Daniele Guilhermano Machado

INVESTIGAÇÃO DO POTENCIAL ANTIDEPRESSIVO DE *ROSMARINUS OFFICINALIS*

Tese de Doutorado submetida ao Programa de Pós-Graduação em Neurociências, da Universidade Federal de Santa Catarina, para obtenção do Grau de Doutora em Neurociências.
Orientadora: Profa. Dra. Ana Lúcia Severo Rodrigues.

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DANIELE GUILHERMANO MACHADO

"INVESTIGAÇÃO DO POTENCIAL ANTIDEPRESSIVO DE *ROSMARINUS OFFICINALIS*".

Esta tese foi julgada e aprovada para a obtenção do Grau de Doutor em Neurociências na área de Neuropsicobiologia no Programa de Pós-Graduação em Neurociências da Universidade Federal de Santa Catarina

Florianópolis, 28 de fevereiro de 2012.

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Ser pesquisador

"De todos os caminhos percorridos, de todos os sonhos trilhados,
A grande vitória diária que permanecerá,
É que valeu a pena lutar para aprender e concretizar
uma ideologia de vida: ser pesquisador..."

E depois de tudo, permanecerão novas perspectivas para a ciência,
novas hipóteses, novas comprovações, pois tudo se renova diariamente...
Somos passageiros, porém podemos contribuir para este progresso,
mesmo que este seja sutil e silencioso.

Somos pequenos, mas podemos nos tornar grandes ao admitir isso!

Façamos a nossa parte, para que amanhã a paz de espírito,
enobreça nossa alma de alegria!

Não importará somente onde nossos passos nos levarão
mas como realizaremos esta caminhada...

Daniele Guilhermano Machado

DEDICATÓRIA

"As palavras o vento leva, mas os exemplos arrastam".

Dedico esta Tese de Doutorado a minha mãe,
Lucia do Amarante Guilhermano,

Que com seus exemplos de conduta moral, fé e coragem impulsionaram-me a percorrer uma estrada voltada ao bem, a verdade e a justiça.

Pessoa despreendida de si mesma e voltada para o bem ao próximo,
Primando sempre pelo auto-burilamento interligado a uma consciência tranquila.

Me ensinaste que os obstáculos na vida, são apenas escadarias invisíveis para alcançarmos os caminhos imperecíveis de nossa auto-superação,
Que a humildade sincera nos possibilita crescer com mais maturidade e em maior amplitude, pois nos conscientiza que somos humanos e que a busca pelo saber é inesgotável.

E ao demonstrar-me na prática de nosso dia-a-dia,
que a paz de espírito tem um valor imensurável.

Obrigada mãe!!!

Por seu doce, firme e incansável apoio diante das adversidades que passamos,
por ter abraçado minha ideologia, como se fosse sua,
e compartilhado todos os momentos de minha vida,
especialmente este.

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Aos meus colegas do Laboratório de Neurobiologia da Depressão, obrigada a todos pela convivência destes anos e pelos inúmeros cafezinhos de todas as horas!!! Desejo-lhes boa sorte e progresso em vossas trajetórias. O meu abraço fraterno!

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Ao professor, Moacir G. Pizzolatti e sua aluna de doutorado Juliana Dalmarco, pelo fornecimento dos extratos, compostos isolados e óleo essencial de *Rosmarinus officinalis*, bem como pelas análises fitoquímicas. Grata, pela colaboração e pelo carinho. Lembrarei com saudades dos anos de convivência!

Aos Professores da Pós-Graduação em Neurociências, em especial ao professor Marcelo Farina, pela disponibilidade e boa vontade com que sempre esclareceu minhas dúvidas de estatística, bem como os professores integrantes de minha qualificação, que me incentivaram e orientaram com sugestões que contribuíram para a finalização desta tese.

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Ao Nivaldo, secretário da Pós-Graduação, por sua colaboração incansável, por sua amizade e incentivo em todos os momentos. Que Deus ampare você e sua família. Serei eternamente grata pelo apoio de todos estes anos!

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Aos índios e a medicina popular em geral, nossos primeiros e incansáveis cientistas. Grata, pela colaboração, pois o início deste trabalho, na busca da comprovação científica do alecrim como antidepressivo, só foi possível através suas evidências etnofarmacológicas preliminares.

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APRESENTAÇÃO

Esta Tese de doutorado encontra-se organizada da seguinte forma:

A introdução contém o embasamento teórico da proposta do trabalho apresentado, a justificativa e os objetivos propostos e desenvolvidos durante o transcorrer da execução desta tese.

Os capítulos contêm os materiais e métodos, os resultados e as discussões, bem como as referências específicas de cada assunto tratado, apresentados na forma de capítulos científicos.

A discussão geral apresenta a integração de todos os estudos realizados.

A seção de referências apresenta especificamente as referências citadas na introdução e discussão desta tese.

Os capítulos pertencentes a esta Tese foram distribuídos da seguinte forma:

Capítulo 1: Refere-se ao artigo 1, intitulado "Antidepressant-like effect of the extract of *Rosmarinus officinalis* in mice: Involvement of the monoaminergic system", sendo a primeira evidência do potencial antidepressivo desta planta em modelos preditivos de atividade antidepressiva (TSC e TNF);

Capítulo 2: Refere-se ao artigo 2, intitulado "Antidepressant-like effects of fractions, essential oil and isolated compounds of *Rosmarinus officinalis* L. in mice". O enfoque deste capítulo foi a investigação dos compostos fitoquímicos responsáveis, pelo menos em parte pelo potencial antidepressivo desta planta.

Capítulo 3: Refere-se ao estudo do potencial antidepressivo do ácido ursólico, composto isolado de *Rosmarinus officinalis* no TSC: "Antidepressant-like effect of ursolic acid isolated from *Rosmarinus officinalis* L. in mice: evidence for the involvement of the dopaminergic system".

Capítulo 4: Investigou o potencial antidepressivo da fluoxetina, avaliando sua habilidade em reverter o comportamento tipo-depressivo (hiperatividade associada ao comportamento anedônico) bem como alterações bioquímicas induzidas pela BO, a fim de melhor compreender este modelo animal de depressão. Este artigo foi intitulado: "Fluoxetine reverses hyperactivity, anhedonic behavior and increased hippocampal acethylcholinesterase activity induced by olfactory bulbectomy".

Capítulo 5: Investigou o potencial antidepressivo de *Rosmarinus officinalis* no modelo da BO e avaliou o desempenho cognitivo no labirinto aquático de Morris de animais submetidos ao modelo da BO e ao tratamento repetido com o extrato. O referido artigo foi intitulado: "*Rosmarinus officinalis* L. hydroalcoholic extract, similarly to fluoxetine, reverses depressive-like behavior without altering learning deficit in olfactory bulbectomized mice".

RESUMO

MACHADO, Daniele Guilhermano. **Investigação do potencial antidepressivo de *Rosmarinus officinalis.*** 2012. Tese (Doutorado em Neurociências) - Universidade Federal de Santa Catarina, Florianópolis.

Rosmarinus officinalis L. (Labiatae), alecrim, possui várias aplicações terapêuticas na medicina popular no tratamento de várias doenças, incluindo a depressão. Primeiramente, o efeito do extrato hidroalcoólico dos talos e folhas desta planta foi investigado em dois testes comportamentais preditivos de atividade antidepressiva, o teste do nado forçado (TNF) e teste de suspensão da cauda (TSC) em camundongos. *Rosmarinus officinalis* produziu um efeito tipo-antidepressivo, pois o tratamento agudo com o extrato desta planta por via oral reduziu significativamente o tempo de imobilidade no TNF e TSC, sem alterar a atividade locomotora no teste de campo aberto. Além disso, a administração repetida (14 dias) do extrato produziu um efeito antidepressivo no TSC. O pré-tratamento com p-clorofenilalanina (PCPA, inibidor da síntese de serotonina, 4 dias consecutivos), NAN-190 (antagonista de receptores 5-HT_{1A}), cetanserina (antagonista de receptores 5-HT_{2A}), 1-(m-clorofenil) biguanida (mCPBG, agonista de receptores 5-HT_3), prazosina (antagonista de α_1-adrenoceptores), SCH23390 (antagonista de receptores dopaminérgicos D_1) ou sulpirida (antagonista de receptores dopaminérgicos D_2), mas não ioimbina (antagonista de α_2-adrenoceptores) reverteu a redução do tempo de imobilidade causada pelo extrato administrado agudamente no TSC. A combinação de MDL72222 (antagonista de receptores 5-HT_3) com uma dose sub-ativa do extrato produziu um efeito anti-imobilidade no TSC. Estes resultados sugerem que a ação antidepressiva do extrato de *Rosmarinus officinalis* é mediada por uma interação com os sistemas monoaminérgicos. Subsequentemente, foi investigado o efeito antidepressivo de frações de *Rosmarinus officinalis*: fração acetato de etila 1 e 2 (AcOEt1 e 2), hexânica (HEX), etanólica (ET), e fração isenta de óleo essencial (IOE), bem como o óleo essencial e dos compostos isolados carnosol e ácido betulínico no TSC. Todas as frações analisadas, óleo essencial e compostos isolados produziram efeito antidepressivo: frações AcOEt1, AcOEt2, HEX, ET e IOE, o óleo essencial, e os compostos isolados carnosol e ácido betulínico. Nenhum efeito psicoestimulante foi mostrado no teste do campo aberto, indicando que os efeitos no TSC são específicos. Estes resultados sugerem que o ácido betulínico e o carnosol podem ser responsáveis,

pelo menos em parte, pelo o efeito anti-imobilidade dos extratos de *Rosmarinus officinalis* e que o óleo essencial pode também contribuir para o efeito antidepressivo desta planta. Adicionalmente, o efeito do ácido ursólico no TSC e o envolvimento do sistema dopaminérgico neste efeito foram investigados. O ácido ursólico reduziu o tempo de imobilidade no TSC. O efeito do ácido ursólico no TSC foi prevenido pelo pré-tratamento de camundongos com SCH23390 (antagonista do receptores dopaminérgicos D_1) e sulpirida (antagonista de receptores dopaminérgicos D_2). A administração de doses sub-efetivas de ácido ursólico e de SKF38393 (agonista de receptores dopaminérgicos D_1), apomorfina (agonista de receptores dopaminérgicos D_2) ou bupropiona (inibidor da recaptação de dopamina) reduziu o tempo de imobilidade no TSC, em comparação com o efeito produzido por cada composto isoladamente. O ácido ursólico e os agentes dopaminérgicos, sozinhos ou em combinação, não causaram alterações significativas nas atividade locomotora e exploratória. Estes resultados sugerem que o efeito antidepressivo de ácido ursólico no TSC seja mediado por uma interação com o sistema dopaminérgico, através da ativação dos receptores dopaminérgicos D_1 e D_2. Em outra etapa experimental, as alterações induzidas pela bulbectomia olfatória (BO), modelo animal de depressão, e a influência do tratamento com fluoxetina (14 dias) sobre as alterações comportamentais induzidas por este modelo foram estudadas. O tratamento com fluoxetina reverteu a hiperatividade induzida pela BO no teste de campo aberto, a hiperatividade locomotora e o aumento do comportamento exploratório induzida pela novidade no teste de objeto novo e no teste da caixa nova e o comportamento anedônico no splash teste. A BO causou um aumento na atividade da acetilcolinesterase (AchE) no hipocampo, mas não no córtex cerebral, efeito que foi revertido pela fluoxetina. Corticosterona sérica foi aumentada em camundongos SHAM e bulbectomizados tratados com fluoxetina. Em conclusão, as alterações comportamentais e neuroquímicas induzidas pela BO foram revertidas pela fluoxetina, validando este modelo de depressão. Subsequentemente, foi investigada a habilidade do extrato de *Rosmarinus officinalis* em reverter alterações comportamentais e bioquímicas induzidas pela BO. A atividade locomotora e exploratória foi avaliada no teste de campo aberto, no teste do objeto novo e no teste da caixa nova, comportamento anedônico foi avaliado no splash teste, o déficit cognitivo foi avaliado no labirinto aquático de Morris. Para a etapa experimental 1, o extrato ou fluoxetina foram administrados por via oral (p.o.) uma vez ao dia durante 14 dias após a BO. Para a etapa experimental 2, foram determinados a glicemia e a atividade da AchE

hipocampal e cerebrocortical em camundongos SHAM e bulbectomizados tratados com o extrato ou fluoxetina. O extrato, de forma semelhante à fluoxetina, reverteu a hiperatividade, o aumento do comportamento exploratório e o comportamento anedônico. Os animais bulbectomizados necessitaram maior número de treinos na sessão de treino para adquirir a informação espacial, porém estes exibiram um perfil semelhante ao dos camundongos SHAM na sessão de teste, demonstrando um déficit seletivo na aprendizagem espacial. Assim, a BO causou um déficit na aprendizagem, mas não na memória no teste do labirinto aquático de Morris, e este resultado não foi revertido pelo extrato ou fluoxetina. A redução dos níveis de glicose sérica e o aumento da atividade da AchE no hipocampo foram observados em camundongos bulbectomizados, mas apenas o último efeito foi revertido pela fluoxetina, enquanto ambos os efeitos foram revertidos pelo extrato de *Rosmarinus officinalis*. Em conclusão, o extrato desta planta, exerce um efeito antidepressivo em camundongos bulbectomizados e reverte a disfunção colinérgica e a hipoglicemia induzida pela BO. Em conjunto, os resultados sugerem o potencial de *Rosmarinus officinalis* para o tratamento de depressão e contribuem para a validação do uso tradicional desta planta para o tratamento desta doença.

Palavras-chave: *Rosmarinus officinalis*. Bulbectomia olfatória. Teste de suspensão pela cauda. Antidepressivo. Sistema monoaminérgico. Sistema colinérgico.

ABSTRACT

MACHADO, Daniele Guilhermano. **Investigation of the potential antidepressant of *Rosmarinus officinalis***. 2012. Thesis (Doctorate in Neuroscience) – Federal of Santa Catarina University, Florianópolis, Brazil.

Rosemary, *Rosmarinus officinalis* L. (Labiatae) has several therapeutic applications in folk medicine in managing a wide range of diseases, including depression. Firstly, in this study, the effect of the hydroalcoholic extract of the stems and leaves of this plant was investigated in two behavioral models predictive of antidepressant activity, the forced swimming test (FST) and tail suspension test (TST) in mice. *Rosmarinus officinalis* hydroalcoholic extract (ROHE) produced an antidepressant-like effect, since the acute treatment of mice with the extract by p.o. route significantly reduced the immobility time in the FST and TST, as compared to the control group, without accompanying changes in ambulation in the open-field test. Moreover, the repeated administration (14 days) of ROHE by p.o. route also produced an antidepressant-like effect in the TST. The pretreatment of mice with p-chlorophenylalanine (PCPA, an inhibitor of serotonin synthesis, for 4 consecutive days), NAN-190 (a 5-HT_{1A} receptor antagonist), ketanserin (a 5-HT_{2A} receptor antagonist), 1-(m-chlorophenyl) biguanide (mCPBG, a 5-HT_3 receptor agonist), prazosin (an α_1- adrenoceptor antagonist), SCH23390 (a dopamine D_1 receptor antagonist) or sulpiride (a dopamine D_2 receptor antagonist), but not yohimbine (an α_2-adrenoceptor antagonist) reversed the anti-immobility effect ROHE in the TST. The combination of MDL72222, (a 5-HT_3 receptor antagonist) with a sub-effective dose of ROHE produced an anti-immobility effect in the TST. These results suggest that the antidepressant action of ROHE is mediated by an interaction with the monoaminergic system. Subsequently, the antidepressant-like effects of fractions from *Rosmarinus officinalis*: ethyl acetate 1 and 2 (AcOEt1 and 2), hexane (HEX), ethanolic (ET), and essential oil free (EOF) fractions, as well essential oil and isolated compounds carnosol and betulinic acid were investigated. All of them produced a significant antidepressant-like effect in the TST: AcOEt1, AcOEt2, HEX, ET and EOF fractions, essential oil and isolated compounds carnosol and betulinic acid. No psychostimulant effect was shown in the open-field test, indicating that the effects in the TST are specific. This study suggests that carnosol and betulinic acid could be responsible, at least in part, for the anti-

immobility effect of extracts from *Rosmarinus officinalis.* The essential oil of this plant can also contribute to this effect. Additionally, the antidepressant-like effect of ursolic acid was investigated, well as the involvement of dopaminergic system in its effect. Ursolic acid reduced the immobility time in the TST. The effect of ursolic acid in TST was prevented by the pretreatment of mice with SCH23390 (a dopamine D_1 receptor antagonist) and sulpiride (a dopamine D_2 receptor antagonist). The administration of a sub-effective dose of ursolic acid in combination with sub-effective doses of SKF38393 (a dopamine D_1 receptor agonist), apomorphine (a dopamine D_2 receptor agonist) or bupropion (a dopamine reuptake inhibitor) reduced the immobility time in the TST as compared with either drug alone. Ursolic acid and dopaminergic agents alone or in combination did not cause significant alterations in the locomotor and exploratory activities. These results indicate that the antidepressant-like effect of ursolic acid in the TST is likely mediated by an interaction with the dopaminergic system, through the activation of dopamine D_1 and D_2 receptors. In another experimental approach the effects of chronic treatment with fluoxetine (14 days) on the behavioral alterations induced by olfactory bulbectomy (OB) were investigated. Fluoxetine reversed OB-induced hyperactivity in the open-field, novel object and novel cage tests, and anhedonic behavior in the splash test. OB caused an increase in hippocampal, but not cerebrocortical AChE activity, an effect reversed by fluoxetine. Serum corticosterone was increased in SHAM and bulbectomized mice treated with fluoxetine. In conclusion, OB-induced behavioral and neurochemical alterations were reversed by fluoxetine, validating this model of depression. In addition, the ability of ROHE, as compared with fluoxetine to reverse behavioral and biochemical alterations induced by OB was investigated. Locomotor and exploratory behavior was assessed in the open-field test, in the novel object test and in the novel cage test, anhedonic behavior was assessed in the splash test, cognitive deficits were evaluated in the water maze. For the first set of experiments, ROHE or fluoxetine was administered once daily for 14 days 2 weeks after OB. For experiment 2, serum glucose and hippocampal and cerebrocortical AChE activity were determined in OB and SHAM-operated mice treated orally with ROHE or fluoxetine. The results show that ROHE, similarly to fluoxetine, reversed OB-induced hyperactivity, and increased exploratory and anhedonic behaviors. OB mice needed significantly more trials in the training session to acquire the spatial information, but they displayed a similar profile to that of SHAM mice in the test session, demonstrating a selective deficit in spatial learning, which was not reversed by extract or fluoxetine. A

reduced serum glucose levels and an increased hippocampal AChE activity were observed in bulbectomized mice, only the latter effect was reversed by fluoxetine, while both effects were reversed by ROHE. In conclusion, ROHE exerts an antidepressant-like effect in bulbectomized mice and is able to reverse OB-induced cholinergic dysfunction and hipoglicemia. Overall, results suggest the potential of *Rosmarinus officinalis* for the treatment of depression and contribute towards validation of the traditional use of this plant for the treatment of this disease.

