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Estudo de moléculas potencialmente antidepressivas e analgésicas de espécies de *Hypericum* nativas do Rio Grande do Sul

Alice Fialho Viana

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Estudo de moléculas potencialmente antidepressivas e analgésicas de espécies de *Hypericum* nativas do Rio Grande do Sul

Tese apresentada por **Alice Fialho Viana**
para obtenção do TÍTULO DE DOUTOR
em CiênciasFarmacêuticas

Orientadores: Prof Dr Stela Maris K. Rates
Dr Jean Claude do Rego
Pr Jean Costentin

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APRESENTAÇÃO

Esta tese está inserida no projeto **Planejamento e Síntese de Substâncias de Origem Natural e Derivados com Potencial Atividade Farmacológica** aprovado pelo acordo CAPES/COFECUB - BRASIL / FRANÇA (Número CAPES 418/03; Ata Aprovação PPG- CF nº 9 de 3 de maio de 2001). O Projeto CAPES/COFECUB tem como objetivo a obtenção de moléculas bioativas, através de síntese orgânica e a partir da flora nativa do Rio Grande do Sul, centrando-se em dois gêneros bem representados no Estado: *Ilex* e *Hypericum*.

Especificamente, esta tese está ligada ao subprojeto **Prospecção de moléculas potencialmente antidepressivas e analgésicas em espécies do gênero *Hypericum* nativas do Rio Grande do Sul,** e foi realizada na modalidade de cotutela entre o Programa de Pós-Graduação em Ciências Farmacêuticas - UFRGS (PPG-CF), sob responsabilidade da Prof Dr Stela Maris Kuze Rates e a Unidade de Neuropsicofarmacologia Experimental da Faculdade de Medicina e Farmácia da Universidade de Rouen – F.R.E. 2735 C.N.R.S – I.F.R.M.P. 23, Rouen, França, sob a responsabilidade de Dr. Jean Claude do Rego e Prof. Dr. Jean Costentin.

PRESENTATION

Ce travail a été réalisé dans le cadre d'une thèse en cotutelle entre le Programme de Post-Graduation en Sciences Pharmaceutiques de la Faculté de Pharmacie de l'Université Fédérale de Rio Grande do Sul (Porto Alegre, Brésil), sous la responsabilité de Prof Dr Stela Maris Kuze Rates et le Laboratoire de Neuropsychopharmacologie Expérimentale, CNRS FRE 2735, à la Faculté de Médecine et Pharmacie de l'Université de Rouen (France), sous la responsabilité de Dr. Jean Claude do Rego et Prof. Dr. Jean Costentin . Il a porté sur l'**Etude de molécules potentiellement antidépressives et analgésiques issues d'espèces d'Hypericum natives du sud du Brésil,** qui est inscrit pleinement dans notre projet commun, sur **l'Etude et la synthèse de substances naturelles ainsi que de leurs dérivées ayant (possédant) des activités pharmacologiques potentielles,** approuvé par l'accord CAPES/COFECUB - BRESIL/FRANCE (sous le numéro 418/03). L'objectif de ce projet est l'obtention, soit par synthèse organique ou à partir de la flore du Rio Grande do Sul, de molecules bioactives à partir d'étude des genres: *Ilex* et *Hypericum*.

"I am among those who think that science has great beauty.
A scientist in the laboratory is not only a technician:
is also a child placed before natural phenomena
which impress him like a fairy tale."
Marie Curie (1867 - 1934)

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SUMÁRIO

LISTA DE ABREVIATURAS

APO	apomorfina
BUP	bupropiona
HC1	Fração com floroglucinóis do extrato ciclo-hexano de *H. caprifoliatum*
HCP	Extrato ciclo-hexano purificado das partes aéreas de *H. caprifoliatum*
HP4	Uliginosina B
MOR	morfina
NAL	naloxona
POL	Extrato ciclo-hexano das partes aéreas de *H. polyanthemum*
SAL	solução salina (NaCl 0,9%)
SCH	SCH 23390
SUL	sulpirida

LISTA DE FIGURAS

RESUMO

Estudo de moléculas potencialmente antidepressivas e analgésicas de espécies de *Hypericum* nativas do Rio Grande do Sul

O objetivo deste trabalho foi estudar a ação antidepressiva e analgésica de extratos ciclo-hexano de *Hypericum caprifoliatum* (HCP) e *Hypericum polyanthemum* (POL) e seus principais constituintes químicos, os derivados floroglucinol, HC1 e uliginosina (HP4), respectivamente. HCP, POL, HC1 e HP4 reduziram significativamente a imobilidade de ratos e camundongos no teste de natação forçada (FST), indicando potencial atividade antidepressiva. Os efeitos de HCP e POL no FST foram prevenidos pelo pré-tratamento com SCH23390 e sulpirida, antagonistas de receptores D_1 e D_2 respectivamente. Os extratos, HC1 e HP4 inibiram de modo dose-dependente a recaptação sinaptossomal de dopamina ([^{3}H]-DA), noradrenalina ([^{3}H]-NA) e serotonina ([^{3}H]-5HT), com uma maior potência para a recaptação de DA. Entretanto, este efeito não parece ser dependente de uma ação direta das substâncias sobre o transportador de monoaminas, uma vez que diferentes concentrações de HC1 e HP4 não afetaram a ligação de [^{3}H]-mazindol, [^{3}H]-nisoxetina e [^{3}H]-citalopram aos sítios de recaptação de DA, NA e 5-HT, respectivamente. Para avaliação do efeito dos extratos sobre os receptores de DA, NA, 5-HT e opióides, foi utilizada a técnica de ligação de [^{35}S]-GTPγS estimulada por agonistas. O tratamento agudo de ratos com HCP ou POL aumentou significativamente a ligação de [^{35}S]-GTPγS estimulada por DA, NA e 5-HT, enquanto 5 dias de tratamento diminuiram esta ligação. Nenhum dos regimes de tratamento afetou a ligação estimulada por DAMGO, agonista opióide. Estes resultados demonstram que os tratamentos com HCP e POL resultam em alterações na transmissão monoaminérgica. Porém estas não se devem a efeitos diretos de HC1 e HP4 sobre os receptores, pois a incubação com HC1 ou HP4 não alterou a ligação de [^{35}S]-GTPγS em nenhuma preparação estudada. Além do estudo sobre o sistema monoaminérgico, foi verificada a ação de HCP e POL sobre o eixo HPA através da medida dos níveis séricos e plasmáticos de corticosterona em camundongos submetidos ou não ao estresse da natação forçada. Três dias de tratamento com HCP e POL reduziram significativamente o aumento do nível cortical de corticosterona induzido pelo nado forçado, sem afetar o nível sérico, evidenciando que o

tratamento repetido com os extratos afeta repostas hormonais ao estresse. Em relação à atividade analgésica, HCP e POL apresentaram efeito antinociceptivo na placa quente, o qual foi prevenido pelo pré-tratamento com naloxona. Entretanto, HC1 e HP4 não inibiram a ligação da [^{3}H]-naloxona, o efeito antinociceptivo de HP4 não foi prevenido pela naloxona, e o tratamento com os extratos, assim como a incubação direta HC1 ou HP4 não modificou a ligação de [^{35}S]-GTPγS ao receptor opióide. Os resultados apresentados confirmam que HCP e POL têm potencial atividade antidepressiva e analgésica, provavelmente com um mecanismo de ação diferente dos fármacos atuais, sendo os derivados floroglucinol os prováveis responsáveis por estes efeitos.

Palavras-chave: *Hypericum caprifoliatum, Hypericum polyanthemum*, antidepressivo, analgésico, teste de natação froçada, placa-quente, recaptação sinaptossomal, ligação aos transportadores, [^{35}S]-GTPγS, corticosterona.

ABSTRACT

Prospective of molecules with antidepressant and analgesic activities from *Hypericum* species native to Rio Grande do Sul.

This work aimed to study the antidepressant and analgesic effect of *Hypericum caprifoliatum* (HCP) and *H. polyanthemum* (POL) cyclohexane extracts, as well as the substances and mechanisms involved on their activities. HCP, POL and their phloroglucinol derivatives (HC1 and HP4, respectively) significantly reduced rats and mice immobility time in the forced swimming test (FST), indicating potential antidepressant effect. The extracts showed some selectivity for activating the dopaminergic system. The antidepressant-like effect of HCP and POL was inhibited by D_1 and D_2 antagonists; and their extracts, HC1 and HP4, inhibited dopamine ([^{3}H]-DA), noradrenaline ([^{3}H]-NA) and serotonin ([^{3}H]-5HT) uptake, more potently of dopamine. However, this effect is not related to the binding to monoamine transporters, since they did not affect the binding of [^{3}H]-mazindol, [^{3}H]-nisoxetine and [^{3}H]-citalopram to DA, NA and 5-HT transporters, respectively. In order to investigate the functional role of DA, NA, 5-HT and opioid receptors in the effects of HCP and POL, we evaluated the extracts effect on monoamine and opioid receptor-stimulated [^{35}S]-GTPγS binding. We demonstrated that acute or 3 treatments within 24h increases [^{35}S]-GTPγS binding to DA, NA and 5-HT sites, while after 5 days of treatment the binding is reduced. Non treatment regimen affected binding to opioid sites. Hence, the results demonstrate that the treatment with HCP and POL induce alterations in monoaminergic transmission. As seen at the binding to monoamine transporters, these effects are not due to HC1 or HP4 interaction with the receptors, since direct incubation of HC1 and HP4 did no affect [^{35}S]-GTPγS binding to membranes. Besides studying HCP and POL effects on the monoaminergic system we investigated their activity on other system related to depression, the hypothalamus-pituitary-adrenal axis. We demonstrated that 3 days treatment with the extract reduce cortical corticosterone but not serum levels increased by FST. Regarding to the analgesic activity, HCP and POL antinociceptive effect in the hot plate were prevented by naloxone. However, HC1 and HP4 did not inhibited [^{3}H]-naloxone binding to opioid receptors. In conclusion, HCP and POL have potencial antidepressant and analgesic activities and the phloroglucinol

derivatives are very likely to be the substances responsible for them. Even though the mechanism of action involves the monoaminergic system, the manner by which the extracts influence this system is different from classical antidepressants.

Keywords: *Hypericum caprifoliatum, Hypericum polyanthemum*, antidepressant, nociception, forced swimming test, hot-plate test, synaptosomal uptake, transporters binding, [^{35}S]-GTPγS, corticosterone level.

RESUME

Etudes de molécules potentiellement antidépressives et analgésiques issues d'espèces d'*Hypericum* natives du Rio Grande do Sul

L'objectif du présent travail était d'étudier les extraits cyclohexaniques de deux espèces d'*Hypericum*, natives du sud du Brésil, *Hypericum caprifoliatum* (HCP) et *Hypericum polyanthemum* (POL), qui joueraient un rôle antidépresseur et un rôle analgésique, afin de caractériser les substances responsables de ces activités, ainsi que leurs mécanismes d'action. Dans l'épreuve de la nage forcée, HCP et POL, ainsi que leurs dérivés phloroglucinols (respectivement nommés HC1 et HP4) réduisent de façon significative la durée d'immobilité des rats et des souris, indiquant un effet de type antidépresseur potentiel de ces extraits. Cet effet est totalement réversé par le SCH23390 et le sulpiride (antagonistes respectifs des récepteurs D_1 et D_2 de la dopamine), suggérant l'implication du système dopaminergique dans l'effet type antidépresseur de HCP et POL. D'autre part, leurs fractions riches en phloroglucinol (HC1 et HP4) inhibent la capture synaptosomale de la dopamine ([^{3}H]-DA), de la noradrénaline ([^{3}H]-NA) et de la sérotonine ([^{3}H]-5HT), avec une certaine primauté d'action sur la capture de la DA. Cependant, cet effet ne semble pas dépendre d'une action directe sur les transporteurs des monoamines, puisque HC1 et HP4 n'affectent ni la liaison du [^{3}H]-mazindol, ni celle de la [^{3}H]-nisoxétine et ni celle du [^{3}H]-citalopram, respectivement aux transporteurs de la DA, de la NA et de la 5-HT. Par ailleurs, nous avons évalué l'effet des extraits sur les adaptations fonctionnelles des récepteurs monoaminergiques et opioïdergiques, grâce à la liaison de [^{35}S]-GTPγS stimulée respectivement par les monoamines et les opioïdes. Nous avons montré qu'administrés à la dose unique ou après trois traitements pendant 24 heures, HCP et POL provoquent une augmentation significative de la liaison de [^{35}S]-GTPγS stimulée par la DA, la NA et la 5-HT, alors qu'après cinq jours de traitement, à raison de deux administrations quotidiennes, ces extraits réduisent de façon significative la liaison de [^{35}S]-GTPγS stimulée par les monoamines. Il est important de noter que quelque soit le traitement, ni HCP, ni POL n'affecte de manière significative la liaison de [^{35}S]-GTPγS stimulée par les opioïdes. Ces résultats démontrent que HCP et POL provoquent une altération fonctionnelle des

transmissions monoaminergiques. Nous nous sommes intéressés, par la suite, aux effets de HCP et de POL sur l'axe corticotrope, et avons montré qu'administré sur trois jours, à raison d'une administration quotidienne, HCP et POL réduisent de façon significative l'augmentation du taux de corticostérone corticale induite par le stress de la nage forcée, sans affectée de manière significative celle du taux de corticostérone sérique, ni les taux de corticostérone plasmatique et corticale basale chez les animaux non soumis aux stress. Par ailleurs, nous avons montré, qu'aux doses où ils exercent un effet type antidépresseur, HCP et POL induisent, dans le test de la plaque chaude, des effets analgésiques réversés par la naloxone (antagoniste des récepteurs opioïdes). Cet effet pourrait être attribué soit à d'autres substances, autres que HC1 et HP4, présentes dans les extraits; soit à une action indirecte de HC1 et HP4 sur les récepteurs opioïdes, puisque ni HC1, ni HP4 n'affectent de manière significative la liaison de la [^{3}H]-naloxone. En conclusion, l'ensemble de ce travail indique que les extraits de *H*. *caprifoliatum* et *H*. *polyanthemum* développent des effets de type analgésique et antidépresseur, qui pourraient être attribués à leurs dérivés phloroglucinols. Ce dernier effet semble résulter de l'activation d'une transmission monoaminergique, selon un mécanisme d'action différent de celui des antidépresseurs de références.

Mots clés: *Hypericum caprifoliatum, Hypericum polyanthemum*, antidépresseur, nociception, test de la nage forcée, test de la plaque chaude, capture synaptosomale, radioliaison aux transporteurs neuronaux, [^{35}S]-GTPγS, taux de corticosterone.

I PARTE

INTRODUÇÃO

E

REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

Os psicofármacos constituem uma classe de fármacos com fundamental necessidade de desenvolvimento. Foram introduzidos na terapêutica no final da década de 50 e propiciaram uma revolução no tratamento das doenças psiquiátricas e uma própria mudança de atitude diante destas, visto a possibilidade de uma maior compreensão de seu substrato biológico (Feighner, 1999). O conhecimento das bases neuroquímicas da depressão e esquizofrenia, por exemplo, está estritamente relacionado com o conhecimento dos mecanismos de ação de antidepressivos e antipsicóticos. No entanto, a totalidade dos fenômenos bioquímicos relacionados com estas doenças está longe de ser completamente elucidada. Da mesma forma, o mecanismo de ação exato de muitos destes fármacos ainda não é totalmente compreendido. Soma-se a isto o fato de que cerca de 35% dos pacientes psiquiátricos não respondem adequadamente ao tratamento farmacológico, e de que, mesmo a resposta terapêutica adequada, é acompanhada, na maioria das vezes, por reações adversas importantes (Graeff *et al.*, 1999 ; Berton e Nestler, 2006).

Entretanto, a grande maioria das pesquisas é ainda alicerçada na abordagem original da teoria monoaminérgica da gênese dos distúrbios psiquiátricos, fundamentada na observação dos efeitos da reserpina, dos inibidores da monoaminoxidase e antidepressivos tricíclicos. A manutenção desta abordagem não resultará em fármacos realmente inovadores e tão pouco auxiliará na evolução do conhecimento sobre as doenças mentais (Nestler e Carlezon, 2006). Este fato torna-se preocupante considerando que distúrbios do humor, como a depressão, têm prevalência mundial de aproximadamente 18%, sendo considerada uma das doenças mentais mais incapacitantes e dispendiosas (Gold e Charney, 2002).

É neste contexto que se insere a pesquisa do gênero *Hypericum.* A espécie *Hypericum perforatum*, conhecida popularmente nos EUA e Inglaterra como *St.*

John's wort e na Alemanha como *Johanniskraut* (erva-de-São-João), apresenta-se como alternativa aos antidepressivos sintéticos no tratamento de depressões leves a moderadas. Estudos clínicos demonstram a eficácia de extratos padronizados de *H. perforatum* nestas situações (Linde *et al.*, 1996; Bilia *et al.*, 2002; Kasper *et al.*, 2006) e estudos sobre seu mecanismo de ação indicam que estes extratos atuam de modo diferente dos antidepressivos atuais (Bhattacharya *et al.,* 1998; Chatterjee *et al.*, 1998a; Sarrell *et al.*, 2001, Kumar *et al.*, 2001). Uma característica interessante de sua atividade é a ação não-específica. O extrato e algumas substâncias isoladas inibem a recaptação sinaptossomal de serotonina, noradrenalina, dopamina, GABA e glutamato (Wonnemann *et al.*, 2000; Roz e Rehavi, 2003). A ligação de histamina, de neurocinina, de corticotropina e opiódes aos seus respectivos receptores também é inibida por extratos de *H. perforatum* (Simmen *et al.*, 2001).

O grupo, coordenado pelas Prof. Dr. Stela Maris Kuze Rates e Prof. Dr. Gilsane Lino von Poser, vem desenvolvendo, desde 1998, uma linha de pesquisa que visa avaliar espécies do gênero *Hypericum*, nativas do Rio Grande do Sul, quanto à taxonomia, constituição química e atividades biológicas, especialmente aquelas relacionadas a uma potencial atividade antidepressiva. Até o momento, foram avaliadas as espécies *H. brasiliense*, *H. caprifoliatum*, *H. conatum, H. myrianthum, H. piriai, H. polyanthemum, H. carinatum* e *H. cordatum*, sendo que *H. caprifoliatum* demonstrou os resultados mais promissores (Viana, 2002). Esta tese de doutorado visou aprofundar o estudo da atividade antidepressiva de *H. caprifoliatum* e iniciar o estudo da atividade farmacológica de *H. polyanthemum*, buscando identificar, em cada espécie, a(s) substância(s) ativa(s) e investigar os respectivos mecanismos da ação.

2. OBJETIVOS

O objetivo geral deste trabalho foi estudar a ação antidepressiva e analgésica de extratos ciclo-hexano de *Hypericum caprifoliatum* (denominado HCP) e *Hypericum polyanthemum* (denominado POL) e dos derivados floroglucinol, HC1 e HP4 (uliginosina B), isolados de *H. caprifoliatum* e *H. polyanthemum*, respectivamente.

2.1. Objetivos específicos

- Avaliar frações da espécie *H. caprifoliatum* e *H. polyanthemum* no modelo animal de depressão teste de natação forçada em ratos e camundongos, em diferentes tratamentos.

- Estudar os possíveis mecanismos envolvidos nos efeitos observados nos modelos animais de depressão, através de:
  - Ensaios neuroquímicos de recaptação sinaptossomal de monoaminas;
  - Ligação a transportadores de monoaminas;
  - Avaliação do efeito sobre a funcionalidade de receptores monoaminérgicos e opióides.
  - Avaliação do efeito sobre os níveis séricos e corticais de corticosterona.

- Avaliar o efeito antinociceptivo de extratos de *H. caprifoliatum* e *H. polyanthemum* nos testes da placa quente e contorções abdominais induzidas por ácido acético.

3. REVISÃO DA LITERATURA

3.1. TRANSTORNOS DEPRESSIVOS

Muito do que sabemos atualmente sobre a depressão e doenças relacionadas foi descrito por médicos da Grécia e Roma antigas, que cunharam termos como melancolia e mania e perceberam sua relação. No século IV a.c, Hipócrates já fazia referência ao sofrimento e à melancolia. Ele descreveu melancolia, “a bile negra” , como um estado de “aversão à comida, falta de sono, irritabilidade, infelicidade e agitação”. Galeno (131-201 d.c.) descreveu a melancolia como “medo, depressão, descontentamento com a vida e desejo de ficar longe das pessoas”. Posteriormente, a medicina greco-romana não apenas reconheceu os sintomas da melancolia na forma de medo, desconfiança, agressão e desejos de morte, mas também a relacionou a fatores ambientais como alto consumo de vinho, “perturbações da alma por paixões”, e distúrbios no sono. Muitos textos gregos originais sobre melancolia foram transmitidos para posteridade através de textos medievais árabes, nos quais conexões entre dois principais estados de humor eram sugeridas, e supunha-se que a causa de doenças era devido às interações entre o ‘temperamento’, o ambiente e quatro ‘humores’: vento, humor, bile amarela e bile negra (WHO, 2006a).

Na era moderna, o livro “Anatomy of Melancholy” de Robert Burton (1621) foi o primeiro grande texto da história ocidental sobre as ciências cognitivas por sistematizar as descrições de muitos padrões de doenças mentais, principalmente a depressão. Burton categoriza várias formas de melancolia e tristeza, e também descreve “melancolias sem causa”. Este último significa que é possível sofrer de melancolia sem haver uma causa aparente, ou seja, evento desencadeador.

No século XIX, foram feitas várias tentativas de melhorar o conceito de melancolia e aproximá-la do que atualmente considera-se depressão. Muitos médicos, como Esquirol (1820), Samuel Tuke (1813) e Henry Maudsley (1868) (WHO, 2006a), tentaram definir a causa, natureza e forma da melancolia. No final

de 1800, a ‘melancolia’ e ‘doença maníaco depressiva’ começaram a ser vistas como doenças independentes das doenças ditas ‘físicas’, de tal modo que o interesse sobre a depressão e mania foi renovado e intensos estudos sobre vários aspectos de suas características clínicas, dinâmica, neurobiologia, epidemiologia, classificação e tratamento (WHO, 2006a).

Após a II Guerra Mundial, a OMS (Organização Mundial de Saúde) criou uma força-tarefa para rever a classificação das doenças psiquiátricas e produzir uma edição revisada da Classificação Internacional de Doenças (CID). Suas revisões subseqüentes, juntamente com edições revisadas do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM) da Associação Psiquiátrica Americana, revolucionaram o modo de estudar doenças mentais. Estes guias oficiais estabeleceram critérios operacionais claros para o diagnóstico, assim como requerimentos para inclusão e exclusão, o que significa maior conhecimento sobre o que constitui a depressão e o que não a constitui (WHO, 2006a).

O Manual Diagnóstico e Estatístico de Transtornos Mentais 4ª edição com texto revisado (DSM-IV-TR, 2002), classifica os transtornos depressivos em:

- *Transtorno Depressivo Maior:* caracteriza-se por um ou mais episódios depressivos maiores, i.e. pelo menos duas semanas de humor deprimido ou perda de interesse, sem história de episódios maníacos ou hipomaníacos, acompanhados por quatro sintomas adicionais de depressão extraídos de uma lista que inclui: alterações no apetite ou peso, sono e atividade psicomotora; diminuição da energia; sentimentos de desvalia ou culpa; dificuldades para pensar, concentrar-se ou tomar decisões; pensamentos recorrentes sobre morte ou ideação suicida, planos ou tentativas de suicídio. O Transtorno Depressivo Maior está associado com uma alta mortalidade. Os indivíduos com Transtorno Depressivo Maior severo que morrem por suicídio chegam a 15%.

- *Transtorno Distímico:* sua característica essencial é um humor cronicamente deprimido que ocorre na maior parte do dia, na maioria dos dias, por pelo menos 2

anos. Os indivíduos descrevem seu humor como triste ou "na fossa". Durante os períodos de humor deprimido, pelo menos dois dos seguintes sintomas adicionais estão presentes: apetite diminuído ou hiperfagia, insônia ou hipersonia, baixa energia ou fadiga, baixa auto-estima, fraca concentração ou dificuldade em tomar decisões e sentimentos de desesperança. Os indivíduos podem notar a presença proeminente de baixo interesse e de autocrítica, freqüentemente vendo a si mesmos como desinteressantes ou incapazes.

- *Transtorno Depressivo Sem Outra Especificação***:** é incluído para a codificação de transtornos com características depressivas que não satisfazem os critérios para Transtorno Depressivo Maior, Transtorno Distímico, Transtorno de Ajustamento com Humor Deprimido ou Transtorno de Ajustamento Misto de Ansiedade e Depressão (ou sintomas depressivos acerca dos quais existem informações inadequadas ou contraditórias).

3.1.1. Dados epidemiológicos

Os Transtornos Depressivos podem acometer pessoas de qualquer gênero, raça e nível sócio-econômico. A OMS estima que atualmente 121 milhões de pessoas sofrem de depressão. Aproximadamente 5,8% dos homens e 9,5% das mulheres sofrerão um episódio depressivo em algum momento da vida, estes valores podem variar entre as diferentes populações (WHO, 2006b). A depressão crônica ou recorrente pode resultar em prejuízos de ordem pessoal e profissional. Ela é principal causa de afastamento do trabalho (medido em YLD, do inglês, *Years Lived with Disability*) e quarta colocada quando se considera os anos de potencial vida produtiva perdidos por morte prematura ou doença (DALY, do inglês Disability Adjusted Life Years), sendo que a OMS projeta que em 2020 a depressão alcançará o segundo lugar como causa de DALY. Além disso, o suicídio permanece uma possível decorrência do Transtorno Depressivo Maior. Os Transtornos Depressivos e a Esquizofrenia são responsáveis por 60% dos suicídios registrados no mundo (WHO, 2006b).

Os Transtornos Depressivos estão fortemente correlacionados com fatores hereditários, considera-se que 40-50 % do risco para o desenvolvimento de depressão transmitido pelos pais, embora os genes responsáveis por este risco não estejam identificados (Berton e Nestler, 2006). A caracterização dos riscos causados pelo meio também permanece escassa, com sugestões para traumas durante a infância, estresse emocional, outras doenças que envolvam ou não o sistema nervoso central (SNC), incluindo doenças virais (Berton e Nestler, 2006).

3.1.2. Tratamentos

A primeira linha de tratamento inclui medicação antidepressiva, psicoterapia ou a combinação de ambos. Outras intervenções efetivas incluem a criação de uma rede de apoio para indivíduos, famílias ou grupos vulneráveis. As evidências sobre a prevenção de episódios depressivos são menos conclusivas. O tratamento com antidepressivos juntamente com psicoterapia é efetivo em 60 - 80 % dos pacientes. Entretanto, menos de 25 % dos indivíduos afetados (em alguns países menos de 10 %) recebem tratamento, algumas razões são a falta de recursos, de pessoal treinado e o estigma associado às doenças mentais que leva os pacientes a não procurar auxílio (WHO, 2006b).

Na década de 50 antidepressivos de duas classes foram descobertos, os tricíclicos (ADT) e os inibidores de monoaminoxidase (IMAO). Apesar de atualmente existirem mais de 25 substâncias utilizadas para fabricação de medicamentos antidepressivos, desde a descoberta dos ADT e IMAO não ocorreu nenhuma grande inovação no mecanismo de ação destes medicamentos, todos ainda agem via transmissão monoaminérgica, apenas com maior especificidade que os tricíclicos e IMAO. A grande vantagem dos antidepressivos de segunda geração e atípicos são efeitos adversos mais brandos e por isso melhor tolerados pelos pacientes. A seguir apresentamos um resumo sobre as principais características das classes de medicamentos antidepressivos disponíveis:

3.1.2.1. Antidepressivos Tricíclicos (ADT)

No final da década de 40, Häflinger e Schindler sintetizaram uma série de mais de 40 derivados de iminodibenzila para possível uso como anti-histamínico, analgésico, anti-parkinson e/ou sedativo (Baldessarini, 1996; Nestler, 1998). Entre as substâncias selecionadas em testes pré-clínicos, por suas propriedades sedativas ou hipnóticas, estava a imipramina. Porém, durante os testes clínicos ela foi relativamente ineficaz em reduzir a agitação de pacientes psicóticos, inesperadamente, ela apresentou uma melhora indiscutível em certos pacientes deprimidos (Baldessarini, 1996). Posteriormente, descobriu-se que todos os antidepressivos tricíclicos ativos clinicamente inibem a recaptação de serotonina e noradrenalina com diferentes potências (Nestler, 1998). Porém, devido a sua origem eles também atuam sobre os receptores de histamina e acetilcolina. Esta última interação causa importantes efeitos tipo atropina, incluindo boca seca e constipação, tontura, visão borrada, sedação, hipotensão ortostática. Outras reações adversas incluem alterações cardiovasculares, em sobre-dose os ADTs diminuem a condução intraventricular podendo causar falência cardíaca ou arritmias ventriculares. Em sobre-dose estes medicamentos também podem causar ataques epiléticos principalmente em pacientes que já apresentaram episódios anteriores. Alguns exemplos são: imipramina, amitriptilina, clorimipramina, desimipramina.

3.1.2.2. Inibidores de monoaminoxidases (IMAO)

Foram os primeiros antidepressivos clinicamente ativos e tiveram um grande impacto no desenvolvimento da moderna psiquiatria biológica (Baldessarini, 1996; Stahl, 2000). Em 1951 a isoniazida e seu derivado isopropila, iproniazida, foram desenvolvidos para o tratamento de tuberculose. Observou-se que a iproniazida melhorava o humor em pacientes tuberculosos com sintomas de depressão. Em 1952, o grupo de Zeller descobriu que, diferente da isoniazida, a iproniazida possuia atividade inibidora da enzima monoamino oxidase (Baldessarini, 1996). Esta classe de antidepressivos aumenta os níveis de

catecolaminas por inibir a MAO, uma das enzimas que degradam as aminas cerebrais. Os primeiros medicamentos com atividade IMAO (fenelzina, tranilcicloprida) são inibidores irreversíveis da enzima, ou seja, sua atividade retorna apenas após a síntese de uma nova enzima. Por esta razão, a ingestão de alimentos que contém tiramina, principalmente os produzidos por fermentação (p.ex. queijos, vinhos, cerveja) deve ser evitada, pois a MAO também oxida outras feniletilaminas, como a tiramina e é importante no metabolismo de primeira passagem. Quando a MAO gastrointestinal e hepática está inibida, uma grande quantidade de tiramina e outras feniletilaminas são absorvidas e alcançam a circulação sistêmica sem serem oxidadas, causando uma liberação massiva de noradrenalina e o que pode levar a hiperpirexia, taquicardia, arritimias cardíacas, crise hipertensiva severa e até mesmo hemorragia intracerebral (Hoffman e Lefkowitz, 1996; Stahl, 2000). Esta interação é menos crítica para IMAOs seletivos para MAO A e reversíveis, como moclobemida. Outras reações adversas são: hipotensão, ganho de peso e disfunção sexual. Apesar disso, alguns pacientes com depressão respondem melhor a IMAOs que a qualquer outra classe de antidepressivos (Feighner, 1999).

3.1.2.3. Inibidores seletivos da recaptação de serotonina (ISRS)

Estes antidepressivos são seletivos, mas não sub-tipo específico, aumentam a biodisponibilidade de 5-HT na fenda sináptica, tanto nos terminais como nos corpos, em todas as regiões cerebrais. Como efeito imediato os ISRS inibem o transportador de serotonina (Stahl, 1998; Yadid et al, 2000). Esta ação causa o aumento repentino de serotonina predominantemente na área somatodendrítica A administração crônica de ISRS, o aumento persistente de serotonina na área somatodendrítica do neurônio leva a desensibilização dos auto-receptores somatodendríticos tipo 5-HT_{1A}. Uma vez que estes auto-receptores fiquem dessensibilizados, o fluxo de impulso neural não é mais rapidamente inibido pela presença de 5-HT. Portanto, o fluxo de impulso neural é `ligado´. Outro modo de dizer-se é que, a neurotransmissão serotonérgica é desinibida, e mais serotonina é liberada do terminal axônico (Stahl, 1998). Sua eficácia, especialmente em depressão maior, não é superior aos ADT, mas o risco de

sobredose é menor. Além disso, a maioria dos pacientes parece tolerar as reações adversas, que muitas vezes são transitórias como náusea, tontura, diarréia, agitação ou sedação. O uso de ISRS pode ocasionar disfunções sexuais em homens e mulheres, incluindo redução da libido, anorgasmia, retardo ejaculatório, impotência (Papakostas e Fava, 2007). Ex: fluxetina, paroxetina, sertralina.

3.1.2.4. Antidepressivos atípicos:

Inibidores seletivos da recaptação de serotonina e noradrenalina (IRSNs) - Os inibidores da recaptação de serotonina e norepinefrina são uma classe recente de antidepressivos, seu primeiro representante foi a velafaxina, mas já é um tratamento de primeira linha para depressão. Eles aumentam a atividade tanto de NA como de 5-HT, além de interagir fracamente com receptores dopaminérgicos e parecem agir como antagonista não-competitivode receptores nicotínicos (Papakostas e Fava, 2007). Parece haver uma relação dose-resposta com este fármaco, e em altas doses, o efeito adrenérgico é aumentado (Feighner, 1999). A velafaxina causa uma *down-regulation* aguda nos receptores β-adrenérgicos, fato que sugere um possível mecanismo de início de ação agudo, porém não existem estudos. Dependendo da dose empregada, seus efeitos adrenérgicos podem causar aumento da pressão sangüínea, entretanto este efeito não é encontrato em todos os estudos. Nos EUA o órgão de controle FDA (do inglês *Food and Drug Administration*) orienta que é necessária a monitorização da pressão sangüínea (Feighner, 1999). Alguns dos efeitos adversos destes antidepressivos, assim como dos ISRS, devem-se a ativação não seletiva de múltiplos receptores 5-HT e NA (Yadid *et al.*, 2000). Outros representantes são, duloxetina e milnacipram (Papakostas e Fava, 2007).

Inibidores da recaptação de serotonina e bloqueador 5-HT$_2$ - A este grupo pertencem a trazodona e seu análogo, a nefazodona. Elas agem como inibidores relativamente fracos da serotonina e noradrenalina (Papakostas e Fava, 2007) e bloqueiam receptores pós-sinápticos 5-HT$_{2A}$/5-HT$_{2C}$ (Feighner, 1999; Millan, 2006). A nefazodona é mais potente e específica para serotonina que a trazodona.

Por bloquear 5-HT_{2A} e inibir a recaptação de serotonina, a nefazodona parece possuir um mecanismo dual de ação sobre o sistema serotonérgico. A trazodona também é um forte bloqueador do receptor adrenérgico α_1, acredita-se que este receptor seja responsável pelo efeito sedativo da trazodona e também pode estar relacionado com a ocorrência de priapismo, efeito menos pronunciado durante o uso de nefazodona (Papakostas e Fava, 2007). Outras reações adversas incluem dificuldade de concentração e letargia. Ela também não possui ação do tipo quinidina, sendo segura na sobre-dose, e tem uma pequena taxa epileptigênica e de disfunção sexual, especialmente quando comparado com ISRS, venlafaxina, ADT e IMAO. A nefazodona não possui atividade anti-histamínica nem anti-colinérgica, o que melhora sua tolerabilidade e segurança. (Feighner, 1999).

Antidepressivos adrenérgico e serotonérgico específicos (NaSSa) - O exemplo de antidepressivo que atua em receptores NA e 5-HT específicos é a mirtazepina. Ela bloqueia os auto-receptores α_2-adrenérgicos e α_2-hetero-receptores serotoninérgicos responsáveis pela regulação na liberação de NA e 5-HT. Além disso, bloqueia os receptores pós-sinápticos 5-HT_{2A} , 5-HT_{2C} e 5-HT_3 (Feighner, 1999; Yadid *et al*, 2000). Este bloqueio resulta num aumento da atividade noradrenérgica e atividade serotoninérgica específica, o que resulta em menores efeitos colaterais do tipo ISRS (distúrbios gastrointestinais, insônia e disfunção sexual) e dos ADT (boca seca, tontura e constipação). Entretanto, a mirtazepina tem ação histaminérgica, o que pode causar sedação e aumento de apetite com aumento de peso (Feighner, 1999; Papakostas e Fava, 2007).

Inibidores da recaptação de noradrenalina (IRNs)- A reboxetina é o mais específico inibidor da recaptação de NA, com fraca afinidade pelo transportador de serotonina e dopamina e receptor muscarínico. Apesar de ser classificada como tricíclico, a desipramina também pode ser incluída nesta classe, devido a sua grande especificidade pela transportados de NA (Millan, 2006; Papakostas e Fava, 2007). O aumento de noradrenalina parece estar relacionado à ativação de receptores α_1- e α_2-adrenérgicos pós-sinapticos na região cortico-limbíca (Millan,

2006). Alguns estudos sugerem que a reboxetina pode se efetiva no tratamento de alterações cognitivas e no funcionamento psicossocial durante a depressão (Yadid *et al*, 2000; Papakostas e Fava, 2007). A reações adversas incluem, cefaléia, insônia, boca seca, hesitação urinária e constipação. Não está associada a reações adversas típicas dos ISRS, como disfunção sexual (Yadid *et al*, 2000; Millan, 2006).

Inibidores da recaptação de noradrenalina e dopamina (IRND)- Seu principal representante é a bupropiona. Uma grande diferença em relação aos ISRS e IRN é a elevação dos níveis de DA no núcleo acumbens, apesar de bloquear fracamente a recaptação de dopamina (Millan, 2006). A ativação de receptores D_1 que facilitam a liberação de NA podem contribuir para elevação de NA no cortex frontal (Feighner, 1999; Millan, 2006). Além disso, bupropiona é rapidamente metabolizada a derivados que inibem a recaptação de NA (Millan, 2006). Não possui efeito sobre os receptores serotoninérgicos, muscarínicos, histamínicos e α_2-adrenérgicos. Porém, a bupropiona parece agir como um antagonista não-competitivo de receptores nicotínicos (Millan, 2006), este efeito pode estar relacionado com seu uso no tratamento do tabagismo (Cordioli *et al.*, 2005). Uma vantagem em comparação aos ISRS é não estar associada com disfunções sexuais nem sedação. As reações adversas mais comuns da bupropiona são, agitação, insônia, perda de peso, boca seca, constipação, cefaléia e tremor. Um efeito adverso importante é a convulsão, principalmente em fórmulas de liberação imediata. Embora possa elevar a pressão sanguínea, este efeito não é muito comum (Papakostas e Fava, 2007).

Tianeptina - Apesar de ainda ser classificada por alguns autores como um potencializador da recaptação de serotonina (Papakostas e Fava, 2007), este efeito parece ser indireto uma vez que a afinidade da tianeptina pelo transportador de 5-HT é muito baixa (Millan, 2006) e não afeta de maneira importante os níveis extracelulares de 5-HT (Malagié *et al.*, 2000). Ainda que o mecanismo de ação da tianeptina não esteja elucidado, vários achados experimentais justificam seu

incontestável efeito antidepressivo. Cronicamente ela aumenta a sensibilidade de receptores α_1-adrenérgicos, porém não modifica os níveis sinápticos de NA (Rogoz *et al.*, 2001). O sistema dopaminérgico também é alterado pelo tratamento crônico com tianeptina, foi verificado um aumento na funcionalidade dos receptores D_2 no núcleo acumbens (Dziedzicka-Wasylewska *et al.*, 2002) e na liberação mesolímbica de dopamina (Invernizzi *et al.*, 1992). O modo pelo qual este antidepressivo aumenta a transmissão dopaminérgica não está claro, pois ela não se liga a transportadores de DA nem aos auto-receptores D_2 e D_3. Outras alterações induzidas pela tianeptina que podem estar relacionadas com seu efeito antidepressivo são: atenuar a influência inibitória de GABA (ácido gama-amino butírico) e glicina sobre a excitabilidade neuronal (Kim *et al.*, 2002); ter efeito neuroprotetor contra os efeitos do estresse causados pelos glicocorticóides e citocinas neurotóxicas e modular a transmissão glutamatérgica (McEwen *et al.*, 2002; Castanon *et al.*, 2004).

3.1.3. Pacientes resistentes

Um terço ou mais dos pacientes não responde e mais da metade não consegue obter ou manter uma remissão completa com qualquer tratamento farmacológico isolado. Ao avaliar a resistência de um paciente ao tratamento é preciso considerar os cinco D: diagnóstico, droga, dose, duração do tratamento e diferentes tratamentos. O médico deve considerar a mudança de todos eles caso o paciente não responda num período de 6-8 semanas. Em casos extremos, como em depressões psicóticas, catatônicas, onde o paciente corre risco de vida, podem ser empregados métodos não farmacológicos como eletroconvulsoterapia, estimulação transcraniana, privação do sono (Potter e Hollister 2006).

3.2. BASES NEUROQUÍMCAS DA DEPRESSÃO

3.2.1. Teoria monoaminérgica

A compreensão da depressão como uma alteração na neurotransmissão cerebral está intimamente relacionada à descoberta de que fármacos podiam melhorar os sintomas de depressão. Assim, as teorias sobre a patofisiologia da depressão são derivadas de estudos sobre o mecanismo de ação de medicamentos antidepressivos e das alterações fisiológicas observadas na depressão (Henn *et al.*, 2004). Como mencionado no item 3.1.2, os primeiros antidepressivos a serem descobertos pertenciam a duas classes de medicamentos: os tricíclicos e os inibidores de monoaminoxidases (IMAO). Os primeiros bloqueiam os transportadores de noradrenalina e serotonina, ocasionam aumento da disponibilidade dos respectivos neurotransmissores na sinapse. Os IMAO também aumentam a disponibilidade dos neurotransmissores, mas por outro mecanismo, inibindo sua metabolização pela enzima monoaminooxidase (Duman, 1999; Berton e Nestler, 2006). Por esta razão, a primeira teoria sobre a etiologia da depressão considerava que os pacientes possuíam uma carência de monoaminas, principalmente noradrenalina e serotonina. As evidências para esta hipótese eram bastante simples: certas drogas que depletavam estes neurotransmissores (p.ex. reserpina) poderiam causar depressão, enquanto os antidepressivos conhecidos na época possuíam efeitos farmacológicos de aumentar a quantidade de neurotransmissores na fenda sinaptica (Duman, 1999; Feighner, 1999; Stahl, 2000), originando a idéia de que as quantidades “normais” de monoaminas eram de algum modo depletadas causando a depressão.

Muitos estudos foram feitos para identificar na prática as deficiências teóricas de monoaminas. Ainda que essas observações sugiram que os níveis de monoaminas e a densidade de receptores e transportadores estão fortemente relacionados s com a causa e tratamento da depressão, algumas contradições sugerem um relacionamento mais complexo entre monoaminas e depressão (Maes e Meltzer, 1995; Duman, 1999). Por exemplo, os níveis de NA e 5-HT se elevam algumas horas ou dias após o início do tratamento, enquanto o efeito clínico é observado após aproximadamente 04 semanas (Duman, 1999); em indivíduos normais a depleção de 5-HT ou NA não induz sintomas de depressão

(Miller *et al.*, 1996). Embora a hipótese monoaminérgica seja uma noção simplificada da depressão, ela teve grande valor ao focalizar a atenção sobre os três neurotransmissores monoaminérgicos, dopamina (DA), noradrenalina (NA) e serotonina (5-HT). Isto levou a um melhor entendimento de suas funções fisiológicas e, principalmente, dos vários mecanismos pelos quais todos os antidepressivos conhecidos agem aumentando a neurotransmissão em uma ou mais destas monoaminas (Stahl, 2000).

O tratamento crônico com antidepressivos, principalmente tricíclicos, causa redução na ligação a sítios β1AR (receptores β adrenérgicos tipo 1) em regiões límbicas. Porém, várias observações mostraram que este efeito não pode ser considerado como o responsável pela ação dos antidepressivos (Duman, 1999). Primeiro, nem todos os antidepressivos possuem este efeito e o tempo para o desenvolvimento desta *down-regulation* é mais curto do que o para observado para o surgimento do efeito clínico. Além disso, o uso de antagonistas de βAR como anti-hipertensivos não melhora estados depressivos e pode até mesmo produzi-los em certos indivíduos, enquanto agonistas βAR produzem efeito antidepressivo em modelos animais e pacientes. O mesmo acontece com o receptor 5-HT_{2A}: alguns antidepressivos reduzem sua expressão, em um tempo menor que o necessário para se observar efeitos clínicos, e antagonistas 5-HT_{2A} não são antidepressivos; mas, nem todos os antidepressivos causam este efeito e o tratamento crônico com eletroconvulsoterapia causa aumento nos níveis destes receptores. Em relação aos receptores 5-HT_{1A}, o tratamento crônico com antidepressivos causa desensibilização dos auto-receptores somatodendríticos o que leva ao aumento na transmissão serotoninérgica via receptor 5-HT_{1A} pós-sináptico (Stahl, 1998).

Apesar dos mecanismos envolvidos na depressão e ação dos antidepressivos terem como foco principal a NA e a 5-HT, os receptores dopaminérgicos também são afetados por estes medicamentos. A maioria das evidências mostra que o tratamento crônico com antidepressivos está relacionado

ao aumento das funções dos receptores tipo D_2 (i.e. D_2 e D_3) e a uma diminuição no número e sensibilidade de receptores D_1. Estas mudanças são mais evidentes nas áreas límbicas, i.e. aquelas áreas inervadas por neurônios dopaminérgicos da área ventral tegmental (D'Aquila *et al.*, 2000).

Considerando-se que, a depressão não pode ser explicada apenas em termos de disfunções na neurotransmissão de NA e 5-HT e que o aumento destas monoaminas na fenda sináptica por antidepressivos é apenas o primeiro passo de uma série de eventos mais complexos, nenhuma hipótese baseada em um único sistema de neurotransmissão pode ser considerada como a explicação final tanto dos efeitos terapêuticos dos tratamentos antidepressivos, como do possível substrato biológico da depressão (Duman, 1999; D'Aquila *et al.*, 2000; Millan 2006). Existem crescentes evidências de que na depressão o sistema monoaminérgico não responde normalmente aos estímulos, sugerindo que uma deficiência na transdução do sinal, a partir da ocupação do receptor pela monoamina, pode ser a base de uma resposta celular deficiente (Stahl, 2000). Além disso, a *down-regulation* de receptores apóia a idéia que os receptores permanecem ativados durante o tratamento crônico com antidepressivos. Na verdade, os níveis destes receptores são reduzidos, não completamente eliminados pelo tratamento crônico com antidepressivos, sugerindo que existe um nível suficiente de receptores para responder aos níveis elevados de monoaminas. A redução no número de receptores na presença de níveis elevados de monoaminas pode ser conseqüência do aumento da funcionalidade dos receptores durante tratamentos longos. Isto pode sugerir que há uma ativação sustentada das cascatas de sinalização intracelular reguladas pelos receptores monoaminérgicos. Estas cascatas regulam muitas proteínas celulares e a expressão de genes que podem ser alvo do tratamento com antidepressivos (Duman, 1999).

