEZ-MATEO *et al*., 2006).

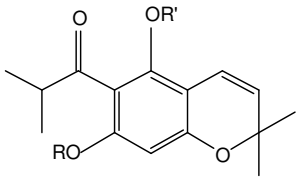
Espécies de *Hypericum* encontradas do sul do país vêm mostrando resultados promissores tanto do ponto de vista químico quanto de atividades farmacológicas sobre o sistema nervoso central. Estudos realizados por nosso grupo mostraram que espécies nativas do Rio Grande do Sul apresentaram efeito antinociceptivo (VIANA *et al*., 2003) e antiimobilidade em roedores (VIANA *et al*., 2005; 2006). A análise química mostra que essas espécies são ricas em flavonóides, como hiperosídeo (**1**), rutina (**2**), quercitrina (**3**) e isoquercitrina (**4**), derivados de floroglucinol, além de benzopiranos, como isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano (HP1) (**5**), 7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano (HP2) (**6**) e 5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano (HP3) (**7**), benzofenonas, taninos, ácidos fenólicos e óleos voláteis em pequenas

concentrações (FERRAZ *et al*., 2001; 2002a; 2005a; DALL'AGNOL *et al*., 2003; BERNARDI *et al*., 2005). Foram identificadas cerca de 20 espécies nativas do gênero *Hypericum* no sul do país. São exemplos *H. carinatum* Griseb., *H. ternum* A. St. Hil., *H. connatum* Lam., *H. myrianthum* Cham. & Schltdl., *H. linoides* A. St. Hil., *H. caprifoliatum* Cham. & Schltdl. e *H. polyanthem*um Klotzsch ex Reichardt (ROBSON, 1981; 1990).

(**1**) R = β-D-galactosil
(**2**) R = α-L-rutinosil
(**3**) R = α-L-ramnosil
(**4**) R = β-D-glicosil

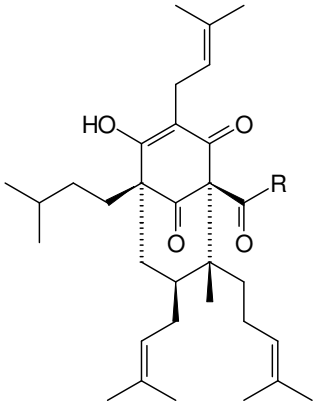


(**5**) R=CH_3 e R'=CH3
(**6**) R=H e R'= CH_3
(**7**) R= CH_3 e R'=H

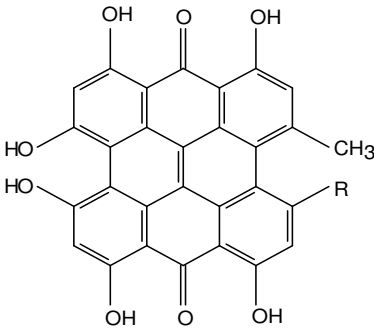


A atividade antidepressiva de *H. perforatum* é atribuída principalmente aos derivados de floroglucinol hiperforina (**8**) e *ad*-hiperforina (**9**), às naftodiantronas hipericina (**10**) e *pseudo*-hipericina (**11**), assim como a vários flavonóides. O papel e o mecanismo de ação dos diferentes compostos ainda estão sob investigação. A visão simplista de que somente um composto através de um mecanismo de ação seria responsável pela atividade parece incorreta, havendo um consenso entre vários autores de que múltiplos compostos bioativos contribuem para a atividade antidepressiva do extrato da planta de forma complexa (AMARAL *et al.,* 1999; MENNINI e GOBBI, 2004; BUTTERWECK e SCHMIDT, 2007).

(**8**) R = H
(**9**) R = CH_3



(**10**) R=CH_3
(**11**) R=CH_2OH



Preparações de *H. perforatum* são indicadas para o tratamento de depressão leve a moderada, e têm sido consideradas também para o tratamento da depressão maior, com efeito comparável ao de antidepressivos clássicos e inibidores da recaptação de serotonina (LINDE *et al*., 2008; RAHIMI *et al*., 2009), além do tratamento de distúrbios de ansiedade (KINRYS *et al*., 2009; SARRIS e KAVANAGH, 2009). Os mecanismos sobre a depressão sugeridos são: inibição fraca da enzima monoaminooxidase (MAO); ligação a receptores benzodiazepínicos no cérebro; inibição da recaptação não-seletiva de neurotransmissores, como serotonina, norepinefrina, dopamina e colina; modulação do eixo hipotálamo-pituitária-adrenal (HPA), levando ao aumento da produção de fator neurotrófico derivado do cérebro (FNDC); e aumento da atividade dopaminérgica no córtex pré-frontal (BUTTERWECK e SCHMIDT, 2007; SARRIS e KAVANAGH, 2009).

Nos últimos anos, houve um enriquecimento do potencial farmacológico de *H. perforatum*, sendo descritas as atividades antiviral, anticancerígena, antiproliferativa, antimicrobiana, antiinflamatória, antioxidante, na cicatrização de ferimentos, entre outras (AVATO *et al*., 2004; MARTARELLI *et al*., 2004; SILVA *et al*.; 2005; SKALKOS *et al*., 2005; SILVA *et al*., 2008; BIRT *et al.,* 2009; MAURY *et al*., 2009; SAMADI *et al.,* 2010; SÜNTAR *et al.,* 2010). Dessa forma, investigações envolvendo outras plantas do gênero têm sido feitas baseadas no valor científico e econômico, buscando identificar fontes alternativas de moléculas ativas, assim como compostos com mecanismos de ação inovadores (TROVATO *et al.,* 2001; MENDES *et al.,* 2002; CAKIR *et al.,* 2003; RABANAL *et al.,* 2005; SÁNCHEZ-MATEO *et al*., 2006; VIANA *et al.,* 2006; FRITZ *et al*., 2007a).

Em uma triagem realizada com sete espécies do gênero, *H. ternum* mostrou atividade antifúngica significativa contra espécies como *Trichophyton rubrum, T. mentagrophytes, Microsporum canis, M. gypseum, Cryptococcus neoformans*, e *Candida albicans* (FENNER *et al.,* 2003). *Hypericum myrianthum* e *H. polyanthemum* inibiram a germinação e o crescimento de alface (*Lactuca sativa* L.), atividade conferida à presença de compostos fenólicos nas mesmas (FRITZ *et al*., 2007b). Para *H. polyanthemum*, foi observada toxicidade significativa dos extratos das partes aéreas sobre o ácaro *Rhipicephalus* (*Boophilus*) *microplus*, possivelmente atribuída aos cromenos presentes na planta (RIBEIRO *et al.,* 2007).

O uso popular das espécies *H. brasiliense* e *H. connatum* para alívio de desordens como angina, câimbras e inflamação oral de faringe apontam possível efeito analgésico para o gênero. Os extratos de *H. caprifoliatum* e de *H. polyanthemum* demonstraram efeito antinociceptivo nos testes da placa quente e contorções induzidas por ácido acético, com envolvimento do sistema opióide (VIANA *et al*., 2003). *Hypericum caprifoliatum* inibiu as contrações induzidas por agonistas em íleo isolado de cobaio (VIANA *et al.,* 2007). Através de estudos farmacológicos *in vivo* e *in vitro* verificou-se que *H. caprifoliatum* apresenta efeito do tipo antidepressivo de forma dose-dependente, com atividades inibidora da

MAO, redutora dos níveis de corticosterona e inibidora da recaptação monoaminérgica, principalmente dopaminérgica (DAUDT *et al*., 2000; GNERRE *et al.,* 2001; VIANA *et al*., 2005; 2006; 2008). Os principais constituintes dessa espécie são os derivados de floroglucinol HC1 (cuja estrutura ainda não foi completamente elucidada), hiperbrasilol B, uliginosina B e o flavonóide hiperosídeo (NÖR *et al.,* 2004; 2008; DALL'AGNOL *et al*., 2005). Diversos trabalhos mostram que compostos com padrão de substituição floroglucinol e flavonóides, podem estar envolvidos no efeito apresentado por essas espécies sobre o sistema nervoso central (BUTTERWECK *et al*., 2003; FERNANDÉZ *et al*., 2005; LINDE e KNÜPPEL, 2005; MACHADO *et al.,* 2008).

Além disso, outras propriedades farmacológicas vêm sendo reveladas para extratos, frações e compostos isolados de exemplares do gênero. Como exemplos para as atividades biológicas demonstradas podem ser citados: a atividade inibidora da MAO de extratos de diferentes espécies, e do benzopirano HP3, isolado de *H. polyanthemum* (GNERRE *et al*., 2001); a ação antiproliferativa de extratos e dos benzopiranos HP1, HP2 e HP3 (FERRAZ *et al*., 2005b; FERRAZ *et al*., 2005c; GRIVICICH *et al.,* 2008); efeito citotóxico de HP1, HP2 e HP3 frente a células envolvidas no processo de angiogênese (NÖR, 2006); e atividade antimicrobiana de frações e compostos isolados (DALL'AGNOL *et al*., 2003; 2005). Além disso, os benzopiranos apresentaram baixa genotoxicidade e mutagenicidade (FERRAZ *et al.,* 2009).

Hypericum polyanthemum se destaca devido ao potencial terapêutico demonstrado por extratos apolares e por substâncias isoladas da planta. Em função da necessidade de padronizar o material vegetal, protocolos para cultivo *in vitro* e aclimatização foram estabelecidos, assim como a análise dos teores dessas substâncias em plantas provenientes de diferentes cultivos e da planta *in natura* (BERNARDI *et al.,* 2007a; BERNARDI *et al.,* 2008).

Outro estudo realizado com essa espécie comparou métodos de extração, objetivando estabelecer técnicas mais eficientes para a obtenção de compostos bioativos. A extração por fluido supercrítico (EFS CO_2) mostrou ser um método vantajoso para a extração de moléculas bioativas com maior rendimento, em condições que proporcionam a não-degradabilidade devido à baixa temperatura, e por utilizar um extrator não-tóxico, facilmente removido do extrato (CARGNIN *et al.,* 2010).

Alguns flavonóides isolados de *H. ternum*, caracterizados nas espécies nativas, mostraram relevante potencial antioxidante (BERNARDI *et al*., 2007b). SILVA e colaboradores (2008) relataram a atividade antioxidante de quercetina e hiperosídeo através de ensaio de captura de $DPPH^{\bullet}$ (2,2-difenil-1-picril-hidrazil), sendo que hiperosídeo apresentou também atividade através do teste de captura de $AAPH^{\bullet}$ (2,2'-azobis (2-amidinopropano) hidrocloreto) e de inibição da peroxidação lipídica por ligação ao ferro.

Estudos indicam que flavonóides atuam em sistemas celulares de animais em diferentes estágios do processo canceroso e na homeostasia, sendo demonstrada ação antiproliferativa, antimutagênica e anticarcinogênica para diversos compostos. Acredita-se que essas atividades podem estar relacionadas com as propriedades antioxidantes dos compostos, protegendo, ainda, os tecidos contra a peroxidação lipídica e agregação plaquetária, envolvidas em patologias como aterosclerose e inflamação crônica (HOLLMAN *et al*., 1997). Dentre os trabalhos desenvolvidos para avaliação de efeitos biológicos de flavonóides, estão os estudos sobre a ação analgésica e antidepressiva desses compostos (COOK e SAMMAN*,* 1996; DI CARLO *et al.,* 1999; HARBORNE e WILLIAMS, 2000; BUTTERWECK *et al.,* 2000; HAVSTEEN, 2002).

Considerando os dados apresentados, torna-se evidente que estas espécies são alvos importantes na busca de estruturas inéditas e de substâncias relevantes do ponto de vista biológico.

2. OBJETIVOS

2.1. Objetivos gerais

Isolar compostos fenólicos lipofílicos de *Hypericum polyanthemum* e o flavonóide hiperosídeo de *H. caprifoliatum,* assim como avaliar atividades biológicas do extrato ciclo-hexano de *H. polyanthemum* e de algumas dessas substâncias.

2.2. Objetivos específicos

¤ Realizar a análise fitoquímica de *Hypericum polyanthemum*, buscando isolar os benzopiranos isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano (HP1), 7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano (HP2) e 5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano (HP3), uliginosina B, e outros compostos não relatados.

¤ Investigar a relação dose-efeito do extrato ciclo-hexano de *H. polyanthemum* na placa quente, assim como o envolvimento do sistema opióide nesse efeito.

¤ Avaliar, em roedores, as atividades do extrato ciclo-hexano de *H. polyanthemum*, dos benzopiranos HP1, HP2 e HP3, e do flavonóide hiperosídeo sobre o Sistema Nervoso Central.

¤ Estudar o mecanismo de ação da atividade farmacológica encontrada para algumas dessas moléculas isoladas.

¤ Investigar a atividade acaricida de derivados benzopirânicos, isoladamente, sobre larvas do carrapato *Rhipicephalus* (*Boophilus*) *microplus*.

3. PARTE EXPERIMENTAL E RESULTADOS

A parte experimental e os resultados obtidos no desenvolvimento dessa dissertação estão apresentados nos manuscritos encartados a seguir:

3.1. **"Benzopyran and xanthone derivatives from *Hypericum polyanthemum"***, em preparação para o periódico *Natural Product Communications*

3.2. **"The anti-immobility effect of hyperoside on the forced swimming test in rats is mediated by the D2-like receptors activation"**, aceito para publicação no periódico *Planta Medica,* sob o código PLAMED-2010-02-0196-OP.

3.3. **"The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone"**, aceito para publicação no periódico *Planta Medica.* DOI: 10.1055/s-0029-1240942.

3.4. **"Acaricidal activity from benzopyrans from *Hypericum polyanthemum* on *Rhipicephalus (Bophilus) microplus*",** em preparação para o periódico *Veterinary Parasitology*.

3.1. MANUSCRITO I:

“Benzopyran and xanthone derivatives from *Hypericum polyanthemum*”

Em preparação para o periódico *Natural Product Communications*

O presente trabalho teve como objetivo o isolamento de compostos fenólicos lipofílicos não relatados para as partes aéreas de *Hypericum polyanthemum* a partir de um extrato obtido por fluido supercrítico CO_2. Para esse fim, foram utilizados métodos cromatográficos, como cromatografia em coluna (CC), e cromatografia em camada delgada (CCD) analítica e preparativa. A identificação dos compostos isolados foi realizada através dos métodos espectroscópicos RMN ^{1}H e ^{13}C.

Além dos compostos já relatados para a planta, um derivado de benzopirano e uma xantona foram isolados. A elucidação dos compostos permitiu identificar a xantona como a 6-desoxijacareubina. Para elucidação da estrutura do derivado benzopirânico, outros estudos são necessários. Desse modo, novos espectros estão sendo providenciados.

Original paper

Benzopyran and xanthone derivatives from *Hypericum polyanthemum*

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From the aerial parts of *Hypericum polyanthemum* Klotzsch ex Reichardt (Guttiferae), besides the three known benzopyrans 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (**1**); 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (**2**) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (**3**), and the phloroglucinol derivative uliginosin B (**4**), the benzoyran derivative HPF7 (**5**) and the xanthone 6-deoxyjacareubin (**6**) were isolated. The structures of these compounds were elucidated by means of NMR spectroscopic methods. The spectral data of **5** and **6** are reported for the first time to the plant.

Keywords: *Hypericum polyanthemum*, Guttiferae, Benzopyrans, Xanthones.

1. Introduction

Hypericum genus is a rich source of phenolic compounds. For these species it has been described the presence of phloroglucinol derivatives, xanthones, benzopyrans and flavonoids (von Poser *et al*., 2006). *Hypericum polyanthemum*, one of the 20 *Hypericum* species native to South Brazil, has been studied showing antinociceptive effect in mice (Viana *et al.,* 2003). Extracts, fractions and isolated compounds from this species also displayed MAOI, antibacterial, antiproliferative and acaricidal activities (von Poser *et al*., 2006). Three benzopyrans, 6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethylbenzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethylbenzopyran (HP3) have previously been isolated from the aerial parts of this plant (Ferraz *et al*., 2001). These benzopyrans were singly tested, showing a significant reduction of the nociceptive responses in the hot plate and in writhing induced by acetic acid tests for HP1. This effect was prevented by the naloxone administration, showing that this compound is involved in the opioid mediated antinociceptive effect of *H. polyanthemum* (Haas *et al.,* 2010).

Some pharmacologically active substances are susceptible to rapid thermal, light-induced and oxidative decomposition, as the phloroglucinol derivative hyperforin, one of the main compounds from *H. perforatum* (Glisic *et al*., 2008). The supercritical fluid extraction using CO_2 (SFE-CO_2) improves the selectivity and the non-degradation of thermolabile molecules, uses a non-toxic and non-explosive extractor, easily removed from the extract, with low cost, confering attractive characteristics as a solvent for the extraction of components from the solid matrix (Taylor, 1996). The raise of extraction temperature showed a negative effect, leading to an increased degradation of phloroglucinol derivatives (Römpp *et al.,* 2004). Recently it was shown that the SFE-CO_2 extracts obtained from *H. polyanthemum* reached higher amounts of secondary metabolites then the *n*-hexane extract obtained by maceration. According to this study, the temperature of 50 °C was considered the best condition to obtain the target compounds (Cargnin *et al*., 2010).

Considering the selectivity of this process, the aim of the present work was to analyse the SFE-CO_2 of *H. polyanthemum* and to isolate some of the minor components of this extract.

2. Methodology

2.1. General experiment procedures

NMR data (600 MHz for ^{1}H and 150 MHz for ^{13}C) were measured on a JEOL Eclipse 400 spectrometer in $CDCl_3$ using the solvent peaks as internal standard; for 2D experiments standard pulse sequences from the Delta software (version 3.2) were used. The HMQC (Heteronuclear Multiple Quantum Coherence) experiment was optimized for J = 140 Hz. The HMBC (Heteronuclear Multiple Bond Correlation) experiment was optimized for J = 8 Hz. TLC: silica gel 60 F_{254} precoated (Merck®), *n*-hexane:dichloromethane, 1:1 v/v (**1**); 3:2 v/v (**2** and **4**); 4:1 v/v (**5** and **3**); dichloromethane (**6**). Bands were detected under UV light (254 nm).

2.2. Plant material

Plant material of *H. polyanthemum* Klotzsch ex Reichardt (aerial parts) was collected in Caçapava do Sul, South Brazil, in November of 2005. The voucher specimen was deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN) (*Hypericum polyanthemum*, Bordignon *et al*. 1405).

2.3. Supercritical extraction

Supercritical extractions were carried out on pilot-scale automated equipment previously described (Cassel *et al*., 2007). The extraction vessel is supplied with a heating jacket and an automated temperature controller. Heating tapes were used throughout the apparatus to maintain constant temperature in the extraction section. To ensure constant and steady solvent delivery the pump head was cooled by a circulating fluid, which passes through a chiller. Flow rates and accumulated gas volumes passing through the apparatus were measured using a flowmeter assay, 1-300 g min^{-1} (Thar 06618-2, USA). Ke (USA) micrometering valves (VC1) were used for flow control throughout the apparatus. Heating

tapes with automated temperature controller were also used around this valve to prevent both freezing of the solvents and solid solute precipitation following depressurization. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of ± 1.0 bar. The temperature controller (TC) was connected to thermocouples (PT-100), with an accuracy of 0.5K. Powdered aerial plant material (180 g) was used and the extractions were conducted at 50 °C and pressure of 90 bar for 60 min. The supercritical carbon dioxide flowed at rate of $6.67x10^{-4}$ kg s^{-1} through the extraction vessel.

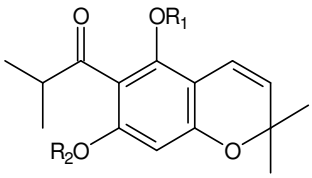
2.4. Isolation procedures

The CO_2 extract was treated with acetone in order to remove waxes and insoluble impurities, evaporated to dryness, and analysed by TLC. The isolation procedures were performed by silica gel column chromatography GF_{60} (Merck®), (50 X 3 cm) using cyclohexane-dichloromethane gradient system as mobile phase. The compounds were purified by preparative-TLC performed on 20cm×20cm glass-supported plates covered with 0.5 mm layers of silica gel GF_{254} (Merck®). The chromatographic eluent used were as follow: cyclohexane:CH_2Cl_2 (1:1 v/v) for (**1**); (3:2 v/v) for (**2**) and (**4**); (4:1 v/v) for (**3**) and (**5**); and CH_2Cl_2 for (**6**). Bands were detected using UV light (254 nm) and the compounds were identified by ^{1}H RMN and ^{13}C RMN.

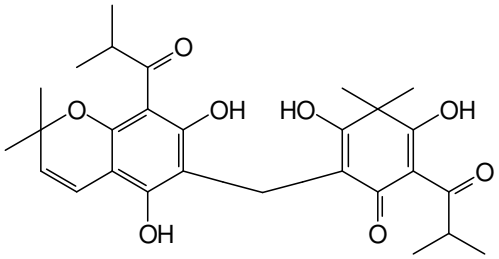
In order to compare the SFE-CO_2 of the aerial parts of *H. polyanthemum* with a *n*-hexane extract obtained by maceration, a TLC analysis was performed, showing similar chromatographic behavior. Considering the selectivity of the former method, this extract, free from carotenoids, chlorophylls and other undesirable compounds, was elected for the isolation of the compounds.

The SFE-CO_2 of the aerial parts of *H. polyanthemum* on silica gel chromatography yielded the four major compounds HP1 (**1**), HP2 (**2**), HP3 (**3**) and uliginosin B (**4**), previously obtained from this plant, and the minor compounds **5** and **6**, showing to be a benzopyran

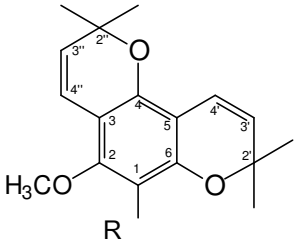
and a xanthone derivatives. The structures of these compounds were elucidated by means of spectroscopic methods. This is the first report of these compounds for *H. polyanthemum*.



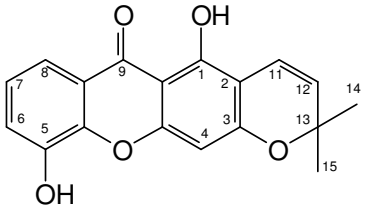
(**1**) HP1 R1=Me R2=Me
(**2**) HP2 R1=Me R2=H
(**3**) HP3 R1=H R2=Me



(**4**) Uliginosin B



(**5**)



(**6**)

The compound **5** (benzopyran derivative) was obtained as a light yellow crystalline solid. The ^{1}H NMR and ^{13}CNMR ($CDCl_3$) are present in Table 1. The ^{1}H NMR spectra showed the presence of two pairs of chromene doublets at δ 6.60 and 5.44 ppm (each 1H, *J*=10.2 Hz), and 6.66 and 5.56 ppm (*J*=10.2 Hz), together with two six-proton singlets at δ 1.44 and 1.49 ppm, indicating the presence of two dimethylpyran ring systems in the molecule. The ^{1}H and ^{13}C signals at δ 3.66 ppm and δ 60.10 ppm, respectively, indicated the presence of a methoxyl group attached to the aromatic ring. A singlet proton at δ H 14.10 ppm revealed the presence of a hydroxyl group. The proposed skeleton has only two possible positions to attach substituents, one of them occupied by the methoxyl group. Considering that the spectrum presents aditional signals compatible with an alkyl chain, it suggests that the hydroxyl could belong to a carboxylic acid group. The assemblage of signals does not

allow defining the structure of compound **5**. Other experiments are necessary to confirm its identity.

Table 1: ^{1}H NMR and ^{13}C NMR chemical shift data for compound **5** (δ, in $CDCl_3$)

Position H	Shift (δ)	Multiplicity	*J* (Hz)	Carbon	Shift (δ)
				C-1	
				C-2	161.1
MeO-2	3.66	s	-	MeO-2	51.46
				C-4	
				C-5	
				C-6	
7-H	2.28	d		C-7	34.38
					34.10
8-H		dd		C-8	
9-COOH	14.16	s		9-CO	174.0
				C-2'	
3'-H	5.43	d	$J_{4'}$=10.2	C-3'	124.52
4'-H	6.65	d	$J_{3'}$=10.2	C-4'	116.26
				C-2"	
3"-H	5.45	d	$J_{4"}$=10.2	C-3"	125.32
4"-H	6.59	d	$J_{3"}$=10.2	C-4"	116.49
	2.28	ddd			
	4.12	dd	Not yet attributed		
	5.37	m			

The family Rutaceae has been proved to be, together with Asteraceae and Guttiferae, a rich source of benzopyrans compounds (Proksch and Rodriguez, 1983; Kamperdick et al., 1997). A compound with similar chemical structure to compound **5**, named eriostemoic acid, was previously isolated from *Eriostemon cymbiformis* and *Eriostemon rhomboideus*, Rutaceae (Sarker *et al*., 1995; Sultana *et al*., 1999).

Compound **6,** 6-deoxyjacareubin, was obtained as a deep yellow crystalline constituent. This xanthone (R_f 0.50 in CH_2Cl_2) is soluble in dichloromethane and poorly soluble in *n*-hexane. The ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) are shown in Table 2.

Table 2: ^{1}H NMR and ^{13}C NMR chemical shift data for compound **6** (δ, in $CDCl_3$)

Position H	Shift (δ)	Multiplicity	*J* (Hz)	Carbon	Shift (δ)	HMBC correlation data
1-OH	12.98 (1H)	s		C-1	160.98	C-2, C-9[a]
				C-2	101.02	
4-H	6.30 (1H)	s		C-4	99.83	C-1, C-2, C-9[a]
				C-5	144.08	
6-H	7.33 (1H)	dd	*J*=7.8; 1.8	C-6	120.38	C-5, C-8
7-H	7.27 (1H)	d	$J_{6,8}$=7.8	C-7	124.23	C-5, C-6, C-8
8-H	7.78 (1H)	dd	$J_{7,6}$=7.8,1.8	C-8	117.14	C-5, C-6, C-9
				9-CO	181.00	
				C-9a	103.55	
11-H	6.78 (1H)	d	J_{11}=9.9	C-11	114.58	C-1, C-2, C-9a, C-13
12-H	5.65 (1H)	d	J_{12}=9.9	C-12	127.75	C-2, C-13, C-14, C-15
				C-13	78.32	
14-Me/15-Me	1.49 (1H)	s		14/15-Me	28.23	C-11, C-12, C-13

The ^{1}H NMR spectral data characterized compound **6** as a chromenoxanthone. The ^{1}H NMR spectrum resonances characterized a dimethylpyran ring and the presence of two protoneted aromatic rings. A singlet proton at δ H 12.98 revealed the presence of a hydroxyl group at C-1, chelated to a carbonyl group of the xanthone. These data are in agreement with literature values for this compound, previously isolated from *H. brasiliense* and *Calophyllum* species (Karunanayake *et al*., 1981; Kumar *et al*., 1982; Goh *et al*., 1991; Rocha *et al*., 1994).

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References

Bennett GJ, Lee HH. (**1989**) Xanthones from Guttiferae. *Phytochemistry*, ***28***, 967-998.

Bowers WS, Ohta T, Cleere JS, Marsella PA. (**1976**) Discovery of insect anti-juvenile hormones in plants. *Science*, ***193***, 542-547.

Cargnin ST, de Matos Nunes J, Haas JS, Baladão LF, Cassel E, Vargas RF, Rech SB, von Poser GL. (**2010**) Supercritical fluid extraction and high performance liquid chromatographic determination of benzopyrans and phloroglucinol derivative in *Hypericum polyanthemum. Journal of Chromatography B*, ***878***, 83–87.

Cassel E, Vargas RMF, Bedinot C. (**2007**) Unidad Piloto de Extracción Supercrítica Automatizada. In *Aplicaciones industriales de los Taninos vegetales: productos y procesos*. EDIPUCRS, Porto Alegre, Brazil.

Ferraz ABF, Bordignon SAL, Staats C, Schripsema J, von Poser GL. (2001) Benzopyrans from *Hypericum polyanthemum*. *Phytochemistry*, ***57***, 1227-1230.

Goh SH, Jantan I. A xanthone from *Calophyllum inophyllum*. (**1991**) *Phytochemistry*, ***30***, 366-367.

Glisic S, Smelcerovic A, Zuehlke S, Spiteller M, Skala D. (**2008**) Extraction of hyperforin and adhyperforin from St. John's Wort (*Hypericum perforatum* L.) by supercritical carbon dioxide. *The Journal of Supercritical Fluids,* ***45***, 332–337.

Haas JS, Viana AF, Heckler APM, von Poser GL, Rates SMK. (**2010**) The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone. *Planta Medica*, doi: 10.1055/s-0029-1240942. *In press.*

Karunanayake S, Sotheeswaran S, Uvais M, Sultanbawa S, Balasubramaniam S. (**1981**) Xanthones and triterpenes of *Calophyllum tomentosum*. *Phytochemistry*, ***20***, 1303-1304.

Kamperdick C, Van NH, Sung TV, Adam G. (**1997**) Benzopyrans from *Melicope ptelefolia* leaves. *Phytochemistry*, ***45***, 1049-1056.

Kumar V, Sotheeswaran S, Surendrakumar S, Balasubramaniam S. (**1982**) Calocalabaxanthone, the putative isoprenyl precursor of calabaxanthone from *Calophyllum calaba. Phytochemistry*, ***21***, 807-809.

Mkounga P, Fomum ZT, Meyer M, Bodo B, Nkengfack AE. (**2009**) Globulixanthone F, a new polyoxygenated xanthone with an isoprenoid group and two antimicrobial biflavonoids from the stem bark of *Symphonia globulifera*. *Natural Product Communications*, ***4***, 803-808.

Morel C, Seráphin D, Teyrouz A, Larcher G, Bouchara J Litaudon M, Richomme P, Bruneton J. (**2002**) New and Antifungal Xanthones from *Calophyllum caledonicum. Planta Medica,* ***68***, 41-44.

Peres V, Nagem TG. (**1997**) Trioxygenated naturally occurring xanthones. *Phytochemistry*, ***44***, 191-214.

Peres V, Nagem TG, de Oliveira FF. (**2000**) Tetraoxygenated naturally occurring xanthones. *Phytochemistry*, ***55***, 683-710.

Proksch P, Rodriguez E. (**1983**) Chromenes and benzofurans of the Asteraceae, their chemistry and biological significance. *Phytochemistry*, ***22***, 2335-2348.