Keywords: *Rosmarinus officinalis*. Olfactory bulbectomy. Tail suspension test. Antidepressant. Monoaminergic system. Cholinergic systems.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AcOEt1= Acetato de Etila 1
AcOEt2= Acetato de Etila 2
AHV=Ácido homovanílico
BO= Bulbectomia Olfatória
DMSO = Dimetilsulfóxido
DA= Dopamina
E.P.M = Erro Padrão da Média
ET= Etanólica
HEX= Hexância
5-HIAA=5-hidroxiindolacético
5-HT = 5-hidroxitriptamina ou serotonina
i.p.= Intraperitoneal
p.o.= Oral
IOE= Isenta de Óleo Essencial
ISRS = Inibidor Seletivo da Recaptação de Serotonina
IRD= Inibidor da Recaptação de Dopamina
MAO = Monoamina Oxidase
IMAO = Inibidor da Monoamina Oxidase
NMDA = N-metil-D-aspartato
NA= Noradrenalina
PCPA = p-clorofenilalanina metil éster
TNF = Teste do Nado Forçado
TSC = Teste da Suspensão pela Cauda
TCA= Teste do Campo Aberto
TON= Teste do Objeto Novo
TCN= Teste da Caixa Nova

SUMÁRIO

1. INTRODUÇÃO

1.1. Depressão e a relação com os antidepressivos

A depressão representa um dos distúrbios psiquiátricos mais prevalentes na população mundial, que acarreta altas taxas de afastamento do trabalho, perda considerável de produtividade e qualidade de vida, além de estar associada com um elevado índice de suicídio (Nemeroff e Owens, 2002; Páez-Pereda, 2005). Estima-se que a depressão será a segunda maior causa de incapacidade até 2020 (WHO, 2009; Pitchot et al., 2010). Neste contexto, no Brasil aproximadamente 54 milhões de pessoas terão algum tipo de depressão, em algum momento de suas vidas, sendo que 7,5 milhões terão episódios agudos e graves (Nardi, 2000).

A depressão caracteriza-se como um distúrbio heterogêneo que causa alterações psicológicas, comportamentais e fisiológicas e pode estar associada a outras condições ou comorbidades clínicas como ansiedade, doenças cardiovarculares, diabetes mellitus tipo 2, câncer e doenças neurodegenerativas (Nemeroff e Owens, 2002; Evans et al., 2005; Krishnan e Nestler, 2008). Notavelmente, a depressão é mais prevalente em mulheres do que em homens - na proporção de 5:2, inclusive no Brasil (Almeida-Filho, 1997; Wong e Licinio, 2001; Licinio et al., 2007).

Em um breve histórico a cerca da depressão, os primeiros relatos deste transtorno de humor foram cerca de 400 anos antes de Cristo, quando Hipócrates descreveu um paradigma menos específico e mais filosófico, a Teoria Humoral Hipocrática: a bílis negra, tradução literal do termo grego "melancolia" referindo-se ao episódio depressivo (Wong e Licinio, 2001; Nestler et al., 2002a). Dentre vários conceitos que surgiram para classificar a depressão, as teorias de Emil Kraepelin (1893) sobre transtornos psiquiátricos baseados em príncipios clínicos e anatômicos destacaram-se na psiquiatria por que auxiliaram no conceito da depressão como doença e contribuiram de forma relevante para vários critérios diagnósticos. O Manual Estatístico e Diagnóstico de Doenças Mentais, quarta edição (DSM-IV) e a classificação de transtornos mentais e do comportamento (CID-10) são os critérios diagnósticos mais utilizados atualmente. Além disso, Kraepelin diferenciou a depressão do episódico maníaco-depressivo, da melancolia, da depressão associada ao medo, agitação e/ou sintomas hipocondríacos, entre outros (Licinio et al., 2007).

Os critérios para o diagnóstico da depressão baseiam-se na observação clínica dos sintomas que incluem alterações somáticas e cognitivas, tais como:1. humor deprimido; 2. anedonia (perda de interesse ou satisfação em quase todas as atividades); 3. perda ou ganho de peso ou de apetite; 4. insônia ou hipersônia; 5. retardo ou agitação psicomotora; 6. fadiga ou perda de energia; 7. sentimentos de desvalia ou culpa; 8. diminuição da concentração e 9. pensamentos recorrentes de morte ou suicídio. Para o diagnóstico de um episódio depressivo é necessária a constatação de no mínimo cinco entre estes nove sintomas, sendo um deles obrigatoriamente humor deprimido ou anedonia, presentes na maior parte do tempo, com uma duração mínima de duas semanas (American Psychiatry Association, DSM-IV-2000).

O tratamento para depressão caracterizou-se inicialmente pela descoberta dos primeiros compostos antidepressivos e teve seu avanço significativo na década de 50, baseando-se em várias evidências científicas. Dentre estas, destacou-se a observação de que a reserpina utilizada para o tratamento de hipertensão arterial, causava depressão como um efeito colateral e que o tratamento com o precursor de catecolaminas L-DOPA, reverteria os sintomas depressivos induzido pelo tratamento deste fármaco em humanos e animais (Iversen, 2007; Licinio et al., 2007).

Posteriormente, a evidência da relação das monoaminas e a depressão, foi reforçada pela descoberta ocasional de que a iproniazida, usada para o tratamento da tuberculose, exercia o efeito de elevar o humor destes pacientes e que este fármaco possuia a propriedade de inibir a monoamina oxidase (MAO), enzima que degrada as monoaminas (serotonina, noradrenalina e dopamina) (Nestler et al., 2002a; Iversen, 2007).

Desta forma, os primeiros fármacos com efeitos comprovadamente benéficos sobre o humor agiam primariamente sobre o sistema monoaminérgico, como inibidores da MAO, ou inibidores da recaptação de monoaminas (Nemeroff e Owens, 2002; Nestler et al., 2002a). Todas estas evidências corroboraram para o surgimento da "Hipótese Monoaminérgica da Depressão", a qual postulava que a depressão resultaria da deficiência de neurotransmissores em sinapses monoaminérgicas ou ainda receptores ineficientes (Wong e Licínio, 2001; Castrén, 2005), conforme ilustrado na **Figura 1**.

Inicialmente, esta hipótese baseou-se na deficiência de noradrenalina (Schildkraut et al., 1965) e serotonina (Coppen, 1967), tendo sido estendida a deficiência de dopamina (Randrup et al., 1975). Esta constatação sugeriu que os antidepressivos atuassem por aumento

da transmissão serotoninérgica, noradrenérgica e dopaminérgica, compensando um possível estado de deficiência destes neurotransmissores.

Além disso, têm sido observado em pacientes com depressão maior, baixos níveis plasmáticos de serotonina ou 5-hidroxitriptamina (5-HT) (Coppen e Doogan, 1988) e de seu metabólito, o ácido 5-hidroxiindolacético (5-HIAA) no líquor (Ricci e Wellman, 1990).

O sistema dopaminérgico tem um papel importante na fisiopatologia da depressão e sobre os mecanismos de ação de antidepressivos, principalmente a bupropiona (D'Aquila et al., 2000; Papakostas, 2006; Dunlop e Nemeroff, 2007). Desta forma, várias evidências confirmam o envolvimento do sistema dopaminérgico neste transtorno de humor, tais como: I. baixos níveis de metabólitos de dopamina, como o ácido homovanílico (AHV) no líquor de pacientes depressivos e em várias regiões do cérebro que medeiam humor e motivação (Brown e Gershon, 1993; Papakostas, 2006). II. a deficiência mesolímbica de dopamina é um dos principais candidatos a etiologia de alguns sintomas da depressão, como anedonia e perda de motivação (Dunlop e Nemeroff, 2007); III. as implicações da ativação dopaminérgica nas respostas de alguns antidepressivos (Waehrens e Gerlach, 1981; Papakostas, 2006); IV) a investigação de novos agentes antidepressivos que atuam sobre o sistema dopaminérgico é de suma importância, visto que tem sido mostrado resultados promissores para o tratamento de pacientes com depressão-resistente (depressão associada a baixa remissão dos sintomas e alta recorrência dos episódios depressivos) (Dunlop e Nemeroff, 2007).

Anedonia, ou hipossensibilidade ao prazer, constitui-se em um dos principais sintomas para o diagnóstico da depressão (Dunlop e Nemerof, 2007). Em estudos pré-clínicos, este comportamento pode ser inferido através de uma redução na ingestão de sacarose ou diminuição do tempo gasto no comportamento de auto-limpeza, evocado pela pulverização de uma solução de sacarose no dorso dos animais no Splash Test (Jancsar e Leonard, 1981; Song e Leonard, 2005; Yalcin et al., 2005).

O comportamento-anedônico pode ser observado em animais submetidos previamente a um modelo animal de depressão, ou seja, modelo que induz o comportamento tipo-depressivo. Entre os vários modelos destacam-se: estresse crônico moderado (Luo et al., 2008; Yalcin et al., 2008; Detanico et al., 2009), estresse crônico (Strekalova et al., 2004) induzido pela administração de substâncias, como a costicorterona (David et al., 2009) e citocinas pró-inflamatórias como

TNF-α (Kaster et al., 2012), ou ainda pela bulbectomia olfatória (Calcagnetti et al., 1996; Stock et al., 2000; Romeas et al., 2009).

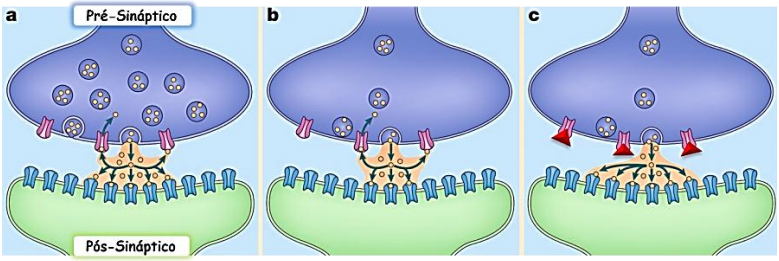

Figura 1 - Hipótese monoaminérgica da depressão:
a) No cérebro normal, neurotransmissores monoaminérgicos (amarelo) são liberados e se ligam a receptores no neurônio pós-sináptico. A neurotransmissão é controlada ou finalizada por recaptação do transmissor;
b) Na depressão, a diminuição da concentração de monoaminas em locais sinápticos produz o transtorno de humor;
c) O bloqueio da recaptação (vermelho) aumenta a concentração dos neurotransmissores monoaminérgicos, aumentando a disponibilidade destes na fenda sináptica e consequentemente, em receptores específicos, restaurando o humor (Adaptado de Castrén, 2005).

Nota-se ainda que, a grande maioria dos fármacos utilizados atualmente para o tratamento da depressão (inibidores da monoamina oxidase, antidepressivos tricíclicos, inibidores seletivos da recaptação da serotonina, inibidores seletivos da recaptação de noradrenalina) aumenta os níveis sinápticos de monoaminas (Risch e Nemeroff, 1992, Taylor et al., 2005).

No entanto a hipótese monoaminégica possui algumas limitações, visto que falha ao não explicar a ação de alguns antidepressivos que não agem sobre o sistema monoaminérgico e ao fato de que, nem toda droga que aumenta as monoaminas na fenda sináptica age como antidepressivo (como por exemplo, a anfetamina e cocaína), além da discrepância temporal que existe entre os eventos bioquímicos rápidos envolvidos no metabolismo de monoaminas e os efeitos clínicos dos antidepressivos (Stahl, 2000, 2002; Torres et al., 2003).

No entanto, apesar de muitos estudos neste sentido, a etiologia da depressão permanece ainda incerta. Evidentemente, novas evidências ampliaram os conhecimentos a cerca da teoria monoaminérgica, visto que mostraram que a fisiopatologia da depressão e o mecanismo de ação dos antidepressivos não se restringiria apenas aos "níveis" de monoaminas, mas também no que estas poderiam desencadear nas membranas pós-sinápticas e em segundos mensageiros e suas cascatas intra-celulares (Duman et al., 2000; Manji et al., 2001; D'Sa e Duman, 2002; Castrén, 2005; Belmaker e Agam, 2008). Assim, atualmente além da hipótese monoaminérgica da depressão e de todas as suas novas abordagens: cascatas de sinalização intracelular, modulação da expressão dos genes, participação de fatores neurotróficos, tais como o BDNF, os quais visam complementar e melhor compreender a fisiopatotologia da depressão. Dentre estas, destacam-se a hipótese neurotrófica da depressão, e aquelas baseadas no enfoque sobre a participação do sistema endócrino e imune (Dantzer e Kelley, 1989; Nestler et al., 2002a; Castrén et al., 2007; Belmaker e Agam, 2008).

Recentemente, vários estudos têm demonstrado que alterações na neuroplasticidade em áreas cerebrais associadas a depressão, aonde destaca-se o hipocampo podem influenciar na predisposição e no tratamento deste transtorno de humor, o que corroborou para o surgimento da "Hipótese neurotrófica da depressão" (Duman et al., 2000; Nestler et al., 2002a; Duman e Monteggia, 2006). A neuroplasticidade engloba diferentes processos como a formação dendrítica, remodelação sináptica, desenvolvimento axonal, extensão neurítica, sinaptogênese e neurogênese, pelos quais o cérebro se adapta e responde a uma variedade de estímulos internos e externos. Estes eventos fornecem o suporte trófico ao SNC permitindo seu desenvolvimento, manutenção e/ou proteção. Desta forma, a falta de fatores tróficos promove disfunções na neuroplasticidade, colaborando com o aparecimento dos sintomas depressivos, enquanto que o reestabelecimento do suporte trófico, após o tratamento crônico com os antidepressivos, tem sido associado ao desaparecimento destes sintomas (**Figura 2**) (Manji et al., 2001; Nestler et al., 2002a; D' Sa e Duman, 2002; Páez-Pereda, 2005; Duman e Monteggia, 2006).

Neste enfoque, a teoria neurotrófica da depressão também pode estar vinculada a ação dos antidepressivos e pode explicar o atraso do efeito clínico dos antidepressivos, pelo tempo necessário para que ocorra a neurogênese (Duman et al., 2000, 2001; Manji et al., 2001; D' Sa e Duman, 2002; Kempermann e Kronenberg, 2003).

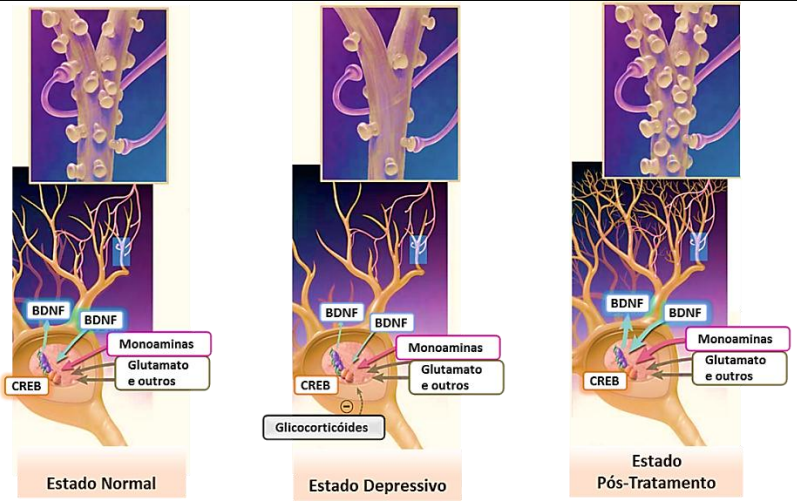

Figura 2 - Hipótese Neurotrófica da depressão:
a) O painel à esquerda mostra um neurônio piramidal do hipocampo em estado normal e sua inervação por neurônios monoaminérgicos e glutamatérgicos. Estes neurônios sofrem regulação pelo BDNF;
b) O estresse severo ou crônico desencadeia várias mudanças nestes neurônios, incluindo uma redução em suas arborizações dendríticas e uma redução na expressão de BDNF (entre outros fatores neurotróficos), que poderia ser um dos fatores mediando estas alterações dentríticas e desta forma diminuindo os contatos sinápticos, bem como o número de sinapses e alterando desta forma a funcionalidade deste evento neuroquímico. A redução no BDNF é mediada em parte por excesso de glicocorticóides, o que poderia interferir com o mecanismo normal de transcrição (por exemplo, CREB) que controla a expressão de BDNF;
c) Antidepressivos revertem os efeitos desencadeados pelo estresse e/ou depressão: aumentam arborizações dendríticas e expressão BDNF desses neurônios do hipocampo. Desta forma, por essas ações, os antidepressivos podem além de reverter, prevenir as ações do estresse sobre o hipocampo e melhorar alguns sintomas da depressão (Adaptado de Nestler et al., 2002a).

Com relação ao envolvimento do sistema neuroendócrino e a fisiopatologia da depressão destaca-se como um achado clínico relevante a disfunção do eixo HPA (hipotálamo-pituitária-adrenal), a qual está associada ao incremento de cortisol sérico em pacientes deprimidos, (Nestler et al., 2002a; Belmaker e Agam, 2008). **Figura 3**.

Corroborando com esta evidência, sabe-se que o surgimento dos episódios depressivos pode estar vinculado a eventos estressantes no decorrer da vida, seja um evento agudo de alta intensidade, ou ainda um estresse crônico, caracterizado pelo excesso de eventos negativos por pelo menos seis meses, sendo que ambos podem gerar a depressão (Blackburn-Munro e Blackburn-Munro, 2001). O estudo de Mello et al. (2003) mostrou que frequentemente o estresse psicológico precede os episódios de perturbação do humor; está correlacionado com a gravidade do episódio, com o risco de recidiva e com a menor resposta aos antidepressivos convencionais.

A hipótese citocinérgica da depressão fundamenta-se nas evidências de que o aumento na produção de citocinas pró-inflamatórias (como TNF-α; IL-1b; IL-6) e um desbalanço na resposta imune, incluindo a diminuição de citocinas anti-inflamatórias (IL-10), entre outras citocinas, poderiam desempenhar um papel relevante na etiologia da depressão (Dantzer et al., 1999, 2002; Leonard et al., 2001, 2007; Schiepers et al., 2005). Sendo assim, essa teoria baseia-se na idéia de que o aumento na produção de citocinas pró-inflamatórias observado na depressão resultaria nos sintomas a ela relacionados, como o aparecimento de um tipo especial e característico de "comportamento doentio". Nesse sentido, as citocinas pró-inflamatórias, atuariam como neuromoduladores, mediando os aspectos neuroquímicos, neuroendócrinos e comportamentais dos transtornos depressivos (Leonard et al., 2001, 2007; Schiepers et al., 2005).

Estudos morfológicos e de neuroimagem funcional de pacientes deprimidos mostram que a depressão está associada a alterações morfológicas de regiões específicas, tais como, córtex pré-frontal, amígdala, córtex cingular anterior e diminuição do volume do hipocampo (Phillips et al., 2003; Pryce et al., 2005). Vale ressaltar, que outros estudos de neuroimagem (RMN e PET), mostraram que quanto maior é a duração da depressão, maior é a perda de volume do hipocampo e maior é a alteração na memória verbal (Sheline et al., 1996, 1999). Esta perda hipocampal está correlacionada com a duração da depressão mas, não com a idade dos doentes, o que pode sugerir que episódios de estresse sucessivos possam provocar perdas de volume

cumulativas nesta estrutura (Rajkowska, 2000; Stockmeier e Rajkowska, 2004).