3.2.2. Teoria neurotrófica

Uma das cascatas de transdução de sinal mais importantes é a que envolve as proteínas G (proteínas ligantes de guanina nucleotídeos), que são os segundos

mensageiros para 80% da sinalização extracelular, incluindo hormônios, neurotransmissores e neuromoduladores (Harrison e Traynor, 2003). Os receptores associados à proteína G (GPCR) mediam respostas à estimulação de hormônios, neurotransmissores, peptídeos e aminoácidos. Quase metade das drogas conhecidas atua por meio dos GPCRs (Landry e Gies, 2003; Alberts, 2004).

As proteínas G são formadas por 03 subunidades: α, β e γ. A ligação de um agonista a um receptor associado à proteína G faz com que a subunidade α desacople GDP (5'-guanosina di-fosfato) permitindo que o GTP (5'-guanosina tri-fosfato)se ligue. A ligação do GTP causa uma mudança conformacional da subunidade α, a qual provoca a liberação do complexo $\beta\gamma$ permite à subunidade α interagir com suas proteínas-alvo. Uma das possíveis proteínas-alvo é a adenilil ciclase, que sintetiza AMPc (3',5'-monofosfato de adenosina cíclico) a partir do ATP (3',5'-trifosfato de adenosina). O AMPc exerce seus efeitos na maioria das células por meio da proteína-cinase dependente de AMPc (PKA). O aumento da concentração de AMPc ativa a PKA no citosol e as subunidades catalíticas liberadas migram para o núcleo, onde fosforilam a proteína reguladora CREB (do inglês *cAMP response element-binding protein*). Uma vez fosforilada, a CREB estimula a transcrição gênica ao ligar-se a uma região reguladora do gene que contém uma seqüência curta de DNA chamada de CRE (do inglês: *cAMP response element*) (Girault e Greengard, 1999; Alberts, 2004).

Duman e col. (1997) propõem que os antidepressivos ativariam o CRE ligado ao BDNF (do inglês: *brain derived neurotrofic factor*) o qual teria sua expressão deficiente em pacientes deprimidos. O BDNF é o fator neurotrófico mais abundante e com maior distribuição no cérebro (Duman, 1999). Além do papel fundamental dos fatores neurotróficos na diferenciação e desenvolvimento celular, eles são necessários para sobrevivência e funcionalidade dos neurônios no sistema nervoso adulto. Diversos estudos sugerem que o BDNF pode ser um alvo de ação dos antidepressivos e que a disfunção deste e outros fatores

neurotróficos pode estar relacionada à patofisiologia da depressão (Duman, 1997; Chen *et al.*, 2001). Sob estresse, o gene para BDNF é reprimido, levando a atrofia e possível apoptose de neurônios vulneráveis no hipocampo (Sheline, 1999). A infusão de BDNF em regiões cerebrais causa efeitos similares aos de antidepressivos em modelos comportamentais como a natação forçada e o desamparo aprendido (Siuciak *et al.*, 1997; Shirayama *et al.*, 2002). Estes efeitos também são observados através do aumento da expressão de CREB no hipocampo (Chen *et al.*, 2001). Além disso, a possibilidade de que os neurônios hipocampais estejam diminuídos em tamanho e prejudicados no funcionamento durante a depressão é apoiada por estudos clínicos de neuro-imagem que mostram uma redução no volume cerebral das estruturas relacionadas (Sapolsky, 2000). Isto conduziu à formulação de uma hipótese neurotrófica da depressão, consistente com um mecanismo pós ligação neurotransmissor-receptor e envolvendo uma anormalidade na expressão gênica (Duman *et al.*, 1997; Sapolsky, 2000; Charney e Manji, 2004). Estudos demonstrando que o estresse e outras agressões do ambiente podem danificar populações específicas de neurônios e, deste modo, contribuir para patofisiologia da depressão em indivíduos vulneráveis apóiam esta hipótese (Chrousous e Gold, 1992. Duman *et al.*, 1997; Monteggia *et al.*, 2004; de Kloet *et al.*, 2005, Duman e Monteggia, 2006). Por sua vez, o aumento de monoaminas na fenda sináptica induzido pelos antidepressivos irá aumentar a ativação de GPCRs levando ao aumento na concentração de AMPc e por conseqüência resultará no aumento da expressão de BDNF, que exercerá ações tróficas em neurônios alvo, aumentando assim a sobrevivência e as funções celulares. da célula (Duman *et al.*, 1997; Duman, 1999; Duman e Monteggia, 2006).

Algumas limitações da hipótese neurotrófica da depressão incluem o fato de nem todos antidepressivos aumentarem os níveis de BDNF e que dependendo da região cerebral onde o BDNF é administrado ele pode ou não ter efeito antidepressivo. Por exemplo, a infusão de BDNF no núcleo acumbens, na área ventro-tegmental (VTA) e no sistema mesolímbico é pró-depressiva (Eisch *et al.*,

2003; Duman e Monteggia, 2006). Outros receptores, fatores neurotróficos e vias de transdução de sinal também podem estar envolvidos na patofisiologia da depressão e mecanismo de ação dos antidepressivos. Um exemplo é o receptor para glutamato NMDA (N-metil D-aspartato), que influencia CREB através da ativação da proteína cinase Ca^{2+}-dependente, sendo que o tratamento crônico com antidepressivos reduz a função deste receptor (Paul *et al.*, 1993; Sheline *et al.*, 1999; Bonanno et al, 2005) e a diminuição de Ca^{2+} intracelular normaliza a atividade neuronal na mania e na depressão (Kristhal *et al.*, 2001). Uma vez que o BDNF e outros fatores de crescimento se ligam ao receptor tirosina cinase (TrkB) a cascata da MAPk (do inglês *mitogen activated protein kinase*) também é ativada (Duman, 1999; Tiraboschi *et al.*, 2004; Tsai, 2006). Tiraboschi *et al.* (2004) demonstraram que o tratamento crônico com fluoxetina não influencia a fosforilação de CREB via PKA, mas que este efeito está relacionado às as cascatas de MAPk e calmodulina cinase (CaMk). Além disso, existem diferentes tipos de subunidades α nas proteínas G que podem estimular (família G_s) ou inibir (família G_i/G_q ou G_i/G_o) a produção de AMPc e, ainda, ativar outras cascatas como a do diacilglicerol (DAG), inositoltrifosfato (IP_3) e fosfolipase C (PLC) (família G_q/G_{11}) (Duman, 1999; Harrison e Traynor, 2003).

3.2.3. Eixo hipotálamo-pituitária-adrenal

Outra hipótese importante sobre a patofisiologia da depressão é a que relaciona a desregulação do eixo hipotálamo-pituiária-adrenal (HPA) como um possível desencadeador de distúrbios do humor. A hiperatividade do eixo HPA é um dos achados mais consistentes na psiquiatria (Barden *et al.*, 1995; Gold *et al.*, 1995; Holsboer e Barden, 1996; Reul e Holsboer, 2002; Pariante at al 2004). Pesquisas realizadas na década de 70 mostraram uma correlação entre distúrbios do humor e níveis anormais de corticóides. Pacientes, principalmente com depressão maior, apresentam alterações como: aumento nas concentrações de cortisol no plasma e urina e de CRH (homônio de liberação da corticotropina) no fluido cérebro-espinhal (CSF); aumento no tamanho das glândulas pituitária e adrenal; aumento de resposta do cortisol ao hormônio adrenocorticotrópico

(ACTH, do inglês *adrenocorticotropic hormone*) (Sachar *et al.*, 1976; Gold *et al.*, 1995; Nemeroff, 1996; Bremner *et al.*, 1997). O tratamento crônico com antidepressivos leva à diminuição plasmática de cortisol (em humanos) e corticosterona (em roedores), afeta marcadores cerebrais como RNAm para CRH, aumenta a expressão de receptores para glicocortióides (GR), aumenta a função de GR e promove a translocação de GRpara o núcleo (Holsboer 2000; Pariante e Miller, 2001).

Sabe-se que o estresse psicossocial pode levar à depressão em indivíduos vulneráveis (Chrousous e Gold, 1998; Tafet e Bernardini, 2003). A adaptação ao estresse modulada por esteróides envolve a integração entre os impulsos excitatórios indicativos de estresse vindos de diferentes receptores sensórios e circuitos neurais inibitórios, que convergem para o núcleo paraventricular (PVN) do hipotálamo, influenciando o eixo HPA. Caso o controle do estresse falhe desenvolve-se um desequilíbrio entre o estímulo estressante e o retro controle negativo no nível do PVN do hipotálamo, resultando na alteração da secreção de peptídeos como o CRH, ACTH e vasopressina. Não é completamente conhecido como o desequilíbrio entre os impulsos excitatórios e inibitórios se desenvolve. O aumento na secreção de cortisol parece induzir uma diminuição de GRs hipocampais (Sapolsky, 2000). Estes receptores são considerados os mais importantes na regulação da resposta ao estresse, enquanto os receptores mineralocorticóides teriam um papel relacionado às flutuações circadianas destes hormônios (Reul e Holsboer, 2002; Pariante, 2006). A diminuição de GRs tem como objetivo contornar a quantidade excessiva de glicocorticóides, e levaria a alteração dos mecanismos de retrocontrole negativo, ou seja os níveis elevados de cortisol circulante poderiam persistir após o estímulo que deu início ao aumento de cortisol haver terminado, podendo ainda resultar em processos degenerativos no hipocampo. Desta maneira, as alterações hipocampais produzidas pela exposição prolongada a níveis excessivos de cortisol, com o conseqüente desajuste da alça de retrocontrole, podem ser responsáveis pela incapacidade dos

glicocorticóides em controlar sua própria secreção durante o estresse crônico (Tafet e Bernardini, 2003).

Holsboer (2000) afirma que a teoria neurotrófica (Duman *et al.*, 1997) e as alterações do eixo límbico-HPA são complementares e não excludentes. O aumento na ativação do receptor GR (i.e. formação de complexos cortisol-GR) levaria à dimuição na transcrição de BDNF. O GR ativado associa-se fisicamente ao CREB e assim diminui sua fosforilação. Como apenas o CREB fosoforilado (p-CREB) pode se ligar ao DNA, a redução de p-CREB resultaria na diminuição da expressão de BDNF, principalmente no hipocampo, o que diminuiria a neuroplasticidade, neurogênese e aumentaria a vulnerabilidade dos neurônios (Holsboer, 2000). Além disso, a ligação de cortisol/corticosterona aos receptores mineralocorticóides ou glicocorticóides pode regular a transcrição de genes que estão envolvidos no controle de receptores ligados à proteína G (GPCR), canais iônicos e receptores ionotrópicos (de Kloet *et al.*, 2005).

Provavelmente a elucidação completa das causas da depressão é ainda mais complexa. Muito especialistas consideram que a depressão deve ser vista como uma síndrome e não uma doença (Gold e Charney, 2002; Tafet e Bernardini, 2003; Evans *et al.*, 2005; Berton e Nestler, 2006; Milan, 2006). Uma das razões para isto é o fato de que diversas moléculas são partilhadas entre os sistemas nervoso central (SNC) e periférico, endócrino e imune, e estes sistemas produzirem regulação recíproca entre si (Chrousos e Gold, 1998; Tafet e Bernardini, 2003).

Assim como o hipocampo, outras estruturas límbicas também regulam o eixo HPA através da liberação de monoaminas. A adrenalina e noradrenalina pertencem ao outro importante sistema de resposta adaptativa ao estresse, o sistema autônomo simpático (SAS). Este sistema e o eixo HPA possuem uma regulação positiva entre si, i.e. a ativação de um envolve a ativação do outro (Abercrombie e Zigmond, 1995).O locus coeruleus (LC) projeta fibras

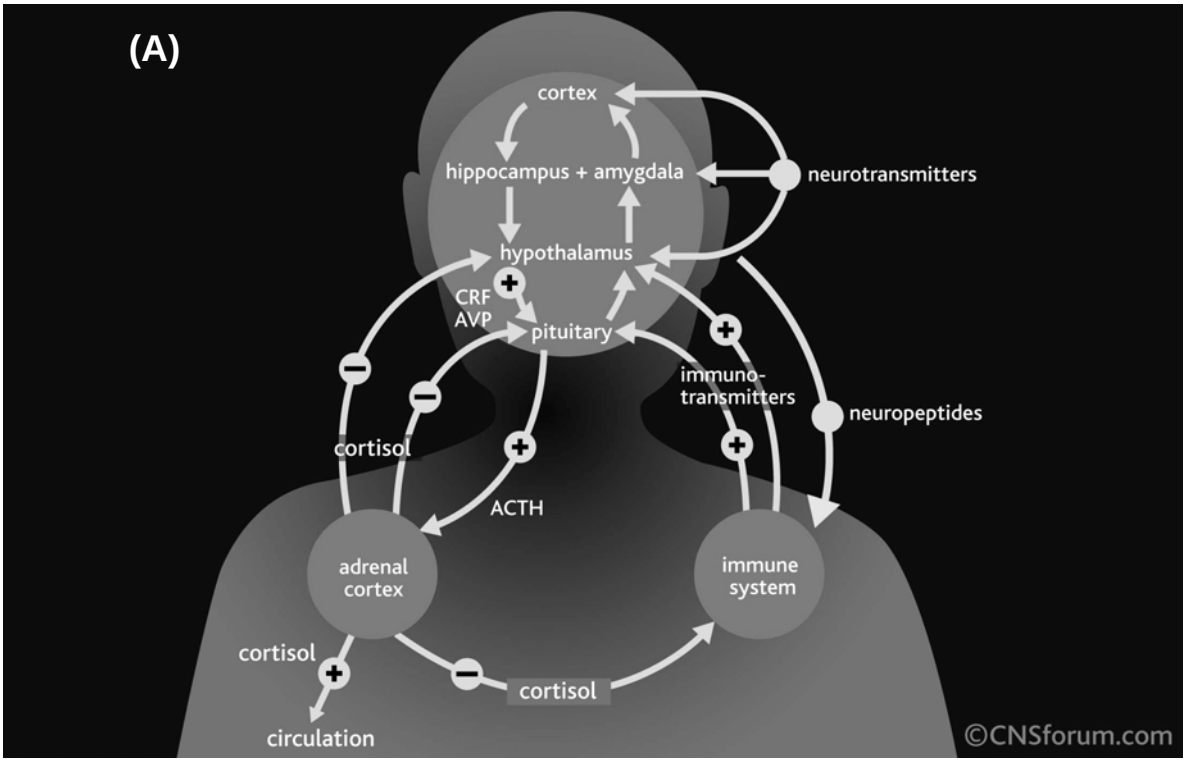
noradrenérgicas para várias estruturas, incluindo a amígdala, hipocampo e PVN do hipotálamo. Juntamente com o LC, o hipotálamo lateral media a ativação do SAS (Charney *et al.*, 1995). O sistema serotoninérgico parece exercer um controle positivo obre o eixo HPA enquanto os glicocorticóides e catecolaminas produzem alterações induzidas pelo estresse no sistema serotoninérgico (Tafet e Bernardini, 2003). As projeções serotoninérgicas a partir dos núcleos mediano (MRN) e dorsal (DRN) da rafe inervam corpos celulares produtores de CRH do PVN hipotalâmico e existem neurônios serotoninérgicos localizados inteiramente no hipotálamo (Azmitia e Whitaker-Azmitia, 1995). O sistema dopaminérgico também está relacionado à regulação em resposta ao estresse do eixo HPA (Nestler e Calerzon, 2006). Existem evidências que agonistas dopaminérgicos exerçam um controle positivo sobre o eixo HPA. A ativação de receptores dopaminérgicos cerebrais, por pergolide e análogos, leva ao aumento da concentração sérica de corticosterona em ratos e este efeito é revertido por antagonistas dopaminérgicos (Fuller *et al.*, 1983; Foreman *et al.*, 1989). As projeções dopaminérgicas mesolímbicas e mesocorticais estão envolvidas nos processos de adaptação. A primeira processa e reforça estimulas relacionados à recompensa e à motivação, enquando a segunda está envolvida em funções cognitivas como a avaliação de situações potencialmente estressantes (le Moal, 1995). O estresse agudo aumenta a liberação mesolímbica de dopamina (Imperato *et al.*, 1993; Lindley *et al.*, 1999; Dazzi *et al.*, 2001). Experimentos de auto-estimulação no hipotálamo demonstraram sua importância nos mecanismos de recompensa (Nestler e Calerzon, 2006).

Existem fortes evidências sobre a regulação recíproca entre o sistema imune e o eixo HPA. Pesquisas mostram que a morbidade associada com depressão vai além dos distúrbios no afeto e humor; e que a mortalidade entre pacientes com depressão maior, em qualquer idade, independentemente de suicídio, uso de drogas e outros fatores prejudiciais à saúde, é duas vezes maior (Chrousos e Gold, 1998; Evans *et al.*, 2005). Várias evidências sugeriem um mecanismo bidirecional entre as desordens do humor e outras doenças. Se aceita

facilmente que doenças incapacitantes são fatores de risco para ocorrência de um episódio depressivo em pessoas vulneráveis; porém um número cada vez maior de estudos vem demonstrando que a depressão pode ser um fator de risco para doenças como cardiopatias, neoplasias, epilepsia, diabetes, dor crônica (Evans *et al.*, 2005). Estudos mostram que pacientes deprimidos têm uma perda significativa de células do córtex frontal, uma área importante para: o discernimento entre recompensa e punição, a mudança do humor de um estado para o outro, e por exercer restrição cortical sobre a amígdala (no controle do medo) da através do eixo HPA e o SNS. Um aumento na secreção do cortisol e da noradrenalina transforma um ambiente estável em um ambiente altamente adverso em termos bioquímicos. Esta condição pode contribuir para diferentes conseqüências adversas incluindo, aumento da gordura visceral, resistência à insulina, aumento na resposta inflamatória, coagulação sanguínea aumentada, fibrólise deficiente, diminuição da formação e aumento na reabsorção óssea (Gold e Charney, 2002).

Os neurônios de CRH hipotalâmicos parecem ser os principais pontos de conexão entre eventos periféricos e respostas do SNC. Assim como a secreção de glicocorticóides é fisiologicamente aumentada em resposta a um processo inflamatório, com o objetivo de restringir o dano tecidual em conseqüência à resposta imune, dados experimentais mostram que mediadores inflamatórios/imunes ativam o eixo HPA via neurônios CRH hipotalâmicos ou via pituitária, podendo levar a respostas patológicas, como por exemplo, à depressão (Ashwell *et al.*, 2000; Sapolsky *et al.*, 2000). Uma hipótese para esta inter-relação seria que as citocinas e outros mediadores imunes seriam ”sensores” moleculares, responsáveis por transformar estímulos não cognitivos (i.e. processos inflamatórios) em estímulos cognitivos, permitindo que o SNC reconheça-os e elabore uma resposta integrada para os eventos periféricos (Chrousos e Gold, 1998). Ao mesmo tempo, o SNC pode reagir a tal estímulo, por exemplo, induzindo ao hipercortisolismo para inibir a resposta inflamatória (Ashwell *et al.*, 2000). Porém, se o aumento nos níveis de glicocorticóides é patologicamente prolongado, por estresse crônico ou em indivíduos deprimidos, a imunossupressão

pode levar à incidência de doenças infecciosas ou auto-imunes e a formação de tumores (Chrousos e Gold, 1998), logo, não é mera casualidade que muitas doenças auto-imunes estão associadas à depressão (Evans *et al.*, 2005). Para facilitar a compreensão deste item por parte dos leitores franceses, um sumário, em inglês, das inter-relações entre o SNC, o eixo HPA e o sistema imune em indivíduos saudáveis e deprimidos está apresentado na figura 1.



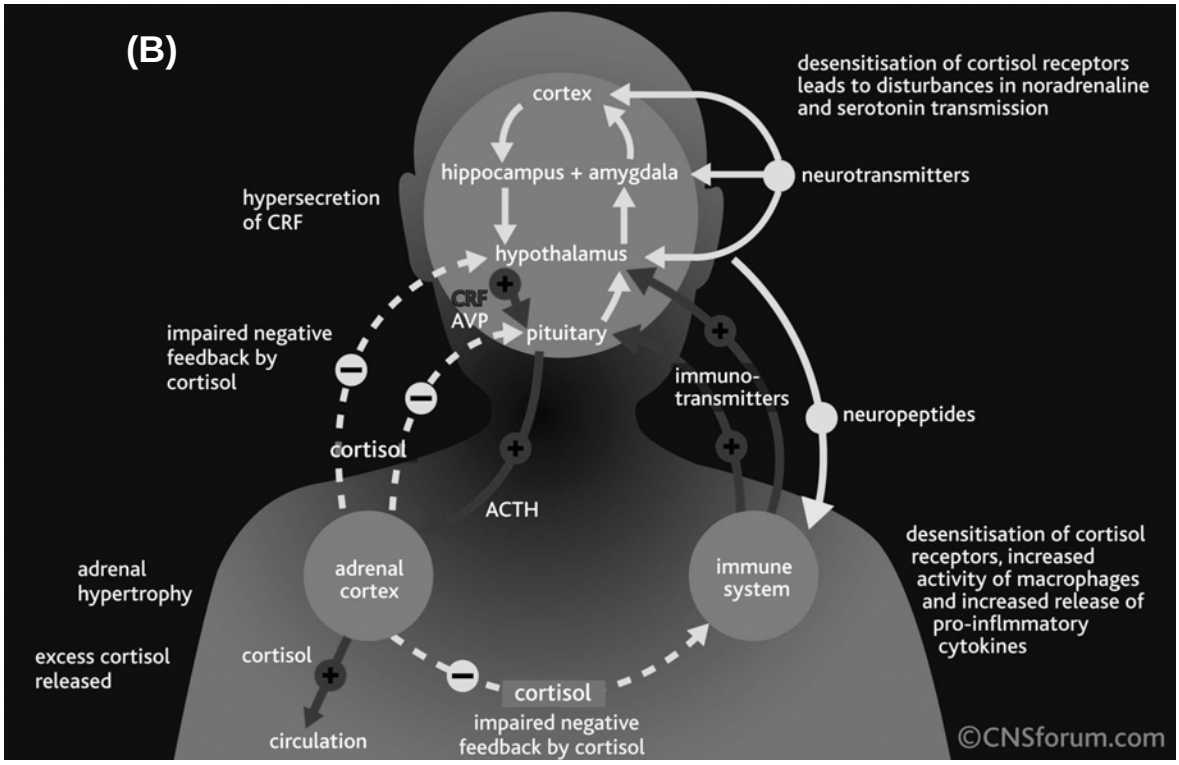


Figure 1: The hypothalamic-pituitary-adrenal (HPA) axis in a normal (A) and depressed (B) person. (A) The hypothalamic-pituitary-adrenal (HPA) axis is a feedback loop that includes the hypothalamus, the pituitary and the adrenal glands. The main hormones that activate the HPA axis are corticotropin-releasing factor (CRF), arginine vasopressin (AVP) and adrenocorticotropin hormone (ACTH). The loop is completed by the negative feedback of cortisol on the hypothalamus and pituitary. The simultaneous release of cortisol into the circulation has a number of effects, including elevation of blood glucose for increased metabolic demand. Cortisol also negatively affects the immune system and prevents the release of immunotransmitters. Interference from other brain regions (eg hippocampus and amygdala) can also modify the HPA axis, as can neuropeptides and neurotransmitters. (B) In depression, the hypothalamic-pituitary-adrenal (HPA) axis is upregulated with a down-regulation of its negative feedback controls. Corticotropin-releasing factor (CRF) is hypersecreted from the hypothalamus and induces the release of adrenocorticotropin hormone (ACTH) from the pituitary. ACTH interacts with receptors on adrenocortical cells and cortisol is released from the adrenal glands; adrenal hypertrophy can also occur. Release of cortisol into the circulation has a number of effects, including elevation of blood glucose. The negative feedback of cortisol to the hypothalamus, pituitary and immune system is impaired. This leads to continual activation of the HPA axis and excess cortisol release. Cortisol receptors become desensitized leading to increased activity of the

pro-inflammatory immune mediators and disturbances in neurotransmitter transmission (From CNSforum Image Bank; http://www.cnsforum.com).

3.3. O GÊNERO *HYPERICUM*

A família Guttiferae é constituída por 50 gêneros e aproximadamente 1000 espécies distribuídas pelas regiões tropicais e subtropicais do planeta (Cronquist, 1981). Entre alguns gêneros importantes da família destacam-se *Calophyllum*, *Garcinia*, *Vismia* e *Hypericum*. Um grande número de espécies desses gêneros tem sido utilizado na medicina tradicional para o tratamento de câncer, doenças de origem viral, bacteriana e fúngica, entre outras. A ampla utilização desses vegetais tem levado à descoberta de inúmeras moléculas com diversas atividades biológicas. Entre estas, destacam-se as substâncias fenólicas. Essas substâncias - em especial as *meta*-diidroxiladas - freqüentemente apresentam-se substituídas por grupamentos prenila, considerados como interessantes grupos farmacofóricos. A prenilação é facilitada pela influência dos grupos hidroxila, os quais aumentam a densidade eletrônica favorecendo energeticamente as reações enzimáticas de substituição eletrofílica com pirofosfato de dimetilialila (Zuurbier *et al.*, 1998). Posteriormente, numa seqüência biossintética, pode ocorrer ciclização da cadeia prenilada originando os derivados dimetil-benzopirânicos correspondentes.

Esse padrão de substituição – hidroxilação, prenilação e posterior ciclização do grupo prenila - é verificado nas diversas substâncias fenólicas encontradas em espécies de Guttiferae como calanolídeos, piranocumarinas com importante atividade anti-HIV-1 (Mckee *et al.*, 1998) e xantonas, as quais têm mostrado ação antiinflamatória, anti-hepatotóxica, antiviral, antimicrobiana, antioxidante, inibidora de monoaminoxidases (IMAO), antiprotozoária e antitumoral (Rocha *et al.*, 1994; Bennet e Lee, 1989). Outras substâncias importantes são benzofenonas, precursores de xantonas, que apresentam atividade antiprotozoária e anti-HIV-1 (Bennet e Lee 1989; Fuller *et al.*, 1999); e derivados de floroglucinol, apresentando atividades antidepressiva, antimicrobiana, cicatrizante, antiproliferativa, entre

outras (Rocha *et al.*, 1994; Ishiguro *et al.*, 1986; Jayasuriya *et al.*, 1991; Rocha *et al.*, 1996).

Em relação aos estudos farmacológicos, o gênero *Hypericum*, constituído por cerca de 400 espécies, tem recebido especial atenção devido à atividade antiviral de quinonas policíclicas - hipericina e pseudo-hipericina - sobre vários retrovirus, *in vitro* e *in vivo*, em particular sobre o HIV (Awang *et al.*, 1991) e pelo emprego terapêutico de *H. perforatum*, a espécie mais conhecida do gênero, como antidepressivo (Linde *et al.*, 1996; Gaster e Holroyd, 2000).

3. 3.1 Dados farmacológicos do gênero *Hypericum*

ATIVIDADE ANTIDEPRESSIVA

A eficácia clínica de *H. perforatum* foi comprovada tanto para medicamentos encontrados no comércio mundial, padronizados em hipericina (Hänsgen *et al.*, 1994; Vorbach *et al.*, 1994; Volz, 1997; Friede *et al.*, 2001), como de extratos padronizados em hiperforina (Biber *et al.*, 1998; Laakman *et al.*, 1998) e em ambos (Philippu *et al.*, 1999). Os medicamentos utilizados nos estudos são basicamente compostos dos seguintes extratos hidroalcóolicos; LI160: 0,2-0,3% de hipericina e 2,5-5% de hiperforina; ZE117 com 0,2% de hipericina; WS5573 e WS5572 com 0,5% e 5% de hiperforina, respectivamente; STW 0,2-0,3% de hipericina e pseudo-hipericina e 2-3% de hiperforina (Viana *et al.,* 2001). Meta-análises (Kim *et al.*, 1999), análise sistemática (Gaster e Holroyd, 2000) e revisões (Kasper, 2001; Käufeler *et al.*, 2001; Bilia *et al.*, 2002) concordam que medicamentos a base de *Hypericum* são mais eficazes do que placebo no tratamento de depressão leve a moderada e apresentam efeitos adversos menos pronunciados que os antidepressivos tricíclicos.

Após o estudo de Shelton e col (2001) publicado no JAMA (*Journal of the American Medical Association*), que não mostrou eficácia de um medicamento

padronizado em hipericina no tratamento de depressão severa, houve grande controvérsia em relação ao uso de extratos padronizados de *Hypericum*. Um estudo brasileiro, empregando medicamento padronizado em hipericina, também demonstrou eficácia menor quando comparado ao grupo fluoxetina e placebo (Moreno *et al.*, 2006). Contudo, estudos recentes utilizando extratos padronizados em hiperforina (WS e STW), demonstraram que estes possuem eficácia maior que placebo (Kasper *et al.*, 2006) e comparável a inibidores seletivos da recaptação de serotonina (ISRS) no tratamento de depressão moderada a severa (Gastpar *et al.*, 2005; Kieser *et al.*, 2005; Szegedi et al 2005). Na revisão sistemática realizada recentemente para base de dados Cochrane, Linde e col (2005) consideram os dados em relação ao uso clínico de *Hypericum* são inconsistentes e confusos, principalmente para o tratamento de depressão maior, pois existe grande heterogeneidade entre os estudos placebo controlados, ainda que os estudos comparando extrato versus antidepressivos sejam mais homogêneos e mostrem eficácia equivalente.

Inicialmente, o principal constituinte a ser considerado como responsável pela atividade antidepressiva da espécie *H. perforatum* foi a hipericina (Suzuki *et al.*, 1984; Wagner e Bladt, 1994; Perovic e Müller, 1995). Posteriormente, acumularam-se evidências de que a ação terapêutica depende de mais de um constituinte, entre os quais destacam-se os floruglucinóis, hiperforina e ad-hiperforina (Bhattacharya *et al.*, 1998; Chatterjee *et al.*, 1998a, b; Müller *et al.*, 1998; Kaehler *et al.*, 1999), compostos fenólicos, como procianidinas e flavonóides (quercetina, quercitrina, e hiperosídeo) (Butterweck *et al.*, 1998, 2000; Calapai *et al.*, 1999) e xantonas (Hölz *et al.*, 1989; Wagner e Bladt, 1994).

O primeiro mecanismo de ação proposto para o extrato bruto foi a inibição de monoamino oxidases (MAO_A e MAO_B), a qual foi atribuída à hipericina (Suzuki *et al.*, 1984). Contudo, estudos utilizando hipericina e hiperforina puras não confirmaram esta ação (Cott, 1997; Chatterjee *et al.*, 1998a). A inibição da MAO pelo extrato bruto também não é farmacologicamente relevante, visto que não foi

confirmada *in vivo*: Wagner e Bladt 1994) não observaram este efeito *ex vivo,* após a administração de 300 mg/kg de extrato de *H. perforatum* em ratos; estudos farmacocinéticos demonstram que os níveis sanguíneos de hipericina são de magnitude muito abaixo das concentrações necessárias para a inibição da MAO (10^{-3} mol/L, para hipericina e 10^{-4} mol/L, para o extrato bruto) (Staffeldt *et al.*, 1994; Thiede e Walper, 1994). A inibição da catecol-o-metil-transferase (COMT) também foi demonstrada com concentrações acima dos fisiologicamente possíveis (500 μg/ml) (Thiede e Walper, 1994).

Outro possível mecanismo de ação sugerido é a inibição da recaptação de serotonina. Para extratos padronizados em hipericina foi verificada concentração inibitória 50% (CI_{50}) de 6,2 μg/ml (Perovic e Müller, 1995). Porém, estudos recentes concluem que esta concentração seria 10 a 20 vezes maior que a dose biodisponível e que a hipericina, quando testada na concentração de 1 μM, a qual seria mais aproximada da concentração disponível *in vivo*, não causa inibição significativa na captação de serotonina (Raffa, 1998). Por outro lado, estudos em ratos demonstraram que o efeito do extrato alcóolico de *H. perforatum* é revertido ao administrar-se antagonistas serotoninérgicos (pindolol e WAY-100635) em modelo de desamparo aprendido (Gambara *et al.*, 1999). Por outro lado, o tratamento subcrônico com extrato metanólico causa uma *down-regulation* de receptores β–adrenérgicos e uma *up regulation* de receptores serotoninérgicos em córtex frontal de ratos (Müller *et al.*, 1997; 1998; Kientsch *et al.*, 2001), efeito análogo ao observado após tratamento com antidepressivos tricíclicos. Nos poucos trabalhos existentes de microdiálise cerebral envolvendo extratos de *H. perforatum* investigam o efeito sobre os níveis cerebrais das monoaminas e seus metabólitos os resultados sobre os níveis cerebrais de DA são os mais consistentes. Diferentes tipos de extrato de *H. perforatum*, após administração aguda ou repetida pelas vias oral ou intraperitoneal, causam aumento nos níveis cerebrais de DA no núcleo acumbens (Di Matteo *et al.*, 2000; Rommelspacher *et al.*, 2001) e córtex frontal (Yoshitake *et al.*, 2004). Apenas tratamentos repetidos foram capazes de verificar aumento nos níveis cerebrais de 5-HT no núcleo

acumbens (Rommelspacher *et al.*, 2001), enquanto no córtex frontal o tratamento agudo causa aumento significativo desta monoamina (Yoshitake *et al.*, 2004). Nenhum trabalho encontrado observou efeito sobre os níveis cerebais de NA.

Também foi sugerido que extratos com diferentes concentrações de hipericina, hiperforina ou flavonóides, e seus respectivos derivados, atuem diferentemente nos receptores relacionados a patofisiologia da depressão (Bhattacharya *et al.*, 1998; Butterweck *et al.*, 1998; Calapai *et al.*, 1999; Philippu, 2001).

A hiperforina, *in vitro*, inibe inespecificamente a recaptação sinaptosomal de serotonina (5-HT), dopamina (DA), noradrenalina (NA), GABA e glutamato, com CI_{50} (μg/ml) de 0,11; 0,06; 0,04; 0,09 e 0,44; respectivamente (Chaterjee *et al.*, 1998b; Kaehler *et al.*, 1999; Müller *et al.*, 1998, 2001). Estudos de microdiálise em ratos indicam que o tratamento com extrato padronizado em hiperforina (4,67 %) aumenta as concentrações extracelulares de dopamina, noradrenalina e serotonina (Philippu, 2001; Rommelspacher *et al.*, 2001) e acetilcolina (Buchholzer, 2002; Kiewert *et al.*, 2004). Este estudo de Phillipu e col. (2001) mostra ainda que extratos sem hiperforina diminuem a concentração de serotonina, enquanto que as de dopamina e noradrenalina continuam aumentadas. Estudos *in vitro*, empregando extratos preparados utilizando-se CO_2 supercrítico (altamente lipofílicos) ricos em hiperforina (4,5 % e 38,8 %), demonstraram inibição do efeito contrátil da serotonina sobre íleo isolado de cobaio e da recaptação de serotonina por células peritoneais, indicando que alguns efeitos centrais de *H. peforatum* possam ser mediados por receptores 5-HT_3 e 5-HT_4 (Chatterjee *et al.*, 1998a). Outros relatos, utilizando extratos alcóolicos padronizados em hipericina, relacionaram a atividade destes extratos aos receptores dopaminérgicos, através de antagonismo farmacológico no teste de natação forçada e desamparo aprendido (Butterweck *et al.* 1997; Gambara *et al.* 1999).

Estudos em animais demonstraram que após tratamento crônico com extrato metanólico, hipericina pura ou hipericina e procianidinas, ocorre diminuição nos níveis de corticosterona e ACTH (hormônio adrenocorticotrófico), sem alteração nos níveis de prolactina (Butterweck *et al.*, 2001). Em um teste em voluntários, utilizando medicamento padronizado em hiperforina, foi verificado um aumento nas concentrações plasmáticas de cortisol e de hormônio do crescimento sem alteração na concentração de prolactina (Schüle *et al.*, 2001). Em outro estudo, voluntários que receberam medicamento padronizado em hipericina e hiperforina apresentaram aumento nos níveis plasmáticos de hormônio do crescimento e diminuição nos de prolactina (Franklin *et al.*, 2001). Esses resultados vão ao encontro de achados pré-clínicos que indicam uma ação sobre o sistema dopaminérgico, pois estas vias facilitam a liberação do hormônio de crescimento e suprimem a secreção de prolactina (Cooper *et al.*, 1996).

Flavonóides glicosilados isolados e frações ricas em procianidinas possuem atividade no teste de natação forçada tanto no tratamento agudo quanto por 14 dias (Butterweck *et al.*, 2000 e 2001). Calapai e col. (1999) avaliaram o efeito da administração, em ratos, de extratos contendo diferentes concentrações de flavonóides, sobre os níveis de 5-HT, NA e DA em três regiões cerebrais (córtex, diencéfalo e tronco cerebral). O extrato com maior concentração de flavonóides causou um aumento significativo nos níveis de 5-HT e NA no tronco e diencéfalo. Segundo os autores, este aumento pode indicar um modo de ação diferente para este extrato de *H. perforatum*, relacionado à atividade sobre eixo hipotálamo-pituitária-adrenal (HPA). Testes in vivo (Franklin et al 2000, 2004) e *in vitro* (Simmen *et al.*, 2001; 2003) demonstraram que a hiperforina, a hipericina e a pseudo-hipericina possuem atividade sobre o eixo HPA, diminuindo os níveis plasmáticos de corticosterona e agindo como antagonistas do fator de liberação de corticotropina, *in vitro*.

Os grupos de Müller (1998, 2001) e Chatterjee (1998 e 2001) propõem que a hiperforina é o principal constituinte neuroativo de *H. perforatum* com potencial

efeito antidepressivo e um mecanismo de ação completamente diferente dos antidepressivos atuais. Este floroglucinol age de maneira diferenciada sobre os sítios de recaptação, inibindo tanto as aminas, NA, DA e 5-HT, como os aminoácidos, L-glutamato e GABA (Chatterjeee *et al.*, 1999). A ad-hiperforina inibe a recaptação destes neurotransmissores com potência equivalente a hiperforina (Jensen *et al.*, 2001; Muller *et al.*, 2001; 2003). Entretanto, estes floroglucinóis são fracos ligantes de sítios de recaptação (Gobbi *et al.*, 2001; Jensen *et al.*, 2001), o que conduziu à hipótese de que eles atuem em canais iônicos ligante dependente, principalmente de sódio e cálcio, e também influenciem mecanismos reguladores de pH (Singer *et al.*, 1999; Wonnemann *et al.*, 2000; Chatterjee *et al.*, 2001; Marsh e Davies, 2002; Roz e Rehavi, 2003). Estes achados explicam sua não seletividade sobre os diferentes transportadores de neurotransmissores (Muller *et al.*, 2001). Embora estes efeitos não estejam completamente caracterizados, é ainda sugerido que esta ação ocorra não nas proteínas ou outro mecanismo associado à membrana, mas sim sobre a vesícula sináptica (Kristhal *et al.*, 2001; Roz *et al.*, 2002) podendo a hiperforina, até mesmo, agir sobre a fluidez das membranas (Eckert e Müller; 2001).

Além da ação dos extratos sobre os receptores relacionados à teoria monoaminérgica da depressão, existem outros mecanismos que podem estar relacionados com a modulação da atividade antidepressiva de *H. perforatum*. Estudos em modelos animais de depressão demonstraram que ao empregarem antagonistas opióides o efeito de extratos padronizados em hipericina é antagonizado (Butterweck *et al.*, 1997; Panocka *et al.*, 2000). Extratos brutos de *H. perforatum* reduzem a expressão de citocinas (Thiele e Walper *et al.*, 1994; Calapai *et al.*, 2001), que têm sido consideradas como relevantes na etiologia da depressão (Nestler, 1998).

Existem poucos relatos sobre a avaliação de outras espécies com possível ação antidepressiva. *H. calycinum*, *H. canariense, H. glandulosum, H.*

grandifolium e *H. reflexum* foram ativos no modelo animal de depressão Teste de Natação Forçada (Özturk *et al.*, 1996; Sanchez-Mateo *et al.* 2002). Entre as espécies brasileiras apenas *H. brasiliense* havia sido estudado, apresentando atividade inibidora de monoamino oxidase in vitro (Rocha *et al.*, 1994).

AÇÃO ANALGÉSICA

Experimentos utilizando extratos alcoólicos de *H. perforatum* demonstraram atividade analgésica no teste de *tail-flick* (Özturk *et al.*, 1996, Kumar *et al.*, 2001), placa aquecida e teste das contorções abdominais induzidas por ácido acético (Kumar *et al.*, 2001). Entretanto, outro estudo utilizando o teste de *tail-flick* e o da placa quente apresentou resultados negativos para o extrato hidro-alcóolico de *H. perforatum* (Gambara *et al.*, 1999). Experimentos *in vitro* indicaram um possível efeito analgésico para hiperforina, uma vez que ela bloqueia canais de sódio e inibe receptores do tipo ATP dependentes, envolvidos na transdução do estímulo de dor (Kristhal *et al.*, 2001). Estudos de *binding* demonstraram afinidade de hipericina (1μM) por receptores opióides tipo σ, com inibição de *binding* específico de 48 % (Raffa, 1998). Em um estudo com medicamento padronizado em hipericina sobre o efeito em dor neuropática em humanos não houve diferença em relação ao placebo (Sindrup *et al.*, 2000).

Outras espécies que demonstraram atividade antinociceptiva em modelos animais, *tail-flick*, teste da formalina e teste das contorções abdominais induzidas por ácido acético foram *H. calycinum* e *H. triquefolium* (Özturk *et al.*, 1996; Apaydin *et al.*, 1999; Trovato *et al.*, 2001). As espécies H. canariense H. glandulosum H. reflexum além do efeito analgésico também apresentaran atividade antiinflamatória (Rabanal *et al.*, 2005; Sanchez-Mateo *et al.*, 2006). *H. brasiliense* apresentou atividade no teste da placa aquecida e *tail-flick* (Mendes *et al.*, 2002).

AÇÃO ANSIOLÍTICA

Estudos de *binding*, com um extrato alcóolico de *H. perforatum*, demonstraram afinidade por receptores de adenosina, benzodiazepínicos, $GABA_A$ e $GABA_B$, com K_i (μg/ml) de 1; 24; 0,075 e 0,006 respectivamente (Cott, 1997). A atividade ansiolítica *in vivo,* foi verificada em extratos de *H. perforatum* padronizados tanto em hiperforina quanto em hipericina (Bhattacharya *et. al*., 1998; Chatterjee *et al.*, 1998a; Vandenbogaerde *et al.*, 2000; Coleta *et al.*, 2001). A hiperforina em baixas doses (1mg/kg) e seu derivado sintético, acetato de hiperforina (3-5 mg/kg) possuem efeito ansiolítico no teste do labirinto em cruz (Chatterjee *et al.*, 1998a; Zanoli *et al.*, 2002) enquanto o extrato lipofílico apresenta este efeito apenas em doses altas (300mg/kg) (Bhattacharya *et al*., 1998). Uma vez que o aumento nos níveis extracelulares de GABA causado por hiperforina *in vitro* (Chatterjee *et al.*, 1998a) não foi observado *in vivo* (Kaehler *et al.*, 1999), Chatterjee e col. (1998a) argumentam que o efeito ansiolítico apresentado pela hiperforina possa ser causado pela ação sobre receptores serotoninérgicos tipo 5-HT_3 e 5-HT_4, relacionados à atividade ansiolítica (Barnes e Sharp, 1999). Esta inferência deve-se ao fato de que, quando testada em preparação de íleo isolado de cobaio, a hiperforina inibiu a contração do órgão induzida por serotonina (Chatterjee *et al.*, 1998a). No íleo são encontrados receptores 5-HT_3 e 5-HT_4, relacionados à atividade ansiolítica (Barnes e Sharp, 1999).

Vandenbogaerde e col. (2000), utilizando técnicas de *patch-clamp*, relacionaram a ação ansiolítica à atividade da hipericina e pseudo-hipericina, demonstrando que esta estimula receptores $GABA_A$ e inibe a transmissão glutamatérgica mediada por receptores NMDA. O extrato aquoso de *H. perforatum* também apresentou atividade ansiolítica, a qual os autores relacionaram a presença de flavonóides e hipericina, uma vez que o extrato não possuía hiperforina (Coleta *et al.*, 2001).

Testes utilizando modelos comportamentais diferenciados, como o labirinto em T, *The Mouse Defense Test Battery* (MDTB) e o teste de esconder esferas

(*marble-burying test*), mostraram que o extrato LI 160 possui potencial efeito no tratamento de síndrome do pânico e transtorno obsessivo compulsivo (Flausino *et al.*, 2002; Beijamini e Andreatini, 2003; Skalisz *et al.*, 2004).

AÇÃO ANTIMICROBIANA E ANTIPARASITÁRIA

Em 1954, Neuwald e Hagenström (*apud* Reichling *et al.*, 2001) reportaram atividade contra *Staphylococcus aureus* para os extratos éter de petróleo e acetona de *H. perforatum*. Na Rússia, extratos acetônicos são usados clinicamente para tratar infecções bacterianas e destes extratos, foi isolada e identificada a hiperforina (Bystrov *et al.*, 1975 *apud* Reichling *et al.*, 2001), a qual foi ativa contra bactérias gram-positivas, mas inativa contra gram-negativas e fungos (Gurevich *et al.*, 1971 *apud* Reichling *et al.*, 2001; Ishiguro *et al.*, 1986; Jayasuriya *et al.*, 1991; Tada *et al.*, 1991; Yamaki *et al.*, 1994; Rocha *et al.*, 1995). Floroglucinóis extraídos de *H. papuanum* foram ativos contra *Bacillus cereus, Staphylococcus epidermidis* e *Micrococcus luteus* (Winkelmann *et al.*, 2000). A atividade antimicrobiana de *H. perforatum* também está relacionada com a presença de óleos voláteis e flavonóides (Maisenbacher e Kovar, 1992; Trifunovic *et al.*, 1998). Reichling e col. (2001) encontraram atividade contra bactérias gram-positivas, até mesmo para *Staphylococcus aureus* resistente a meticilina, em infusões preparadas com as partes aéreas de *H. perforatum*.

Saroaspidina A, B, C e sarotroleno C e D, floroglucinóis de *H. japonicum*, apresentaram efeito antibiótico *in vitro* (Ishiguro *et al.*, 1987; Ishiguro *et al.*, 1994). Em *H. calycinum*, foi identificado um floroglucinol com atividade antimalárica e antifúngica (Decosterd *et al.*, 1991). Um extrato alcóolico de *H. brasiliense* demostrou atividade inibitória sobre bactéria *B. subtillis* em cromatografia bioautográfica (Rocha *et al.*, 1995; Rocha *et al.*, 1996).