Rocha L, Marston A, Kaplan MA, Stoeckli-Evans H, Thull U, Testa B, Hostettmann K. (**1994**) An antifungal gamma-pyrone and xanthones with monoamine oxidase inhibitory activity from *Hypericum brasiliense*. *Phytochemistry, **36***, 1381-1385.

Sarker SD, Waterman PG. (**1995**) Coumarin glycosides from two species of *Eriostemon. Journal of Natural Products*, ***58***, 1109-1115.

Sultana N, Armstrong JA, Waterman PG. (**1999**) Benzopyran derivatives from the aerial parts of *Eriostemon rhomboideus*. *Phytochemistry*, ***52***, 895-900.

von Poser GL, Rech SB, Rates SMK. (**2006**) Chemical and pharmacological aspects of Southern Brazilian *Hypericum* species. In: *Jaime Teixeira da Silva. (Org.). Floriculture, Ornamental and Plant Biotechnology*. Global Science Books, London UK. 509-515.

3.2. MANUSCRITO II:

"The anti-immobility effect of hyperoside on the forced swimming test in rats is mediated by the D2-like receptors activation"

Aceito para publicação no periódico *Planta Medica*, sob o código PLAMED-2010-02-0196-OP.

Hiperosídeo é encontrado com abundância em espécies de *Hypericum*, sendo relatadas diversas atividades para o composto, tais como antioxidante, antibacteriana, antinociceptiva e antiimobilidade. Neste trabalho foram realizados o isolamento da substância e uma avaliação de algumas atividades farmacológicas utilizando modelos animais.

A administração do flavonóide diminuiu a atividade locomotora, aumentou o tempo de sono e mostrou efeito antiimobilidade em camundongos. Em ratos a atividade do tipo antidepressiva foi reproduzida, sendo verificado que esse efeito é mediado pelo sistema dopaminérgico. Não foi verificado efeito antinociceptivo nos modelos testados.

Carta de aceite do manuscrito:

Preview

From: butterwk@cop.ufl.edu

To: ratessmk@hotmail.com

CC: luc.pieters@ua.ac.be

Subject: Planta Medica - Decision on PLAMED-2010-02-0196-OP

Body:

03-Apr-2010

Dear Dr. Rates,

Manuscript PLAMED-2010-02-0196-OP entitled "The anti-immobility effect of hyperoside on the forced swimming test in rats is mediated by the D2-like receptors activation" submitted to Planta Medica has been reviewed. The comments of the reviewer(s) are attached below.

The reviewer(s) have recommended publication, but also suggest major revisions to your manuscript.

Original paper

The anti-immobility effect of hyperoside on the forced swimming test in rats is mediated by the D2-like receptors activation

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Abstract

The crude extracts of *Hypericum* species native to South Brazil showed analgesic and antidepressant-like effects in rodents. The chemical characterization of these species revealed that they are rich in flavonoids and phloroglucinol derivatives. In the present study a detailed investigation was performed on the activities of hyperoside (HYP), a common flavonoid in the genus *Hypericum.* Hyperoside was obtained from the aerial parts of *H. caprifoliatum* by chromatographic procedures. Mice treated with single doses (10, 20 and 40 mg/kg i.p.) did not present signs of toxicity or weight loss. At 20 and 40 mg/kg i.p. the mice exploratory behavior in the open field test was reduced. At 20 mg/kg i.p. the pentobarbital sleeping time increased, but not the sleeping latency. No activity was found neither on the hot plate (10 and 20 mg/kg i.p.) nor in the acetic acid-induced writhing test (20 and 40 mg/kg p.o.). Nevertheless, an antidepressant-like effect in forced swimming test in mice and rats was observed (HYP 10 and 20 mg/kg i.p. in mice; HYP 1.8 mg/kg/day p.o. in rats). The antidepressant-like effect in rats was prevented by the administration of sulpiride (50 mg/kg i.p.) a D2 antagonist. In conclusion, hyperoside was found to present a depressor effect on the Central Nervous System as well as an antidepressant-like effect in rodents which is, at least in part, mediated by the dopaminergic system.

Keywords: *Hypericum*, Guttiferae, *Hypericum caprifoliatum,* hyperoside, antidepressant-like activity

Abbreviations

ACTH	Adrenocorticotropic hormone
DBH	Dopamine-beta-hydroxylase
FST	Forced Swimming Test
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HP1	6-Isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran
HPA	Hypothalamic-Pituitary-Adrenal
HYP	Hyperoside
SULP	Sulpiride

Introduction

Hyperoside (quercetin-3-O-galactoside) (HYP) is one of the major components of *Hypericum perforatum* (St John's Wort) and also of South Brazilian *Hypericum* species [1]. The efficacy of *H. perforatum* as antidepressant has been demonstrated in numerous clinical studies and meta-analyses [2-6]. Nowadays, the pharmacological actions of this species are attributed to a variety of constituents rather then to a single compound [7-8]. The phloroglucinol derivatives hyperforin and adhyperforin, the naphthodianthrones hypericin and pseudohypericin, the flavonoids hyperoside, rutin, quercitrin and isoquercitrin have all been considered as active ingredients of the plant. Some data strongly indicate hyperforin and derivatives as the most active compounds [9-13] while other studies pointed out the flavonoids, such as hyperoside and rutin, as responsible for the activity [14].

In South Brazil, 20 *Hypericum* species have been identified. Although in popular medicine the use of these species for antidepressant purposes has not been documented, previous experiments carried out in our laboratory demonstrated that the methanol [15-16] and cyclohexane extracts [17-18] of the native *Hypericum* species presented antidepressant-like action in rodents *via* dopaminergic neurotransmission [17]. *H. polyanthemum* and *H. caprifoliatum* extracts also displayed antinociceptive effects. The effect of the methanolic extract from *H. caprifoliatum* was only partially prevented by naloxone suggesting that this extract contains at least two groups of substances acting by different mechanisms [19]. The chemical characterization revealed that the abovementioned species are rich in phloroglucinol derivatives, benzopyrans and flavonoids, being hyperoside the major component of the methanol extracts [1,20,21]. HP1 (6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran), the main benzopyran isolated from the cyclohexane extract of *H. polyanthemum* showed an antinociceptive effect in the hot plate and writhing tests in mice, which was counteracted by naloxone, thus indicating that it acts through the opioid system [22].

Rylski and co-workers [23] demonstrated the activity of hyperoside in the hot plate test at doses ranging from 3.5 - 10 mg/kg i.p. Several studies attributed anti-inflammatory

properties to hyperoside. It presented a low topical anti-inflammatory activity in croton-oil-induced mice ear edema [24], reduced prostaglandin E2 (PGE2) production [25], inhibited the increase of acetic acid-induced vascular permeability in mice [26], and also suppressed LPS-induced nitrite production [27]. A mixture of hyperoside and isoquercitrin inhibited *p*-benzoquinone-induced writhings in mice in 25.8% as well as reduced carrageenan-induced paw edema in 26.8–29.7%, and also inhibited 12-O-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema in 32.8% [28].

In this study we have carried out a reassessment of the antinociceptive and antidepressant-like effects of hyperoside testing doses and animal species different from those evaluated by others [14,23]. Hyperoside was evaluated in the hot plate and acetic acid-induced writhing tests in mice, as well as in the forced swimming test (FST) in mice and rats. The involvement of D2-like dopamine receptors on the antidepressant-like effect in the forced swimming test was also investigated.

Materials and Methods

Plant material

Aerial parts of *H. caprifoliatum* Cham. & Schlecht. were collected in November 2007, in the region of Viamão, South Brazil. The plant material was identified by Dr. Sérgio Bordignon (ULBRA-RS-BRASIL) and voucher specimens were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon 1496). The plant collection was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (Nº 03/2008).

Extraction and Isolation

The dried and powdered plant material (300 g of aerial parts) was successively extracted by maceration with dichloromethane (1:10 w/v; 3x24h), to remove the lipophilic compounds, and then with methanol (1:10 w/v; 3x24h) at room temperature (25 ºC). The methanol extract, concentrated under reduced pressure, afforded a fraction rich in flavonoids, in

which the most abundant compound was hyperoside. The compound was purified by precipitation and subsequent silica gel column chromatography GF_{60} (Merck®) (50 x 3 cm) by using ethyl acetate-methanol gradient system as mobile phase. Hyperoside (1200 mg: 0.4 g/100g raw material) was identified by TLC, ^{1}H-NMR and ^{13}C-NMR.

NMR data (500 MHz for ^{1}H and 125 MHz for ^{13}C) were measured on a JEOL Eclipse 400 spectrometer in MeOD using the solvent peaks as internal standard; for 2D experiments standard pulse sequences from the Delta software (version 3.2) were used. The HMQC (Heteronuclear Multiple Quantum Coherence) experiment was optimized for J = 140 Hz. The HMBC (Heteronuclear Multiple Bond Correlation) experiment was optimized for J = 8 Hz. UV data was obtained in a Hewlett Packard UV-VIS model HP8452-A, using methanol as solvent. TLC: silica gel 60 F_{254} precoated, ethyl acetate-acetic acid-formic acid-water, 100:11:11:26 v/v/v/v). Bands were detected under UV light (254 nm).

Behavioral experiments

Animals

Adult male CF1 mice (25-30 g) and male Wistar rats (weight 200-300 g) purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS – RS - Brazil) colony were used. The animals were housed in plastic cages in groups of eight mice (17 x 28 x 13 cm) or five rats (42 x 28 x 16 cm). All animals were kept under a 12-h light/dark cycle (lights on at 7:00 a.m.) at a constant room temperature of 22±1 °C and humidity (60%) with free access to standard certified rodent diet (Nuvital®) and tap water. All behavioral experiments were performed according to the guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998) and Brazilian Law [29], which are in compliance with the International Guiding Principles for Biomedical Research Involving Animals [30]. All protocols were approved by the UFRGS Ethical Committee (N° 2008008).

Drugs and treatments

The following drugs were used: pentobarbital (Cristália, Brazil), diazepam (Cristália, Brazil), morphine (Cristália, Brazil), dipyrone (Sanofi-Aventis, Brazil), imipramine (Galena, Brazil). Hyperoside was dissolved in saline solution containing 1% polysorbate 80. The final solutions' pH was 5.0. The negative control group received vehicle i.p. or p.o. (saline containing 1% polysorbate 80). The other drugs were dissolved in saline (NaCl 0.9%) solution immediately before using. All administered drugs presented pharmaceutical grade of purity (> 97%). The volume administered was 10 mL/kg for mice and 1 mL/kg for rats. Hyperoside doses were chosen based on Butterweck and co-workers [14], Rylski and co-workers [23] and Chang and co-workers [31].

Gross behavior observation

Groups of mice were treated with a single dose of hyperoside 10, 20 or 40 mg/kg i.p. or vehicle i.p. and observed for 2 h with no interruption. After that, animals were observed 6 and 12 h after treating and everyday for 14 days. Death occurrence and signs such as piloerection, palpebral ptoses, abdominal contortions, locomotion, hypothermia, muscular tonus, shacking, posterior paws paralisation, salivation, bronchial secretion and convulsions were considered. The body weight was also registered.

Locomotor activity

The Swiss CF1 mice were exposed to a 45 x 30 x 30 cm open field (black acrylic floor divided into 24 equal quadrangles by white lines; transparent acrylic walls). Immediately after the treatments (HYP 10, 20 or 40 mg/kg i.p., or vehicle i.p.), animals were left to explore the open field freely for 20 min. The number of line crossings, rearings and groomings were counted. The first 5 min were not considered (habituation period).

Pentobarbital sleeping time

Groups of male Swiss mice were treated with HYP 10, 20 or 40 mg/kg i.p., or vehicle i.p. Thirty minutes after treating all groups received pentobarbital (40 mg/kg i.p.) and the time elapsed between the loss and voluntary recovery of the righting reflex was recorded as

sleeping time. A ceiling of 240 s was imposed in this measure, i.e. animals whose sleeping time was over 240 min were counted as 240 min. Sleep latency was also recorded.

Hot plate test

Before actual testing on the hot plate, the mice were habituated to the nonfunctioning apparatus for 1 min. Thirty minutes later, the animals were placed on the functioning hot plate (Ugo Basile, Comerino, Italy) (55 ± 1ºC) to determine baseline responsiveness. The time elapsed until the animal lick one of its hind paws or jump was recorded (latency time, in seconds = basal latency). Mice that presented baseline reaction of more than 20 s were eliminated from the test. After the determination of the baseline responsiveness the animals received HYP 10 or 20 mg/kg i.p, vehicle i.p. or morphine 4 mg/kg i.p (positive control). Thirty minutes after treating they were placed again on the hot plate and the latency time was measured. In the second session a maximum latency time of 40 s was imposed in order to avoid tissue damage.

Writhing test

The animals were treated with HYP 20 or 40 mg/kg p.o., dipyrone 150 mg/kg p.o (positive control) or vehicle p.o. 45 min before receiving an intraperitoneal acetic acid injection (0.8%, 10 mL/kg) (Merck AG, Germany). Mice were then individually placed in glass observation chambers and observed during 15 min in which the number of abdominal writhes was counted. The percentage analgesic activity was calculated as follows:

$$\text{Percentage analgesic activity} = \left(\frac{N - N' \text{ x } 100}{N} \right)$$

N represents the average number of writhing of control group and *N*' the average number of stretching of test group.

Forced swimming test

Rats and mice were submitted to forced swimming according to Porsolt's procedure [32-33] with minor modifications. The animal was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect.

In rats. In this test an acrylic box with four sections of 30 x 30 x 40 cm was used. The external walls and the cover were transparent, but the inside sections were dark allowing the isolation of each one of the four quadrants. The rats were submitted to swimming for 15 min in water with temperature between 22±1 °C and height of 30 cm (Porsolt and co-workers [33], employed 15 cm). The ambient temperature was approximately 22 °C. At the end of the swimming exposition, the animals were removed from the water and gently dried. The treatment was administered 5 min, 19 and 23 h after the first swimming exposition; the first administration was carried out between 2:00 and 5:00 p.m.; the second, between 9:00 and 12:00 a.m.; and the third, between 1:00 and 4:00 p.m. One hour after the last injection (24 h after the first swimming session), the animals were submitted to a second swimming exposition (5 min), and their immobility time was measured.

Different groups were treated with HYP 1.8 mg/kg/day, p.o. (three adminstration of 0.6mg/kg) according to Butterweck and co-workers [14], imipramine (positive control) 60 mg/kg/day p.o. (three adminstration of 20 mg/kg) or vehicle p.o. To assess the involvement of the dopaminergic neurotransmission on the antidepressant-like effect of hyperoside in the FST, rats were treated with sulpiride (50 mg/kg, i.p.) [17] 30 min before receiving the last dose of HYP (0.6 mg/kg p.o.).

In mice. Mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 22±1 °C; the total duration of immobility was recorded during 6-min period. Different groups of mice were treated with HYP (10 or 20 mg/kg i.p.), imipramine (20 mg/kg i.p.), or vehicle i.p.

Statistical Analysis

The statistical analysis was performed according to the data distribution. The data from pentobarbital sleeping time and FST in rats were analyzed by Kruskall-Wallis, followed by Dunn's *post hoc* test. The results of the writhing and FST in mice were analyzed by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test, while statistical comparison of hot plate data was performed by two-way repeated measures ANOVA followed by Student-Newman-Keuls *post hoc* test. The analyses were performed using Sigma Stat 2.03 software (Jandel Scientific Corporation). Differences were considered statistically significant at $p<0.05$.

Results and discussion

In this study we have validated previous rather qualitative reports by Dall'Agnol and co-workers [1] that reported hyperoside (Fig 1) as one of main compounds from *H. caprifoliatum* methanol extract. It constitutes 0.4% of the dried aerial parts. The identity and purity of the chemical structure was confirmed by ^{1}H and ^{13}C-NMR experiments and by comparing these spectroscopic data with those cited in the literature [35].

We have evaluated this flavonoid in several behavioral experiments in rats and mice at different routes and doses since pharmacokinetic data were scarce and mainly from rats. Chang and co-workers [31] showed that when administered at 6.0 mg/kg p.o. hyperoside could not be detected in plasma of rats neither as unchanged form nor as its aglycone or conjugated aglycone form. Observations from *in vitro* Caco-2 monolayer model and *in situ* intestinal perfusion model indicated that hyperoside has quite limited permeability [36, 37]. Juergenliemk and co-workers [37] using three *in vitro* membrane barrier cell systems (Caco-2 cell line, porcine cell cultures of brain capillary endothelial cells and epithelial cells of the plexus chorioidei) demonstrated that the main metabolite of hyperoside, miquelianin (Fig. 1), crossed all barriers being able to reach the CNS after oral administration. In addition, many hyperoside pharmacological findings were obtained from

in vitro assays, or topical application, that do not involve systemic metabolism and absorption [24-27].

Hyperoside did not induce neither mice gross behavior changes nor weight loss (data not shown) or deaths up to 40 mg/kg (i.p.) indicating that this compound does not present acute toxicity. In the open field test hyperoside 20 and 40 mg/kg i.p. reduced significantly crossing, rearing and grooming (Fig. 2); at 20 mg/kg i.p. it increased mice sleeping time induced by pentobarbital but not the sleep latency (Fig. 3). Altogether these results suggest that this compound present a CNS depressor effect.

Hyperoside is highlighted as one of the active compounds of St John's Wort methanolic crude extract, showing antidepressant-like effect in the Porsolt's forced swimming test in rats after acute and repeated treatments [14]. In the present study we have reproduced the antidepressant-like effect previously demonstrated by Butterweck and co-workers [14] in rats (1.8 mg/kg/day p.o.) (Fig. 4), and also showed the antidepressant-like effect in mice (10 and 20 mg/kg i.p.) (Fig. 5). Interestingly, Butterweck and co-workers [14] also observed a miquelianin (1.8 mg/kg/day p.o) antidepressant-like effect.

The anti-immobility effect of hyperoside was not related to a non-specific behavioural stimulation, given that it reduced motor activity. This observation corroborate with the hypothesis that the treatment with hyperoside could be antidepressant, since to be considered as a potential antidepressant, a drug must reduce immobility in the FST at doses that do not stimulate locomotion. This result is also in accordance with studies showing that many antidepressants tend to decrease motor activity [31, 34] as well as to present sedative effects [38].

Previous studies in search of the mode of action of hyperoside indicated that this flavonoid down-regulated circulating plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone, which play an important role in the modulation of hypothalamic-pituitary-adrenal (HPA) axis function, that is altered in patients with major depression [39] and reduced beta2-adrenergic sensitivity in C6 cells, which was demonstrated by the reduction

of intracellular cAMP concentration [40]. Denke and co-workers [41] reported that hyperoside and other flavonoids from *H. perforatum* fairly inhibited dopamine-beta-hydroxylase (DBH) which catalyzes conversion of dopamine into noradrenaline. This observation is controversial since some reports indicate that the DBH activity is important to the antidepressant effect. DBH blood levels were reduced in mice submitted to chronic mild stress [42], and these alterations were normalized by imipramine treatment. Mice that are unable to synthesize norepinephrine and epinephrine due to targeted disruption of the DBH gene did not respond to several antidepressants in the forced swimming test [43].

In this study we showed that the hyperoside antidepressant-like effect was prevented by sulpiride (50 mg/kg i.p.), while sulpiride itself did not affect the immobility time (Fig. 4). This result indicates that the activation of D2-like receptors accounts for the anti-immobility effect of hyperoside. To our knowledge this is the first evidence that the treatment with hyperoside acts on dopaminergic neurotransmission. Viana and co-workers [17] showed that the antidepressant-like effect of an *H. caprifoliatum* extract enriched in phloroglucinol derivatives on FST results from an inhibition of neuronal monoamine uptake, mainly dopamine. Dopamine agonists are known to be effective in the FST [44] The chronic treatment with antidepressants increases D2 receptors functioning [45] and some drugs used to treat human depression act through the dopaminergic system [46]. Altogether these data stress the potential antidepressant effect of *H. caprifoliatum* as well as point to hyperoside as a new chemical feature with dopaminergic properties.

Hyperoside did not display antinociceptive effect neither on the hot plate (10 and 20 mg/kg i.p.) (Fig. 6) nor in the acetic acid-induced writhing test (20 and 40 mg/kg p.o.) (Table 1). These results are in disagreement with Rylski and co-workers [23] that reported antinociceptive properties of hyperoside at lower doses (3.5 – 10 mg/kg) in the hot plate test. These conflicting results could be explained by biological and/or inter-laboratory variability or by a bell shaped dose-response curve. By any means the antinociceptive effect of hyperoside seems to be not robust enough to be reproducible.

In summary, the treatment with hyperoside produces in rats and in mice an antidepressant-like effect in FST, a model predictive of antidepressant properties. This effect seems to depend on the D2-like receptor activation. These results strongly suggest that hyperoside has an important contribution to the previously reported antidepressant-like activity of *H. caprifoliatum* [15], along with the phloroglucinol derivatives [17]. Therefore it is in agreement with the conception that the antidepressant effect of *H. perforatum* is due to a variety of constituents rather then to a single compound. Besides, this study indicated that the contribution of hyperoside to the *Hypericum* antinociceptive activity is uncertain. Other constituents such as benzopyrans, previously reported by our group [22], seem to be more relevant to this effect.

Acknowledgements

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References

1. *Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nör C, Sarmento L, Lamb L, Hass M, von Poser G, Schapoval EES*. Antimicrobial activity of some *Hypericum* species. Phytomedicine 2003; 10: 511-516.

2. *Kasper S, Anghelescu IG, Szegedi A, Dienel A, Kieser M*. Superior efficacy of St John's wort extract WS® 5570 compared to placebo in patients with major depression: a randomized, double-blind, placebo-controlled, multi-center trial [ISRCTN77277298]. BMC Med 2006; 4: 4-26.

3. *Kasper S, Gastpar M, Müller WE, Volz HP, Dienel A, Kieser M, Möller HJ*. Efficacy of St. John's wort extract WS® 5570 in acute treatment of mild depression. A reanalysis of data from controlled clinical trials. Eur Arch Psychiatry Clin Neurosci 2008; 258: 59–63.

4. *Linde K, Knüppel L*. Large-scale observational studies of *Hypericum* extracts in patients with depressive disorders-a systematic review. Phytomedicine 2005; 12: 148-157.

5. *Linde K, Berner MM, Kriston L*. St John's wort for major depression. Cochrane Database Syst Rev 2008; 8: CD000448.

6. *Rahimi R, Nikfar S, Abdollahi M*. Efficacy and tolerability of *Hypericum perforatum* in major depressive disorder in comparison with selective serotonin reuptake inhibitors: a meta-analysis. Psychopharmacol Biol Psychiatry 2009; 33: 118-127.

7. *Butterweck V, Schmidt M*. St. John's wort: Role of active compounds for its mechanism of action and efficacy. Wien Med Wochenschr 2007; 157: 356–361.

8. *Sarris J, Kavanagh DJ*. Kava and St. John's Wort: Current evidence for use in mood and anxiety disorders. J Altern Complement Med 2009; 15: 827–836.

9. *Butterweck V, Petereit F, Winterhoff H, Nahrstedt A*. Solubilized hypericin and pseudohypericin from *Hypericum perforatum* exert antidepressant activity in the forced swimming test. Planta Med 1998; 64: 291-294.

10. *Butterweck V, Korte B, Winterhoff H*. Pharmacological and endocrine effects of *Hypericum perforatum* and hypericin after repeated treatment. Pharmacopsychiatry 2001; 34: S2-S7.

11. *Butterweck V, Christoffel V, Nahrstedt A, Petereit F, Spengler B, Winterhoff H*. Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. Life Sci 2003; 20: 627-639.

12. *Zanoli P*. Role of hyperforin in the pharmacological activities of St. John's Wort. CNS Drug Rev 2004; 10: 203-218.

13. *Beerhues L*. Hyperforin. Phytochemistry 2006; 67: 2201–2207.

14. *Butterweck V, Jürgenliemk G, Nahrstedt A, Winterhoff H*. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. Planta Med 2000; 66: 3-6.

15. *Daudt R, von Poser GL, Neves G, Rates SMK*. Screening for the antidepressant activity of some species of *Hypericum* from South Brazil. Phytotherapy Res 2000; 15: 344-346.

16. *Gnerre C, von Poser GL, Ferraz A, Viana AF, Testa B, Rates SMK*. Monoamine oxidase inhibitory activity of some *Hypericum* species native to South Brazil. J Pharm Pharmacol 2001; 53: 1273-1279.

17. *Viana AF, Rego JC, von Poser G, Ferraz A, Heckler AP, Costentin J, Rates SMK*. The antidepressant like effect of *Hypericum caprifoliatum* Cham & Schlecht (Guttiferae) on forced swimming test results from an inhibition of neuronal monoamine uptake. Neuropharmacology 2005; 49: 1042-1052.

18. *Viana AF, do Rego JC, Munari L, Dourmap N, Heckler AP, Costa TD, von Poser GL, Costentin J, Rates SMK*. *Hypericum caprifoliatum* (Guttiferae) Cham. & Schltdl.: a species native to South Brazil with antidepressant-like activity. Fundam Clin Pharmacol 2006; 20: 507–514.

19. *Viana AF, Heckler AP, Fenner R, Rates SMK*. Antinociceptive activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae). Braz J Med Biol Res 2003; 36: 631-634.

20. *Nör C, Albring D, Ferraz ABF, Schripsema J, Pires V, Sonnet P, Guillaume D, von Poser GL*. Phloroglucinol derivatives from four *Hypericum* species belonging to the Trigynobrathys section. Biochem Syst Ecol 2004; 32: 517-519.

21. *Nör C, Bernardi APM, Haas JS, Schripsema J, Rech SB, von Poser GL*. Phenolic constituents of *Hypericum* flowers. Nat Prod Commun 2008; 3: 237-240.

22. *Haas JS, Viana AF, Heckler APM, von Poser GL, Rates SMK*. The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone. Planta Med, advance online publication 17 February 2010; doi: 10.1055/s-0029-1240942.

23. *Rylski M, Duriasz-Rowinka H, Rewerski W*. The analgesic action of some flavonoids in the hot plate test. Acta Physiol Pol 1979; 30: 385-388.

24. *Sosa S, Pace R, Bornancin A, Morazzoni P, Riva A, Tubaro A, Della Loggia R.* Topical anti-inflammatory activity of extracts and compounds from *Hypericum perforatum* L. J Pharm Pharmacol 2007; 59: 703-709.

25. *Hammer KDP, Hillwig ML, Solco AKS, Dixon PM, Delate K, Murphy PA, Wurtele ES, Birt DF*. Inhibition of prostaglandin E2 production by anti-inflammatory *Hypericum perforatum* extracts and constituents in RAW264.7 mouse macrophage cells. J Agric Food Chem 2007; 55: 7323–7331.

26. *Lee S, Jung SH, Lee YS, Yamada M, Kim BK, Ohuchi K, Shin KH*. Antiinflammatory activity of hyperin from *Acanthopanax chiisanensis* roots. Arch Pharm Res 2004; 27: 628-632.

27. *Lee S, Park HS, Notsu Y, Ban HS, Kim YP, Ishihara K, Hirasawa N, Jung SH, Lee YS, Lim SS, Park EH, Shin KH, Seyama T, Hong J, Ohuchi K*. Effects of hyperin, isoquercitrin and quercetin on lipopolysaccharide-induced nitrite production in rat peritoneal macrophages. Phytother Res 2008; 22: 1552–1556.

28. *Erdemoglu N, Akkol EK, Yesiladab E, Caliş I*. Bioassay-guided isolation of anti-inflammatory and antinociceptive principles from a folk remedy, *Rhododendron ponticum* L. leaves. J Ethnopharmacol 2008; 119: 172–178.

29. *Brasil*. Congresso Nacional. Lei n. 11794. Regulamenta o inciso VII do § 1º do artigo 225 da Constituição Federal, estabelecendo procedimentos para o uso científico de animais. Revoga a lei nº 6638, de 8 de maio de 1979 e dá outras providências. D.O.U., Brasília, 8 de outubro de 2008.

30. *Bankowski Z*. CIOMS. Council for International Organization of Medical Sciences International guiding principles for biomedical research involving animals. 1985. Avaiable at http://www.cioms.ch/frame_1985_texts_of_guidelines.htm. Accessed September 30. 2009.

31. *Chang Q, Zuo Z, Chow MS, Ho WK*. Difference in absorption of the two structurally similar flavonoid glycosides, hyperoside and isoquercitrin, in rats. Eur J Pharm Biopharm 2005; 59: 549-555.

32. *Porsolt RD, Bertin A, Jalfre M*. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977; 229: 327-336.

33. *Porsolt RD, Le Pichon M, Jalfre M*. Depression: a new animal model sensitive to antidepressant treatments. Nature 1978; 266: 730–732.

34. *Porsolt RD, Anton G, Blavet N, Jalfre M*. Behavioural despair in rats: A new model sensitive to antidepressant treatments. Eur J Pharmacol 1978; 47: 379-391.

35. *Zhou T, Chen B, Fan G, Chai Y, Wu Y*. Application of high-speed counter-current chromatography coupled with high-performance liquid chromatography-diode array detection for the preparative isolation and purification of hyperoside from *Hypericum perforatum* with online purity monitoring. J Chromatogr A 2006; 1116: 97-101.

36. *Zuo Z, Zhang L, Zhou L, Chang Q, Chow M*. Intestinal absorption of hawthorn flavonoids-in vitro, in situ and in vivo correlations. Life Sci 2006; 79: 2455-2462.

37. *Juergenliemk G, Boje K, Huewel S, Lohmann C, Galla HJ, Nahrstedt A*. In vitro studies indicate that miquelianin (quercetin 3-O-beta-D-glucuronopyranoside) is able to reach the CNS from the small intestine. Planta Med 2003; 69: 1013-1017.

38. *Mayers AG, Baldwin DA*. Antidepressants and their effect on sleep. Hum Psychopharmacol 2005; 20: 533-559.

39. *Butterweck V, Hegger M, Winterhoff H*. Flavonoids of St. John's Wort reduce HPA axis function in the rat. Planta Med 2004; 70: 1008-1011.

40. *Prenner L, Sieben A, Zeller K, Weiser D, Häberlein H*. Reduction of high-affinity β2-adrenergic receptor binding by hyperforin and hyperoside on rat C6 glioblastoma cells measured by fluorescence correlation spectroscopy. Biochemistry 2007; 46: 5106-5113.