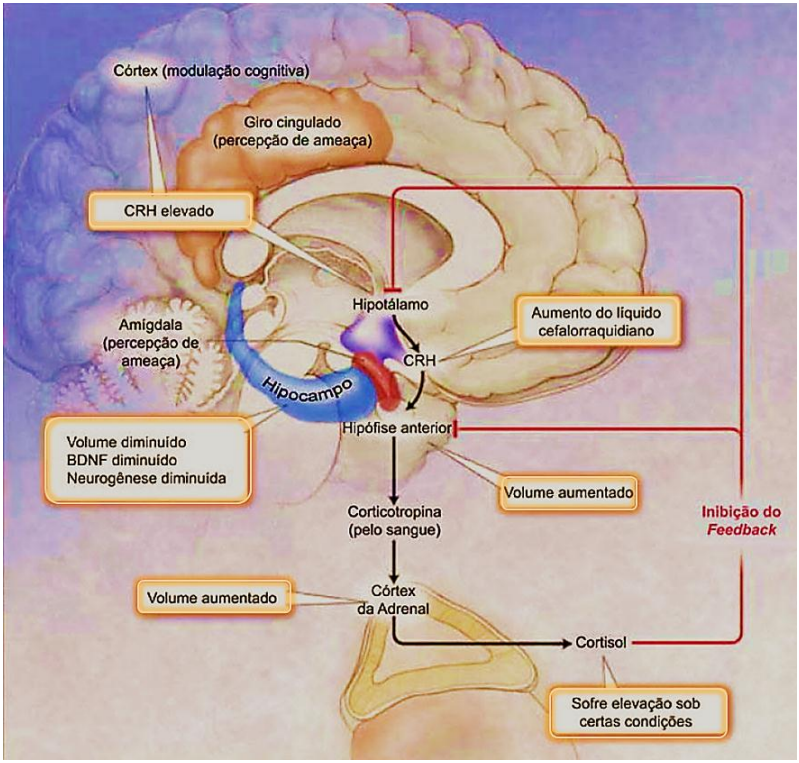

Figura 3. Hipótese de desregulação do eixo HPA na etiologia da depressão:
Esta hipótese sugere que anormalidades na resposta do cortisol ao estresse poderiam explicar a depressão. As setas pretas indicam que, em resposta ao estresse, o qual é percebido pelo córtex e amígdala, e transmitido ao hipocampo, o hormônio liberador de corticotropina (CRH) é liberado, induzindo a glândula pituitária anterior a secretar corticotropina na corrente sanguínea. As setas vermelhas mostram a inibição no sistema de retro-alimentação no hipotálamo e na pituitária pelo cortisol, suprimindo a produção de CRH e corticotropina, respectivamente (Adaptado de Belmaker e Agam, 2008).

Além disso, a depressão está associada a déficits cognitivos, visto que pacientes deprimidos apresentam déficit de aprendizagem e memória (Kuzis et al., 1997; Dolan, 2002).

As principais alterações patofisiológicas e morfológicas nas regiões cerebrais vinculadas ao transtorno depressivo são: a redução de tamanho destas regiões, vulnerabilidade seletiva à morte celular de subpopulações de neurônios, alterações neuroquímicas envolvendo receptores, na sinalização intracelular e na regulação da expressão gênica (Pryce et al., 2005; Tsankova et al., 2007). Apesar do mecanismo celular preciso das alterações estruturais em transtornos depressivos não estarem completamente compreendidas, progressos tem sido feito na caracterização de cascatas de transdução de sinais que promovem a atrofia neuronal e morte celular programada (Duman et al., 2000; Manji et al., 2001; Duman e Monteggia, 2006). Além disso, o tratamento crônico com antidepressivos pode influenciar a quantidade e/ou a viabilidade de células hipocampais e gerar adaptações na sinalização de proteínas intracelulares e de genes, contribuindo desta forma, para o mecanismo de ação do tratamento com antidepressivos (Duman et al., 2000; Wong e Licinio, 2001; Duman e Monteggia, 2006).

Os fármacos usados para o tratamento da depressão causam vários efeitos colaterais e são efetivos somente em cerca de 50-70% dos pacientes (Wong e Licinio, 2001; Nestler et al., 2002a; Brunello et al., 2002; Taylor e Stein, 2005). Além disso, muitos pacientes possuem uma remissão parcial dos sintomas depressivos e alguns permanecem refratários (Wong e Licinio, 2001; Nestler et al., 2002a; Páez-Pereda, 2005). Desta forma, considerando as limitações da terapêutica antidepressiva convencional, torna-se relevante a identificação de novas alternativas terapêuticas para o tratamento deste distúrbio psiquiátrico, no intuito de melhorar clinicamente as possibilidades de remissão dos sintomas de maneira mais expressiva nestes pacientes, gerando uma melhor qualidade de vida.

1.2. A utilização de plantas medicinais

A medicina tradicional representa a soma de diversos conhecimentos, sabedoria e práticas das mais diversas culturas, usada desde os tempos mais remotos da humanidade, seja na sua manutenção da saúde, prevenção e/ou tratamento de diversos males. De acordo com a Organização Mundial da Saúde, cerca de 80% da população mundial nos países em desenvolvimento, dependem essencialmente das plantas para seus cuidados primários de saúde (WHO, 2002). Além disso, o

conhecimento de plantas medicinais representa muitas vezes, a única opção terapêutica para muitas comunidades e grupos étnicos em países pobres (Revene et al., 2008).

Neste contexto, o conhecimento popular advindo dos benefícios das plantas medicinais foi construído empiricamente através de várias gerações em diversas civilizações, constituindo um crescente patrimônio etnofarmacológico pela experimentação sistemática e constante (Elizabetsky, 1997; Zhang, 2004). O estudo etnofarmacológico consiste em associar o conhecimento popular a cerca da utilização das plantas medicinais, com os estudos químicos e farmacológicos, permitindo a investigação científica quanto às atividades farmacológicas e os princípios ativos responsáveis pelas ações terapêuticas relatadas (ANVISA, RDC-48/04). Nesse contexto, cerca de 25% das drogas convencionais utilizadas atualmente são originadas diretamente ou indiretamente de plantas, sendo que estas descobertas surgiram através de estudos em comunidades indígenas (De Smet, 1997; Zhang et al., 2004).

É importante notar que o Brasil é o país com maior número de espécies de plantas do mundo. Dos diversos ecossistemas brasileiros (pantanal, floresta amazônica, caatinga, cerrado, mata atlântica, mata de restinga, manguezal e pampa) estima-se que o país possua aproximadamente 25% dentre as 350 mil espécies presentes no mundo, fornecendo um potencial imenso para a pesquisa e desenvolvimento de novos medicamentos a partir das plantas medicinais (Yunes e Calixto, 2001; Pinto, 2002).

As plantas medicinais são designadas como uma espécie vegetal que possui substâncias biologicamente ativas com propriedades terapêuticas, profiláticas ou paliativas. Desta forma, constituem-se basicamente naqueles vegetais que oferecem efeito terapêutico para uma ou mais patologias, através de alguma parte da planta denominada tecnicamente de droga vegetal. Esta parte contém uma ou várias substâncias, chamadas de princípios ativos, que é o componente responsável por determinado efeito biológico e irá proporcionar o efeito curativo. A droga vegetal pode ser qualquer parte da planta, seja a casca, folha, flor, fruto, raiz ou semente (Di Stasi, 1995).

O termo fitoterápico foi escolhido para designar a droga vegetal, quando esta assume uma forma farmacêutica, pronta para o uso do consumidor, que também poderá ser denominado de fitomedicamento (Di Stasi, 1995; Vieira, 2008). Os fitomedicamentos são produtos terapêuticos feitos a partir de plantas medicinais, extratos integrais ou concentrados de princípios ativos vegetais. Estão disponíveis em

forma sólida e líquida, como tinturas, xaropes, óleos, extratos alcoólicos e sucos de plantas. A fitoterapia consiste no estudo, pesquisa e aplicação terapêutica de produtos que contenham plantas medicinais. Apesar de sua caracterização como uma "terapia alternativa", a fitoterapia é uma modalidade de tratamento cientificamente testada e aprovada que deu origem à farmacoterapia moderna (Schulz et al., 2001; Vieira, 2008).

1.2.1. Plantas medicinais como alternativa terapêutica para o tratamento da depressão

A terapia com plantas medicinais pode ser uma alternativa efetiva para o tratamento de vários transtornos psiquiátricos, incluindo a depressão, como no caso do *Hypericum perforatum* (erva de São João) que apresenta potencial antidepressivo, sem a severidade dos efeitos colaterais apresentados pelos antidepressivos convencionais (Akhondzadeh e Maleki, 2006; Linde e Knüppel, 2005; McGarry et al., 2006). Linde et al. (1996) demonstraram que os extratos de *Hypericum* são mais eficientes do que placebo para o tratamento de transtornos depressivos leves e moderados. A hipericina e a hiperforina são os principais principios ativos associados ao potencial antidepressivo desta planta, visto que modulam o sistema monoaminérgico, inibindo a recaptação de serotonina, noradrenalina e dopamina, e consequentemente, aumentando as suas concentrações na fenda sináptica (Chatterjee et al.,1998; Butterweck et al., 2003; Wurglics e Schubert-Zsilavecz, 2006). O estudo de Noldner e Schotz (2002) mostra que o flavonóide rutina também é fundamental para o potencial antidepressivo de extratos de *Hypericum perforatum* no TNF.

O estudo etnofarmacológico tem progredido significativamente na última década, principalmente no que se refere à pesquisa de novos agentes terapêuticos dotados de potencial antidepressivo, provenientes de plantas medicinais e seus compostos isolados (Zhang, 2004). Neste sentido, dados da literatura mostram uma diversidade de plantas e/ou principios ativos isolados de plantas com propriedades antidepressivas cientificamente comprovadas em ensaios pré-clinicos, tais como: *Aloysia gratissima* (Zeni et al., 2012); *Curcuma longa* (Yu et al., 2002), *Ginko biloba* (Sakakibara et al., 2006; Rojas et al., 2011), *Morinda officinalis* (Li et al., 2001), *Polygala paniculata* (Bettio et al., 2011), *Salvia elegans* (Herrera-Ruiz et al., 2006), *Schinus molle* (Machado et al., 2007), *Siphocampylus verticillatus* (Rodrigues et al., 2002), *Tabebuia avellanedae* (Freitas et al., 2010), *Valeriana glechomifolia* (Müller et al., 2012), *Origanum vulgarea* (Mechan et al.,

2011); e os compostos isolados, tais como: ácido ferulíco (Zeni et al., 2012), berberina (Peng et al., 2007), escopoletina (Capra et al., 2010), rutina (Machado et al., 2008), curcumina (Huang et al., 2011; Kulkarni et al., 2008), trans-resveratrol (Xu et al., 2010).

No presente trabalho será investigado o potencial antidepressivo de *Rosmarinus officinalis*.

1.2.2. *Rosmarinus officinalis* L. (alecrim)

As plantas medicinais são amplamente utilizados como remédios caseiros no Brasil, sendo que várias espécies são nativas de outros continentes e foram introduzidas aqui desde a colonização, aproximadamente em 1500 (Brandão et al., 2008). Dentre uma variedade de plantas usadas pela medicina popular em todo o mundo para cuidados com a saúde, destaca-se *Rosmarinus officinalis*, também conhecido popularmente no Brasil como"alecrim", sendo comumente utilizado na culinária como tempero e conservante natural para certos alimentos (Shahidi, 2000).

Rosmarinus officinalis é uma planta pertencente à família Lamiaceae (**Figura 4**) originária nos países do Mediterrânio e amplamente cultivado em várias regiões do mundo, incluindo o Brasil (Al-Sereiti et al., 1999; Heinrich et al., 2006).

Vários relatos na literatura mostram os usos etnofarmacológicos de *Rosmarinus officinalis* para o tratamento de doenças inflamatórias, de fadiga física e mental, melhora da memória e tratamento de agitação nervosa, histeria e depressão, entre outras aplicações (Duke, 2000; Negraes, 2003; Heinrich et al., 2006; Franco e Fontana, 2007).

Vale ressaltar que, o uso etnofarmacológico de *Rosmarinus officinalis* como antidepressivo, até o presente estudo, não havia sido estudado cientificamente.

Estudos pré-clinicos reportam que o extrato desta planta exerce várias atividades farmacológicas tais como: hepatoprotetora (Sotelo-Félix et al., 2002), antibacteriana (Del Campo et al., 2000), antitrombose (Yamamoto et al., 2005), antiulcerogênico (Dias et al., 2000), antimutagênico (Minnunni et al., 1992), diurético (Haloui et al., 2000), anti-diabética (Bakirel et al., 2008), antioxidante (Bakirel et al.,2008; Ozcan, 2003), antinociceptiva (González-Trujano et al., 2007) e antiinflamatória (Altinier et al., 2007; Benincá et al., 2011).

Figura 4: *Rosmarinus officinalis* L. (Alecrim): Talos e folhas (à esquerda) e flores (à direita).

Estudos fitoquímicos identificaram vários componentes ativos em *Rosmarinus officinalis*, tais como: flavonóides (diosmetina, quercetina, apigenina, luteonina), composto fenólicos (ácido rosmarínico e cafeico) e predominantemente terpenos (carnosol, ácido oleoanólico, ácido betulínico, ácido ursólico) (Frankel et al., 1996; Barnes et al., 2001; Altinier et al., 2007; González-Trujano et al., 2007; Benincá et al., 2010). Dentre estes, há relatos de que o ácido rosmarínico e ácido cafeico apresentaram efeito antidepressivo no teste do nado forçado (Takeda et al., 2002).

Estudos in vitro com o carnosol, revelaram que este composto possui um efeito protetor na neurotoxicidade induzida por rotenona em culturas de células dopaminérgicas (Kim et al., 2006). Dados na literatura mostraram que o ácido betulínico, outro composto encontrado em *Rosmarinus officinalis*, possui propriedades antitumorais, antiinflamatórias e antinociceptiva (Yogeeswari e Sriram, 2005; Mullauer et al., 2010).

Recentemente, tem sido enfatizada a propriedade anti-cancer do extrato desta planta, bem como de seus compostos isolados, tais como

carnosol, ácido carnósico, ácido rosmarinico e ácido ursólico (Ngo et al., 2011). Destaca-se que um estudo recente demonstrou o potencial neuroprotetor do extrato de *Rosmarinus officinalis* em cultura de células dopaminérgicas (SH-SY5Y) (Park et al., 2010).

O ácido ursólico é dos compostos fitoquímicos que se destacam por seus diversos efeitos biológicos, tais como: antioxidante (Tsai e Yin, 2008), antiinflamatório (Baricevic et al., 2001, Benincá et al., 2010), antitumoral (Yan-xia et al., 2010), antidiabético (Jang et al., 2009) e neuroprotetor (Lu et al., 2007). Além disso, foi relatado que o ácido ursólico protege células PC12 contra o dano induzido pela neurotoxina dopaminérgica 1-metil-4-fenilpiridinio (MPP+) (Tsai e Yin, 2008).

Tendo em vista o uso etnofarmacológico de *Rosmarinus officinalis* para depressão, bem a necessidade de se investigar outros compostos com potencial terapêutico, esta planta bem como seus compostos isolados (carnosol, ácido betulínico e ácido ursólico) foram investigadas quanto ao seu potencial antidepressivo. Para este fim foram utilizados os testes preditivos de atividade antidepressiva (TNF e/ou TSC) e o modelo animal de depressão induzida pela bulbectomia olfatória bilateral em camundongos.

1.3. Modelos animais para o estudo da depressão

Os modelos animais são amplamente utilizados para a compreensão dos mecanismos responsáveis pela etiologia e tratamento de diversas doenças, incluindo a depressão. Estes modelos são sensíveis aos fármacos utilizados clinicamente ou mimetizam um ou alguns dos sintomas associados à doença. Desta maneira, é possível se estabelecer uma análise comparativa entre os efeitos comportamentais induzidos pelos fármacos com os sinais clínicos ou neurofisiológicos encontrados em humanos, visando contribuir para a elucidação das bases etiológicas das várias doenças mentais.

Modelos animais com validade preditiva são designados com esta terminologia, por serem baseados exclusivamente no efeito comportamental dos fármacos utilizados clinicamente, contudo não mimetizam sintomas ou bases neurobiológicas da doença. Os testes preditivos de atividade antidepressiva são amplamente utilizados para seleção de novos compostos antidepressivos e possibilitam uma melhor compreensão dos mecanismos ou vias responsáveis pelo seu efeito biológico, ou seja, pelo mecanismo de ação dos mesmos (Cryan et al., 2002, 2005; Nestler et al., 2002a,b). Entre eles, podemos destacar o teste

do nado forçado (TNF) e o teste de suspensão pela cauda (TSC) (Nestler et al., 2002a,b). Estes testes comportamentais caracterizam-se pela exposição do animal à uma situação de estresse inescapável, acarretando um estado de "desespero comportamental", observado pela tentativa inicial de escape ou luta para sair desta situação (postura de reatividade ou movimentação inicial). Porém, após os primeiros minutos, o animal desenvolve um comportamento de "desistência ou fracasso" na tentativa de escapar do estímulo estressante, permanecendo quase imóvel diante desta situação inescapável (Willner, 1984; Steru et al., 1985; Porsolt et al., 1977). Sendo assim, o comportamento de imobilidade exibido em roedores quando submetidos à uma situação de estresse inescapável, reflete um estado de desespero comportamental, que é comparado e relacionado ao estado depressivo em humanos.

Os antidepressivos clássicos têm a habilidade de reverter esta imobilidade comportamental, ou seja, de aumentar a reatividade do animal à esta situação, observado pela diminuição do tempo de imobilidade, representando desta forma o potencial antidepressivo de determinado composto (Cryan et al., 2002, Nestler et al., 2002a,b). Há uma correlação signicativa entre a potência clínica e eficácia dos antidepressivos no TNF e no TSC (Porsolt et al., 1977; Steru et al., 1985; Cryan et al., 2002).

Contudo, apesar da alta reprodutilibidade, facilidade na execução e serem testes altamente difundidos e utilizados, os testes preditivos apresentam algumas limitações ou desvantagens, tais como: a) testes preditivos são sensíveis ao tratamento agudo com os antidepressivos, fato este que difere do que é observado no tratamento com os antidepressivos em seres humanos, para o qual é necessário 2-4 semanas para que os efeitos terapêuticos sejam observados (Cryan et al., 2002a); b) estes testes podem apresentar resultados "falso positivos" ou "falso negativos". Compostos que aumentam a atividade locomotora (efeito psicoestimulante) podem diminuir o tempo de imobilidade, caracterizando-se como um resultado "falso positivo", enquanto que compostos ou drogas que diminuem a locomoção afetam o desempenho nestes testes, acarretando resultados "falso negativos" (Borsini e Meli, 1988).

Desta forma, são necessários experimentos complementares que visem avaliar a atividade locomotora, possibilitando comprovar que o potencial antidepressivo de determinado composto seja específico e não devido a um incremento na locomoção, ou seja, um efeito estimulante. Neste intuito, o teste do campo aberto é utilizado para avaliação da atividade locomotora e exploratória dos animais e permite uma

avaliação da atividade estimulante ou depressora de um determinado composto em estudo (Hall,1936; Siegel, 1946).

O potencial antidepressivo de extratos vegetais e de seus compostos isolados tem sido amplamente detectado nestes testes comportamentais (Rodrigues et al., 2002; Zhang et al., 2004; Machado et al., 2007, 2008; Capra et al., 2010; Freitas et al., 2010; Bettio et al., 2011; Zeni et al., 2011, 2012).

Vale ressaltar, que além dos testes preditivos para detectar o potencial antidepressivo de uma variedade de compostos, há outros modelos animais de depressão que apresentam validade fenomenológica e/ou de constructo, além da validade preditiva. Estes modelos, além de serem sensíveis aos fármacos utilizados clinicamente, mimetizam em animais sintomas ou efeitos neurobiológicos associados à doença.

Os critérios mínimos estabelecidos para satisfazer os critérios de um adequado modelo animal de depressão são: I) ter analogia com a depressão humana em sua manifestação ou sintomatologia; II) existir mudança comportamental no animal que possa ser observada e monitorada objetivamente; III) as alterações comportamentais observadas no animal, devem ser revertidas pelo tratamento com antidepressivo clássico, comumente utilizado na terapia antidepressiva em seres humanos; IV) os ensaios pré-clínicos devem ter reprodutibilidade entre os investigadores (McKinney e Bunney, 1969; Cryan et al., 2002). Neste contexto, destacam-se como modelo animal de depressão, a bulbectomia olfatória, estresse crônico moderado, estresse de separação maternal e isolamento social, e aquele induzido por citocina TNF-α, entre outros (Kelly e Leonard, 1997; Nestler et al., 2002a,b; Cryan et al., 2005; Willner, 2005; McArthur e Borsini, 2006; Kaster et al., 2012).