As xantonas identificadas em *H. roeperanum* possuem atividade antifúngica contra *Candida albicans* (Rath *et al.*, 1996), e as xantonas presentes em *H. patulum* demonstraram atividade antimicrobiana (Ishiguro *et al.*, 1996). Extrato

metanólico de *H. patulum* também apresentou eficácia comparável ao nitrofural em modelo de cicatrização em ratos (Mukherjee *et al.*, 2000) e floroglucinóis isolados desta espécie apresentaram atividade antimicrobiana (Winkelmann *et al.*, 2001). Os óleos voláteis de *H. scabrum*, *H. scabroides*, *H. triquetrifolium* (Kizil et al, 2004), *H. rumeliacum* (Couladis *et al.*, 2003), *H. hyssopifolium* e *H. lysimachioides* (Toker *et al.*, 2006) também possuem atividade antibacteriana *in vitro.*

AÇÃO ANTIVIRAL

Estudos mostraram que hipericina e pseudo-hipericina inibem uma variedade de vírus encapsulados, inclusive herpes simples dos tipos 1 e 2, vírus de imunodeficiência humana tipo 1 (HIV-1), vírus *para*-influenza 3 e citomegalovírus (Wood *et al.*, 1990; Taylor *et al.*, 1996; Axarlis *et al.*, 1998). Estudos clínicos com hipericina em pacientes com SIDA (síndrome da imunodeficiência adquirida) demonstraram redução na carga viral, sem sinais de toxicidade nem fotosensibilidade cutânea. Infusões de *H. perforatum*, contendo flavonóides, saponinas, ácidos fenólicos, taninos e polissacarídeos(Serkedjieva *et al.*, 1990), e extratos de *H. mysorense, H. hookerianum* (Vijayan *et al.*, 2004) apresentaram atividade contra vírus de herpes simples tipo 1 e influenza tipo A e B.

ATIVIDADE CITOTÓXICA

A hipericina é a principal substância fototóxica de *H. perforatum* (Wilhelm *et al.*, 2001). Quando foto-ativada, a hipericina é citotóxica *in vitro* e *in vivo*, sendo um potencial agente fotossensibilizador para o tratamento de tumores na terapia fotodinâmica (Vandenbogaerde *et al.*, 1996; Ali *et al.*, 2001). Existem relatos de que a hipericina inibe o crescimento de linhagens de células de glioma através da inibição da proteína cinase C (Couldwell *et al.*, 1997). Ensaios *in vitro* indicam que a hipericina é um potente antagonista da ligação de topoisomerase II e/ou da clivagem de DNA (Peebles *et al.*, 2001). Porém também existem certas linhagens de células tumorais contra as quais o extrato foi ativo, mas hipericina e hiperforina

não, como a K562 (células eritroleucêmicas) (Roscetti *et al.*, 2004), T24 e NBT-II (câncer de bexiga) (Skalkos *et al.*, 2005). Outras espécies que apresentaram efeito citotóxico foram *H. papuanum* e *H. sampsonii* (Hu *et al.*, 1998; Winkelmann *et al.*, 2001).

ATIVIDADE SOBRE O SISTEMA ENZIMÁTICO CITOCROMO P450 E GLICO PROTEÍNA P

Em 1999 Ernst questionou a segurança do uso medicamentos a base de *Hypericum perforatum* devido a vários relatos de caso mostrando interações entre estes e medicamentos, principalmente contraceptivos orais, imunossupressores, anticoagulantes anti-histamínicos e digitálicos. Estudos clínicos verificaram que pacientes utilizando *H. perforatum* apresentam diminuição nos níveis plasmáticos de medicamentos metabolizados pelo sistema enzimático citocromo P450 (CYP450), indicando que os extratos causam uma indução deste sistema (Mai *et al.*, 2004; Herbert *et al.*, 2004). Estudos *in vitro* demonstraram que esta indução poderia ser causada pela ativação do receptor nuclear pregnano X, o qual induz a expressão da sub-enzima CYP3A4 (Moore *et al.*, 2000). Contudo, as conseqüências da ação de *H. perforatum* sobre o CYP450 ainda são controversas (Jobst *et al.*, 2000; de Smet e Touw, 2000; Wheatley, 2000; Yue *et al.*, 2000). Obach (2000) verificou que o extrato bruto, hiperforina, hipericina e I3-II8-biapigenina inibem competitiva e não-competitivamente a atividade de diferentes enzimas do CYP450. Existem estudos em animais que verificaram ativação das enzimas do sistema CYP450 (Durr *et al.*, 2000; Krusekopf *et al.*, 2003) enquanto outro obteve resultado negativo (Nölder & Chatterjee, 2001). Um estudo em humanos, demonstrou que a co-administração de medicamento a base de *H. perforatum* não alterou o perfil farmacocinético de dextrometofano (metabolizado pelo CYP2D6) nem de alprazolam ou midazolam (metabolizados pelo CYP3A4) (Markowitz *et al.*, 2000; Hall *et al.*, 2003). Entretanto, no estudo de Hall e col. (2003) assim como em outros (Pfrunder *et al.*, 2003, Murphy *et al.*, 2005) a co-administração de *H. perforatum* causou a redução da área sobre a curva, da meia-vida de contraceptivos orais e sangramentos no meio do ciclo. Além do efeito

sobre CYP3A4, não pode ser descartada a possibilidade de inibição das outras isoenzimas do complexo CYP450 e da influência em processo de fase II do metabolismo (sulfatação, acetilação ou glicuronidação) (Marowitz *et al.*, 2000). Outro evento que pode determinar a redução da biodisponibilidade de alguns medicamentos co-administrados com *H. perforatum* é sua atividade indutora da glicoproteína P, ocasionando a redução na absorção intestinal, como é o caso da digoxina (Durr *et al.*, 2000; Wang *et al.*, 2004; Tian *et al.*, 2005).

Revisões recentes confirmam que extratos de *H. perforatum* e alguns de seus principais metabólitos, hipericina e hiperforina, induze a glicoproteína P intestinal e a enzima CYP 450 hepática e intestinal, reduzindo a concentração plasmática de alguns medicamentos como, agentes antineoplásicos, anti-coagulantes, anti-hitamínicos, contraceptivos orais, digitálicos, imunossupressores, (Zhou *et al.*, 2004; Mannel, 2005; Singh, 2005). Apesar disto, os extratos são seguros quando prescritos por médicos que conheçam suas possíveis interações medicamentosas (Knuppel e Linde, 2004; Mannel, 2005).

OUTRAS ATIVIDADES

Extratos de *H. perforatum* tem efeito nootrópico (Kumar *et al.*, 2000; Khalifa 2001). Klusa e col (2001) demonstraram que baixas doses de extrato etanólico (50 mg/kg, v.o.) de *H. perforatum* facilitam a consolidação de memória em animais e que a hiperforina pura (1,25 mg/kg, v.o.) apresenta maior potência como agente nootrópico do que como antidepressivo no modelo de esquiva passiva (Klusa *et al.*, 2001). Em experimentos *in vitro*, a hiperforina em concentrações nanomolares parece apresentar efeito neuroprotetor por inibir canais voltagem-dependente de Ca^{++}, NMDA, AMPA e purinérgicos, que são alvos farmacologicamente importantes para prevenir a excitotoxicidade causada pela saturação de Ca^{++}. Enquanto que em altas concentrações (micromolar), este metabólito secundário aumenta o cálcio intracelular (Chatterjee *et al.*, 1999; Krishtal *et al.*, 2001). Um estudo piloto, em pacientes sofrendo de fatiga, demonstrou redução dos sintomas após 6 semanas de tratamento com medicamento a base de extrato metanólico de

H. perforatum padronizado em hipericina. Juntamente com as medidas de fadiga foi empregada uma escala de ansiedade e depressão (*Hospital anxiety and depression scale*) a qual foi significativamente reduzida durante este período (Stevinson *et al.,* 1998).

Neary e col. (2001) demonstraram que o extrato LI 160 (5 % de hiperforina) ativa uma das proteínas da cascata da MAPkinase (*mitogen induced protein kinase*), envolvida na regulação gênica relacionada à proliferação e sobrevivência celular (Kolch, 2000).

Extratos hidroalcóolicos de *H. perforatum* reduzem o consumo de álcool em linhagens de ratos geneticamente selecionadas para preferirem etanol (10 %) a água. Nestas linhagens, que normalmente possuem alta tendência à depressão, o tratamento com os extratos diminuiu o tempo de imobilidade no teste de natação forçada, juntamente com a redução de consumo de álcool, podendo, portanto, seu efeito sobre o consumo de etanol estar relacionado com sua ação antidepressiva (De Vry *et al.*,1999; Carai *et al.*, 2000; Pandocka *et al.*, 2000; Perfumi *et al.*, 2005).

Floroglucinóis de *H. chinense* e *H. erectum* demontraram atividade inibitória de leucotrieno D_4 e tromboxano A_2, o que pode indicar uma atividade antialérgica (Tada *et al.*, 1991). Frações de *H. perforatum* ricas em procianidinas antagonizaram o efeito de vasocontração induzido por histamina e prostaglandina $F_{2\alpha}$ em artérias, podendo este efeito estar relacionado a inibição de fostodiesterases e/ou da enzima conversora de angiotensina (Melzer *et al.*, 1991).

3.4. REVISÃO DOS DADOS DE ESPÉCIES DE *HYPERICUM* NATIVAS DO RIO GRANDE DO SUL

3.4.1 Dados químicos

Os estudos fitoquímicos, realizados no laboratório de Farmacognosia da Faculdade de Farmácia - UFRGS, verificaram a pesença das seguintes substâncias:

Derivados de floroglucinol: Até o momento, todos os derivados de floroglucinol identificados apresentam estrutura dimérica consistindo de um ácido filicínico ligado a um floroglucinol. A partir do extrato ciclo-hexano das partes aéreas de *H. myrianthum*, foram isolados os floroglucinóis já descritos: japonicina A (Dall' Agnol *et al.*, 2003), presente em *H. japonicum* (Ishiguro *et al.*, 1987) e *H. brasiliense* (Rocha *et al.*, 1995), e uliginosina B (Ferraz *et al.*, 2002a) também presente em *H. carinatum* e *H. polyanthemum* (Nör *et al.*, 2004). Hiperbrasilol B, previamente isolado de *H. brasiliense* (Rocha *et al.*, 1996), foi identificado em *H. carinatum* e *H. caprifoliatum.* A partir de *H. caprifoliatum* foi também isolado um conjunto de tautômeros, cuja análise espectroscópica por ressonância magnética nuclear (RMN) demonstrou tratar-se de um derivado de floroclucinol ligado ao ácido filicínico (Viana, 2002). A separação e elucidação estrutural destes tautômeros estão ainda em andamento.

Benzopiranos: A partir do extrato clorofórmico das partes aéreas de *H. polyanthemum,* três benzopiranos de estrutura inédita foram isolados: **HP1** (6-isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano), **HP2** (7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano) e **HP3** (5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano) (Ferraz *et al.*, 2001). Posteriormente, estes benzopiranos também foram isolados de *H. ternum* (Ferraz *et al.*, 2005c). Utilizando a técnica de micropropagação *in vitro*, Bernardi e col. (2005a) conseguiram que as plântulas de *H. polyanthemum* produzissem benzopiranos. Estas plântulas foram aclimatizadas com sucesso e mostraram o mesmo perfil de benzopiranos encontrado na planta selvagem (Bernardi *et al.* 2005a).

Taninos e flavonóides: A avaliação de taninos demonstrou teores entre 5 a 16%; *H. myrianthum* (5,1%), *H. caprifoliatum* (6,4%), *H. polyantemum* (6,7%), *H. carinatum* (9,1%), *H. connatum* (11,5%), *H. ternum* (16,7%) (Dall' Agnol *et al.*, 2003). A fração fenólica não-tanante destas espécies foi analisada quanto à presença de flavonóides glicosilados. Foram encontrados os compostos

normalmente citados na literatura: hiperosídeo, quercitrina, isoquercitrina, guaijaverina (Dall' Agnol *et al.*, 2003).

Benzofenonas: A partir das partes aéreas de *H. carinatum,* foram isoladas duas benzofenonas inéditas, carifenona A e carifenona B (Bernardi *et al.*, 2005b).

Óleos voláteis: As espécies de *Hypericum* não são caracteristicamente aromáticas, nas espécies *H. caprifoliatum*, *H. polyanthemum*, *H. myrianthum*, *H. carinatum*, *H. connatum* and *H. ternum* a quantidade de óleos voláteis varia de 0,1% a 0,5%. Nestas espécies, sesquiterpenos estão em maior concentração que monoterpenos e todas possuem alcanos, principalmente nonano e undecano (Ferraz *et al.*, 2005a).

Após investigação fitoquímica de oito espécies, *H. brasiliense, H. carinatum, H. caprifoliatum, H. connatum, H. cordatum, H. myrianthum, H. polyanthemum* e *H. piriai*, Ferraz *et al.* (2002b) verificaram a ausência de hipericina em todas as espécies. Este resultado está de acordo com a divisão quimiotaxonômica proposta por Robson (1990), onde a produção de hipericina é relacionada com a presença de glândulas negras nas folhas. As espécies brasileiras, que pertencem às seções *Brathys* e *Trigynobrathys* possuem apenas glândulas pálidas, que não produzem hipericina.

3.4.2 Dados biológicos

ATIVIDADE ANTIPROLIFERATIVA E ANTIOXIDANTE

Ferraz e colaboradores (2005 b, c), analisaram o efeito antiproliferativo dos extratos brutos e frações (hexano, clorofórmio e metanol) de seis espécies de *Hypericum* nativas do sul do Brasil (*H. caprifoliatum*, *H. carinatum*, *H. connatum*, *H. myrianthum*, *H. polyanthemum* e *H. ternum*) e de três benzopiranos (HP1, HP2 e HP3) de *H. polyanthemum*. Extratos e frações de *H. caprifoliatum*, *H. myrianthum*, *H. ternum* e os benzopiranos apresentaram atividade antiproliferativa

contra as células de carcinoma de cólon humano HT-29, células não pequenas de carcinoma de pulmão H-460 e células de glioma humano maligno U-373.

As benzofenonas carifenona A e B, isoladas de *H. carinatum,* foram testadas no teste de TRAP (do inglês: *total radical-trapping parameter assay*), apenas carifenona A apresentou atividade antioxidante (Bernardi *et al.*, 2005b).

ATIVIDADE ANTIMICROBIANA E ANTIVIRAL

Extratos metanólicos totais de *H. caprifoliatum, H. carinatum, H. connatum, H. myrianthum, H. polyanthemum* , *H. ternum*, benzopiranos e derivados de floroglucinol, nas concentrações de 100 e 200 μg/ml, foram testados, através do método de difusão em agar, contra as seguintes bactétias: *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Micrococcus luteus, Staphylococcus aureus*, *S. epidermidis*; e os fungos *Candida albicans* e *Saccharomyces cerevisiae* (Dall'Agnol *et al.*,2003; 2005). Os extratos que apresentaram maiores halos de inibição contra *S. aureus* foram *H. caprifoliatum* e *H. myrianthum*, em ambas as concentrações. *H. polyanthemum* (100 e 200 μg/ml) e *H. ternum* (200 μg/ml) foram ativos contra *B.subtilis* e *M. luteus*

Fenner e col. (2005) utilizaram o método de diluição em ágar para testar extratos de seis espécies (*H. caprifoliatum, H. carinatum, H. connatum, H. myrianthum, H. piriai, H. polyanthemum* e *H. ternum*) contra fungos dos gêneros *Candida, Saccharomyces, Cryptococcus, Aspergillus, Epidermophyton, Trichophyton* e *Microsporum*. O extrato clorofórmico de *H. ternum* apresentou maior atividade antifúngica principalmente contra espécies do gênero *Candida* (MICs varando de 250 a 1000 μg/ml) e *T. mentagrophytes* e *T. rubrum* (MIC_{50}=250 μg/ml).

O extrato metanólico de *H. connatum* apresentou atividade inibitória in vitro sobre o crescimento de vírus da imunodeficiência felina (Schmitt, 2000; Schmitt *et al.*, 2001).

ATIVIDADE ANALGÉSICA

O efeito antinociceptivo dos extratos ciclo-hexânico e metanólico de *H. caprifoliatum* (90 mg/kg) foi avaliado nos testes da placa aquecida (i.p. e v.o) e no teste de contorções abdominais induzidas por ácido acético 0,8%. Ambos os extratos apresentaram atividade no teste da placa aquecida. O efeito dos extratos ciclo-hexânicos de ambas as espécies parece estar relacionado com o sistema opióide, pois o efeito no teste da placa aquecida foi completamente bloqueado com o pré-tratamento com naloxona (antagonista opióide). Por outro lado, o efeito analgésico do extrato metanólico é apenas parcialmente relacionado a este sistema, uma vez que o bloqueio causado por naloxona foi apenas parcial para via i.p. e que o efeito analgésico v.o. não foi afetado. No teste de contorções abdominais induzidas por ácido acético 0,8% apenas o extrato ciclo-hexânico apresentou atividade (Viana, 2002). Estes resultados juntamente com os obtidos nesta tese encontram-se encartados no Capítulo 1 (Viana *et al.*, 2003).

ATIVIDADE ANSIOLÍTICA

O extrato ciclo-hexânico de *H. caprifoliatum* (ECH 90 mg/kg v.o.) foi testado em três modelos animais: labirinto em cruz elevada, potenciação do sono barbitúrico (pentobarbital 40 mg/kg, i.p.) e proteção a convulsões induzidas por pentilenotetrazol (PTZ, 80 mg/kg, i.p.). ECH não alterou significativamente os parâmetros observados no labirinto em cruz elevada, apesar de o tempo de permanência e o número de entradas no braço aberto terem sido maiores que para o controle negativo. O extrato prolongou significativamente a duração do sono, mas não alterou o tempo de indução. ECH não protegeu contra as convulsões induzidas por PTZ (Viana, 2002). Paralelamente foram realizados ensaios com íleo isolado de cobaio com o extrato ciclo-hexânico sem cera (HCP). O extrato (10 μg/ml) demonstrou ser um antagonista não-competitivo de receptores serotoninérgicos periféricos (5-HT_3 e 5-HT_4). Este resultado pode indicar que ECH possui um efeito ansiolítico, porém com mecanismo de ação diferente dos benzodiazepínicos (Viana, 2002), uma vez que o bloqueio de

receptores 5-HT_3 está relacionado ao efeito ansiolítico de algumas substâncias (Graeff, 1999). Estes resultados encontram-se no Capítulo 1 (Viana *et al.*, 2006).

ATIVIDADE ANTIDEPRESSIVA

As espécies *H. caprifoliatum, H. carinatum, H. connatum, H. myrianthum, H. piriai, H. polyanthemum* e *H. cordatum*, e três benzopiranos isolados de *H. polyanthemum* (HP1; HP2; e HP3) foram testadas quanto à atividade inibidora de monoamino oxidase A e B em preparações de mitocôndrias de cérebro de ratos. Os extratos que apresentaram inibição significativa apenas para MAO_A, os com maior atividade na concentração de $1,5x10^{-2}$ mg/ml foram o extrato clorofórmico de *H. caprifoliatum* (83%)e de *H. polyanthemum* (82%) e o extrato éter de petróleo de *H. piriai* (90%), entre os benzopiranos apenas HP3 (IC_{50} MAO_Ade 22,2μM) apresentou atividade significativa (Gnerre *et al.* 2001). As observações *in vitro* não foram confirmdas *in vivo*, a única espécie que apresentou redução no tempo de imobilidade dos ratos foi *H. caprifoliatum*, mas não o extrato clorofórmico, e sim o éter de petróleo (Daudt *et al.*, 2000; Gnerre *et al.* 2001).

O possível mecanismo de ação da espécie *H. caprifoliatum* está sendo investigado e, até o momento, os dados indicam o envolvimento do sistema dopaminérgico. Verificou-se que o efeito antiimobilidade do extrato lipofílico (270 mg/kg/dia, v.o.) é prevenido pelo pré-tratamento com sulpirida (50 mg/kg, i.p.) (antagonista D2) e que este extrato (90 mg/kg, v.o.) potencializa a hipotermia causada por apomorfina (16 mg/kg, s.c.) (Viana, 2002). Estes resultados juntamente com os obtidos nesta tese encontram-se encartados no Capítulo 1 (Viana *et al.*, 2005).

Para facilitar a compreensão deste item por parte dos leitores franceses, um resumo, em inglês, das atividades farmacológicas das espécies de *Hypericum* nativas do Rio Grande do Sul e de *H. perforatum* estão apresentadas na tabela 1.

Table 1: Pharmacological activities of *Hypericum* species.

***HYPERICUM* SPECIES**	**PHARMACOLOGICAL ACTIVITY**
H. brasiliense .	**EtOH:** antibacterial (Rocha *et al.*, 1995; Rocha *et al.*, 1996) and antinociceptive (Mendes *et al.*, 2002)
H. caprifoliatum	**HCP:** antinociceptive (Viana *et al.*, 2003), antidepressant (Viana *et al.*, 2005) and antibacterial (Dall'Agnol *et al.*, 2003, 2005) **HC1:** antidepressant (Viana *et al.*, 2005) **MET:** antinociceptive (Viana *et al.*, 2003), antiproliferative (Ferraz *et al.*, 2005 b) and antibacterial (Dall'Agnol *et al.*, 2003)
H. carinatum	**benzophenone Carifenone A:** antioxidative (Bernardi *et al.*, 2005b)
H. connatum	**MET:** antiviral (Schmitt *et al.*, 2001)
H. myrianthum	**MET:** antiproliferative (Ferraz *et al.*, 2005 b) and antibacterial (Dall'Agnol *et al.*, 2003)
H. polyanthemum	**POL and HP4**: antidepressant (Chapter 3) and antinociceptive (Viana *et al.*, 2003) **HP2, HP3:** antiproliferative (Ferraz *et al.*, 2005 c) and antibacterial (Dall'Agnol *et al.*, 2005)
H. ternum	**MET:** antiproliferative (Ferraz *et al.*, 2005 b), antibacterial (Dall'Agnol *et al.*, 2003) and antifungal (Fenner *et al.*, 2005)
H. perforatum	**extracts, essential oils, xanthones hyperforin:** antimicrobial (for review see Reichling *et al.*, 2001) **LI 160, hyperforin:** antidepressant (Linde *et al*., 1996; Kasper *et al.*, 2006), anxiolytic (Chatterjee *et al.*, 1998a; Beijamini e Andreatini, 2003; Skalisz *et al.*, 2004), CYP450 and Pgp inducer (Herbert et al., 2004; Tian et al., 2005) **EtOH:** antinociceptive(Özturk *et al.*, 1996, Kumar *et al.*, 2001), nootropic (Klusa et al., 2001) **hypericin:** antidepressant (Linde *et al*., 1996), anxiolytic (Chatterjee *et al.*, 1998a; Beijamini e Andreatini, 2003; Skalisz *et al.*, 2004), CYP450 and Pgp inducer (Herbert et al., 2004; Tian et al., 2005), citotoxic (Vandenbogaerde *et al.*, 1996; Ali *et al.*, 2001) and antiviral (Axarlis *et al.*, 1998).

EtOH: ethanolic extract
HCP: *H. caprifoliatum* cyclohexane extract
HC1: *H. caprifoliatum* phloroglucinol derivative
MET: methanolic extracts
POL: *H. polyanthemum* cyclohexane extract
HP4: *H. polyanthemum* phloroglucinol derivative
LI 160: *H. perforatum* standardized extract
CYP450: cytochrome 450
Pgp: glycoprotein P

II PARTE

RESULTADOS EXPERIMENTAIS

CAPÍTULO 1: Artigos publicados

1.1. INTRODUCTION

In this chapter three papers are enclosed. The first one that has been published in 2003 in Brazilian Journal of Medical and Biological Research concerns the antinociceptive effects of *Hypericum caprifoliatum* and *Hypericum polyanthemum* extracts. The second paper, published in 2005 in Neuropharmacology, reports the activity and mechanism of action of *Hypericum caprifoliatum* cyclohexane extract as antidepressant agent. The last paper (Fundamental and Clinical Pharmacology) reviews chemical and pharmacological published results as well as original data describing the effects of *Hypericum caprifoliatum* extracts on depression, anxiety and analgesia.

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Antinociceptive activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae)

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Abstract

The aim of the present study was to assess the analgesic activity of the aerial parts of two *Hypericum* species native to Southern Brazil, *H. caprifoliatum* and *H. polyanthemum.* The antinociceptive effect of the *H. polyanthemum* cyclohexane extract (POL; 180 mg/kg) and of the *H. caprifoliatum* methanol (MET) and cyclohexane (CH) extracts (90 mg/kg) was evaluated in the hot-plate (*ip* and *po*) and writhing (*po*) tests using male Swiss CF1 mice weighing 22-27 g (N = 10 per group). All extracts displayed antinociceptive effects in the hot-plate test (MET *ip* = 48%, MET *po* = 39%, CH *ip* = 27%, CH *po* = 50%, POL *ip* = 74%, and POL *po* = 49% compared to control). Pretreatment with naloxone (2.5 mg/kg, *sc*) abolished the effects of CH and POL, and partially prevented the analgesia induced by MET administered by the *ip* (but not by the *po*) route. POL and CH (*po*) significantly reduced the number of writhes induced by acetic acid, while MET was ineffective in this regard. We conclude that the antinociceptive effects of the *H. caprifoliatum* (CH) and *H. polyanthemum* (POL) hexane extracts seem to be mediated by the opioid system. Moreover, the antinociceptive activity of the *H. caprifoliatum* MET extract seems to depend on at least two chemical substances (or groups of substances) with distinct pharmacokinetic profiles and mechanisms of action. Only the naloxone-insensitive component of MET activity showed good bioavailability following oral administration.

Key words
- *Hypericum caprifoliatum*
- *Hypericum polyanthemum*
- Analgesia
- Antinociception
- Hot-plate test
- Writhing test

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The chemical investigation of the genus *Hypericum* (Guttiferae), which comprises approximately 400 species (1), has led to the isolation of more than 100 compounds from about 20 species with various different biological activities, especially antiviral, antimicrobial and antidepressant properties. *H. perforatum* extracts are widely used in Europe, in the United States, and also in Brazil, for the treatment of mild to moderate depression (2).

The Southern Brazilian *Hypericum* species *H. brasiliensis* and *H. connatum* are popularly used for relief of disorders such as angina, cramps and oral and pharyngeal inflammations, which suggests an analgesic property for this genus (3).

Previous reports published by our group

have shown interesting biological activities for the *Hypericum* species native to the State of Rio Grande do Sul, Brazil. A crude lipophilic extract of *H. caprifoliatum* induces an anti-immobility effect in the forced swimming test (4), which is considered to indicate an antidepressant action (5), as well as an antinociceptive effect in the hot-plate test (6). *H. caprifoliatum*, *H. piriai* and *H. polyanthemum* showed *in vitro* monoamine oxidase A-inhibitory activity (7). The aim of the present study was to investigate further the antinociceptive effects of *H. caprifoliatum* and to start the characterization of the antinociceptive properties of *H. polyanthemum*.

Air-dried and powdered aerial parts of *H. caprifoliatum* and *H. polyanthemum* were extracted with cyclohexane using an ultra-turrax apparatus (3 x 5 min; plant/solvent ratio 1:10, w/v), yielding extracts termed CH and POL, respectively. In order to obtain an extract rich in polar substances, *H. caprifoliatum* was also extracted consecutively in a Soxhlet apparatus with petroleum ether, chloroform and methanol (MET) and only the MET extract was used. All solvents were evaporated to dryness under reduced pressure. The extract was dissolved in saline solution containing 2.5% (w/v) polysorbate 80. The pH of the final solutions was 6.5 to 7.0. The volume administered was 1 ml/100 g body weight for the analgesic tests.

Male Swiss CF1 mice (22-27 g) from the 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 a cage, under a 12-h 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. The experiments were performed according to the guidelines of the National Ethics Committee on Research, Brazilian National Health Council. Ten mice per group were used for all experiments.

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) to determine baseline responsiveness 10 min before treatment with 90 mg/kg CH or MET or 180

Table 1. Antincociceptive effect of cyclohexane (CH) and methanol (MET) extracts of the aerial parts of *Hypericum caprifoliatum* and of the cyclohexane extract of aerial parts of *H. polyanthemum* (POL) in mice submitted to the hot-plate test.

Treatment (administration)	Latency (s)		% Analgesia
	Before treatment	After treatment	
Control (saline + 2.5% polysorbate 80)	12.1 ± 0.8	12.0 ± 1.4	0
MOR (6 mg/kg, *sc*)	10.2 ± 1.2	27.9 ± 1.9*	100
MET (90 mg/kg, *ip*)	12.0 ± 1.8	20.5 ± 3.7*	48.4
CH (90 mg/kg, *ip*)	9.4 ± 1.5	14.2 ± 1.8*	27.3
POL (180 mg/kg, *ip*)	10.2 ± 1.8	23.1 ± 3.9*	73.8
NAL (2.5 mg/kg, *sc*) + MOR (6 mg/kg, *sc*)	10.3 ± 1.3	10.8 ± 1.5	3.1
NAL (2.5 mg/kg, *sc*) + CH (90 mg/kg, *ip*)	11.4 ± 1.3	11.2 ± 2.0	0
NAL (2.5 mg/kg, *sc*) + MET (90 mg/kg, *ip*)	9.7 ± 1.4	15.6 ± 2.4*	30.8
NAL (2.5 mg/kg, *sc*) + POL (180 mg/kg, *ip*)	12.37 ± 1.3	12.6 ± 3.1	1.5
CH (90 mg/kg, *po*)	9.2 ± 0.9	17.8 ± 1.7*	50
MET (90 mg/kg, *po*)	10.5 ± 0.8	17.4 ± 2.1*	39.2
POL (180 mg/kg, *po*)	12.26 ± 1.8	20.7 ± 2.5*	48.6
NAL (2.5 mg/kg, *sc*) + CH (90 mg/kg, *po*)	12.8 ± 1.5	11.1 ± 0.6	0
NAL (2.5 mg/kg, *sc*) + MET (90 mg/kg, *po*)	10.2 ± 1.0	16.6 ± 2.7*	36.5

Data are reported as mean ± SEM. MOR: morphine; NAL: naloxone.
*P<0.005 compared to the latency of the same mouse before treatment (paired Student *t*-test).

mg/kg POL (*ip* and *po*). Treatment-induced changes in responsiveness to the hot plate were determined 30 and 45 min after *ip* and *po* administration, respectively. The negative control group received an equal volume of vehicle (saline + 2.5% (w/v) polysorbate 80). Morphine (6 mg/kg, *sc*) was administered to the positive control group. To determine the possible involvement of opioid-mediated mechanisms, some groups of animals were pretreated with naloxone (2.5 mg/kg, *sc*), a nonspecific opioid receptor antagonist, immediately after evaluating baseline responsiveness, 10 min before extract administration.

For the hot-plate test, mice were placed on a metal surface kept at 53 ± 1ºC. The time elapsed until the animal licked one of its hind paws or jumped was recorded (latency time, in s) and considered to be the reaction time in both exposures. Mice that presented baseline reaction times of more than 15 s in the first session were not used. In the second session, a maximum latency time of 30 s was imposed in order to avoid tissue damage.

The data were analyzed by the paired Student *t*-test, considering the animal as its own control (second measure *vs* first measure). The results obtained in the hot-plate test are reported as the mean ± SEM absolute latency time or as the percent of antinociceptive effect relative to morphine (6 mg/kg, *sc*) according to the following formula: % analgesia = $(test_{after} - test_{before})/(morphine_{after} - morphine_{before}) \times 100$.

The animals were treated with CH, MET (90 mg/kg, *po*) or POL (180 mg/kg, *po*) for the writhing test 45 min before receiving an *ip* injection of 0.8% acetic acid. Mice were then placed individually in glass observation chambers and the number of abdominal writhes was counted over a period of 15 min. The control group received an equal volume of vehicle (saline + 2.5% (w/v) polysorbate 80, *po*). Dipyrone (150 mg/kg, *po*) was the positive control treatment. Previous experiments carried out in our laboratory have revealed that none of the extracts caused any signs of pain or writhes *per se*, in mice, when injected *ip* (8). The results obtained in the writhing test are reported as median values and their respective interquartile intervals, and were analyzed by the Kruskal-Wallis test.

All extracts displayed antinociceptive effects in the hot-plate test (Table 1). Pretreatment with naloxone abolished the effects of CH and POL, indicating that these effects are produced by opioid-mediated mechanisms. Conversely, antinociception produced by MET administered *ip* was only partially prevented by naloxone, whereas the *po* antinociceptive activity was not modified, indicating that opioid-like substances present in this extract were not absorbed by the gastrointestinal tract or suffered single-pass inactivation by the liver. In addition, the percent of analgesia was higher when MET was administered by the *ip* route compared to the *po* route.

Administration of CH and POL significantly reduced the number of abdominal writhes induced by acetic acid, whereas MET did not have a significant effect (Table 2). Interestingly, the magnitude of the antinociceptive effect of CH in the writhing test (oral route) was similar to that observed in the

Table 2. Analgesic effects of methanol (MET) and cyclohexane (CH) extracts of aerial parts of *Hypericum caprifoliatum* (90 mg/kg, *po*) and of the cyclohexane extract of aerial parts of *H. polyanthemum* (POL, 180 mg/kg, *po*) on writhing induced by 0.8% acetic acid (*ip*) in mice.

Treatment	Number of writhes#	% Reduction of abdominal writhing compared to control
Control	58 (55-65)	–
DIP	0 (2-16)*	100
MOR	0 (0-3)*	100
CH	24 (0-34.5)*	58.6
MET	40.5 (16-59)	30.2
POL	0 (0-10)*	100

Control (saline + 2.5% polysorbate 80); DIP (dipyrone, 150 mg/kg, *po*); MOR (morphine, 10 mg/kg, *po*).
#Values are reported as medians (interquartile intervals).
*P<0.001 compared to control (Kruskal-Wallis, H = 30.235).

hot-plate test, while the effect of POL was more pronounced in the writhing test. Thus, the antinociceptive properties of POL might be due to actions on both central and peripheral pain systems. Apparently, the antinociceptive activity is not correlated with the antidepressant activity previously reported. Although lipophilic extracts of *H. caprifoliatum* were active in the Porsolt test (1,6), the same was not true for MET or POL (4,7).

The CH extract is rich in phloroglucinol (7) and the POL extract contains benzopyrans as its main constituent (9). Phloroglucinol derivatives may be responsible for the opioid-like effect since Simmen et al. (10) have reported that hyperforin - a phloroglucinol isolated from *H. perforatum* - inhibited binding to opioid receptors. With respect to the benzopyrans as well as the flavonoid derivatives, which are present in MET (Dall'Agnol R, Ferraz A, Schapoval ES and von Poser G, unpublished data), we are unaware of any previous reports on their influence/action on opioid systems. None of the extracts (CH, POL or MET) contains hypericin (11).

In conclusion, extracts obtained from both species, *H. caprifoliatum* and *H. polyanthemum*, contain compounds with substantial antinociceptive properties related, at least in part, to activation of opioid-mediated mechanisms. Further studies are in progress in order to elucidate the mechanisms underlying the antinociceptive effects of these species.

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The antidepressant-like effect of *Hypericum caprifoliatum* Cham & Schlecht (Guttiferae) on forced swimming test results from an inhibition of neuronal monoamine uptake

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Abstract

A crude (ECH) and a purified cyclohexane extract (HCP) of *Hypericum caprifoliatum* and their main phloroglucinol derivative (HC1) were evaluated regarding their action on monoaminergic systems, more precisely on dopamine. In rats and mice forced swimming test, ECH and HCP dose-dependently reduced the immobility time. The effect of the highest dose was prevented by a prior administration of either sulpiride or SCH 23390 (D_2 and D_1 dopamine receptor antagonist, respectively). HCP (360 mg/kg) decreased the locomotor activity of mice. ECH (90 mg/kg) caused hypothermia and potentiated apomorphine-induced (16 mg/kg) hypothermia in mice. HCP and HC1 inhibited, in a concentration-dependent and monophasic manner, the [^{3}H]-DA, [^{3}H]-NA and [^{3}H]-5HT synaptosomal uptakes, but did not prevent the binding of specific ligands to the monoamine transporters. Moreover, when tested at the concentrations corresponding to its IC_{50} on [^{3}H]-DA uptake, HC1 did not induce a significant [^{3}H]-DA release, while at a higher concentration (200 ng/ml) it enhanced significantly (by 12%) the synaptosomal DA release. These data suggest that the antidepressant-like effect of *H. caprifoliatum* on the forced swimming test is due to an increase in monoaminergic transmission, resulting from monoamine uptake inhibition, more potently of dopamine, which may be related to their phloroglucinol contents.

Keywords: Depression; Monoaminergic system; Forced swimming test; *Hypericum caprifoliatum*; Phloroglucinol derivatives

1. Introduction

Hypericum (Guttiferae) is a large genus of herbaceous or shrubby plants, which widely occurs in temperate regions of the world. This genus encompasses approximately 400 species (Nör et al., 2004). Chemical investigation has led to the isolation of more than 100 compounds from about 20 species, with various biological activities, which are primarily antiviral, antimicrobial and antidepressant (Wu et al., 1998). *Hypericum perforatum* extracts are used in Europe, USA, as well as in South America, for the treatment of mild to moderate depression. A substantial amount of experimental and clinical data demonstrate that this plant is an effective antidepressant (Linde et al., 1996; Butterweck et al., 1997; Müller et al., 1998; Müller, 2003). However, neither the mechanisms of action nor the identity of the active constituents are completely understood. The

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naphthodianthrone hypericin has been considered as the most active constituent of *H. perforatum* (Butterweck et al., 1997). In fact, some reports still provide evidences for the role of hypericin in the antidepressant activity of *H. perforatum* (Butterweck et al., 1998) and the majority of the phytomedicines available are standardized on this naphthodianthrone. Nevertheless, nowadays most researchers consider that the antidepressant effects are due to a variety of constituents rather than a single one (Chatterjee et al., 1998a; Butterweck et al., 2000). Xanthones (Rocha et al., 1994), flavonoids (Butterweck et al., 2000), and especially hyperforin (Chatterjee et al., 1998b; Müller et al., 2001), a phloroglucinol derivative, are also considered as pharmacologically relevant compounds.

H. perforatum mechanism of action is still unknown, whereas early studies report that hypericin inhibits MAO in the concentration range of 50 μg/ml (Suzuki et al., 1984), other authors have failed to confirm this effect (Yu, 2000). In fact, the inhibitory activity was reached at concentrations considered too high ($K_i = 2$ μg/ml for MAO_A and 3.2 μg/ml for MAO_B) to be therapeutically relevant (Suzuki et al., 1984). Presently, several reports have showed that the main therapeutically used *H. perforatum* extract (LI 160, standardized at 0.3% of hypericin) is a potent but unspecific inhibitor of the neuronal uptake of several biogenic amines and amino acid neurotransmitters (Müller et al., 1998). Currently, some reports have attempted to link this nonselective profile to an effect of hyperforin on sodium channels (Singer et al., 1999; Müller et al., 2001). In agreement with this hypothesis, Buchholzer et al. (2002) have demonstrated that hyperforin inhibits the sodium dependent high-affinity choline uptake and thereby the striatal acetylcholine release. Furthermore, Roz and Rehavi (2003) have reported that the effect of hyperforin on the uptake of monoamines could be explained by its action on the pH gradient in the synaptic vesicle membrane.

In Southern Brazil, 20 *Hypericum* species have been identified (Robson, 1990). However, scientific reports regarding these species are scarce. Xanthones, flavonoids and phloroglucinols were isolated from *Hypericum brasiliense* and its antimicrobial and monoamine oxidase inhibitory activities were demonstrated by Rocha et al. (1994, 1995). Schmitt et al. (2001) have reported an antiviral activity in vitro against feline immunodeficiency virus (FIV) of *Hypericum connatum*.

As regards a possible antidepressant effect, *Hypericum caprifoliatum* showed promising results in the forced swimming test, which predicts antidepressant activity (Daudt et al., 2000). The in vitro monoamine oxidase inhibitory (MAOI) activity of the extracts of *H. caprifoliatum* and some other Brazilian species has also been demonstrated (Gnerre et al., 2001). Nevertheless, the effect of *H. caprifoliatum* in the forced swimming test (FST) does not appear to be related to the MAOI activity, since the extracts which displayed this activity were not active in the FST (Gnerre et al., 2001). After phytochemical investigation of eight species, including *H. caprifoliatum*, Ferraz et al. (2002) verified the absence of hypericin in all species. Recent study, carried out by Nör et al. (2004), has shown that the phloroglucinol derivatives from *Hypericum* species, native to southern Brazil, have a dimeric structure consisting of filicinic acid and phloroglucinol moiety. This molecular feature has been proposed as chemotaxonomic marker for the species of *Hypericum*, and differs from hyperforin and adhyperforin, which are polyisoprenylated phloroglucinol derivatives. By means of bioassay-guided fractionation through forced swimming test, it was concluded that the active substance(s) are present only in the most lipophilic (light petroleum) fraction, which primarily consists of phloroglucinol derivatives (Daudt et al., 2000). This class of secondary metabolites has been reported as being one of the main compounds responsible for *H. perforatum* activity, in pre-clinical as well as clinical assays (Chatterjee et al., 1998a; Müller, 2003).

The aim of this study was to further investigate the *H. caprifoliatum* antidepressant-like effect in order to evaluate its mechanism of action. Therefore, after attempting to address the in vivo monoaminergic effects of *H. caprifoliatum* purified lipophilic extracts on experimental model of depression, the forced swimming test, we attempted to assess their effects on the dopaminergic, noradrenergic and serotoninergic neuronal systems.

2. Materials and methods

2.1. Plant material

The aerial parts of *H. caprifoliatum* were collected in the region of Viamão, in the state of Rio Grande do Sul – Brazil (August/2001). The voucher specimens were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon 1496).

2.2. Preparation of extracts

The dried and powdered plant material (120 g of aerial parts) was extracted with cyclohexane (plant/solvent ratio 1:10 w/v) by turbo-extraction during 5 min followed by evaporation to dryness under reduced pressure at 45 °C yielding an extract termed ECH (ca. 5.0 g). To obtain the purified extract, ECH was treated with acetone, according to Rocha et al. (1995), producing an insoluble fatty residue (10% w/w), which was eliminated through a paper filter. Then, chlorophyll was removed by mixing the acetone soluble extract with

powdered charcoal. This mixture was filtered and the solvent evaporated under reduced pressure at 45 °C yielding 4.0 g of a wax and chlorophyll free extract termed HCP.

2.3. Chemical characterization of the extracts

The ECH and HCP were analyzed by preparative Thin Layer Chromatography (TLC) using silica gel GF_{254} as stationary phase, chloroform/hexane (1:1 v/v) as mobile phase and Godin's reagent as chromogenic agent characteristic for phloroglucinol derivatives (Rocha et al., 1995). Both extracts showed a main spot (Rf 0.10–0.40), reactive to Godin's reagent, which was removed by preparative TLC (90 mg) and characterized by NMR (^{1}H and ^{13}C, 400 MHz). This compound was termed HC1. All solvents (pro-analysis) were obtained from MERCK Kga (Darmstadt, Germany).

2.4. Behavioral experiments

2.4.1. Animals

Adult male Wistar rats (weight 200–300 g) and male CF1 Swiss mice (25–30 g) purchased from Fundação Estadual de Produção e Pesquisa em Saúde – RS (Brazil) colony were used. The animals were housed 5 rats or 20 mice in plastic cages (*L*: 42 cm, *W*: 27.5 cm, *H*: 16 cm). All animals were kept under a 12-h light/dark cycle (lights on at 7:00 a.m.) at a constant temperature of 23 ± 1 °C with free access to standard certified rodent diet (Nuvilab CR-1) and tap water.

All the behavioral experiments were approved by CONEP – Brazil (National Commission of Research Ethics) and performed according to the guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998), which are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.4.2. Drugs

Bupropion HCl (Glaxo Wellcome, São Paulo, Brazil), imipramine HCl (Galena, São Paulo, Brazil), sulpiride (DEG, São Paulo, Brazil), apomorphine (Sigma, St. Louis, MO), dimethylsulfoxide (DMSO), cyclohexane, polysorbate 80 (Merck, Darmstadt, Germany), SCH 23390 (Sigma–Aldrich, Saint Quentin Fallavier, France).

2.4.3. Forced swimming test (FST)

2.4.3.1. In rats. The Porsolt's procedure (Porsolt et al., 1978) was used with minor modifications. In this test an acrylic box with four sections of 30 × 30 × 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 23 ± 2 °C and height of 30 cm (Porsolt et al., 1978 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 exposure (5 min), and their immobility time was measured.

In preliminary dose–response studies, we observed that ECH 270 mg/kg/day, p.o. (three administrations of 90 mg/kg), afforded the maximal anti-immobility effect in rats (data not shown). Thus, this dose was selected for continuing the study. The ECH and HCP were diluted to a concentration of 90 mg/ml (270 mg/kg/day, p.o.) in water with 2% of polysorbate 80 or 10% DMSO, respectively. Imipramine hydrochloride 20 mg/kg (60 mg/kg/day, p.o.) was used as the antidepressant reference drug and 2% of polysorbate 80 or 10% DMSO in saline were used as the negative control. For evaluating the dopaminergic action of the extracts, the animals were treated with sulpiride (50 mg/kg, i.p.) 30 min prior to the last administration of the solutions to be tested. In this case, bupropion was used at the 20 mg/kg dose (60 mg/kg/day, p.o.) as the dopaminergic antidepressant reference drug. All treatments were administered at 1 ml/kg body weight.

2.4.3.2. In mice. The apparatus consisted of a glass cylinder (10 cm internal diameter, 25 cm height) filled with water (19 cm height) at 23 ± 2 °C. The HCP was diluted to concentrations between 9 and 36 mg/ml in water with 2% of polysorbate 80. Different groups of mice were treated with HCP (90, 180, 270 or 360 mg/kg, p.o.), imipramine (20 mg/kg, p.o.), bupropion (30 mg/kg, p.o.), or 2% polysorbate 80 solution in water. For evaluating the dopaminergic action, the different groups were pre-treated with sulpiride (50 mg/kg, i.p.) or SCH 23390 (15 μg/kg, s.c.) 30 min before the administration of the most effective dose of HCP. All treatments were administered at 10 ml/kg body weight. After 60 min, the animals were placed into the cylinder and the total duration of immobility, during a 6-min test period, was measured. A mouse was considered immobile when it remained floating in the water, and only makes movements necessary to keep its head above the water.

2.4.4. Locomotor activity

Locomotor activity was accessed automatically in a digiscan photocell activity meter box (45 × 30 × 30 cm) (Omnitech Electronics Inc, Columbus, OH). The

response to HCP 360 mg/kg, administered by gavage (10 ml/kg body weight) immediately before the test, was expressed as the number of beams crossed between 5 and 45 min after treatments.

2.4.5. Apomorphine-induced hypothermia

Temperature measurements were performed in a temperature-controlled room (23 ± 1 °C) between 9:30 and 11:30 a.m. The mice colonic temperature was recorded using commercially available thermometer (Pró-check®), which was dipped in Vaseline and inserted about 1 cm in the gently hand-restrained mouse.

After recording their initial colonic temperature ($t = 0$) different groups of mice received either ECH (90 mg/kg, p.o.) or solvent (polysorbate 80, 2% in water) (1 ml/100 g). Thirty minutes later, all animals were treated with apomorphine (16 mg/kg, s.c.). Their colonic-temperature was recorded 15 and 30 min after the last treatment.