41. *Denke A, Schempp H, Weiser D, Elstner EF*. Biochemical activities of extracts from *Hypericum perforatum* L. 5th communication: dopamine-beta-hydroxylase-product

quantification by HPLC and inhibition by hypericins and flavonoids. Arzneimittelforschung 2000; 50: 415-419.

42. *Grabowska M, Schlegel-Zawadzka M, Papp M, Nowak G*. Effect of imipramine treatment on plasma dopamine beta-hydroxylase activity in chronic mild stress in rats. Pol J Pharmacol 2004; 56: 825-829.

43. *Cryan JF, Dalvi A, Jin SH, Hirsch BR, Lucki I, Thomas SA*. Use of dopamine-beta-hydroxylase-deficient mice to determine the role of norepinephrine in the mechanism of action of antidepressant drugs. J Pharmacol Exp Ther 2001; 298: 651-657.

44. *Duterte-Boucher D, Leclère JF, Panissaud C, Costentin J*. Acute effects of direct dopamine agonists in the mouse behavioral despair test. Eur J Pharmacol 1988; 154: 185-190.

45. *D'Aquila PS, Collu M, Pani L, Gessa GL, Serra G*. Antidepressant-like effect of selective dopamine D1 receptor agonist in the behavioral despair animal model of depression. Eur J Pharmacol 1994; 262: 107-111.

46. *Stahl SM*. Essential Psychopharmacology - Neuroscientific Basis and Practical Applications, 2nd edition. New York: Cambridge University Press; 2000: 241-243.

Figure Legends

Figure 1: Chemical structure of hyperoside and miquelianin

Figure 2: Effect of hyperoside (10, 20 and 40 mg/kg i.p.) on locomotor activity. Data are presented as mean ± SEM (n = 6 - 8 mice/group). Significantly different values were detected by one-way ANOVA, crossings $F_{3,27} = 8.92$ $^{ccc}p<0.001$, rearings $F_{3,27} = 4.70$ $^{***}p<0.001$, groomings $F_{3,27} = 9.73$ $^{###}p<0.001$, followed by Dunn's *post hoc* test: compared to vehicle.

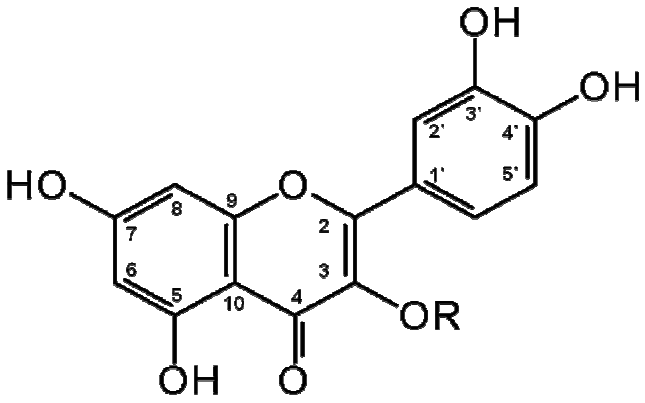
Figure 3: Effect of hyperoside (10, 20 and 40 mg/kg i.p.) and diazepam (1 mg/kg i.p.) on the potentiation of sodium pentobarbital sleeping latency **(a)** and duration **(b)**. Data are presented as medians (interquartile intervals) (n = 7 - 8 mice/group). Significantly different values were detected by Kruskal-Wallis, (**a**) $H = 11.78$ (**b**) $H = 16.81$, followed by Dunn's *post-hoc* test: * $p<0.05$, compared to vehicle.

Figure 4: Effect of hyperoside 1.8 mg/kg/day p.o. on the forced swimming test in rats. Data are presented as median ± 25% (n = 8 - 12 mice/group). Significantly different values were detected by Kruskal-Wallis $H = 29.85$, followed by Dunn's *post-hoc* test: $^{*}p<0.05$, between groups.

Figure 5: Effect of hyperoside 10 and 20 mg/kg i.p. on the forced swimming test in mice. Data are presented as mean ± SEM (n = 8 mice/group). Significantly different values were detected by one-way ANOVA $F_{3,31} = 14.92$, followed by Student-Newman-Keuls *post-hoc* test: $^{***}p<0.001$.

Figure 6: Effect of hyperoside (10 and 20 mg/kg i.p.) on the hot plate test. Data are presented as mean ± SEM (n = 6 – 8 mice/group). Significantly different values were detected by two-way repeated measures ANOVA followed by Student-Newman-Keuls *post-hoc* test: treatment factor $F_{3,59} = 15.21$; latency factor $F_{1,59} = 58.20$; latency *vs*

treatment factor $F_{3,59}$ = 37.75. ***p<0.001 significant difference from vehicle (second latency); ###p<0.001 significant difference from respective basal latency.



R = β-D-galactosyl **Hyperoside**
R = β-D-glucoronosyl **Miquelianin**

Figure 1

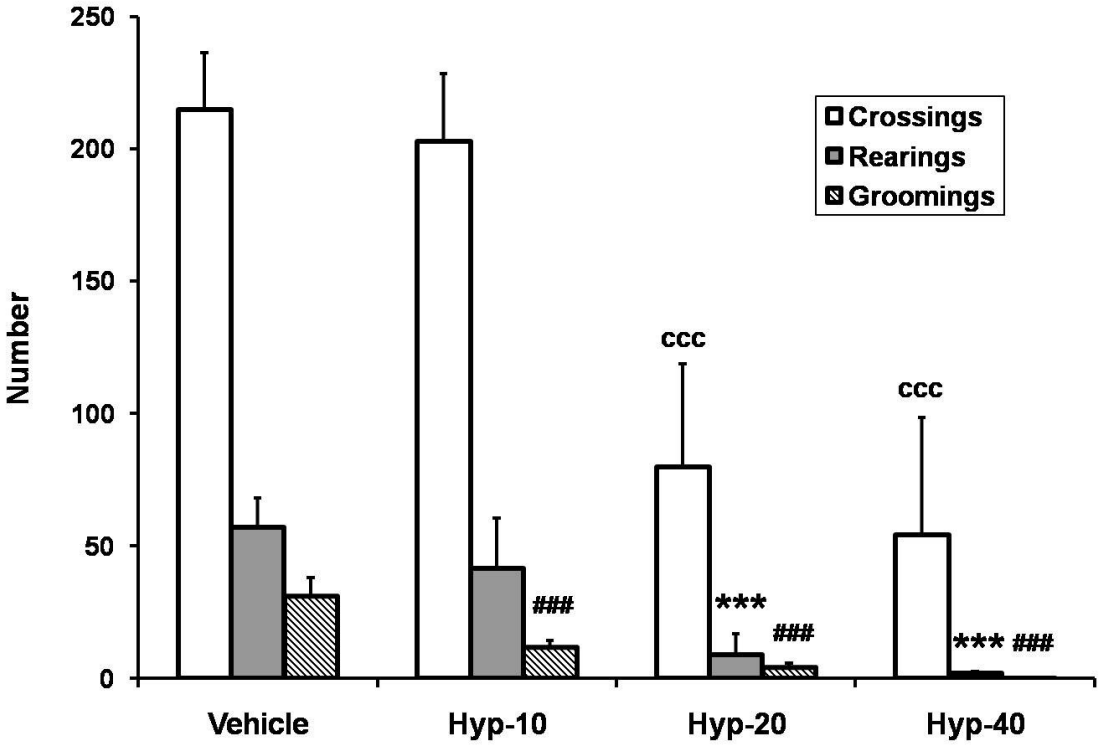

250
200
150
100
50
0
Number
Crossings
Rearings
Groomings
ccc
ccc
###
***
###
*** ###
Vehicle
Hyp-10
Hyp-20
Hyp-40

Figure 2

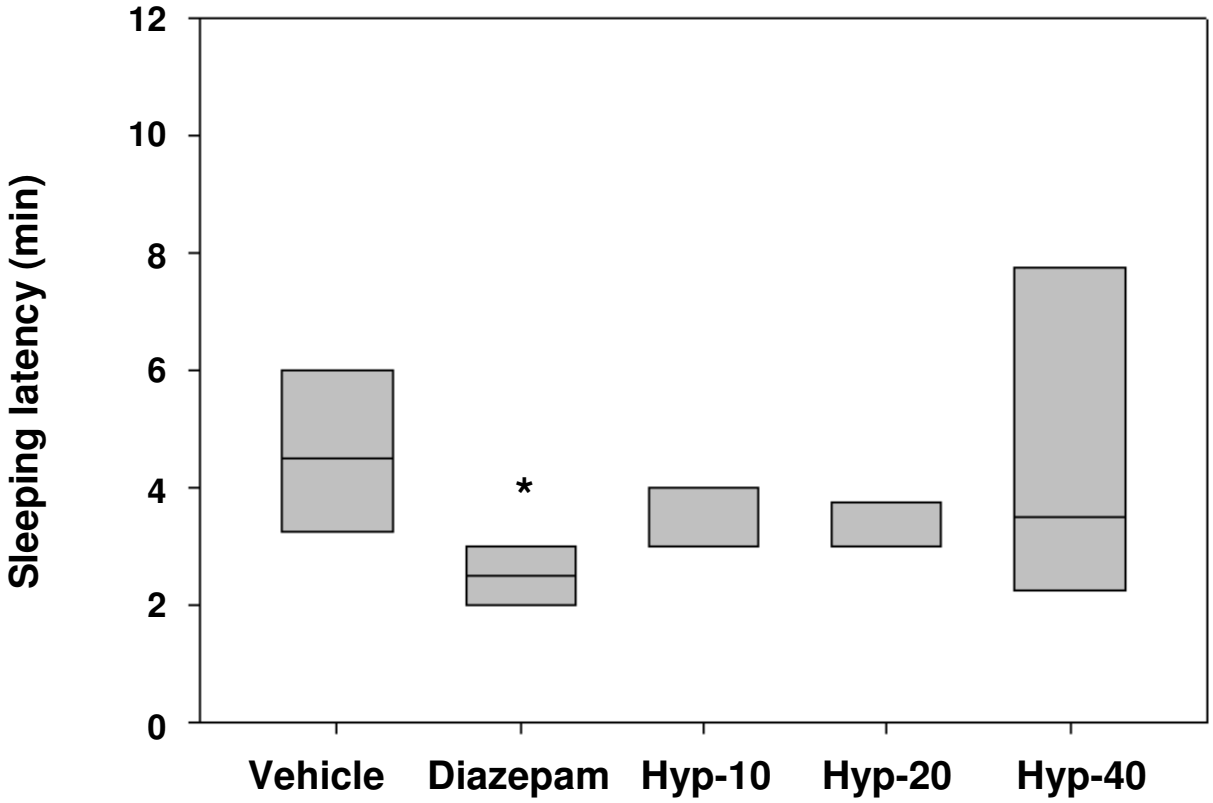

Sleeping latency (min)
12
10
8
6
4
2
0
*
Vehicle
Diazepam
Hyp-10
Hyp-20
Hyp-40

Figure 3 (a)

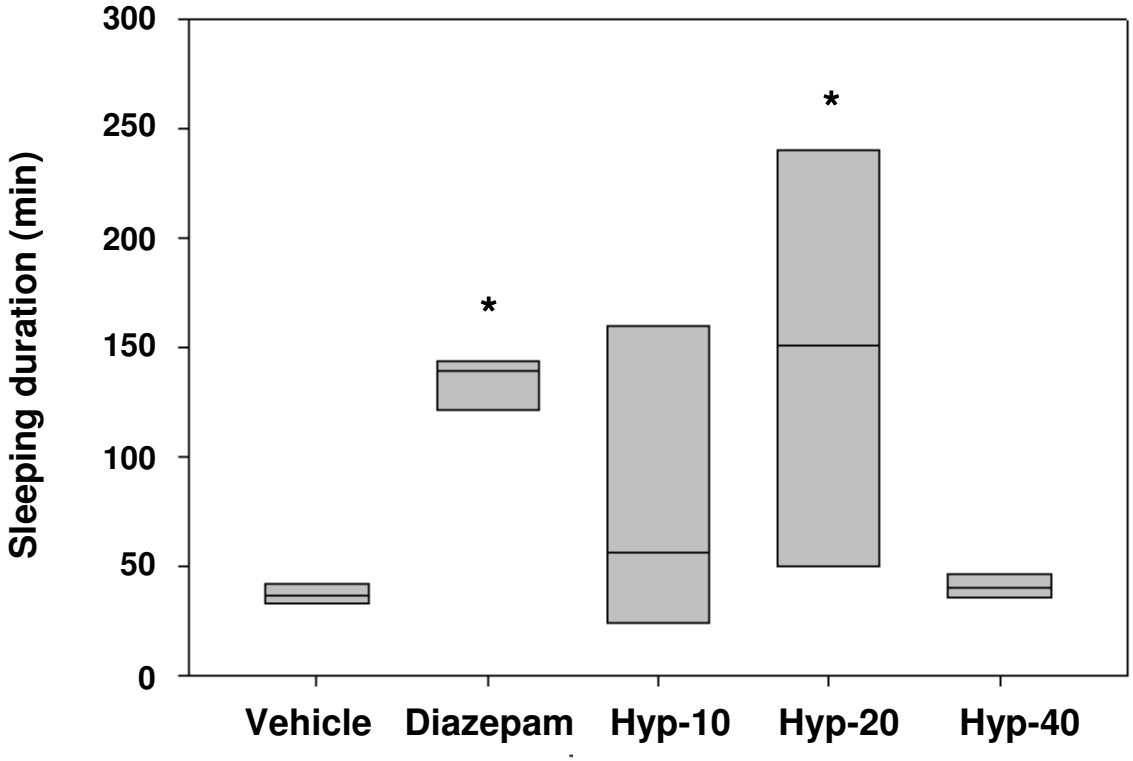

Sleeping duration (min)
300
250
200
150
100
50
0
*
*
Vehicle
Diazepam
Hyp-10
Hyp-20
Hyp-40

Figure 3 (b)

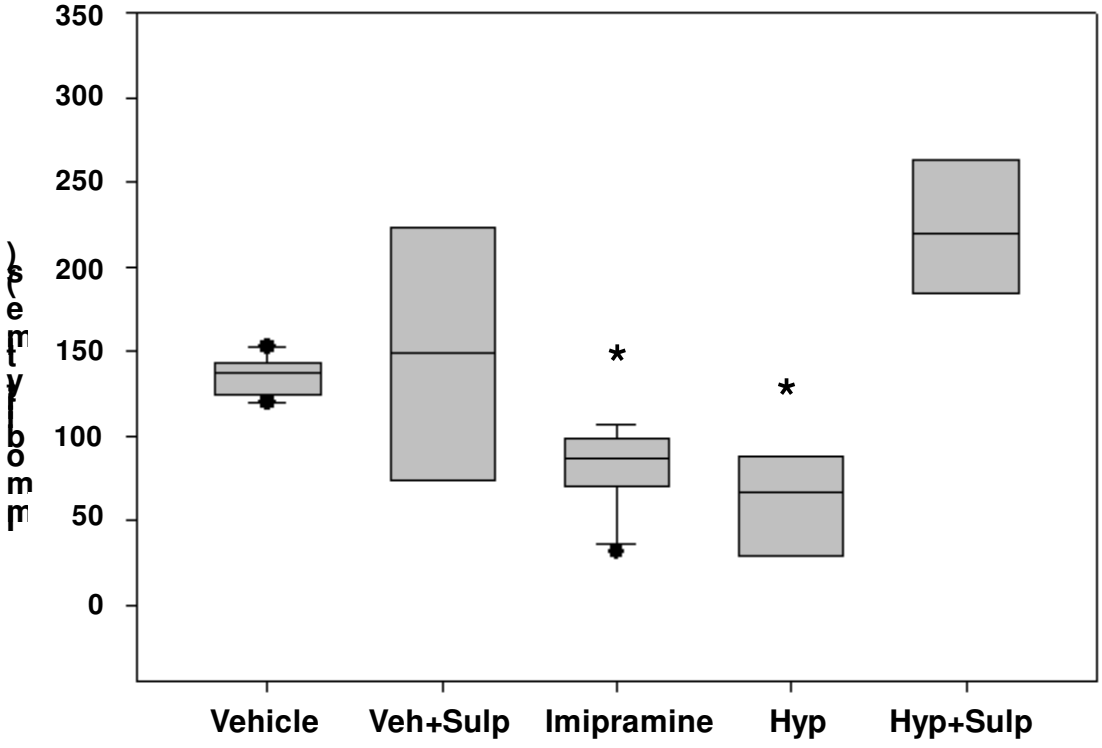

350
300
250
200
150
100
50
0
Vehicle
Veh+Sulp
Imipramine
Hyp
Hyp+Sulp

Figure 4

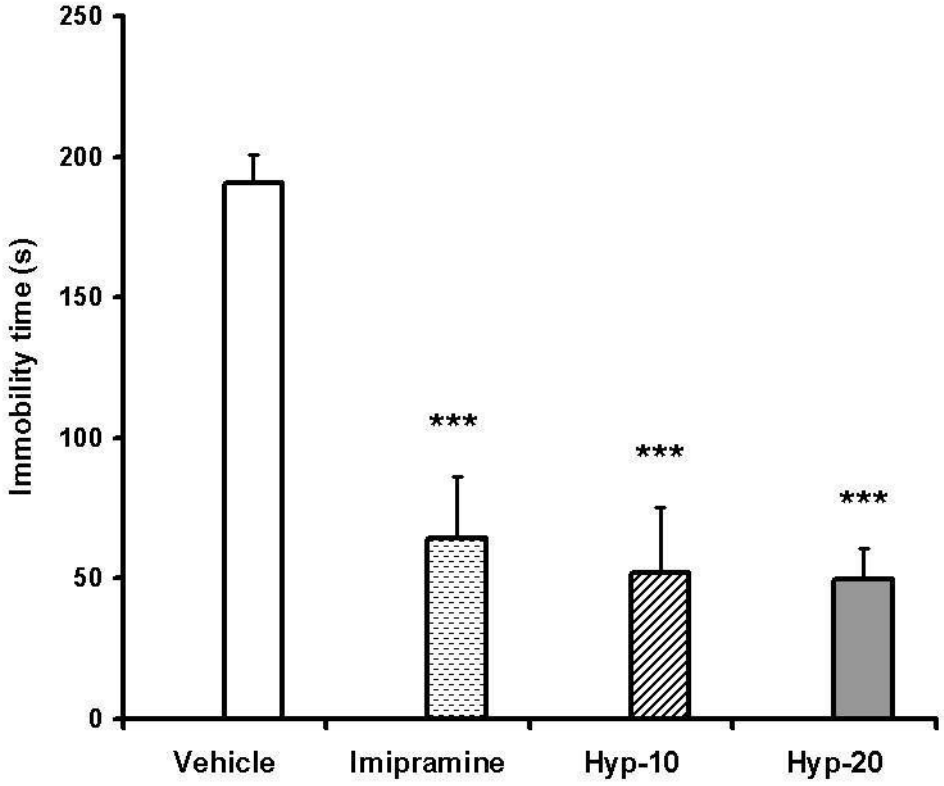

250
200
150
100
50
0
Immobility time (s)
***
***
***
Vehicle
Imipramine
Hyp-10
Hyp-20

Figure 5

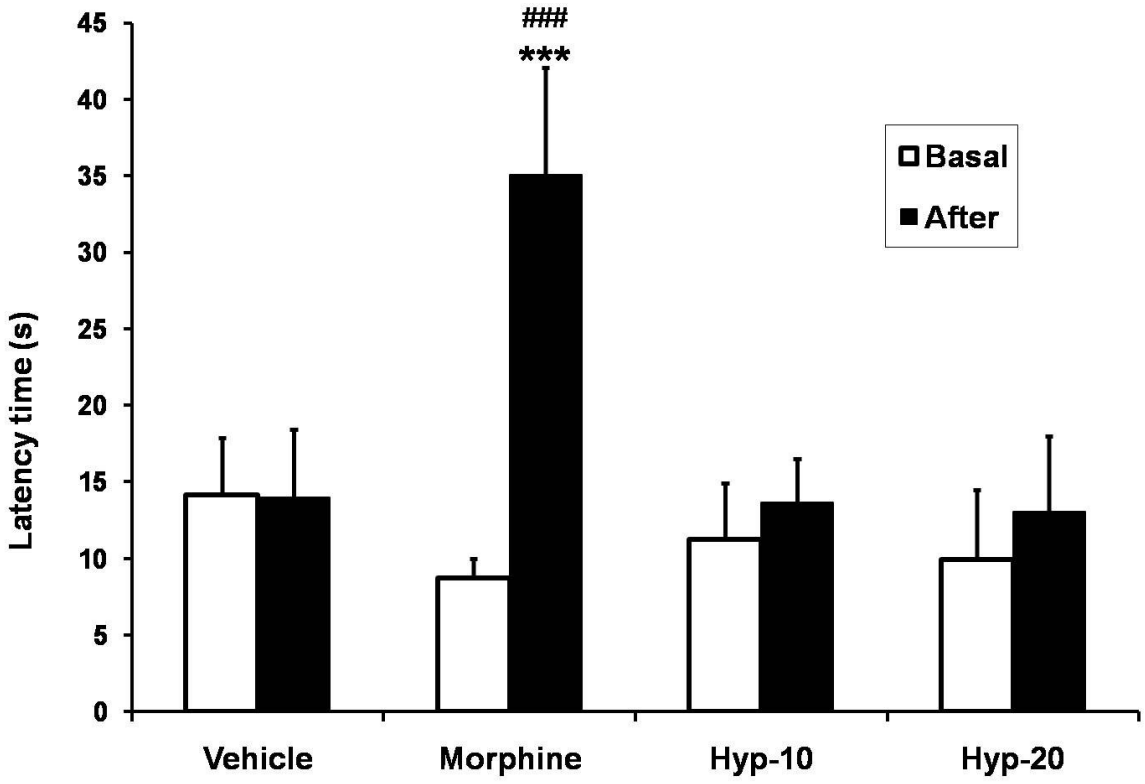

###
***
Basal
After
Latency time (s)
45
40
35
30
25
20
15
10
5
0
Vehicle
Morphine
Hyp-10
Hyp-20

Figure 6

Tables

Table 1: Effect of hyperoside (20 and 40 mg/kg, p.o.) on the writhing induced by acetic acid 0.8% in mice:

Test samples	Dose (p.o.)	Number of writhings ± S.E.M.	Inhibitory ratio compared to control (%)
Vehicle	**10 mL/kg**	**28.0 ± 4.9**	**-**
Dipyrone	**150 mg/kg**	**9.7 ± 2.5** ***	**65**
Hyperoside	**20 mg/kg**	**16.4 ± 3.4**	**41**
Hyperoside	**40 mg/kg**	**20.4 ± 4.2**	**27**

Effect of hyperoside (20 and 40 mg/kg p.o) on the writhing test. Data are presented in mean ± SEM (n = 13 mice/group). Significantly different values were detected by one-way ANOVA $F_{3,51}$ = 3.91 followed by Student–Newman–Keuls *post hoc* test: ***p<0.001.

3.3. MANUSCRITO III:

"The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone"

Aceito para publicação no periódico *Planta Medica.* DOI: 10.1055/s-0029-1240942.

O presente trabalho apresenta a avaliação da atividade analgésica dos benzopiranos isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano (HP1), 7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano (HP2) e 5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano (HP3) isolados de *Hypericum polyanthemum*. Os compostos foram testados isoladamente no modelo da placa quente e no teste de contorções induzidas por ácido acético. O extrato ciclo-hexano da planta também foi testado em diferentes doses. O envolvimento do sistema opióide na atividade dos compostos e do extrato foram verificados.

O extrato ciclo-hexano mostrou atividade do tipo dose-efeito. Dentre os compostos isolados, o benzopirano HP1 mostrou efeito antinociceptivo nos testes da placa quente, prevenido pela administração de naloxona, e de contorções induzidas por ácido acético. A observação do desempenho dos animais no rota-rod mostrou que a administração de HP1 e do extrato ciclo-hexânico não causam prejuízo motor.

Carta de aceite do manuscrito:

Preview

From: calixto@farmaco.ufsc.br

To: ratessmk@hotmail.com

CC: claudia.schaerer@unibas.ch

Subject: Planta Medica - Decision on PLAMED-2009-10-0857-OP.R1

Body:

03-Feb-2010

Dear Dr. Rates,

It is a pleasure to accept your revised manuscript entitled "The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone" in its current form for publication in Planta Medica. The comments of the reviewer(s) who have reviewed your manuscript are included at the bottom of this letter

According to the comment by reviewer 1, the bottom line of page 7/24 will be modified to: " ... yielding 200 mg of HP1 (11.1%), 100 mg of HP2 (5.6%) and 150 mg of HP3 (8.3%)." at the Editorial Office.

Thank you for your fine contribution. I look forward to your continued contribution to the Journal.

Sincerely,

Dr. João Calixto
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Prof. Luc Pieters
Editor-In-Chief Planta Medica
luc.pieters@ua.ac.be

Original paper

The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone

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Abstract

Several species of the genus *Hypericum* (Guttiferae) have been used for analgesic purposing over the world and some of them have demonstrated to possess this effect in rodents. This study describes the antinociceptive effect of the cyclohexane extract from aerial parts of *H. polyanthemum* (POL) as well as of benzopyrans, 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) which are the main components of POL. The antinociceptive effect was evaluated through hot plate and writhing tests in mice. The opioid system involvement was assessed by using naloxone (2.5 mg/kg, s.c.) antagonism on the hot plate test. POL (45, 90, 180 mg/kg p.o) showed a dose-dependent effect in the hot plate. Out of the benzopyrans only HP1 (30, 60, 90 mg/kg, i.p.) was active in the hot plate test. This effect was dose-dependent, with the maximal effect reached at 60 mg/kg. HP1 60 mg/kg (p.o.) also inhibited acetic acid-induced writhing in 58%. The pre-treatment with naloxone abolished the antinociceptive effect of HP1 60 mg/kg (i.p) in the hot plate. Furthermore, the *H. polyanthemum* cyclohexane extract and HP1 did not affect the mice performance in the rota-rod apparatus suggesting that at antinociceptive doses they do not present gross neurotoxicity neither induce motor impairment. From these data it is reasonable to assume that the benzopyran HP1 accounts for the *H. polyanthemum* cyclohexane extract antinociceptive effect, and this effect is at least in part mediated by an opioid-like mechanism.

Key words: *Hypericum polyanthemum,* Guttiferae, benzopyrans, antinociceptive effect

Abbreviations

HP1 - 6-Isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran
HP2 - 7- Hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran
HP3 - 5-Hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran
POL – Cyclohexane extract from *H. polyanthemum*
POL 45 - Cyclohexane extract from *H. polyanthemum* 45 mg/kg p.o.
POL 90 - Cyclohexane extract from *H. polyanthemum* 90 mg/kg p.o.
POL 180 - Cyclohexane extract from *H. polyanthemum* 180 mg/kg p.o.
S.E.M. - Standard Error of the Mean
THC - delta-9-tetrahydrocannabinol

Introduction

Different species of the genus *Hypericum* (Guttiferae) have been used for the analgesic purpose all over the world and some of them have demonstrated to possess this effect in pre-clinical studies. *Hypericum perforatum, H. reflexum*, *H. brasiliense, H. canariense* and *H. glandulosum* are examples [1-3]. Among Brazilian species*, H. brasiliensis* and *H. connatum* are popularly used for relieving disorders such as angina, cramps and oral-pharyngeal inflammations [4] which could be associated with potential analgesic and anti-inflammatory properties of the genus *Hypericum*. In fact, *H. caprifoliatum* and *H. polyanthemum*, species native to South Brazil, exhibited antinociceptive activity in rodents [5].

The cyclohexane extract of *H. polyanthemum* (180 mg/kg, i.p. and p.o.) displayed antinociceptive effect in the hot-plate and writhing tests that seems to be, at least in part, mediated by the opioid system [5]. From this extract three benzopyrans, 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) (Figure 1) were obtained [6]. These compounds showed bioactivities

such as monoamine oxidase inhibition (MAOI) [7], antibacterial [8] and potent growth inhibitory activity in several cell lines (human colon carcinoma cells, non-small cell lung carcinoma, and human malignant glioma cells), and also induced alterations in cell cycle distribution in non-small cell lung carcinoma [9]. Recently it was demonstrated that HP1, HP2 and HP3 present a low propensity to induce genotoxicity [10-11].

In this study we have carried out the evaluation of antinociceptive effect of HP1, HP2 and HP3 which are the main compounds found in *H. polyanthemum.* The antinociceptive effect was evaluated by hot plate and writhing testing in mice. The opioid system participation in the antinociceptive effect was assessed by using naloxone antagonism on the hot plate test.

Materials and Methods

Plant material

Aerial parts of *H. polyanthemum* Klotzsch ex Reichardt were collected in the state of Rio Grande do Sul (Brazil) in October 2005. Plant was identified by Dr. Sérgio Bordignon (ULBRA-RS-BRASIL) and the voucher was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN) (*H. polyanthemum*, Bordignon *et al*. 1429).

Extraction and Isolation procedures

Air dried and powdered plant material (aerial parts, 80 g) was extracted by maceration in cyclohexane (3 x 24h at 20°C). The extract (1.8 g) was evaporated to dryness under reduced pressure and submitted to silica gel column chromatography GF_{60} (Merck®), (50 X 3 cm) using cyclohexane-dichloromethane gradient system as the mobile phase. The benzopyrans HP1, HP2 and HP3 were purified by preparative TLC performed on 20cm×20cm glass-supported plates covered with 0.5 mm layers of silica gel GF_{254} (Merck®) yielding 200 mg of HP1, 100 mg of HP2 and 150 mg of HP3. The chromatographic eluent used were as follow: cyclohexane:CH_2Cl_2 (1:1) for HP1, (3:2) for HP2 and (4:1) for HP3. Bands were detected using UV light (254 nm) and the compounds

were identified by ^{1}H RMN and ^{13}C RMN. The spectral data are described elsewhere [6]. The benzopyrans' purity and yield were also determined by HPLC [12].