1.3.1. Bulbectomia olfatória bilateral (BO)

A bulbectomia olfatória (BO) consiste em um modelo animal de depressão, caracterizada pela destruição bilateral do bulbo olfatório, resultando em alterações comportamentais, neuroquímicas, neuroendócrinas, neuroimunológicas e morfológicas, características compatíveis e comparáveis ao que é observado em pacientes com depressão (Kelly et al., 1997; Pause et al., 2001; Cryan e Mombereau, 2004; Song e Leonard, 2005). Interessantemente, estudos clínicos mostram que pacientes depressivos apresentam uma redução da

sensibilidade olfatória associada a uma redução do volume do bulbo olfatório, mostrando desta forma a relação existente entre o sistema olfatório e a depressão (Pollatos et al., 2007; Atanasova et al., 2008; Negoias et al., 2010). Além disso, foi observada uma correlação siginificativamente negativa entre o volume de bulbo olfatório e a depressão, ou seja, quanto maior o estado depressivo, menor o volume do bulbo olfatório. Além disso, estas evidências podem ser relacionadas à redução da neurogênese na depressão, oque pode ser indicativo como a causa para a redução do bulbo olfatório (Negoias et al., 2010).

O modelo da BO acarreta modificações em várias regiões do cérebro, como conseqüência da interrupção das conexões neurais existentes entre os bulbos olfatórios e outras regiões do cérebro, principalmente o circuito olfatório-límbico (Jesberger e Richardson, 1988; Kelly et al., 1997; Song e Leonard, 2005), apesar do fato dos bulbos olfatórios representarem apenas 4% da massa cerebral total em ratos (Cain et al., 1974). A **Figura 5** ilustra a bulbectomia olfatória e as principais regiões cerebrais afetadas.

Desta forma, a avulsão dos bulbos olfatórios bilateralmente induz um processo de reorganização nas áreas límbicas e corticais que parece ser responsável pelas anormalidades comportamentais em roedores, as quais aparecem depois de duas semanas da cirurgia (Van Riezen e Leonard, 1990; Zueger et al., 2005; Jarosik et al., 2007).

Além disso, as alterações induzidas pela BO são quantificáveis, replicáveis e revertidas pela administração crônica de antidepressivos ativos terapeuticamente, incluindo os ISRS (Inibidores Seletivos da Recaptação de Serotonina) (Kelly et al., 1997). Sendo assim, a BO em roedores tem sido proposta como um modelo animal de depressão, sendo caracterizado por um modelo eficaz não somente para detectar compostos com potencial antidepressivo, mas também para estudar as inter-relações entre as áreas cerebrais que apresentam alterações funcionais em pacientes com depressão (Leonard, 1984; Song e Leonard, 2005).

A hiperatividade de animais bulbectomizados no teste do campo aberto, teste utilizado para verificar a atividade locomotora, é revertida quase que exclusivamente pelo tratamento crônico, mas não agudo, com antidepressivos, mimetizando o lento início de ação antidepressiva relatado em estudos clínicos (Leonard e Tuite, 1981; Van Riezen e Leonard , 1990; Kelly et al., 1997; Mar et al., 2000).

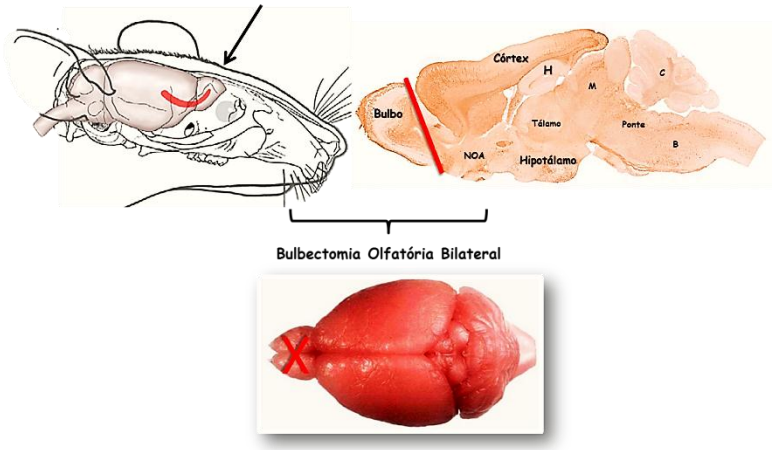

Figura 5: Bulbectomia olfatória e as principais regiões afetadas por este procedimento (destacadas em negrito). Regiões neuroanatômicas: NOA: núcleo olfatório anterior; H, hipocampo; M: mesencéfalo; C: cerebelo; B: bulbo do tronco cerebral.

Além desta hiperatividade, os ratos bulbectomizados apresentam anedonia, déficits de aprendizagem e memória, alterações no comportamento exploratório e de ingesta alimentar, os quais são revertidos por antidepressivos (Kelly et al., 1997; Harkin et al., 2003; Zueger et al., 2005).

Entretanto, existem resultados escassos na literatura com relação ao comportamento anedônico associado com hiperatividade em animais bulbectomizados (Romeas et al., 2009), visto que a maioria dos estudos avalia apenas hiperatividade nestes roedores (Leonard, 1984; Kelly et al., 1997; Harkin, et al., 2003; Song e Leonard, 2005; Zueger et al., 2005).

Estudos pré-clínicos revelaram a similaridade entre o comportamento no modelo da BO e a sintomatologia da depressão agitada, principalmente com base em algumas características como agitação psicomotora (Kelly et al., 1997; Harkin et al., 2003; Romeas et al., 2009) .

Neste contexto, a BO tem sido considerado um modelo de boa validade de face com transtorno depressivo humano, especialmente no que refere-se à depressão agitada (Lumia et al., 1992; Kelly et al., 1997; Harkin et al., 2003; Romeas et al., 2009).

Outra importante alteração comportamental induzida pela BO é a maior vulnerabilidade e responsividade ao estresse, principalmente aquele induzido pela novidade. Este déficit comportamental pode resultar de uma reatividade aumentada e inadequada em resposta a uma nova situação e/ou ambiente (Leonard e Tuite, 1981; Van Riezen e Leonard, 1990; Song e Leonard, 2005).

Além disso, a BO em roedores também tem sido associada a alterações bioquímicas, como a hipofunção serotoninérgica e noradrenérgica, caracterizada tanto pela redução dos níveis dos neurotransmissores serotonina e noradrenalina no cérebro, quanto de seus metabólitos (Lumia et al., 1992; Kelly et al., 1997; Song e Leonard, 2005; Hellweg et al., 2007); a desregulação do eixo hipotálamo-pituitária-adrenal (HPA), com um aumento níveis séricos de corticosterona nos animais bulbectomizados (Cairncross et al., 1977; Marcilhac et al., 1999) e disfunção colinérgica (Moriguchi et al., 2006; Nakajima et al ., 2007).

A disfunção colinérgica também tem sido implicada na neurobiologia da depressão e está envolvida em algumas alterações desencadeadas pelo modelo da BO. Dados na literatura mostram que camundongos bulbectomizados apresentaram redução da densidade de marcação para a enzima acetilcolinesterase (AchE) e redução da expressão da colina acetil transferase no hipocampo (Nakajima et al., 2007).

A maioria das alterações bioquímicas encontradas em roedores bulbectomizados mencionadas acima é consistente com a hipofunção monoaminérgica e desregulação do eixo HPA que estão implicados na fisiopatologia da depressão (Nestler et al., 2002a; Krishnan e Nestler, 2008). Além disso, uma maior atividade da AchE sérica foi relatada em pacientes depressivos (Tiwari et al., 1982).

Embora as disfunções colinérgicas possam contribuir para o desenvolvimento da depressão (Dagytė et al., 2011) e possam estar associadas com mudanças comportamentais encontradas em roedores bulbectomizados, há poucos dados sobre a atividade da AchE em estruturas cerebrais implicadas com a depressão, tais como hipocampo e córtex cerebral em camundongos bulbectomizados (Yamada et al., 2011).

Importante notar que uma disfunção no sistema colinérgico pode estar relacionada à etiologia da depressão, tanto no que se refere a uma hiperatividade colinérgica quanto hipofunção colinérgica.

Neste contexto, estudos pré-clinicos mostram que tanto o aumento exarcebado de acetilcolina na fenda sináptica induzido pela exposição de pesticidas, desencadeada pela inibição da enzima AchE (Assini et al., 2005), quanto modelos animais da doença de Alzheimer em que há um hipofunção colinérgica, foi observado o comportamento tipo-depressivo (Colaianna et al., 2010). Sendo que, nesta última, os inibidores da enzima AchE apresentam propriedades terapêuticas por aumentar a disponibilidade de acetilcolina na fenda sináptica. Desta forma, compostos que modulam este sistema, reestabelecendo sua funcionabilidade, podem apresentar potencial terapêutico interessante para o tratamento da depressão.

1.4. JUSTIFICATIVA

Considerando que: a) a depressão é um distúrbio psiquiátrico altamente incapacitante na qualidade de vida dos indivíduos (Nemeroff e Owens, 2002; Páez-Pereda, 2005); b) o tratamento com antidepressivos convencionais, promove uma remissão parcial dos sintomas depressivos e alguns pacientes permanecem refratários (Wong e Licinio, 2001; Nestler et al. 2002a), c) a terapia antidepressiva convencional produz inúmeros efeitos colaterais (Brunello et al., 2002), reduzindo, assim, a adesão de pacientes ao tratamento (MacGillivray et al., 2003); d) existe uma carência de novas alternativas terapêuticas que aumentem a eficácia clínica no tratamento da depressão; e) as plantas medicinais podem ser utilizadas como ferramentas promissoras para o tratamento deste transtorno psiquiátrico (Linde e Knüppel, 2005); f) apesar do uso etnofarmacológico de *Rosmarinus officinalis* para o tratamento da depressão (Heinrich et al., 2006), não há evidências de comprovação científica da atividade antidepressiva desta planta em modelos animais de depressão; este estudo visa investigar o potencial antidepressivo do extrato de *Rosmarinus officinalis* em testes preditivos de atividade antidepressiva (TSC e TNF), bem como analisar a possível participação do sistema monoaminérgico nesta ação antidepressiva no TSC.

Além disso, como a BO é proposta como um modelo animal de depressão apropriado para o estudo do sintoma relacionado à agitação psicomotora dos pacientes com depressão, especialmente na "depressão agitada", visto que a característica comportamental marcante deste modelo é hiperatividade associado com anedonia

(Kelly et al., 1997; Romeas et al., 2009), o presente trabalho visa avaliar o efeito do tratamento repetido com o extrato desta planta frente à esta intervenção.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Investigar o potencial antidepressivo de *Rosmarinus officinalis* em modelos animais preditivos de atividade antidepressiva (TNF e/ou TSC) e no modelo animal de depressão induzida pela Bulbectomia Olfatória Bilateral (BO) através de ensaios farmacológicos e neuroquímicos.

2.2. OBJETIVOS ESPECÍFICOS:

a) Verificar o efeito antidepressivo da administração aguda por via oral (p.o.) do extrato hidroalcoólico de *Rosmarinus officinalis*, no teste do nado forçado (TNF) e no teste da suspensão pela cauda (TSC) em camundongos; bem como o efeito do tratamento sob a atividade locomotora e exploratória no TCA.

b) Verificar o efeito antidepressivo da administração repetida (14 dias) por via oral (p.o.) do extrato hidroalcoólico de *Rosmarinus officinalis* no TSC em camundongos; bem como o efeito do tratamento sob a atividade locomotora e exploratória no TCA.

c) Investigar a participação dos sistemas serotoninérgico, noradrenérgico e dopaminérgico no efeito antidepressivo do extrato hidroalcoólico de *Rosmarinus officinalis* no TSC.

d) Avaliar o efeito da administração aguda de diferentes frações de *Rosmarinus officinalis*, bem como do óleo essencial desta planta, no potencial antidepressivo TSC e na atividade locomotora no TCA.

e) Analisar os possíveis constituintes químicos isolados de *Rosmarinus officinalis* que produzem ação antidepressiva no TSC e sua ação sob a atividade locomotora no TCA.

f) Investigar a participação do sistema dopaminérgico no efeito antidepressivo do composto isolado de *Rosmarinus officinalis* que apresentar melhor potencial antidepressivo no TSC.

g) Padronizar a técnica da bulbectomia olfatória e investigar o efeito da administração repetida do antidepressivo clássico fluoxetina (ISRS), administrado por via oral (p.o.), nas possíveis alterações comportamentais induzidas por este modelo (hiperatividade e anedonia); e nas alterações bioquímicas, como determinação dos níveis séricos de corticosterona e a atividade da enzima AchE no córtex anterior e hipocampo em animais bulbectomizados. Esta fase preliminar visa possibilitar a melhor caracterização deste modelo, bem como, a realização das etapas subsequentes.

h) Investigar o efeito da administração repetida do extrato hidroalcóolico de *Rosmarinus officinalis* sobre a hiperatividade locomotora e exploratória de camundongos submetidos à BO sobre a atividade locomotora no TCA.

i) Verificar o potencial antidepressivo da administração repetida do extrato hidroalcóolico de *Rosmarinus officinalis* no comportamento tipo-depressivo (anedonia no Splash test) em camundongos submetidos à BO.

j) Investigar o efeito da administração repetida do extrato hidroalcóolico de *Rosmarinus officinalis* de camundongos submetidos previamente à BO sobre o comportamento nos testes do objeto novo (TON) e no teste da caixa nova (TCN).

l) Investigar o desempenho cognitivo (aprendizado e/ou memória) no labirinto aquático de Morris de camundongos submetidos à BO e ao tratamento repetido com o extrato hidroalcóolico de *Rosmarinus officinalis*.

m) Determinar a atividade da enzima acetilcolinesterase (AchE) em córtex anterior e hipocampo de camundongos submetidos à BO e ao tratamento repetido com o extrato hidroalcóolico de *Rosmarinus officinalis*.

n) Determinar os níveis séricos de glicose de camundongos submetidos à BO e ao tratamento repetido com o extrato hidroalcóolico de *Rosmarinus officinalis*.

Em suma, o presente trabalho visa investigar o potencial antidepressivo de *Rosmarinus officinalis*, bem como a participação dos sistemas monoaminérgicos neste efeito. Subsequentemente, investigar os possíveis compostos fitoquímicos responsáveis, pelo menos em parte, por seu possível efeito antidepressivo. Após as evidências encontradas nos testes preditivos (TNF e TSC), o extrato desta planta foi avaliado em um modelo de depressão, como a bulbectomia olfatória. Este estudo teve como objetivo investigar os efeitos da administração crônica do

extrato de *Rosmarinus officinalis* em três principais mudanças de comportamento induzidas por BO em camundongos: hiperatividade em teste de campo aberto e hiperatividade induzida pela novidade nos testes do objeto novo e na caixa nova, comportamento anedônico no "Splash Test" e o déficit cognitivo no labirinto aquático de Morris. Além disso, foram investigados os efeitos da BO nos níveis séricos de glicose e a atividade da acetilcolinesterase hipocampal e cérebro-cortical, bem como, a capacidade do tratamento repetido com o extrato dessa planta em reverter as possíveis alterações induzida pela BO nestes parâmetros bioquímicos.

CAPÍTULO 1

Antidepressant-like effect of the extract of *Rosmarinus officinalis* in mice: Involvement of the monoaminergic system

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Antidepressant-like effect of the extract of *Rosmarinus officinalis* in mice: Involvement of the monoaminergic system

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Tail suspension test

ABSTRACT

Rosemary, *Rosmarinus officinalis* L. (Labiatae) has several therapeutic applications in folk medicine in curing or managing a wide range of diseases, including depression. In this study, the effect of the hydroalcoholic extract of the stems and leaves of this plant was investigated in two behavioral models, the forced swimming test (FST) and tail suspension test (TST) in mice. The extract of *R. officinalis* produced an antidepressant-like effect, since the acute treatment of mice with the extract by p.o. route significantly reduced the immobility time in the FST (100 mg/kg) and TST (10–100 mg/kg), as compared to a control group, without accompanying changes in ambulation in the open-field test. Moreover, the repeated administration (14 days) of the hydroalcoholic extract of *R. officinalis* by p.o. route also produced an antidepressant-like effect in the TST (100–300 mg/kg). The pretreatment of mice with p-chlorophenylalanine (PCPA, 100 mg/kg, i.p., an inhibitor of serotonin synthesis, for 4 consecutive days), NAN-190 (0.5 mg/kg, i.p., a 5-HT_{1A} receptor antagonist), ketanserin (5 mg/kg, i.p., a 5-HT_{2A} receptor antagonist), 1-(m-chlorophenyl) biguanide (mCPBG, 10 mg/kg, i.p., a 5-HT_3 receptor agonist), prazosin (1 mg/kg, i.p., an α_1-adrenoceptor antagonist), SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist) or sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist), but not yohimbine (1 mg/kg, i.p., an α_2-adrenoceptor antagonist) was able to reverse the anti-immobility effect of the extract (10 mg/kg, p.o.) in the TST. The combination of MDL72222, (0.1 mg/kg, i.p., a 5-HT_3 receptor antagonist) with a sub-effective dose of the extract of *R. officinalis* (1 mg/kg, p.o.) produced an anti-immobility effect in the TST. The results suggest that the antidepressant action of the extract of *R. officinalis* is mediated by an interaction with the monoaminergic system and that this plant should be further investigated as an alternative therapeutic approach for the treatment of depression.

1. Introduction

Depression is a disorder characterized by a broad range of symptoms, including altered mood and cognitive functions, and recurrent thoughts of death or suicide. In contrast with the normal experiences of sadness, clinical depression is a chronic disease that can interfere significantly in the individual's life quality. Current antidepressant treatments are efficacious, however, just for 70–80% of the patients and it often takes more than 5–8 weeks until the patients respond to the treatment. Moreover, conventional treatment modalities are hindered by adverse effects and produce only a partial remission (Páez-Pereda, 2005; Taylor and Stein, 2005). Plant extracts have been used for the treatment of some psychiatric disorders, including St. John's wort that is largely studied for the treatment of depression (Nathan, 2001; Rodríguez-Landa and Contreras, 2003; Sakakibara et al., 2006). Because of the limitations of the antidepressant therapy, there has been renewed interest in other alternative therapies with medicinal plants, which may have comparable efficacy to prescription medications while lacking their severe side effects (Bilia et al., 2002; McGarry et al., 2007).

There are also a large number of herbal medicines whose therapeutic potential have been assessed in a variety of animal models. Most assessments of herbal antidepressant activity were conducted using the rodent forced swimming test (FST) and the tail suspension test (TST). These studies have provided useful information for the development of new pharmacotherapies from medicinal plants for use in clinical psychiatry for the treatment of depression (Zhang, 2004). Literature data have shown the potential of several herbal extracts and

Abbreviations: ANOVA, analysis of variance; mCPBG, 1-(m-chlorophenyl) biguanide; DMSO, dimethylsulfoxide; FST, forced swimming test; MAOi, monoamine oxidase inhibitor; MDL72222, tropanyl 3, 5-dichlorobenzoate; NAN-190, 1-(2-methoxyphenyl)-4 [-(2-phthalimido)butyl]piperazine); NA, noradrenaline; PCPA, p-chlorophenylalanine methyl ester; SCH23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; 5-HT, serotonin; SSRI, selective serotonin reuptake inhibitor; TST, tail suspension test.

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constituents as antidepressant agents, whose mechanism of action involves an interaction with the monoaminergic system (Machado et al., 2007; Müller, 2003; Rodrigues et al., 2002; Zhang, 2004).

Rosemary, *Rosmarinus officinalis* L. (Labiatae) is native to Europe, but has been cultivated in all Brazilian states. Some studies have reported that the extract of this plant exerts a number of pharmacological activities, such as hepatoprotective (Sotelo-Félix et al., 2002), antibacterial (Del Campo et al., 2000), antithrombotic (Yamamoto et al., 2005), antiulcerogenic (Dias et al., 2000), diuretic (Haloui et al., 2000), antidiabetic (Bakirel et al., 2008), antioxidant (Bakirel et al., 2008), antinociceptive (González-Trujano et al., 2007) and antiinflammatory (Altinier et al., 2007). An ethnopharmacological use of *R. officinalis* in the treatment of depression, among other uses, was reported (Heinrich et al., 2006).