2.5. In vitro assays

2.5.1. Animals

Male Sprague–Dawley rats, weighing 180–200 g, were purchased from IFFA-CREDO/Charles River Laboratories (Domaine des Oncins, Saint-Germain sur L'Arbresle, France). They were housed by four in Makrolon cages (*L*: 40 cm, *W*: 25 cm, *H*: 18 cm), with free access to water and food (U.A.R., France) and kept in a well ventilated room, at a temperature of 21 ± 1 °C, under a 12-h light/dark cycle (lights on between 7:00 hour and 19:00 hour). The procedures used in this study are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.5.2. Drugs

[^{3}H]-dopamine ([^{3}H]-DA, 48 Ci/mmol) and [^{3}H]-nisoxetine (86 Ci/mmol) were purchased from Amersham (Les Ulis, France). [^{3}H]-noradrenaline ([^{3}H]-NA, 12.5 Ci/mmol), [^{3}H]-serotonin ([^{3}H]-5HT, 25.5 Ci/mmol), [^{3}H]-citalopram (84.2 Ci/mmol) and [^{3}H]-mazindol (24.5 Ci/mmol) were purchased from Perkin–Elmer–NEN Life Science Products (Paris, France). Cocaine hydrochloride was obtained from la Coopérative Pharmaceutique Française (Melun, France). Desipramine hydrochloride, fluoxetine hydrochloride and GBR 12783 were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Solutions of HCP and HC1 extracts (10^{-4}–10^{-11} g/ml) were prepared in an incubation medium containing 5% cremophor and 5% DMSO. Millimolar solutions of GBR 12783 were prepared in distilled water. Subsequent dilutions and solutions of other agents were performed in the incubation medium.

2.5.3. Synaptosomal uptake of [^{3}H]-DA, [^{3}H]-5HT and [^{3}H]-NA

All procedures necessary to prepare synaptosomal suspensions were performed at 0–2 °C. Animals were sacrificed by decapitation; their striata (for [^{3}H]-DA uptake), frontal cortex (for [^{3}H]-5HT uptake) and hypothalamus (for [^{3}H]-NA uptake) were dissected out and homogenized with 12 up and down strokes of a Teflon–glass homogenizer (800 r.p.m.) in 10 volumes (w/v) of ice-cold 0.32 M sucrose solution containing 0.1 mM pargyline. The nuclear material was removed by centrifugation at 1000 *g* for 10 min, and the supernatant (crude synaptosomal fraction) was used in uptake experiments.

Aliquots (100 μl) of the crude synaptosomal fraction were preincubated for 5 min at 37 °C in a Krebs–Ringer medium, containing (mM): NaCl 109, KH_2PO_4 1, $CaCl_2$ 1, $NaHCO_3$ 27, glucose 5.4, pH 7.4 ± 0.1, in the presence or absence of HCP or HC1. The incubation was continued for 5 min in the same medium, in the presence of [^{3}H]-DA, [^{3}H]-5HT or [^{3}H]-NA (10 nM; 1 ml final volume). The reaction was stopped by adding 3 ml of ice-cold incubation medium and immediate centrifugation (7000 *g*, 10 min, 4 °C). The pellet was washed with 1 ml of the latter medium and centrifuged in the same condition. The final pellet was sonicated in 250 μl distilled water and aliquots of the homogenate were used for the determination of radioactivity and protein concentrations. The radioactivity was determined by liquid scintillation spectrometry (Betamatic V, KONTRON, Trapes, France) in 4 ml of Optiphase Highsafe II with 33–36% counting efficiency. Protein concentrations were determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

The specific uptake of DA, 5HT or NA was defined as the difference between the total uptake at 37 °C and the non-specific accumulation observed at 0 °C, respectively, in the presence of 100 μM cocaine for [^{3}H]-DA, 1 μM fluoxetine for [^{3}H]-5HT or 0.3 μM desipramine for [^{3}H]-NA. [^{3}H]-5HT and [^{3}H]-NA uptake assays were performed in the presence of 30 nM 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)-piperazine, dihydrochloride (GBR 12783) in order to block 5HT and NA transporters operated by DA nerve terminals present, respectively, in cortical and hypothalamic preparations.

The monoamine synaptosomal uptake was analyzed using the equation of the sigmoidal dose–response curve (variable slope). IC_{50} and *n* Hill values were calculated using the equation of the curve-fitting programs Microcal Origin (Microcal Software).

2.5.4. Binding assays to monoamine transporters

The binding to dopamine (DAT), noradrenaline (NAT) or serotonin (SERT) transporters was assessed,

respectively, on striatal, cortex and hypothalamus membranes of rats. The crude synaptosomal suspensions, prepared as described in Section 2.5.3, were centrifuged at 17,000 *g* for 30 min at 4 °C. The pellet was resuspended by sonication in: 10 mM Na^+ medium (0.30 mM NaH_2PO_4, 9.70 mM $NaHCO_3$, pH 7.5 ± 0.1) for the binding to DAT or 50 mM Tris–HCl (containing 120 mM NaCl and 5 mM KCl, pH 7.4 ± 0.1) for NAT and SERT and centrifuged (50,000 *g*, 10 min, 4 °C). The final pellet was resuspended by sonication in the same respective medium for the binding to DAT and SERT, for NAT it was resuspended in 50 mM Tris–HCl (containing 300 mM NaCl and 5 mM KCl, pH 7.4 ± 0.1).

[^{3}H]-mazindol (2 nM final concentration) and membranes (100 μg protein) were incubated at 0 °C, for 2 h, to evaluate the binding to DAT. For the binding to NAT 300 μg of protein were incubated with [^{3}H]-nisoxetine (1 nM) at 25 °C during 2 h; and for SERT, 200 μg of protein were incubated with [^{3}H]-citalopram (1 nM) during 1 h at 25 °C. All incubations were carried out in the presence of different HC1 concentrations (3×10^{-7}–3×10^{-11} g/ml), in a final volume of 500 μl. Incubations were stopped by dilution with 3 ml of ice-cold incubation medium and immediate filtration through GF/B filters, previously soaked, for at least 1 h, in 0.5% polyethyleneimine. Each tube and filter was rinsed once or twice with 3 ml of ice-cold incubation medium. Filters were counted for radioactivity by liquid scintillation spectrometry (Tri-carb 2100TR, Packard BioScience Co.) in 4 ml of Ultima Gold (Perkin–Elmer). The non-specific binding was determined by incubation with cocaine (100 μM, for DAT), desipramine (10 μM, for NAT) or fluoxetine (10 μM, for SERT). Protein concentrations were determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.5.5. Synaptosomal [^{3}H]-DA release

The DA release was determined on synaptosomes previously loaded with [^{3}H]-DA. The striata synaptosomes were prepared as described in Section 2.5.3. The crude synaptosomal suspension was centrifuged for 30 min at 17,000 *g* and the resulting pellet was resuspended in the uptake incubation Krebs–Ringer medium (described in Section 2.5.3) corresponding to the total synaptosomal fraction (S1). An aliquot (1 ml) of S1 was diluted in 2900 μl of Krebs–Ringer and was incubated for 10 min at 37 °C in the presence of 100 μl of [^{3}H]-DA (100 nM final concentration). The [^{3}H]-DA loading was stopped by centrifugation (7000 *g*, 10 min, 4 °C) followed by two washings of the pellet in 4 ml of Ca^{2+} free modified Krebs–Ringer medium (NaCl 109 mM, KH_2PO_4 1 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 27 mM, glucose 5.4 mM, ascorbic acid 1 mM and pargyline 0.05 mM at pH 7.5 ± 0.1) to remove radioactivity not uptaken into the synaptosomes. The final pellet was then suspended in 4 ml of modified Krebs–Ringer medium, corresponding to [^{3}H]-DA loaded synaptosomal fraction (S2).

To assess the [^{3}H]-DA releasing effect of HC1 the procedure was performed according to Carruba et al. (1977), by incubating (10 min at 37 °C) 250 μl aliquots of synaptosomal samples S2, in Eppendorf tubes containing Ca^{2+} free modified Krebs–Ringer medium, in the absence (control) or presence of HC1 (100 ng/ml or 200 ng/ml final concentrations), in a total volume of 500 μl. The releasing effect was stopped by centrifugation (7000 *g*, 10 min, 4 °C). Aliquots of 400 μl from the last supernatant, as well as 250 μl of synaptosomal samples S2, were measured by liquid scintillation spectrometry (Betamatic V, KONTRON, Trapes, France) in 4 ml of Optiphase Highsafe II (33–36% counting efficiency). The amount of [^{3}H]-DA released is expressed as percentage of the total radioactivity contained in 250 μl synaptosomal samples S2. The values obtained represent the means ± SEM of three independent experiments, each performed in triplicate.

2.6. Statistical analysis

The data were evaluated using one or two-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test or Student's *t*-test depending on the experimental design. *p*-Values less than 0.05 were considered as statistically significant.

3. Results

3.1. Chemical characterization

HC1 appears to be the main compound of both ECH and HCP extracts, representing 8% of HCP. TLC and spectral data revealed that HC1 is a phloroglucinol derivative. The NMR spectra gave hydroxyl resonance at ca. δ 18, 16, 11, and 10. The unusually low field shifted signals (ca. δ 18) suggest the presence of hydroxyl protons that participate in rather strong hydrogen bonds. This signal is frequently observed in NMR spectra of phloroglucinol derivatives consisting of acyl filicinic acid and phloroglucinol moieties linked by a methylene bridge. Furthermore, the spectra revealed several signals at ca. δ 18 which could indicate a mixture of tautomeric forms (Verotta et al., 2000). The purification and complete structural elucidation of this compound are in progress.

3.2. Behavioral experiments

3.2.1. Forced swimming test

Two different sets of experiments were performed. The first was carried out to confirm whether the

immobility time reported by Daudt et al. (2000) in rats treated intraperitoneally with the *H. caprifoliatum* petroleum–ether extract, was also observed in animals treated per os with the cyclohexane extract (ECH) or the cyclohexane purified extract (HCP) of *H. caprifoliatum* per os. Both ECH and HCP were effective on rat FST when compared to solvent (ANOVA $F_{4,62} = 15.39$, $p < 0.001$) (Fig. 1). In mice, the immobility time was dose-dependently reduced by HCP administration and the highest tested dose was the most effective (360 mg/kg) (ANOVA, $F_{6,57} = 15.5$, $p < 0.001$) (Fig. 2). The second set of experiments was performed in order to evaluate whether the dopaminergic system was involved in the anti-immobility effect. The injection of sulpiride, 30 min prior to the last administration of the extract or bupropion, impaired the immobility time reduction caused by either bupropion or ECH treatment in rats (two-way ANOVA, pre-treatment × treatment factor, $F_{2,64} = 12.71$, $p < 0.001$) (Fig. 3). The anti-immobility effect in mice was prevented by sulpiride (50 mg/kg) (two-way ANOVA, pre-treatment × treatment factor, $F_{1,33} = 14.77$, $p < 0.001$) (Fig. 4) or by SCH 23390 (15 μg/kg) pre-treatment (two-away ANOVA, pre-treatment × treatment factor, $F_{1,30} = 8.87$, $p < 0.01$) (Fig. 4).

3.2.2. Locomotor activity

The treatment with HCP, 360 mg/kg, p.o., significantly reduced the locomotor activity of mice. The mean number of beams crossed by control animals was 814 ± 87 while it was 520 ± 98 for HCP treated animals (Student 's *t*-test, $t = 2.24$; $p < 0.05$).

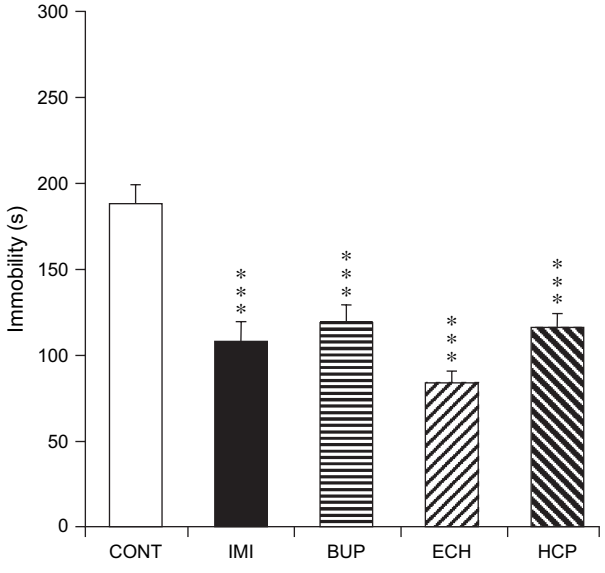

Fig. 1. Anti-immobility effect of imipramine 60 mg/kg/day, p.o. (IMI), bupropion 60 mg/kg/day, p.o. (BUP), cyclohexane extract (ECH) and purified ECH (HCP) of *H. caprifoliatum* (270 mg/kg/day, p.o.) in rat forced swimming test. The results are presented in mean ± SEM ($n = 12$–14 rats/group). Student–Newman–Keul's post hoc comparisons: ***$p < 0.001$ significant difference in relation to control (CONT).

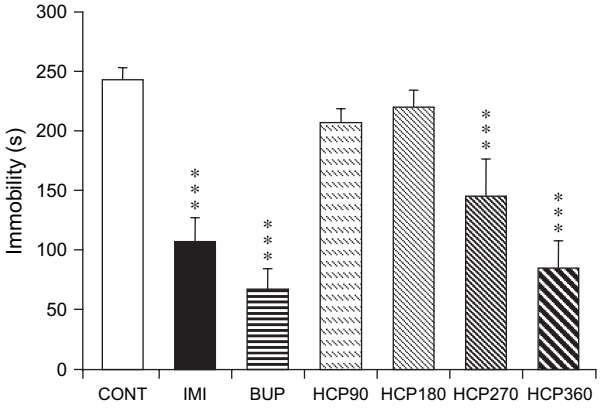

Fig. 2. Anti-immobility effect of imipramine 20 mg/kg, p.o. (IMI), bupropion 30 mg/kg, p.o. (BUP) and *H. caprifoliatum* purified cyclohexane extract (HCP 90, 180, 270 and 360 mg/kg, p.o.) in mice forced swimming test. The results are presented in mean ± SEM ($n = 8$–12 mice/group). Student–Newman–Keul's post hoc comparisons: ***$p < 0.001$ compared with control (CONT).

3.2.3. Apomorphine-induced hypothermia

ECH alone provoked significant hypothermia. Furthermore, in mice pre-treated with ECH, an enhancement of apomorphine-induced hypothermia was observed (two-way ANOVA, time factor, $F_{2,88} = 89.3$;

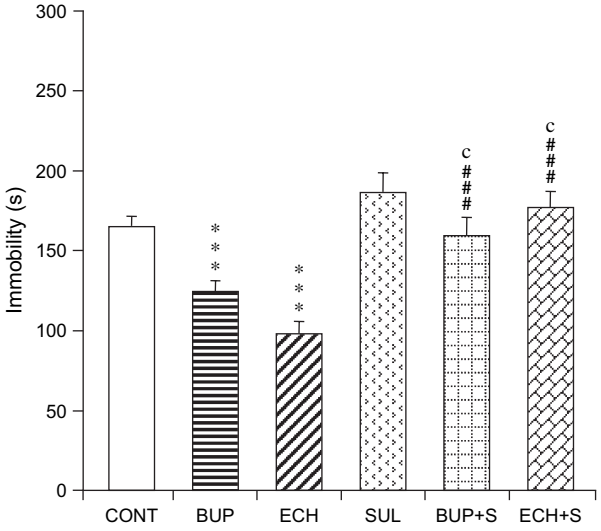

Fig. 3. Effect of SUL (sulpiride 50 mg/kg, i.p.) administration on the anti-immobility action of ECH 270 mg/kg/day (p.o.) in rat forced swimming test. BUP = bupropion 60 mg/kg/day, p.o., ECH = cyclohexane extract, BUP + S = bupropion + sulpiride, ECH + S = cyclohexane extract + sulpiride. The results are presented in mean ± SEM ($n = 12$–14 rats/group). $^{c}p < 0.001$ (Two-way ANOVA, BUP or ECH × SUL interaction). Student–Newman–Keul's post hoc comparisons: ***$p < 0.001$ significant difference in relation to control (CONT). $^{###}p < 0.001$ significant difference in relation to the respective group without SUL.

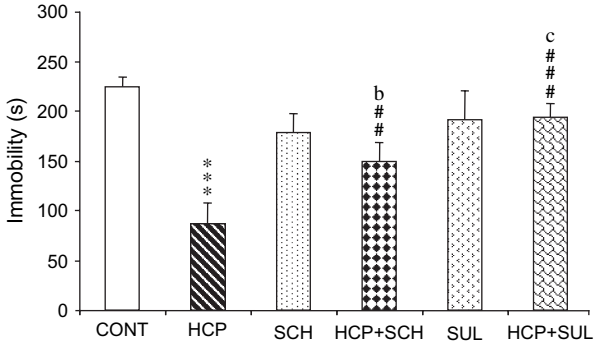

Fig. 4. Effect of SCH 23390 (15 μg/kg, s.c.) or SUL (sulpiride 50 mg/kg, i.p.) administration on the anti-immobility action of HCP (*H. caprifoliatum* cyclohexane extract, 360 mg/kg, p.o.) in mice forced swimming test. The drugs were injected 30 min before the extract treatment. The results are presented in mean ± SEM ($n = 8$–12 mice/group). $^{b}p < 0.01$; $^{c}p < 0.001$ (Two-way ANOVA, HCP × SCH interaction or HCP × SUL interaction). $^{***}p < 0.001$ compared with control (CONT). $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared with HCP.

treatment factor, $F_{3,88} = 23.3$; time × treatment factor, $F_{6,88} = 14.75$, $p < 0.001$ for all factors) (Table 1).

3.3. Neurochemical experiments

3.3.1. Effects of HCP and HC1 on the uptake of biogenic amines

HCP and HC1 inhibited [^{3}H]-DA, [^{3}H]-5HT and [^{3}H]-NA uptake by crude synaptosomal suspensions prepared from rat striatum (for DA), rat frontal cortex (for 5HT) and hypothalamus (for NA), in a concentration-dependent and monophasic manner. The IC_{50} values with respective Hill coefficients (*n* Hill), which were close to unity, are summarized in Table 2. It should be pointed out that HCP and HC1 potently inhibited [^{3}H]-DA uptake ($IC_{50} = 98 \pm 18$ ng/ml and 85 ± 19 ng/ml), being, respectively, five and seven times less effective against [^{3}H]-5HT and 2.5 (HC1) and 10 (HCP) times less effective on [^{3}H]-NA than on [^{3}H]-DA uptake. Nevertheless, no significant difference was found between the IC_{50} [^{3}H]-DA and [^{3}H]-5HT uptake values of HCP and HC1. In contrast, concerning the [^{3}H]-NA uptake, HC1 was more effective, with IC_{50} value about five times lower than the IC_{50} of HCP.

Table 1
Effect of the cyclohexane extract of aerial parts of *Hypericum caprifoliatum* (ECH) (90 mg/kg, p.o.) on apomorphine-induced hypothermia (16 mg/kg, i.p.)

Treatments	Temperatures (°C)		
	$t = 0$	$t = 15$ min	$t = 30$ min
SAL	38.2 ± 0.1	37.8 ± 0.1	38 ± 0.1
APO	38.1 ± 0.1	35.8 ± 0.25$^{***\#\#\#}$	35 ± 0.3$^{***\#\#\#}$
ECH	38.3 ± 0.5	37.5 ± 0.3$^{\#\#}$	37.2 ± 0.3$^{\#\#}$
ECH + APO	38.1 ± 0.15	34.9 ± 0.35$^{***\#\#\#a}$	33.8 ± 0.3$^{***\#\#\#b}$

SAL = saline; APO = apomorphine 16 mg/kg; ECH = cyclohexane extract 90 mg/kg; ECH + APO = cyclohexane extract 90 mg/kg followed by apomorphine 16 mg/kg. The results are presented in mean ± SEM of 8–15 mice/group.Two-way ANOVA, time factor $F_{2,88} = 89.3$; treatment factor $F_{3,88} = 23.3$; time × treatment factor $F_{6,88} = 14.75$, $p < 0.001$ for all factors. $^{***}p < 0.001$ significant difference in relation to saline. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant difference in relation to $t = 0$ within the group. $^{a}p < 0.05$, $^{b}p < 0.01$ significant difference in relation to apomorphine within the same time.

Table 2
Comparison of the inhibition of monoamine uptake by HCP and HC1

	Uptake of [^{3}H]-DA		Uptake of [^{3}H]-5HT		Uptake of [^{3}H]-NA	
	IC_{50} (ng/ml)	*n* Hill	IC_{50} (ng/ml)	*n* Hill	IC_{50} (ng/ml)	*n* Hill
HCP	98 ± 18	1.0 ± 0.15	520 ± 32	0.8 ± 0.1	971 ± 98	0.74 ± 0.1
HC1	85 ± 19	1.2 ± 0.25	599 ± 60	0.7 ± 0.1	206 ± 24	0.70 ± 0.1

Data were calculated using the equation of the curve-fitting programs Microcal Origin. IC_{50} and *n* Hill values represent the mean ± SEM of four independent experiments, carried out in duplicate.

3.3.2. Effects of HC1 on the binding of uptake inhibitors to monoamine transporters

The different tested concentrations of HC1 did not inhibit, in a statistically significant manner, the binding of [^{3}H]-mazindol, [^{3}H]-nisoxetine, [^{3}H]-citalopram to their respective monoamine transporters (Table 3).

3.3.3. Effects of HC1 on synaptosomal [^{3}H]-DA release

The level of spontaneous [^{3}H]-DA release by synaptosomes, previously loaded with [^{3}H]-DA and incubated in Ca^{2+} free Krebs–Ringer medium, was not significantly modified in the presence of 100 ng/ml HC1. In contrast, at the 200 ng/ml concentration, HC1 increased significantly, by about 12% (ANOVA, $F_{2,8} = 16.66$, $p < 0.05$) the [^{3}H]-DA release. The percentages of [^{3}H]-DA released by pre-loaded synaptosomes were 31.5 ± 1.6% in absence (control), 38 ± 2.4% in the

Table 3
Effect of HC1 on the binding of [^{3}H]-mazindol, [^{3}H]-nisoxetine and [^{3}H]-citalopram

Concentration of HC1 (g/ml)	Binding of [^{3}H]-mazindol (fmol/mg prot)	Binding of [^{3}H]-nisoxetine (fmol/mg prot)	Binding of [^{3}H]-citalopram (fmol/mg prot)
0	2834 ± 452	48 ± 2	243 ± 15
3×10^{-7}	3120 ± 418	49 ± 2	246 ± 18
10^{-7}	3110 ± 431	52 ± 3	250 ± 18
3×10^{-8}	2918 ± 370	54 ± 4	245 ± 17
10^{-8}	3026 ± 465	53 ± 3	248 ± 15
3×10^{-9}	2901 ± 391	53 ± 4	252 ± 15
10^{-9}	2815 ± 397	53 ± 3	242 ± 19
3×10^{-10}	2785 ± 390	51 ± 3	253 ± 23
10^{-10}	2794 ± 396	50 ± 4	243 ± 17
3×10^{-11}	2885 ± 437	49 ± 2	240 ± 17

Mean ± SEM of four (for [^{3}H]-nisoxetine and [^{3}H]-citalopram binding) and six (for [^{3}H]-mazindol binding) different experiments.

presence of 100 ng/mg HC1 and 43.2 ± 3.2% in the presence of 200 ng/ml HC1.

4. Discussion

The results of our study demonstrate that the antidepressant-like effect of *H. caprifoliatum* petroleum light crude extract, previously observed in rats by Daudt et al. (2000), is reproduced by other lipophilic and purified extracts rich in phloroglucinols. We also observed that this effect is reproducible since it was dose-dependently reproduced when the same extracts were evaluated on mice FST. Furthermore, this anti-immobility effect was not related to a non-specific behavioral stimulation, given that HCP reduced motor activity. This observation supports the hypothesis that, to be considered as a potential antidepressant, a drug must reduce immobility in FST at doses that do not stimulate locomotion (Porsolt et al., 1978). This is in agreement with previously reported studies, which show that many antidepressants tend to decrease motor activity (Duterte-Boucher et al., 1988; Bourin, 1990). Moreover, we also observed that ECH enhanced the hypothermic effect of apomorphine 16 mg/kg and displayed a mild intrinsic hypothermic effect. This result was unexpected because the hypothermia induced by a high dose of apomorphine is strongly antagonized by antidepressants such as imipramine-like drugs (Puech et al., 1978; Menon et al., 1988).

Puech et al. (1981) suggested that apomorphine-induced hypothermia results from two effects: (i) the first, observed at small doses and antagonized by neuroleptics, is related to stimulation of D_2 dopaminergic receptor and (ii) the second, induced by high doses and antagonized by imipramine, is probably not related to dopaminergic system, but to the β-adrenergic system. Furthermore, DA agonists are known to be effective on FST (Duterte-Boucher et al., 1988) as well as in depressed patients (Post et al., 1978). These results prompted us to evaluate the involvement of the dopaminergic system in the ECH and HCP effects on the FST.

The anti-immobility effects of ECH and HCP were entirely prevented by sulpiride (D_2 antagonist) and partially prevented by SCH 23390 (D_1 antagonist), at doses devoid of locomotor impairment (Barghon et al., 1981; Vaugeois et al., 1996), suggesting that the anti-immobility effect of *H. caprifoliatum* is mediated by D_2 and possibly by D_1 dopamine receptor activation. Nevertheless, the impairment caused by SCH 23390 on the effect of HCP was puzzling, since Vaugeois et al. (1996) have demonstrated that effects of indirect DA agonists on the despair test depend on the stimulation of D_2 but not D_1 dopamine receptors. In fact, the effectiveness of D_2 receptor stimulation on the anti-immobility effect in FST is generally accepted, while the role of D_1 receptors are more controversial. D_1 dopamine agonists have been reported to be effective in animal models of depression by some authors (D'Aquila et al., 1994; Gambarana et al., 1995) but not by others (Duterte-Boucher et al., 1988; Geofroy and Christensen, 1993). In contrast, functional interaction between D_1 and D_2 receptors has been well documented in various behaviors, they are known to interact synergistically in some behaviors (Menon et al., 1988). Furthermore, SCH 23390, in addition to being a highly potent D_1 receptor antagonist, binds with a high-affinity to $5HT_2$ and $5HT_{1C}$ receptors (Bourne, 2001); it is also effective in blocking 8-OH-DPAT ($5HT_{1A}$ agonist) effects in FST (Luscombe et al., 1993). It would be possible to postulate that 5HT plays a role in the effect of *H. caprifoliatum* in the FST, despite the fact that this test frequently fails to detect antidepressant action of serotonergic drugs (Porsolt et al., 1991). However, our results demonstrate that HCP and its main phloroglucinol rich extract (HC1) more potently inhibit the DA synaptosomal uptake than those of NA and 5-HT, confirming involvement of the dopaminergic transmission in the antidepressant-like effect of *H. caprifoliatum* lipophilic extracts (ECH, HCP). It appears that these effects are related to the phloroglucinol derivative present in HC1 since, although this fraction constitutes only 8% of HCP, it inhibited [^{3}H]-DA synaptosomal uptake with potency equivalent to HCP. Contrary to all other known antidepressant drugs, the inhibition of monoamine uptake operated by *H. caprifoliatum* extracts is not due to a binding of HC1 or a blockage of DA, NA or 5-HT transporters at the binding sites of [^{3}H]-mazindol, [^{3}H]-nisoxetine, [^{3}H]-citalopram, respectively. These observations suggest that the mechanism of action of *H. caprifoliatum* extract is probably not associated with a specific binding on each of the different monoamine transporters, but could depend on mechanisms involved in neurotransmitter transport in general. This assumption had been previously reported for other species of *Hypericum*, such as *H. perforatum* (Chatterjee et al., 1998b; Müller et al., 1997, 1998) and *H. triquetrifolium* (Roz et al., 2002). In these reports, several in vivo and in vitro animal studies have demonstrated that the antidepressant properties and the inhibitory effects of the extracts from these species on the neuronal uptake of the monoamines were due to the lipophilic phloroglucinol derivative, hyperforin (Chatterjee et al., 1998a,b; Müller et al., 1998; Jensen et al., 2001; Roz et al., 2002), which is considered as the main active substance of *H. perforatum* and *H. triquetrifolium*. These studies have considered that hyperforin operates in a non-specific manner since it inhibits the uptake of each biogenic amine with an almost identical potency (Chatterjee et al., 1998b; Müller et al., 1998; Roz et al., 2002) that extends to GABA and L-glutamate uptakes (Chatterjee

et al., 1998b; Singer et al., 1999; Wonnemann et al., 2000). In contrast, HCP and HC1 were more effective on DA than NA and 5-HT accumulation in synaptosomes. However, analyzing the data of Müller et al. (1997, 1998) and Chatterjee et al. (1998b), it appears that the difference in the IC_{50} values to the uptake inhibition of DA are 3–5 times lower than IC_{50} values to NA and 5-HT uptake inhibition, which is statistically different. Thus, in these studies, the phloroglucinol rich extract from *Hypericum* species behaves like an antidepressant with a preferential impact on the dopaminergic system, which was not considered. In addition, although the IC_{50} for [^{3}H]-DA uptake inhibition of HCP and HC1 is approximately twice as high as that of hyperforin and hyperforin enriched CO_2 extract (Chatterjee et al., 1998b), *H. caprifoliatum* appears to be in the same range of potency as other *Hypericum* species in the FST. In order to obtain an effect similar to imipramine 30 mg/kg/day in the rat FST, a dose of 270 mg/kg/day of ECH and HCP were required, while the effective doses of alcoholic extracts from other species ranged from 300 to 3000 mg/kg/day in rats (Butterweck et al., 1997; Chatterjee et al., 1998a). In the mice FST, the effective doses of other *Hypericum* extracts are between 250 and 1000 mg/kg (Öztürk et al., 1996; Sanchez-Mateo et al., 2002), for HCP the lowest effective dose, which promotes an effect almost equivalent to imipramine 20 mg/kg is 270 mg/kg.

The molecular mechanisms by which HC1 or other phloroglucinol derivatives induce antidepressant-like activity remain unclear. Nevertheless, recently reported studies have suggested that the phloroglucinol enriched extract from *H. perforatum* does not act as an detergent-like substance on the membrane, since it did not affect synaptosomal membrane microviscosity up to 30 mM (Gobbi et al., 1999), but through the inhibitory effect on vesicular storage by an interference with the driving force of the vesicular uptake (Roz et al., 2002). This prompted us to investigate the effects of HC1 on synaptosomal preparations pre-loaded with [^{3}H]-DA, in a Ca^{2+} free modified Krebs–Ringer medium. We demonstrated that exposure of the pre-loaded synaptosomes to HC1 induced a small dose-dependent increase in the efflux of [^{3}H]-DA. Moreover, the HC1 concentration needed to induce the efflux of [^{3}H]-DA was higher than that needed to inhibit the DA uptake. Similar difference in potencies was previously observed by Gobbi et al. (1999) in the effects of the hydromethanolic extract of *H. perforatum* and hyperforin on synaptosomal [^{3}H]-DA uptake and release. In this study, the concentration of hydromethanolic extract of *H. perforatum* (4000 ng/ml) or hyperforin (440 ng/ml) necessary to inhibit 50% of [^{3}H]-DA into rat striatal synaptosomes induced, respectively, only $10.3 \pm 1.4\%$ and $13.9 \pm 0.6\%$ release from synaptosomes pre-loaded with [^{3}H]-DA. Furthermore, using in vivo microdialysis technique in the prefrontal cortex of awake rats, Yoshitake et al. (2004) showed that a single dose of hydro-alcoholic extracts of *H. perforatum* (60 mg/kg i.p. or 300 mg/kg p.o.) caused: a marked increase in the extracellular levels of DA (165 and 140%, respectively); a moderate increase in the extracellular level of 5HT (about 135%); whereas NA levels remained unchanged. Based on the present study and other studies (Roz et al., 2002; Gobbi et al., 1999; Yoshitake et al., 2004) the low magnitude though significant efflux of [^{3}H]-monoamines triggered by phloroglucinol rich extracts is probably due to diffusion of the monoamines across the plasmatic membrane following their concentration gradient.

In conclusion, the results obtained in our study are in accordance with previous reports regarding the effect of lipophilic extracts of *H. caprifoliatum* in the forced swimming test (FST) (Daudt et al., 2000; Gnerre et al., 2001), an animal model for selecting antidepressant drugs with a good predictive value (Porsolt et al., 1991). They suggest a mechanism of action that could be related to the inhibition of neuronal monoamine uptake, most potently of dopamine. This effect could be caused by an indirect effect of HC1 on monoamine transporters, most likely due to the elevated neurotransmitter levels in the synaptic clefts, thus leading to an alteration of monoamine transporter function different from the mechanism of action of reference monoamine uptake inhibitors. Our further investigation are focused on evaluating the effects of *H. caprifoliatum* extracts on cerebral extracellular monoamines levels, in order to characterize the monoaminergic system responsible for the antidepressant-like effect of *H. caprifoliatum*.

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REVIEW ARTICLE

Hypericum caprifoliatum (Guttiferae) Cham. & Schltdl.: a species native to South Brazil with antidepressant-like activity

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ABSTRACT

In this work, previously published and unpublished results on biological activity of *Hypericum caprifoliatum*, a native specie to South Brazil, are presented. Lipophilic extracts obtained from this species showed an antidepressant-like activity in mice and rat forced swimming test. Results from in vivo experiments suggest an effect on the dopaminergic transmission. Besides that, in vitro experiments demonstrated that the extract and its main component (a phloroglucinol derivative) inhibit monoamine uptake in a concentration-dependent manner, more potently to dopamine, but this effect is not related to direct binding at the uptake sites. It was also observed that a 3-day treatment with lipophilic extract prevents stress-induced corticosterone rise in mice frontal cortex but not in plasma. The lipophilic and methanolic *H. caprifoliatum* extracts also demonstrated antinociceptive effect, which seems to be indirectly mediated by the opioid system. These results indicate that *H. caprifoliatum* presents a promising antidepressant-like effect in rodents which seems to be related to a mechanism different from that of other classes of antidepressants.

INTRODUCTION

In the last 10 years, interest in the genus *Hypericum* (Guttiferae) has increased considerably all over the world. *Hypericum* is a large genus of herbaceous or shrubby plants which widely occur in temperate regions of the world. This genus encompasses approximately 400 species [1]. Chemical investigation of the genus *Hypericum* has led to the isolation of more than 100 compounds, such as flavonoids, xanthones and phloroglucinol derivatives [2]. Among the last type of compounds, some are related to the well-known hyperforin isolated from *Hypericum perforatum* L. while others possess a phloroglucinol unit conjugated with a filicinic acid moiety [2,3].

From the clinical point of view *H. perforatum* is the main species. In Europe, in the USA and South America *H. perforatum* extracts are used for treating mild to moderate depression. However, neither the mechanisms of action nor the identity of the active constituents are completely understood. Most authors agree that it is not only one component but a combination of some of them, such as hypericin, flavonoid derivatives and mainly hyperforin [4–8] that are responsible for the antidepressant activity. With regard to the mode of action, lipophylic extracts and some components (e.g. hyperforin) have been shown to inhibit the uptake of noradrelanine (NA), serotonin (5-HT), dopamine (DA), gamma-aminobutyric acid (GABA) and glutamate by a mechanism not directly related to binding at the uptake sites [9–11]. Nowadays other approaches attempting to explain the pharmacological properties of *H. perforatum* have shown that hyperforin alters sodium conductive pathways [12], affects the physico-chemical properties of

neuronal membranes [13,14] and acts on hypothalamic–pituitary–adrenal (HPA) axis [15–17].

As the chemical composition and the pharmacological profile of *Hypericum* species are comparable, the study of the genus *Hypericum* could be helpful in searching for alternative sources of antidepressant molecules and to identify drugs with innovative mechanisms of action.

Brazilian *Hypericum* species occur predominantly at southern regions, where 20 of them have been identified. Species growing in Rio Grande do Sul, the southernmost state in Brazil, belong to the sections Brathys and Trigynobrathys, the latter with a greater number of native species [18].

In the course of our ongoing project aiming to detect bioactive molecules in southern Brazilian species of the genus *Hypericum*, we selected the most widespread ones to begin the screening of their chemical composition and pharmacological properties. Until now eight species have been studied: *H. brasiliense* Choisy, *H. caprifoliatum* Cham. & Schltdl., *H. carinatum* Griseb., *H. connatum* Lam., *H. cordatum* (Vell.) N. Robson subsp. *kleinii* N. Robson, *H. myrianthum* Cham. & Schltdl., *H. polyanthemum* Klotzsch ex Reichardt and *H. ternum* A. St. Hil. As far as we know, none of the species is used in folk medicine for antidepressant purposes. *Hypericum brasiliensis* and *H. connatum* are popularly used for relief of disorders such as angina, cramps and ora-pharyngeal inflammations, which could be associated with the analgesic properties of these plants [19]. To the best of our knowledge, only *H. brasiliense* and *H. cordatum* have been previously investigated for antidepressant activity. Rats' immobility time in forced swimming test (FST) was not reduced by hydroalcoholic extracts of both species [20]; conversely, *H. brasiliense* extract inhibited monoamine oxidases [21,22].

Regarding chemical composition, these species are rich in flavonoids [23] and dimeric phloroglucinols conjugated with a filicinic acid moiety [24]. These phloroglucinol derivatives are proposed as chemotaxonomic markers for the southern Brazilian species [25]. None of the studied species presented hypericin [24].

In this review, we brought together both published and unpublished data pertaining to *H. caprifoliatum*. This species was evaluated concerning its chemistry, toxicity, general activity on central nervous system and effects on animal models of anxiety, nociception and depression. The most promising results were found on antidepressant-like activity which prompted us to carry out a deeper investigation of this subject.

HYPERICUM CAPRIFOLIATUM

Botanic

Hypericum caprifoliatum is a subshrub 0.4–1 m tall found in forest clearings, pastures and roadsides. It has characteristic orange-yellow to orange pentamer small flowers with 9–15 mm diameter stellate; buds ovoid, acute and sub-acute. Leaves are ovate-deltoid to oblong (10–22 × 7–10 mm) margin recurved to revolute, perfoliate, spreading, with sparse pale but not dark glands. The slenderer stems, strongly connate leaves, and smaller sepals distinguish *H. caprifoliatum* from its closest relative *H. teretiusculum*, which has a more northern distribution [18].

Chemistry

All studies were carried out with flowering aerial parts collected between August and December, 2001, in the region of Viamão, in the state of Rio Grande do Sul, Brazil. The voucher specimens were deposited at the herbarium of the Federal University of Rio Grande do Sul (ICN-Bordignon 1496).

The presence of tannins and flavonoids in *H. caprifoliatum* total methanolic extract was determined by using standard phytochemical methods to characterize the most usual plant secondary metabolites [23]. The tannin fraction (6.1%) includes triflavonoids and heptaflavonoids (three to seven condensed monomers, formed by flavan-3-ol or flavan-3,4-diol units). The non-tannin fraction contains phenolic compounds (monomeric flavonoid compounds and hydroxycinnamic acid derivatives), carbohydrates (glucose, fructose, saccharose and hydrocolloidal gums) and nitrogen compounds (such as amino and imino acids). The main flavonoids are hyperoside and other glycosides of quercetin. This was expected as flavonoids are abundant in *Hypericum* species [25], being quercitrin, isoquercitrin, hyperoside and rutin (all derived from quercetin) the most commonly reported glycosides [22].

Hypericum species have a strong tendency to accumulate phenolic compounds with the phloroglucinol substitution pattern. Recently two dimeric structures consisting of filicinic acid and a phloroglucinol moiety were isolated from *H. caprifoliatum*: hyperbrasilol [25] and a tautomeric mixture still unresolved, termed HC1 [26]. HC1 corresponds to 8% of a purified cyclohexane extract and seems to be the active fraction on central nervous system [26]. This type of compound has already been found in other species belonging to the sections Trigynobrathys – *H. brasiliense* [21,22], *H. carin-*

atum, *H. connatum*, *H. myrianthum*, *H. polyanthemum* [25], *H. uliginosum* [27], *H. japonicum* [28] – and Brathys – *H. drummondii* [29,30].

Although *Hypericum* species are not characteristically aromatic, usually presenting no more than 1% of essential oil, the presence of these compounds was also assessed. The Brazilian species, *H. caprifoliatum*, *H. polyanthemum*, *H. myrianthum*, *H. carinatum*, *H. connatum* and *H. ternum* afforded essential oils in the range of 0.1–0.5%. In these species, the sesquiterpene are present in higher concentration than monoterpenes and all of them contain alkanes in the volatile fraction [24].

To carry out the psychopharmacological study, several extracts were obtained by continuous extraction with solvents of increasing polarity [petroleum ether, chloroform and methanol (MET)] or by maceration with cyclohexane. Details of the extraction and purification methods were presented elsewhere [26].

Pharmacology

At the beginning of our project we tested the total methanolic extracts of aerial parts of several *Hypericum* species in the FST which predicts antidepressant activity [31,32]. Only *H. caprifoliatum* extract was active. Therefore, we assayed petroleum ether, chloroform and MET extracts in the FST, hot plate test and for monoamino oxidase inhibitory (MAOI) activity [26,31,32].

The most promising extract in the FST was the petroleum ether which is rich in phloroglucinols. However, in subsequent experiments it showed a significant toxicity, thus another liphofilic extract was prepared by maceration with cyclohexane. This cyclohexane extract (ECH) was investigated specially toward the antidepressant activity. In addition, ECH was purified yielding an extract free of chlorophyll and waxes termed HCP. In vivo experiments were carried out with MET and cyclohexane extracts. In vitro studies were performed with HCP and with a phloroglucinols tautomeric mixture HC1, the main constituent of ECH and HCP.

All experiments were approved by CONEP, Brazil (National Commission of Research Ethics) and performed according to guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998), which are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Toxicity and general activity

Rats treated with petroleum ether extract by intraperitoneal route (270 mg/kg/day) showed signs of toxicity (piloerection, hypothermia, apnea and prostration) and some of them died between 24 and 48 h after the last treatment. During autopsy we verified the presence of ascitic liquid in the abdomen. Histological and biochemical analysis revealed superficial liver necrosis and low levels of albumin. These findings suggest that the death was caused by circulatory collapse, i.e. in the attempt to dilute the extract there was a permeation of liquid into the peritoneum. The lethal dose 50% (LD_{50}) determined in mice by intraperitoneal route was 197 mg/kg. No signs of toxicity were found when rats or mice were treated orally (50–2000 mg/kg) with petroleum ether and cyclohexane extracts.

For the assessment of cyclohexane extract (ECH) nonspecific effects on the central nervous system, locomotor activity, motor coordination, pentobarbital sleeping time and hypothermia were evaluated in mice treated with doses ranging from 90 to 360 mg/kg p.o. Treatment with ECH 90–360 mg/kg reduced locomotor activity [26,34] but did not interfere with motor coordination, demonstrating that ECH neither presents a gross neurotoxicity nor affects the neuromuscular junction. The extract increased the sleeping time (saline = 31.6 ± 7.8 min; ECH 90 mg/kg = 77 ± 16 min) of mice treated with pentobarbital but not the sleep latency (saline = 4.4 ± 0.2 min; ECH 90 mg/kg = 9 ± 3.4 min). ECH 90 mg/kg caused a significant hypothermia by itself and an enhanced apomorphine-induced (16 mg/kg, s.c.) hypothermia [26]. These results suggest that ECH has a depressor effect on the central nervous system or affects the metabolism of depressor substances. This latter assumption is based on studies that verified an interaction of *H. perforatum* extracts and isolated substances with metabolizing systems such as cytochrome P-450 enzyme system and pregnane X receptor [33,34]. The evaluation of *H. caprifoliatum* action on cytochrome P-450 system is under way.

Anxiety and seizures

The ECH was tested in the models of anxiety such as black and white compartments and plus maze as well as pentylenetetrazole induced seizures. All tests were performed with acute treatments with doses ranging from 90 to 360 mg/kg p.o. The extract showed neither anxiolytic nor anticonvulsive effects (*Tables I* and *II*).

Nociception

Methanol (MET) and the cyclohexane (ECH) extracts (90 mg/kg, i.p. and p.o.) presented an antinociceptive effect in the hot plate test [35]. The pre-treatment with

Table I Effect of *Hypericum caprifoliatum* cyclohexane extract (ECH) on plus maze and dark/light compartments scores.

	Plus maze Time in the open arm (s)		Dark/light compartments Time in the light compartment (s)
Treatment per os (dose)	Rat[a]	Mice[b]	Mice[c]
NaCl 0.9% ($n = 10$)	142 ± 50	23 ± 15	131 ± 23
Diazepam (2 mg/kg; $n = 12$)	213 ± 53*	84 ± 19***	150 ± 25***
ECH (90 mg/kg; $n = 10$)	171 ± 79	26 ± 14	NT
ECH (180 mg/kg; $n = 10$)	NT	28 ± 14	NT
ECH (360 mg/kg; $n = 10$)	NT	29 ± 7	103 ± 34

Values expressed as mean ± SD.

NT, dose not tested.

[a]One way ANOVA $F_{2,27} = 3.4$ followed by multiple comparison test. Student–Newman–Keuls, $*P < 0.05$.

[b]One way ANOVA $F_{4,51} = 36.7$ followed by multiple comparison test. Student–Newman–Keuls, $***P < 0.001$.

[c]One way ANOVA $F_{2,29} = 11.2$ followed by multiple comparison test. Student–Newman–Keuls, $***P < 0.001$.

Table II Effect of *Hypericum caprifoliatum* cyclohexane extract (ECH) on pentylenetetrazole (80 mg/kg, i.p.)-induced seizures parameters in mice.

Treatment per os	Convulsion latency (s)[a]	Number of convulsions[b]	Number of deaths[c]
Diazepam (2 mg/kg; $n = 10$)	–	0	0
NaCl 0.9% ($n = 10$)	62 (54–74)	11.5 ± 5	3
ECH (90 mg/kg; $n = 10$)	57 (53–59)	18.33 ± 8	5

Values expressed as: [a]median (inter-quartiles intervals), ANOVA on ranks $H = 4.2$, [b]mean ± SD, one-way ANOVA $F_{1,19} = 1.2$; [c]absolute number.

naloxone (2.5 mg/kg, s.c.) abolished the effect of the ECH i.p. and p.o., indicating that it may be mediated by the opioid system. Conversely, antinociception produced by MET given by i.p. route was only partially prevented by naloxone and the antinociceptive activity per os (p.o.) was not modified. Furthermore, ECH significantly reduced the number of abdominal writhing induced by acetic acid while MET showed only a mild and not significant effect.

As mentioned in the section 'Chemistry', the methanolic extract is rich in flavonoids while the main compound of the cyclohexane extract (ECH) is a phloroglucinol derivative tautomeric mixture (HC1). Therefore we tested the fractions from each extract, enriched in flavonoids or HC1, in the hot plate test. Unexpectedly, none of the fractions had antinociceptive effect. In addition, we verified that the phloroglucinol mixture HC1 (10^{-6}–3×10^{-11} g/mL) did not affect the binding of [^{3}H]-naloxone (1 nM) to the rats' total brain membrane.

The summation of results indicates that the antinociceptive effect of ECH and MET may be attributed to a synergistic effect of substances rather than the effect of a single one. MET seems to have at least two groups of substances with distinct pharmacokinetic profiles and mechanisms of action [35]. The effect of ECH on the opioid receptors is not directly related to HC1, but we cannot rule out the possibility that HC1 metabolites, that are not formed in vitro, might be responsible for the analgesic activity. Furthermore, we can reason that the antinociceptive effect of ECH is indirect by acting in other neurotransmitter systems (e.g. dopaminergic system) rather than modulated by the opioid functions.