Pharmacological Study

Animals

Male CF1 mice (25 – 30 g) from breeding colony of Fundação Estadual de Pesquisa e Ensino em Saúde (FEPPS, RS, Brazil) were used. The animals were housed in plastic cages, 5 to cage, under a 12h light/dark cycle (lights on at 7:00 h) at constant temperature (23 ± 1ºC) with free access to standard certified rodent diet and tap water. All protocols were approved by a Local Research Ethical Committee (UFRGS) (Nº2008008) and are in compliance with Brazilian law [13] and CIOMS (Council for International Organization of Medical Sciences International) guiding principles for biomedical research involving animals [14].

Drugs and treatments

The following drugs were used: naloxone, morphine (Cristália, Brazil), dypirone (Sanofi-Aventis, Brazil), codeine (Janssen-Cilag, Brazil) and haloperidol (Galena, Brazil). In all experiments, POL and benzopyrans were dissolved in saline with 2.5% polysorbate 80 and the negative control group received 10 mL/kg of vehicle (saline with 2.5% polysorbate 80). The other drugs were dissolved in saline (NaCl 0.9%) solution immediately before using. All administered drugs presented pharmaceutical grade of purity (> 97%).

Hot-plate test

Before actual testing on the hot plate, the mice were habituated to the nonfunctioning apparatus for 1 min. Thirty minutes later, the animals were placed on the functioning hot plate (Ugo Basile, Comerino, Italy) (53 ± 1ºC) to determine baseline responsiveness. The time elapsed until the animal lick one of its hind paws or jump was recorded (latency time, in seconds). Mice that presented baseline reaction of more than 15 s were eliminated from the test. After the baseline responsiveness determination the animals received the different

treatments. Thirty minutes later they were placed again in the hot plate and the latency time was measured. In the second session, a maximum latency time of 30 s was imposed in order to avoid tissue damage.

The study was carried out through four experiments. First, we evaluated the effect of the *H. polyanthemum* cyclohexane extract (POL) 45, 90 and 180 mg/kg p.o. Second, the single administration of HP1, HP2 and HP3 (30 mg/kg, i.p.) was tested. Afterward we determined a dose-response profile of HP1 (30, 60, 90 mg/kg, i.p.). Finally we investigated the influence of naloxone (2.5 mg/kg, s.c) on the antinociceptive effect of HP1 (60 mg/kg, i.p.). The naloxone was administered immediately after evaluating baseline responsiveness, 10 min before the HP1 administration. Morphine (6mg/kg, s.c.) was used as positive control.

Acetic Acid-Induced Writhing Test

Different groups of mice received HP1 60 mg/kg, p.o., vehicle p.o. or dypirone (150 mg/kg, p.o.) 45 min before an intraperitoneal injection of acetic acid (Merck AG, Germany) (0.8 %, 10 mL/kg). Then mice were observed for 15 min in individual glass observation chambers, and the number of writhing motions was counted. The percentage analgesic activity was calculated as follows:

$$\text{Percentage of analgesic activity} = \left(\frac{N - N' \text{ x } 100}{N} \right)$$

N represents the average number of writhing of control group and *N*' the average number of stretching of test group.

Motor coordination test

The procedure was adapted from Dunham & Miya [15]. Briefly, the apparatus consisted of a cylinder of 3 cm of diameter rotating at 5 rpm. One day before testing the animals were trained once during five minutes. On the test day the mice that were able to stay 90 seconds balanced on the rotating rod were selected for testing. The selected mice received POL 180

mg/kg, i.p., HP1 (60 mg/kg, i.p.), codeine (30 mg/kg, i.p.), haloperidol (4 mg/kg, i.p.), or vehicle (i.p.). Mice performance was measured before and 45 minutes after the drug administration. The integrity of motor coordination was assessed on the basis of the longest time of permanence and the number of falls in a 5 min period.

Statistical Analysis

The data from hot plate and rota-rod performance were analyzed by the paired Student *t*-test, considering the animal as its own control: second (after) latency *vs* first (basal) latency; T0 *vs* T45. Writhing results were analyzed by ANOVA one way followed by Student-Newman-Keuls test. All results were expressed in mean ± S.E.M. The analyses were performed using Sigma Stat 2.03 software (Jandel Scientific Corporation). Differences were considered statistically significant at $p<0.05$.

Results and discussion

The *H. polyanthemum* cyclohexane extract showed a dose dependent antinociceptive effect, and the maximal effect was reached at 90 mg/kg (Figure 2). These results are in agreement with those previously reported by Viana et al. [5]. From all benzopyrans (30 mg/kg, i.p.) only HP1 presented antinociceptive effect in the hot plate test (Figure 3). This effect was dose-dependent, with significant increase in the latency to avoid the nociceptive stimulus at 30 and 60 mg/kg (i.p.) but no significant effect at 90 mg/kg, indicating the possibility of a bell-shape dose-effect curve (Figure 4). HP1 60 mg/kg (p.o.) also inhibited acetic acid induced writhing in 58% (Table 1). Abdominal writhing induced by acetic acid represents a peripheral nociception model and is frequently used for new antinociceptive compounds screening, but it is not a specific model. Several drugs such as opioid analgesics, adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors and neuroleptics inhibit acetic acid induced writhing whereas hot plate test presents selectivity for drugs that affect supraspinally integrated responses as opioid-derived analgesics [16]. The pre-treatment with naloxone abolished the antinociceptive effect of HP1 60 mg/kg (i.p) in the hot plate (Figure 5), indicating that this effect is mediated by the opioid system.

Furthermore, the *H. polyanthemum* cyclohexane extract and HP1 did not affect the mice performance in the rota-rod apparatus suggesting that at antinociceptive doses they do not present gross neurotoxicity neither induce motor impairment [17] (Figure 6). From these data it is reasonable to assume that the benzopyran HP1 accounts for the opioid-like antinociceptive effect previously reported for *H. polyanthemum* cyclohexane extract. An accurate measurement of the contribution of HP1 to the analgesic effect of POL should be adressed by further studies. Other experiments performed by our group have demonstrated that phloroglucinol derivatives are also implicated in the POL antinociceptive effect, but their effect is not mediated by opioid system [18].

The benzopyran skeleton formed by the cyclization of a prenyl unit with an adjacent phenolic hydroxyl group cover a wide range of potential pharmacologically active compounds. Several molecules presenting this nucleus, either natural or synthetic, have shown important pharmacological activities: tonabersat (SB-220453), a benzopyran with anticonvulsant properties, attenuates trigeminal nerve-induced neurovascular reflexes [19]; compounds with 7-hydroxy-2H-1-benzopyran-2-one moiety, 2,3-diaryl benzopyrans and hallogenated benzopyran derivatives present anti-inflammatory and antipyretic activity through cyclooxygenases inhibition [20-22]; and cannabinoids such as delta-9-tetrahydrocannabinol (THC). Cannabinoids enhances the potency of opioids such as morphine in animal models [23]. The analgesic effect of THC is, at least in part, mediated through delta and kappa opioid receptors, indicating an intimate connection between cannabinoid and opioid signaling pathways in the modulation of pain perception (for reviewing see Cichewicz [23]). In addition THC releases endogenous opioids and endocannabinoids, such as anandamide, and also alters endogenous opioid tone [24].

In genus *Hypericum* the benzopyran skeleton can appear as monomeric benzopyrans, as benzophenones, as xanthones, as dimeric phloroglucinol derivatives, among others. Several studies have reported that the opioid system could be involved in the antidepressant effect of the phloroglucinol hyperforin and *H. perforatum* extracts through interaction with sigma 1 receptor [25]. Chen et al. have shown that *H. perforatum* produces a significant and region-specific change in μ-opioid receptors in a number of rodent mesolimbic regions

[26]. Perfumi et al. have shown that a *H. perforatum* CO_2 supercritical extract and low doses of opiate receptor antagonists, naloxone and naltrexone, act synergistically to induce a pronounced and selective reduction of voluntary ethanol consumption in msP rats [27].

In conclusion, the results obtained in this work provide evidences that the benzopyran skeleton deserves further studies aiming to identify new analgesic scaffold as well as strong point to the importance of plants from the genus *Hypericum* in providing natural compounds with relevant activities on the central nervous system.

Acknowledgements

The authors are grateful to the Brazilian agencies CAPES, CNPq and FAPERGS for financial support.

References

1. *Mendes FR, Mattei R, Carlini ELA*. Activity of *Hypericum brasiliense* and *Hypericum cordatum* on the central nervous system in rodents. Fitoterapia 2002; 73: 462-471.

2. *Rabanal RM, Bonkanka CX, Hernández-Pérez M, Sánchez-Mateo CC*. Analgesic and topical anti-inflammatory activity of *Hypericum canariense* L. and *Hypericum glandulosum* Ait. J Ethnopharmacol 2005; 96: 591–596.

3. *Sánchez-Mateo CC, Bonkanka CX, Hernández-Pérez M, Rabanal RM*. Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. fil. J Ethnopharmacol 2006; 107: 1–6.

4. *Mentz LA, Lutzemberger LC, Schenkel EP*. Da flora medicinal do Rio Grande do Sul: notas sobre a obra de D'Avila (1910). Caderno de Farmácia 1997; 15: 25–47.

5. *Viana AF, Heckler AP, Fenner R, Rates SMK*. Antinociceptive activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae). Braz J Med Biol Res 2003; 36: 631-634.

6. *Ferraz ABF, Bordignon SAL, Staats C, Schripsema J, von Poser GL*. Benzopyrans from *Hypericum polyanthemum*. Phytoche 2001; 57: 1227-1230.

7. *Gnerre C, von Poser GL, Ferraz A, Viana A, Testa B, Rates SMK*. Monoamine oxidase inhibitory activity of some *Hypericum* species native to South Brazil. J Pharm Pharmacol 2001; 53: 1273-1279.

8. *Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nör C, Schapoval EES, von Poser GL*. Bioassay-guided isolation of antimicrobial benzopyrans and phloroglucinol derivatives from *Hypericum* species. Phytother Res 2005; 19: 291-293.

9. *Ferraz ABF, Grivicich I, von Poser GL, Faria DH, Kayser GB, Schwartsmann G, Henriques AT, da Rocha AB*. Antitumor activity of three benzopyrans isolated from *Hypericum polyanthemum*. Fitoterapia 2005; 76: 210-215.

10. *Grivicich I, Ferraz A, Faria DH, Regner A, Schwartsmann G, Henriques AT, von Poser GL, da Rocha AB*. Synergistic effect of three benzopyrans isolated from *Hypericum polyanthemum* in U-373 MG glioblastoma cell line. Phytother Res 2008; 22: 1577–1580.

11. *Ferraz ABF, da Silva J, Deimlimg LI, Santos-Mello R, Sharlau A, von Poser GL, Picada JN*. Genotoxicity evaluation of three benzopyrans from *Hypericum polyanthemum*. Planta Med 2009; 75: 37–40.

12. *Cargnin ST, de Matos Nunes J, Haas JS, Baladão LF, Cassel E, Vargas RF, Rech SB, von Poser GL.* Supercritical fluid extraction and high performance liquid chromatographic determination of benzopyrans and phloroglucinol derivative in *Hypericum polyanthemum*. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878: 83-87.

13. *Brasil*. Congresso Nacional. Lei n. 11794. Regulamenta o inciso VII do § 1º do artigo 225 da Constituição Federal, estabelecendo procedimentos para o uso científico de animais. Revoga a lei nº 6638, de 8 de maio de 1979 e dá outras providências. D.O.U., Brasília, 8 de outubro de 2008.

14. *Bankowski Z*. CIOMS. Council for International Organization of Medical Sciences International guiding principles for biomedical research involving animals. 1985. Avaiable at http://www.cioms.ch/frame_1985_texts_of_guidelines.htm. Accessed September 30. 2009.

15. *Dunham NW, Miya TS*. A note on a simple apparatus for detecting neurological deficit in rats and mice. J Am Pharm Assoc Am Pharm Assoc (Baltim) 1957; 46: 208-209.

16. *Le Bars D, Gozariu M, Cadden SW*. Animal models of nociception. Pharmacol Rev 2001; 53: 597–652.

17. *Pearl J, Stander H, McKean DB*. Effects of analgesics and other drugs on mice in phenylquinone and Rotorod tests. J Pharmacol Exp Ther 1969; 167: 9–13.

18. *Heckler APM, Viana AF, Fenner R, Nor C, Kliemann M, do Rego JC, Costentin J, von Poser GL, Rates SMK*. Atividade antinociceptiva de uliginosina isolada de *Hypericum polyanthemum* (Guttiferae). 20ª Reunião da Federação de Sociedades de Biologia Experimental, Águas de Lindóia; 2005.

19. *Parsons AA, Bingham S, Raval P, Read S, Thompson M, Upton N*. Tonabersat (SB-220453) a novel benzopyran with anticonvulsant properties attenuates trigeminal nerve-induced neurovascular reflexes. Br J Pharmacol 2001; 132: 1549-1557.

20. *Prasanna S, Manivannan E, Chaturvedi SC*. Quantitative structure–activity relationship analysis of a series of 2,3-diaryl benzopyran analogues as novel selective cyclooxygenase-2 inhibitors. Bioorg Med Chem Lett 2004; 14: 4005–4011.

21. *Gierse J, Nickols M, Leahy K, Warner J, Zhang Y, Cortes-Burgos L, Carter J, Seibert K, Masferrer J*. Evalaution of COX-1/COX-2 selectivity and potency of a new class of COX-2 inhibitors. Eur J Pharmacol 2008; 588: 93–98.

22. *Eissa AAM, Farag NAH, Soliman GAH*. Synthesis, biological evaluation and docking studies of novel benzopyranone congeners for their expected activity as anti-inflammatory, analgesic and antipyretic agents. Bioorg Med Chem 2009; 17: 5059–5070.

23. *Cichewicz DL*. Synergistic interactions between cannabinoid and opioid analgesics. Life Sci 2004; 74: 1317-1324.

24. *Welch SP*. Interaction of the cannabinoid and opioid systems in the modulation of nociception. Int Rev Psychiatry 2009; 21: 143-151.

25. *Mennini T, Gobbi M*. The antidepressant mechanism of *Hypericum perforatum*. Life Sci 2004; 75: 1021–1027.

26. *Chen F, Rezvani AH, Lawrence AJ*. Autoradiographic quantification of neurochemical markers of serotonin, dopamine and opioid systems in rat brain mesolimbic regions following chronic St. John's wort treatment. Naunyn Schmiedebergs Arch Pharmacol 2003; 367: 126-133.

27. *Perfumi M, Mattioli L, Cucculelli M, Massi M*. Reduction of ethanol intake by chronic treatment with *Hypericum perforatum*, alone or combined with naltrexone in rats. J Psychopharmacol 2005; 19: 448-454.

Figure Legends

Figure 1: Benzopyrans from *H. polyanthemum.*

Figure 2: Effect of *H. polyanthemum* cyclohexane extract (45, 90 and 180 mg/kg, p.o.) and morphine (6 mg/kg, s.c.) on the hot-plate test. Basal: latency before the treatment. After: latency after treatment. Data are presented in mean ± S.E.M. (n = 8 - 10 mice/group). Significantly different values were detected by paired Student *t* test: **p*<0.05; ****p*<0.001 compared to their respective basal latency.

Figure 3: Effect of benzopyrans (HP1, HP2, HP3, 30 mg/kg, i.p.) from *Hypericum polyanthemum* and morphine (6 mg/kg, s.c.) on the hot-plate test. Basal: latency before the treatment. After: latency after treatment. Data are presented in mean ± S.E.M. (n = 8 - 10 mice/group). Significantly different values were detected by paired Student *t* test: ***p*<0.01; ****p*<0.001 compared to their respective basal latency.

Figure 4: Dose-effect response of the benzopyran HP1 (30, 60 and 90 mg/kg, i.p.) and morphine (6 mg/kg, s.c.) on the hot-plate test. Basal: latency before the treatment. After: latency after treatment. Data are presented in mean ± S.E.M. (n = 8 - 10 mice/group). Significantly different values were detected by paired Student *t* test: ***p*<0.01; ****p*<0.001 compared to their respective basal latency.

Figure 5: Effect of naloxone (2.5 mg/kg, s.c.) on the antinociceptive effect of the benzopyran HP1 (60 mg/kg, i.p.) and morphine (6 mg/kg, s.c.) on the hot-plate test. Basal: latency before the treatment. After: latency after treatment. Data are presented in mean ± S.E.M. (n = 8 - 10 mice/group). Significantly different values were detected by paired Student *t* test: ***p*<0.01; ****p*<0.001 compared to their respective basal latency.

Figure 6: Effect of the cyclohexane extract of *H. polyanthemum* (180 mg/kg, i.p.) and the benzopyran HP1 (60 mg/kg, i.p.) on the rotarod apparatus: (A) Permanence time; (B) Number of falls. Data are presented in mean ± S.E.M. (n = 10 - 16 mice/group).

Significantly different values were detected by paired Student *t* test: ***$p<0.001$ compared to their respective T0.

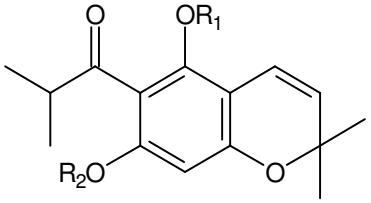
Tables

Table 1: Effect of HP1 (60 mg/kg p.o.) on writhing induced by 0.8% acetic acid (10 mL/kg, i.p.) in mice.

Test samples	Dose (mg/kg)	Number of writhings ± S.E.M.	Inhibitory ratio compared to control (%)
Control	**10 mL/kg**	**56.0 ± 2.7**	**-**
Dipyrone	**150**	**4.4 ± 1.4** ***	**92**
HP1	**60**	**23.5 ± 8.2** ***	**58**

Data are presented in mean ± S.E.M. (n = 9 - 19 mice/group). Significantly different values were detected by ANOVA one way followed by *post hoc* Dunnett's Method: ***$p<0.001$, as compared to their control group. $F_{2,44}=65.3$.

Figures



HP1	R1=Me	R2=Me
HP2	R1=Me	R2=H
HP3	R1=H	R2=Me

Figure 1

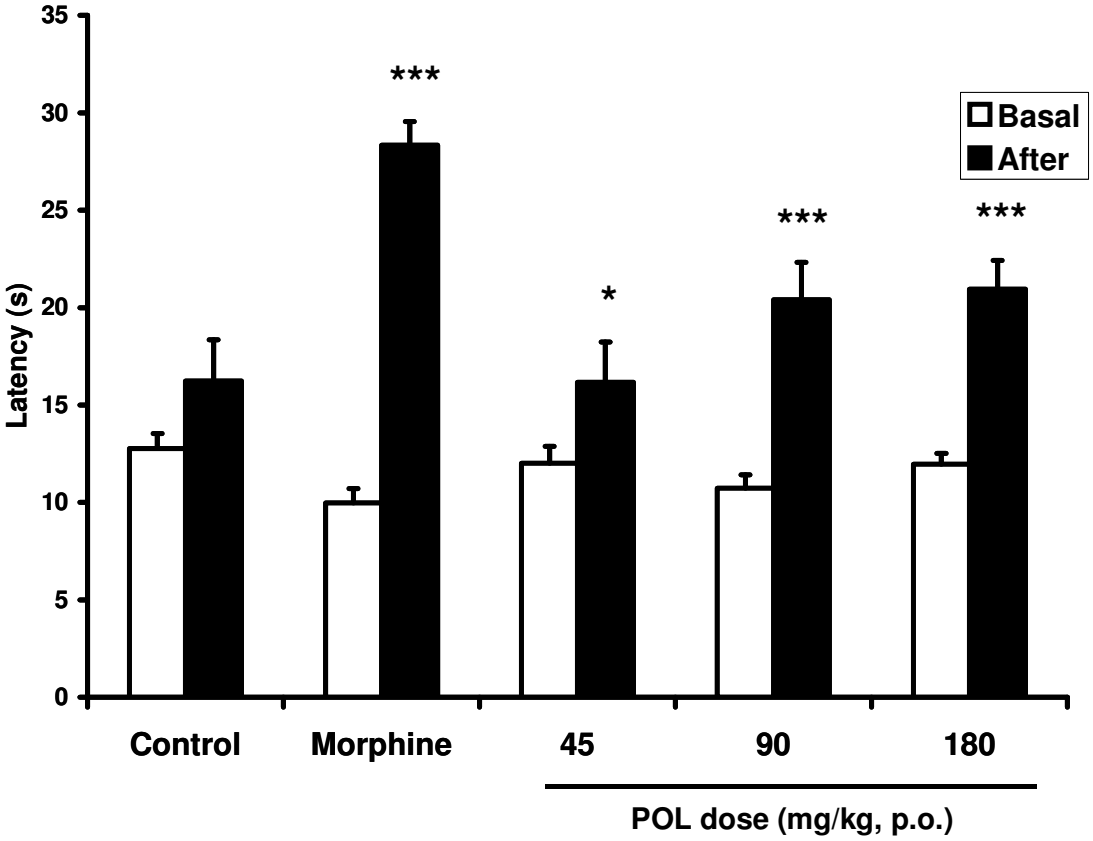

35
30
25
20
15
10
5
0
Latency (s)
***
*
***
***
Basal
After
Control
Morphine
45
90
180
POL dose (mg/kg, p.o.)

Figure 2

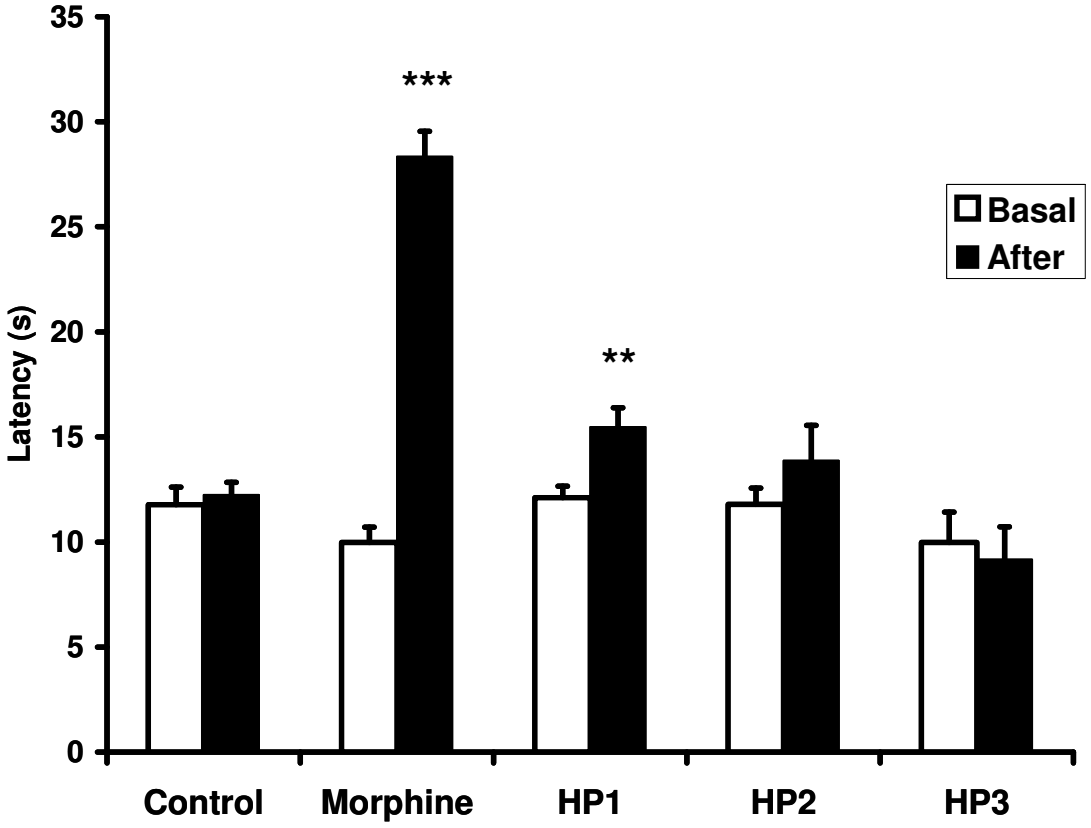

35
30
25
20
15
10
5
0
Latency (s)
***
**
Basal
After
Control
Morphine
HP1
HP2
HP3

Figure 3

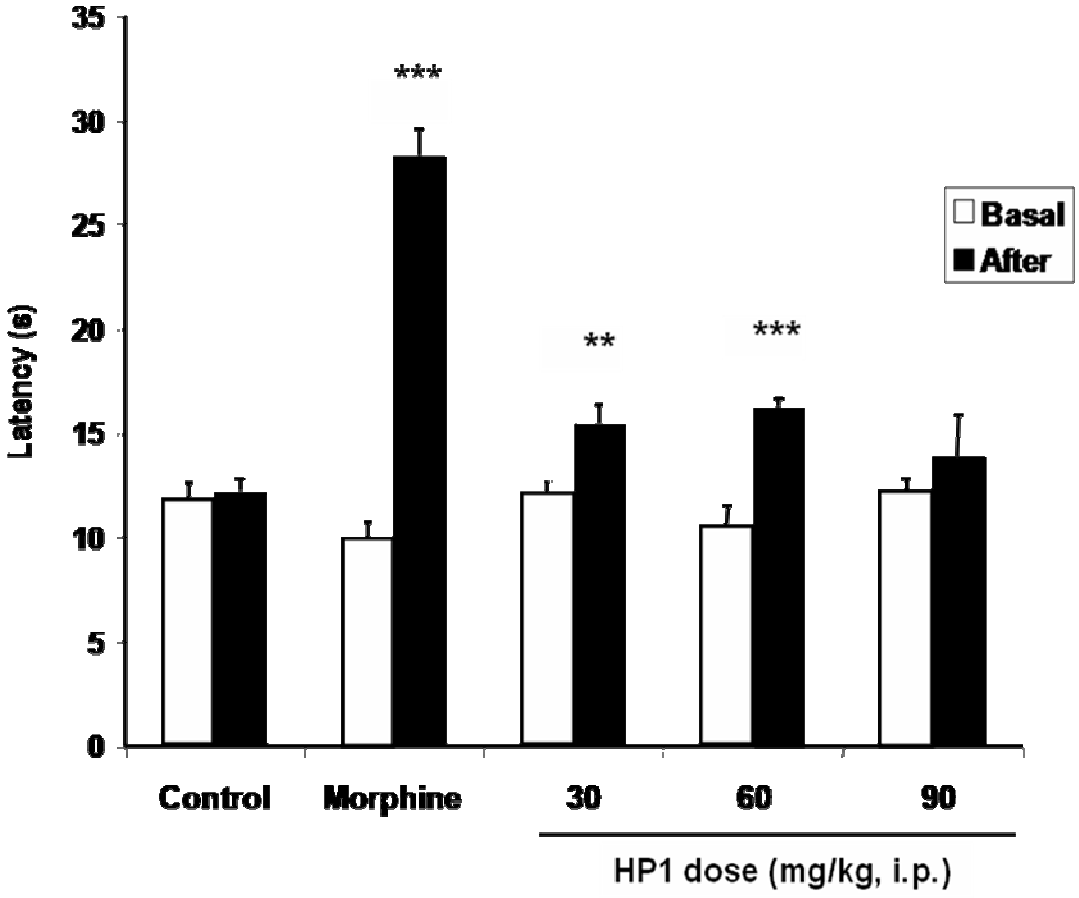

35
30
25
20
15
10
5
0
Latency (s)
***
**
***
Basal
After
Control
Morphine
30
60
90
HP1 dose (mg/kg, i.p.)

Figure 4

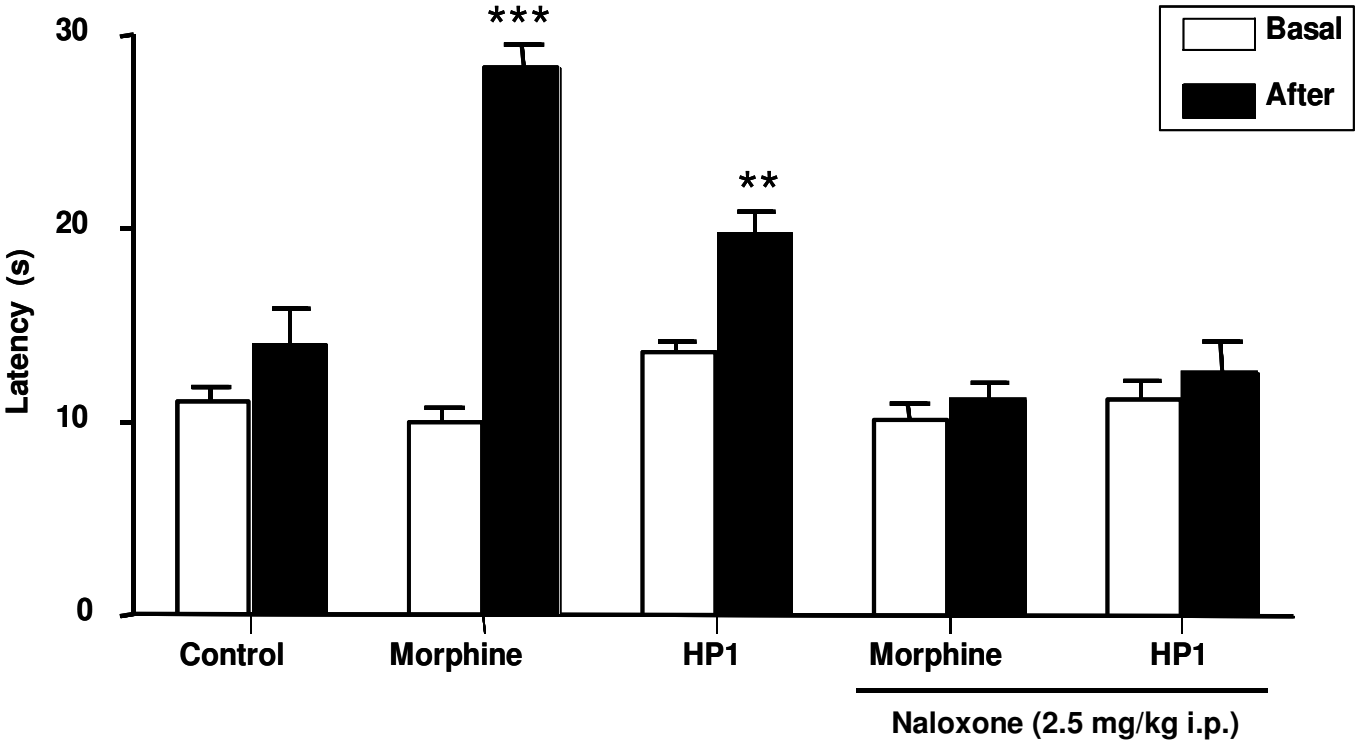

30
20
10
0
Latency (s)
***
**
Basal
After
Control
Morphine
HP1
Morphine
HP1
Naloxone (2.5 mg/kg i.p.)