Considering the therapeutic application of *R. officinalis* in folk medicine in the management of mood disorders, the present work sought to investigate the effect of the hydroalcoholic extract of this plant in FST and TST, predictive models of antidepressant activity. Additionally, the involvement of the monoaminergic system in its antidepressant-like action through the use of pharmacological procedures was also investigated.

2. Methods

2.1. Plant material and preparation of the hydroalcoholic extract of *R. officinalis*

Stems and leaves of *R. officinalis* (Labiatae) were collected in Santo Amaro do Imperatriz, Santa Catarina, and identified by Dr. Daniel Falkenberg, from the Department of Botany, Federal University of Santa Catarina. A voucher specimen (Excicata number 34918) was deposited in the Herbarium of the Department of Botany, Federal University of Santa Catarina, Santa Catarina, Brazil. Dried aerial parts of *R. officinalis* (600 g), was submitted to maceration in ethanol (96%) during fifteen days at room temperature (25 ± 2 °C). Thereafter, the extract was filtered and then concentrated under reduced pressure (at approximately 60°). The maceration was repeated three times. After removing the solvent by liophylization, this procedure gave 61 g of a green solid and dry ethanolic crude extract (10.2% w/w yield).

2.2. Animals

Male Swiss mice (60 to 80 days old weighing 40–50 g) were maintained at constant room temperature (22–25 °C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 48 h before the behavioral procedure. Animals were randomly distributed into specified experimental groups. All experiments were carried out between 11:00 and 16:00 h, with each animal used only once ($N = 6$–11 animals per group). The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.3. Drugs and treatment

The drugs used in the present study were: ketanserin tartarate, 1-(2-methoxyphenyl)-4[-(2-phthalimido)butyl]piperazine) (NAN-190), p-chlorophenylalanine methyl ester (PCPA), tropanyl 3, 5-dichlorobenzoate (MDL72222), 1-(m-chlorophenyl) biguanide hydrochloride (mCPBG), sulpiride, prazosin, yohimbine, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390), fluoxetine (all from Sigma Chemical Company, St. Louis, MO, U.S.A.). All drugs were administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight, except SCH23390 that were administered by subcutaneous (s.c.) route (10 ml/kg body weight). Drugs were dissolved in saline except NAN-190 and MDL72222, that were diluted in saline with 1% Tween 80, sulpiride that was diluted in saline with 5% dimethylsulfoxide (DMSO) and fluoxetine that was dissolved in distillated water. Control animals received appropriate vehicle.

The extract of *R. officinalis* (1–300 mg/kg, p.o.) was dissolved in distillated water and administered acutely by oral route (p.o.) 60 min before the FST, TST or open-field test. Alternatively, mice were administered daily with the extract for 14 days by p.o. route 24 h before the TST or open-field test. The dissolution of the extract was freshly done from the liophylized power immediately before its administration by gavage. A control group received distillated water as vehicle. Fluoxetine (10 mg/kg, p.o., a conventional antidepressant) was used as a positive control.

To address some of the mechanisms by which the extract of *R. officinalis* causes antidepressant-like action in the TST, animals were pretreated with different pharmacological agents.

To investigate a possible contribution of the serotonergic system to the effect of the extract of *R. officinalis* in reducing the immobility time in the TST, animals were pretreated with PCPA (100 mg/kg, an inhibitor of serotonin synthesis) or vehicle, once a day, for 4 consecutive days (Brocardo et al., 2008; Eckeli et al., 2000; Kaster et al., 2005; Machado et al., 2007; Rodrigues et al., 2002). Then, 24 h after the last PCPA or saline injection, animals were acutely treated with the extract of *R. officinalis* (10 mg/kg, p.o.), fluoxetine (10 mg/kg, p.o., positive control) or vehicle and were tested in the TST 60 min later. The dose of fluoxetine was chosen based on previous studies from our group (Cunha et al., 2008; Machado et al., 2007).

In order to investigate the possible involvement of the 5-HT receptor subtypes system in the antidepressant-like effect of the extract, mice were pretreated with NAN-190 (0.5 mg/kg, i.p. a 5-HT_{1A} receptor antagonist), ketanserin (5 mg/kg, i.p., a 5-HT_{2A} receptor antagonist), or vehicle and after 30 min they received the extract of *R. officinalis* (10 mg/kg, p.o.) or vehicle injection before being tested in the TST 60 min later. To assess the possible involvement of the 5-HT_3 receptor in its antidepressant-like effect, the animals were pretreated with 1-(m-chlorophenyl) biguanide hydrochloride (mCPBG) (10 mg/kg, i.p., a selective 5-HT_3 receptor agonist) or vehicle and after 30 min they received the extract of *R. officinalis* (10 mg/kg, p.o.) or vehicle injection before being tested in the TST 60 min later. In addition, in another set of experiment mice were pretreated with sub-effective dose of MDL72222 (0.1 mg/kg, i.p., a selective 5-HT_3 receptor antagonist) or vehicle and after 30 min they were treated with a sub-effective dose of the extract of *R. officinalis* (1 mg/kg, p.o.) or vehicle injection before being tested in the TST 60 min later.

To investigate the possible involvement of the noradrenergic and dopaminergic systems on the antidepressant-like effect of the extract in the TST, animals were pretreated with prazosin (1 mg, i.p., an α_1-adrenoceptor antagonist), yohimbine (1 mg/kg, i.p., an α_2-adrenoceptor antagonist), SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist) or sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist), and after 30 min they received the extract of *R. officinalis* (10 mg/kg, p.o.) or vehicle and were tested in the TST 60 min later.

The administration schedule and the doses of the drugs used were chosen on the basis of experiments previously performed in our laboratory and literature data confirm the efficacy of the above-mentioned protocols (Brocardo et al., 2008; Kaster et al., 2005; Machado et al., 2007; O'Neill and Conway, 2001; Redrobe and Bourin, 1997; Rodrigues et al., 2002; Yamada et al., 2004).

2.4. Forced swimming test (FST)

The FST has been used as a model predictive of antidepressant effect (Cryan et al., 2002). The test procedure was carried out

according to the previously standardized and validated animal model in our laboratory (Brocardo et al., 2008; Eckeli et al., 2000; Kaster et al., 2005; Zomkowski et al., 2004). Mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; the total duration of immobility was recorded during a 6-min period. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al., 1977). After the initial 2–3 min of vigorous activity the animals showed a period of immobility by floating with minimum movements. An animal is considered to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its nose above the water surface. The total immobility period for the period of 6 min was recorded.

2.5. Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was chronometered by an observer during a 6 min period (Cunha et al., 2008; Machado et al., 2007; Rodrigues et al., 2002).

2.6. Open-field test

To assess the possible effects of the extract of *R. officinalis* on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Machado et al., 2007; Rodrigues et al., 1996). Mice were individually placed in a wooden box (40 × 60 × 50 cm) with the floor divided into 12 rectangles. The number of rectangles crossed by the animal with its four paws was considered as indicative of locomotor activity and number of rearings was an indicative of the exploratory behavior (Felipe et al., 2007; Rodrigues et al., 1996). The number of crossings and rearings was registered during a period of 6 min. Animals were treated with the extract of *R. officinalis* (1, 10, 100 and 300 mg/kg, p.o.) or with vehicle by p.o. route 60 min before the experiments.

2.7. Statistical analysis

Comparisons between experimental and control groups were performed by one (dose–response curves) or two-way ANOVA (study of the mechanism of action) followed by Tukey's HSD test when appropriate. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Effect of acute treatment with the hydroalcoholic extract of *R. officinalis* on the immobility time in the FST and TST and locomotor and exploratory activity in the open-field test

The effects of the oral administration of the hydroalcoholic extract of *R. officinalis* on the immobility time in the FST and TST were shown in Fig. 1A and B, respectively. The extract given by oral route at 100 mg/kg decreased the immobility time in the FST as compared to the control group (Fig. 1A). It also significantly decreased the immobility time in the TST when administered at doses of 10 and 100 mg/kg (Fig. 1B). The percent of reduction in the immobility time was 49.5% in FST and 22.9%, 28.0% in TST, respectively. The one-way ANOVA revealed a significant effect of the extract [$F(4,31) = 6.24$, $P<0.01$] in the FST and in TST [$F(4,29) = 6.80$, $P<0.01$]. However, the extract did not cause any change in the locomotor activity of mice as compared to control group (Fig. 1C), as shown by one-way ANOVA [$F(4,40) = 0.51$, $P = 0.73$]. The results depicted in Fig. 1D show that the extract of *R. officinalis* (dose range 1–300 mg/kg, p.o.) did not significantly alter the number of the rearings of mice in the open-

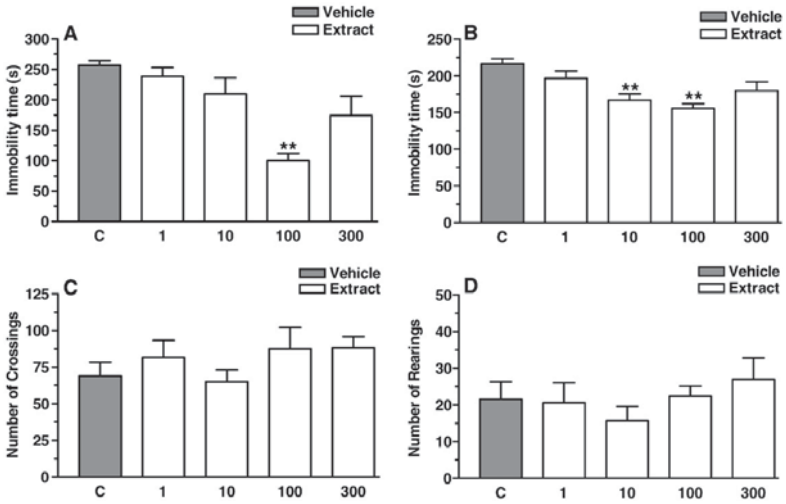

Fig. 1. Effect of the acute treatment of mice with the hydroalcoholic extract of *Rosmarinus officinalis* (1–300 mg/kg, p.o.) on the immobility time in the FST (A) and TST (B) and on the number of crossings (C) and rearings (D) in the open-field test. Each column represents the mean + S.E. of 6–10 animals. ** $P<0.01$ compared with the vehicle-treated control (C).

field test as compared to the control group [$F(4,41) = 0.47$, $P = 0.76$].

3.2. Effect of repeated treatment with the hydroalcoholic extract of *R. officinalis* on the immobility time in the TST and locomotor and exploratory activity in the open-field test

In order to investigate if the antidepressant-like effect of the extract of *R. officinalis* continues after a chronic administration (14 days), a dose–response curve in the TST and open-field test were carried out. Fig. 2A shows that the extract given by oral route at 100 and 300 mg/kg decreased the immobility time in the TST, as compared to the control group. The percent of reduction in the immobility time in TST was 29.7% and 27.8%, respectively. Fluoxetine (10 mg/kg, p.o.), used as a positive control, also produced a significant reduction in the immobility time (22.8% of reduction) in the TST. The one-way ANOVA revealed a significant effect of the treatment in the TST [$F(4,35) = 6.366$, $P<0.01$]. However, either the extract or fluoxetine caused no change in the locomotor activity of mice as compared to control group (Fig. 2B), as shown by one-way ANOVA [$F(4,44) = 0.187$, $P = 0.943$]. The results depicted in Fig. 2C show that the treatment with extract of *R. officinalis* (dose range 10–300 mg/kg, p.o.) and with fluoxetine did not significantly alter the number of the rearings of mice in the open-field test as compared to control group [$F(4,44) = 0.33$, $P = 0.85$].

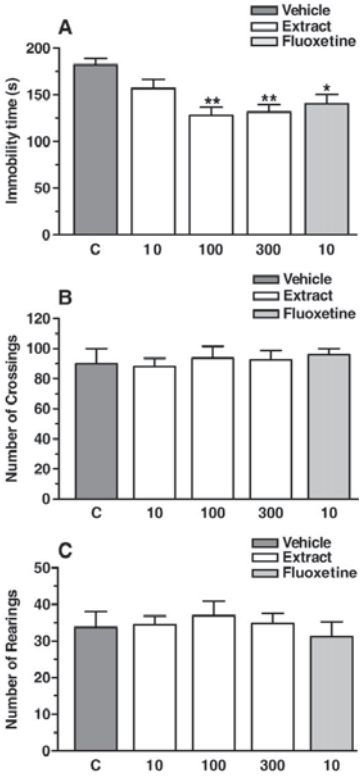

Fig. 2. Effect of the repeated treatment of mice (14 days) with the hydroalcoholic extract of *Rosmarinus officinalis* (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on the immobility time in the TST (A), on the number of crossings (B) and rearings (C) in the open-field test. Each column represents the mean + S.E. of 9–10 animals. * $P<0.05$, ** $P<0.01$ compared with the vehicle-treated control (C).

3.3. Investigation of some possible mechanisms underlying the antidepressant-like effect of the extract of R. officinalis in the TST

Since 10 mg/kg of the extract of *R. officinalis* was the lowest acute effective dose, all the experiments regarding the investigation of the mechanisms underlying the antidepressant-like effect of the extract of *R. officinalis* were performed in the TST using this dose.

3.3.1. Involvement of the serotonergic system

Fig. 3A shows that the pretreatment of mice with the inhibitor of serotonin synthesis PCPA (100 mg/kg, i.p., once a day for 4 consecutive days) significantly prevented the decrease in the immobility time elicited by the extract (10 mg/kg, p.o.). The results obtained in this experiment were analyzed by a two-way ANOVA. There was a significant effect of PCPA pretreatment [$F(1,35) = 18.31$, $P<0.01$], extract or fluoxetine treatment [$F(2,35) = 21.39$, $P<0.01$] and of extract or fluoxetine X PCPA interaction [$F(2,35) = 5.76$, $P<0.01$]. Fluoxetine (10 mg/kg, p.o.), used as a positive control, produced a significant reduction in the immobility time in the TST, which was significantly prevented by PCPA pretreatment. Moreover, the pretreatment of mice with NAN-190 (0.5 mg/kg, i.p.) also prevented the antidepressant-like effect elicited by the extract. A two-way ANOVA showed significant differences for NAN-190 [$F(1,30) = 8.26$, $P<0.01$], extract [$F(1,30) = 9.04$, $P<0.01$] and extract × NAN-190 interaction [$F(1,30) = 16.65$, $P<0.01$](Fig. 3B). Fig. 3C shows that the pretreatment of mice with ketanserin (5 mg/kg, i.p.) also prevented the action of the extract in the TST. The two-way ANOVA revealed a main effect of the ketanserin [$F(1,33) = 20.19$, $P<0.01$], extract[$F(1,33) = 17.38$, $P<0.01$] and extract × ketanserin interaction [$F(1,33) = 25.39$, $P<0.01$]. The results depicted in Fig. 3D shows that the pretreatment of mice with mCPBG (10 mg/kg, i.p.) prevented the anti-immobility effect elicited by the extract. A two-way ANOVA showed significant differences for the extract [$F(1,36) = 5.28$, $P<0.05$] and extract × mCPBG interaction [$F(1,36) = 7.89$, $P<0.01$], but not mCPBG [$F(1,36) = 3.71$, $P = 0.06$]. Fig. 3E shows that the administration of MDL72222 administered at a dose (0.1 mg/kg, i.p.) that per se produced no effect in the TST, in combination with a sub-effective dose of the extract (1 mg/kg, p.o.), reduced the immobility time in the TST as compared with each one alone. The results were analyzed by a two-way ANOVA that showed a significant effect of MDL72222 [$F(1,31) = 16.23$, $P<0.01$], extract [$F(1,31) = 13.14$, $P<0.01$] and extract × MDL72222 interaction [$F(1,31) = 4.24$, $P<0.05$].

3.3.2. Involvement of the noradrenergic system

The results depicted in Fig. 4A shows that pretreatment of mice with prazosin (1 mg/kg, i.p) was able to reverse the antidepressant-like effect of the extract of *R. officinalis* (10 mg/kg, p.o.) in the TST. The two-way ANOVA revealed a main effect of the prazosin [$F(1,31) = 20.05$, $P<0.01$], extract [$F(1,31) = 20.26$, $P<0.01$] and extract × prazosin interaction [$F(1,31) = 4.11$, $P<0.01$]. Fig. 4B shows that the pretreatment of mice with yohimbine (1 mg/kg, i.p.) was not able to prevent the anti-immobility effects of the extract of *R. officinalis* (10 mg/kg, p.o.) in the TST. The two-way ANOVA revealed a main effect of the extract [$F(1,35) = 26.87$, $P<0.01$], but not of yohimbine [$F(1,35) = 0.70$, $P = 0.79$] and the extract × yohimbine interaction [$F(1,35) = 0.45$, $P = 0.51$].

3.3.3. Involvement of the dopaminergic system

The anti-immobility effect of the extract of *R. officinalis* (10 mg/kg, p.o.) was prevented by pretreatment of mice with SCH23390 (0.05 mg/kg, s.c., Fig. 5A). The two-way ANOVA revealed a main effect of the SCH23390 [$F(1,27) = 19.26$, $P<0.01$], extract [$F(1,27) = 17.22$, $P<0.01$] and extract × SCH23390 interaction [$F(1,27) = 37.76$, $P<0.01$]. The pretreatment of the animals with sulpiride (50 mg/kg, i.p., Fig. 5B) was

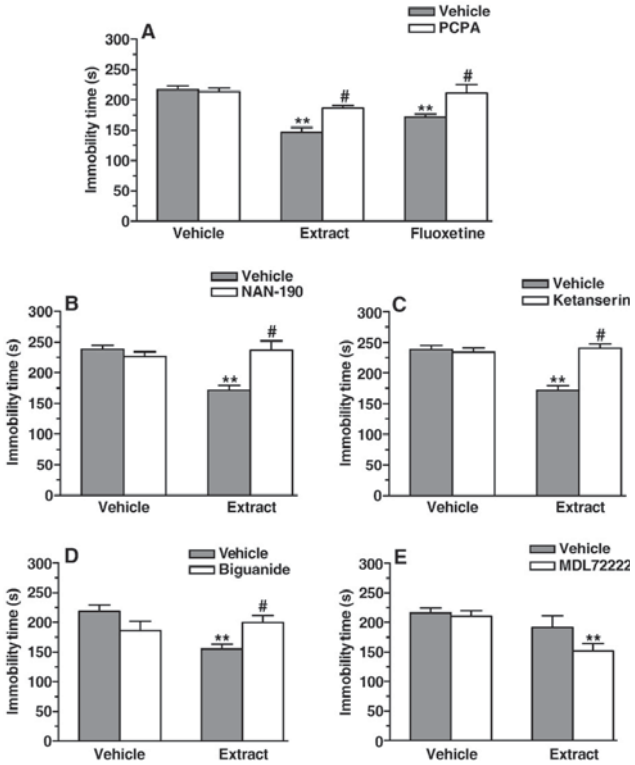

Fig. 3. Effect of pretreatment of mice with PCPA (100 mg/kg, i.p. once a day for 4 consecutive days, panel A), NAN-190 (0.5 mg/kg, i.p., panel B), ketanserin (5 mg/kg, i.p., panel C) or mCPBG (10 mg/kg, i.p., panel D) on the hydroalcoholic extract of *Rosmarinus officinalis* (10 mg/kg, p.o.)-induced reduction in immobility time in the TST. Effect of combined treatment of mice with the extract of *Rosmarinus officinalis* (1 mg/kg, p.o) and MDL72222 (0.1 mg/kg, i.p.) on the immobility time in the TST (panel E). Each column represents the mean + S.E. of 6–10 animals. ** $P<0.01$ compared with the vehicle-treated control. # $P<0.01$ as compared with the extract or fluoxetine alone (not with the extract alone)

also able to prevent the anti-immobility effect of the extract in the TST. The two-way ANOVA revealed a main effect of the sulpiride [$F(1,24) = 10.48$, $P<0.01$], extract [$F(1,24) = 6.77$, $P<0.05$] and of the extract × sulpiride interaction [$F(1,24) = 24.21$, $P<0.01$].