Depression

Some of us have reported the effect of *H. caprifoliatum* in the rat FST [31]. This study revealed that among the investigated extracts (petroleum ether, chloroform and MET), the only one displaying an antidepressant-like effect was the petroleum ether (270 mg/kg/day, i.p.). This extract (1.5×10^{-2} mg/mL) was submitted to in vitro monoamino oxidase (MAO) inhibition assays and revealed an inhibition of no more than 25% on both MAO-A and MAO-B. This magnitude of effect was considered too low to explain the antidepressant-like effect observed in the FST [32].

The antidepressant-like effect of *H. caprifoliatum* petroleum ether extract is retained by other lipophilic and purified extract rich in phloroglucinols (ECH). We observed that ECH shows a dose-dependent (90–360 mg/kg, p.o.) effect when evaluated on rat and mice

FST [26]. Furthermore, the anti-immobility effect on FST was not due to a nonspecific behavioral stimulation as ECH 90 mg/kg p.o. also reduces mice locomotor activity as shown in general activity studies. This observation reinforces the hypothesis that ECH has antidepressant properties. Given this, to be considered as a potential antidepressant, a drug must reduce immobility in FST at doses that do not stimulate locomotion [36]. This result is also in accordance with studies which show that many antidepressants tend to decrease motor activity [37,38].

Aiming to study the mode of action of *H. caprifoliatum* lipophilic extracts, the second set of experiments was performed to evaluate whether the monoaminergic system is involved in anti-immobility effect. The summary of the results is presented next. Methodological details and full data are presented elsewhere [26]. The effect of ECH in mice FST was prevented by a prior administration of either sulpiride or SCH 23390 (D_2 and D_1 dopamine receptor antagonist, respectively). These results led us to further investigate ECH and HC1 regarding their action on monoaminergic system, more precisely on dopamine. ECH was purified as previously described [26] furnishing HCP which has a chemical profile comparable to ECH: both extracts present HC1 as the main constituent. HCP and HC1 inhibited, in a concentration-dependent and monophasic manner, [^{3}H]-DA, [^{3}H]-NA and [^{3}H]-5HT synaptosomal uptakes (*Table III*). This inhibition was stronger for DA than NA and 5-HT confirming the dopaminergic feature of *H. caprifoliatum* antidepressant-like effect. It appears that these effects are related to the phloroglucinol derivative present in ECH as it inhibited [^{3}H]-DA synaptosomal uptake with a potency equivalent to ECH although HC1 constitutes only 8% of ECH. When tested at concentrations corresponding to its IC_{50} on [^{3}H]-DA uptake, HC1 did not induce a significant [^{3}H]-DA release, while at a higher concentration (200 ng/mL) it significantly enhanced (by 12%) the synaptosomal DA release [26]. Despite the effects on dopamine uptake and release, acute treatment with ECH (270 mg/kg, p.o.) did not increase dopamine, homovanilic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) extracellular levels in the accumbens nucleus (coordinates from bregma: A +2.2; L −1.5; D −6) measured by microdialysis in awake rats, using high-pressure liquid chromatography with electrochemical detection. This fact could be a consequence of low ECH or HC1 systemic levels. The evaluation on striatum DA levels as wells as other treatment regimens and the pharmacokinetic plasma profile of ECH is in progress. Moreover, contrary to all other known antidepressant drugs, the inhibition of monoamine uptake operated by *H. caprifoliatum* extracts is not due to HC1 binding or DA, NA or 5-HT transporters blockage at the binding sites of [^{3}H]-mazindol, [^{3}H]-nisoxetine and [^{3}H]-citalopram, respectively [26]. These observations suggest that the mechanism of action of *H. caprifoliatum* extract is probably not associated with a specific binding to either one of the different monoamine transporters, but could depend on a mechanism involved in neurotransmitters transport in general.

This assumption had been previously reported for other species of *Hypericum*, such as *H. perforatum* [39–42] and *H. triquetrifolium* [44]. In these reports, several in vivo and in vitro animal studies have demonstrated that the antidepressant properties and the inhibitory effects of the extracts from these species on the neuronal uptake of the monoamines were due to the lipophilic phloroglucinol derivative, hyperforin [39,41,43–45], which is considered the main active substance of *H. perforatum* and *H. triquetrifolium*. Comparing our results with *H. perforatum* data, we can point out that although ECH and HC1 IC_{50} for [^{3}H]-DA uptake inhibition (98 and 85 ng/mL, respectively) are about twice as high as that of hyperforin- (55 ng/mL) and hyperforin-enriched CO_2 extract (56 ng/mL) [44], *H. caprifoliatum* appears to have the same range of potency of other *Hypericum* species in

Table III Comparison of the inhibition of monoamine uptake by HCP and HC1.

	Uptake of					
	[^{3}H]-DA		[^{3}H]-5HT		[^{3}H]-NA	
	IC_{50} (ng/mL)	n Hill	IC_{50} (ng/mL)	n Hill	IC_{50} (ng/mL)	n Hill
HCP	98 ± 18	1.0 ± 0.1	520 ± 32	0.8 ± 0.1	971 ± 98	0.7 ± 0.1
HC1	85 ± 19	1.2 ± 0.2	599 ± 60	0.7 ± 0.1	206 ± 24	0.7 ± 0.1

Data were calculated using the equation of the curve-fitting programs MICROCAL ORIGIN (Microcal Software Inc., Northampton, MA, USA). IC_{50} and n Hill values represent the mean ± SEM of four independent experiments, carried out in duplicate. Data from our earlier results [26].

the FST. In order to obtain an effect similar to imipramine 30 mg/kg/day in the rat FST, a dose of 270 mg/kg/day of ECH was required, while the effective doses of alcoholic extracts from other species ranged from 300 to 3000 mg/kg/day in rats [41,45]. In the mice FST the effective doses of other *Hypericum* extracts are between 250 and 1000 mg/kg [46,47], while for ECH the lowest effective dose is 270 mg/kg.

Nevertheless, despite all studies on the antidepressant-like activity of the genus *Hypericum*, the molecular mechanisms by which phloroglucinol derivatives, such as HC1, induce antidepressant-like activity remain unclear. Recently, a second line of investigation alongside the monoaminergic approach has been focusing on the action of antidepressants in the HPA axis [48].

We investigated the effect of ECH (360 mg/kg, p.o.) on serum and brain corticosterone levels of mice, stressed or not by FST [A. F. Viana, unpublished data]. In brief, cortical but not serum corticosterone FST-induced rise was diminished by repeated (3 days) ECH treatment; acute (1 day) treatment did not reduce FST-augmented corticosterone; basal corticosterone levels (non-stressed animals) were not reduced by either treatment regimes. In parallel, we demonstrated that repeated administration of imipramine and bupropion reduces both cortical and serum corticosterone levels. Thus, the mechanism by which *H. caprifoliatum* reduces corticosterone levels seems to be different from that of antidepressants, whereas the extract had no effect in non-stressed animals and its protection against FST-induced corticosterone rise is observed only in the cortex. Several reports have demonstrated that the antidepressants reduce corticosterone basal levels [49].

CONCLUSION

Among the Brazilian species studied until now, *H. caprifoliatum* showed the most attractive results. The lipophilic extracts contain substances which are phloroglucinol derivatives (HC1) with potential antidepressant activity [26]. Results from in vivo experiments suggest an effect on the dopaminergic transmission. This assumption was reinforced by in vitro experiments, in which both extract and HC1 inhibited monoamine uptake, in particular of dopamine. However, the fact that *Hypericum* extracts and isolated substances do not bind to monoaminergic transporters led research groups, including ours, to conclude that the antidepressant-like activity is related to a mechanism different from that reported for other classes of antidepressants [11,26,39,43]. Besides that, the fact that *Hypericum* phloroglucinol derivatives do not have nitrogen atoms in their structures is remarkably against the structure–activity relationship studies for monoamines receptors and transporters [50,51] which may account for its particular mechanism of action. Consequently, the research on substances from this genus could result in antidepressant molecules with innovative mechanisms of action and also give insights into the pathophysiology of depression.

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CHAPTER 2: Effects of *Hypericum caprifoliatum* Cham. & Schltdt. (Guttiferae) cyclohexane extract on serum and brain corticosterone levels

2.1. INTRODUCTION

In this chapter, we continued the investigation of *Hypericum caprifoliatum* cyclohexane extract (HCP) mechanism of action. Now we carried out a less conventional approach, by studying HCP influence on the hypothalamic-pituitary-adrenal axis (HPA). As highlighted in the review section, mood disorders often coincide with abnormal levels of corticosteroids. Likewise, it has long been observed that patients with Cushing's syndrome, hyperactivity of the HPA axis that can be induced pharmacologically or by pituitary or adrenal tumors, have high comorbity of psychiatric disturbances, including depression (Starkman *et al.*, 1981; Becker *et al.*, 1983). During the past decade, several research groups formulated a hypothesis relating aberrant stress hormone deregulation to causality of depression and submitted that antidepressants may act through normalization of these HPA changes (Gold *et al.*, 1995; Holsboer and Barden 1996; Nemmeroff, 1996). Therefore HPA axis may be an important target to develop antidepressant action (Barden *et al.*, 1995; Gold *et al.*, 1995; Holsboer and Barden, 1996; Pariante *et al.*, 2004).

In essence, stress underlies most animal models predictive of antidepressant acitivity. Stressful conditions such as electrical paws stimulations performed in the learned helplessness test (Foa et al., 1992; Henn et al., 1993; Petty et al., 1997), chronic mild stress (Willner et al., 1987, 1997, 2005; Moreau et al., 1992, 1994; Auriacombe et al., 1997), forced swimming test (Porsolt et al., 1978), are known to induce in rodents depressive like symptoms that are reversed by antidepressants. Furthermore, models that use animals selectively bred for presenting neurochemical and behavior alterations parallel to those found in human depressed patients, present alterations in the HPA axis, that are corrected after antidepressant treatment (El Yacoubi *et al*., 2003; Morley-Fletcher *et al*., 2004). Accordingly, it has been observed that the hyperactivity of the HPA axis in animals and depressed patients was corrected by antidepressant drugs such as imipramine, moclobemide, fluoxetine (Raap and van de Kar, 1999; Holsboer, 2000; Pariante and Miller, 2001).

The antidepressant plant *H. perforatum* have demonstrated interesting effects on HPA axis. Franklin and colleagues showed that the acute treatment of rats with *H. perforatum* causes an elevation of plasma corticosterone (Franklin *et al.*, 2000), whereas 15 days

treatment reduces brain levels of cortisol and corticosterone (Franklin *et al.*, 2004). The treatment of rats with flavonoids isolated from *H. perforatum* for two weeks down-regulated circulating plasma levels of ACTH and corticosterone (Butterweck *et al.*, 2004). The salivary concentration of cortisol under cortisol infusion was decreased in patients treated with *H. perforatum* when compared to placebo (Murck *et al.*, 2004). Long-term pre-treatment of rats with *H. perforatum* extracts and hypericin significantly decreased levels of corticotropin-releasing hormone (CRH) mRNA in the hypothalamic paraventricular nucleus and reduced the stress-induced increases in gene transcription of proopiomelanocortin (POMC) in the anterior pituitary, glutamic acid decarboxylase (GAD 65/67) in the bed nucleus of the stria terminalis (BST) and cyclic AMP response element binding protein (CREB) in the hippocampus (Butterweck *et al.*, 2001). Conflicting results were reported by Webber *et al.* (2006) for LI160 *H. perforatum* extract, both acute and 2 weeks treatments increased brain and plasma corticosterone levels in non-stressed mice.

Thus, the results obtained for *H. perforatum*, together with the importance of HPA axis for most of the comercial available antidepressant drugs, prompted us to study the effects of acute and repeated administration of *H. caprifoliatum*, compared to that of imipramine and bupropion, on serum and brain frontal cortex corticosterone levels in mice submitted or not to forced swimming test (FST).

2.2. BACKGROUND

The successful behavioral adaptation to stress modulated by steroid effects on higher brain functions involves negative feedback action via the paraventricular nucleus (PVN) of hypothalamus and neural inhibitory circuits that overcome the excitatory extra-hypothalamic influences on HPA axis. If stress control fails, an imbalance between stress stimuli and negative feedback develops at the different levels of the HPA axis such as: altered expression of corticotrophin releasing hormone (CRH) and vasopressin (VP) at the PVN of hypothalamus, and adrenocorticotropic hormone (ACTH) at pituitary. The extention of the alterations is dependent on the nature and context of the stress. How the imbalance between

excitatory and inhibitory signals develops is not known. Whatever the cause, in the end, after the adaptation of HPA axis, the imbalance leads to altered sensitivity to corticoids hormones at the adrenal level, down-regulation of hipocamppal glucocorticoid receptors (GR), and alterations on the negative feedback mechanisms resulting in incresead levels of hormones and peptides (de Kloet *et al.*, 1998; Sapolsky, 2001; Reul and Holsboer, 2002). These modifications are observed in patients with mood disorders, being documented the hypersecretion of CRH, ACTH and cortisol, exaggerated cortisol response to ACTH as well as blunted ACTH response to a CRH and a relative resistance to the dexamethasone suppressive effect on cortisol (Sachar *et al.*, 1976; Gold *et al.*, 1996; Holsboer and Barden, 1996). Postmortem samples revealed enlargement of pituitary and adrenal glands, increased CRH messenger RNA (mRNA) and downregulation of CRH receptors in the frontal cortex of victims of suicide (Nemeroff 1996).

Cortisol and corticosterone actions in the brain are mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs occur everywhere in the brain but are most abundant in hypothalamic CRH neurons and pituitary corticotropes. Most MRs in the brain is located in the hippocampus where they may be co-expressed with GR by some neurons (de Kloet *et al.*, 1998). Hippocampal MRs bind the glucocorticoid cortisol and corticosterone with approximately tenfold higher affinity than GRs. This “nonselectivity” of brain MRs is determined by the fact that unlike in peripheral cells, a different isoform of the steroid-metabolizing enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is present in the hippocampus, which does not effectively exclude corticosterone or cortisol from MR-targets in this structure (Seckl, 1997). Since hippocampal MRs have about tenfold higher affinity for corticosterone, these receptors are already almost completely occupied at basal levels of corticosteroid secretion. On the other hand, hippocampal GRs are only occupied when corticosteroid levels increase under stress conditions or at the peak of the circadian rhythm of corticosteroid secretion (Holsboer, 2000). The coexistence of MRs activated at low corticosteroid concentrations, and of GRs activated only at high concentrations, allows the brain to differentially respond to the wide range of concentrations over which corticosteroids are secreted. At low concentrations, cortisol/corticosterone maintains neuronal excitability

which is a predominantly MR governed effect, whereas at higher hormone concentrations this is opposed by increasing GR activation (Joëls and de Kloet 1992; Reul and Holsboer, 2002).

Chronic stress increases glucocorticoid levels and decreases BDNF (brain derived neurotrophic factor) and thereby causes atrophy or, in severe cases, death of hipocamppus CA3 neurons. These effects of stress could contribute to the observations of decreased volume and function of hippocampus in depressed patients. In addition, subtle damage of neurons by prior exposure to a neuronal insult could explain the selective vulnerability of certain individuals to become depressed when exposed to stress. Genetic factors could also contribute to such selective vulnerability. Studies with depressed patients, animals and cellular models have demonstrated that antidepressants increase expression and function of GR, and promote its nuclear translocation, these effects being associated with normalization of GR-mediated negative feedback by endogenous glucocorticoids (Pariante and Miller, 2001). Chronic antidepressant treatment increases the expression of BDNF and TrkB, and prevents the down-regulation of BDNF in response to stress. This could reverse the atrophy of hippocampal neurons, as well as protect these neurons from further damage. Up-regulation of BDNF and TrkB occurs via increased 5-HT and NE neurotransmission and up-regulation of the cAMP–CREB cascade (Duman *et al.*, 1998; Duman and Monteggia, 2006). This cascade of events is depicted in the figure 2.1.

Therefore, antidepressant's effect on monoaminergic systems may lead to the normalization of HPA axis through limbic system connections. Serotonin has been implicated in the stress-related regulation of the HPA axis. The dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) innervate both limbic and HPA axis structures (Cooper, 1996). Serotonergic fibers from the DRN and the MRN innervate CRH producing cell bodies in the PVN of hypothalamus and there are serotonergic neurons located entirely in the hypothalamus. There is evidence that central serotonergic systems exert a positive control on the HPA axis and that reciprocally, glucocorticoids and catecholamines mediate stress-induced alterations in the central serotonergic systems (Tafet and Bernardini, 2003). Adrenaline and noradrenaline belong to the other main system of adaptative response to stress, the sympatho-adrenergic-noradrenergic (SAN) axis. The SAN and HPA axis produce

mutual positive regulation; the activation of one involves the activation of the other and vice-versa (Abercrombie and Zigmond, 1995). The locus coeruleus (LC) projects noradrenergic fibers to various structures, including amygdala, hippocampus and PVN of hypothalamus (Charney *et al.*, 1995). In association with the LC, the lateral hypothalamus mediates the activation of the sympathetic component of the autonomic nervous system (ANS). Dopaminergic system is also implicated in stress-related regulation of HPA axis and depression (Nestler and Carlezon, 2006). The mesolimbic and mesocortical dopaminergic projections are involved in adaptational process; the first one processes and reinforces rewarding and motivational stimuli; and the second one is involved in cognitive functions such as evaluation potentially stressful situations (Le Moal, 1995). The role of hypothalamus in reward mechanisms is well recognized by means of self stimulation experiments (Nestler and Carlezon, 2006). Acute stress increases mesolimbic dopamine (DA) release (Imperato *et al.*, 1993; Lindley *et al.*, 1999; Dazzi *et al.*, 2001). There is evidence that dopamine agonists exert a positive control on the HPA axis. Activation of brain dopaminergic receptors, by pergolide and chemical analogs, leads to increased serum corticosterone concentration in rats, and this effect is reversed by dopamine antagonists (Fuller and Snoddy, 1983, 1984; Foreman *et al.*, 1989).

Pariante *et al.* (2001) suggested that the interactions between antidepressants and glucocorticoids also may be mediated via the multiple drug resistance (MDR) P-glycoprotein (Pgp). The MDR-Pgp expression in endothelial cells of capillary blood vessels at the blood brain barrier (BBB) was first reported by Cordon-Cardo *et al.* (1989), and associated to brain protection against apolar xenobiotics and resistance to some cancer chemotherapies. Many other substances as modern histamine H-1 receptor antagonists, calcium channel blockers, antidepressants are also Pgp substrates and/or inhibitors (Pariante *et al.*, 2001; Weiss *et al.*, 2003; Loscher and Potschka, 2005). Nowadays it is established that some GR ligands, like cortisol and dexamethasone, are actively excreted from cells by the MDR-Pgp and other membrane transporters also belonging to the ATP-binding cassette transport family (Muller *et al.*, 2003). Based on these findings, a relationship between Pgp activity at BBB and the regulation of the HPA system under both basal and stress conditions has been suggested. It is proposed that antidepressants could inhibit steroid transporters localized on the BBB in

humans, increasing the access of cortisol to the brain and, in consequence, the glucocorticoid-mediated negative feedback on the HPA axis (Pariante and Miller, 2001; Juruena *et al.*, 2004; Pariante *et al.*, 2004). Other factors that control the access of natural or synthetic corticosteroids to the brain receptors as the corticosteroid-binding globulin (CBG) and the isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD 1 and 2), were not yet clearly correlated with depression (Webber *et al.*, 2000; Poor *et al.*, 2004; Holmes *et al.,* 2006).

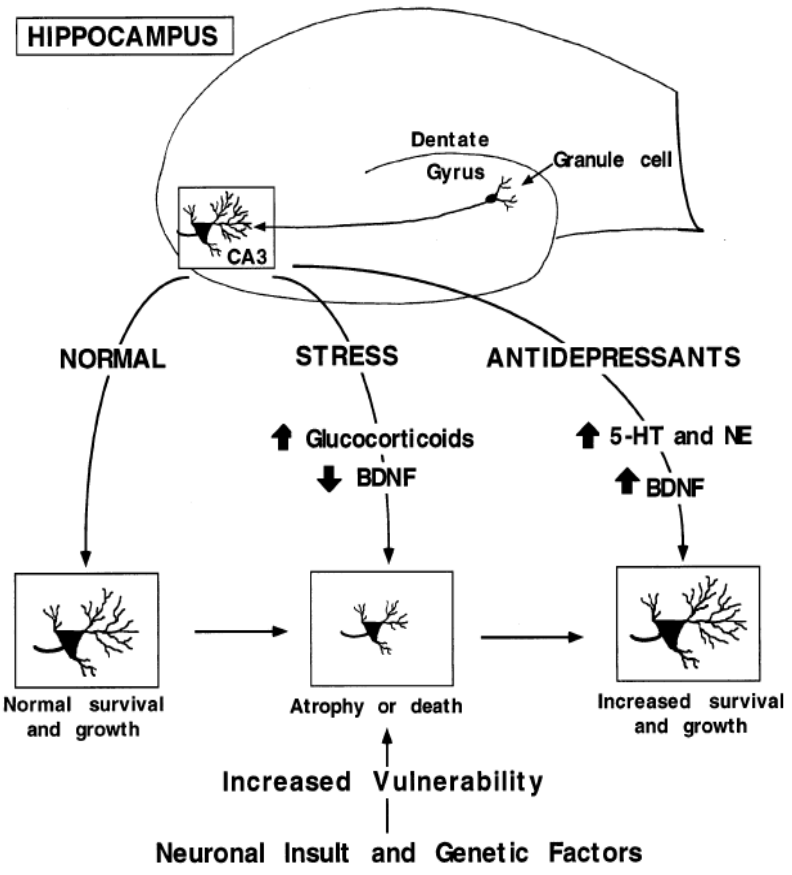

Figure 2.1. The influence of stress and antidepressant treatments on CA3 pyramidal neurons in the hippocampus (Duman *et al*.,1997).

2.3. MATERIALS AND METHODS

2.3.1. Animals

Male Swiss albinos CD1 mice (IFFA-CREDO/Charles River, Saint-Germain sur L'Arbresle, France), weighing 25-30 g, were housed 20 in Makrolon cages (L : 40 cm, W : 25 cm, H : 18 cm), with free access to standard semi-synthetic laboratory diet (U.A.R., Villemoisson sur Orge, France) and tap water ad libitum. The animals were kept in a ventilated room, at a temperature of 22 ± 1°C, under a 12-h light/12-h dark cycle (light on between 7:00 a.m. and 7:00 p.m.). On the day before sacrifice, in order to become familiar with the environment, mice were placed in an experimental room with food and water available ad libitum and with the same housing conditions.

All the experiments were carried out between 8:00 a.m. 12:00 a.m. in testing rooms adjacent to the animal rooms. Animal manipulations were performed according to the European Communities Council Directive of November 24th 1986 (86:609:EEC), approved by Regional Ethical Committee for Animal Experimentation (Normandy; no. 09-04-04-11) and conducted by authorized investigators.

2.3.2. Drugs

Hypericum caprifoliatum extract (HCP): The aerial parts of *H. caprifoliatum* were collected in the region of Viamão in the state of Rio Grande do Sul - Brazil (August/2003). The voucher specimen was deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon, 1496). The dried and powdered plant material (120 g of aerial parts) was extracted with cyclohexane (plant/solvent ratio 1:10 w/v) by maceration (3x24h), followed by evaporation to dryness under reduced pressure at 45ºC yielding an extract named HCP (c.a. 5.0 g).

Bupropion HCl, imipramine HCl, corticosterone and rabbit antibodies against corticosterone were obtained from Sigma Aldrich, Saint-Quentin Fallavier (France); cyclohexane, and polysorbate 80 from Merck, Darmstadt/DE; tritiated corticosterone ([1,2,6,7(N)]3H-corticosterone), specific activity 81 Ci/mmol from Amersham, Orsay, (France).

2.3.3. Treatments and procedures

Acute treatment: groups of 30 animals were treated by gavages with HCP (360 mg/kg), imipramine (IMI - 20 mg/kg), bupropion (BUP - 30 mg/kg) or saline (with 10% polysorbate 80). *Repeated treatment:* groups of 30 animals were treated once daily with the same doses as in the acute treatment, during 3 days.

In each of preceding groups half of the animals (n = 15) were submitted to a single 6 min forced swimming session (FSS), 1h after the single or the third treatment on the repeated one.

All procedures (mice isolation, forced swimming session and decapitation) were performed respectively in separated rooms.

2.3.4. Forced-swimming test

The forced-swimming test was essentially similar to that described by Porsolt *et al.* (1977). The apparatus consisted of two Plexiglas cylinders (20 cm height, 14 cm internal diameter) placed side by side in a Makrolon cage (38 x 24 x 18 cm), filled with water, maintained at 22 ± 1°C, to a height of 12 cm instead of 6 cm suggested by Porsolt *et al.* (1977). Two mice were tested simultaneously for a 6-min period inside vertical Plexiglas cylinders; an opaque screen placed between the two cylinders prevented mice from seeing each other. The total duration of immobility was measured, during a 6 minutes test period, with an automated image analysis system (Videotrack MV 45 system).

2.3.5. Corticosterone assay

Cortical and serum corticosterone levels were determined by radioimmunoassay (RIA). Serum corticosterone level was assayed as described by Le Cudennec *et al.* (2002). Mice submitted to FSS were sacrificed 30 min after the swimming session. Mice not submitted to FSS were sacrificed 1h after the last treatment. Trunk blood was collected and was centrifuged (13,000 *g*; 4°C; 15 min) after clotting. Serum was collected, centrifuged again (13,000g, 4°C, 15 min) and aliquots of 100 µl were stored at – 40°C until RIA.

For cortical corticosterone measurements, the frontal cortex, dissected according to Glowinski and Iversen (1966), of 3 mice was weighed (approximately 125 mg) and homogenized in 1 ml phosphate buffer (pH 7.4) containing 1% bovine serum albumin (BSA) and stored at – 40°C until RIA.

Cortex homogenates (1 ml) and serum aliquots (11 µl) were extracted by mixing with 2 ml of ethyl acetate, followed by centrifugation (3000 g; 4°C; 5 min) to speed up phase separation. Aliquots of 1.5 ml of the ethyl acetate phase were evaporated to dryness. Samples and standards (39 - 10,000 pg/tube) were incubated at 4°C overnight in a final volume of 1 ml with rabbit antibodies against corticosterone and tritiated corticosterone. The separation of free and bound corticosterone was performed, at 4°C, by adding 500 µl of dextran-coated charcoal suspension. Ten minutes later, samples were centrifuged (3300 *g*; 4°C; 15 min) and radioactivity contained in the supernatant was measured by liquid scintillation (Tri-carb 2100TR, Packard Bioscience Co). The intra-assay and inter-assay coefficients of variation were 3.6 and 5.2% respectively.

2.6. Statistical analysis

Results are expressed as means ± S.E.M. Statistical of differences between groups was performed by using ANOVA (one or two factors) followed by Newman-Keuls multiple comparison tests. A probability levels of 0.05 or smaller was used to indicate statistical significance.

2.4. RESULTS

2.4.1. Effect of acute and repeated treatment with IMI, BUP and HCP on swim stress-induced immobility

As expected (Viana *et al.*, 2005), acute administration of IMI (20 mg/kg, per os), BUP (30 mg/kg, per os) or HCP (360 mg/kg, per os) significantly ($p < 0.05$ - 0.001) reduced the immobility time induced by forced swimming test, compared to saline treated mice (Figure

2.2). When administrated daily during three days, IMI (20 mg/kg, daily), BUP (30 mg/kg, daily) or HCP (360 mg/kg, daily) also provoked a significant ($p < 0.01$) reduction in the immobility time (Figure 2.2). The repeated treatment effect of IMI and HCP was somewhat more important than that measured in mice treated acutely. The percentage of immobility time reduction in mice treated three days with IMI (52.7 ± 5.1%) or HCP (39.6 ± 2.5%) differs significantly ($p < 0.05 – 0.01$) from that of mice treated acutely (36.3 ± 2.4% and 29.2 ± 2.2% for IMI and HCP respectively). The percentage of immobility time reduction in mice treated with BUP in the acute treatment was 63.2 ± 5.1% and did not change after three days treatment 63.3 ± 6.6%.

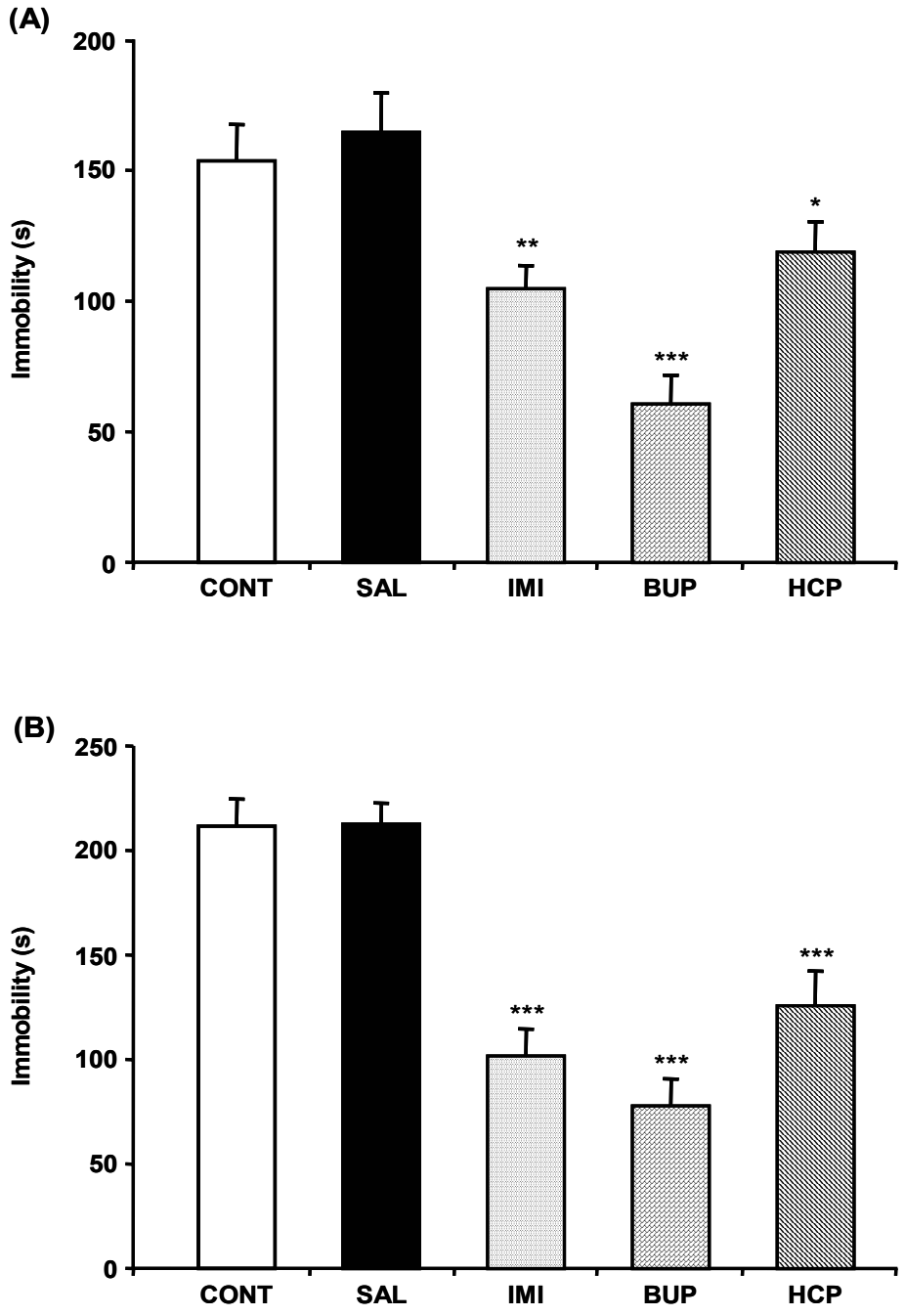

Figure 2.2. Effect of (A) acute (1 day) or (B) repeated treatment (3 days) with imipramine (IMI 20 mg/kg), bupropion (BUP 30 mg/kg), *H. caprifoliatum* (HCP 360 mg/kg) or saline (SAL), CONT (naive mice) on forced swimming test. 60 minutes after the last treatments, the animals were submitted to the swimming test, and the total duration of immobility was measured during a 6-min test period. Data are presented in mean ± SEM (n = 15 mice/group). Significantly different values were detected by one way ANOVA followed by post hoc Student-Newman-Keuls test : *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to their respective saline (SAL) group.

2.4.2. Effect of forced swimming test on serum and cortical corticosterone levels

The FST significantly ($p < 0.001$, Student- Newman-Keuls test) increased both serum (+400%) (Figure 2.3A, Figure 2.4A) and cortical (+600%) (Figure 2.3B, Figure 2.4B) corticosterone levels, measured 30 min later. These effects were similar whether animals were treated with saline or were naïve mice (CONT).

2.4.3. Acute effect of IMI, BUP or HCP on serum and cortical corticosterone levels

Acute treatment with IMI (20 mg/kg, *p.o.*) or BUP (30 mg/kg, *p.o*.) did not have significant effect neither on serum (Figure 2.3A) nor on cortical (Figure 2.3B) corticosterone levels, both in mice submitted or not to a FST. Acute treatment with HCP (360 mg/kg, *p.o*.), also did not cause significant effect on FST-induced increase of serum and cortical corticosterone levels, but produced a marked ($p < 0.001$) increase in basal serum corticosterone level (Figure 2.3A). It is noteworthy that, although not significantly, HCP also induced a slight increase of cortical corticosterone level (Figure 2.3B).

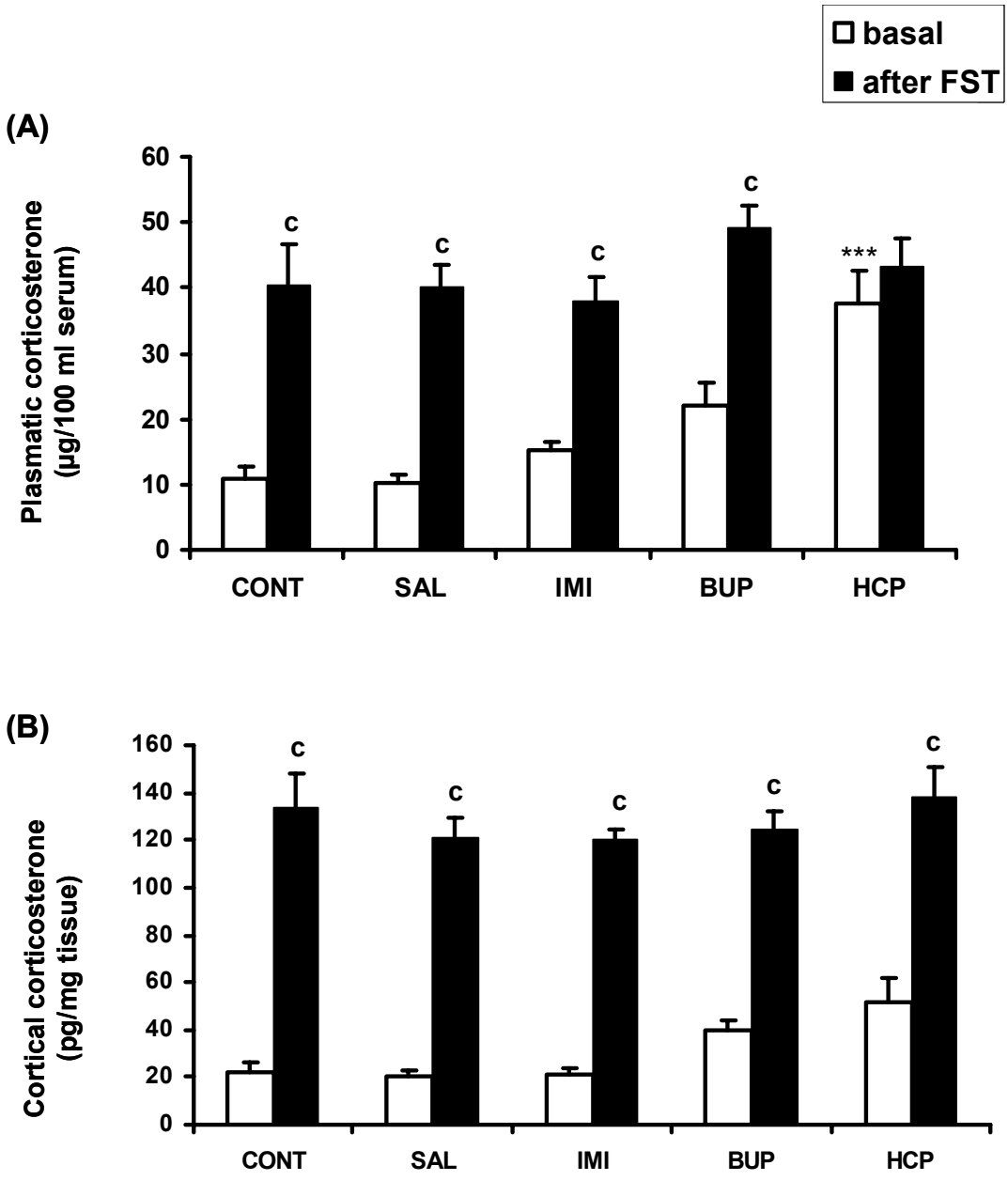

Figure 2.3. Effect of acute treatment (1 day) with imipramine (IMI 20 mg/kg), bupropion (BUP 30 mg/kg), *H. caprifoliatum* (HCP 360 mg/kg) or saline (SAL) on serum (A) and cortical (B) corticosterone levels, in mice submitted or not to forced swim stress. Data are presented in mean ± SEM (n = 15 mice/group). Significantly different values were detected by two way ANOVA followed by post hoc Student-Newman-Keuls test : $^{c}p < 0.001$ compared to respective no swim stressed group; ***$p < 0.001$ compared to respective saline (SAL) group.

2.4.4. Repeated treatment effect of IMI, BUP or HCP on serum and cortical corticosterone levels

After three days of treatment, the increase of both serum and cortical corticosterone levels elicited by FST was significantly (p < 0.01-0.001) reduced by IMI (20 mg/kg, daily) and BUP (30 mg/kg, daily), while they decreased slightly but not significantly (two way ANOVA) both serum and cortical basal corticosterone levels in mice without swim stress (Figure 2.3). It is noteworthy that, when compared with one way ANOVA, separately from the stress factor, there are a significant difference (p < 0.05) among the basal corticosterone levels of group treated with saline and IMI or BUP. The repeated treatment with HCP (360 mg/kg, daily) significantly reduced only the cortical corticosterone level (Figure 2.3B) but not serum corticosterone level (Figure 2.3A). In this treatment group, the basal serum corticosterone level in mice without stress was not markedly modified (Figure 2.3A), whereas the basal cortical corticosterone level in mice without stress was slightly, but not significantly, increased (Figure 2.3B). In percentage terms, the reductions in serum and cortical corticosterone levels-induced by FST were respectively: 58.9 and 57.2% for IMI; 36 and 46.2% for BUP; and 24 and 49.9% for HCP, relatively to respective saline treated mice.

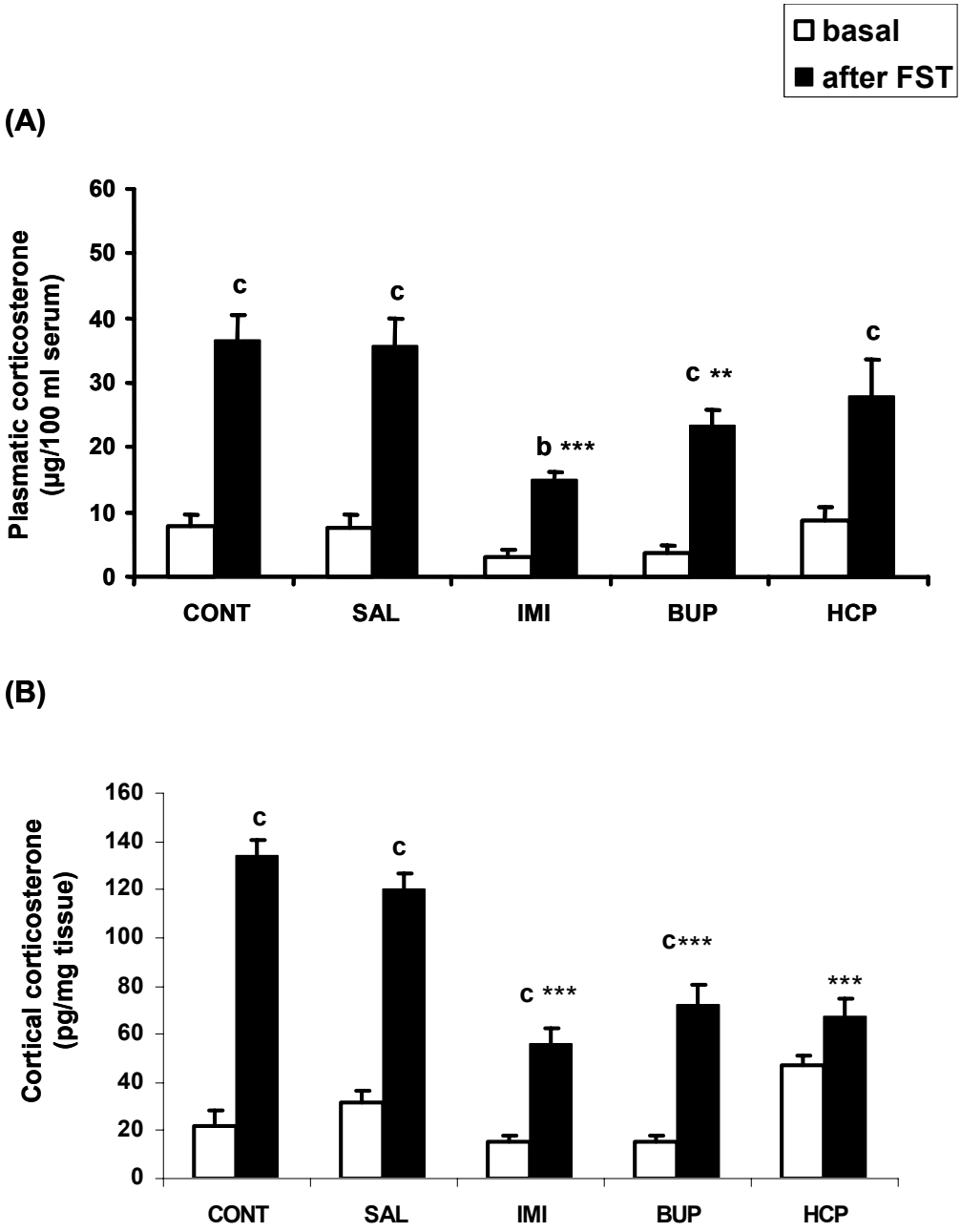

Figure 2.4. Effect of repeated treatment (3 days) with imipramine (IMI 20 mg/kg daily), bupropion (BUP 30 mg/kg daily), *H. caprifoliatum* (HCP 360 mg/kg daily) or saline (SAL) on serum (A) and cortical (B) corticosterone levels in mice submitted or no to forced swim stress. Data are presented in mean ± SEM (n = 15 mice/group). Significantly different values were detected by two way ANOVA followed by post hoc Student-Newman-Keuls test : $^{b}p < 0.01$, $^{c}p < 0.001$ as compared to respective no swim stressed group; $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to respective saline (SAL) group.

2.5. DISCUSSION

In this study we have confirmed that a forced swimming test (FST) is a major stressor in mice (Marek *et al.*, 1993; Grosel *et al.*, 1993; Mogil *et al.*, 1996). In all experiments, cortical and serum corticosterone levels were significantly increased by forced swimming session in control animals or in mice treated with saline. This marked increase in serum and brain corticosterone levels reflects an activation of the hypothalamo-pituitary-adrenal (HPA) axis. This neuroendocrine activity induced by forced swimming was significantly reduced, in both serum and frontal cortex, by repeated treatments (3 days) with imipramine and bupropion. To the best of our knowledge, this is the first report of a prevention of a stress-induced increase in serum or cortical corticosterone levels elicited by antidepressants after a so brief treatment. Previous studies had practiced acute (Delbende *et al.*, 1991) and chronic treatments (14 – 30 days) and measured only plasma or serum corticosterone (Holsboer, 2000; Pariante and Miller, 2001) with few reports to brain levels (Weber *et al.*, 2006).

When administered acutely none of the antidepressants was effective in reducing FST-induced increase in serum or cortical corticosterone levels, despite of their anti-immobility effects. Thus, it appears that the reduction in FST immobility time after the acute administration of drugs is not linked to a decrease in either serum or cortical corticosterone. This is reinforced by the fact that BUP reduces serum and cortical corticosterone response to FST only when administered during three days; it reduces the immobility in the FST with the same magnitude in both acute and repeated treatment. Conversely, the reduction of FST immobility time induced by IMI and HCP is more pronounced after repeated than after acute treatment. The lack of more pronounced decrease in immobility time, in mice repeatedly treated with BUP makes uneasy the establishment of a correlation between the two effects. One may notice that, among the three tested drugs, BUP is the only to induce stimulant locomotor effect (Nielsen *et al.*, 1986; Zarrindast and Hosseini-Nia, 1988; Martin *et al.*, 1990; Ascher *et al.*, 1995), which may interfere with the immobility time in the FST. Considering all these points, one cannot establish a relationship between the decrease in corticosterone response to FST and the reduction in immobility time during the FST after the third injection of tested drugs.

Similarly to IMI and BUP, HCP had no acute effect on stressed mice corticosterone levels and was effective in reducing the immobility time in the FST in both treatment regimens, confirming data observed by Daudt *et al.* (2000) and Viana *et al.* (2005). Differently from IMI and BUP, after a three days treatment, HCP decrease cortical but not serum corticosterone levels. It should be remarked that, under acute treatment with HCP, there was no significant difference in the corticosterone levels between mice submitted or not to a FST. However, this does not represent a true protection against stress since animals treated with HCP already presented hormone basal levels somewhat higher than animals receiving other treatments. Our results are in agreement with those reported by Franklin *et al.* (2000) and Webber *et al.* (2006) for *H. perforatum* extracts, an effective antidepressant (Linde *et al.*, 1996; Butterweck *et al.*, 1997; Müller *et al.*, 1998; Brenner *et al.*, 2000; Schrader, 2000; Müller, 2003). They showed that acute (Franklin *et al.* 2000; Webber *et al.*, 2006) or two weeks (Webber *et al.*, 2006) treatment with *H. perforatum* extracts causes an elevation of plasma and brain corticosterone. Furthermore, several studies report elevation in plasmatic HPA axis hormones in men and animals after antidepressant treatment with citalopram, fluoxetine, reboxetine, mirtazepine, *H. perforatum* extract WS 5570 and LI 160 (Seifritz *et al.*, 1996; Duncan *et al.*, 1998; Schule *et al.*, 2004a, b; Webber *et al.*, 2006). Some authors support that monoamines elevation in the synaptic clef activate of post-synaptic receptors in the hypothalamus and stimulates the secretion of several hormones, postulating that antidepressant's effect on monoaminergic systems may lead to the normalization of HPA axis through limbic system connections (Raap and van de Kar, 1999; Tafet and Bernardini, 2003; Schule *et al.*, 2004b).