Figure 5

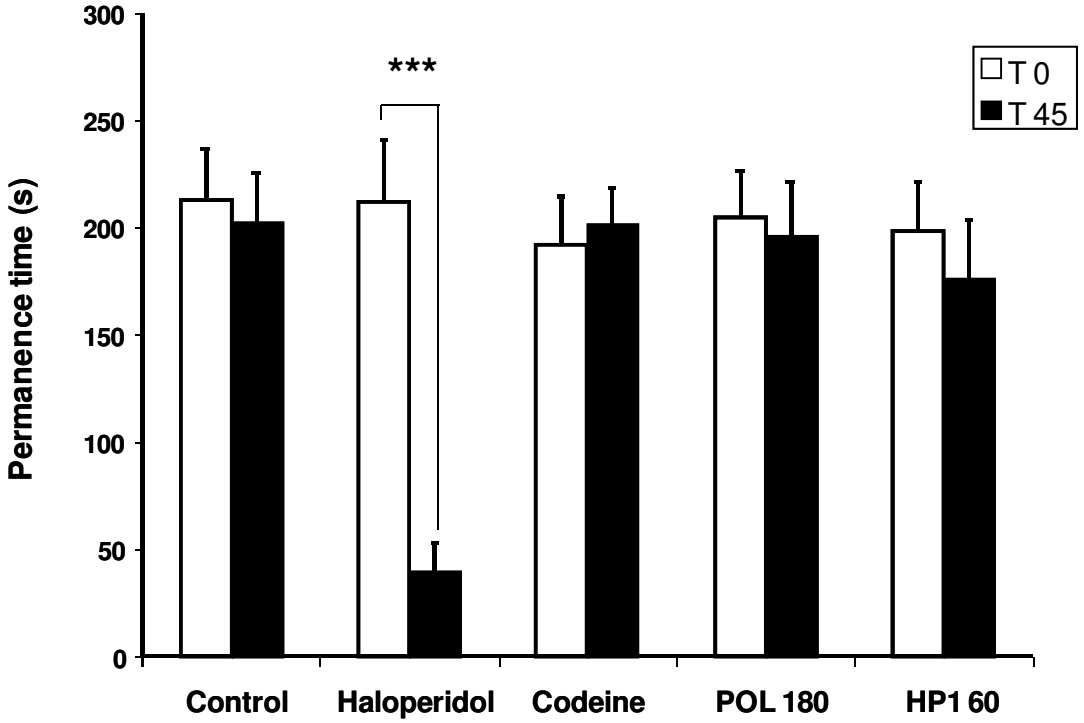

300
250
200
150
100
50
0
Permanence time (s)
***
T 0
T 45
Control
Haloperidol
Codeine
POL 180
HP1 60

Figure 6 (A)

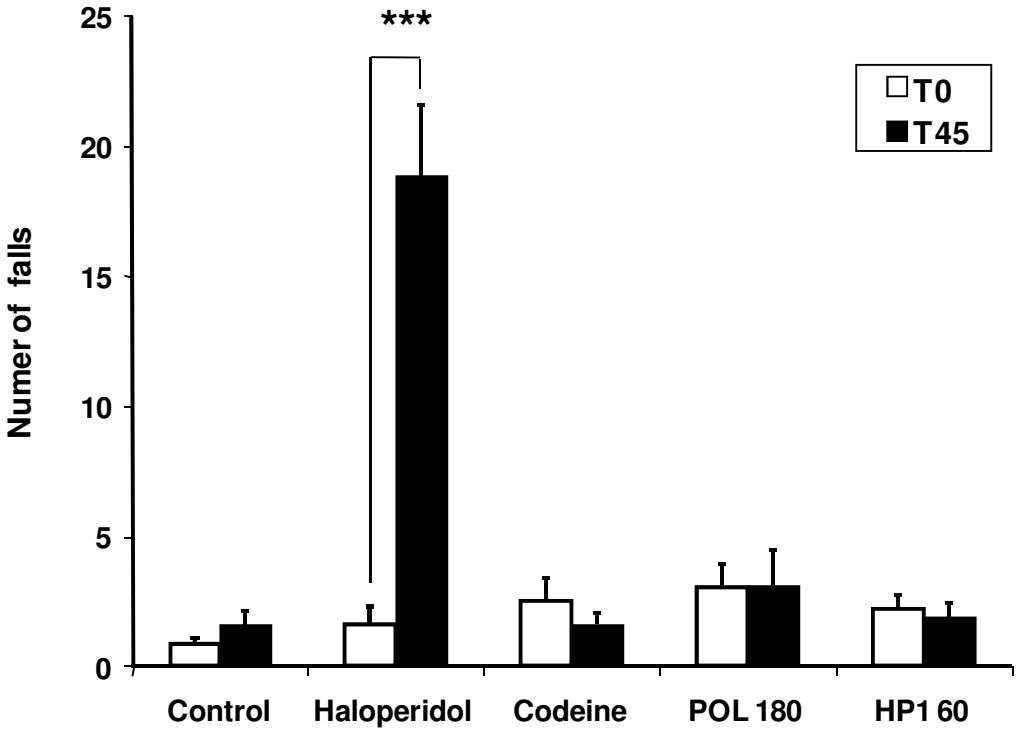

25
20
15
10
5
0
Numer of falls
***
T0
T45
Control
Haloperidol
Codeine
POL 180
HP1 60

Figure 6 (B)

3.4. MANUSCRITO IV:

"Acaricidal activity of benzopyrans from *Hypericum polyanthemum* on *Rhipicephalus (Bophilus) microplus*"

Em preparação para o periódico *Veterinary Parasitology*

Em trabalhos anteriores, extratos de *Hypericum polyanthemum* e *Calea serrata* (Asteraceae) mostraram importantes propriedades acaricidas frente às espécies de carrapato *Rhipicephalus* (*Boophilus*) *microplus* e *Rhipicephalus sanguineus*. A fração lipofílica de ambas as espécies vegetais é rica em benzopiranos (também referidos como cromenos), compostos com reconhecida atividade inseticida.

No manuscrito apresentado, os benzopiranos de *H. polyanthemum* foram isolados e testados separadamente frente a larvas de *Rhipicephalus* (*Boophilus*) *microplus* em diferentes concentrações.

Acaricidal activity from benzopyrans from *Hypericum polyanthemum* on *Rhipicephalus (Bophilus) microplus*

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Abstract

Laboratory tests were carried out on larvae of the cattle tick *Rhipicephalus* (*Bophilus*) *microplus* to determine the toxicity of three benzopyrans 6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) isolated from the *n*-hexane extract of *Hypericum polyanthemum* (Guttiferae) using the larval immersion test (LIT). The compounds demonstrated to be highly toxic to the larvae, being HP1 slightly more active.

Keywords: *Rhipicephalus* (*Boophilus*) *microplus; Hypericum polyanthemum*; Benzopyrans; Chromenes; Precocenes

1. Introduction

Preliminary studies have showed that extracts from *Calea serrata* (Asteraceae) and *Hypericum polyanthemum* (Guttiferae), both species native to South Brazil, have acaricidal activity, being highly toxic to larvae and engorged females of the cattle tick *Rhipicephalus* (*Bophilus*) *microplus* (Ribeiro et al., 2007; Ribeiro et al., 2008). The lipophilic fractions of both plants, which were the most active, present benzopyrans as major components. From *C. serrata*, eupatoriochromene and preconene II were obtained (Steinbeck et al., 1997), while *H. polyanthemum* afforded the compounds 6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) (Figure 1) (Ferraz et al., 2001).

The insecticidal activity of some benzopyrans is well documented. Precocene I (7-methoxy-2,2-dimethylbenzopyran) and the 6,7-dimethoxy derivative (precocene II) as well as encecalin and demethylencecalin have been reported to have antijuvenile hormonal activity. These compounds are highly selective chemical substances that attack specific aspects of

the endocrine systemand affect development and reproduction of the insect (Bowers et al., 1976; Pamo et al., 2004).

The structural similarity between the insecticidal benzopyrans and the compounds isolated from *H. polyanthemum* suggested that the benzopyrans could have insecticidal or acaricidal activity. Thus, the aim of this work was to isolate and to investigate the activity of the benzopyrans HP1, HP2 and HP3 on the cattle tick *R.* (*B.*) *microplus* in order to corroborate with the above statement.

2. Materials and methods

2.1. Plant material

Plant material of *H. polyanthemum* Klotzsch ex Reichardt were collected in October 2007, in the city of Caçapava do Sul, South Brazil. Plant material was identified by Dr. Sérgio Bordignon (ULBRA-RS-BRASIL) and voucher specimens were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (H. polyanthemum, Bordignon 1429). The vegetal harvest was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (Nº 03/2008).

The aerial parts of the plant (80 g) were dried and powdered in apropriated conditions, and extracted by maceration with *n*-hexane (3 x 24h at 20°C). The extract (1.8 g) was evaporated to dryness under reduced pressure.

2.2. Isolation procedures

The obtained extract was fractionated under silica gel column chromatography GF_{60} (Merck) using *n*-hexane-dichloromethane gradient system as the mobile phase, followed by isolation of the obtained enriched fractions on preparative-TLC performed on 20cm×20cm glass-supported plates covered with 0.5 mm layers of silica gel GF_{254} (Merck) . The chromatographic systems used were as follow: *n*-hexane:CH_2Cl_2 (1:1 v/v) for HP1, *n*-hexane:CH_2Cl_2 (3:2 v/v) for HP2, *n*-hexane:CH_2Cl_2 (4:1 v/v) for HP3, yielding 200 mg of

HP1, 100 mg of HP2 and 150 mg of HP3. Bands were detected using UV light (254 nm) and the compounds were identified by ^{1}H RMN and ^{13}C RMN. The spectral data are described elsewhere (Ferraz at al., 2001).

2.3. Preparation of ticks

Engorged females of *Rhipicephalus* (*Boophilus*) *microplus* were collected from infested cattle, washed with water and dried in paper toweling. The average weight of engorging ticks was 0.30 g. These females were incubated at 27–28 °C and 70–80 % relative humidity for ca. 2 weeks until the egg laying. These eggs provided the larvae used for the larval immersion test (LIT).

2.4. Larvae Imersion Test (LIT)

The modified-LIT was conducted by placing approximately 200 embryonated eggs (0.01 g) into pockets made with pieces (3 cm X 3 cm) of TNT fabric. The pockets were incubated at 27–28 °C and 70–80% relative humidity for ca. 14 days, until the eggs started hatching. After another 14 days the pockets containing the larvae ready for the test were immersed for 5 min in 10-15 ml of the test solutions. Ethanol 96° was used as control. After ca. 1 h to allow the solvents to evaporate, the pockets were incubated at 27–28 °C and 70–80% relative humidity for 48 h and then larvae (alive and dead) were counted to assess percent mortality. Each treatment contained three replicates.

2.5. Statistical analysis

The data were evaluated using ANOVA one-way followed by Holm-Sidak's Method. All results were expressed in mean ± S.E.M. The analyses were performed using Sigma Stat 2.03 software (Jandel Scientific Corporation). Differences were considered statistically significant at $p<0.05$, compared to control group.

3. Results and discussion

Results obtained with the larvae of *R.* (*B.*) *microplus* treated with the benzopyrans isolated from the lipophilic extract of the aerial parts of *H. polyanthemum* are shown in Table 1.

The larvicidal activity of the extracts of *H. polyanthemum* was attributed to the benzopyrans structurally related to precocenes found in the plant. As stated before, precocenes are specific chemical substances, which cause not only toxic effects but also disturb the development process and reproduction of the insects. The pronounced activity verified in the treatment with the benzopyrans reinforces this supposition. In fact, the isolated compounds demonstrated to be active in a concentration 10 times lower than the last concentrations of the *n*-hexane extract from *H. polyanthemum*.

Benzopyrans are known insecticides but their acaricidal activity has not been elucidated. Precocene activity has been demonstrated previously in ticks using engorged females. The essential oil of *Ageratum houstonianum* flowers showed an acaricidal effect on the tick *Rhipicephalus lunulatus*. The main components of this essential oil are the benzopyrans precocenes I and II. The toxicity of the oil to *R. lunulatus* was attributed to these two substances or to a synergistic interaction between these components and the other constituents of the essential oil (Pamo et al., 2004; 2005). When argasid ticks were treated with precocene, oviposition was disrupted (Pound and Oliver, 1979). Other studies suggested that precocene was detrimental to tick development and reproduction (Gaber et al., 1983; Khalil et al., 1983), although this may have represented sublethal toxicity rather than true hormonal activity (Dees et al., 1982).

The study conducted by Booth et al. (1986) demonstrated that the treatment of engorged females of *R.* (*B.*) *microplus* with precocene resulted in desiccated nonviable eggs due to the absence of a waterproofing wax layer. Electron microscopy showed that precocene had a destructive effect on the glandular cells of Gene's organ. The precocene also inhibited in vitro wax synthesis by the gland cells, indicating a selective cytotoxic effect. In an experiment with *Ornithodoros moubata* females treated with precocenes I and II oviposition was reduced in ticks that survived repeated treatment with high doses of precocene II (Taylor et al., 1992). The same compounds were applied topically to *R.* (*B.*)

microplus females and prevented oviposition in 20–50% of the treated females. The remaining females oviposited a reduced number of desiccated eggs (Connat, 1988). Ethoxy-precocene and analogues were applied topically to *O. moubata* females 1 day after feeding resulting in the rapid death of the females (Connat and Nepa, 1990).

Although the precocenes have their insecticidal effect attributed to antijuvenile hormonal activity, the results obtained in this work cannot be explained by this mechanism since the experiments were performed with tick larvae and adult. Thus, the effect on the moulting was not investigated.

4. Conclusions

The isolated compounds showed to be, at least in part, responsible for the presented toxicity of the extracts of *H. polyanthemum*, being the benzopyran HP1 slightly more toxic among the three compounds tested.

Acknowledgments

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References

Bernardi, A.P.M., Maurmann, N., Rech, S., von Poser, G.L., 2007. Benzopyrans in *Hypericum polyanthemum* Klotzsch ex Reichardt cultured in vitro. *Acta Physiol Plant.* 29, 165-170.

Booth, T.F., Beadle, D.J., Hart, R.J., 1986. The effects of precocene treatment on egg wax production in Gene's organ and egg viability in the cattle tick *Boophilus microplus* (Acarina Ixodidae): an ultrastructural study. Exp. Appl. Acarol. 2, 187–198.

Bowers, W.S., Ohta, T., Cleere, J.S., Marsella, P.A., 1976. Discovery of insect anti-juvenile hormones in plants. *Science* 193, 542–547.

Connat, J.L., 1988. Effects of different anti-juvenile hormone agents on the fecundity of the female cattle tick *Boophilus microplus*. *Pesticide Biochem. Physiol*. 30, 28–34.

Connat, J.-L., Nepa, M.-C., 1990. Effects of different anti-juvenile hormone agents on the fecundity of the female tick *Ornithodoros moubata. Pesticide Biochem. Physiol*. 37, 266–274.

Dees, W.H., Sonenshine, D.E., Briedling, E., Buford, N.P., Khalil, G.M., 1982. Toxicity of precocene-2 for the American dog tick, *Dermacentor variabilis* (Say). *J. Med. Entomol*. 19, 734–742.

Ferraz, A.B.F., Bordignon, S.A.L., Staats, C., Schripsema, J., von Poser, G.L., 2001. Benzopyrans from *Hypericum polyanthemum*. *Phytochemistry* 57, 1227-1230.

Ferraz, A.B., Grivicich, I., von Poser, G.L., Faria, D.H., Kayser, G.B., Schwartsmann, G., Henriques, A.T., da Rocha, A.B., 2005. Antitumor activity of three benzopyrans isolated from *Hypericum polyanthemum*. *Fitoterapia.* 76, 210-215.

Ferraz, A.B.F., da Silva, J., Deimlimg, L.I., Santos-Mello, R., Sharlau, A., von Poser, G.L., Picada, J.N., 2009. Genotoxicity Evaluation of Three Benzopyrans from *Hypericum polyanthemum. Planta Med.* 75, 37–40.

Gaber, S.H.A., Khalil, G.M., Sonenshine, D.E., Moez, M.A., 1983. Precocene-2 effects on the camel tick, *Hyalomma dromedary*. I. Adult responses. *J. Med. Entomol*. 20, 534–540.

Khalil, G.M., Gaber, S.H.A., Sonenshine, D.E., Gad, S.M., 1983. Precocene-2 effects on the camel tick *Hylaomma dromedarii.* 2. Larval and nymphal responses. *J. Med. Entomol*. 20, 541–550.

Pamo, T.E., Tendonkeng, F., Kana, J.R., Tenekeu, G., Tapondjou, L.A., Khan Payne, V., 2004. The acaricidal effect of the essential oil of *Ageratum houstonianum* Mill. flowers on ticks (*Rhipicephalus lunulatus*) in Cameroon. *S. Afr. J. Anim. Sci*. 34, 244–247.

Pamo, T.E., Tendonkeng, F., Kana, J.R., Khan Payne, V., Boukila, B., Lemoufouet, J., Miegoue, E., Nanda, A.S., 2005. A study of the acaricidal properties of an essential oil extracted from the leaves of *Ageratum houstonianum. Vet. Parasitol*. 128, 319–323.

Pound, J.M., Oliver Jr., J.H., 1979. Juvenile hormone: evidence of its role in the reproduction of ticks. *Science* 206, 355–357.

Ribeiro, V.L.S., Toigo, E., Bordignon, S.A.L., Gonçalves, K., von Poser, G., 2007. Acaricidal properties of extracts from the aerial parts of *Hypericum polyanthemum* on the cattle tick *Boophilus microplus. Vet Parasitol.* 147, 199–203.

Ribeiro, V.L.S., Avancini, C., Gonçalves, K., Toigo, E., von Poser, G.L., 2008. Acaricidal activity of *Calea serrata* (Asteraceae) on *Boophilus microplus* and *Rhipicephalus sanguineus*. *Vet Parasitol.* 151, 351-354.

Steinbeck, C., Spitzer, V., Starosta, M., von Poser, G., 1997. Identification of two chromenes from *Calea serrata* by semiautomatic structure elucidation. *J. Nat. Prod.* 60, 627-628.

Taylor, D., Chinzei, Y., Miura, K., Ando, K., 1992. Effects of precocenes on vitellogenesis in the adult female tick, *Ornithodoros moubata* (Acari: Argasidae). *Exp. Appl. Acarol*. 14, 123–136.

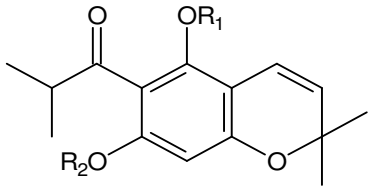
Viana, A.F., Heckler, A.P., Fenner, R., Rates, S.M.K., 2001. Antinociceptive activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae). *Braz J Med Biol Res.* 36, 631-634.

Table 1: Toxic effect of the benzopyrans HP1, HP2 and HP3 on the larvae of *R*. (*B*.) *microplus* (percentage %):

	Ethanol 96°	**0.625 mg/mL**	**1.250 mg/mL**	**2.500 mg/mL**	**5.000 mg/mL**
HP1		94.00 ± 3.05***	70.00 ± 5.77***	89,67 ± 3.28***	97.00 ± 1.00***
HP2		81.67 ± 7.26***	85.00 ± 2.88***	93.33 ± 6.67***	97.67 ± 1.45***
HP3		36.67 ± 8.82*	46.67 ± 8.82**	11.67 ± 1.67*	71.67 ± 7.26***
Control	3.00 ± 1.00				

Data are presented as mean ± SEM (three replicates). Significantly different values were detected by ANOVA one-way followed by *post hoc* Holm-Sidak's Method: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, as compared to their control group.

Figure 1: Benzopyrans from *Hypericum polyanthemum*:



HP1 R1=Me R2=Me
HP2 R1=Me R2=H
HP3 R1=H R2=Me

4. DISCUSSÃO GERAL

Os estudos realizados com espécies de *Hypericum* nativas do sul do Brasil reforçam o potencial do gênero tanto do ponto de vista químico quanto farmacológico. Espécies de *Hypericum* de todo o mundo se destacam pela presença de constituintes químicos estruturalmente diferenciados, dotados de pronunciadas ações terapêuticas. Visto isso, as informações obtidas nessa dissertação ressaltam a importância das espécies estudadas como fonte de moléculas base para o desenvolvimento de novos fármacos.

As espécies do gênero *Hypericum* estão distribuídas em regiões temperadas e subtropicais. Representantes do gênero são encontrados na Ásia, no Mediterrâneo, na Europa, nos Estados Unidos e no sul do Brasil. Várias espécies são utilizadas na medicina popular. O exemplo mais difundido é o de *H. perforatum*, para o tratamento da depressão leve a moderada. As atividades farmacológicas de *H. perforatum*, relatadas em grande número de trabalhos científicos, têm motivado a pesquisa de espécies nativas do Rio Grande do Sul. Os efeitos farmacológicos da planta européia são atribuídos a um conjunto de compostos encontrados na fração apolar das partes aéreas do vegetal, como às naftodiantronas hipericina e *pseudo*-hipericina, aos derivados de floroglucinol hiperforina e *ad*-hiperforina, e a compostos polares, como os flavonóides hiperosídeo, rutina, isoquercitrina e miquelianina (BUTTERWECK e SCHMIDT, 2007).

Espécies de outras regiões, como *Hypericum glandulosum, H. grandifolium, H. reflexum*, *H. canariense* das Ilhas Canárias (Espanha), mostraram atividade do tipo antidepressiva (SÁNCHEZ-MATEO *et al.,* 2005; 2007; 2009) e analgésica (SÁNCHEZ-MATEO *et al.,* 2006). O extrato metanólico de *H. empetrifolium,* encontrado no Mediterrâneo, demonstrou atividade antiinflamatória e analgésica em roedores (TROVATO *et al.,* 2001). No sul do país, as espécies *H. caprifoliatum* e *H. polyanthemum* apresentaram atividade antinociceptiva (VIANA *et al.,* 2003), enquanto *H. caprifoliatum* mostrou atividade do tipo antidepressiva para os extratos ciclo-hexano e metanol (DAUDT *et al.,* 2000; VIANA *et al.,* 2005).

Algumas características como distribuição geográfica, mecanismos de polinização e a presença de glândulas pardas e negras diferenciam as espécies de *Hypericum,* sendo utilizadas para classificá-las em diferentes seções. As espécies de *Hypericum* nativas do sul do Brasil de espécies são caracterizadas pela ausência de glândulas negras. Nas glândulas negras, é observada grande quantidade de hipericina e *pseudo*-hipericina. Já nas glândulas pardas, é observada a presença de taninos, ceras e óleos essenciais (FERRAZ *et al.,* 2002a). Hipericina e derivados são relatados para espécies das seções *Hypericum, Drosacarpium, Oligostema, Taeniocarpium* e *Adenosepalum*. Para espécies do Rio Grande do Sul, pertencentes às seções *Brathys* e *Trigynobrathys*, foi caracterizada a ausência de naftodiantronas, assim como a ausência de glândulas negras, nas espécies *H. brasiliense, H. caprifoliatum, H. carinatum, H. connatum, H. cordatum, H. myrianthum, H. piriai* e *H. polyanthemum* (FERRAZ *et al.,* 2002b).

Em estudo realizado por SMELCEROVIC e colaboradores (2007), a determinação da composição dos óleos essenciais em 9 espécies de *Hypericum* nativas da Sérvia (*H. barbatum, H. hirsutum, H. linarioides, H. maculatum, H. olympicum, H. perforatum, H. richeri, H. rumeliacum e H. tetrapterum*) revelou a presença de 98 substâncias nos óleos das plantas. Foi identificada a presença não-terpenos, como ácidos carboxílicos e hidrocarbonetos, monoterpenos e sesquiterpenos. *Hypericum barbatum, H. richeri, H. rumeliacum* (seção *Drosocarpium*) apresentaram alta quantidade de ácidos graxos, enquanto a constituição de não-terpenos e sesquiterpenos de espécies da seção *Hypericum* (*H. maculatum*, *H. perforatum* e *H. tetrapterum*) foi semelhante. Em espécies nativas do Rio Grande do Sul, foram encontrados óleos voláteis em pequenas concentrações (FERRAZ *et al*., 2002a).

Uma característica freqüente em espécies de *Hypericum* é a presença de compostos fenólicos. Nas espécies do sul do país, esses compostos apresentam a presença de prenilas, importantes dos pontos de vista quimiotaxonômico e biológico (BOTTA *et al*., 2005; DO REGO *et al.,* 2007; VIANA *et al.,* 2008). As prenilas

podem se apresentar na forma aberta ou ciclizada, ligando-se à hidroxila fenólica adjacente, levando à formação do derivado benzopirânico correspondente. Esse padrão estrutural é encontrado em grande parte dos compostos lipofílicos obtidos das espécies nativas. Como exemplos de derivados benzopirânicos podem ser citados os benzopiranos isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano (HP1), 7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano (HP2) e 5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano (HP3), isolados de *H. polyanthemum* (FERRAZ *et al*., 2001); os derivados de floroglucinol hiperbrasilol B e uliginosina B, encontrados em diferentes espécies (FERRAZ *et al.,* 2002a; NÖR *et al.,* 2004; 2008); as benzofenonas carifenona A e carifenona B, de *H. carinatum* (BERNARDI *et al.,* 2005); e a xantona 6-desoxijacareubina, relatada pela primeira vez para *H. polyanthemum* no presente trabalho.

O benzopirano HP1 mostrou atividade antinociceptiva em diferentes modelos animais. Esse efeito foi bloqueado pela pré-administração de naloxona. O derivado de floroglucinol uliginosina B também vem sendo estudado pelo nosso grupo por suas propriedades antinociceptivas. Este composto apresentou efeito mediado pela neurotransmissão dopaminérgica (STOLZ *et al.,* 2008). Essas estruturas podem representar novas entidades com propriedades farmacológicas. Apesar da comum presença do núcleo benzopirano nessas moléculas, as mesmas apresentam ação por mecanismos distintos.

Os derivados de floroglucinol diméricos, são biomarcadores das seções *Brathys* e *Trigynobrathys*. Os derivados de floroglucinol são divididos em dois grupos biogenéticos principais: os monoméricos e os diméricos. Estruturalmente, os derivados diméricos são constituídos por uma porção floroglucinol e uma porção ácido filicínico. Em espécies dessas seções, como *H. ulignosum, H. japonicum, H. drummondii, H. brasiliense, H. myrianthum, H. carinatum, H. polyanthemum, H. caprifoliatum* e *H. connatum*, cerca de 25 compostos diméricos foram identificados (NÖR *et al*., 2004). Algumas dessas moléculas mostraram atividade antimicrobiana (ROCHA *et al*., 1996; DALL'AGNOL *et al.,* 2005), além de

serem, pelo menos em parte, responsáveis pela ação antinociceptiva e do tipo antidepressiva encontradas para *H. caprifoliatum* e *H. polyanthemum* em roedores (HECKLER *et al*., 2005; VIANA *et al*., 2005; STOLZ *et al*., 2008). Os derivados monoméricos podem apresentar grupamento aromático ou não, e são encontrados em *H. perforatum.* Os acilfloroglucinóis hiperforina e *ad*-hiperforina, caracterizados pela presença de prenilas em sua estrutura, são exemplos.

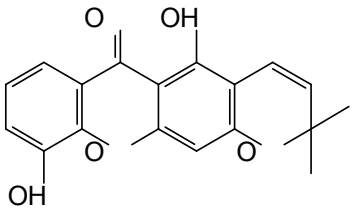
Devido à distribuição altamente restrita e ao alto potencial terapêutico de *H. polyanthemum*, protocolos *in vitro* (BERNARDI *et al.,* 2007a) e *ex vitro* (BERNARDI *et al*., 2008) foram desenvolvidos para seu cultivo. As técnicas de extração aplicadas aos materiais vegetais influenciam a qualidade dos extratos obtidos, afetando as concentrações encontradas dos componentes ativos, podendo alterar a eficácia e segurança de preparações vegetais (SCHILTER *et al*., 2003).

Algumas substâncias farmacologicamente ativas são sensíveis à temperatura, à luz e à oxidação, como o derivado de floroglucinol hiperforina, encontrado em *H. perforatum* (GLISIC *et al*., 2008). Os extratos obtidos por maceração utilizam evaporador rotatório sob pressão reduzida para remoção do solvente, usando banho-maria com temperatura em torno de 50 a 60 °C. São necessárias repetidas macerações (cerca de 3 por período de 24h), com repetidas evaporações. Esse processo expõe o material vegetal a possíveis processos de degradação pela exposição ao calor, aos solventes e à oxidação, podendo levar à perda de compostos bioativos de interesse (CARGNIN *et al.,* 2010).

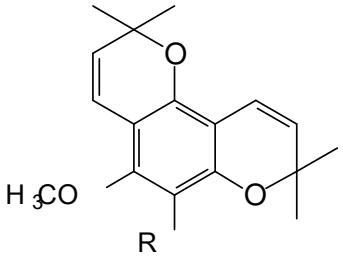
A extração por fluido supercrítico (EFS CO_2) é uma técnica usada para separação, com seletividade para compostos lipofílicos. O dióxido de carbono (CO_2), solvente usado, é não-tóxico e não-explosivo, apresenta baixo custo e pode facilmente ser removido do extrato. A EFS CO_2 apresenta propriedades como alta difusibilidade, baixa viscosidade e baixa tensão superficial, que conferem características atrativas para seu uso como extrator. Além disso, a EFS CO_2 melhora a seletividade e reduz a degradação de moléculas termolábeis (TAYLOR,

1996). A aplicação dessa técnica de extração objetiva reduzir o uso de solventes, o tempo de extração e aumentar a concentração de compostos bioativos, levando a um extrato sem resíduos (HERRERO *et al.,* 2010).