4. Discussion

There is an increasing interest in the study of the antidepressant effect of herbs, since treatment of depression with conventional antidepressants (monoamine oxidase inhibitors, tricyclics, selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors) provides a complete remission just for 50% of the individuals (Nestler et al., 2002). Research reports have been indicated that herbal extracts and their constituents including *Hypericum perforatum*, *Curcuma longa*, *Ginkgo biloba*, *Schinus molle*, among others, exert antidepressant-like effect in animal models of depression (Machado et al., 2007; McGarry et al., 2007; Peng et al., 2007; Rodrigues et al., 2002; Sakakibara et al., 2006; Wang et al., 2008; Zhang, 2004; Zhang et al., 2007). The FST and TST are widely used for screening potential antidepressants. Antidepressants reduce the immobility time in both FST and TST. The immobility behavior displayed in rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. There is, indeed, a significant correlation between clinical potency and effectiveness of antidepressants in both models (Cryan et al., 2002; Porsolt et al., 1977; Steru et al., 1985).

In the present study, our results demonstrate that the acute treatment with hydroalcoholic extract of *R. officinalis* produced a significant antidepressant-like response in both FST and TST. Moreover, our study showed that the repeated administration (14 days) of the hydroalcoholic extract of *R. officinalis* was also able to produce an antidepressant-like effect in the TST. These results are in accordance with the reported ethnopharmacological effect of this plant (Heinrich et al., 2006). It is noteworthy that the effect produced by the extract of this plant was comparable to the one produced by the classical antidepressant fluoxetine (10 mg/kg, p.o.). The antidepressant-like effect of the acutely administered extract was observed at a lower dose in the TST (10 mg/kg, p.o.) than in the FST (100 mg/kg, p.o.). The underlying principle measuring the lack of active coping behavior is identical in the TST and FST, but their variability in response to certain antidepressants indicates potentially different substrates and neurochemical pathways mediating performance in these tests. These issues may underlie the observed behavioral differences (Bai et al., 2001). Furthermore, one of the most important differences between

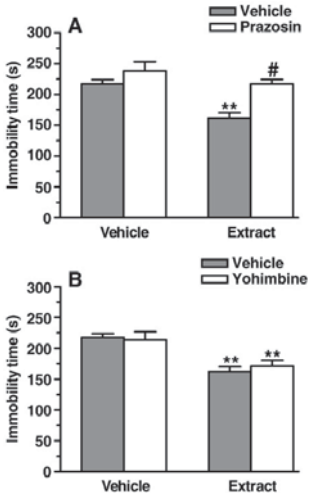

Fig. 4. Effect of pretreatment of mice with prazosin (1 mg/kg, i.p., panel A) or with yohimbine (1 mg/kg, i.p., panel B) on the hydroalcoholic extract of *Rosmarinus officinalis* (10 mg/kg, p.o.)-induced reduction in immobility time in the TST. Each column represents the mean + S.E. of 6–10 animals. ** $P<0.01$ compared with the vehicle-treated control. # $P<0.01$ as compared with the extract alone.

these two models is the response to drugs in both tests and the apparent increased sensitivity of the TST. The mouse FST has not traditionally been viewed as a consistently sensitive model for detecting selective serotonin reuptake inhibitor activity, whereas these antidepressants are generally reported as active in the TST (Cryan et al., 2005). Moreover, the TST was proposed to have a greater pharmacological sensitivity as compared with the FST (Thierry et al., 1986; Cryan et al., 2005).

To avoid false positive results in the FST and TST, it is important to rule out the possibility that reductions in immobility time were not merely a result from psychostimulant effects of the extract. In our study, either acute or repeated treatment with *R. officinalis* did not increase locomotor and exploratory activity at doses that produced an antidepressant-like effect, indicating a specific effect of this plant on behavioral models predictive of antidepressant activity.

The monoamine hypothesis based on the deficiency of one or several monoamines is commonly evoked to explain the physiopathology of depression. This hypothesis initially based on noradrenaline (Schildkraut et al., 1965) and serotonin deficiency (Coppen, 1967) has been extended to dopamine (Randrup et al., 1975). Most of the antidepressants currently used today exert their primary biochemical effects by regulating synaptic concentrations of serotonin, noradrenaline and/or dopamine (Elhwuegi, 2004; Páez-Pereda, 2005). Drugs inhibiting the uptake of serotonin, noradrenaline and dopamine (triple reuptake inhibitors) that have been recently developed could produce a more rapid onset of action and possess greater efficacy than traditional antidepressants (Chen and Skolnick, 2007). Hence, considering that the monoaminergic system is implicated in the pathophysiology and treatment of human depression (Elhwuegi, 2004; Holsboer, 2004), the present study aimed to investigate the influence of pharmacological agents that modulate the monoaminergic system on the antidepressant-like activity of the extract *R. officinalis* (10 mg/kg, p.o.) in the TSC.

The serotonergic system has long been implicated in the etiology of depression. Some of the most compelling evidence involves the alleviation of depression caused by serotonin selective reuptake inhibitors (SSRIs). Moreover, the tryptophan depletion studies confirmed the relationship between serotonin and this psychiatric disorder. Numerous studies have been conducted in an effort to uncover how antidepressants operate on the serotonergic system (Taylor et al., 2005). In our study, the antidepressant-like effect of the extract of *R. officinalis* was completely prevented by pretreatment of mice with the neuronal serotonin store depletor, PCPA as well as the 5-HT_{1A}, 5-HT_{2A} receptor antagonists NAN-190 and ketanserin, respectively. In addition, the pretreatment of mice with 5-HT_3 receptor agonist mCPBG was able to reverse the antidepressant-like effect of the extract, whereas the 5-HT_3 receptor antagonist MDL72222 administered in combination with the extract produced a synergistic antidepressant-like effect. These results provide evidence that the antidepressant-like effect of the extract of *R. officinalis* is dependent on an interaction with the serotonergic system.

PCPA, an inhibitor of the enzyme tryptophan hydroxylase, administered to mice at the dose employed in the present study for four consecutive days is able to deplete the endogenous store of serotonin by about 60% without affecting the noradrenaline and dopamine levels (Redrobe et al., 1998a,b). In our study, PCPA pretreatment prior to the administration of the extract of *R. officinalis*, was effective in causing a blockade of the anti-immobility effect of the extract in the TST. This result clearly suggests that an intact serotonergic system plays a critical role in the effect of the extract in the TST. PCPA was also previously shown by our group to completely prevent the antidepressant-like effect of fluoxetine, used here as a positive control (Machado et al., 2007; Rodrigues et al., 2002), but not of imipramine in the TST (Rodrigues et al., 2002). In addition, a study from O'Leary et al. (2007) has recently shown that the pretreatment with PCPA in mice alters the response to the fluoxetine and citalopram in the TST, but not noradrenaline reuptake inhibitors.

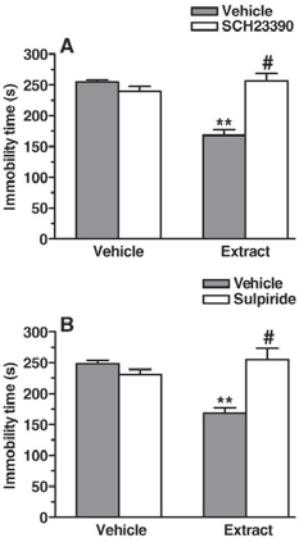

Fig. 5. Effect of pretreatment of mice with SCH23390 (0.05 mg/kg, s.c., panel A) or with sulpiride (50 mg/kg, i.p., panel B) on the hydroalcoholic extract of *Rosmarinus officinalis* (10 mg/kg, p.o.) -induced reduction in immobility time in the TST. Each column represents the mean + S.E. of 6–8 animals. ** $P<0.01$ compared with the vehicle-treated control. # $P<0.01$ as compared with the with the extract alone.

A decrease in 5-HT_{1A} binding potential has been reported, as determined by positron emission tomography, in depressed patients in multiple forebrain areas, including the frontal cortex and hippocampus (Drevets et al., 1999; Sargent et al., 2000). Therefore, a deficiency in the function and expression of 5-HT_{1A} receptors is an important factor in the development of depression (Leitch et al., 2003). In addition, the mechanism of action of several classes of antidepressant drugs, such as tricyclics, SSRIs (selective serotonin reuptake inhibitors), MAOi (monoamine oxidase inhibitors) occur by participation of the 5-HT_{1A} receptors (Hensler, 2002), including the antidepressant-like action of some plants (Kim et al., 2007; Machado et al., 2007). Additionally, 5-HT_{1A} receptors appear to be necessary for the effects of SSRI antidepressant drugs in both acute and chronic behavioral models (Mayorga et al., 2001). The SSRIs fluoxetine and paroxetine failed to decrease immobility in the TST in 5-HT_{1A} receptor mutant mice at a test dose that was active in wild-type and 5-HT_{1B} receptor mutant mice. However, 5-HT_{1A} receptor mutant mice still demonstrated an antidepressant-like response to the noradrenaline reuptake inhibitor desipramine. These data suggest that the presence of 5-HT_{1A} receptors may be critical for the expression of the antidepressant-like behavioral responses of SSRIs in the TST (Mayorga et al., 2001). In our study, the pretreatment of mice with the 5-HT_{1A} antagonist NAN-190 abolished the anti-immobility effect elicited by the extract of *R. officinalis* in the TST, suggesting the involvement of 5-HT_{1A} receptors in the antidepressant-like effect of this plant.

Preclinical and clinical studies have reported a key role for 5-HT_2 receptors in the pathophysiology of depression as well as the action of many antidepressants (Boothman et al., 2006; Cryan and Lucki, 2000; Wang et al., 2008). Moreover, many established antidepressants are effective 5-HT_2 receptor antagonists (Deakin, 1988). However, the preferential 5-HT_{2A} receptor partial agonist DOI was reported to enhance the antidepressant-like effect of some compounds (Khisti and Chopde, 2000; Zomkowski et al., 2004). In our study, the pretreatment with ketanserin prevented the anti-immobility effect of the extract of *R. officinalis*, which suggests that its effect in the TST is mediated through an interaction with 5-HT_{2A} receptors, since ketanserin has a higher affinity for 5-HT_{2A} receptors than for other 5-HT_2 receptor subtypes (Baxter et al., 1995). Data from our group have shown that ketanserin was able to reverse the antidepressant-like effects of some compounds and plant extracts, such as: agmatine (Zomkowski et al., 2004), *S. molle* (Machado et al., 2007), folic acid (Brocardo et al., 2008) and magnesium chloride (Cardoso et al., 2009).

The involvement of 5-HT_3 receptors in the pathophysiology of depression is less reported in the literature, but some reports have indicated that different classes of antidepressants act as functional antagonists at the 5-HT_3 receptors, indicating that the suppression of 5-HT_3 receptor activity may contribute to the action of antidepressants (Eisensamer et al., 2003). The 5-HT_3 receptor antagonists administered acutely, decreased the duration of immobility in the FST (Bravo and Maswood, 2006). While a 5-HT_3 receptor agonist attenuated the decrease in immobility produced by imipramine, desipramine and mianserin, 5-HT_3 receptor antagonists, potentiated antidepressant-like effects of several SSRIs in the FST (Nakagawa et al., 1998). In the present study, mCPBG, a 5-HT_3 receptor agonist, attenuated the decreased duration of immobility induced by *R. officinalis*, although mCPBG did not affect the duration of immobility when it was given alone. This result suggests that the antidepressant-like effect of the extract is dependent on a decrease in the 5-HT_3 receptor activation. It is somewhat in accordance with the study of Kos et al. (2006), which has shown that MDL72222, a 5-HT_3 receptor antagonist, administered at a higher dose (3 mg/kg, i.p.) than the one employed in the work, produced an antidepressant-like effect in the TST. Moreover, the hypothesis that the suppression of 5-HT_3 receptor activity contribute to the antidepressant-like effect of the extract of *R. officinalis* was reinforced by the synergistic antidepressant-like effect observed when mice were treated with MDL72222 (0.1 mg/kg, i.p.) in combination with a sub-effective dose of the extract of *R. officinalis* (1 mg/kg, p.o.).

In parallel with the serotonergic system, the noradrenergic system has been a valuable target for antidepressants. Depression seems to be associated with a hypofunction of the noradrenergic system, and some antidepressants act by increasing the synaptic availability of norepinephrine (Elhwuegi, 2004; Taylor et al., 2005). In our experiments, the pretreatment of mice with prazosin (an α_1-adrenoceptor antagonist) was able to reverse the antidepressant-like effect of the extract of *R. officinalis*, whereas yohimbine (an α_2-adrenoceptor antagonist) was ineffective in reversing the immobility period in mice. This result indicates that the extract may exert its effect in the TST by interacting with α_1, but not with α_2-adrenoceptors.

In animal studies, the implication of the dopaminergic system in depression has been essentially studied via the use of agonists that increase dopaminergic activity resulting in antidepressant-like effects in animal model of depression (Basso et al., 2005; Renard et al., 2001). Moreover, several studies, including post mortem investigations, particularly of subjects with severe depression, have demonstrated reduced concentrations of dopamine metabolites both in the cerebrospinal fluid and in brain regions that mediate mood and motivation (Papakostas, 2006). Furthermore, the neuroimaging findings support the hypothesis that major depression is associated with a state of reduced dopaminergic transmission, possibly reflected by a compensatory up-regulation of D_2 receptors. A deficiency of mesolimbic dopamine is a leading candidate for the etiology of certain symptoms of depression (e.g., anhedonia and loss of motivation) (Dunlop and Nemeroff, 2007). In our study, the selective dopamine D_1 receptor antagonist SCH23390 and the dopamine D_2 receptor antagonist, sulpiride, were able to reverse the antidepressant-like effect of the extract of *R. officinalis*. These results are in accordance with literature data indicating that both dopamine D_1 and D_2 receptors might play a role in depression (Basso et al., 2005; Machado et al., 2007; Papakostas, 2006; Yamada et al., 2004).

Altogether, our results firstly indicate that the extract of *R. officinalis* causes an antidepressant-like effect that seems to be mediated by an interaction with the monoaminergic system. Phytochemical studies have identified active components in this plant, such as flavonoids including diosmetin, diosmin, luteolin, apigenin, quercetin and kaempherol; phenols such as caffeic and rosmarinic acids and terpenoids like, carnosol, carnosic acid, rosmanol, oleanolic and ursolic acids (Altinier et al., 2007; Barnes et al., 2001; Bentayeb et al., 2007; Frankel et al., 1996; González-Trujano et al., 2007; Newall et al., 1998; Wellwood and Cole, 2004). Screening of the ethanol extract of *R. officinalis* aerial parts has reported the presence of flavonoids, tannins and saponins, but not the presence of alkaloids as detected in an aqueous extract (Hosseinzadeh and Nourbakhsh, 2003). In our study, a preliminary chemical composition of the hydroalcoholic extract by thin layer chromatography, infrared spectroscopic method and NMR spectrum revealed the presence of carnosol, carnosic acid and oleanolic acid (data not shown). Further chemical and pharmacological analysis of the extract will be conducted to isolate and characterize the active principles responsible for the antidepressant-like effect.

5. Conclusion

In conclusion, the present study indicates that *R. officinalis* produces a specific antidepressant-like effect in animal models predictive of antidepressant properties, forced swimming test and tail suspension test. Moreover, the effect of the acute or repeated administration of this extract was similar to the action produced by the classical antidepressant fluoxetine. In addition, it was also shown that its antidepressant-like effect is dependent on its interaction with the serotonergic (5-HT_{1A}, 5-HT_{2A} and 5-HT_3 receptors), noradrenergic (α_1-receptor) and dopaminergic (D_1 and

D_2 receptors) systems. Further studies are necessary to elucidate which isolated compounds are responsible for the antidepressant-like effects of the extract of *R. officinalis*.

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CAPÍTULO 2

Antidepressant-like effect of fractions, isolated compounds and essential oil from *Rosmarinus officinalis* L. in mice

Manuscrito submetido à revista Food Chemistry

Antidepressant-like effects of fractions, essential oil and isolated compounds of *Rosmarinus officinalis* L. in mice

[Running title: Antidepressant-like effects of *Rosmarinus officinalis*]

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Abstract

The aim of this study was to investigate the antidepressant-like effect of fractions from *Rosmarinus officinalis* L.: ethyl acetate 1 and 2 (AcOEt1 and 2), hexane (HEX), ethanolic (ET), and essential oil free (EOF) fractions, as well essential oil, the isolated compounds carnosol and betulinic acid in the tail suspension test, a predictive test of depression in mice. All of them produced a significant antidepressant-like effect: AcOEt1, ET, EOF fractions and essential oil (0.1-100 mg/ kg, p.o); HEX (0.1-10 mg/ kg, p.o) and AcOEt2 fraction (0.1-1 mg/ kg, p.o), carnosol (0.01-0.1 mg/ kg, p.o.) and betulinic acid (10 mg/ kg, p.o.). No psychostimulant effect was shown in the open-field test, indicating that the effects in the tail suspension test are specific. This study suggests that carnosol and betulinic acid could be responsible, at least in part, for the anti-immobility effect of extracts from *Rosmarinus officinalis.*

*Keywords**: Rosmarinus officinalis*; carnosol; betulinic acid; essential oil; tail suspension test; antidepressant.

Abbreviations: ANOVA, analysis of variance; ethyl acetate 1, AcOEt1; ethyl acetate 2, AcOEt 2; hexane, HEX; ethanolic, ET; essential oil free, EOF; TST, tail suspension test.

1. Introduction

Depression is one of the major mental disorders associated with considerable morbidity and mortality, unfortunately with a consistently high prevalence worldwide. The lifetime prevalence of depression has been estimated to be as high as 21% of the general population in some developed countries (Wong & Licinio, 2001).

Despite the introduction of various classes of antidepressants, including tricyclics, selective reversible inhibitors of monoamine oxidase, selective serotonin reuptake inhibitors and specific serotonin-noradrenaline reuptake inhibitors, the treatment of depression is not entirely satisfactory, since this conventional treatment modalities are hindered by adverse effects and generally produce only a partial remission (Páez-Pereda, 2005; Richelson, 1994; Taylor & Stein, 2005).

Herbal therapies may be effective alternatives in the treatment of depression. Moreover, pharmacotherapy with medicinal plants can offer advantages in terms of safety and tolerability, possibly also improving patient compliance (Richelson, 1994). The search of extracts and isolated compounds of plants has progressed significantly in the past decade (Zhang, 2004) and this fact could be due to, in part, the need to identify new therapeutic alternative for treatment of psychiatric illnesses, including depression.

Rosemary, *Rosmarinus officinalis* L. (Labiatae) is an evergreen perennial shrub, native to Europe that has been cultivated in many parts of the world, including Brazil (Balmé, 1978; Duke, 2000). The leaves of this plant are commonly used as a condiment to season food, and as a source of antioxidant compounds for food conservation (Zeng et al., 2001). The essential oil produced by *Rosmarinus officinalis* is colourless or pale yellow with the characteristic of rosemary and warm camphoraceous taste. The oil of this plant is used in the perfume industry and as a flavor agent and the majority of its constituents have been reported to be inhibitory to microorganisms (Deans & Svoboda, 1993).