Franklin *et al.* (2004) showed that reduced brain levels of corticosterone and cortisol were verified in rats fed during two weeks with pellets containing *H. perforatum* extracts. The authors suggest that *H. perforatum* extracts reduce intracerebral glucocorticoid concentration possibly by its action to induce the expression of the multiple drug resistance protein P-glycoprotein. Recent works suggest a possible role of membrane steroid transporters, like Multiple Drug Resistance protein P-glycoprotein (MDR-Pgp), in the regulation of glucocorticoids receptors function during antidepressant treatment (Juruena *et al.*, 2004; Pariante *et al.*, 2004). The hypothesis that antidepressants could exert their clinical effects

through a direct modulation of the glucocorticoids receptor was one of the most innovative for the mechanism of action of this class of drugs (Holsboer, 2000; de Kloet *et al.*, 2005). The MDR-Pgp inhibition operated by antidepressants would inhibit glucocorticoids extrusion from the cell, and thus increase the glucocorticoid-mediated negative feedback on the HPA axis (Pariante *et al.*, 2004). We can assume that the results found in this work with tested drugs, i.e. the lack of acute effect of IMI and BUP on serum corticosterone and their reduction in serum and cortical corticosterone after repeated treatments is due to negative feedback enhancement of the HPA axis.

Contrary to antidepressants, *H. perforatum* extracts and isolated substances, hypericin, hyperforin and flavonoids, are inducers of this glycoprotein at gastro-intestinal levels (Durr *et al.*, 2000; Perloff *et al.*, 2001; Hesseney *et al.*, 2002; Webber *et al.*, 2006). Even, if Webber *et al.* (2004) demonstrated that *H. perforatum* extract, as well as hypericin or hyperforin inhibit MDR-Pgp in a human lymphocytic leukaemia cell line and in porcine brain capillary cells (which model blood brain barrier), in their most recent paper (Webber *et al.*, 2006), a two weeks treatment with *H. perforatum* extract (LI 160) significantly increased the expression of Pgp in mice brain homogenate. In addition, they observed a linear relation between plasma and brain concentrations of corticosterone in mice treated acute and chronically with different antidepressants, indicating passive corticosterone diffusion into the brain and minor influence of Pgp. The authors support that antidepressant effect on HPA axis should be via monoaminergic transmission. The HPA axis receives and integrates many inputs indicative of stress from different brain areas that converge in the paraventricular nucleus of hypothalamus (Chrousos and Gold, 1998; Reul and Holsboer, 2002). The cooperative interplay between HPA axis and central nervous system provides the means through which thoughts and emotions may regulate hormone secretion (Tafet and Bernadini, 2003).

At present, the mechanism by which HCP reduces brain corticosterone levels remains to be elucidated. Although the possibility that HCP operates via MDR Pgp inhibition cannot be ruled out, the effect of *H. caprifoliatum* on this protein merits to be performed. It is plausible that ability of HCP to inhibit monoamines uptake could lead to modifications that reduce stress-induced corticosterone rise. In any case, the mechanism by which HCP reduces corticosterone levels seems to be different from the one of antidepressants, since the

repeated treatment with HCP has no effect in non stressed animals and its protection against FST induced corticosterone raise is observed only in the cortex. In order to elucidate the mechanisms involved in HCP effect on corticosterone level, further investigations should be performed.

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CHAPTER 3: Study of antidepressant and antinociceptive activities of *Hypericum polyanthemum* Klotzsch ex Reichardt

3.1. INTRODUCTION

In this chapter, we present the study of *Hypericum polyanthemum* antidepressant profile. As *H. caprifoliatum, H. polyanthemum* is native to South Brazil and presents high concentration of phloroglucinol derivatives (Nör *et al.* 2004). Furthermore, some extracts and pure compounds obtained from this species demonstrated monoamine oxidase inhibitory and antinociceptive activities (Gnerre et al, 2001; Viana *et al.*, 2003).

The aim of this chapter was to investigate the potential antidepressant and analgesic effect of *H. polyanthemum* cyclohexane extract in animal models and search its mode of action by studying the extract effect on the monoaminergic and opioid systems, and its influence on the hypothalamic-pituitary-adrenal (HPA) axis.

3.2. BACKGROUND

Guttiferae is a large family with more than 1,000 species. According to some authors the family is divided into six subfamilies, the Hypericoideae - including the genera *Hypericum* (tribe Hypericeae), *Cratoxylum* (tribe Cratoxyleae), *Harungana*, *Psorospermum* and *Vismia* (tribe Vismieae) (Cronquist 1981, Bennet and Lee 1989) - frequently being considered as an independent family by some authors (Robson, 1990).

The genus *Hypericum* has more than 400 species accommodated in 30 sections (Robson, 1990). The species growing in Rio Grande do Sul, Southern Brazil, belong to the sections *Brathys* and *Trigynobrathys* (Robson 1990), the latter with a greater number of native representatives.

The section *Brathys* includes 88 species of shrubs (rarely small trees), subshrubs, shrublets, and wiry annuals (rarely perennials) distributed into four subsections. This section is predominantly encountered in two centers, Belize and Cuba, and the Venezuela-Colombia

border. From the latter area, the mainly shrubby part has radiated eastward to Roraima, westward to Costa Rica, and southwestward along the Andes and Bolivia, with a disjunct species (*H. piriai* Arechav.) in southeastern Brazil and Uruguay. Besides *H. piriai*, the other species from this section found in southern Brazil is *H. gentianoides* (L.) Britton *et al.* (Robson,1990).

The section *Trigynobrathys* comprises 52 species of shrubs, subshrubs, shrublets, as well as perennial and annual herbs. The primary area of speciation has been southeastern Brazil, with a primary radiation (to the south of Amazon) northward to eastern Brazil (Bahia), and to southeastern USA, southward to Uruguay and northern Argentina, westward to Bolivia, Peru, and northern Chile and then north along the Andes to southern Colombia and the Galapagos Islands, and west to New Zealand, Australia, New Caledonia, and New Guinea and to scattered occurrences in south-east Asia. The species of this section are distributed over two subsections: *Connatum* and *Knifa*. The species growing in southern Brazil belong to the former (Robson, 1990).

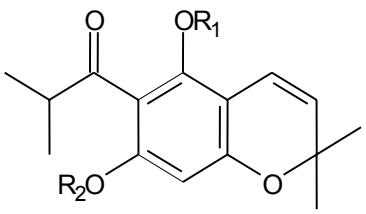
Since 1998, our group studies the chemical and pharmacological features of South Brazilian *Hypericum* species (Dall'agnol *et al* 2005, Ferraz et al, 2001; Schmitt, 2001; Daudt *et al*, 2000; Viana et al, 2003, 2005, 2006). After phytochemical investigation of eight species of *Hypericum* native to southern Brazil, Ferraz *et al.* (2002a) verified the absence of hypericin and pseudohypericin in all species. As there is a good correlation between dark glands and hypericin production (Robson, 1990), the absence of hypericins in the Brazilian native species is consistent with the absence of these glands in species from sections *Brathys* and *Trigynobrathys* (Robson, 1990, Ferraz et al, 2002a).

South Brazilian *Hypericum* species have a strong tendency to accumulate phenolic compounds with benzopyran nucleus. Normally, free benzopyrans or chromenes are rare in *Hypericum* species; these compounds were isolated from few species such as *H. revolutum* Vahl. (Décosterd *et al.,* 1986). From the chloroform extract of *H. polyanthemum* aerial parts, three new 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) were isolated (Ferraz *et al.,* 2001) (Figure 3.1). A

protocol for *in vitro* propagation of *H. polyanthemum* was established by Bernardi e coll (2005). In vitro regenerated plantlets were successfully acclimatized and displayed the same benzopyrans, quantified by HPLC, were the same found in the field-grown plants.

At the same time, the benzopyran nucleus is a common constituent of other phenolic compounds present in *Hypericum* species, such as benzophenones, xanthones, phloroglucinol derivatives, due to the cyclization of a prenyl radical with a vicinal aromatic hydroxyl group, both frequently found in these substances. Differently from the well-known hyperforin and adhyperforin isolated from *H. perforatum*, that are polyisoprenilated phloroglucinols, Brazilian species are a source of dimeric structures, consisting of filicinic acid and a phloroglucinol moiety (von Poser *et al.*, 2006). Uliginosin B was isolated from *H. myrianthum* (Ferraz *et al.,* 2002b), *H. carinatum* and *H. polyanthemum* (Nör *et al.,* 2004); hyperbrasilol B from *H. caprifoliatum* and *H. connatum* (Nör *et al.,* 2004). A phloroglucinol named HC1 was isolated from *H. caprifoliatum* but its chemical structure remains not completely elucidated (Viana *et al.*, 2005) (Figure 3.1). From these results we can point out that Southern Brazilian *Hypericum* species differ chemically from *H. perforatum*.

Benzopyrans

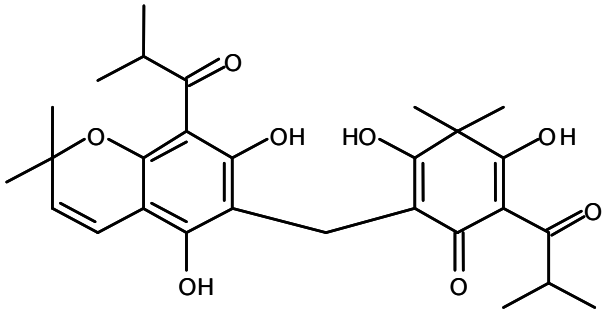


HP1 R_1=Me R_2=Me

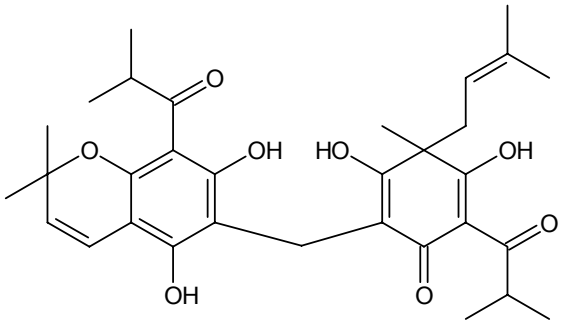
HP2 R_1=Me R_2=H

HP3 R_1=H R_2=Me

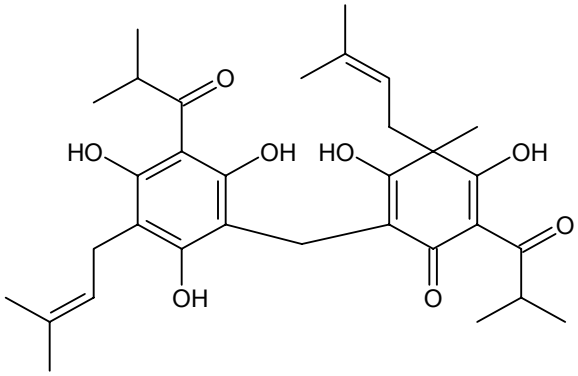
Phloroglucinols



Uliginosin B (HP4)



Hyperbrasilol B



Putative HC1 structure

Figure 3.1.: Molecules isolated from the lipophilic extracts of Southern Brazil *Hypericum* species.

With regard to pharmacological activity, especially at the central nervous system (CNS), *H. piriai* petroleum ether extract, *H. caprifoliatum* and *H. polyanthemum* chloroform extract and the benzopyrans (Gnerre *et al.* 2001) showed *in vitro* MAOI (monoamino oxidase inhibitory) activity (Gnerre *et al.* 2001). However, this activity does not appear to be relevant for antidepressant-like effect, since the extracts which displayed this activity were not active in the forced swimming test (FST) (Gnerre *et al.* 2001). After screening eight *Hypericum* species using the FST, we had promising results with *H. caprifoliatum* lipophilic extracts (Daudt *et al.*, 2000; Viana, 2002). *H. polyanthemum* crude methanolic extract showed slight but not significant reduction in forced swimming test immobility time (Gnerre et al, 2001). On the other hand, *H. polyanthemum* and *H. caprifoliatum* cyclohexane extract demonstrated antinociceptive effect in the hot plate and writhing tests (Chapter 1: Viana et al, 2003).

3.3. MATERIALS AND METHODS

3.3.1. Plant material

The aerial parts of *Hypericum polyanthemum* Klotzsch ex Reichardt were collected in Caçapava do Sul, in the state of Rio Grande do Sul - Brazil (August/2003). The *voucher specimens* were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon1 429).

3.3.2. Preparation of extracts and compounds isolation

The dried and powdered plant material (120 g of aerial parts) was extracted with cyclohexane (plant/solvent ratio 1:10 w/v) by maceration (3x24h), followed by evaporation to dryness under reduced pressure at 45^{o}C yielding an extract termed POL (c.a. 5.0 g). The benzopyrans (HP1, HP2, HP3) and the phloroglucinol derivative, uliginosin B (HP4), were isolated by means of thin layer and column chromatography as described elsewhere (Ferraz *et al.* 2001; Nör *et al.*, 2004).

3.3.3. Animals

Adult male Wistar or Sprague Dawley rats (weight 200-300 g) and male CF1 or CD1 Swiss mice (25–30 g) purchased from Fundação Estadual de Produção e Pesquisa em Saúde – RS (Brazil) or IFFA-CREDO/Charles River Laboratories (Domaine des Oncins, Saint-Germain sur L'Arbresle, France) colony were used. The animals were housed five rats or twenty mice in plastic cages (L: 42 cm, W: 27.5 cm, H: 16 cm). All animals were kept under a 12 hours light/dark cycle (lights on at 7:00 a.m.) at constant temperature of $23^{\circ} \pm 1^{\circ}C$ with free access to standard certified rodent diet and tap water.

All behavioural experiments were approved by CONEP - Brazil (National Commission of Research Ethics (Nº project approval number 01-188 at 07/26/2001) and performed according to guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998), which are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.3.4. Drugs and Reagents

Desipramine hydrochloride, fluoxetine hydrochloride, imipramine, naloxone, hydrochloride, corticosterone, rabbit antibodies against corticosterone and SCH 23390 (Sigma Aldrich, Saint Quentin Fallavier, France). Cocaine hydrochloride was obtained from la Coopérative Pharmaceutique Française (Melun, France). Cyclohexane, polysorbate 80 (Merck, Darmstadt/ DE), sulpiride (DEG, São Paulo/ BR).

[^{3}H]-dopamine ([^{3}H]-DA, 48 Ci/mmol), [^{3}H]-naloxone (56 Ci/mmol), [^{3}H]-nisoxetine (86 Ci/mmol) and [1,2,6,7(N)]^{3}H-corticosterone (81 Ci/mmol) were purchased from Amersham (Orsay, France). [^{3}H]-noradrenaline ([^{3}H]-NA, 12.5 Ci/mmol), [^{3}H]-serotonin ([^{3}H]-5HT, 25.5 Ci/mmol), [^{3}H]-citalopram (84.2 Ci/mmol) and [^{3}H]-mazindol (24.5 Ci/mmol) were purchased from Perkin-Elmer-NEN Life Science Products (Paris, France). Subsequent dilutions and solutions of these agents were performed in the incubation medium.

3.3.5. Forced Swimming Test (FST)

In rats:

The Porsolt's procedure (Porsolt *et al.*, 1978) was used with minor modifications. 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 minutes in water with temperature between 23 ± 2°C and height of 30 cm (Porsolt *et al.*, employed 15 cm). The ambient temperature was approximately 24°C. At the end of the swimming exposition, the animals were removed from the water and gently dried. The treatment was administered 5 minutes, 19 and 23 hours after the first swimming exposition. One hour after the last injection (24 hours after the first swimming session), the animals were submitted to a second swimming exposure (5 minutes), and their immobility time was measured.

Rats were treated with POL 270 mg/kg/day, p.o. (3 administrations of 90 mg/kg). This dose was chosen based on results from *H. caprifoliatum* (Viana *et al.*, 2005). POL was diluted to a concentration of 90 mg/ml in water with 2% of polysorbate 80. Imipramine hydrochloride 20 mg/kg (60 mg/kg/day, p.o.) was used as the antidepressant reference drug and 2% of polysorbate 80 in water was used as the negative control. All treatments were administered at 1 ml/kg body weight.

In mice:

The apparatus consisted of a glass cylinder (10 cm internal diameter, 25 cm height) filled with water (19 cm height) at 23 ± 2°C. Different groups of mice were acutely treated, per os (10 ml/1kg), with POL 180, 270 or 360 mg/kg, uliginosin B 90 mg/kg, imipramine 20 mg/kg, or 2% polysorbate 80 solution in water.

For evaluating the dopaminergic action, different groups were pre-treated with sulpiride (50 mg/kg, i.p.) or SCH 23390 (15 μg/kg, s.c.) 30 minutes before the administration of POL 270 mg/kg p.o. Sixty minutes later, the animals were placed into the cylinder and the total duration of immobility, during a 6 minutes test period, was measured.

3.3.6. Hot-plate test

The test was performed as described by Viana et al (2003) (Chapter 1). Shortly, the animals were placed on the hot-plate (54 ± 2 ºC) to measure baseline responsiveness, and immediately treated with HP1, HP2, HP3 or HP4 (90 mg/kg, i.p.), the positive control was morphine (6 mg/kg, s.c.) and the negative control was 2 % polysorbate 80 in water (SAL). Treatment-induced changes in responsiveness to heat were observed 30 min later. To evaluate the possible involvement of opioid-mediated mechanisms, groups showing antinociceptive effect were pretreated with naloxone (2.5 mg/kg, s.c.), a nonspecific opioid receptor antagonist, immediately after evaluating baseline responsiveness and 10 min before extract administration.

3.3.7. Locomotor activity

Locomotor activity was accessed automatically in a digiscan photocell activity meter box (45 x 30 x 30 cm) (Omnitech Eletronics Inc, Columbus, OH). The response to POL 270 mg/kg, administered by gavage (10 ml/kg body weight) immediately before the test, was expressed as the number of beams crossed between the 5^{th} and 45^{th} minutes after treatments.

3.3.8. Synaptosomal uptake of [3H]-DA, [3H]-5HT and [3H]-NA

The procedures were identical from Viana et al (2005). Shortly, rats striata (for [^{3}H]-DA uptake), frontal cortex (for [^{3}H]-5HT uptake) and hypothalamus (for [^{3}H]-NA uptake) were dissected out and homogenized in a teflon-glass homogenizer (800 r.p.m.) in 10 volumes (w/v) of ice-cold 0.32 M sucrose solution containing 0.1 mM pargyline. The nuclear material was removed by centrifugation at 1,000 g for 10 minutes, and the supernatant (crude synaptosomal fraction) was used in uptake experiments.

Aliquots (100 µl) of the crude synaptosomal fraction were preincubated for 5 minutes at 37°C in a Krebs-Ringer medium, containing (mM): NaCl 109, KH_2PO_4 1, $CaCl_2$ 1, $NaHCO_3$ 27, glucose 5.4, pH 7.4 ± 0.1, in presence or absence of HP4. The incubation was continued

for 5 minutes in the same medium, in the presence of [^{3}H]-DA, [^{3}H]-5HT or [^{3}H]-NA (10 nM; 1 ml final volume). The reaction was stopped by adding 3 ml of ice-cold incubation medium and immediate centrifugation (7,000 g, 10 minutes, 4°C). The pellet was washed with 1 ml of the latter medium and centrifuged in the same condition. The final pellet was sonicated in 250 µl distilled water and aliquots of the homogenate were used for the determination of radioactivity and protein concentrations. The radioactivity was determined by liquid scintillation spectrometry (Tri-carb 2100TR, Packard BioScience Co.) in 4 ml of Ultima Gold (Perkin Elmer). Protein concentrations were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

The specific uptake of DA, 5HT or NA was defined as the difference between the total uptake at 37°C and the non-specific accumulation observed at 0°C, respectively in the presence of 100 µM cocaine for [^{3}H]-DA, 1 µM fluoxetine for [^{3}H]-5HT or 1 µM desipramine for [^{3}H]-NA.

The monoamine synaptosomal uptake was analyzed using the equation of the sigmoidal dose-response curve (variable slope). IC_{50} and n Hill values were calculated using the equation of the curve-fitting programs Microcal Origin (Microcal Software).

3.3.9. Binding assays to monoamine transporters and opioid receptors

This assay also followed the binding protocol cited by Viana et al (2005; Chapter 1). Dopamine (DAT), noradrenaline (NAT), serotonin (SERT) transporters and opiod receptors binding was assessed, respectively, on striatum, cortex, hypothalamus and forebrain membranes of rats. The crude synaptosomal suspensions, prepared as described in 2.7., were centrifuged at 17,000 g for 30 minutes at 4 °C. The pellet was resuspended by sonication in: 10 mM Na^+ medium (0.30 mM NaH_2PO_4, 9.70 mM $NaHCO_3$, pH 7.5 ± 0.1) for the binding to DAT, 50 mM Tris HCl (containing 120 mM NaCl and 5 mM KCl, pH 7.4 ± 0.1) for NAT and SERT or 50 mM Tris HCl pH 7.4 ± 0.1 for opioid receptors; and centrifuged (50,000 g, 10 minutes, 4 °C). The final pellet was resuspended by sonication in the same respective medium for the binding to DAT and SERT, for NAT it was resuspended in 50 mM Tris HCl (containing 300 mM NaCl and 5 mM KCl, pH 7.4 ± 0.1) and for opioid receptors in 50 mM Tris HCl (containing $MgCl_2$ 20 mM, pH 7.4 ± 0.1) .

[^{3}H]-Mazindol (2 nM final concentration) and membranes (100 µg protein) were incubated at 0°C, for 2 h, to evaluate the binding to DAT. For the binding to NAT, 300 µg of protein were incubated with [^{3}H]-nisoxetine (1nM) at 25°C during 2 h; for SERT, 200 µg of protein were incubated with [^{3}H]-citalopram (1 nM) during 1 h at 25°C and for opioid receptors, 400 µg of protein were incubated with [^{3}H]-naloxone (1nM) at 25°C during 2 h. All incubations were carried out in the presence of different HP4 concentrations (3 x 10^{-7} – 3 x10^{-11} g/ml), in a final volume of 500 µl for monoamine transporters or 1 ml for opioid receptors. Incubations were stopped by dilution with 3 ml of ice-cold incubation medium and immediate filtration through GF/B filters, previously soaked, for at least 1 h, in 0.5% polyethyleneimine. Each tube and filter were rinsed once or twice with 3 ml of ice-cold incubation medium. Filters were counted for radioactivity by liquid scintillation spectrometry (Tri-carb 2100TR, Packard BioScience Co.) in 4 ml of Ultima Gold (Perkin Elmer). The non-specific binding was determined by incubation with cocaine (100 µM, for DAT), desipramine (10 µM, for NAT), fluoxetine (10 µM, for SERT) or naloxone (10 µM, for opioid receptors). Protein concentrations were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

3.3.10. Corticosterone assay

3.3.10.1 Treatments and procedures

Acute treatment: groups of 30 animals were treated by gavage with POL (360 mg/kg), imipramine (IMI 20 mg/kg) or saline (with 10% polysorbate 80).

Repeated treatment: groups of 30 animals were treated once daily with the same doses as in the acute treatment, during 3 days.

In each of preceding groups half of the animals (n = 15) were submitted to the forced swimming test (FST), 1h after the single or the last of the three treatments. Mice immobility time was counted in both treatment regimens. All procedures (mice treatment, forced swimming session and decapitation) were performed in separated rooms.

3.3.10.2. Radioimmunoassay (RIA)

Cortical and serum corticosterone levels were determined by radioimmunoassay (RIA). Mice submitted to FST were sacrificed 30 min after the swimming session. Mice not submitted to FST were sacrificed 1h after the last treatment. Trunk blood was collected and was centrifuged twice (13,000 g; 4°C; 15 min). For cortical corticosterone measurements, the frontal cortex of 3 mice was dissected, according to Glowinski and Iversen (1966), weighed (approximately 125 mg) and homogenized in 1 ml phosphate buffer (pH 7.4) containing 1% bovine serum albumin (BSA). All samples were stored at – 40°C until RIA.

Cortex homogenates (1 ml) and serum aliquots (11 µl) were extracted by mixing with 2 ml of ethyl acetate, followed by centrifugation (3,000 g; 4°C; 5 min) to speed up phase separation. Aliquots of 1.5 ml of the ethyl acetate phase were evaporated to dryness. Samples and standards (39 - 10,000 pg/tube) were incubated at 4°C overnight in a final volume of 1 ml with rabbit antibodies against corticosterone and tritiated corticosterone. The separation of free and bound corticosterone was performed, at 4°C, by adding 500 µl of dextran-coated charcoal suspension. Ten minutes later, samples were centrifuged (3,300 g; 4°C; 15 min) and radioactivity contained in the supernatant was measured by liquid scintillation (Tri-carb 2100TR, Packard Bioscience Co).

3.3.11. Statistical analysis

The data were evaluated using one or two-way analysis of variance (ANOVA) followed by Student-Newman Keulls test or Student's t-test depending on the experimental design. P-values less than 0.05 were considered as statistically significant.

3.4. RESULTS

3.4.1. Forced Swimming Test

The cyclohexane extract (POL) reduced the immobility time in both rats and mice tests. POL 270 mg/kg significantly reduced rats immobility time (ANOVA $F_{2,30}$ = 4.55; $p < 0.05$) (Figure 3.2).

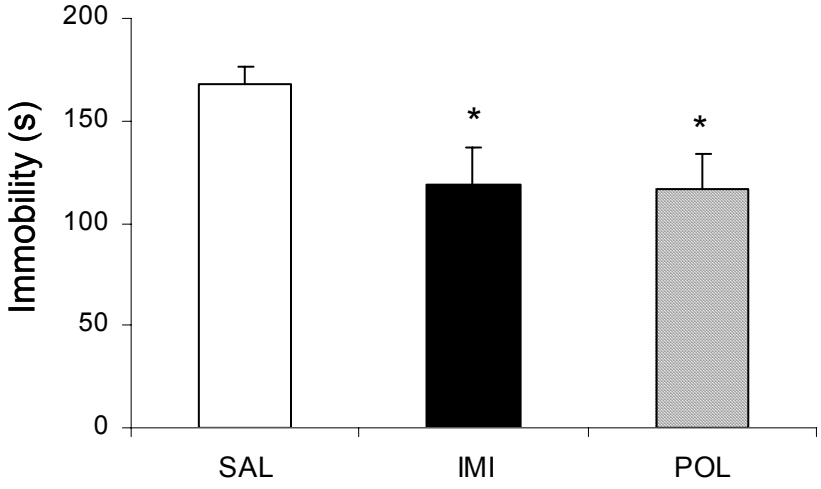


Figure 3.2. Anti-immobility effect of imipramine 60 mg/kg/day, p.o. (IMI) and *H. polyanthemum* (270 mg/kg/day, p.o.) in rat forced swimming test. The results are presented in mean ± SEM (n = 12-14 rats/group). ANOVA followed by Student Newman-Keuls post-hoc comparisons: *$p < 0.05$ significant difference in relation to saline (SAL).

In mice, all tested doses reduced significantly the immobility time (ANOVA, $F_{4,52}$= 12.13; $p < 0.001$) (Figure 3.3). The phloroglucinol derivative, uliginosin B (HP4 90 mg/kg), significantly reduced the immobility time when administered in half of POL lowest dose (180 mg/kg) (Figure 3.4; ANOVA, $F_{2,29}$= 15.5; $p < 0{,}001$).

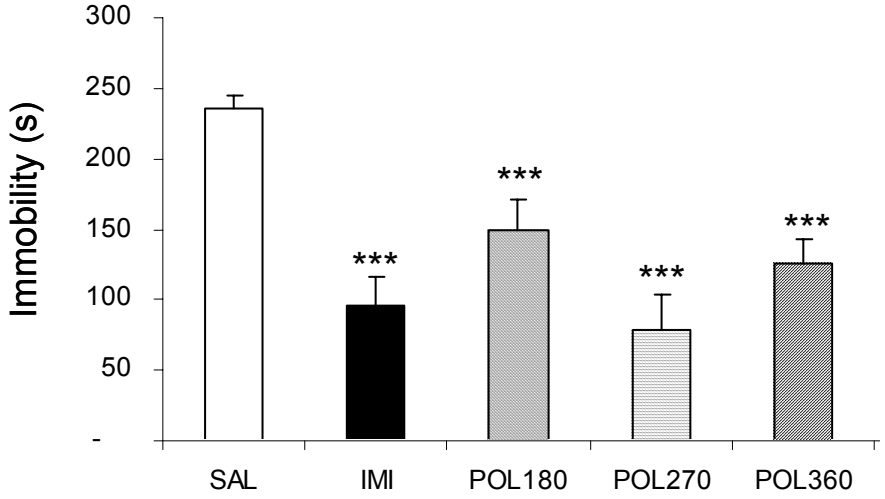


Figure 3.3. Anti-immobility effect of imipramine 20 mg/kg, p.o. (IMI), and *H. polyanthemum* cyclohexane extract (POL 180, 270 and 360 mg/kg, p.o.) in mice forced swimming test. The results are presented in mean ± SEM (n = 8–12 mice/group). ANOVA followed by Student Newman-Keuls post-hoc comparisons: ***$p < 0.001$ compared to saline (SAL).

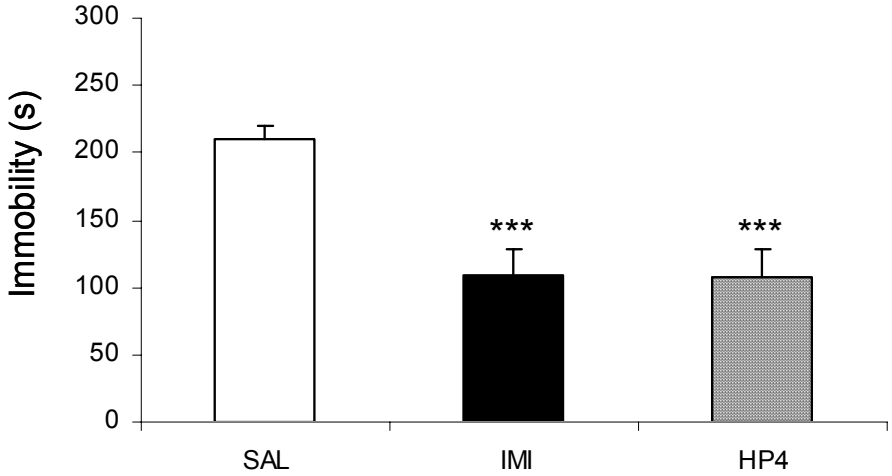


Figure 3.4. Anti-immobility effect of imipramine 20 mg/kg, p.o. (IMI), and uliginosin B (HP4 90 mg/kg, p.o.) in mice forced swimming test. The results are presented in mean ± SEM (n = 8–12 mice/group). ANOVA followed by Student Newman-Keuls post-hoc comparisons: ***$p < 0.001$ compared to saline (SAL).

Three days treatment with POL were effective in reducing the immobility time in FST (Figure 3.5, $F_{2,44}$= 20.9; $p < 0.001$).

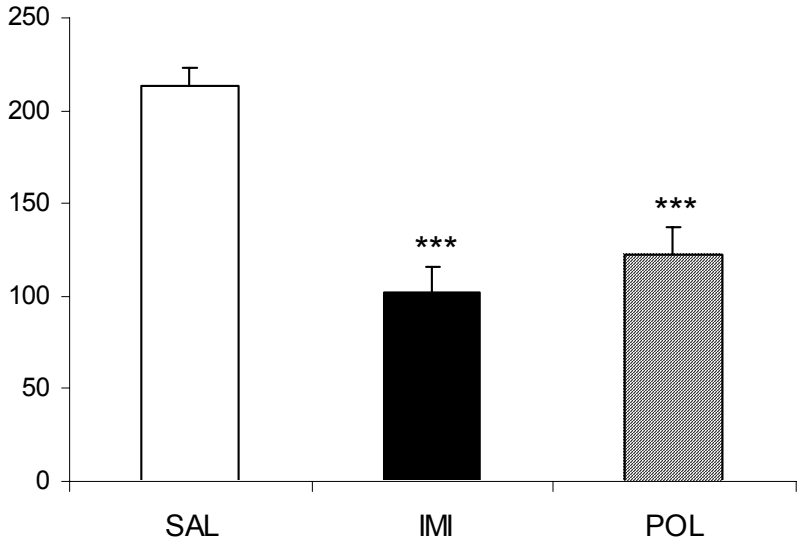


Figure 3.5. Anti-immobility effect of three days treatment with imipramine 20 mg/kg, p.o. (IMI) or *H. polyanthemum* cyclohexane extract (POL, 360 mg/kg, p.o.) in mice forced swimming test. The results are presented in mean ± SEM (n = 15 mice/group). ANOVA followed by Student Newman-Keuls post-hoc comparisons: ***$p < 0.001$ compared to the respective saline (SAL) group.

Even though there were no statistically significant differences among POL doses, at 270 mg/kg the anti-immobility effect was more pronounced. Thus this dose was chosen for the pharmacological antagonism test. POL anti-immobility effect in mice was prevented by sulpiride (50 mg/kg) and by SCH 23390 (15 µg/kg) (two away ANOVA, pre-treatment x treatment factor, $F_{2,48} = 7{,}58$; $p < 0.01$) (Figure 3.6).

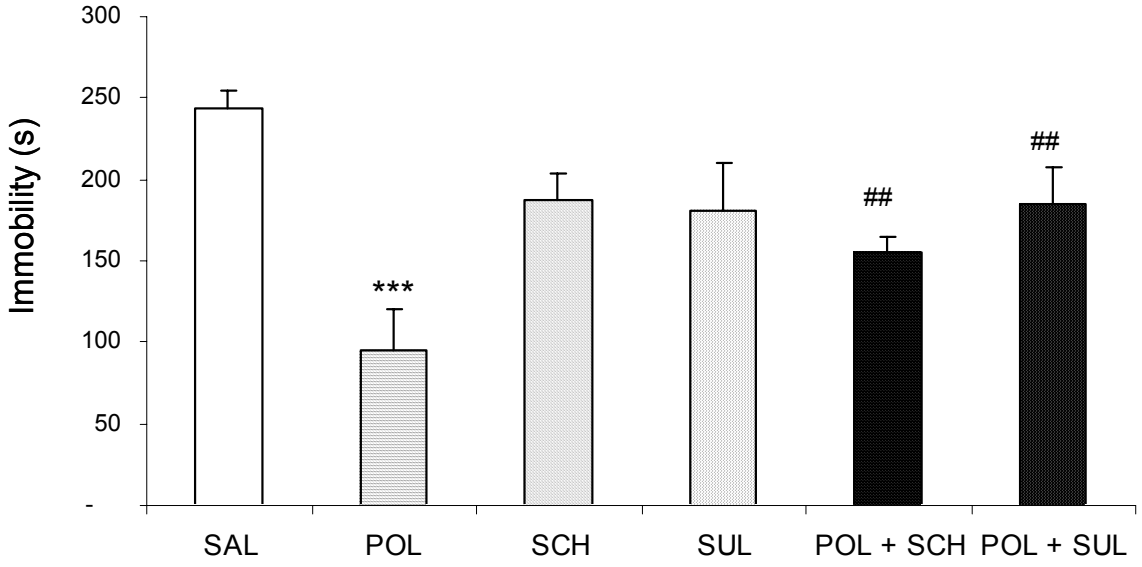


Figure 3.6. Effect of SCH 23390 (SCH 15 µg/kg, s.c.) or sulpiride (SUL 50 mg/kg, i.p.) administration on the anti-immobility action of POL (*H. polyanthemum* cyclohexane extract, 270 mg/kg, p.o.) in mice forced swimming test. The drugs were injected 30 minutes before the extract treatment. The results are presented in mean ± SEM (n = 8-12 mice/group). Two way ANOVA followed by Student Newman-Keuls post-hoc comparisons. ***$p < 0.001$ compared to saline (SAL). ##$p < 0.01$ compared to POL.

3.4.2 Locomotor activity

The treatment with POL 270 mg/kg, p.o., did not significantly modify the locomotor activity of mice, even if we can observe a reduction on mice locomotion. The mean number of beams crossed by control animals was 814 ± 87 while it was 653 ± 94 for POL treated animals.

3.4.3. Effect of HP4 on the uptake of biogenic amines

HP4 inhibited [^{3}H]-DA, [^{3}H]-5HT and [^{3}H]-NA uptake in crude synaptosomal suspensions in a concentration-dependent and monophasic manner (Figure 3.7). It should be pointed out that [^{3}H]-DA uptake (IC_{50} = 90 ± 38 nM) was more potently inhibited by HP4 than [^{3}H]-5HT and [^{3}H]-NA uptake (IC_{50} = 252 ± 13 nM and 280 ± 48 nM, respectively).

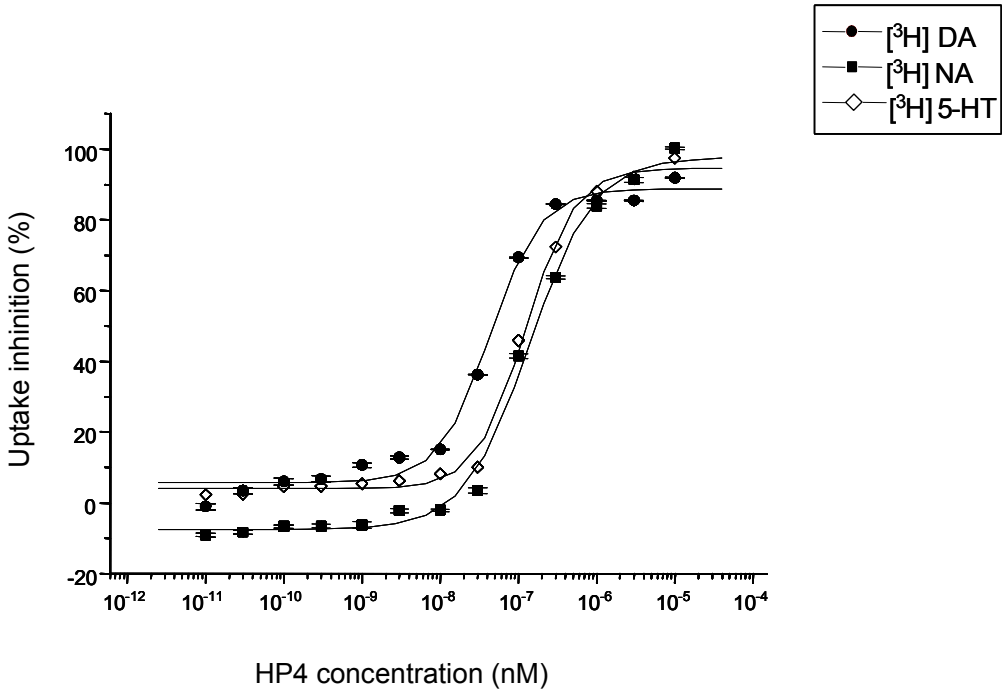


Figure 3.7. Effect of HP4 on [^{3}H]-dopamine (•), [^{3}H]-noradrenalin (■) and [^{3}H]-serotonin (◊) synaptosomal uptake. The data are presented as percentage of uptake inhibition over basal (mean ± SEM from 4 separated experiments performed in duplicate).

3.4.4. Effects of HP4 on the binding of uptake inhibitors to monoamine transporters

The different tested concentrations of HP4 did not inhibit, in a statistically significant manner, the binding of [^{3}H]-mazindol, [^{3}H]-nisoxetine, [^{3}H]-citalopram to their respective monoamine transporters (Figure 3.8).

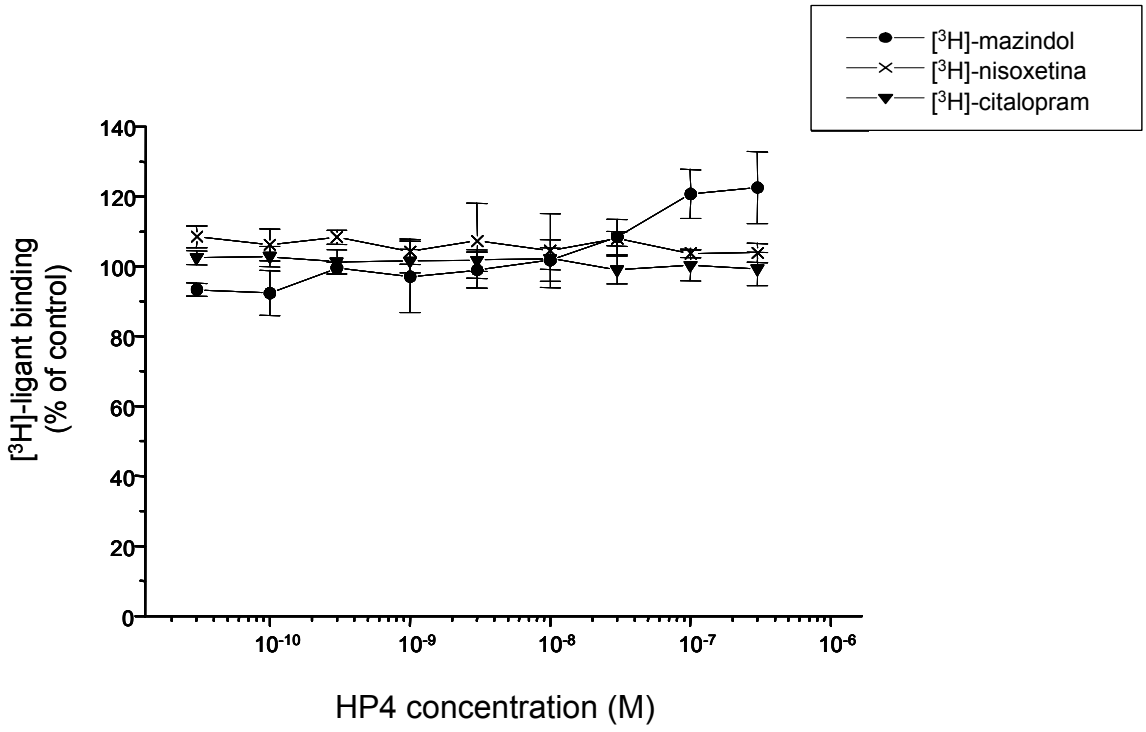


Figure 3.8. Effect of HP4 on [^{3}H]-mazindol, [^{3}H]-nisoxetine, [^{3}H]-citalopram binding to dopamine (DAT), noradrenalin (NAT) or serotonin (SERT) transporters. The data are presented as percentage of uptake inhibition over basal (mean ± SEM from 4 separated experiments performed in duplicate).

3.4.5 Corticosterone assay

The forced swimming test significantly increased both serum (Fig. 3.9A, Fig. 3.10A) and cortical (Fig. 3.9B, Fig. 3.10B) corticosterone levels measured 30 min later. This corticosterone raise was not prevented neither by acute treatment with imipramine or POL (Figure 3.9). As expected, like HCP (Chapter 2), repeated treatment with IMI or POL significantly reduced the hormone raise induced by FST (Figure 3.10). When compared to CONT or SAL groups imipramine significantly reduced corticosterone levels both in serum and cortex while POL was effective only in reducing cortex levels. The imipramine had no effect in corticosterone levels of non-stressed mice in either treatment regimens whereas, acutely, POL induces an increase in basal levels of serum corticosterone (Figure 3.9A)

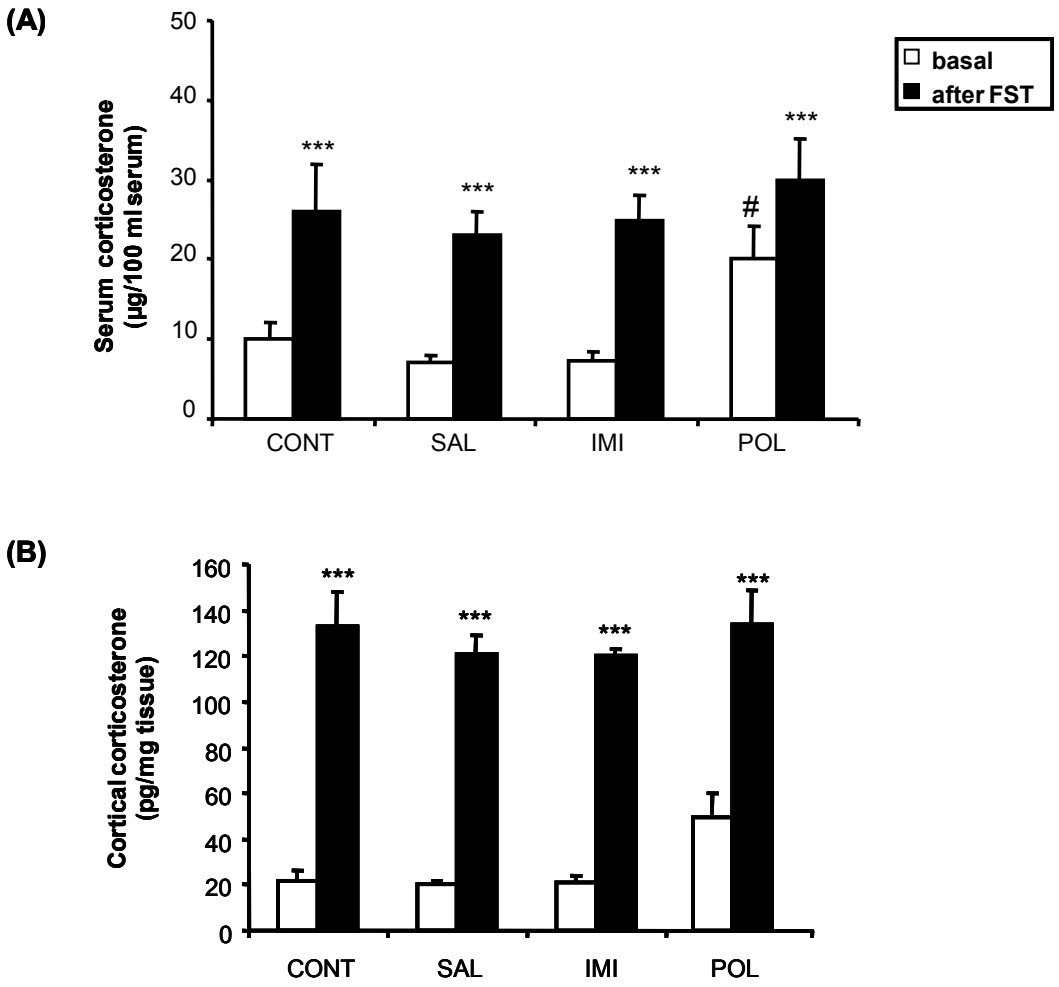


Figure 3.9. Effect of acute per os treatment with imipramine (IMI 20 mg/kg), *H. polyanthemum* cyclohexane extract (POL 360 mg/kg) or saline (SAL) on serum (A) and cortical (B) corticosterone levels, in mice submitted or not to forced swim test. Data are presented in mean ± SEM (n = 15 mice/group). Two way ANOVA followed by Student-Newman-Keuls post hoc comparisons: ***$p < 0.001$ compared to the respective non stressed group; # $p < 0.05$ compared to the respective control groups.