Em estudo recente, a EFS CO_2 mostrou-se vantajosa para a extração de componentes bioativos de *H. polyanthemum* (CARGNIN *et al.,* 2010). No presente trabalho, utilizou-se fluido supercrítico para extração dos compostos lipofílicos das partes aéreas de *H. polyanthemum*. Através da utilização de técnicas cromatográficas, foram isolados e identificados os benzopiranos já conhecidos para a espécie HP1, HP2 e HP3, assim como o derivado de floroglucinol uliginosina B. Foram também isolados dois compostos ainda não relatados para a planta: uma xantona, identificada como 6-desoxijacareubina (**12**) e um derivado benzopirânico (**13**). Ambos os compostos apresentam prenila ciclizada, o que é evidenciado no espectro de RMN pela presença de dubletos na região de δ 5,5 e 6,5 ppm. Ainda caracterizando este núcleo, verifica-se, nas duas substâncias, a presença de sinais referentes às metilas geminais na região de δ 1,50 ppm. O isolamento desses compostos, com núcleo benzopirano, reforça o valor quimiotaxonômico desse padrão de substituição.



(**12**)



(**13**)

Nos espectros do derivado de benzopirano verificou-se a presença de sinais referentes a dois anéis dimetilpirano. Verificou-se também a presença de uma metoxila, indicada pela presença de sinais de ^{1}H e ^{13}C a δ 3.66 ppm e δ 60.10 ppm, respectivamente. Um sinal simples em δ H 14.10 ppm revelou a presença de um grupamento hidroxila. No esqueleto proposto existem somente

duas posições no anel aromático com possibilidade de substituição. Considerando que os espectros apresentam sinais adicionais referentes a uma cadeia alquila, considerou-se que a hidroxila estaria fazendo parte de um grupamento ácido carboxílico. Assim, o conjunto de sinais permitiu propor a estrutura do composto como um derivado benzopirânico ainda não completamente elucidado, semelhante ao ácido eriostemóico, previamente isolado de *Eriostemon cymbiformis* e *E. wonganensis*, espécies pertencentes à família Rutaceae (SARKER *et al*., 1995). Essa família tem provado ser, juntamente com Asteraceae e Guttiferae, uma rica fonte de benzopiranos (PROKSCH e RODRIGUEZ, 1983; KAMPERDICK *et al*., 1997).

A estrutura da xantona 6-desoxijacareubina foi definida pelos sinais de δH que indicam a presença de dois conjuntos de hidrogênios caracterizando dois núcleos aromáticos, desblindados pela influência dos orbitais π. O sinal em δ 12,80 ppm sinaliza a presença de uma hidroxila quelada a uma carbonila adjacente. O δH na região de 7,8 ppm é característico de H-8, altamente desprotegido por ser vizinho à carbonila.

A presença de xantonas em espécies da família Guttiferae é amplamente difundida na literatura, embora ainda não houvesse sido relatada para *H. polyanthemum* (BENNETT e LEE, 1989; PERES e NAGEM, 1997; PERES *et al*., 2000). A xantona isolada no presente trabalho é derivada da xantona tetraoxigenada jacareubina, descrita na literatura pela primeira vez por KING e colaboradores (1953), isolada de *Calophyllum brasiliense*, nomeada de acordo com a árvore tropical de onde foi isolada, conhecida como “Santa Maria” ou “jacareuba” (PERES *et al.,* 2000). O derivado 6-desoxijacareubina havia sido descrito para *H. brasiliense*, sendo este o primeiro relato do composto para o gênero *Hypericum*. No mesmo trabalho, o derivado tetracíclico demonstrou atividade inibitória considerável contra MAO-A e MAO-B, além de atividade antigúngica frente a *Cladosporium cucumerinum* (ROCHA *et al*., 1994). A propriedade inibidora da MAO está envolvida com o mecanismo de ação de alguns antidepressivos, descrita para

extratos de espécies nativas *Hypericum* e para *H. perforatum* (GNERRE *et al.,* 2001; PRENNER *et al.,* 2007; SARRIS e KAVANAGH, 2009).

Outros compostos da classe mostraram importantes propriedades biológicas, como atividades antifúngica contra *Aspergillus fumigatus* e *Candida albicans* (MOREL *et al*., 2002), antibacteriana contra bactérias Gram-positivas e Gram-negativas, antileishmania (AZEBAZE *et al*., 2008; MKOUNGA *et al.*, 2009), supressora do crescimento tumoral e de metástase em modelo de câncer mamário em animais, e efeito citotóxico sobre linhagens de células cancerosas humanas (DOI *et al*., 2009; TANAKA *et al.,* 2009).

Hiperosídeo (quercetina-3-*O*-galactosídeo), outra substância isolada nesse trabalho, é encontrado com abundância em espécies nativas do Rio Grande do Sul. Primeiramente, o composto foi identificado em *H. myrianthum, H. carinatum, H. caprifoliatum, H. ternum,* e *H. polyanthemum* através de comparação com amostra conhecida utilizando CCD (DALL'AGNOL *et al*., 2003). No presente trabalho, a identidade química do flavonóide foi confirmada através de técnicas espectroscópicas de RMN de ^{1}H e ^{13}C, e análise de correlação bidimencional (HMBC e HMQC), comparando os dados espectroscópicos obtidos com dados publicados na literatura. O composto foi isolado a partir da fração acetato de etila do extrato metanólico, obtido através de maceração estática (partição água: acetato de etila).

As propriedades antioxidantes demonstradas por extratos de *H. perforatum* podem ser em parte responsáveis por alguns efeitos terapêuticos nos quais a geração de radicais livres está implicada, tais como as propriedades antiinflamatórias e a ação no sistema nervoso central (SILVA *et al*., 2005). Vários trabalhos demonstram a capacidade de diferentes extratos desta espécie em seqüestrar radicais livres. Esta mesma ação foi verificada para extratos enriquecidos em flavonóides (ZOU *et al*., 2004; SILVA *et al*., 2005; SILVA *et al*., 2008), bem como para flavonóides isolados desta e de outras espécies do gênero,

que apresentaram atividade de forma dose-dependente (ZOU *et al.,* 2004; BERNARDI *et al.,* 2007b; PIAO *et al.,* 2008).

Hiperosídeo foi submetido a experimentos comportamentais em ratos e camundongos, com diferentes doses e vias de administração, já que os dados farmacocinéticos da molécula são escassos e provêm principalmente de ratos (JUERGENLIEMK *et al*., 2003; CHANG *et al*., 2005; ZUO *et al*., 2006). Esse flavonóide atinge o sistema nervoso central após administração oral em baixas proporções, enquanto seu principal metabólito, a miquelianina apresenta maior captação (JUERGENLIEMK *et al*., 2003). Outros trabalhos mostraram não foi possível detectar a presença de hiperosídeo em sua forma inalterada ou em sua forma conjugada no plasma após sua administração oral (6,0 mg/kg) (CHANG *et al*., 2005). Observou-se também que o composto tem baixa permeabilidade intestinal em modelos *in vitro* e *in situ* (ZUO *et al*., 2006).

O tratamento com hiperosídeo 20 e 40 mg/kg i.p., reduziu significativamente os parâmetros *de crossing, rearing e grooming* no teste de atividade locomotora espontânea. No teste de potenciação do sono barbitúrico, hiperosídeo aumentou o tempo de duração do sono (20 mg/kg i.p.), mas não a latência. Além disso, não foram observadas alterações no comportamento geral dos animais, perda de peso ou mortes, indicando que o composto não apresenta toxicidade aguda (10, 20 e 40 mg/kg i.p.).

Hiperosídeo é considerado um dos responsáveis pela atividade antidepressiva de *H. perforatum.* O efeito antiiimobilidade do composto foi demonstrado em ratos após administração aguda e repetida (BUTTERWECK *et al*., 2000). No presente trabalho, a atividade do tipo antidepressiva foi reproduzida em ratos (0,6 mg/kg v.o.) e demonstrada em camundongos (10 e 20 mg/kg i.p.). Curiosamente, esse efeito foi evidenciado também para o metabólito miquelianina por BUTTERWECK e colaboradores (2000), na dose de 0,6 mg/kg v.o.

Esses dados sugerem um efeito depressor do SNC e corroboram com a hipótese de que hiperosídeo apresenta propriedades antidepressivas, já que para ser considerado um potencial antidepressivo, um fármaco deve reduzir o tempo de imobilidade no teste da natação forçada em doses que não estimulem a locomoção. Esse resultado está de acordo também com estudos que mostram que os antidepressivos tendem a reduzir a atividade locomotora (PORSOLT *et al.,* 1978) e a apresentar efeitos sedativos (MAYERS e BALDWIN, 2005).

O efeito do tipo antidepressivo de hiperosídeo foi prevenido pela administração de sulpirida (50 mg/kg i.p.), enquanto a administração de sulpirida somente não alterou o tempo de imobilidade dos animais no nado forçado. Esse resultado indica que a ativação de receptores do tipo D2 está envolvida no efeito antiimobilidade do composto, sendo a primeira evidência do envolvimento da neurotransmissão dopaminérgica na atividade do tipo antidepressiva da molécula. Em outro estudo realizado por nosso grupo, o extrato ciclo-hexano de *H. caprifoliatum* e uma fração enriquecida em HC1, tiveram efeito antiimobilidade com maior impacto no sistema dopaminérgico, mostrado pela prevenção do efeito com a administração de sulpirida e SCH 23390, e inibição da recaptação sinaptossomal de dopamina ([^{3}H]-DA) (VIANA *et al.,* 2005).

Em estudo realizado por RYLSKI e colaboradores (1979), a atividade analgésica de hiperosídeo no modelo da placa quente foi relatada na faixa de 3,5 – 10 mg/kg i.p. No presente trabalho, não foi verificado efeito antinociceptivo para hiperosídeo no teste da placa quente (10 e 20 mg/kg i.p.). Além disso, não foi observada atividade no modelo de contorções induzidas por ácido acético (20 e 40 mg/kg v.o.). A diferença nos resultados obtidos pode ser justificada por variabilidade biológica e/ou interlaboratorial, ou ainda por uma curva dose-efeito em formato de sino. Assim, o efeito antinociceptivo de hiperosídeo não parece ser robusto o suficiente para ser reproduzido.

Benzopiranos representam uma classe de produtos naturais com propriedades biológicas interessantes. Em nosso grupo de pesquisa, as substâncias isoladas vêm despertando atenção nos diferentes ensaios realizados. Inicialmente, caracterizou-se o efeito inibitório de HP3 sobre as enzimas monoamino oxidase A e B em preparações de mitocôndrias de cérebro de ratos (GNERRE *et al.*, 2001). Dando continuidade, evidenciou-se o efeito antimicrobiano promissor de HP1 e HP3 (DALL'AGNOL *et al.*, 2005). Além disso, foi demonstrado que HP1, HP2 e HP3 apresentam ação antiproliferativa frente a HT-29 (células de carcinoma de cólon humano), H-460 (células de carcinoma de pulmão) e U-373 (glioma maligno humano) (FERRAZ *et al.*, 2005b; 2005c), assim como efeito citotóxico frente às células envolvidas no processo de angiogênese (NÖR, 2006). Recentemente, HP1, HP2 e HP3 apresentaram baixa propensão de induzirem genotoxicidade (FERRAZ *et al.,* 2009).

Diversas moléculas contendo o esqueleto benzopirano, tanto naturais quanto sintéticas, mostraram atividades farmacológicas importantes: tonabersat (SB-220453), um benzopirano com propriedades anticonvulcivantes, atenua reflexos neurovasculares induzidos no nervo trigêmeo (PARSONS *et al.,* 2001); compostos com a porção 7-hidroxi-2H-1-benzopirano-2-ona, 2,3-diarilbenzopirano, e derivados de benzopirano halogenados evidenciaram atividade antiinflamatória e antipirética através da inibição das ciclooxigenases (PRASANNA *et al.,* 2004; GIERSE *et al.,* 2008; EISSA *et al.,* 2009).

O extrato ciclo-hexano de *H. polyanthemum* reduziu significativamente o estímulo nociceptivo na dose de 180 mg/kg i.p. e v.o., efeito este mediado em parte pelo sistema opióide. No presente trabalho, o extrato ciclo-hexano mostrou efeito dose-dependente, atingindo maior efeito na dose de 90 mg/kg i.p. Os principais constituintes desse extrato, os benzopiranos HP1, HP2 e HP3 foram avaliados no modelo da placa quente. De todos os benzopiranos testados, HP1 (30 mg/kg i.p.) foi o único efetivo. Esse efeito foi dose-dependente, com atividade nas doses de 30 e 60 mg/kg i.p., mas sem efeito na dose de 90 mg/kg i.p.,

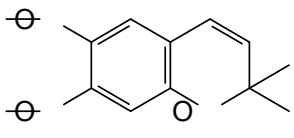
indicando curva dose-resposta em forma de “sino”. O pré-tratamento com naloxona aboliu o efeito de HP1 60 mg/kg i.p., indicando que a ação da substância é mediada pelo sistema opióide.

Vários estudos mostram que o sistema opióide pode estar relacionado com a ação antidepressiva de hiperforina e de extratos de *H. perforatum*, através da interação com o receptor sigma I (MENNINI e GOBBI, 2004). A administração de extrato CO_2 de *H. perforatum,* junto com a administração de baixas doses de antagonistas opióides, induz a redução do consumo voluntário de etanol em animais (PERFUMI *et al.*, 2005). Já CHEN *et al.* (2003), evidenciaram que a planta produz alterações significativas e região-específicas em receptores opióides μ na região mesolímbica em roedores.

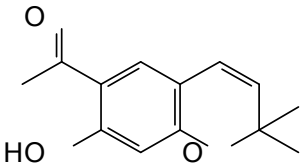
HP1 60 mg/kg v.o. inibiu as contorções induzidas por ácido acético em 58%. Avaliado no aparelho de rota-rod, HP1 não afetou o desempenho dos animais, sugerindo que na dose antinociceptiva esses não apresentam prejuízo motor ou neurotoxicidade. Assim, pode-se sugerir que o composto contribui para o efeito antinociceptivo de *H. polyanthemum* mediado pelo sistema opióide previamente descrito (VIANA *et al*., 2003).

Outros estudos revelam atividade inseticida, incluindo atividade acaricida para alguns benzopiranos. O óleo essencial das folhas de *Ageratum houstonianum* apresentou efeito tóxico sobre o carrapato *Rhipicephalus lunulatus*. Essa atividade pode ser atribuída à presença do cromeno precoceno I (7-metóxi-2,2-dimetilbenzopirano), ou à sua ação sinérgica com outras substâncias presentes no óleo vegetal (PAMO *et al*., 2005). Precocenos não somente provocam efeitos tóxicos, como promovem distúrbios no desenvolvimento e na reprodução de insetos. A ação anti-hormônio juvenil dos cromenos precoceno I e precoceno II (6,7-dimetóxi-2,2-dimetilbenzopirano) (**14**) é documentada (BOWERS *et al.,* 1976; PAMO *et al.,* 2004). Em ácaros, a atividade de precocenos não foi elucidada, embora experimentos conduzidos tenham mostrado que esses compostos interferem no desenvolvimento e na reprodução de carrapatos, reduzindo sua

oviposição. Cromenos como precoceno II e eupatoriocromeno (**15**) podem ser encontrados em *Calea serrata* (STEINBECK *et al.,* 1997)*,* espécie com atividade tóxica frente aos carrapatos *Rhipicephalus* (*Boophilus*) *microplus* e *Rhipicephalus sanguineus* (RIBEIRO *et al.,* 2008).



(**14**)



(**15**)

Os benzopiranos HP1, HP2 e HP3 encontrados em *H. polyanthemum* são estruturalmente semelhantes aos precocenos citados. Os extratos *n*-hexano e metanólico da espécie, ricos nesses compostos foram altamente tóxicos a larvas e fêmeas adultas de *Rhipicephalus* (*Boophilus*) *microplus* (RIBEIRO *et al.,* 2007).

No presente trabalho, os benzopiranos foram testados isoladamente frente a larvas do carrapato bovino*,* mostrando-se significativamente tóxicos nas concentrações testadas. O composto que provocou maior taxa de mortalidade foi HP1, com 97% de mortalidade na concentração de 5 mg/mL, 90% de mortalidade na concentração de 2,5 mg/mL, 70% de mortalidade na concentração de 1,25 mg/mL, e 94% de mortalidade na concentração de 0,625 mg/mL. Apesar de todas as concentrações terem se mostrado significativamente ativas, observou-se que a taxa de mortalidade não foi linear. É possível que o composto não tenha solubilizado completamente, ou que a solução não tenha entrado totalmente em contato com o ácaro. Em seqüência, HP2 mostrou-se significativamente tóxico após as 48h, atingindo taxa de mortalidade de 97% na maior concentração testada (5 mg/mL), 93% na concentração de 2,5 mg/mL, 85% na concentração de 1,25 mg/mL e 82% na menor concentração (0,625 mg/mL). HP3 teve letalidade de 72% na concentração de 5 mg/mL, com menor eficácia nas menores diluições.

A atividade evidenciada pelos extratos de *H. polyanthemum* parece estar relacionada com a presença dos benzopiranos testados, que mostraram alta toxicidade nos experimentos realizados. O mecanismo de ação dos extratos não está relacionado ao efeito do tipo hormônio antijuvenil, já que as amostras não foram avaliadas em fases de muda do carrapato (RIBEIRO *et al.,* 2007). Assim, os benzopiranos apresentam grande potencial acaricida a ser explorado, sendo importante que outros estudos sejam realizados buscando avaliar a atividade de soluções com menores diluições para definição da DL50 (dose letal 50%). É importante também que o mecanismo de ação desses cromenos seja investigado.

5. CONCLUSÕES

O extrato das partes aéreas de *Hypericum polyanthemum*, obtido por extração com fluido supercrítico, forneceu o derivado de floroglucinol uliginosina B e os benzopiranos HP1, HP2 e HP3 previamente isolados dessa espécie. Além desses, foram obtidos a xantona 6-desoxijacareubina e um benzopirano de estrutura similar a do ácido eriostemóico. Ambos os compostos estão presentes em quantidades minoritárias e não haviam sido relatados para esta planta.

Hiperosídeo, isolado de *H. caprifoliatum*, pode ser considerado um potencial antidepressivo, já que mostrou efeito do tipo antidepressivo em doses que não estimulam a locomoção. Esse efeito foi mediado pela neurotransmissão dopaminérgica, com ativação de receptores do tipo D2. No campo aberto e no teste de potenciação do sono barbitúrico, o composto apresentou atividade depressora central. Assim, hiperosídeo contribui para o efeito do tipo antidepressivo previamente descrito para o extrato metanólico de *H. caprifoliatum.*

O benzopirano HP1, isolado de *H. polyanthemum,* mostrou significativo efeito antinociceptivo. O composto contribuiu para a atividade do extrato ciclo-hexano da planta, mediado por mecanismo do tipo opióide. Assim, o esqueleto benzopirano identificado em espécies de *Hypericum* pode ser um alvo para a busca de outras moléculas com potencial antinociceptivo.

Os benzopiranos HP1, HP2 e HP3 foram altamente tóxicos frente a larvas de *Riphicephalus* (*Bophilus*) *microplus*, mostrando ser componentes ativos do extrato *n*-hexano de *H. polyanthemum.*

6. REFERÊNCIAS

AMARAL, A.C.F.; Kuster, R.M.; Gonçalves, J.L.S.; Wigg, M.D. Antiviral investigation on the flavonoids of *Chamaesyce thymifolia.* **Fitoterapia**, v. 10, p. 293-295, 1999.

AVATO, P.; Raffo, F.; Guglielmi, G.; Vitali, C.; Rosato A. Extracts from St John's Wort and their antimicrobial activity. **Phytotherapy Research***,* v. 18, p. 230–232, 2004.

AZEBAZE, A.G.B.; Ouahouo, B.M.W.; Vardamides, J.C.; Valentin, A.; Kuete, V.; Acebey, L.; Beng, V.P.; Nkengfack, A.E.; Meyer, M. Antimicrobial and antileishmanial xanthones from the stem bark of *Allanblackia gabonensis* (Guttiferae). **Natural Product Research**, v. 22, p. 333-341, 2008.

BALUNAS, M.J.; Kinghorn, A.D. Drug discovery from medicinal plants. **Life Sciences**, v. 78, p. 431-441, 2005.

BENNETT, G.J.; Lee, H.H. Xanthones from Guttiferae. **Phytochemistry**, v. 28, p. 967–998, 1989.

BERNARDI, A.P.M.; Ferraz, A.B.F.; Albring, D.V.; Bordignon, S.A.L.; Schripsema, J.; Bridi, R.; Dutra Filho, C.S.; Henriques, A.T.; von Poser, G. Benzophenones from *Hypericum carinatum*. **Journal of Natural Products**, v. 68, n. 5, p. 784-786, 2005.

BERNARDI, A.P.M.; Maurmann, N.; Rech, S.; von Poser, G.L. Benzopyrans in *Hypericum polyanthemum* Klotzsch ex Reichardt cultured in vitro. **Acta Physiologiae Plantarum***,* v. 29, p. 165-170, 2007a.

BERNARDI, A.P.M.; López-Alarcón, C.; Aspee, A.; Rech, S.; von Poser, G.L.; Bridi, R.; Lissp, E. Antioxidant activity of flavonoids isolated from *Hypericum ternum.* **Journal of the Chilean Chemical Society**, v. 52, p. 1326-1329, 2007b.

BERNARDI, A.P.; de Matos Nunes, J.; Marchioro, M.K.; Rosa, L.M.; von Poser, G.L.; Rech, S.B. Phenolic compounds profiles during ex vitro acclimatization of micropropagated *Hypericum polyanthemum*. **Plant Physiology and Biochemistry**, v. 46, p. 694-700, 2008.

BILIA, A.R.; Gallori, S.; Vincieri, F.F. St. John's wort and depression, efficacy, safety and tolerability - an update. **Life sciences**, v. 70, p. 3077-3096, 2002.

BIRT, D.F.; Widrlechner, M.P.; Hammer, K.D.P.; Hillwig, M.L.; Wei, J.; Kraus, G.A.; Murphy, P.A.; McCoy, J.; Wurtele, E.S.; Neighbors, J.D.; Wiemer, D.F.; Maury, W.J.; Price, J.P. *Hypericum* in infection: Identification of anti-viral and anti-inflammatory constituents. **Pharmaceutical Biology**, v. 47, p. 774–782, 2009.

BOTTA, B.; Delle Monache, G.; Menendez, P.; Boffi, A. Novel prenyltransferase enzymes as a tool for flavonoid prenylation. **Trends in Pharmacological Sciences***,* v. 26, p. 606-608, 2005.

BOWERS, W.S.; Ohta, T.; Cleere, J.S.; Marsella, P.A. Discovery of insect anti-juvenile hormones in plants. **Science**, v. 193, p. 542-547, 1976.

BUTTERWECK, V.; Jürgenliemk, G.; Nahrstedt, A.; Winterhoff, H. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. **Planta Medica**, v. 66, p. 3-6, 2000.

BUTTERWECK, V.; Christoffel, V.; Nahrstedt, A.; Petereit, F.; Spengler, B.; Winterhoff, H. Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. **Life Sciences**, v. 73, p. 627–639, 2003.

BUTTERWECK, V.; Schmidt, M. St. John's wort: Role of active compounds for its mechanism of action and efficacy. **Wiener Medizinische Wochenschrift**, v. 157, n. 13–14, p. 356–361, 2007.

CAKIR A, Mavi A, Yildirim A, Duru ME, Harmandar M, Kazaz C. Isolation and characterization of antioxidant phenolic compounds from the aerial parts of *Hypericum hyssopifolium* L. by activity-guided fractionation. **Journal of Ethnopharmacology**, v. 87, p. 73-83, 2003.

CARGNIN, S.T.; Nunes, J.M.; Haas, J.S.; Baladão, L.F.; Cassel, E.; Vargas, R.F.; Rech, S.B.; von Poser, G.L. Supercritical fluid extraction and high performance liquid chromatographic determination of benzopyrans and phloroglucinol derivative in *Hypericum polyanthemum*. **Journal of Chromatography B,** v. 878, p. 83–87, 2010.

CHANG, Q.; Zuo, Z.; Chow, M.S.; Ho, W.K. Difference in absorption of the two structurally similar flavonoid glycosides, hyperoside and isoquercitrin, in rats. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 59, p. 549-555, 2005.

CHEN, F.; Rezvani, A.H.; Lawrence, A.J. Autoradiographic quantification of neurochemical markers of serotonin, dopamine and opioid systems in rat brain mesolimbic regions following chronic St. John's wort treatment. **Naunyn Schmiedeberg's Archives of Pharmacology**, v. 367, p. 126-133, 2003.

COOK, N. C.; Samman, S. Flavonoids – Chemistry, metabolism, cardioprotective effects, and dietary sources. **Journal of Nutritional Biochemistry**, v. 7, p. 66-76, 1996.

DALL'AGNOL, R.; Ferraz, A.; Bernardi, A.P.; Albring, D.; Nör, C.; Sarmento, L.; Lamb, L.; Hass, M.; von Poser, G.; Schapoval, E.E.S. Antimicrobial activity of some *Hypericum* species. **Phytomedicine***,* v. 10, p. 511-516, 2003.

DALL'AGNOL, R.; Ferraz, A.; Bernardi, A.P.; Albring, D.; Nör, C.; Schapoval, E.E.S.; von Poser, G.L. Bioassay-guided isolation of antimicrobial benzopyrans and phloroglucinol derivatives from *Hypericum* species. **Phytotherapy Research**, v. 17, p. 291-293, 2005.

DAUDT, R.; von Poser, G.L.; Neves, G.; Rates, S.M.K. Screening for the antidepressant activity of some species of *Hypericum* from South Brazil. **Phytotherapy Research**, v. 15, p. 344-346, 2000.

DI CARLO, G.; Mascolo, N.; Izzo, A.A. Flavonoids: old and new aspects of a class of natural therapeutic drugs. **Life Sciences**, v. 65, p. 337-353, 1999.

DO REGO, J.C.; Benkiki, N.; Chosson, E.; Kabouche, Z.; Seguin, E.; Costentin, J. Antidepressant-like effect of hyperfoliatin, a polyisoprenylated phloroglucinol derivative from *Hypericum perfoliatum* (Clusiaceae) is associated with an inhibition of neuronal monoamines uptake. **European Journal of Pharmacology**, v. 569, p. 197-203, 2007.

DOI, H.; Shibata, M.A.; Morimoto, J.; Akao, Y.; Iinuma, M.; Tanigawa, N.; Otsuki, Y. Panaxanthone isolated from pericarp of *Garcinia mangostana* L. suppresses tumor growth and metastasis of a mouse model of mammary cancer. **Anticancer Research**, v. 29, p. 2485-2495, 2009.

DUGOUA, J.J.; Mills, E.; Perri, D.; Koren, G. Safety and efficacy of St. John's wort (*Hypericum*) during pregnancy and lactation. **The Canadian Journal of Clinical Pharmacology**, v. 13, p. 268-276, 2006.

EISSA, A.A.M.; Farag, N.A.H.; Soliman, G.A.H. Synthesis, biological evaluation and docking studies of novel benzopyranone congeners for their expected activity as anti-inflammatory, analgesic and antipyretic agents. **Bioorganic & Medicinal Chemistry**, v. 17, p. 5059–5070, 2009.

ERNST, E. Herbal remedies for anxiety – a systematic review of controlled clinical trials. **Phytomedicine**, v. 13, p. 205–208, 2006.

FENNER, R.; Sortino, M.; Rates, S.M.K.; Dall'Agnol, R.; Ferraz, A.; Bernardi, A.P.; Albring, D.; Nör, C.; von Poser, G.; Schapoval, E.; Zacchino, S. Antifungal activity of some Brazilian *Hypericum* species. **Phytomedicine***,* v. 12, p. 236-240, 2005.

FERNANDÉZ, S.; Wasowski, C.; Paladini, A.C.; Marder, M. Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. **European Journal of Pharmacology**, v. 512, p.189– 198, 2005.

FERRAZ, A.B.F.; Bordignon, S.A.L.; Staats, C.; Schripsema, J.; von Poser, G.L. Benzopyrans from *Hypericum polyanthemum*. **Phytochemistry**, v. 57, p. 1227-1230, 2001.

FERRAZ, A.B.F.; Schripsema, J.; Pohlmann, A.; von Poser, G.L. Uliginosin B from *Hypericum myrianthum*. **Biochemical Systematics and Ecology**, v. 30, p. 989-991, 2002a.

FERRAZ, A.; Bordignon, S.; Mans, D.R.A.; Schmitt, A.; Ravazzolo, A.P.; von Poser, G.L. Screening for the presence of hypericins in Southern Brazilian species of *Hypericum* (Guttiferae). **Pharmaceutical Biology**, v. 40, p. 294-297, 2002b.

FERRAZ, A.; Limberger, R.P.; Bordignon, S.A.L.; von Poser, G.L.; Henriques, A.T. Essential oil composition of six *Hypericum* species from southern Brazil. **Flavour and Fragrance Journal***,* v. 20, p. 335-339, 2005a.

FERRAZ, A.; Faria, D.H.; Benneti, M.N.; da Rocha, A.; Schwartsmann, G.; Henriques, A.; von Poser, G.L. *Screening* for antiproliferative activity of six southern Brazilian species of *Hypericum*. **Phytomedicine***,* v. 12, p.112-115, 2005b.

FERRAZ, A.; Grivicich, I.; von Poser, G.L.; Faria, D.H.; Kayser, G.B.; Schwartsmann, G, Henriques, A.T.; da Rocha, A.B. Antitumor activity of three benzopyrans isolated from *Hypericum polyanthemum*. **Fitoterapia***,* v. 76, p. 210-215, 2005c.

FERRAZ, A.B.F.; da Silva, J.; Deimlimg, L.I.; Santos-Mello, R.; Sharlau, A.; von Poser, G.L.; Picada, J.N. Genotoxicity evaluation of three benzopyrans from *Hypericum polyanthemum*. **Planta Medica**, v. 75, p. 37-40, 2009.

FRITZ, D.; Venturi, C.R.; Cargnin, S.; Schripsema, J.; Roehe, P.M.; Montanha, J.A.; von Poser, G.L. Herpes virus inhibitory substances from *Hypericum connatum* Lam., a plant used in southern Brazil to treat oral lesions. **Journal of Ethnopharmacology***,* v. 113, p. 517–520, 2007a.