Furthermore, the ethnopharmacological uses of *Rosmarinus officinalis* include the treatment of several disorders, as inflammatory diseases, physical and mental fatigue, and treatment of nervous

82 agitation and depression, among other applications (Balmé, 1978; Duke, 2000; Heinrich, Kufer,
83 Leonti & Pardo-de-Santayana, 2006).
84 Pharmacological studies carried out with the extract and essential oil from *Rosmarinus*
85 *officinalis* show that this plant exerts several biological effects, such as: antioxidant, antidiabetic,
86 antinociceptive and anti-inflammatory, among others applications (Bakirel, Bakirel, Keles, Ülgen &
87 Yardibi, 2008; Benincá, Dalmarco, Pizzolatti & Fröde, 2011; González-Trujano et al., 2007;
88 Mangena & Muyima, 1999; Takaki et al., 2008). We have recently shown that the hydroalcoholic
89 crude extract of *Rosmarinus officinalis* (orally administered to mice) produces an antidepressant-like
90 effect in the in forced swimming test (FST) and tail suspension test (TST), predictive tests of
91 antidepressant activity, by a mechanism dependent on the interaction with the monoaminergic
92 systems (Machado et al., 2009).
93 Phytochemical studies reported the biologically active compounds in this plant, such as:
94 terpenoids - like carnosol, carnosic acid, rosmanol, oleanolic and ursolic acids; flavonoids including
95 diosmin, luteolin, apigenin and quercetin and phenols as caffeic and rosmarinic acids. Furthermore,
96 these compounds have shown antioxidant, anti-inflammatory and antinociceptive properties in pre-
97 clinical studies (Altinier et al., 2007; Barnes, Anderson & Phillipson, 2001; Benincá, Dalmarco,
98 Pizzolatti & Fröde, 2011; Frankel, Huang, Aeschbach & Prior, 1996; González-Trujano et al., 2007).
99 Considering the recent evidence of the antidepressant-like effect of *Rosmarinus officinalis* by
100 our group, the ethnopharmacological use this plant for treatment of the depression and that the herbal
101 medicines include a range of pharmacologically active compounds, the aim of this study was to
102 verify the effects of the several fractions and essential oil of this plant in the TST in mice.
103 Additionally, this study isolated and identified the major components of *Rosmarinus officinalis*, that
104 may be contributing, at least in part, for its antidepressant potential.
105
106 ***2. Materials and methods***
107 ***2.1. Plant material and obtention of crude extract***

Stems and leaves of *Rosmarinus officinalis* (Labiatae) were collected in Santo Amaro do Imperatriz, Santa Catarina, and identified by Dr. Daniel Falkenberg, from the Department of Botany, Federal University of Santa Catarina. A voucher specimen (Excicata number 34918) was deposited in the Herbarium of the Department of Botany, Federal University of Santa Catarina, Santa Catarina, Brazil. The preparation of crude extract was carried out as described previously Machado et al. (2009). Briefly, dried aerial parts of *Rosmarinus officinalis* (600 g) was submitted to maceration in ethanol (96%) during fifteen days at room temperature (25±2 °C). Thereafter, the extract was filtered and then concentrated under reduced pressure (at approximately 60°). The maceration was repeated three times. After removing the solvent by liophylization, this procedure gave 61 g of a green solid and dry hydroalcoholic crude extract (10.2% w/w yield).

2.2. Obtention of fractions and isolation of compounds

The crude extract (61 g) was subjected to passage on a short silica gel 60 (Vetec – 0.063–0.2 mesh) column with hexane, ethyl acetate and ethanol in order of polarity, to give the hexane (HEX: 13.3 g, 21.8%), ethyl acetate (AcOEt1:8.83g, 14.5% and AcOEt2:15.48g, 25.3%) and ethanolic (ET: 13 g, 21%) fractions. Part of the HEX fraction (9.37 g) was subjected to a chromatographic procedure on a silica gel 60 (Vetec –0.063–0.2 mesh) column using hexane–ethyl acetate solutions with increasing polarity as eluents, to afford 13 fractions. Fractions8–9, which were eluted with hexane–ethyl acetate (75:25, v/v),were purified by crystallization in acetone to give the phenolic diterpene carnosol (CA) (Compound 1) (76 mg, 0.8%).This isolated compound presents as colour less crystals, with a melting point (m.p.) of 215–219 C (Figure 1). Part of the AcOEt fraction (8.83 g) was also submitted to the same silica gel column using hexane-ethyl acetate solutions in increasing order of polarity as eluent, to give 33 fractions. Fractions7–9 were met and eluted with hexane–ethyl acetate (75:25, v/v) and purified by crystallization in ethanol, yielding the triterpene betulinic acid (BA) (Compound 2) (43 mg, 0.48%). BA presented as a white powder, with an m.p. of 296–298 C (Figure 1). The structures of the known compounds were identified by spectroscopic data (1 H NMR, 13 C NMR (Varian AS-400 – Palo Alto,CA, USA), and IR – Perkin–Elmer FTIR 16 PC, Beaconsfield,

England). The results were compared with spectral data obtained from the literature (Mahato & Kundu, 1994; Pukalskas, Van Beek & Waard, 2005), as well as co-thin layer chromatography with authentic samples.

[Figure 1 near here]

2.3. HPLC profile of Rosmarinus officinalis fractions

The liquid chromatography (HPLC) profile was obtained using Varian ProStar 310 equipment with a UV/vis Detector (monitoring 210 nm) (Walnut Creek, CA, USA), a manual injector, and the StarFinder version 5.5 software. The HPLC apparatus was equipped with a ChromSpher 5 C18 column (4.6 mm 250 mm i.d.) (Walnut Creek, CA, USA). In the mobile phase the following substances were used: methanol (A), acetonitrile (B) and water (C) with a flow rate of 1.0 ml/min. The following elution profile was used: 0–7.5 min 60:0:40 (A:B:C) (isocratic); 7.5–20 min 0:100:0 (linear); 20–25 min 0:100:0 (isocratic). An equilibration period of 10 min was also included between runs. The carnosol used as standard for quantification was obtained according to Benincá, Dalmarco, Pizzolatti & Fröde (2011). The triterpenes betulinic acid, oleanolic acid, ursolic acid and rosmarinic acid (Sigma–Aldrich, Steinheim, Germany) also was used as standard.

2.4. Distillation of essential oil and preparation of essential oil free fraction

Fresh aerial parts (200g) were ground prior to the operation and then ground rosemary was submitted to water distillation for 4 h using a Clevenger apparatus. The distilled essential oil were dried over anhydrous sodium sulfate, filtered and stored at 4°C. After removing the essential oil, the leaves were re-extracted with alcohol 96% to obtain the essential oil free fraction (13.2g, 6.6%).

2.5. Chromatographic analysis

The quantification of essential oil was performed on a Gas Chromatograph (GC) -Shimadzu 14 B with a Flame Ionization Detector (GC-FID), column OV-5 (30 m x 0.25 mm i.d. x 0.25 µm film), N_2 as flow gas with constant pressure of 80 kPa. The split ratio was 1/150 and injection volume

was 0.3 µL of the oil. Injector and detector were held at 250°C and 300°C, respectively. The following program was: 50 ^{o}C for 3 min, rate of 5°C min^{-1} until 270^{o}C and hold for 8 min. The identification of the compounds was performed on a Gas Cromatograph coupled with Spectrometer Mass (GC-MS) Varian®CP 3800-Saturn 2000, Scanning (1 scan s^{-1}) was performed in the range of 39– 400 m/z and using electron impact ionization at 70 eV. The column used was CPSil 8CB (30 m x 0.25 mm i.d., 0.25 µm film). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL min $^{-1}$ in a split ratio of 1:20 and the following program rate was: 50^{o} C hold for 1 min, rate of 3^{o} C min^{-1} until 240^{o} C; injector: 250^{o} C. The compounds were identified by using three different analytical methods: (1) Arithmetic indices (AI), (2) GC–MS retention indices (authentic chemicals), and (3) mass spectra (authentic chemicals and NIST98 spectral library collection). The Sigma retention index standard (Sigma Aldrich, USA), used in this study, consisted of a mixture of aliphatic hydrocarbons ranging from C10 through C30, dissolved in hexane. It is designed to be used to obtain Kovats or Arithmetic-type gas chromatographic retention indices, which are useful for preliminary identification of unknown compounds. In this study the arithmetic retention index value is calculated for a peak by comparing its retention characteristics to those of the two closest eluting aliphatic hydrocarbons from the retention index standard, analyzed under identical conditions. Presumptive identifications can often be made by comparing the Arithmetic retention index value to a value previously published in literature references (Adams, 2007).

2.6. Animals

Male Swiss mice (60 to 70 days old weighing 45-50 g) were maintained at constant room temperature (21±1°C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 24 h before the behavioral procedure. Animals were randomly distributed into specified experimental groups. All experiments were carried out between 9:00 and 16:00 h, with each animal used only once (N=6-9 animals per group). The procedures in this study were performed in accordance with the National

188 Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics
189 Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the
190 number of animals used in the experiments.
191
192 ***2.7. Drugs and treatment***
193 The fractions: hexane (HEX), ethyl acetate (AcOEt), ethanolic (ET), and essential oil free
194 fraction (EOF) of *Rosmarinus officinalis* (0.1-100 mg/kg, p.o.) were administered acutely by oral
195 route (p.o.) 60 min before the TST or open- field test. To address some of the compounds isolated
196 from the extract of *Rosmarinus officinalis* as possible active principles responsible for the
197 antidepressant-like effect or that causes antidepressant-like action in the TST, animals were treated
198 with: carnosol (0.01-10 mg/kg, p.o.) and betulinic acid (0.1-10 mg/kg, p.o.), which were dissolved in
199 distillated water with 10 % Tween 80 and administered acutely by oral route (p.o.), 60 min before the
200 TST or open- field test. In another set of experiments, the essential oil was dissolved in mineral oil
201 and administered acutely by oral route (p.o.) 60 min before the TST or open- field test. The
202 dissolution of the fractions, isolated compounds and essential oil was freshly done immediately
203 before its administration by gavage. A control group received appropriate vehicles (distillated water
204 or mineral oil). . Fluoxetine (10 mg/kg, p.o.) from Sigma Chemical Company, St. Louis, MO, U.S.A.
205 was used as antidepressant classical and positive control. Drugs were dissolved in distillated water
206 with 10% Tween 80, except ethanolic fraction (ET) that was diluted in saline with 10% ethanol. All
207 fractions and compounds were administered by gavage, in a constant volume of 10 ml/kg body
208 weight.
209
210 ***2.8. Behavioral Tests***
211 ***2.8.1. Tail suspension test (TST)***
212 The total duration of immobility induced by tail suspension was measured according to the
213 method described by Steru, Chermat, Thierry & Simon (1985). Briefly, mice both acoustically and

visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was registered during a 6 min period (Machado et al., 2009).

2.8.2. Open-field test

To assess the possible effects of the fractions, isolated compounds and essential oil of *Rosmarinus officinalis* on locomotor activity mice was evaluated in the open- field paradigm as previously described (Machado et al., 2009). Mice were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 squares. Number of crossings (number of squares crossed by the animal with the four paws) was used to evaluate locomotor activity (Machado et al., 2009). These parameters were registered in a 6-min period.

2.9. Statistical analysis

Comparisons between experimental and control groups were performed by one-way-ANOVA, (dose–response curves) followed by Tukey's HSD post hoc test when appropriate. A value of $P<0.05$ was considered to be significant.

3. Results

Several fractions, essential oil and isolated compounds were submitted to a biological and phytochemical investigation to study their antidepressant-like activity following a bioassay-oriented fractionation and compound isolation procedure.

3.1. Phytochemical analysis and High- performance liquid chromatographic profile (HPLC)

The spectroscopic data of carnosol and betulinic acid used in this experiment was previously reported by Benincá, Dalmarco, Pizzolatti & Fröde (2011). Calibration curves of were prepared by analysis of calibration solutions of investigated compounds in the concentration range from 0.01 to 1.5 mg mL–1. Five calibration solutions were injected in triplicate. Curves were constructed by linear regression of the peak-area ratios (y) of each analyte, versus concentrations (x). The *r2* values were

241 in the range from 0.990 to 0.999 which confirmed the linearity of the method. The correlation
242 coefficients (r2) and their regression equations of calibration curves for silylated investigated
243 compounds were: rosmarinic acid (0.9900; y=2E+07x -12203), carnosol (0.9980; y=2E+07 +33239);
244 betulinic acid (0.9990; y= 2E+06 + 20006), oleanolic acid (0.9980; y= 3E+06 + 19108) and ursolic
245 shown in Table 1. The compounds quantified were: carnosol, ursolic acid, oleanolic acid, betulinic
246 acid and rosmarinic acid. In this context, as observed in Table 1, carnosol is as major compound in
247 the Acoet 1 (37.06%), EOF (12.80%) and HEX (5.85%) fractions. However, ursolic acid is more
248 pronounced in the Acoet 2 (48.32%) and ETOH (18.48%) fractions. Although present at a lower
249 concentration, the triterpene betulinic acid was found mainly in Acoet 2 (10.18%), ETOH (3.06%)
250 and EOF (2.14%) fractions. The phenolic acid rosmarinic acid was found mainly in the EOF (4.74%)
251 fraction and oleanolic acid (7.93%) in Acoet 2 fraction.
252 [Table 1 near here]
253
254 ***3.2. Essential oil analysis***
255 Eleven compounds representing 86.97% of the total oil was identified (Table 2). The main volatiles
256 are 1,8-cineole (45.1%), camphor (21.75%), α-pinene (4.62%), borneol (4.77%), α-terpineole
257 (4.57%).
258 [Table 2 near here]
259
260 ***3.3. Behavioral analysis***
261 ***3.3.1. Effect of acute treatment with the fractions of Rosmarinus officinalis on the immobility time***
262 ***in the TST and locomotor activity in the open-field test***
263 The effects of the oral administration of the all fractions of *Rosmarinus officinalis* on the
264 immobility time in the TST were shown in Table 3. The AcOEt 1 fraction of *Rosmarinus officinalis*,
265 by oral route at 0.1, 1, 10, 100 mg/kg decreased the immobility time in the TST as compared to the
266 control group. The one-way ANOVA revealed a significant effect of this fraction [$F(4,42)=6.29$,
267 $P<0.01$] in the TST. However, the AcOEt1 fraction did not cause any change in the locomotor

activity of mice as compared to control group, as shown by one-way ANOVA [F(4,26) = 0.26, P= 0.90].

[Table 3 near here]

The AcOEt 2 fraction of *Rosmarinus officinalis*, given by oral route at the dose of 0.1 and 1 mg/kg decreased the immobility time in the TST, as compared to the control group. The one-way ANOVA revealed a significant effect of the AcOEt 2 fraction in TST [F(4,35)= 23.19, P<0.01]. The oral administration of the AcOEt 2 fraction caused a decreased number of crossings (0.1 mg/kg) in the open-field test, as can be observed in Table 3. The one-way ANOVA revealed a significant effect of this treatment in the locomotor activity: [F(4,32)= 2.81, P<0.05] of mice in open-field test as compared to the control group.

The HEX fraction of *Rosmarinus officinalis*, administered by oral route at 0.1, 1 and 10 mg/kg decreased the immobility time in the TST as compared to the control group. The one-way ANOVA revealed a significant effect of HEX fraction [F(4,40)=22.99, P<0.01] in TST. Furthermore, the HEX fraction (100 mg/kg, p.o.) decreased locomotor activity of mice in the open-field test, when compared to control group. The one-way ANOVA showed a significant effect of locomotion [F(4,32)=5.12, P<0.01].

The results depicted in Table 3 also show that ETOH fraction of *Rosmarinus officinalis*, by oral route at 0.1, 1, 10 and 100 mg/kg decreased the immobility time in the TST as compared to the control group. The one-way ANOVA revealed a significant effect of the ETOH fraction [F(4,39)=4.78, P<0.01] in TST. The results show that the acute administration of this fraction did not cause any significant change in the locomotion activity of mice as compared to control group [F(4,30)=1.51, P=0.22].

3.3.2. Effect of acute treatment with essential oil and essential oil free fraction (EOF) of Rosmarinus officinalis on the immobility time in the TST and locomotor activity in the open-field test

As can be observed in Table 3, the acute administration by oral route at 0.1, 1, 10 and 100 mg/kg of essential oil and of EOF fraction were similarly able to decrease the immobility time in the TST, as compared to the control group. The one-way ANOVA revealed a significant effect of essential oil [$F(4,35)=12.52$, $P<0.01$] and EOF fraction [$F(4,39)=6.93$, $P<0.01$] in the TST. Additionally, it was shown that the treatment of the essential oil and EOF fraction did not cause any significant change in the locomotion activity of mice as compared to control group in the open-field test. The one way ANOVA did not show significant effect the treatment which essential oil in the locomotion: [$F(4,25)=0.98$, $P=0.43$]. Similarly, the treatment the EOF fraction did not alter the locomotion :[$F(4,35)=0.58$, $P=0.67$].

3.3.3. Effect of acute treatment with isolated compounds of Rosmarinus officinalis on the immobility time in the TST and locomotor activity in the open-field test

The effects of the oral administration of carnosol, a compound isolated from *Rosmarinus officinalis*, on the immobility time in the TST are shown in Table 3. Carnosol given by oral route at the dose of 0.01 and 0.1mg/kg decreased the immobility time, as compared to the control group. The one-way ANOVA revealed a significant effect of carnosol in the TST [$F(4,36)= 4.59$, $P<0.01$]. The oral administration of carnosol decreased the locomotion activity (10 mg/kg, p.o.) of mice as compared to control group, [$F(4,38)=3.09$, $P<0.05$] in the open-field.

The results show that betulic acid, another compound isolated from *Rosmarinus officinalis*, administered by oral route at 10 mg/kg decreased the immobility time in the TST as compared to the control group. The one-way ANOVA revealed a significant effect of betulic acid [$F(3,28)=3.03$, $P<0.05$] in the TST. The results show that the acute administration of this compound did not cause any significant change in the locomotion of mice as compared to control group [$F(3.31)=0.52$, $P=0.66$].

We also show that the antidepressant fluoxetine (10 mg/kg, p.o.), used here as a positive control, produced a significant reduction in the immobility time in the TST (Table 3). The one-way

ANOVA revealed a main effect of treatment [$F(1,15)= 29.25$, $P<0.01$] in the TST, but not in the locomotion [$F(1,12)=0.05$, $P=0.81$] in the open-field-test.

4. Discussion

The TST is a behavioral model predictive of antidepressant activity that is sensitive to antidepressants from different pharmacological classes, extracts and isolated compounds of plants (Capra et al., 2010; Cryan, Mombereau & Vassout, 2005; Machado et al., 2009; Steru, Chermat, Thierry & Simon, 1985).

The present study showed that the all fractions derived from the crude extract of *Rosmarinus officinalis* demonstrated antidepressant-like effect in TST, as well as carnosol and betulic acid, main compounds isolated and identified from this plant that could be, in part, responsible for its antidepressant activity. Interestingly, the essential oil also demonstrated anti-immobility effect in this predictive test in mice. Noteworthy, the effect of the extract of *Rosmarinus officinalis* in the TST was similar to the effect produced by the oral administration of fluoxetine, used as a positive control. These results are consistent with the ethnopharmacological use of this plant for the treatment of depression (Balmé, 1978; Duke, 2000; Heinrich, Kufer, Leonti & Pardo-de-Santayana, 2006), reinforcing the previous evidence from our group which demonstrates the potential antidepressant effect of the hydroalcoholic crude extract in this same experimental protocol (Machado et al., 2009). Furthermore, the present study is the first work which demonstrated the antidepressant-like effect of carnosol and betulic acid, as well of the essential oil of *Rosmarinus officinalis* when administered orally to mice.

Important to note, this study provide convincing evidence that the fractions, isolated compounds (carnosol and betulinic acid) and essential oil of *Rosmarinus officinalis* administered by oral route produces a specific antidepressant-like effect in TST, since the reduction of immobility time elicited by these administrations cannot be attributable to any psychostimulant effect (increased locomotion when as assessed in open-field test).