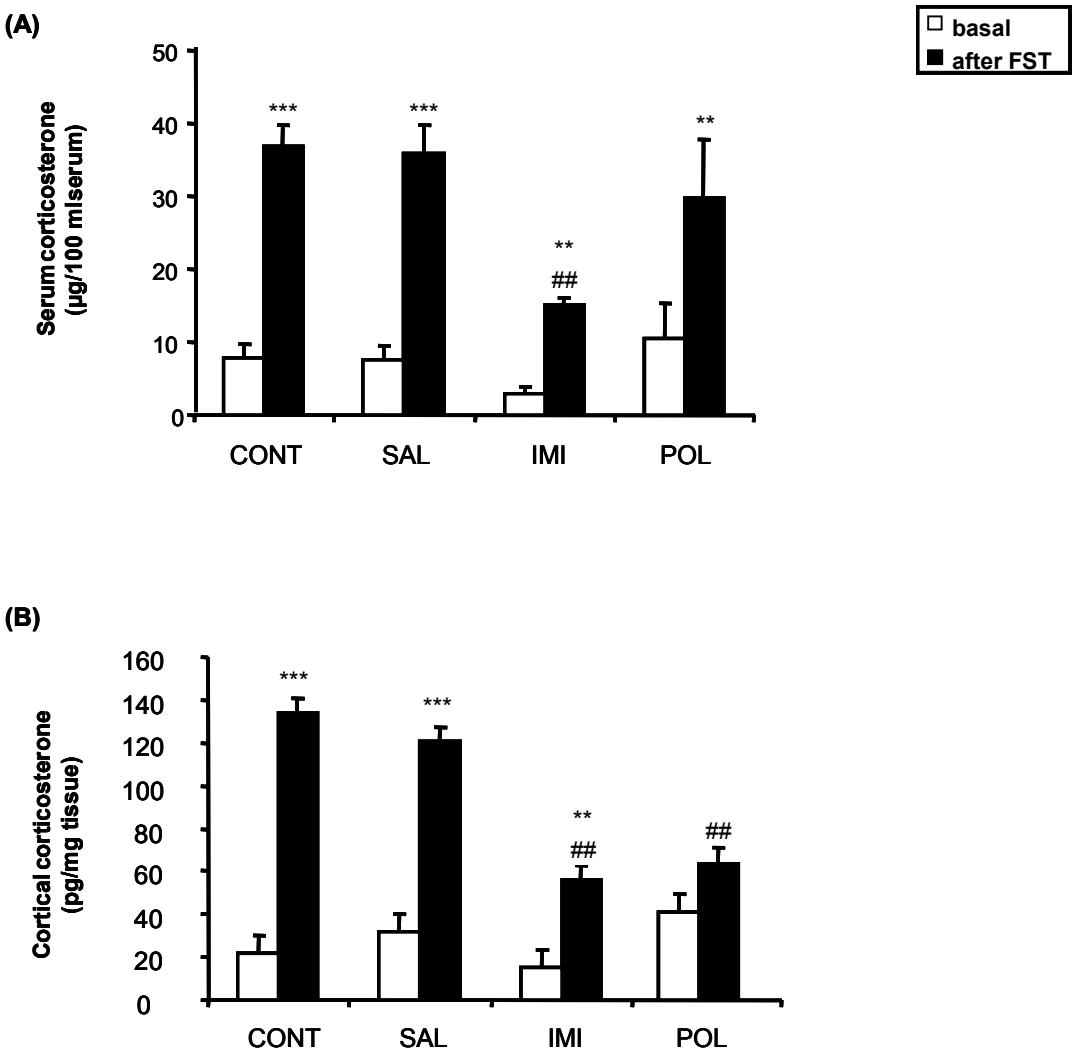


Figure 3.10. Effect of repeated treatment (3 days) with imipramine (IMI 20 mg/kg, p.o.), *H. polyanthemum* cyclohexane extract (POL 360 mg/kg, p.o.) or saline (SAL) on serum (A) and cortical (B) corticosterone levels in mice submitted or not to forced swim test. Data are presented in mean ± SEM (n = 15 mice/group). Two way ANOVA followed by Student-Newman-Keuls post hoc comparisons: $^{**}p < 0.01$, $^{***}p < 0.001$ compared to respective non stressed group; $\#p < 0.01$, compared to the respective saline (SAL) group.

3.4.6. Antinociceptive effect

In order to continue the study of *H. polyanthemum* antinociceptive effect reported in the Chapter 1 (Viana *et al.*, 2003) we tested the main substances isolated from the cyclohexane extract, benzopyrans (HP1, HP2, and HP3) and uliginosin B (HP4). The antinociceptive effect was observable only in mice treated with HP4 (90 mg/kg, i.p.) (Figure 3.11. One away ANOVA, $F_{5,66} = 10.44$; $p < 0.001$).

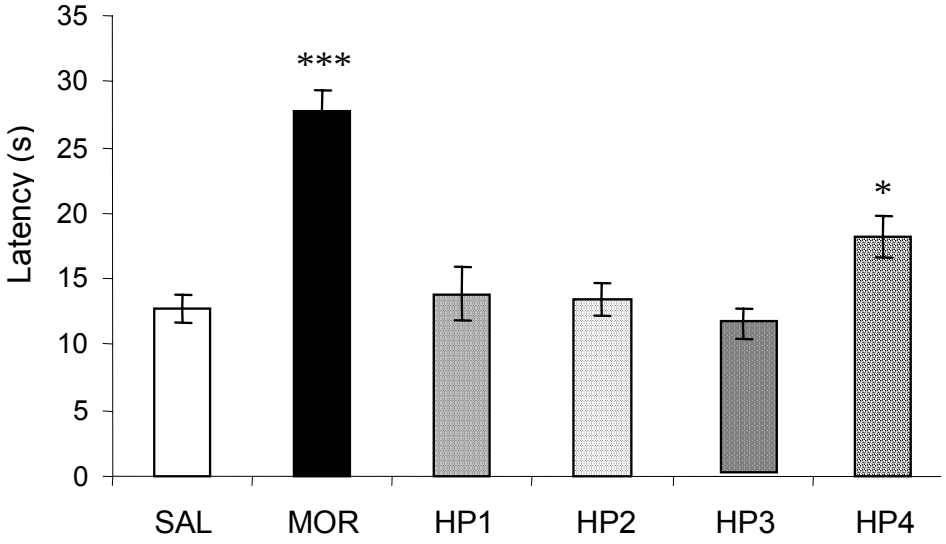


Figura 3.11: Antinociceptive effect of morphine (MOR 10 mg/kg, s.c.), benzopyrans HP1-3 and uliginosin B (90 mg/kg, i.p.) in the hot plate test. Results are presented in mean ± SEM (n = 8–12 mice/group). One way ANOVA followed by Student Newman-Keuls post-hoc comparisons.: ***$p < 0.001$, * $p < 0.05$ compared to saline (SAL).

Contrary to POL, pretreatment with naloxone did not prevent HP4 antinociceptive effect (two away ANOVA, pre-treatment x treatment factor, $F_{2,41} = 24.98$; $p < 0.001$) (Figure 3.12). This indicates that, although HP4 may be responsible for POL's analgesia, it does not interact with opioid receptors. The binding assay also indicates that *H. polyanthemum* antinociceptive effect is not due to a direct action on opioid receptors, since HP4 did not affected [^{3}H]-naloxona binding in membrane preparations (Figure 3.13).

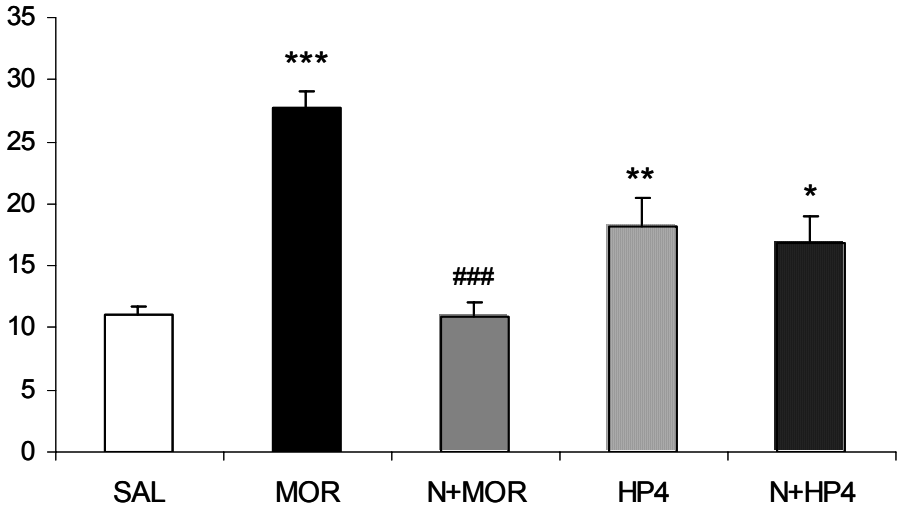


Figure 3.12. Effect of naloxone (2.5 mg/kg, s.c.) administration on the antinociceptive effect of HP4 (90 mg/kg,i.p.) and morphine (6 mg/kg, s.c.) in the hot plate test. The opioid antagonist was injected 10 minutes before the other treatments. Results are presented in mean ± SEM (n = 8–12 mice/group). Two way ANOVA followed by Student Newman-Keuls post-hoc comparisons.: ***p < 0.001, * p < 0.05 compared to saline (SAL), ###p < 0.01 compared to MOR.

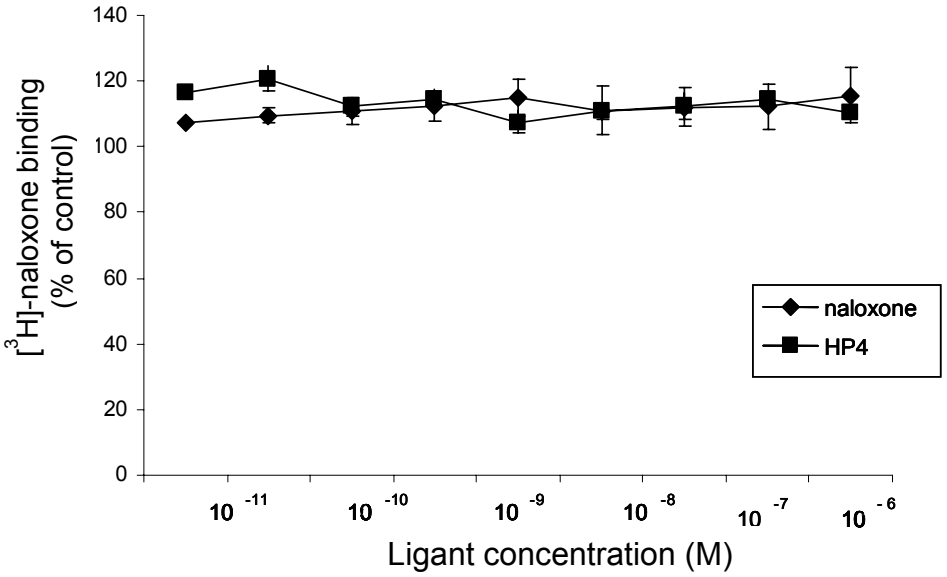


Figure 3.13. Effect of naloxone or HP4 on [^{3}H]-naloxone binding to opioid receptors. The data are presented as percentage of uptake inhibition over basal (mean ± SEM from 4 separated experiments performed in duplicate).

3.5. DISCUSSION

In this work we carried on the pharmacological investigation of *Hypericum* species, focusing on the potential antidepressant and antinociceptive effect of *H. polyanthemum* cyclohexane extract (POL) and isolated compounds. The results demonstrated that POL has antidepressant-like effect in the forced swimming test (FST) both in rats and mice, that is sustained after repeated treatment (3 days). The reduction in immobility time is not related to motor stimulating activity, which is important to differentiate antidepressants from stimulants in the FST (Bourin, 1990; Porsolt *et al.*, 1991). Moreover, pre-treatment with dopaminergic receptors antagonists, SCH 23390 and sulpiride prevented POL antiimmobility effect demonstrating an involvement of dopaminergic system in the FST activity. Since uliginosin B (HP4) at 90 mg/kg was as effective in the FST as POL 360 mg/kg and inhibited monoamine uptake, we presume that HP4 can be the molecule responsible for the POL antiimmobility effect. In addition, HP4 demonstrated antinociceptive effect in the hot-plate test, whereas the other major substances (benzopyrans) present in the cyclohexane extract were inactive.

Research groups working with *Hypericum* species still debate on the substance or substances responsible for antidepressant effect. Some authors support that *H. perforatum* hypericin and flavonoids are the main antidepressant constituents (Butterweck *et al.*, 1998 and 2004) and others say that active substances are the phloroglucinol derivatives, hyperforin and ad-hyperforin (Chatterjee *et al.*, 1998a; Muller *et al.*, 2003; Mennini and Gobbi, 2004; Wurglics and Schubert-Zsilavecz, 2006). Our results confirm that, as hyperforin, HP4 and *H. caprifoliatum* phloroglucinol, HC1, exhibit potential antidepressant activity and are very likely to be the substance with antidepressant-like activity. This assumption is reinforced by the fact that cyclohexane extracts are devoid of flavonoids, which remain in the hydrophilic extracts (Dall'agnol et al, 2003) and Southern Brazilian *Hypericum* species do not have hypericin producing glands (Robson, 1990, Ferraz et al, 2002a).

Besides studying *H. polyanthemum* antidepressant effect via monoaminergic system, we investigated its influence on serum and cortex corticosterone levels. Disturbances in mood often coincide with abnormal levels of corticosteroids. It has been observed that the

hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis observed in depressed patients was corrected by clinically effective treatments using classical antidepressant drugs (Barden *et al.*, 1995; Gold *et al.*, 1995; Holsboer and Barden, 1996; Pariante *et al.*, 2004). In the Chapter 2, we demonstrated that 3 days treatment with imipramine or bupropion reduce FST-induced corticosterone levels raise in serum and brain. However, *H. caprifoliatum* cyclohexane extract reduced corticosterone levels increase in brain but not in serum. POL effects were similar to that of *H. caprifoliatum*, i.e. 3 days treatment with the extract affects only cortical corticosterone levels in mice submitted to FST. In addition, in non stressed mice acute treatment causes corticosterone levels raise in cortex and serum, but only the later is significant. Curiously we can observe a tendency of IMI in reducing serum and cortex corticosterone levels in non-stressed mice. The ability of antidepressants in reducing basal levels of corticosterone has been already reported (Shimoda *et al.*, 1988; Duncan *et al.*, 1998). Taken together these data corroborate with the idea of a diverse mechanism of action for *Hypericum* extracts.

The possibility of a mechanism of action different from classical antidepressants has already been proposed by us for Brazilian *Hypericum* species (Chapter 1: Viana *et al.*, 2005; 2006) and by others for *H. perforatum* extracts (Gobbi *et al.*, 2001; Roz *et al.*, 2002; Yoshitake *et al.*, 2004). Hyperforin as well as HP4 and HC1 inhibit monoamines uptake without binding to their respective transporters (Chatterjee *et al.*, 1998b; Müller *et al.*, 1997, 1998; Jensen *et al.*, 2001), and affect corticosterone levels in a different manner than antidepressants. Franklin *et al.* (2004) also observed reduction only in cortical levels of corticosterone. In Chapter 1 (Viana *et al.*, 2005) we raised the possibility that the mechanism of action could be related to the inhibition of neuronal monoamine uptake, most potently of dopamine. This effect could be caused by an indirect effect on monoamine transporters, most likely due to the elevated neurotransmitter levels in the synaptic clefts, thus leading to an alteration of monoamine transporter function different from the mechanism of action of reference monoamine uptake inhibitors.

Although the mechanism by which the activation of monoaminergic system occurs is not elucidated yet, this effect seems to be responsible not only for the antidepressant-like

activity but also for the antinociceptive effect. This hypothesis was considered since HP4 activity in the hot-plate test is not prevented by naloxone and both HP4 and HC1 do not inhibit [^{3}H]-naloxone binding to opioid receptors (Chapter 1: Viana *et al.*, 2003; 2006).

Antidepressants, mainly noradrenergic (tricyclics and venlafaxine), are widely prescribed for the treatment of chronic and neuropathic pain (Brandão, 1997; Schereiber *et al.*, 1998; Atkinson *et al.*, 1999; Lynch, 2001). The nature and underlying mechanisms of antidepressant analgesia is not clear, there is evidence showing that antidepressants may induce endogenous opioid peptides, and that this effect is independent of their antidepressant effect (Gray *et al.*, 1998; Atkinson *et al.*, 1999). Besides noradrenergic neurons, studies evidence that large neurons of the dopaminergic network of the PAG (periaquedutal grey) participate in supraspinal nociceptive responses after opiates treatment through the involvement of D1 dopamine receptors (Flores *et al.*, 2004). On the other hand, some authors support that the analgesic effect of antidepressants like nomifensina and dopaminergic agonists (amphetamine, apomorphine, bromocriptine) are related only to the dopaminergic system (Michael-Titus *et al.*, 1990; Morgan and Franklin, 1991; Frussa-Filho *et al.*, 1996; Altier and Stewart, 1999; Gilbert and Franklin, 2001). Several studies suggest that the activation of mesolimbic dopamine (DA) neurons arising from the cell bodies of the ventral tegmental area (VTA) and projecting to the nucleus accumbens plays an important role in mediating the suppression of tonic pain (Altier and Stewart, 1999). Therefore since HP4 and HC1 monoamine uptake inhibition is somewhat more potent to dopamine, it is possible that the dopaminergic system is involved in the antinociceptive effect of HCP and POL. Other experiments will be necessary to confirm this hypothesis.

Finally, this study highlights the importance of the accurate knowledge on plants chemical constitution in previewing pharmacological effects. In a first screening procedure several *Hypericum* total methanol extracts were tested in the FST and *H. polyanthemum* have not shown antidepressant like effect (Gnerre *et al.*, 2001). After chemical analysis of Southern Brazilian *Hypericum* species and identification of analogous phloroglucinols in lipophilic extracts of *H.caprifoliatum,* that have already demonstrated antidepressant-like activity (Daudt *et al.*, 2000), and *H. polyanthemum* (Nor *et al.*, 2004), we investigated the potential

antidepressant activity of *H. polyanthemum* cyclohexane extract. The results obtained in this study are in accordance with our previous reports regarding the effect of lipophilic extracts of *H. caprifoliatum* (Chapter 1; Chapter 2). Thus we can point out that the phloroglucinol derivatives represent a molecular pattern promising to the development of antidepressants with innovative mechanism of action.

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CHAPTER 4: Effect of *Hypericum caprifoliatum* and *Hypericum polyanthemum* cyclohexane extracts on monoamine and opioid receptor-stimulated [^{35}S]GTPγS binding in rat brain membranes.

4.1. INTRODUCTION

In previous chapters we demonstrated that *Hypericum caprifoliatum* (HCP) and *H. polyanthemum* (POL) cyclohexane extracts have potential antidepressant and antinociceptive effects (Chapter 1 and 3). The extracts and their main phloroglucinol derivatives (HC1 and HP4) act on pre-synapse by inhibiting monoamine uptake but differently from antidepressants, they did not bind to the monoamine transporters. Furthermore, the conventional antidepressant like-effect in the forced swimming test was inhibited by D_1 and D_2 antagonists while the antinociceptive effect was abolished by naloxone, a nonselective opioid receptor antagonist. These latter observations suggest an action also at post synaptic level.

In this chapter we deal with a functional binding approach for investigating post synaptic effects. We investigated the effect of acute or repeated administration of *H. caprifoliatum* and *H. polyanthemum* cyclohexane extracts on immobility time in FST and on monoamine and opioid receptor-stimulated [^{35}S]GTPγS binding in rat brain membranes. We have also investigated the effect of HC1 and HP4 on mice FST and [^{35}S]GTPγS binding assay.

4.2. BACKGROUND

To date, all the accumulated knowledge about mood disorders treatments (including non medication ones) did not provide a unique and definitive explanation for the antidepressant action (Duman *et al.*, 1997; Nestler, 1998; Stahl, 2000) and it also still lacks a clear understanding of the neural substrates that are abnormal in depression and related syndromes (Nestler and Carlezon, 2006). Biochemical research in mood disorders has focused, along the cascade of events involved in signal transduction, from studies at the level of the primary messenger, the monoamine neurotransmitter, to the level of the neurotransmitter receptors, and lately to information transduction mechanisms beyond receptors, involving the coupling of receptors with signal transducers (Schatzberg and Schildkraut, 1995; Duman *et al.*, 1997; Gould and Manji, 2002).

Targeting multiple neurochemical mechanisms such as serotonin, noradrenaline and dopamine systems in the brain region is hypothesized to be part of the mechanism underlying the efficacy of many conventional antidepressants, including tricyclic antidepressants, monoamine oxidase inhibitors and selective serotonin reuptake inhibitors in the treatment of depression (Millan *et al.*, 2006; Pacher *et al.*, 2001; Chen and Lawrence, 2003). However, it is well known that all antidepressants need a delay of at least two weeks to elicit their therapeutic effect in human, while the raise in monoamines is immediate; therefore, adaptive changes in the monoaminergic system are believed to underlie the therapeutic effectiveness of a variety of antidepressant drugs. It has been suggested that one of such adaptations is the desensitization of monoamine receptors (Blier and de Montigny, 1994; Le Poul *et al.*, 2000; Avissar and Schreiber, 2006). The conflicting observations regarding the regulation of monoamine receptor sensitivity following antidepressant treatment may be due to the effects of these drug treatments on complex neuronal circuits, or differences in the regulation of monoamine receptors function in specific brain regions.

The monoaminergic receptors belong to the superfamily of heptahelical G protein coupled receptors (GPCRs). It means that they have the capacity to activate guanine nucleotide-binding proteins (G proteins), localized in the inner surface of the plasma membrane (Gould and Manji, 2002). G proteins are a ubiquitous family of heterotrimeric proteins, formed by α,β and γ subunits. They have a crucial role as information transducers across the plasma membrane, coupling receptors to various effectors. It is estimated that about 80% of all known hormones, neurotransmitters, and neuromodulators elicit cellular responses through G proteins coupled to a variety of intracellular effectors (Chen *et al.*, 1999; Hermans, 2003).

Growing clinical and pre-clinical evidence suggest that GPCR and its regulation, may be involved in both the pathogenesis and treatment of mood disorders (for review, see Avissar and Schreiber, 2002; Gould and Manji, 2002; Schreiber and Avissar, 2003). The available evidence relies on: *(i)* effect of lithium on receptor-G protein coupling (Avissar and Schreiber, 1992; Donati *et al.*, 2001; Shen *et al.*, 2002; Pejchal *et al.*, 2002); *(ii)* differential alterations in the concentration and/or function of G proteins of patients with bipolar disorder, major depression and seasonal affective disorder (for review, see Avissar and Schreiber, 2002;

Schreiber and Avissar, 2003); *(iii)* alterations in the levels of G proteins in postmortem tissues of patients with mood disorders (Young *et al.*, 1991, 1993; Friedman and Wang, 1996); *(iv)* findings concerning G protein encoding genes, p.ex. G□3, as susceptibility locus for major depressive disorder and seasonal affective disorder and as an indicator for antidepressant treatment response (Lee *et al.*, 2004); *(v)* evidence that activated Gα subunit-adenylyl cyclase coupling plays a major role in mediating the actions of chronic antidepressant treatment, including eletroconvulsive shock (Ozawa and Rasenick, 1989; Gould and Manji, 2002; Donati and Rasenick, 2003, 2005). Antidepressants effects on G-protein functionality are variable depending of the protocol, brain area, antidepressant and duration of treatment. Chronic treatment with fluoxetine and imipramine reduces 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding, in dorsal raphe (Hensler, 2002; Pejchal *et al.*, 2002; Castro *et al.*, 2003) and lateral septum (Shen *et al.*, 2002) but not in striatum or hippocampus (Pejchal *et al.*, 2002). Others studies have shown an increase in $G\alpha_s$ coupling to the catalytic subunit of adenylate cyclase (Chen and Rasenick, 1995; Chen *et al.*, 1999). Finally, some studies have shown no change in G protein subunit levels following chronic treatment with desipramine or amitriptyline (Emamghoreishi *et al.*, 1996. Dwivedi and Pandey,1997; Hensler, 2002).

A method that allows measuring agonist effect on the GPCR is the [^{35}S]GTPγS binding assay to brain structures. This assay is considered convenient, easy, quick and quite accurate to measure the level of G protein activation following agonist occupation of a GPCR (Lazareno, 1999). Since Gα subunit holds GTPase activity, the endogenous GTP is replaced by its nonhydrolyzable analog [^{35}S]-guanosine-5'-*O*-(3-thio) triphosphate, that irreversibly binds to Gα (Bymaster *et al.*, 2001; Harrison and Traynor 2003). This assay allows the construction of concentration-effect curves and therefore potency (EC_{50}) and relative efficacy (E_{max}) measures, with the advantage that agonist measures are not subjected to amplification or other controls that have to be considered when measuring downstream systems, such as adenylyl cyclase, phospholipase C or gene expression, and so provide the best source of information about ligand-induced events at the receptor. Although this assay determines the degree of binding of [^{35}S]GTPγS it is truly a functional assay because it is the activation of a receptor by an agonist that induces [^{35}S]GTPγS binding to Gα subunit (Harrison and Traynor 2003).

4.3. MATERIALS AND METHODS

4.3.1. Plant material

The aerial parts of *Hypericum caprifoliatum* Cham. and Schltdl. were collected in the region of Viamão, while the ones from *Hypericum polyanthemum* Klotzsch ex Reichardt were collected in the region of Caçapava do Sul, both in the state of Rio Grande do Sul - Brazil. The *voucher specimens* were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon 1496 and 1429, respectively).

4.3.2. Preparation and purification of the extract

H. caprifoliatum (HCP) and *H. polyanthemum* (POL) extracts were prepared as described by Viana *et al.* (2003, 2005). Shortly, the dried and powdered plant material was extracted with cyclohexane (Merck, Darmstadt/ DE) followed by evaporation purification (wax elimination). Thin layer and column chromatography were used to isolate the phloroglucinol derivative fraction, HC1 from *H. caprifoliatum* and HP4 from *H. polyanthemum*.

4.3.3. Animals

Adult male Sprague Dawley rats, weighing 180-200 g, were purchased from IFFA-CREDO/Charles River Laboratories (Domaine des Oncins, Saint-Germain sur L'Arbresle, France). Male CF1 mice (25–30 g) were purchased from Fundação Estadual de Produção e Pesquisa em Saúde – RS (Brazil). The animals were housed by five rats or ten mice in Makrolon cages (L : 42 cm, W : 27.5 cm, H : 18 cm). All animals were kept under a 12 hours light/dark cycle (lights on between 7:00 hour and 19:00 hour) in a well ventilated room, at constant temperature of 22 $\pm$ 1°C, with free access to standard certified rodent diet and tap water *ad libitum*.

All the behavioral experiments were performed according to guidelines of The Brazilian Research Ethical Committee (published by National Heath Council – MS, 1998), which are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

4.3.4. Treatments

For the GTP assays, groups of rats (n=4) were submitted to 4 different treatment regimens: T1) one treatment by oral gavage 1 h before sacrifice; T2) three treatments during 24h (23, 5 and 1 h before sacrifice); T3) two treatments per day during 5 days; T4) two treatments per day during 5 days followed by a 3 days wash-out before assays. The extracts were dissolved in water with 5% polisorbate 80 to a dose of 90 mg/kg.

4.3.5. Forced Swimming Test (FST)

FST was performed as previously reported (Viana *et al.*, 2005). In brief, before treatment beginning rats were submitted to swimming for 15 minutes in water 30 cm depth with temperature between 23 $\pm$ 2°C. The ambient temperature was approximately 22°C. At the end of the swimming exposition, the animals were removed from the water and gently dried. One hour after the last treatment (T2, T3) or three days after treatment discontinuation (T4), the animals were submitted to a second swimming exposure (5 minutes), and their immobility time was measured.

In mice, the FST apparatus consisted of a glass cylinder (10 cm internal diameter, 25 cm height) filled with water (19 cm height) at 23 ± 2°C. The animals were acutely treated with HC1, HP4 (90 mg/kg) or 2% polysorbate 80 solution in water, per os (10 ml/1kg). One hour later (T1), the animals were placed into the cylinder and the total duration of immobility, during a 6 minutes test period, was measured.

4.3.6. [^{35}S]GTPγS binding assay

Crude membrane preparations were isolated according to the modified method described elsewhere (Viana *et al.*, 2005). Rats were sacrificed by decapitation one hour after the treatment for T1 and last treatment T2 and T3, or three days later for T4. Frontal cortex, striatum, hypothalamus and thalamus were removed and homogenized in 20 vol. of 0.32M sucrose. The same brain structures were removed from non-treated rats and used for direct incubation with HC1 or HP4. The homogenates were centrifuged (1,000g for 15 min); the

supernatants from two centrifugations were combined and centrifuged (17,000g for 30 min). The resulting pellet was then suspended in Tris buffer (50 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.4), sonicated, and centrifuged (50,000g for 10 min). The final protein concentration was determined by the method of Lowry (Lowry *et al.* 1951).

[^{35}S]GTPγS binding assays were performed as follows. Membranes (15-20µg/100µl) were incubated at 25ºC for 2 h in the assay buffer (Tris buffer added with 1mM dithiothreitol (DTT) and 0,1% metabisulphite) with 10 µM GDP (Sigma–Aldrich Co.), 10^{-4}-10^{-8} M of dopamine (DA), noradrenalin (NA) and serotonin (5-HT), 10^{-5}-10^{-9} of DAMGO (Sigma–Aldrich Co.), or 10^{-6}-10^{-10} M of HC1 or HP4, in the presence of 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol; Perkin-Elmer, Courtaboeuf, France) in a total volume of 1 ml. Basal binding was assessed in the absence of peptide analogue and presence of GDP, and the non-specific binding was assessed in the presence of 10 µM guanosine-5'-*O*-(3-thio)triphosphate (GTPγS; Sigma–Aldrich Co.). The entire mixture was incubated at 25 ºC for 2 h and filtered through Whatman GF/B glass fiber filters, which had been pre-soaked for 2 h in Tris buffer, and washed three times with 4 ml of ice-cold Tris buffer, using a Millipore Sampling Manifold (Billerica, MA, USA). Bound radioactivity was determined in Tri-Carb 2100TR liquid scintillation counter (Packard) after overnight extraction of the filters in 4 ml of Ultima Gold scintillation fluid (Perkin-Elmer). Four independent experiments for each assay were carried out in duplicate.

4.3.7. Data analysis

Data are expressed as means ± SEM. The percent stimulation of [^{35}S]GTPγS binding was calculated according to the following formula: (S - B)/B x 100%, where S is the stimulated level and B is the basal level of [^{35}S]GTPγS binding in the presence of GDP. Individual dose–response curves were obtained by a non-linear regression analysis.

The data were evaluated using one analysis of variance (ANOVA) followed by Student-Newman Keulls test or Student's t-test depending on the experimental design. P-values less than 0.05 were considered as statistically significant.

4.4. RESULTS

4.4.1. Forced Swimming Test

All treatment regimens with HCP significantly reduced immobility time (One way ANOVA, $F_{5,53}$ = 17.46). Five days treatment (T3) was not more effective than the classical Porsolt protocol, i.e. 3 treatments within 24h (T2). The antiimobility effect remains even after 3 days wash-out (T4) although less pronounced than those observed in T2 and T3 (Figure 4.1).

Similarly to HCP all treatment regimens with POL were effective (One way ANOVA, $F_{5,54}$ = 4.89). Even though there are no statically significant differences between POL and HCP treatments it can be observed that the POL effects are weaker than those of HCP (Figure 4.1).

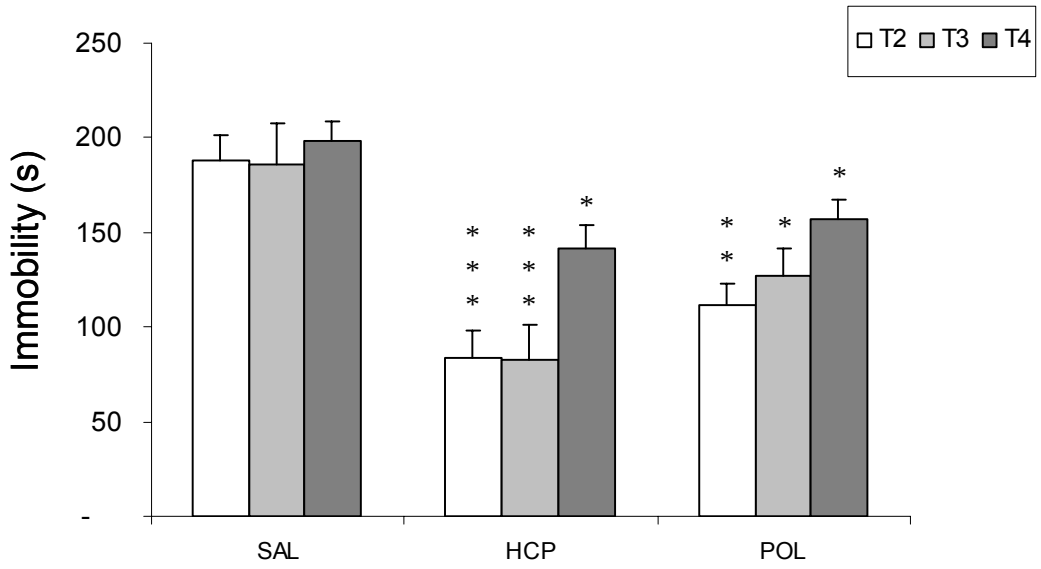

Figure 4.1: Effect of T2 (three treatments during 24h), T3 (two treatments per day during 5 days) and T4 (two treatments per day during 5 days followed by a 3 days wash-out) with 90 mg/kg of *H. caprifoliatum* (HCP), *H. polyanthemum* (POL) or saline (SAL 1ml/kg) on rat forced swimming test. Data are presented in mean ± SEM (n = 8 rats/group). Significantly different values were detected by one way ANOVA followed by post hoc Student-Newman-Keuls test : **p* < 0.05; ***p* < 0.01; ****p* < 0.001 as compared to their respective saline (SAL) group.

Since the phloroglucinols isolated from *H. caprifoliatum* (HC1) and from *H. polyanthemum* (HP4) dose dependently inhibited monoamines uptake (as shown in Chapter 1), here we tested them in the FST to investigate if they have in vivo effect. Both HC1 and HP4 were effective in reducing mice immobility time (One way ANOVA, $F_{2,27} = 12.36$) (Figure 4.2).

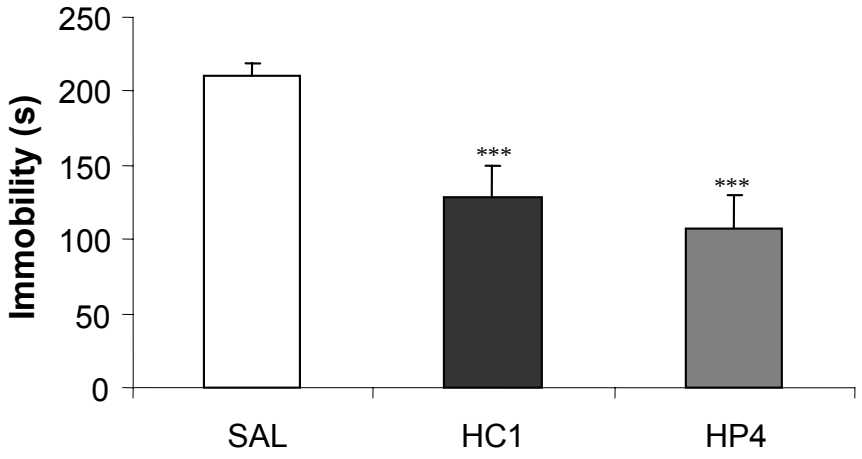

Figure 4.2: Effect of acute treatment with the phloroglucinols HC1 (*H. caprifoliatum*) and HP4 (*H. polyanthemum*) 90 mg/kg p.o.; or saline (SAL 100 ml/kg) on mice forced swimming test. Data are presented in mean ± SEM (n = 10 mice/group). Significantly different values were detected by one way ANOVA followed by post hoc Student-Newman-Keuls test : ****p* < 0.001 as compared to their respective saline (SAL) group.

4.4.2. [^{35}S]GTPγS binding assay

Ex vivo experiments

The different treatment regimens induced contrasting results. Treatments T1 and T2 with HCP and POL increased the maximal effect (E_{max}) of [^{35}S]GTPγS binding induced by all monoamines (Figures 4.3 and 4.4) and did not affect DAMGO curves features. The monoamine potency (EC_{50}) to induce [^{35}S]GTPγS binding was also increased (Table 4.1).

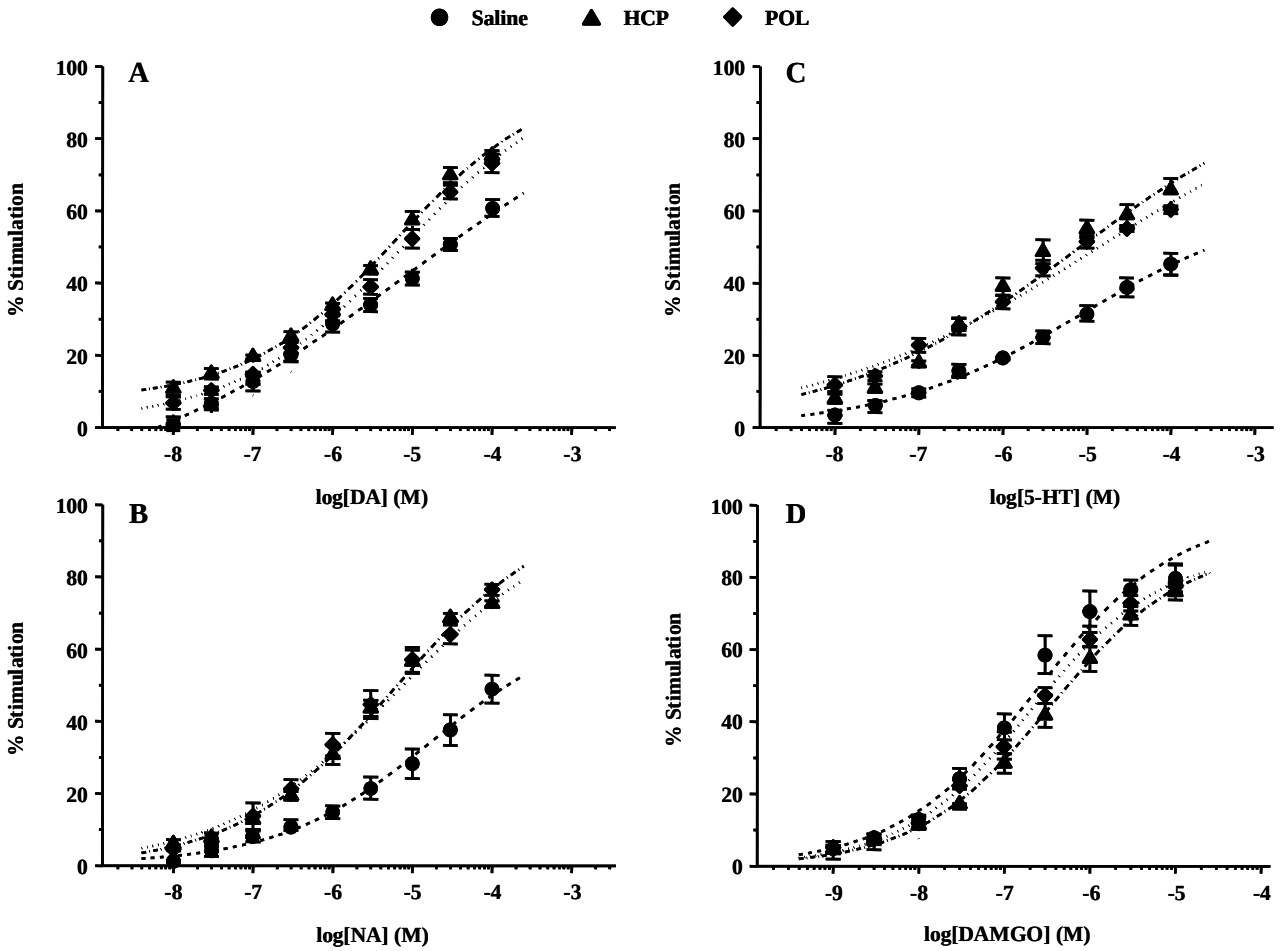

Figure 4.3: Effect of T1 (single administration) with HCP, POL (90 mg/kg, p.o.) or saline on agonist-stimulated [^{35}S]GTPγS binding measured ex vivo. Membranes prepared from rat striatum (A), hypothalamus (B), frontal cortex (C) and thalamus (D) were incubated with increasing doses of dopamine (A), noradrenaline (B), serotonin (C) or DAMGO (D) in the presence of 0.1 nM [^{35}S]GTPgS and 10 mM GDP. Data are presented as percentage of binding stimulation over GDP basal stimulation (absence of agonist). Mean ± SEM from 4 separated experiments carried out in duplicate.

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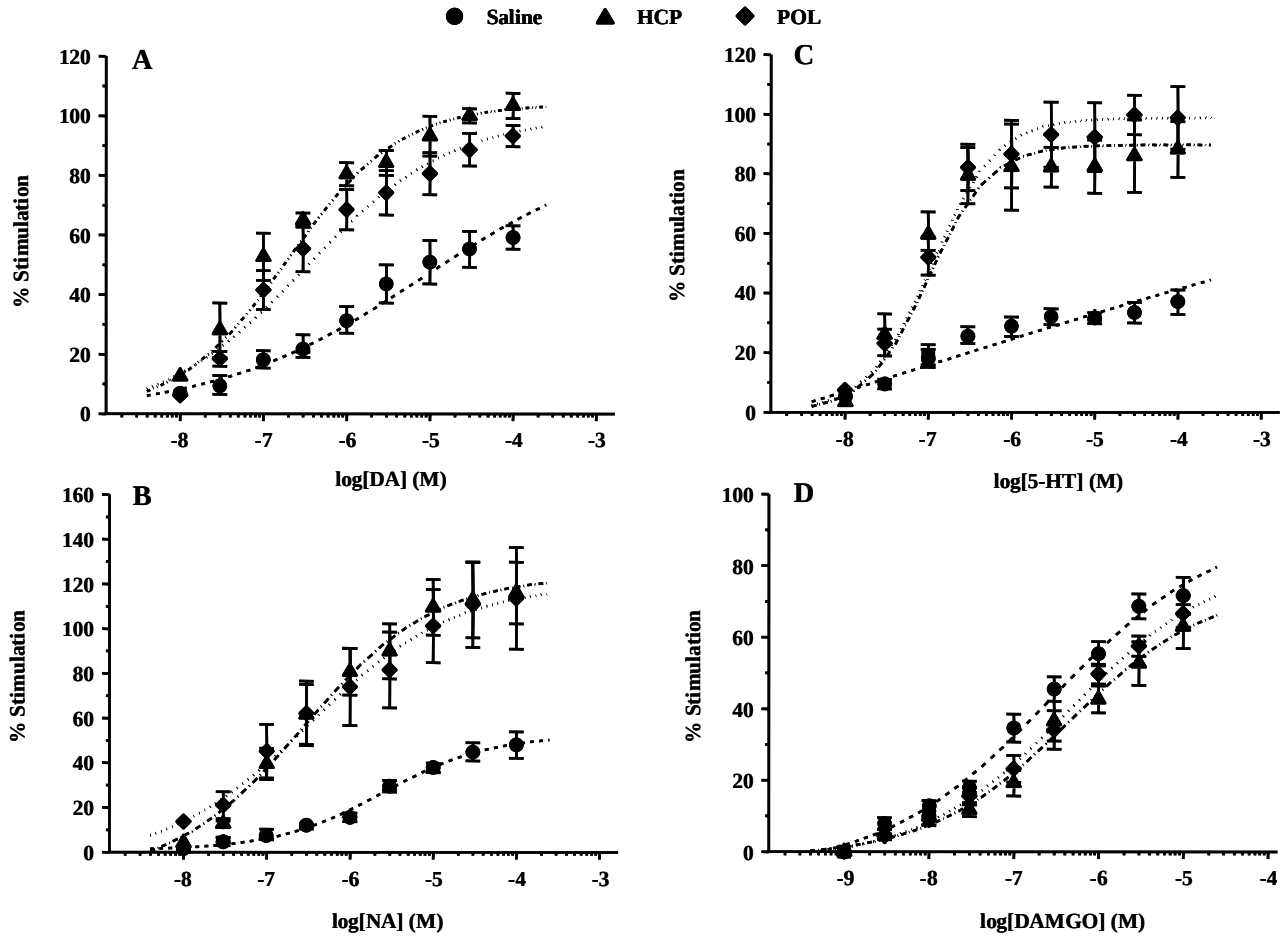

Figure 4.4: Effect of T2 (three treatments during 24h (23, 5 and 1 h before assay)) with HCP, POL (90 mg/kg, p.o.) or saline on agonist-stimulated [^{35}S]GTPγS binding measured ex vivo. Membranes prepared from rat striatum (A), hypothalamus (B), frontal cortex (C) and thalamus (D) were incubated with increasing doses of dopamine (A), noradrenaline (B), serotonin (C) or DAMGO (D) in the presence of 0.1 nM [^{35}S]GTPgS and 10 mM GDP. Data are presented as percentage of binding stimulation over GDP basal stimulation (absence of agonist). Mean ± SEM from 4 separated experiments carried out in duplicate.

Repeated treatment (T3 and T4) with HCP or POL induced a reduction in the E_{max} and potency (EC_{50}) of [^{35}S]GTPγS binding stimulated by of monoamines (Figure 4.5 and 4.6). This reduction was significant for the three monoamines when membranes were prepared one hour after the last treatment (T3) and even following three days wash-out (T4) the it was still observable (Table 4.1). DAMGO-stimulated curves were not affected by the treatments.