FRITZ, D.; Bernardi, A.P.; Haas, J.S.; Ascoli, B.M.; Bordignon, S.; von Poser, G. Germination and growth inhibitory effects of *Hypericum myrianthum* and *H. polyanthemum* extracts on *Lactuca sativa* L. **Revista Brasileira de Farmacognosia**, v. 17, p. 44-48, 2007b.

GIERSE, J.; Nickols, M.; Leahy, K.; Warner, J.; Zhang, Y.; Cortes-Burgos, L.; Carter, J.; Seibert, K.; Masferrer, J. Evaluation of COX-1/COX-2 selectivity and potency of a new class of COX-2 inhibitors. **European Journal of Pharmacology***,* v. 588, p. 93–98, 2008.

GLISIC, S.; Smelcerovic, A.; Zuehlke, S.; Spiteller, M.; Skala, D. Extraction of hyperforin and adhyperforin from St. John's Wort (*Hypericum perforatum* L.) by supercritical carbon dioxide. **The Journal of Supercritical Fluids***,* v. 45, p. 332–337, 2008.

GNERRE, C.; von Poser, G.L.; Ferraz, A.; Viana, A.F.; Testa, B.; Rates, S.M.K. Monoamine oxidase inhibitory activity of some *Hypericum* species native to South Brazil. **Journal of Pharmacy and Pharmacology**, v. 53, p.1273-1279, 2001.

GRIVICICH, I.; Ferraz, A.; Faria, D.H.; Regner, A.; Schwartsmann, G.; Henriques, A.T.; von Poser, G.L.; da Rocha, A.B. Synergistic effect of three benzopyrans isolated from *Hypericum polyanthemum* in U-373 MG glioblastoma cell line. **Phytotherapy Research***,* v. 22, p. 1577-1580, 2008.

HARBORNE, J.B.; Williams, C.A. Advances in flavonoid research since 1992. **Phytochemistry**, v. 55, p. 481-504, 2000.

HAVSTEEN, B.H. The biochemistry and medical significance of the flavonoids. **Pharmacology & Therapeutics**, v. 96, p. 67–202, 2002.

HECKLER, A.P.M.; Viana, A.F.; Fenner, R.; Nör, C.; Kliemann, M.; do Rego, J.C.; Costentin, J.; von Poser, G.L.; Rates, S.M.K. **Atividade antinociceptiva de uliginosina isolada de *Hypericum polyanthemum* (Guttiferae)**. 20ª Reunião da Federação de Sociedades de Biologia Experimental, Águas de Lindóia, Brasil, 2005.

HERRERO, M.; Mendiola, J.A.; Cifuentes, A.; Ibáñe, A. Supercritical fluid extraction: Recent advances and applications. **Journal of Chromatography**, *in press*.

HOLLMAN, P.C.H.; KATAN, M.B. Absorption, metabolism, and health effects of dietary flavonoids in man. **Biomedicine & Pharmacotherapy**, v. 51, p. 305-310, 1997.

JUERGENLIEMK, G.; Boje, K.; Huewel, S.; Lohmann, C.; Galla, H.J.; Nahrstedt, A. In vitro studies indicate that miquelianin (quercetin 3-O-beta-D-glucuronopyranoside) is able to reach the CNS from the small intestine. **Planta Medica**, v. 69, p. 1013-1017, 2003.

KAMPERDICK, C.; Van, N.H.; Sung, T.V.; Adam, G. Benzopyrans from *Melicope ptelefolia* leaves. **Phytochemistry**, v. 45, p. 1049-1056, 1997.

KASPER, S.; Anghelescu, I.G.; Szegedi, A.; Dienel, A.; Kieser, M. Superior efficacy of St John's wort extract WS® 5570 compared to placebo in patients with major depression: a randomized, double-blind, placebo-controlled, multi-center trial [ISRCTN77277298]. **BMC Medicine**, v. 4, p. 1-13, 2006.

KASPER, S.; Gastpar, M.; Muller, W.E.; Volz, H.P.; Dienel, A.; Kieser, M.; Möller, H.J. Efficacy of St. John's wort extract WS® 5570 in acute treatment of mild depression. A reanalysis of data from controlled clinical trials. **European Archives of Psychiatry and Clinical Neuroscience**, v. 258, p. 59–63, 2008.

KING, F.E.; King, T.J.; Manning, L.C. The chemistry of extractives from hardwoods. Part XIV. The constitution of jacareubin, a pyranoxanthone from *Calophyllum brasiliense*. **Journal of the Chemical Society**, p. 3932-3937, 1953.

KINRYS, G.; Coleman, E.; Rothstein, E. Natural remedies for anxiety disorders: potential use and clinical applications. **Depression and Anxiety**, v. 26, p. 259-265, 2009.

LINDE, K.; Knüppel, L. Large-scale observational studies of hypericum extracts in patients with depressive disorders--a systematic review. **Phytomedicine***,* v. 12, p. 148-157, 2005.

LINDE, K.; Berner, M.M.; Kriston, L. St John's wort for major depression. **Cochrane Database of Systematic Reviews**, artigo n. CD000448, 2008.

MACHADO, D.G.; Bettio, L.E.B.; Cunha, M.P.; Santos, A.R.S.; Pizzolatti, M.G.; Brighente, I. M.C.; Rodrigues, A.L.S. Antidepressant-like effect of rutin isolated from the ethanolic extract from *Schinus molle* L. in mice: Evidence for the involvement of the serotonergic and noradrenergic systems. **European Journal of Pharmacology***,* v. 587, p. 163–168, 2008.

MARTARELLI, D.; Martarelli, B.; Pediconi, D.; Nabissi, M.I.; Perfumi, M.; Pompei, P. *Hypericum perforatum* methanolic extract inhibits growth of human prostatic carcinoma cell line orthotopically implanted in nude mice. **Cancer Letters**, v. 210, p. 27-33, 2004.

MAURY, W.; Price, J.P.; Brindley, M.A.; Oh, C.; Neighbors, J.D.; Wiemer, D.F.; Wills, N.; Carpenter, S.; Hauck, C.; Murphy, P.; Widrlechner, M.P.; Delate, K.; Kumar, G.; Kraus, G.A.; Rizshsky, L.; Nikolau, B. Identification of light-independent inhibition of human immunodeficiency virus-1 infection through bioguided fractionation of *Hypericum perforatum*. **Virology Journal**, v. 6, p. 101-113, 2009.

MAYERS, A.G.; Baldwin, D.A. Antidepressants and their effect on sleep. **Human Psychopharmacology: Clinical and Experimental**, v. 20, p. 533-559, 2005.

MENDES, F.R.; Mattei, R.; Carlini, E.L.A. Activity of *Hypericum brasiliense* and *Hypericum cordatum* on the central nervous system in rodents. **Fitoterapia**, v. 73, p. 462–471, 2002.

MENNINI, T.; Gobbi, M. The antidepressant mechanism of *Hypericum perforatum*. **Life Sciences***,* v. 75, p. 1021–1027, 2004.

MESSINA, B.A.M. Herbal Supplements: Facts and Myths—Talking to Your patients about herbal supplements. **Journal of PeriAnesthesia Nursing**, v. 21, p. 279-281, 2006.

MISCHOULON, D. Update and critique of natural remedies as antidepressant treatments. **Psychiatric Clinics of North America***,* v. 30, p. 51-68, 2007.

MISCHOULON, D. Update and critique of natural remedies as antidepressant treatments. **Obstetrics and Gynecology Clinics of North America**, v. 36, p. 789-807, 2009.

MKOUNGA, P.; Fomum, Z.T.; Meyer, M.; Bodo, B.; Nkengfack, A.E. Globulixanthone F, a new polyoxygenated xanthone with an isoprenoid group and two antimicrobial biflavonoids from the stem bark of *Symphonia globulifera*. **Natural Product Communications**, v. 4, p. 803-808, 2009.

MOREL, C.; Seráphin, D.; Teyrouz, A.; Larcher, G.; Bouchara, J.; Litaudon, M.; Richomme, P.; Bruneton, J. New and antifungal xanthones from *Calophyllum caledonicum.* **Planta Medica***,* v. 68, p. 41-44, 2002.

NÖR, C.; Albring, D.; Ferraz, A.B.F.; Schripsema, J.; Pires, V.; Sonnet, P.; Guillaume, D.; von Poser, G.L. Phloroglucinol derivatives from four *Hypericum* species belonging to the *Trigynobrathys* section. **Biochemical Systematics and Ecology**, v. 32, p.517-519, 2004.

NÖR, C. **Análise química e taxonômica de espécies de Hypericum e avaliação da atividade angiogênica***.* Dissertação (Mestrado) – Programa de Pós Graduação em Ciências Farmacêuticas, Porto Alegre: UFRGS, 2006, 186 p.

NÖR, C.; Bernardi, A.P.M.; Haas, J.S.; Schripsema, J.; Rech, S.B.; von Poser, G.L. Phenolic constituents of *Hypericum* flowers. **Natural Product Communications**, v. 3, p. 237-240, 2008.

PAMO, T.E.; Tendonkeng, F.; Kana, J.R.; Tenekeu, G.; Tapondjou, L.A.; Payne, V.K. The acaricidal effect of the essential oil of *Ageratum houstonianum* Mill. flowers on ticks (*Rhipicephalus lunulatus*) in Cameroon. **South African Journal of Animal Science**, v. 34, p. 244-247, 2004.

PAMO, E.T.; Tendonkeng, F.; Kana, J.R.; Payne, V.K.; Boukila, B.; Lemoufouet, J.; Miegoue, E.; Nanda, A.S. A study of the acaricidal properties of an essential oil extracted from the leaves of *Ageratum houstonianum.* **Veterinary Parasitology**, v. 128, p. 319–323, 2005.

PARSONS, A.A.; Bingham, S.; Raval, P.; Read, S.; Thompson, M.; Upton, N. Tonabersat (SB-220453) a novel benzopyran with anticonvulsant properties attenuates trigeminal nerve-induced neurovascular reflexes. **British Journal of Pharmacology**, v. 132, p. 1549-1557, 2001.

PERES, V.; Nagem, T.G. Trioxygenated naturally occurring xanthones. **Phytochemistry**, v. 44, p. 191-214, 1997.

PERES, V.; Nagem, T.G.; de Oliveira, F.F. Tetraoxygenated naturally occurring xanthones. **Phytochemistry**, v. 55, p. 683-710, 2000.

PERFUMI, M.; Mattioli, L.; Cucculelli, M.; Massi, M. Reduction of ethanol intake by chronic treatment with *Hypericum perforatum*, alone or combined with naltrexone in rats. **Journal of Psychopharmacology**, v. 19, p. 448-454, 2005.

PIAO, M.J.; Kang, K.A.; Zhang, R.; Ko, D.O.; Wang, Z.H.; You, H.J.; Kim, H.S.; Kim, J.S.; Kang, S.S.; Hyun, J.W. Hyperoside prevents oxidative damage induced by hydrogen peroxide in lung fibroblast cells via an antioxidant effect. **Biochimica et Biophysica Acta**, v. 1780, p. 1448–1457, 2008.

PORSOLT, R.D.; Le Pichon, M.; Jalfre, M. Depression: a new animal model sensitive to antidepressant treatments. **Nature**, v. 266, p. 730–732, 1978.

PRASANNA, S.; Manivannan, E.; Chaturvedi, S.C. Quantitative structure–activity relationship analysis of a series of 2,3-diaryl benzopyran analogues as novel selective cyclooxygenase-2 inhibitors. **Bioorganic & Medical Chemistry Letters**, v. 14, p. 4005–4011, 2004.

PRENNER, L.; Sieben, A.; Zeller, K.; Weiser, D.; Häberlein, H. Reduction of high-affinity β2-adrenergic receptor binding by hyperforin and hyperoside on rat C6 glioblastoma cells measured by fluorescence correlation spectroscopy. **Biochemistry**, v. 46, p. 5106-5113, 2007.

PROKSCH, P.; Rodriguez, E. Chromenes and benzofurans of the Asteraceae, their chemistry and biological significance. **Phytochemistry**, v. 22, p. 2335-2348, 1983.

QUEIROZ, E.F.; Wolfender, J.L.; Hostettmann, K. Modern approaches in the search for new lead antiparasitic compounds from higher plants. **Current Drug Targets**, v. 10, p. 202-211, 2009.

RABANAL, R.M.; Bonkanka, C.X.; Hernández-Pérez, M.; Sánchez-Mateo, C.C. Analgesic and topical anti-inflammatory activity of *Hypericum canariense* L. and *Hypericum glandulosum* Ait. **Journal of Ethnopharmacology**, v. 96, p. 591–596, 2005.

RAHIMI, R.; Nikfar, S.; Abdollahi, M. Efficacy and tolerability of *Hypericum perforatum* in major depressive disorder in comparison with selective serotonin reuptake inhibitors: a meta-analysis. **Progress in Neuro-psychopharmacology & Biological Psychiatry**, v. 33, p. 118-127, 2009.

RATES, S.M.K. Plants as source of drugs. **Toxicon**, v. 39, p. 603–613, 2001.

RIBEIRO, V.L.S.; Toigo, E.; Bordignon, S.A.L.; Gonçalves, K.; von Poser, G.L. Acaricidal properties of extracts from the aerial parts of *Hypericum polyanthemum* on the cattle tick *Boophilus microplus.* **Veterinary Parasitology***,* v. 147, p. 199-203, 2007.

RIBEIRO, V.L.S.; Avancini, C.; Gonçalves, K.; Toigo, E.; von Poser, G.L. Acaricidal activity of *Calea serrata* (Asteraceae) on *Boophilus microplus* and *Rhipicephalus sanguineus*. **Veterinary Parasitology**, v. 151, p. 351-354, 2008.

ROBSON, N.K.B. Studies in the genus *Hypericum* L. (Guttiferae) 2. **Bulletin of the British Museum**, v. 8, p. 55–226, 1981.

ROBSON, N.K.B. Studies in the genus *Hypericum* L. (Guttiferae) 8. Sections 29. Brathys (part 2) and 30. Trigynobrathys. **Bulletin of the British Museum**, v. 20, p. 1–151, 1990.

ROCHA, L.; Marston, A.; Kaplan, M.A.; Stoeckli-Evans, H.; Thull, U.; Testa, B.; Hostettmann, K. An antifungal gamma-pyrone and xanthones with monoamine oxidase inhibitory activity from *Hypericum brasiliense*. **Phytochemistry***,* v. 36, p. 1381-1385, 1994.

ROCHA, L.; Marston, A.; Potterat, O.; Kaplan, M.A.C.; Hostettmann, K. More phloroglucinols from *Hypericum brasiliense.* **Phytochemistry**, v. 42, p. 185-188, 1996.

RYLSKI, M.; Duriasz-Rowinka, H.; Rewerski, W. The analgesic action of some flavonoids in the hot plate test. **Acta Physiologica Polonica**, v. 30, p. 385-388, 1979.

SAMADI, S.; Khadivzadeh, T.; Emami, A.; Moosavi, N.S.; Tafaghodi, M.; Behnam, H.R. The effect of *Hypericum perforatum* on the wound healing and scar of cesarean. **The Journal of Alternative and Complementary Medicine**, v. 16, p. 113-117, 2010.

SÁNCHEZ-MATEO, C.C.; Bonkanka, C.X.; Prado, B.; Rabanal, R.M. Antidepressant properties of some *Hypericum canariense* L. and *Hypericum glandulosum* Ait. extracts in the forced swimming test in mice. **Journal of Ethnopharmacology**, v. 97, p. 541-547, 2005.

SÁNCHEZ-MATEO, C.C.; Bonkanka, C.X.; Hernández-Pérez, M.; Rabanal, R.M. Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. fil. **Journal of Ethnopharmacology**, v. 107, p. 1-6, 2006.

SÁNCHEZ-MATEO, C.C.; Bonkanka, C.X.; Prado, B.; Rabanal, R.M. Antidepressant activity of some *Hypericum reflexum* L. fil. extracts in the forced swimming test in mice. **Journal of Ethnopharmacology**, v. p. 115-121, 2007.
SÁNCHEZ-MATEO, C.C.; Bonkanka, C.X.; Rabanal, R.M. *Hypericum grandifolium* Choisy: A species native to Macaronesian Region with antidepressant effect. **Journal of Ethnopharmacology**, v. 121, p. 297–303, 2009.

SARKER, S.D.; Waterman, P.G. Coumarin glycosides from two species of *Eriostemon.* **Journal of Natural Products**, v. 58, p. 1109-1115, 1995.

SARRIS, J.; Kavanagh, D.J. Kava and St. John's Wort: Current evidence for use in mood and anxiety disorders. **The Journal of Alternative and Complementary Medicine**, v. 15, p. 827–836, 2009.

SCHILTER, B.; Andersson, C.; Anton, R.; Constable, A.; Kleiner, J.; O'Brien, J.; Renwick, A.G.; Korver, O.; Smit, F.; Walker, R.. Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. **Food and Chemical Toxicology**, v. 41, p. 1625-1649, 2003.

SILVA, B.A.; Ferreres, F.; Malva, J.O.; Dias, A.C.P. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. **Food Chemistry**, v. 90, n. 1-2, p. 157-167, 2005.

SILVA, B.A.; Malva, J.O.; Dias, A.C.P. St. John's Wort (*Hypericum perforatum*) extracts and isolated phenolic compounds are effective antioxidants in several *in vitro* models of oxidative stress. **Food Chemistry**, v. 110, p. 611–619, 2008.

SKALKOS, D.; Stavropoulos, N.E.; Tsimaris, I.; Gioti, E.; Stalikas, C.D.; Nseyo, U.O.; Ioachim, E.; Agnantis, N.J. The lipophilic extract of *Hypericum perforatum* exerts significant cytotoxic activity against T24 and NBT-II urinary bladder tumor cells. **Planta Medica**, v. 71, p. 1030-1035, 2005.

SMELCEROVIC, A.; Spiteller, M.; Ligon, A.P.; Smelcerovic, Z.; Raabe, N. Essential oil composition of *Hypericum* L. species from Southeastern Serbia and their chemotaxonomy. **Biochemical Systematics and Ecology**, v. 35, p. 99-113, 2007.

STEINBECK, C.; Spitzer, V.; Starosta, M.; von Poser, G. Identification of two chromenes from *Calea serrata* by semiautomatic structure elucidation. **Journal of Natural Products**, v. 60, p. 627-628, 1997.

STOLZ, E.D.; Haas, J.S.; Grazziotin, L.; Wild, L.; von Poser, G.L; Rates, S.M.K. **Uliginosina, um floroglucinol isolado de *Hypericum polyanthemum*, apresenta efeito antinociceptivo mediado pela neurotransmissão dopaminérgica.** V Oficina de Neurociências - Fronteiras da Investigação, Garibaldi, Brasil, 2008.

SÜNTAR, I.P.; Akkol, E.K.; Yılmazer, D.; Baykal, T.; Kırmızıbekmez, H.; Alper, M.; Yeşilada, E. Investigations on the in vivo wound healing potential of *Hypericum perforatum* L. **Journal of Ethnopharmacology**, v. 127, p. 468-477, 2010.

TAGBOTO, S.; Townson, S. Antiparasitic properties of medicinal plants and other naturally occurring products. **Advances in Parasitology**, v. 50, p. 199-295, 2001.

TANAKA, N; Kashiwada, Y.; Kim, S.; Sekiya, M.; Ikeshiro, Y.; Takaishi, Y. Xanthones from *Hypericum chinense* and their cytotoxicity evaluation. **Phytochemistry**, v. 70, p. 1456–1461, 2009.

TAYLOR, L.T. **Supercritical Fluid Extraction**. 1th Ed. New York: John Wiley & Sons Inc., 1996.

TROVATO, A.; Raneri, E.; Kouladis, M.; Tzakou, O.; Taviano, M.F.; Galati, E.M. Anti-inflammatory and analgesic activity of *Hypericum empetrifolium* Willd. (Guttiferae). **Il Farmaco**, v. 56, p. 455-457, 2001.

VIANA, A.F.; Hecler, A.P.; Fenner, R.; Rates, S.M.K. Antinociceptive activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae). **Brazilian Journal of Medical and Biological Research**, v. 36, p. 631-634, 2003.

VIANA, A.F.; Rego, J.C.; von Poser, G.; Ferraz, A.; Heckler, A.P.; Costentin, J.; Rates, S.M.K. The antidepressant like effect of *Hypericum caprifoliatum* Cham & Schlecht (Guttiferae) on forced swimming test results from an inhibition of neuronal monoamine uptake. **Neuropharmacology**, v. 49, p. 1042-1052, 2005.

VIANA, A.F.; do Rego, J.C.; Munari, L.; Dourmap, N.; Heckler, A.P.; Costa, T.D.; von Poser, G.L.; Costentin, J.; Rates, S.M.K. *Hypericum caprifoliatum* (Guttiferae) Cham. &

Schltdl.: a species native to South Brazil with antidepressant-like activity. **Fundamental & Clinical Pharmacology**, v. 20, p. 507–514, 2006.

VIANA, A.F.; Heckler, A.P.M.; von Poser, G.L.; Langeloh, A.; Rates, S.M.K. Efeito de *Hypericum caprifoliatum* Cham. & Schltdl. (Guttiferae) sobre contrações em íleo isolado de cobaio induzidas por diferentes agonistas. **Revista Brasileira de Farmacognosia**, v.17, p. 378-383, 2007.

VIANA, A.; Rates, S.; Naudin, B.; Janin, F.; Costentin, J.; do Rego, J.C. Effects of acute or 3-day treatments of *Hypericum caprifoliatum* Cham. & Schltdt. (Guttiferae) extract or of two established antidepressants on basal and stress-induced increase in serum and brain corticosterone levels. **Journal of Psychopharmacology**, v. 22, p. 681-690, 2008.

WHO. Depression. Disponível em http://www.who.int/topics/depression/en/. Visitado em dezembro de 2009.

ZOU, Y.; Lu, Y.; Wei, D. Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. in vitro. **Journal of Agricultural and Food Chemistry**, v. 52, p. 5032-5039, 2004.

ZUO, Z.; Zhang, L.; Zhou, L.; Chang, Q.; Chow, M. Intestinal absorption of hawthorn flavonoids – in vitro, in situ and in vivo correlations. **Life Sciences***,* v. 79, p. 2455–2462, 2006.

7. ANEXOS

Parecer do Comitê de Ética em Pesquisa da UFRGS:

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

pro pesq

PRÓ-REITORIA DE PESQUISA

COMITÊ DE ÉTICA EM PESQUISA

CARTA DE APROVAÇÃO

O Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul analisou o projeto:

Número : **2008008**

Título : **Isolamento e avaliação da atividade biológica de compostos fenólicos de espécies de Hypericum nativas do Rio Grande do Sul**

Pesquisador (es) :

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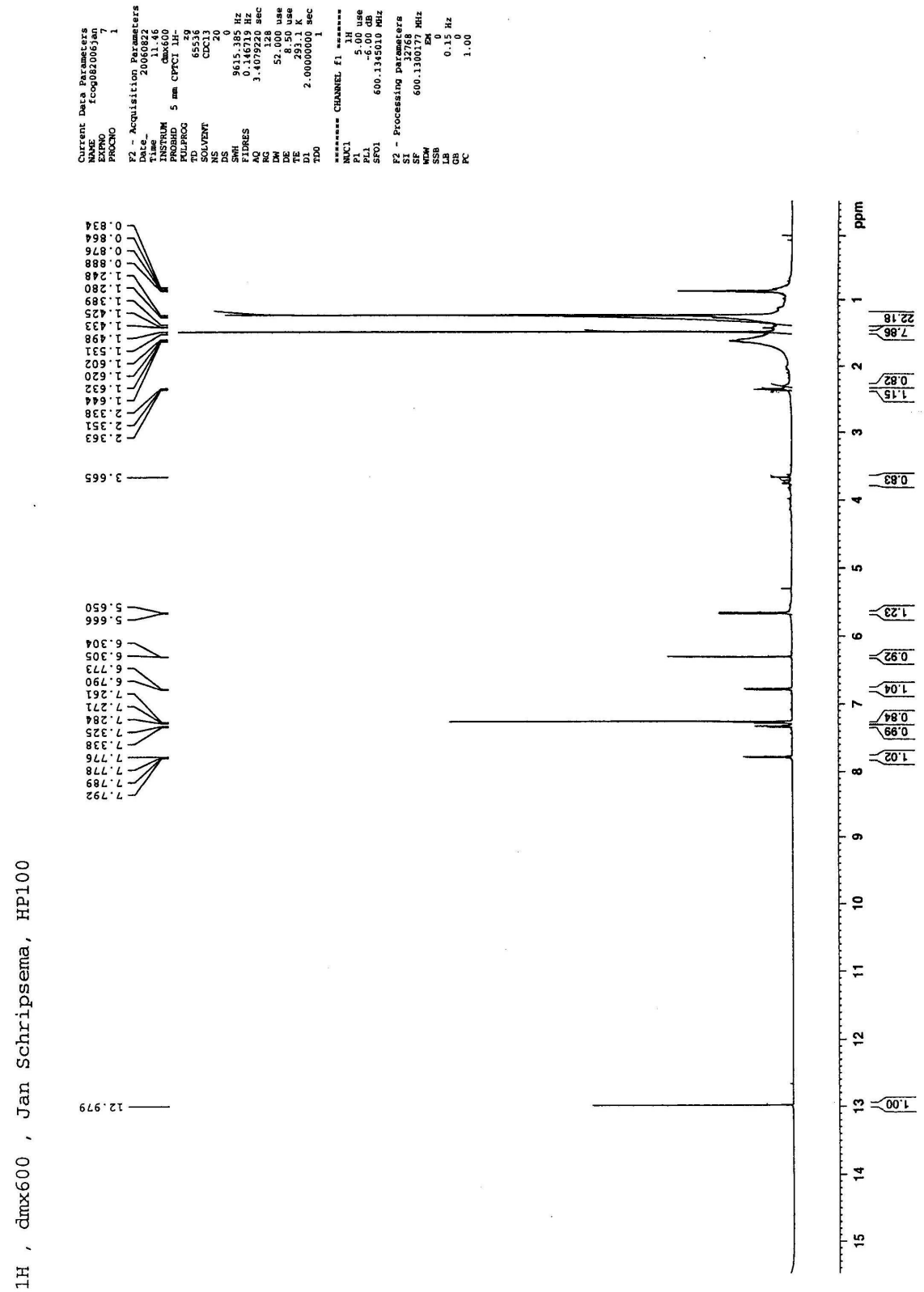
O mesmo foi aprovado pelo Comitê de Ética em Pesquisa da UFRGS, reunião nº 46 , ata nº 126 , de 19/3/2009 , por estar adequado ética e metodologicamente e de acordo com a Resolução 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, segunda-feira, 23 de março de 2009

ILMA SIMONI BRUM DA SILVA
Coordenador do CEP-UFRGS



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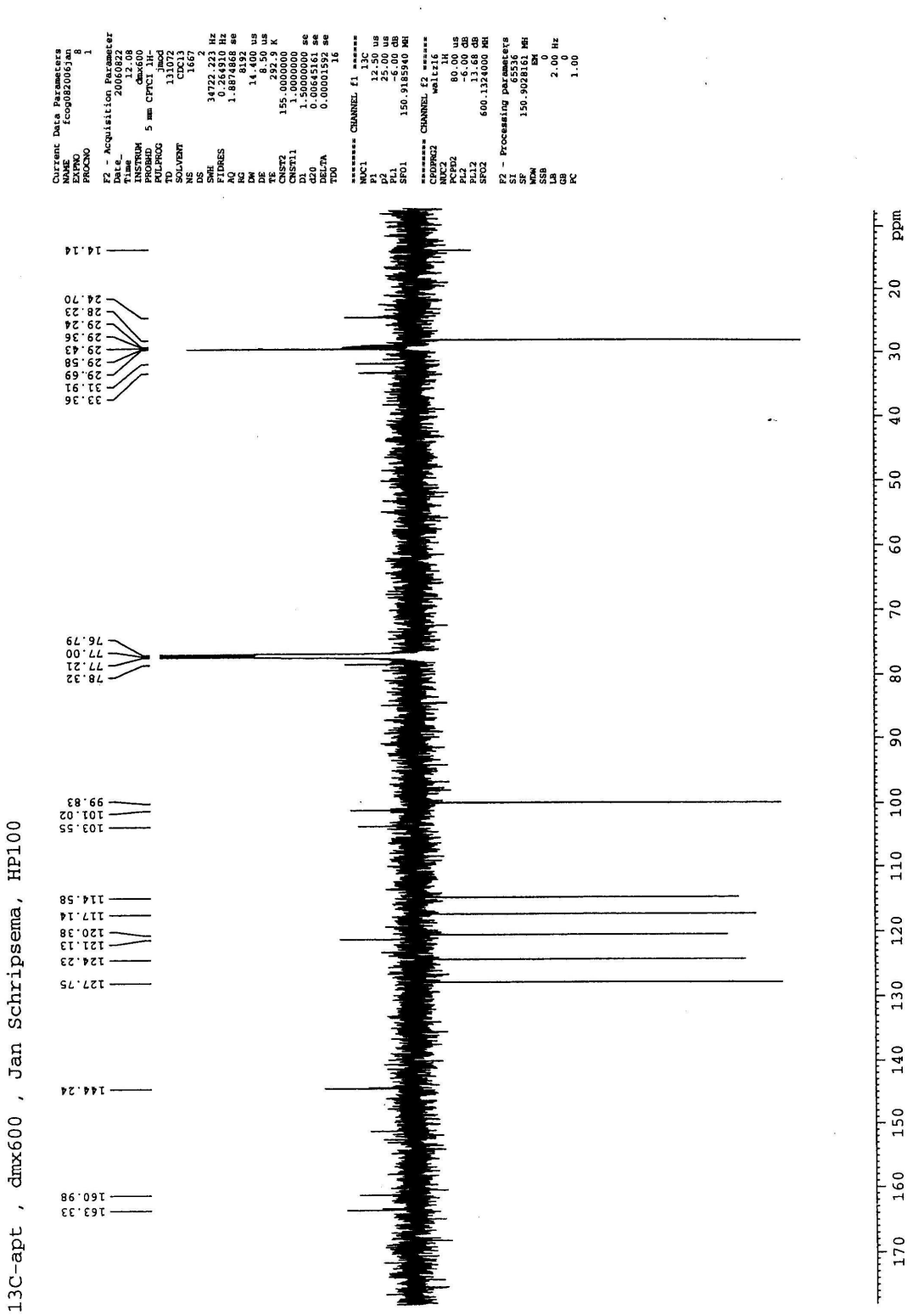