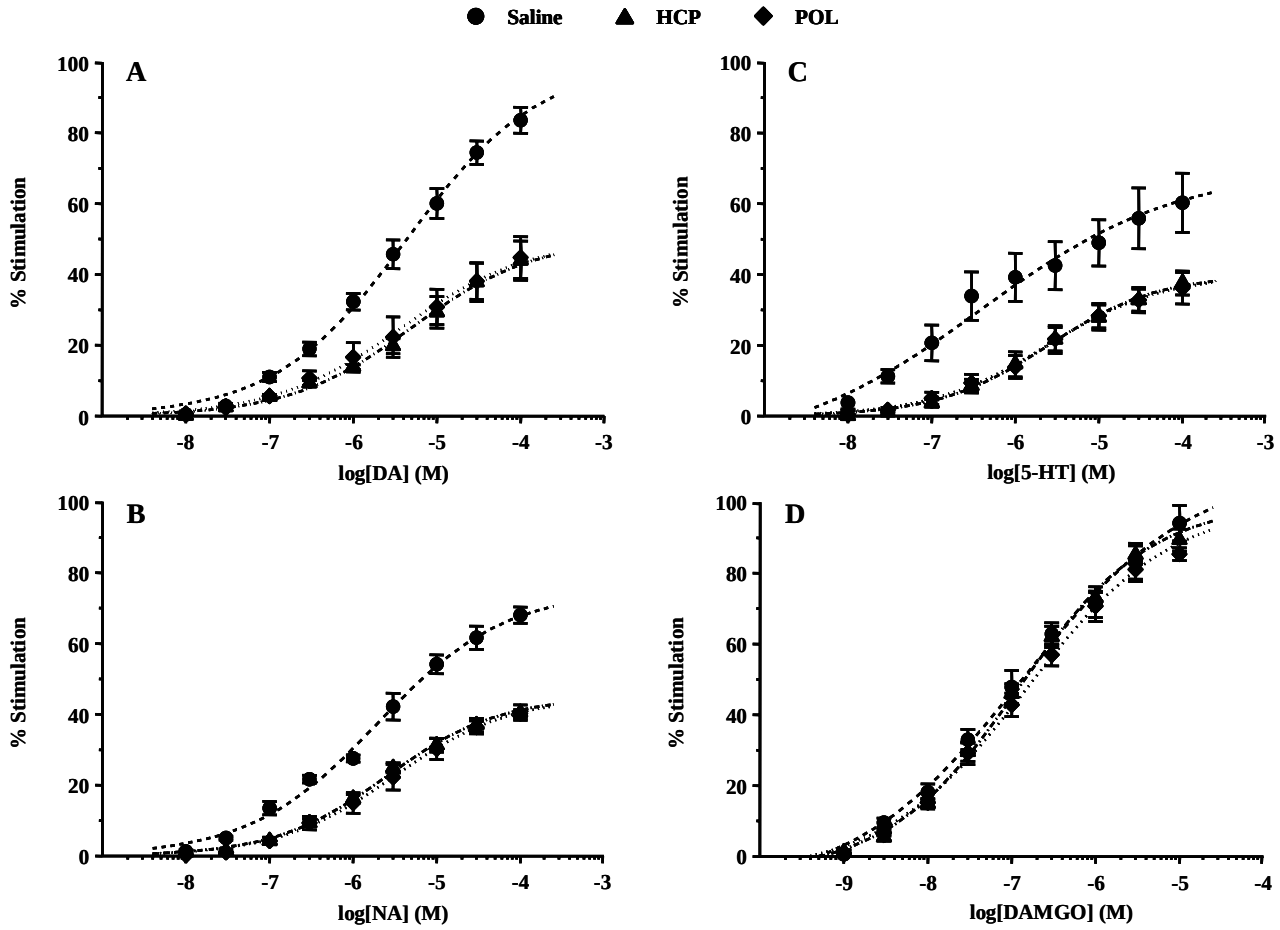

Figure 4.5: Effect of T3 (two treatments per day during 5 days) with HCP, POL (90 mg/kg, p.o.) or saline on agonist-stimulated [^{35}S]GTPγS binding measured ex vivo. Membranes prepared from rat striatum (A), hypothalamus (B), frontal cortex (C) and thalamus (D) were incubated with increasing doses of dopamine (A), noradrenaline (B), serotonin (C) or DAMGO (D) in the presence of 0.1 nM [^{35}S]GTPgS and 10 mM GDP. Data are presented as percentage of binding stimulation over GDP basal stimulation (absence of agonist). Mean ± SEM from 4 separated experiments carried out in duplicate.

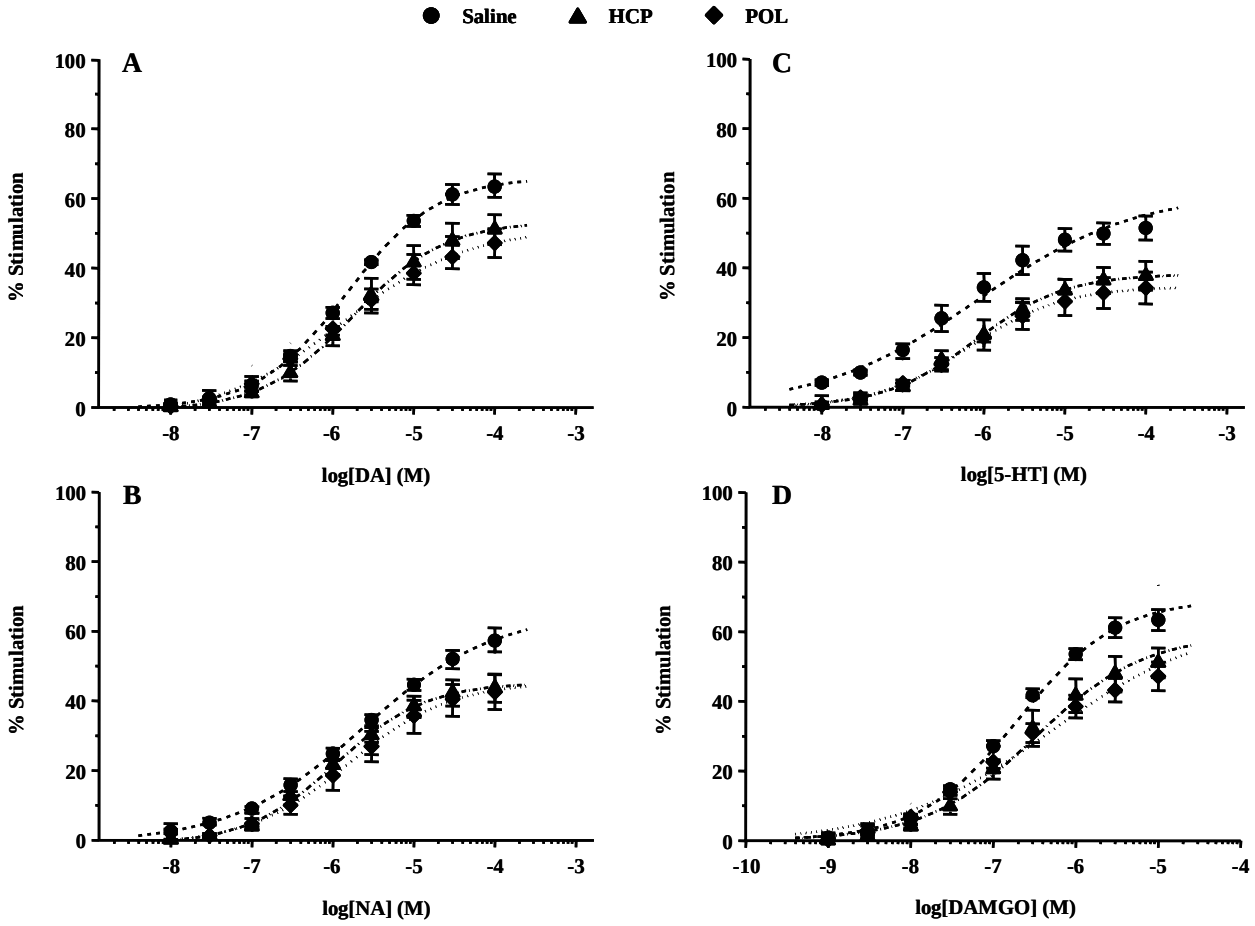

Figure 4.6: Effect of T4 (two treatments per day during 5 days followed by a 3 days wash-out) with HCP, POL (90 mg/kg, p.o.) or saline on agonist-stimulated [^{35}S]GTPγS binding measured ex vivo. Membranes prepared from rat striatum (A), hypothalamus (B), frontal cortex (C) and thalamus (D) were incubated with increasing doses of dopamine (A), noradrenaline (B), serotonin (C) or DAMGO (D) in the presence of 0.1 nM [^{35}S]GTPgS and 10 mM GDP. Data are presented as percentage of binding stimulation over GDP basal stimulation (absence of agonist). Mean ± SEM from 4 separated experiments carried out in duplicate.

Table 4.1: Effect of HCP and POL different treatment regimens on potency (EC_{50}) and relative efficacy (Emax) of [^{35}S]GTPγS binding.

		EC_{50} (μM)				Emax			
		DA	**NA**	**5-HT**	**DAMGO**	**DA**	**NA**	**5-HT**	**DAMGO**
Saline	**T1**	25 ± 6.2	42.5 ± 3.6	156 ± 25	0.24 ± 0.04	60.6 ± 2.2	48.9 ± 3.9	45.3 ± 3.3	79.7 ± 4.5
	T2	18.1 ± 2.4	39.4 ± 6.5	135 ± 26	0.37 ± 0.07	59.3 ± 3.9	47.9 ± 5.9	33.8 ± 2.7	71.6 ± 5.2
	T3	5 ± 1	10.4 ± 2.6	63.2 ± 25.9	1.6 ± 0.3	83.6 ± 3.7**c**	68 ± 2.3	60.3 ± 8.3	70.6 ± 5
	T4	11.3 ± 1.7	25.7 ± 4.7	83.9 ± 10.5	0.39 ± 0.07	63.4 ± 3.1	57.3 ± 3.6	51.9 ± 3.4	74.9 ± 2.7
HCP	**T1**	4.7 ± 0.5 **	6.3 ± 0.6 ** **c**	7.8 ± 1.1 ** **b**	0.37 ± 0.07	75.3 ± 1.5* **c**	72.6 ± 7.9 **b**	61.4 ± 3.2 **	75.9 ± 2.2
	T2	0.19 ± 0.05 *** **c**	0.28 ± 0.07 *** **c**	0.11 ± 0.04 ** **c**	1.52 ± 0.5	103.3 ± 4.2 *** **c**	115.9 ± 13.8 *** **c**	85.9 ± 10.2 *** **b**	62.9 ± 6.1
	T3	228 ± 74 ***	202 ± 59 ***	426 ± 225 ***	1.6 ± 0.3	43.9 ± 5.6 ***	40.5 ± 2.2*	37.6 ± 3.4*	66.9 ± 3
	T4	44.7 ± 5.3 *	88.4 ± 21.1 *	247 ± 24 **	0.76 ± 0.14	51.1 ± 4.3*	43.7 ± 4*	37.7 ± 2.1*	67.7 ± 3.5
POL	**T1**	7.6 ± 0.8 **	7.2 ± 1.3 **	13.4 ± 2.6 **	0.35 ± 0.08	73.2 ± 2.6* **c**	66.9 ± 1.5	57.1 ± 1.1 **a**	77.4 ± 1.3
	T2	0.32 ± 0.05 *** **c**	0.28 ± 0.11 *** **b**	0.13 ± 0.03 *** **c**	1.46 ± 0.47	93.2 ± 3.6 *** **c**	113.5 ± 17.1 *** **c**	93.1 ± 10.9 *** **c**	66.6 ± 3.9
	T3	307 ± 92 ***	189± 40 **	534 ± 225 ***	2.1 ± 0.6	44.8 ± 5.9 ***	40.2 ± 1.1*	36.1 ± 4.5*	64.1 ± 1.8
	T4	71.2 ± 15.2 **	115 ± 24.4 **	353 ± 52.7 ***	1.1 ±0.5	47.2 ± 4.1*	42.5 ± 5.6*	34.3 ±4.6 *	64.8 ± 2.9

T1 - one treatment by oral gavage 1 h before assays;
T2 - three treatments during 24h (23, 5 and 1 h before sacrifice);
T3 - two treatments per day during 5 days;
T4 - two treatments per day during 5 days followed by a 3 days wash-out before assays.
Data represent mean ± SEM from 4 separated experiments carried out in duplicate. Statistical difference calculated by one way ANOVA followed by Student-Newman-Keuls pairwise multiple comparison method. *p < 0.05; **p< 0.01; ***p< 0.001, significant difference in comparison to SAL in the same treatment regimen. ^{a}p < 0.05; ^{b}p< 0.01; ^{c}p< 0.001, significant difference in the same treatment group in comparison to others treatment regimen.

In vitro experiments

In order to verify if the effects observed *ex vivo* were related to direct phloroglucinols action on receptor functionality, we evaluated the [^{35}S]GTPγS binding stimulation by HC1 and HP4 in the absence of agonists. HC1 and HP4 were not able to stimulate the binding of [^{35}S]GTPγS to any of the receptors in membranes prepared from non-treated animals (Figure 4.7).

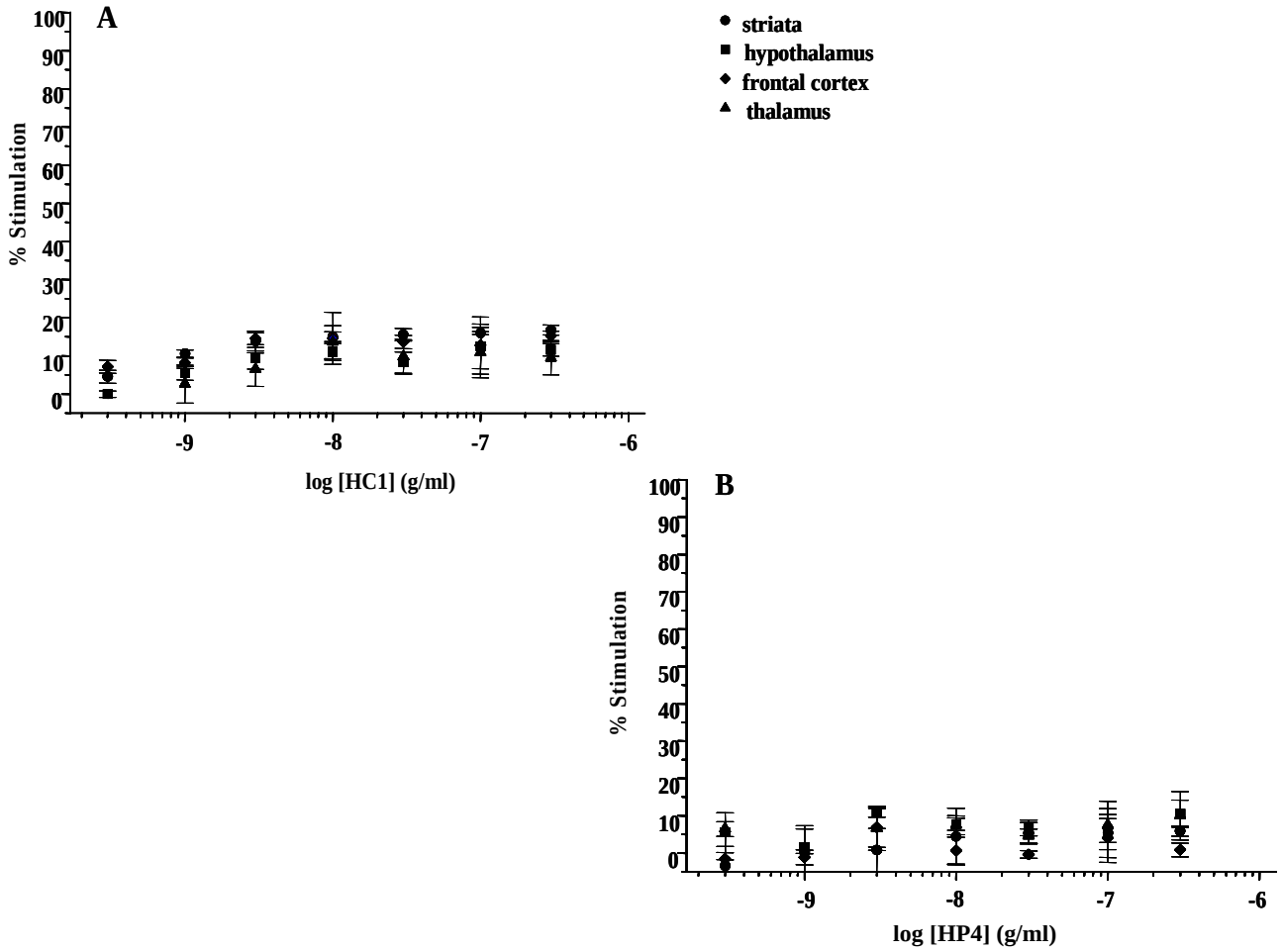

Figure 4.7: Concentration-response effect of (A) HC1 and (B) HP4 on [^{35}S]GTPγS binding. The data are presented as percentage of binding stimulation over GDP basal stimulation. Mean ± SEM from 4 separated experiments carried out in duplicate.

4.5. DISCUSSION

The knowledge concerning alterations in receptor-G protein coupling in the depressive disorder (Chen *et al.*, 1999; Gould and Manji, 2002; Donati and Rasenick, 2003), together with the findings that antidepressant agents may be effective on GPCR function (Hermans, 2003; Avissar and Schereiber, 2006) prompted us to investigate the effect *H. caprifoliatum* (HCP) and *H. polyanthemum* (POL) cyclohexane extracts on monaminergic and opioid receptors response in rat brain by measuring agonist-induced [^{35}S]GTPγS binding. This was the first time that *Hypericum* species were evaluated regarding to post-synaptic biochemical responses after acute and repeated administration at doses they exert antidepressant-like activity in animal models.

Acute (T1) or three repeated treatment within 24h (T2) of rats with HCP or POL increased the binding of [^{35}S]GTPγS to dopamine noradrenaline and serotonin receptors. In contrast five days treatment (T3) and five days with wash-out (T4) reduced the binding of [^{35}S]GTPγS. The EC_{50} values followed the same direction of E_{max}, T1 and T2 increased monoamine potency to stimulate the [^{35}S]GTPγS binding; T3 EC_{50} values were increased by 15 times or more, indicating a reduction on monoamine potency to stimulate the [^{35}S]GTPγS binding, and even after 3 days wash-out (T4) this reduction was still present but in a lesser extent. Incubation of membranes with HC1 and HP4 without agonists did not influence [^{35}S]GTPγS binding. Therefore the effects observed in the [^{35}S]GTPγS binding after treating rats with POL and HCP could not be credited to agonist effect by HP4 or HC1 on monoaminergic receptors. Nevertheless, HC1 and HP4 inhibit monoamines uptake (Chapter 1 and 3), thus we speculate that the observed effects could be a consequence to monoamines increase in the synaptic cleft following the uptake inhibition. Hence, in T1 and T2, the increase in [^{35}S]GTPγS binding could result from receptors adaptations intending to respond to the elevated levels of monoamines in the synapse. After 5 days treatment (T3 and T4), we can imagine that the decrease in [^{35}S]GTPγS binding may be due to a desensitization or down regulation induced by the increased levels of monoamines in the synaptic clef (Stahl, 2000, Hermans, 2003; Spiegel, 2003). This result is in accordance with the majority

experimental results that show a reduction in receptor-G protein coupling after chronic treatment with several classes of antidepressant drugs (Gould and Manji, 2002).

In fact, repeated exposure of a GPCR to agonist leads to a diminished responsiveness, that occurs through a variety of mechanisms, including desensitization, down-regulation and redistribution of cell surface receptors (trafficking) (Ferguson, 2001; Harrison and Traynor, 2003. Hermans, 2003). Donati and Rasenick (2003) point out several possible targets for antidepressant action via G proteins: *(i)* the number or affinity of receptors could be altered; receptors might show decreased affinity for agonist or they may be internalized; *(ii)* the ability of an agonist to activate a G protein might be decreased; *(iii)* the number of G proteins could be changed, p.ex. the activation of G protein genes or the stability of G protein at the cell surface could be affected. The intrinsic properties of a given G protein (e.g. affinity for GTP or rate of GTP hydrolysis) could be modified; *(iv)* the coupling between G proteins and their effectors could be altered, in this case, the ability of $G\alpha_s$ to activate (or $G\alpha_i$ to inhibit) adenylyl cyclase might be modified; *(v)* the effectors (p.ex. adenylyl cyclase) expression or activity could be altered by antidepressant treatment. Even though antidepressants' effects on G protein functionality are variable, it appears that GTP-binding properties of G proteins are unaffected by antidepressant treatment (Emamghoreishi *et al.*, 1996; Dwivedi and Pandey, 1997) and their effects on G protein signaling do not include changes in receptor or G protein gene expression (down regulation) (Hensler, 2002, 2003; Pejchal *et al.*, 2002). Consequently, desensitization seems to play an important role in antidepressants effects on receptor-G protein coupling (Hermans, 2003; Spiegel, 2003).

Regarding the FST results, the effects of the extracts, HC1 and HP4 on [^{35}S]GTPγS binding assay seem not to be directly related to antidepressant effects since T1, T2, T3 and T4 regimens were effective on forced swimming test. However, this observation does not necessarily mean that the antidepressant effect is not linked to the alterations observed in the [^{35}S]GTPγS binding. It is known that FST is a test with good "predictive validity" but with low face and construct (Willner, 1990; Porsolt et al, 1991) this means that the model responds well to the same drugs as depressed humans even though its ability to simulate a depressive state is low. Accordingly, antidepressants that reduce immobility time acutely in the FST show

their effects on GPCRs only after 14 -21 days of treatment (Hensler, 2002; Pejchal *et al.*, 2002; Shen *et al.*, 2002; Castro *et al.*, 2003). Likewise, Galeotti *et al.* (2002) demonstrated that the inactivation of $G\alpha_i$ produces antiimmobility effect comparable to that produced by antidepressants, but this effect was observable only 11 days after pertussis toxin intracerebroventricular (i.c.v.) injection or 18-24h after antisense oligodeoxynucleotides directed against $G\alpha$ (i.c.v.), while antidepressants were effective 30 minutes following treatment.

Our findings indicate that, at doses for which antidepressant-like activity have been demonstrated in animal models, *H. caprifoliatum* and *H. polyanthemum* extracts bring about adaptive changes in monoamine receptors. While within 24h the observed increase in [^{35}S] GTPγS binding may result from receptors adaptations to cope with elevated levels of monoamines in the synapse. The reduced functional coupling of monoamine receptors to G proteins by 5 days treatment is in accord with the antidepressant mode of action. Furthermore, if the reduction in [^{35}S] GTPγS binding after 5 days treatment with the extracts results from a desensitization process, this would explain the persistent antiimmobility observed after 3 days of wash-out.

Finally, the lack of influence over DAMGO-stimulated [^{35}S]GTPγS binding curves stands for extracts selective effect on monoaminergic system. This result also fits with previous observation that, although the extracts present analgesic effects prevented by naloxone, their phloroglucinols do not inhibit [^{3}H]naloxone binding (Chapter 1). From the [^{35}S]GTPγS binding assay results we can infer that the cross-talk between monoaminergic and opioid systems would induce HCP and POL antinociceptive properties (Schereiber *et al.*, 1998; Atkinson *et al.*, 1999; Lynch, 2001), as already discussed in the Chapter 3.

In conclusion, the effects of *H. caprifoliatum* and *H. polyanthemum* on [^{35}S]GTPγS confirms their prominent action on monoaminergic system and are consistent with antidepressants' effects and may have some role in the antidepressant-like effect of these *Hypericum* species.

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III PARTE

DISCUSSÃO GERAL,
CONCLUSÕES E PERSPECTIVAS

GENERAL DISCUSSION

Throughout this thesis, we carried out a study concerning to cyclohexane extracts of two *Hypericum* species native to South Brazil. *Hypericum* species have gained popularity for the treatment of mild to moderate depression (Linde *et al.*, 1996; Butterweck *et al.*, 1997; Müller *et al.*, 1998; Müller, 2003). It has been shown that the antidepressant activity of *Hypericum* species would be due to several compounds such as naphthodianthrone, flavonoids, xanthones and phloroglucinol derivatives (Rocha *et al.*, 1994; Chaterjee *et al.*, 1998a; Butterweck *et al.*, 2000). Studies, carried out by Chatterjee *et al.* (1998b) and Müller *et al.* (2001, 2003) groups, suggest that the *Hypericum* antidepressant-like effect would be assigned to the phloroglucinol derivatives, and more precisely to hyperforin. From these literature data and previous results obtained with *Hypericum* species native to Southern Brazil (Daudt *et al.*, 2000; Gnerre *et al.*, 2001; Viana 2002; Nör *et al.*, 2004), the aim of our research was to investigate the potential antidepressant and analgesic effect of Brazilian *Hypericum* species, as well as to identify the substances responsible for these activities and to elucidate their mechanisms of action.

We have demonstrated that the antidepressant-like effect of *H. caprifoliatum* at rat FST, verified by Daudt *et al.* (2001) and Viana (2002) is reproducible, since it was also observed in mice and with different lipophilic extracts, i.e. petroleum ether and cyclohexane. We have also verified that *H. polyanthemum* cyclohexane extract (POL) has a pharmacological profile similar to that of *H. caprifoliatum* (HCP). Recent study carried out by Nor *et al.* (2004) revealed that phloroglucinol derivatives from *Hypericum* species, native to Southern Brazil have similar molecular pattern which differs from hyperforin and analogues obtained from *H. perforatum.* In spite of that, HC1, from *H. caprifoliatum*, and HP4, from *H. polyanthemum,* showed antidepressant like effect in the forced swimming test.

In order to investigate HCP and POL mechanism of action we performed pharmacological antagonism *in vivo* and neurochemical assays. *In vivo* pre-treatment with SCH 23390 or sulpiride (D_1 and D_2 antagonists, respectively) abolished the anti-immobility effect of the extracts suggesting that the extracts antidepressant-like activity is associated with an effect on the monoaminergic systems. This assumption was reinforced by *in vitro*

experiments in which the main phloroglucinol derivatives of *H. caprifoliatum* (HC1) and *H. polyanthemum* (HP4) dose dependently inhibited monoamine synaptosomal uptake, with more potency for dopamine, without binding to monoaminergic transporters. Our results are in agreement with the ones for other *Hypericum* species, *H. perforatum* (Chatterjee *et al.*, 1998a; Müller *et al.*, 1997, 1998) and *H. triquetrifolium* (Roz *et al.*, 2002). These species also demonstrated antidepressant-like effect in the FST and monoamine uptake inhibition, related to the presence hyperforin, without affecting ligand specific binding to transporters (Chatterjee *et al.*, 1998a, b; Müller *et al.*, 1998, 2001; Jensen *et al.*, 2001; Roz *et al.*, 2002). Taken together, these latter observations and our results, strongly suggest that the antidepressant-like activity of *Hypericum* species is related to a mechanism different from classical antidepressants. However, the molecular mechanisms by which extracts of *Hypericum* species induce antidepressant-like activity, through monoamine uptake inhibition, remain unclear.

One hypothesis that could explain this effect is an alteration of monoamine transporters function or the reduction in the number of these transporters. Transporters are not quiescent plasma membrane proteins, plentiful evidence supports that they dynamically traffic to and from membrane, constitutively and in response to psychostimulant exposure, receptor activation and neuronal activity (do Rego, 2000; Chi and Reith, 2003; Melikian, 2004). Thus, incubation with HC1 and HP4 may reduce DA, NA and 5-HT uptake by reducing transporters in the membrane, this increases monoamines in the synaptic clef, but in a different way from psychostimulants, since they do not bind to the transporter. It is conceivable that DAT endocytosis occurs due to dopamine efflux as protective mechanism against dopamine metabolism that is known to be neurotoxic (Rabinovic *et al.*, 2000).

The fact that *Hypericum* phloroglocinols derivatives influence monoamine uptake by a mechanism different from typical transporters inhibitors is coherent with their chemical structure. These molecules do not have nitrogen atom in their structure which is remarkably against structure activity relationship studies for monoamines transporters (Appell *et al.*, 2004). Muller et coll have been searching alternative manners by which hyperforin influences neurotransmitters extracellular concentrations, firstly they found that it alters sodium

conductive pathways (Singer *et al.*, 1999) but the mechanism responsible for this effect remained uncertain. Recently, this group demonstrated that rats treated with hyperforin had the physico-chemical properties of brain neuronal membranes affected, increasing the flexibility of membrane acyl-chains and decreasing annular- as well as bulk-fluidity, which can be responsible for its pharmacological properties (Eckert *et al.*, 2001, 2004).

It is a consensus among the field that the increase of monoamine levels in the synaptic clef caused by uptake inhibition is only the first from a series of neurochemical modifications induced by antidepressant. This increase evidently will lead to post synaptic modifications at different level of the signaling cascade (Stahl, 2000; Schreiber and Avissar, 2003; Millan, 2006). In addition, depressive patients present alterations in the hypothalamo-pituitary-adrenal (HPA) axis, like abnormal levels of corticosteroids, which are normalized by antidepressants; thus HPA axis has been considered as a target for antidepressant actions (Sachar *et al.*, 1976; Gold *et al.*, 1995; Holsboer and Barden, 1996; Nemeroff 1996; Pariante *et al.*, 2004). For these reasons, we carried out the studies on receptor functionality through [^{35}S]GTPγS binding assay and on corticosterone levels after different treatments regimens.

In relation to the effects on corticosterone levels, we demonstrated that only repeated treatment with antidepressants, imipramine and bupropion, or *Hypericum* extracts reduce forced swimming test induced corticosterone raise. Furthermore, differently from the antidepressants, HCP and POL decreased only cortical and not serum corticosterone levels. This result is in accordance with Franklin *et al.* (2004), who found decreased levels of corticosterone and cortisol after 14 days feeding rats with pellets containing *H. perforatum* extract. As discussed in Chapter 2, the authors suggest that this effect is related to induction of MDR P-glycoprotein, but the theory that antidepressant could restore the balance of HPA axis implies in the inhibition and not induction of this glycoprotein (Muller *et al.*, 2003; Pariante *et al.*, 2004; Pariante, 2006). Given that *H. perforatum* extracts and isolated substances, hypericin, hyperforin and flavonoids, are inducers of this glycoprotein at gastro-intestinal levels (Durr *et al.*, 2000; Perloff *et al.*, 2001; Hennessy *et al.*, 2002; Webber *et al.*, 2006), we consider more likely that extracts effect on HPA axis are via the monoaminergic activation follow-on uptake inhibition.

Neverthless one should keep in mind that this set of experiments represents only a preliminary study of the Brazilian *Hypericum* species influence on the HPA axis functioning. Other parameters as CRH, POMC, ACTH, AVP, GR and MR should be evaluated in order to achieve more reliable conclusions.

Acutely the extracts increase both cortical and serum corticosterone levels in non-stressed mice, and although not significant, bupropion also has the same effect. Several studies report elevation in plasmatic HPA axis hormones in men and animals after acute or sub-chronic antidepressant treatment, p.ex. citalopram, fluoxetine, reboxetine, mirtazepine, *H. perforatum* extract WS 5570 and LI 160 (Seifritz *et al.*, 1996; Duncan *et al.*, 1998; Schule *et al.*, 2004 a,b; Webber *et al.*, 2006), and pergolide, a dopamine agonist (Fuller and Snoddy, 1984; Foreman *et al.*, 1989). As discussed in Chapter 2, the monoamine elevation in the synaptic clef activates post-synaptic receptors in the hypothalamus and stimulates the secretion of several hormones (Raap and van de Kar 1999; Schule *et al.*, 2004b). Moreover, one possible pathway involved glucocorticoid receptor (GR) resistance is the cAMP/PKA cascade, which is activated by monoamines (Pariante, 2006). There is now considerable evidence that phosphorylation of the GR by cAMP-dependent protein kinase has a relevant role in the regulation of GR function and depressed patients had been found to exhibit reduced cAMP-dependent protein kinase activity in cultured fibroblasts (Manier *et al.*, 2000). Therefore, it is possible that disruption in the cAMP/PKA cascade described in major depression is linked to GR resistance in this disorder, and that antidepressants may overcome these receptor alterations via a direct effect on this pathway (Pariante, 2006). It should also be remembered that non-phosphortylated GR can physically associate to CREB, decreasing its CREB's phosphorylation and thus reducing neuron survival (Holsboer, 2000), and neuronal death is one of the important features of the neurotrophic theory of depression (Duman *et al.*, 1997). Major depression is associated with a selective loss of hippocampal volume that involves hypersecretion of glucocorticoids (Duman *et al.*, 1997; Sapolsky, 2000).

The results discussed until this point show that HCP, POL and their phloroglucinols derivatives have antidepressant effect in animal models comparable to tricyclics, bupropion and SSRI, but extracts' neurochemical substrate is to some extent different from classic antidepressants. On the other hand, the results obtained after repeated treatments with the extracts in the [^{35}S]GTPγS binding assay are in agreement with the majority of antidepressants chronic treatment studies, which demonstrate a decrease in receptor G-protein coupling (Gould and Manji, 2002) as consequence of a desensitization process (Hermans, 2003; Spiegel, 2003). As discussed in Chapter 4, the mechanism by which HCP and POL affects [^{35}S]GTPγS binding induced by monoamines is not through direct binding of their phloroglucinols derivatives HC1 and HP4, since direct incubation with the phloroglucinols did not modify [^{35}S]GTPγS binding. Differently from 5 days treatments (two treatments per days), acute (T1) and 3 treatments within 24h (T2) caused an increase in [^{35}S]GTPγS binding induced by the three monoamines. Therefore, these divergent modifications according to the treatment regimen probably are adaptations to the elevation of monoamines in the synaptic clef resultant from uptake inhibition.

Considering the results by Eckert et al (2004) on membrane properties alterations after *H. perforatum* treatment, it is reasonable to propose that phloroglocinol derivatives, hyperforin, HC1 and uliginosin B, may behave in the plasma membrane similarly to cholesterol, modifying its ordering. Steroids modifications on membrane fluidity (Brann *et al.*, 1995; Helmreich, 2003) can result in receptor or ion channel proteins conformational change and thereby influence its physiological function, for example neurotransmitter uptake, neurotransmitter synthesis, receptor binding and second messenger systems as well as signal transduction pathways (Clarke et al 1999, Helmreich, 2003). Membrane physico-chemical environment is important for G-protein activity, since these proteins are associated to the membrane and must move during the GTP cycle after agonist binding to the GPCRs (Donati and Rasenick, 2003; Helmreich, 2003; Hermans, 2003). It was demonstrated that cholesterol inclusion to the membrane matrix reduces its fluidity by restricting phospholipids mobility, hampering G-protein activation and so Gα association to adenylyl cyclase (Ropero *et al.*, 2003).

Although *Hypericum* phloroglocinols do not have the steroid skeleton, their massive prenylation renders the phloroglocinol nucleus highly lipophilic and makes it presumably apt to plunge in the plasma membrane. This could influence not only neurotransmitter uptake but also brain penetration of substances, as corticosterone. If this hypothesis is correct one may ask: why there is certain specificity for HC1 and uliginosin B to cause monoaminergic effects? It should be remembered that dopamine (DA), noradrenalin (NA) and serotonin (5-HT) belong to the same phylogenetic family (Figure 2) (Goldberg *et al.*, 2003) and so they have more prone to be affected in the same way by modifications in their specific lipid environment.

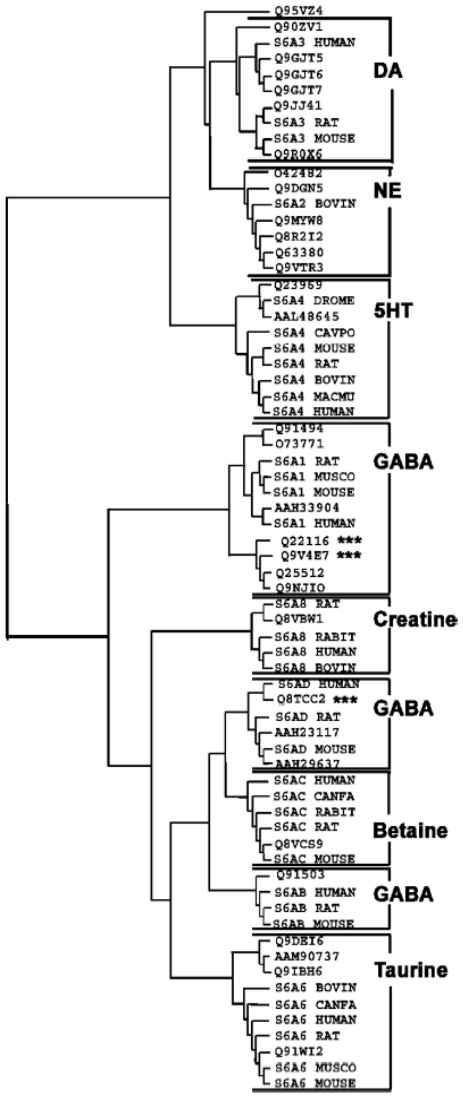

Figure 2: Phylogenetic tree and predictions of the evolutionary model. (A) Phylogenetic tree of transporter proteins that were used to create the evolutionary model. This is a consensus tree from 2000 trees sampled during a Markov Chain Monte Carlo run, using Mr. Bayes, state-of-the-art tree inference software. The tree is rooted at the midpoint of the longest branch. Transporters forming a monophyletic subfamily are indicated as, DA; dopamine transporters, NE; norepinephrine transporters, 5HT; serotonin transporters, GABA, taurine, betaine, and creatine. The single sequence that does not fall under any of the subfamilies is an octopamine transporter from trichoplusia ni (cabbage looper) (SWISSPROT ID: Q95VZ4). Sequences at the end of branches indicated with 3 stars-Q22116, Q9V4E7, Q8TCC2 - are orphan transporters from C. elegans, drosophila, and human, respectively. (From Goldber et al., 2002)

Besides investigating the mechanism involved on the antidepressant-like effect of HCP and POL we also studied their analgesic activity. We can assume that extracts antinociceptive effect is likely to be related to the interaction between monoaminergic and opioid system. Although HCP and POL antinociceptive effect in the hot plate is prevented by naloxone, HC1 and uliginosin B did not affect [^{3}H]-naloxone binding in membranes and the effect of DAMGO-induced [^{35}S]GTPγS binding are not influenced when rats treated with the extracts. In addition, uliginosin B showed antinociceptive activity that was not prevented by naloxone pre-treatment. It is known that antidepressants have analgesic effect, mainly in chronic pain, without direct interaction binding to opioid receptors (Morgan and Franklin, 1991; Frussa-Filho *et al.*, 1996; Altier and Stewart, 1999; Gilbert and Franklin, 2001). Moreover, morphine analgesia is increased by dopaminergic agonists and some of these agonists induce analgesia independently of opioid receptor (Morgan and Franklin, 1991; Altier and Stewart, 1998).

The results obtained for the extracts and phloroglucinol derivatives tested in this thesis as well as the extraction methods are summarized in Figure 3.

Abbreviations:

HCP, POL= cyclohexane extract from *H. caprifoliatum* and *H. polyanthemum*, respectively
HC1, HP4 = phloroglucinol derivatives present in HCP and POL, respectively
CC= column chromatography
TLC = thin layer chromatography
MA = monoamines
OPI = opioid
⊕ = positive result
Θ = negative result

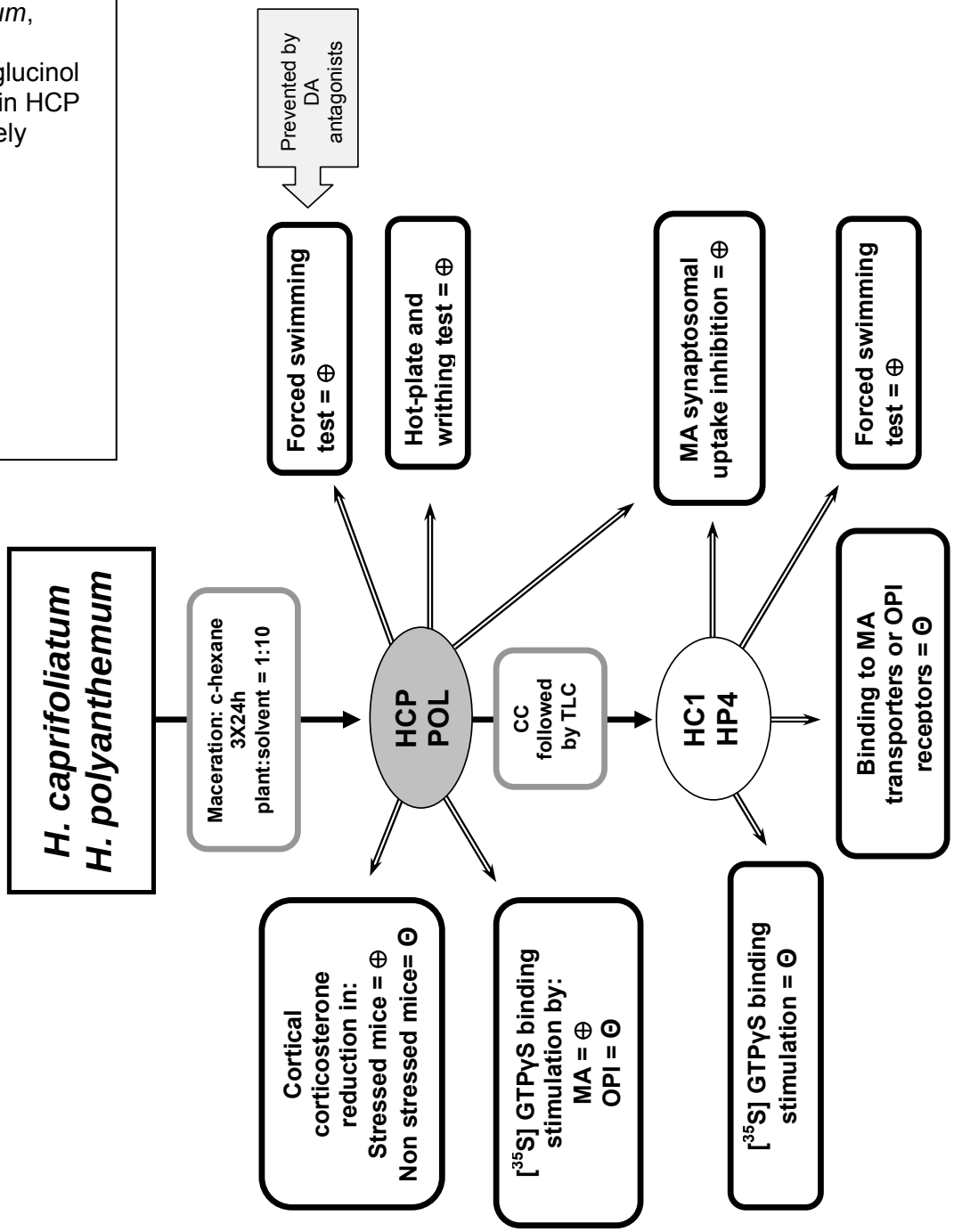

Figure 3: *Hypericum caprifoliatum* and *H. polyanthemum* extraction scheme and pharmacological activities resume.

Notably, this study corroborates with the idea that plant-derived substances have, and will certainly continue to have, a relevant place in the process of drug discovery (Calixto *et al.*, 2000). Plants can be used as therapeutic resources in several ways: (*i*) herbal teas or other home made remedies; (*ii*) crude extracts or "standard enriched fractions" in phytopharmaceutical preparations or herbal medicines; (*iii*) subjected to successive extraction and purification procedures to isolate the compounds of interest, which can themselves be active and used directly as a drug, or as models for total synthesis, determining a prototype drug (Rates, 2001). Since item (*iii*) is the objective our work, we selected *H. caprifoliatum* and *H. polyanthemum* regarding their chemical content; the phloroglucinol derivatives, which have shown antidepressant like activity. We were able to demonstrate that *H. caprifoliatum* and *H. polyanthemum* and their phloroglucinol derivatives, HC1 and HP4, show antinociceptive effect and antidepressant-like activities in vivo and in vitro. Nevertheless, the mechanism by which these effects occur is different from the one of classical antidepressants. As discussed above, considering phloroglucinols molecular pattern and structural requirements to monoamine transporter binding, like presence of nitrogen atom, these results are not surprising (Appel *et al.*, 2004).

Even so, phloroglucinols molecular peculiarities and lack of high selectivity for monoamine uptake do not prevent them from originating antidepressant drugs. Most experts agree that depression should be viewed as a syndrome, not a disease, since it affects various biological mechanisms and has a bidirectional link with co-morbidies (Charney and Manji, 2004; Evans *et al.*, 2005; Berton and Nestler, 2006). In addition, drug discovery programs devoted in multi-target drugs strategies for treating depressive states and other complex CNS disorders, have been launched (Bymaster *et al.*, 2001; for review see Millan, 2006). Drugs like the antidepressant tianeptine and the psychotropic olanzapine (used for the treatment of psychoses, schizophrenia and bipolar disorder) have their effect attributed to a complex, multitarget mechanism of action embracing modulation of monoaminergic, glutamatergic, and GABAergic networks, as well as other central acting systems (Bymaster *et al.*, 2001; Millan, 2006). Natural products can be a source of substances with innovative mechanisms of action, still the challenges of developing and characterizing new drugs should not be neglected, and

careful planned method for studying their extracts and isolated substances are decisive (Rates, 2001; Rollinger *et al.*, 2006).

CONCLUSION AND PERSPECTIVES

The experimental results reported in this thesis provide evidences that plants remain a valuable source for new drugs search. We demonstrated that *H. caprifoliatum* (HCP) and *H. polyanthemum* (POL) cyclohexane extracts have potential antidepressant and analgesic activities. However they seem to have a pharmacological profile somewhat diverse from the classical antidepressant and opioid-like analgesics. Differently from tricyclics and monoamine selective reuptake inhibitors, the main extracts' phloroglucinols, HC1 and HP4, inhibited dopamine ([^{3}H]-DA), noradrenaline ([^{3}H]-NA) and serotonin ([^{3}H]-5HT) uptake but did not affect the binding of [^{3}H]-mazindol, [^{3}H]-nisoxetine and [^{3}H]-citalopram to DA, NA and 5-HT transporters binding sites. Although the anti-immobility effect in the FST was inhibited by D_1 and D_2 antagonists and extracts treatment modify [^{35}S]GTPγS binding profiles, HC1 and HP4 do not modify the [^{35}S]GTPγS binding indicating that these phloroglucinols do not interact directly with monoaminergic receptors. Extracts' effect on stress-induced corticosterone levels increase was also different from the antidepressants, while these drugs decrease cortical and serum corticosterone levels HCP and POL decreased only cortical levels. As well, the antinociceptive effect seems to be indirect. Naloxone prevented HCP and POL antinociceptive effect in the hot plate. Nonetheless, HC1 and HP4 did not inhibit [^{3}H]-naloxone binding to opioid receptors, extracts treatment did no affect [^{35}S]GTPγS binding nor HP4 antinociceptive effect was abolished by naloxone. In conclusion, *H. caprifoliatum* and *H. polyanthemum* analgesic and antidepressant effects engage the monoaminergic system and HPA axis, even though the manner extracts influence this system is different from classical antidepressants.

This conclusion is rather interesting since the research field for new antidepressants is also interested in new mechanisms of action. In order to substantiate the antidepressant potential HCP and POL will be tested on other animal models predictive of antidepressant

action, like tail suspension test and learned helplessness, as well as on models with more construct validity, like depressed animals selective breeding. Several experiments will be clearly required to elucidate their mechanism of action. Microdialysis studies will be performed to verify extracts treatment effect on interstitial brain monoamine levels. Extracts' effect on monoaminergic transporters and receptors number, after different treatment regimens, will be assessed by autoradiography binding. To refine the involvement of the monoaminergic system in the antinociceptive effect, pharmacological antagonism will be performed. In addition, the observation of some conflicting results between in vivo and in vitro assays as well as for extracts and isolated compounds make necessary a deeper chemical investigation and pharmacokinetic study since the effect at molecular level could be related to the minor substances or eventual metabolites. Finally, as the results clearly indicate that HCP and POL - and their main phloroglucinols HC1 and HP4 - activate the dopaminergic neurotransmission, the study of other illnesses related to the dopaminergic functions, such as Parkinson disease and substance abuse, is also planned for future investigations.

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BIOGRAFIA

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