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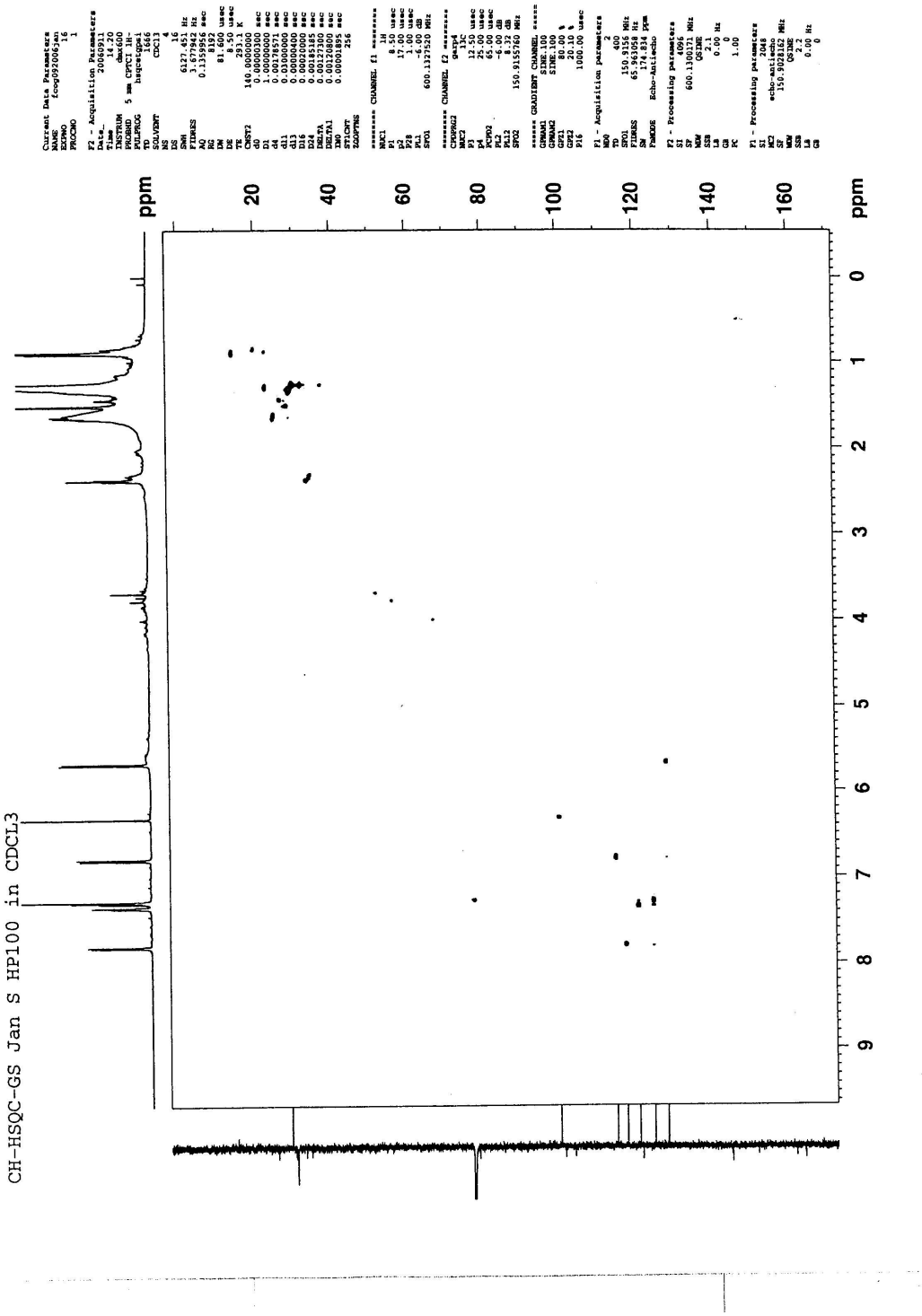

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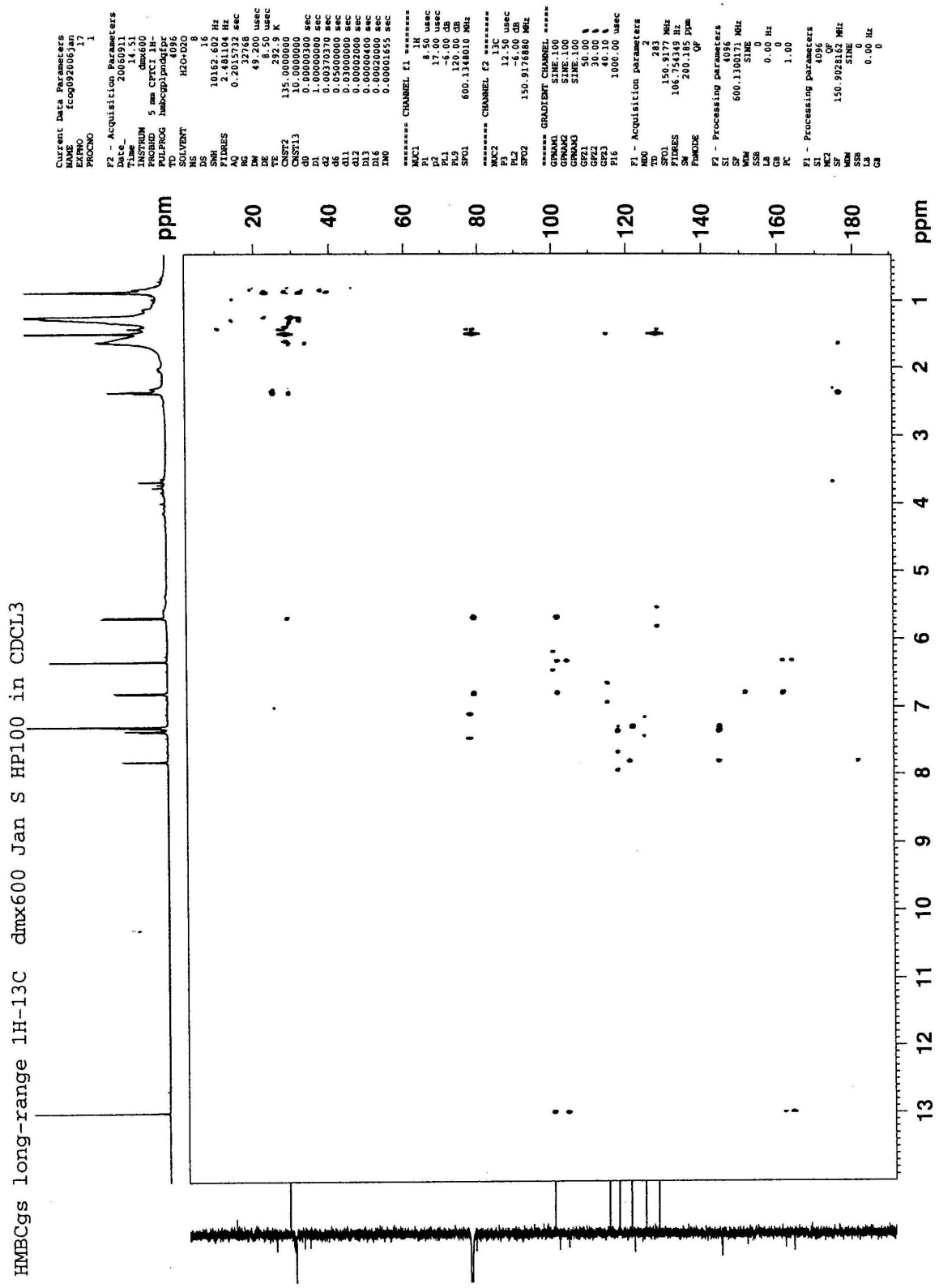

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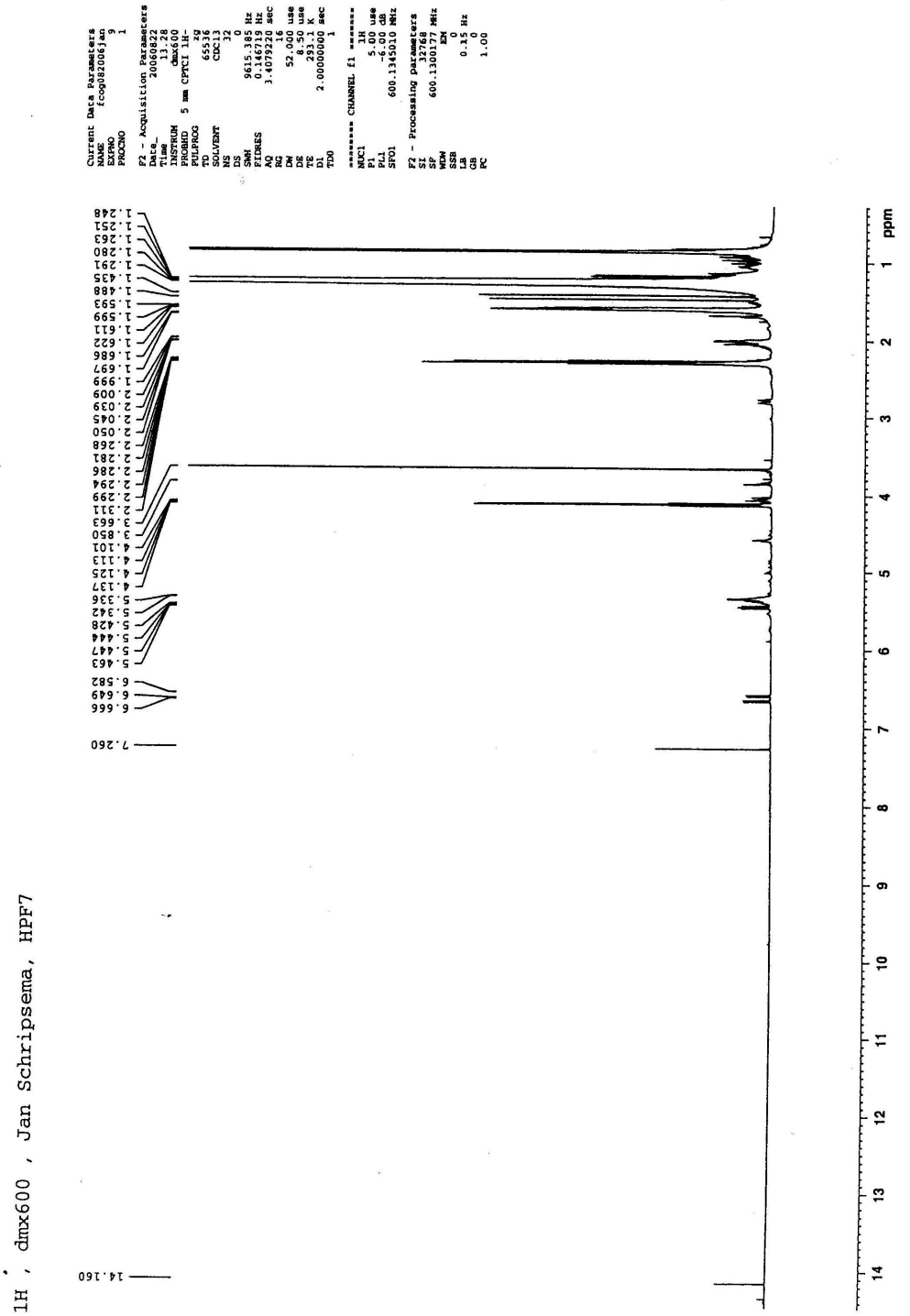

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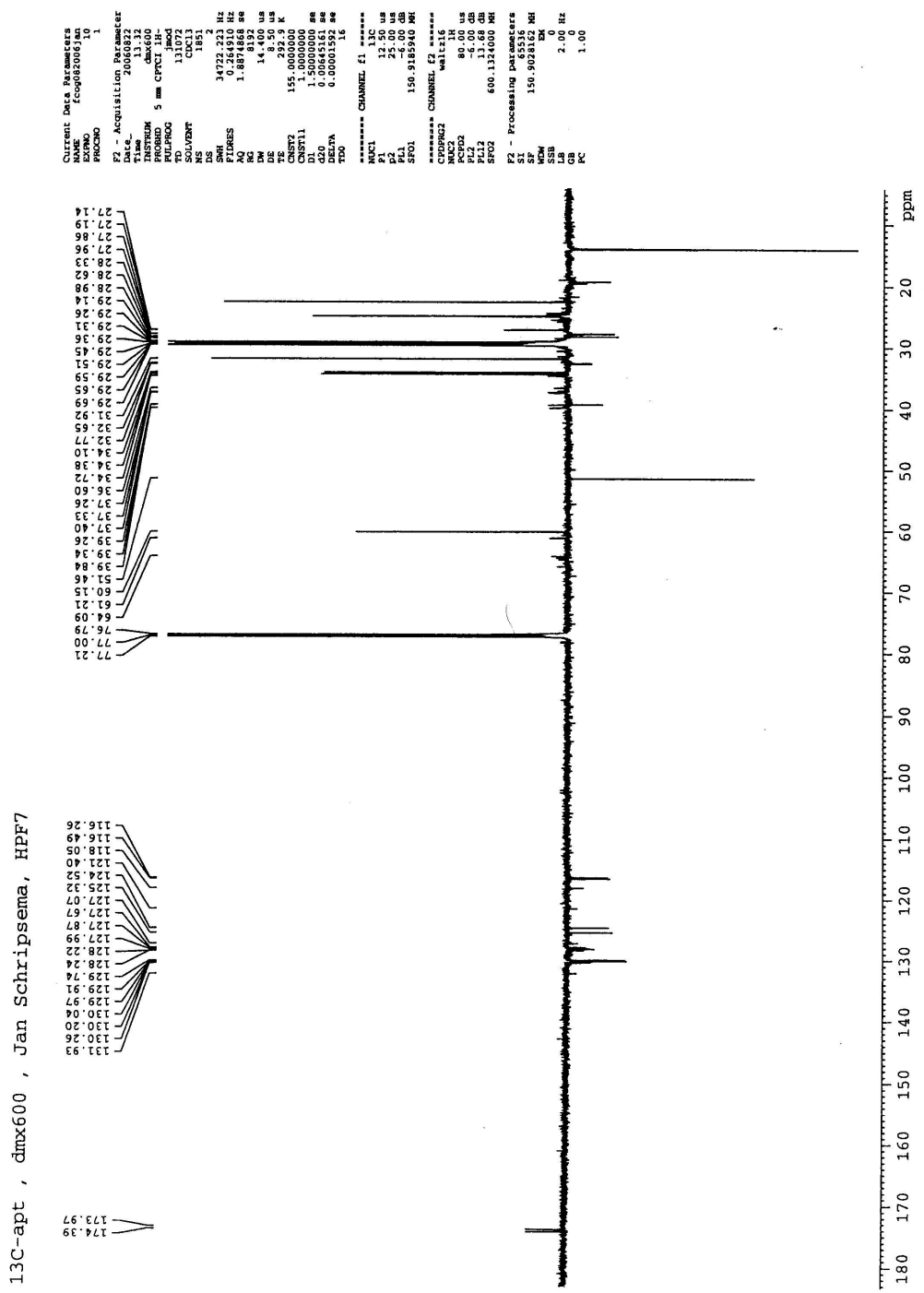

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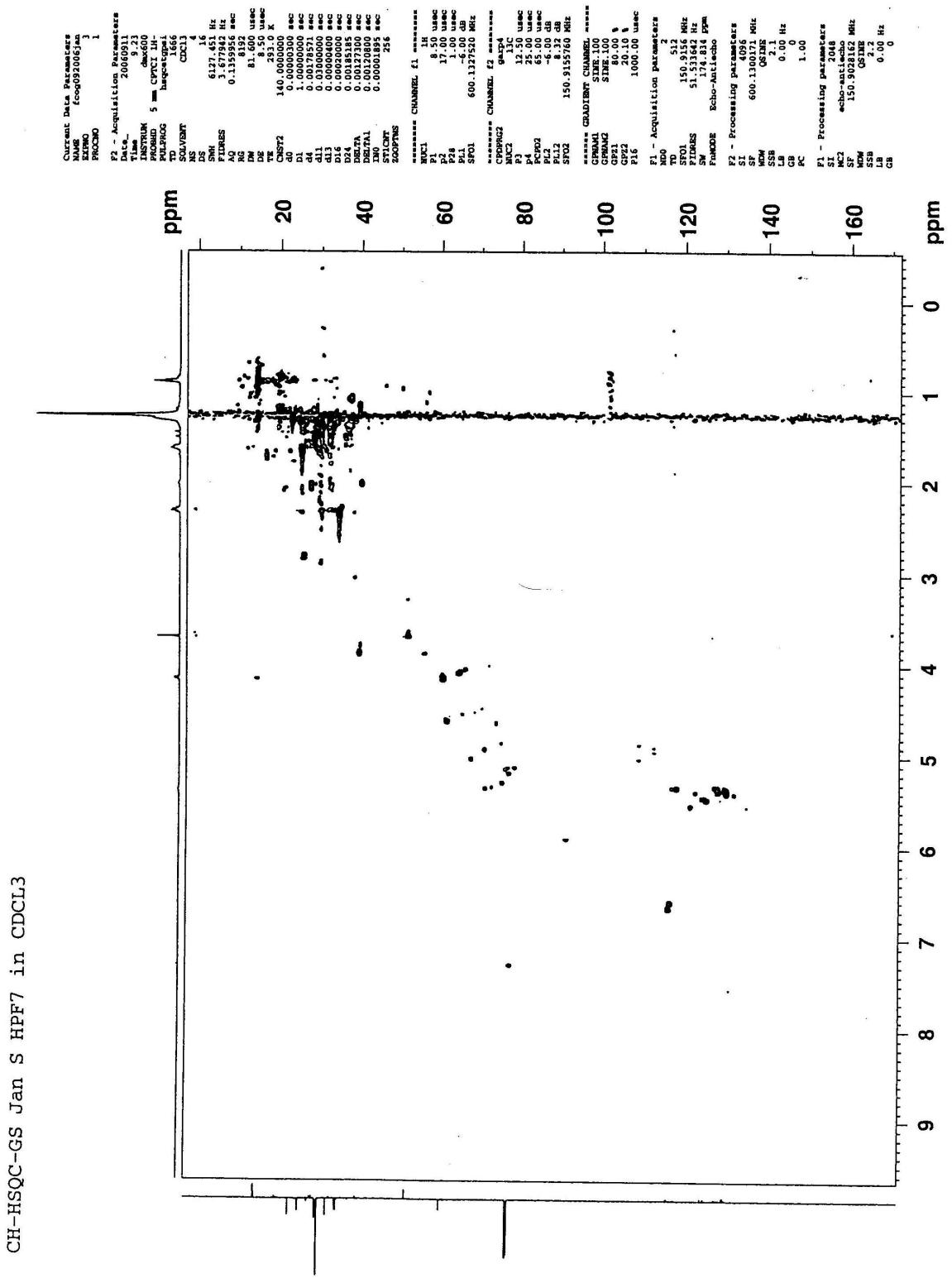

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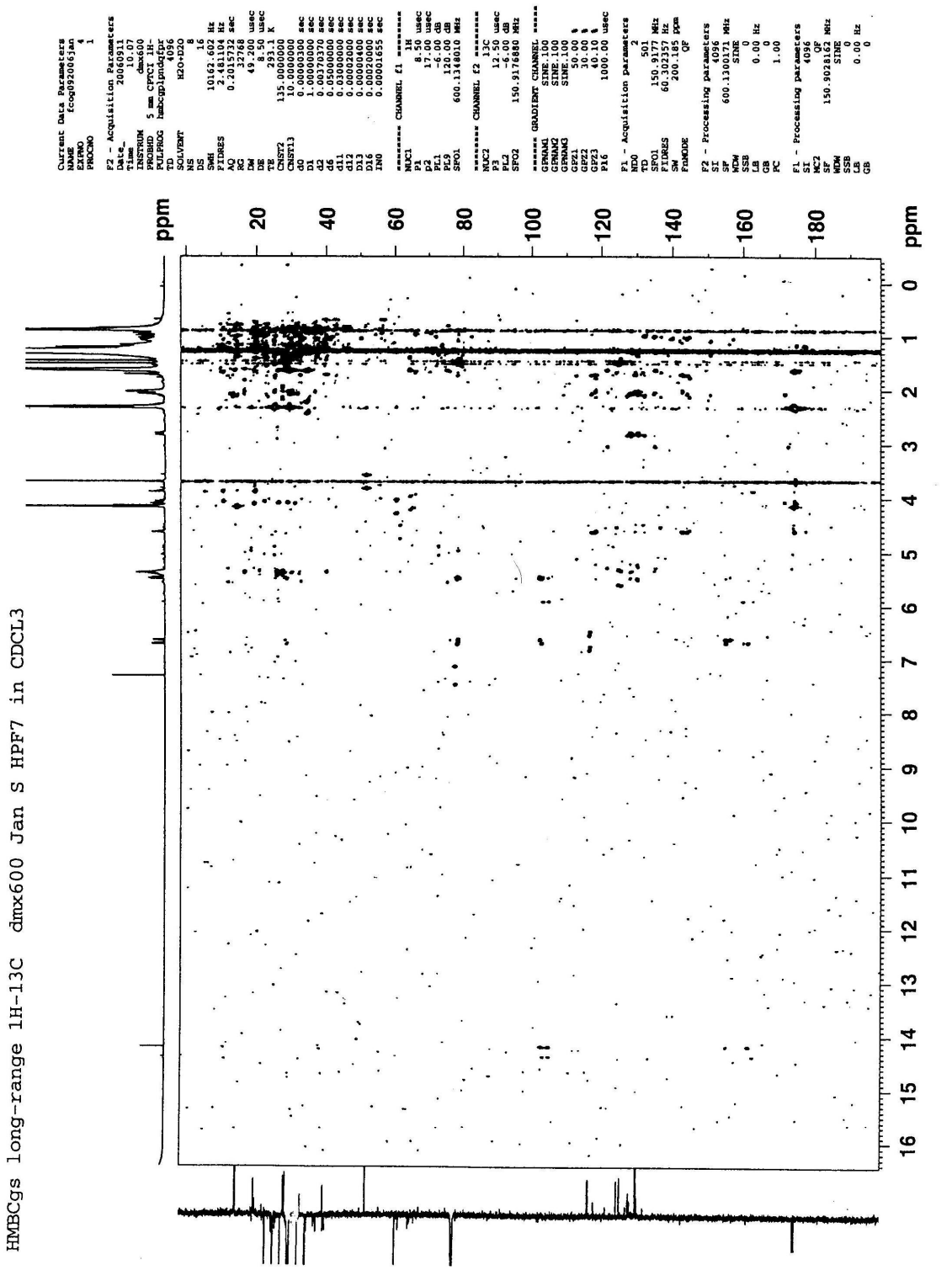

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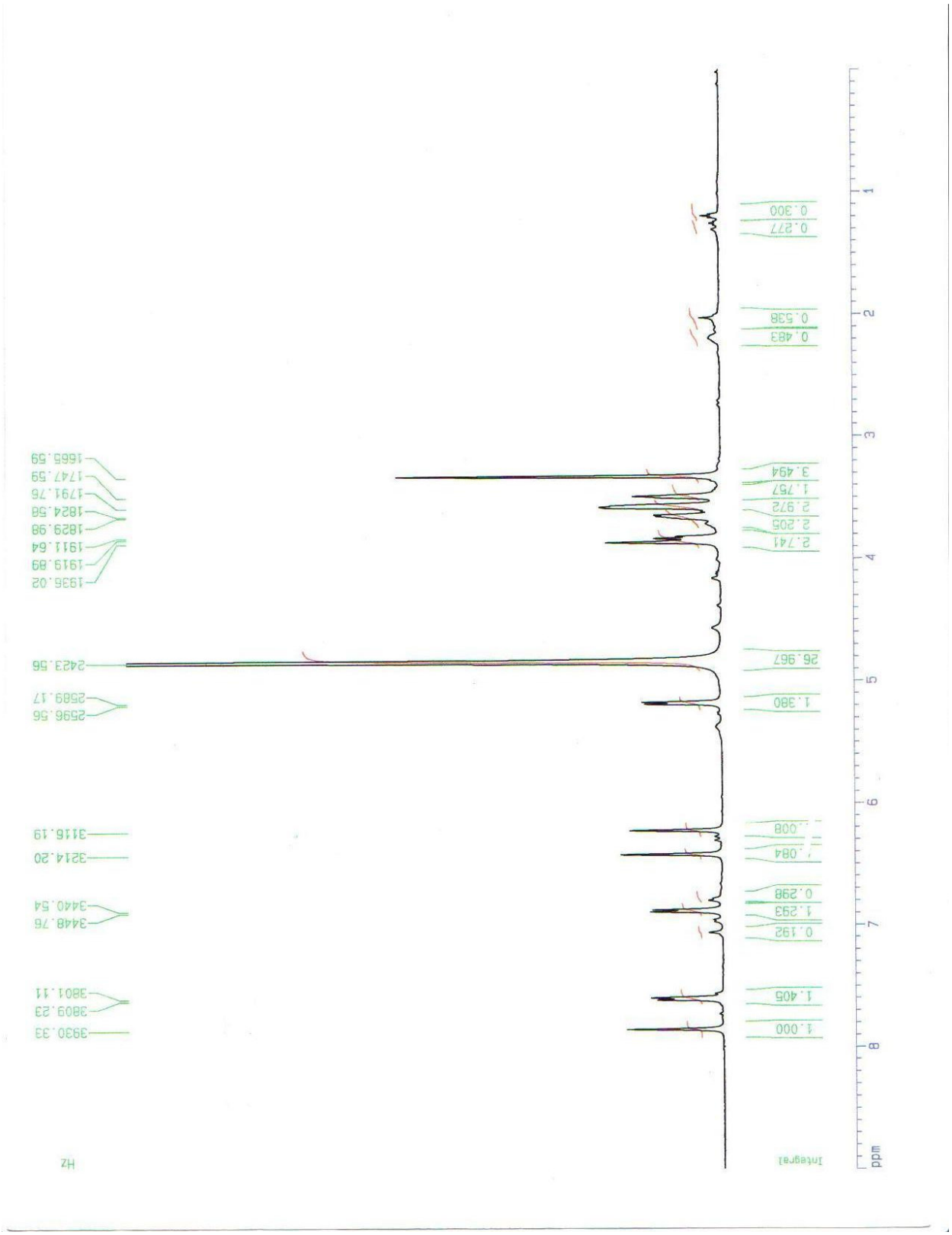

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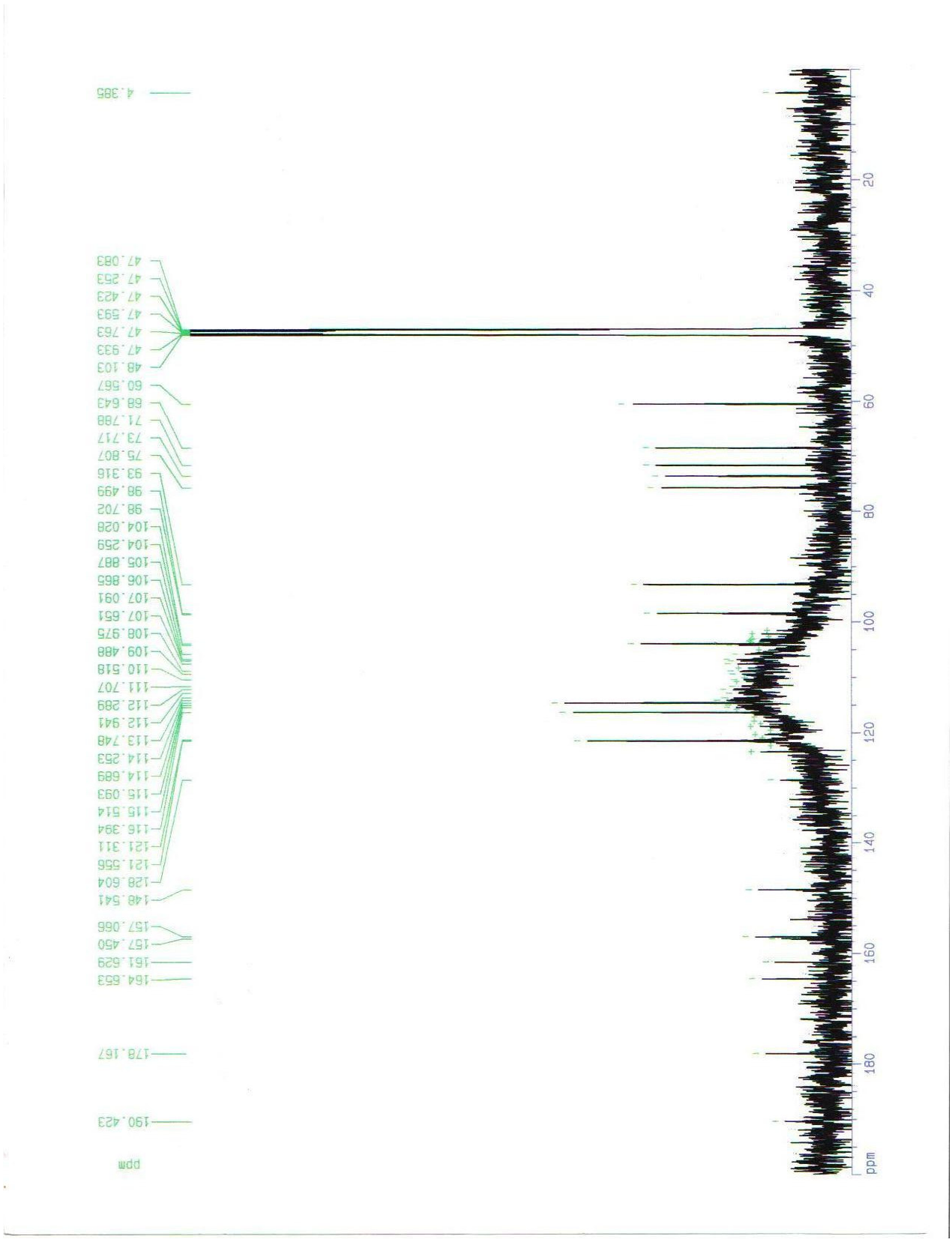

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