In our study, the fractions Acoet 1, ET and EOF fraction, isolated from *Rosmarinus officinalis* L., showed similar antidepressant-like effect in the range dose of 0.1-100 mg/kg, p.o. (with percent of reduction of 18-34% of immobility time in TST). However, it was also observed a difference in dose range and percent of reduction in the Acoet 2 (0.1-1 mg/kg, p.o.; 27-43%) and HEX (0.1-10 mg/kg, p.o.; 28-45%) fraction in the TST. Thus, these fractions showed an effect at lower concentrations and affording a higher percentage of reduction, as compared with the other fractions tested. Probably, the cause by such distinct biological effects may be linked to the differences in the chemical composition of each fraction. Important to note, the examination of the fractions of this plant using HPLC revealed the presence of carnosol, betulinic acid and ursolic acid, as some of the main compounds. The compounds that predominate in each fraction can be decisive for the antidepressant potential of the fractions. Therefore, carnosol is the major constituent of the Acoet 1, HEX and EOF fraction; whereas ursolic acid is the major compound in the Acoet 2 and ETOH fractions. It should be considered that other compounds were also detected in these fractions such as betulinic acid, found mainly in Acoet 2, ETOH and EOF fractions; rosmarinic acid in the EOF fraction and oleanolic acid in Acoet 2 fraction.

The antidepressant-like effect of the acutely administered carnosol (0.01-0.1 mg/kg, p.o.) and betulinic acid (10 mg/kg, p.o.) was observed at low doses in the TST. However, despite the present study indicates that carnosol and betulinic acid may be responsible for the antidepressant action of the fractions from *Rosmarinus officinalis*, we cannot rule out the participation of other phytochemical compounds in this biological effect. It is worth noting that ursolic acid isolated from *Rosmarinus officinalis* was also effective in producing an antidepressant-like effect in TST in mice and this action may be due to involvement of the dopaminergic system (Machado et al., submitted). Furthermore, rosmarinic and caffeic acids were shown to produce an antidepressant effect in the FST (Takeda, Tsuji, Inazu, Egashira & Matsumiya, 2002).

The present study showed that the essential oil of *Rosmarinus officinalis* also produced antidepressant-like effect (0.1-100 mg/kg, p.o.) in TST, without altering the locomotion in open-field test. Interestingly, our results are in agreement with a recent study that reported that the essential oil

of *Rosmarinus officinalis* produced an antidepressant-like effect in the FST, another test predictive of antidepressant potential, in rats (Seol et al., 2010). The chemical analysis revealed that the essential oil of this plant contained as main compounds: 1,8-cineole (45.14%), camphor (21.75%), borneol (4.77%), α-pinene (4.62%), α-terpineole (4.57%). In general, the essential oil of *Rosmarinus officinalis* can be classified in three chemotypes, based on a chemical analysis of its predominant compounds, namely, *cineoliferum* (high content in 1,8-cineole), *camphoriferum* (camphor >20%) and *verbenoniferum* (verbenone >15%). Important to note, the chemical analysis is complex and many others chemotypes have been recognized according to the relative abundance of other relevant compounds such as α-pinene and others constituents (Napoli, Curcuruto & Ruberto, 2010). Moreover, the diversity of chemotypes can be explained by the fact that this plant is cultivated in different regions, with a variety of soil conditions and climate that interfere with the phytochemical composition of the essential oil, as well as their chemotype. In present study, considering the high content of 1,8-cineole (45.14%) in the essential oil, it may be characterized as *cineoliferum* chemoype. It remains to be established if 1,8-cineole is the compound responsible for the antidepressant-like effect of the essential oil.

5. Conclusion

This study suggests that the antidepressant potential of several fractions of *Rosmarinus officinalis* L may be attributed, at least in part, to the presence of carnosol and betulinic acid. Moreover, the essential oil of this plant also produced antidepressant-like effect and its main compound, 1,8-cineole may be involved in this action. However, other compounds could play a role to the antidepressant-like of *Rosmarinus officinalis* L. and further studies are in progress in order to clarify the bioactive principles responsible for these activities, as well as the mechanisms underlying their action. The present study clearly reinforces the notion that *Rosmarinus officinalis* has a therapeutical potential as an antidepressant, since the effects of all fractions, isolated compounds and essential oil in the TST is similar to the one produced by the classical antidepressant fluoxetine.

399 Considering the widespread use of this plant as a condiment, its antidepressant property may be of
400 pharmacological and nutraceutical interest and should be confirmed in future clinical studies.
401
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Legend to the Figures

Figure 1. Scheme of fractionation and isolation of the compounds and obtention of the essential oil of *Rosmarinus officinalis*

Table 1. Contents of phenolic and terpenes compounds in fractions of *Rosmarinus officinalis* in % (w/w). Each value is the mean of three replications.

Compound	HEX	Acoet 1	Acoet 2	ETOH	EOF
Rosmarinic acid	1.71	n.d.	0.79	0.49	4.74
Carnosol	5.85	37.06	8.27	n.d.	12.80
Betulinic acid	n.d.	n.d.	10.18	3.06	2.14
Oleanolic acid	n.d	n.d.	7.93	0.95	1.21
Ursolic acid	n.d	n.d.	48.32	18.48	4.95

n.d. = not detected

Table 2. Mean percentage composition of essential oil of *Rosmarinus officinalis*.

Compound	RT	%REL	AI t	AI c
α-pinene	11.42	4.62	932	931
Camphene	12.13	1.37	946	948
β-pinene	13.61	1.07	974	972
β-mircene	14.57	0.54	988	986
p-cimeneo	16.23	0.76	1020	1018
1,8 cineole	16.61	45.14	1026	1024
β-linalool	20.34	1.26	1095	1093
Camphor	22.49	21.75	1141	1139
Borneol	23.65	4.77	1165	1164
4-terpineole	24.25	1.12	1174	1171
α-terpineole	24.95	4.57	1186	1185

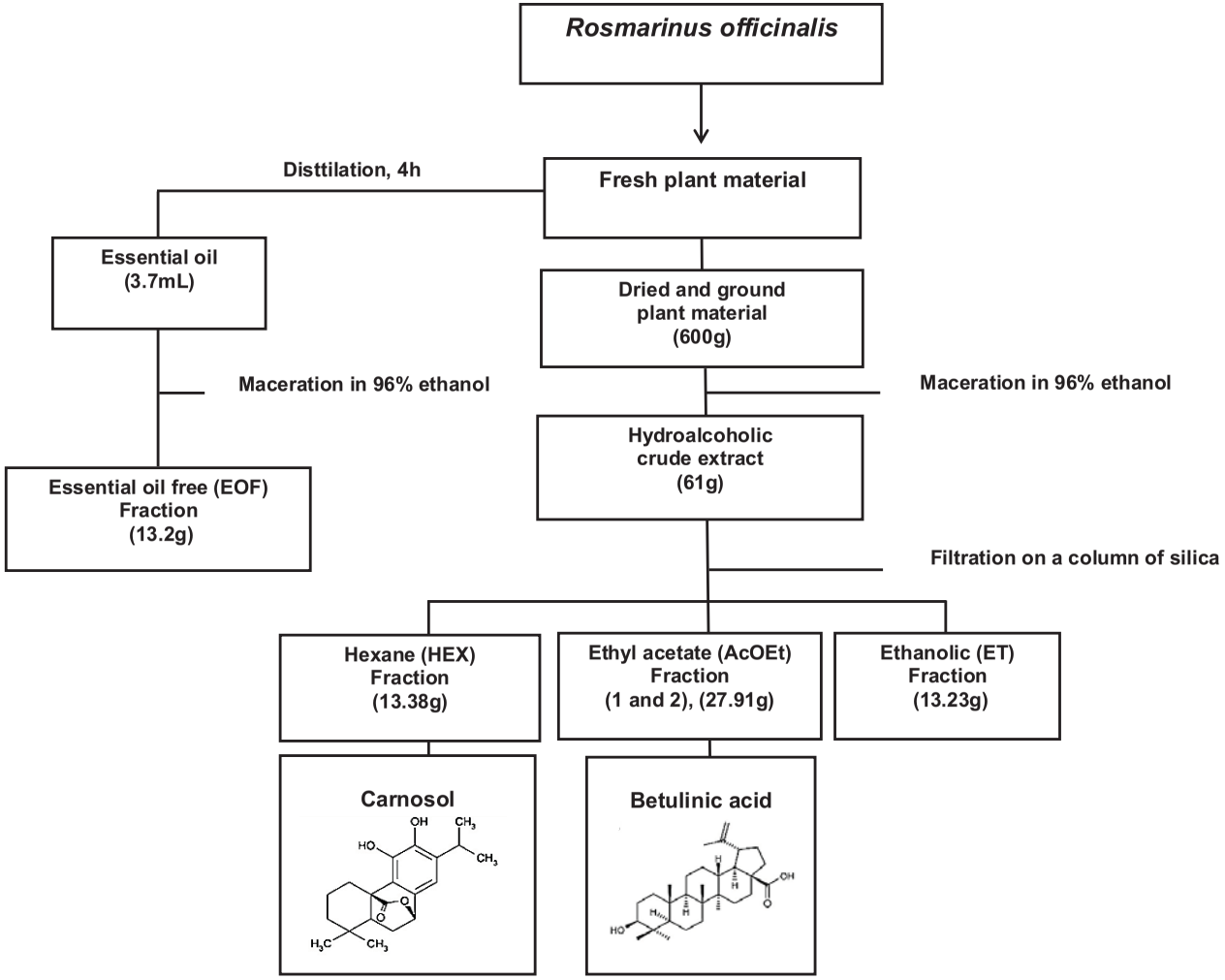
RT=retention time; %REL= relative percentage; AIt=Teoric Arithmetic Index; AIc= Calculated Arithmetic Index

Table 3. Effect of the acute treatment of mice with fractions, essential oil and isolated compounds of *Rosmarinus officinalis* on the immobility time in the tail suspension test and on the number of crossings in the open-field test.

Compound	Dose (mg/kg, p.o.)	Immobility Time (s)	% of reduction in the TST	Crossings
Vehicle	**-**	227.3±7.3	-	80.0±11.9
AcOEt1	**0.1**	165.4 ±7.9**	**27.2**	90.6±9.4
AcOEt1	**1**	166.0 ±12.1**	**26.9**	81.0±9.0
AcOEt1	**10**	179.8±8.7*	**20.8**	82.8±13.9
AcOEt1	**100**	184.7±14.3*	**18.7**	74.1±12.2
Vehicle	**-**	194.2±4.6	**-**	83.6±10.0
AcOEt2	**0.1**	141.1±8.8**	**27.3**	33.8±12.9*
AcOEt2	**1**	109.5±10.5**	**43.6**	53.8±16.6
AcOEt2	**10**	196.0±7.2	**-**	80.8±10.8
AcOEt2	**100**	181.0±8.1	**-**	72.0±10.0
Vehicle	**-**	218.1±4.2	**-**	82.0±11.9
HEX	**0.1**	153.1±7.9**	**29.8**	111.1±13.1
HEX	**1**	118.8±15.7**	**45.5**	71.1±14.7
HEX	**10**	128.6±7.0**	**41.0**	79.7±14.1
HEX	**100**	186.2±9.4	**-**	27.8±6.9*
Vehicle	**-**	203.5±4.8	**-**	100.4±10.3
ETOH	**0.1**	153.6±17.5**	**24.5**	105.0±7.2
ETOH	**1**	165.1±3.4*	**18.8**	71.2±20.1
ETOH	**10**	162.6±10.2*	**20.1**	77.4±14.7
ETOH	**100**	164.1±7.3*	**19.3**	105.1±9.3
Vehicle	**-**	200.8±5.5	**-**	39.2±16.7
EOF	**0.1**	134.3±6.1**	**33.1**	22.7±7.9
EOF	**1**	131.7±13.6**	**34.4**	30.8±9.9
EOF	**10**	140.5±15.4**	**30.0**	41.5±13.9
EOF	**100**	131.0±14.3**	**34.8**	45.1±7.4
Vehicle	**-**	236.1±6.9	**-**	60.1±18.3
EO	**0.1**	193.1±8.6*	**18.2**	82.1±8.9
EO	**1**	197.2±11.6*	**16.4**	92.0±8.3
EO	**10**	173.7±10.8**	**26.4**	69.8±17.4
EO	**100**	149.8±7.2**	**36.5**	67.3±5.2
Vehicle	**-**	197.1±2.7	**-**	53.9±9.7
Carnosol	**0.01**	168.8±7.7*	**14.6**	22.7±6.1
Carnosol	**0.1**	165.7±6.5**	**15.7**	25.0±8.5
Carnosol	**1**	177.1±8.0	**-**	32.8±12.5
Carnosol	**10**	179.1±5.9	**-**	11.6±8.4*
Vehicle	**-**	168.7±5.4	**-**	51.0±17.2
Betulinic acid	**0.1**	131.5±15.4	**-**	52.1±12.7
Betulinic acid	**1**	137.0±13.7	**-**	34.0±9.9
Betulinic acid	**10**	118.7±11.8*	**14.6**	38.4±9.7
Vehicle	**-**	185.7±6.5	**-**	43.5±10.8
Fluoxetine	**10**	131.6±7.6**	**29.1**	47.7±13.7

The fractions and the isolated compounds were acutely administered by p.o. route at the dose range indicated in the table. The classical antidepressant fluoxetine was used as a positive control. Each column represents the mean +S.E. of 6–9 animals. *P<0.05, ** P<0.01 as compared with the vehicle-treated control group. The percent of reduction of the immobility time in the TST is shown for the groups in which a significant effect was revealed by ANOVA followed by Tukey's HSD post hoc test.

Figure 1


Rosmarinus officinalis
Disttilation, 4h
Fresh plant material
Essential oil
(3.7mL)
Dried and ground
plant material
(600g)
Maceration in 96% ethanol
Maceration in 96% ethanol
Hydroalcoholic
crude extract
(61g)
Essential oil free (EOF)
Fraction
(13.2g)
Filtration on a column of silica
Hexane (HEX)
Fraction
(13.38g)
Ethyl acetate (AcOEt)
Fraction
(1 and 2), (27.91g)
Ethanolic (ET)
Fraction
(13.23g)
Carnosol
Betulinic acid

CAPÍTULO 3

Antidepressant-like effect of ursolic acid isolated from *Rosmarinus officinalis* L. in mice: evidence for the involvement of the dopaminergic system

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Antidepressant-like effect of ursolic acid isolated from *Rosmarinus officinalis* L. in mice: evidence for the involvement of the dopaminergic system

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Abstract

Ursolic acid, a constituent from *Rosmarinus officinalis*, is a triterpenoid compound which has been extensively known for its anticancer and antioxidant properties. In the present study, we investigated the antidepressant-like effect of ursolic acid isolated from this plant in a predictive test of antidepressant property, the tail suspension test (TST). Furthermore, the involvement of dopaminergic system in its antidepressant-like effect was investigated in this test in mice. Ursolic acid at a dose of 0.1 mg/kg, p.o. reduced the immobility time in the TST. The effect of ursolic acid (0.1 mg/kg, p.o.) in TST was prevented by the pretreatment of mice with SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist) and sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist). The administration of a sub-effective dose of ursolic acid (0.001 mg/kg, p.o.) in combination with sub-effective doses of SKF38393 (0.1 mg/kg, s.c., a dopamine D_1 receptor agonist), apomorphine (0.5 μg/kg, i.p., a dopamine D_2 receptor agonist) or bupropion (1 mg/kg, i.p., a dopamine reuptake inhibitor) reduced the immobility time in the TST as compared with either drug alone. Ursolic acid and dopaminergic agents alone or in combination did not cause significant alterations in the locomotor and exploratory activities. These results indicate that the antidepressant-like effect of ursolic acid in the TST is likely mediated by an interaction with the dopaminergic system, through the activation of dopamine D_1 and D_2 receptors.

Keywords: Antidepressant; Dopaminergic System; Ursolic acid; Tail Suspension Test

Abbreviations: ANOVA, analysis of variance; DMSO, dimethylsulfoxide; SCH23390, (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetra-hydro-1H-3-benzazepine hydrochloride; SKF 38393, (1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride; TST, tail suspension test.

1. Introduction

Depression is projected to become the second biggest contributor to the global burden of disease and disability by the year 2020 (Pitchot et al., 2010). It is a heterogeneous disorder often manifested with symptoms at the psychological, behavioral and physiological levels. Noteworthy, there are high rates of comorbidity of psychiatric and neurodegenerative disorders, type 2 diabetes mellitus, cancer, cardiovascular disease and depression (Evans et al., 2005; Krishnan and Nestler, 2008).

The treatment of depression with conventional antidepressants (i.e., monoamine oxidase inhibitors, tricyclics, selective serotonin reuptake inhibitors and selective noradrenaline reuptake inhibitors) has several drawbacks. The heterogeneity of clinical response to antidepressant and susceptibility to adverse effects are major clinical problems and support the research for new therapeutical agents to treat depression (Brunello et al., 2002; MacGillivray et al., 2003; Wong and Licinio, 2001). Herbal therapies may be effective alternatives in the treatment of depression, as include the case of St John's wort (Akhondzadeh and Maleki, 2006; Bilia et al., 2002; Whiskey et al., 2001) and the search for novel pharmacotherapy from medicinal plants and compounds isolated from plant extracts for this psychiatric disorder has progressed significantly (Zhang, 2004).

Aiming at searching new antidepressant agents, our group has studied the antidepressant potential of some plant extracts (Freitas et al., 2010; Machado et al., 2007, 2009; Rodrigues et al., 2002) and bioactive compounds (Capra et al., 2010; Machado et al., 2008). We have recently demonstrated that the extract of *Rosmarinus officinalis* produces an antidepressant-like effect in the mouse tail suspension test (TST) by a mechanism dependent on the interaction with the monoaminergic systems (Machado et al., 2009). One of the main constituent present in

Rosmarinus officinalis is ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid compound, that has a broad range of biological effects, such as antioxidant (Tsai and Yin, 2008), anti-inflammatory (Baricevic et al., 2001; Benincá et al., 2010), anti-tumoral (Yan-xia et al., 2010), anti-diabetic (Jang et al., 2009) and neuroprotective (Lu et al., 2007). Noteworthy, ursolic acid was shown to be able to protect PC12 cells against the dopaminergic neurotoxin MPP^+ (Tsai and Yin, 2008).

Several studies have indicated that the dopaminergic system plays a significant role in the pathophysiology of depression and on the mechanisms of action of some current prescribed antidepressant agents, mainly bupropion (D'Aquila et al., 2000; Dunlop and Nemeroff, 2007; Papakostas, 2006). The investigation of novel antidepressant agents that act on this system is justified to improve outcomes for patients with treatment-resistant and non remitting depression (Dunlop and Nemeroff, 2007). Therefore, this study aims, firstly, to examine the antidepressant-like action of ursolic acid isolated from *Rosmarinus officinalis* in the mouse TST, and secondly, to investigate by the use of pharmacological procedures the possible participation of the dopaminergic system in its antidepressant-like action.

2. Methods

2.1. Isolation of ursolic acid from *Rosmarinus officinalis*

Stems and leaves of *Rosmarinus officinalis* (Labiatae) were collected in Santo Amaro do Imperatriz, Santa Catarina, and identified by Dr. Daniel Falkenberg, from the Department of Botany, Federal University of Santa Catarina. A voucher specimen (Excicata number 34918) was deposited in the Herbarium of the Department of Botany, Federal University of Santa Catarina, Santa Catarina, Brazil. The procedure to obtain the crude extract from *Rosmarinus officinalis* was described previously (Machado et al, 2009). The crude extract was subjected to passage on a short

silica gel 60 (Vetec -63-230 mesh) column with hexane, ethyl acetate and ethanol in order of polarity, to give three fractions: the hexane (HEX), ethyl acetate (AcOEt) and ethanolic (EtOH). Part of the AcOEt fraction (8.83 g) was submitted to the silica gel column eluted with hexane-ethyl acetate gradient in increasing polarity, to give 33 fractions. Fractions 20-33 eluted with hexane-acetone solution (8:2 v/v) were met and purified by flash chromatography (silica gel 60 column; Vetec- 230-400 mesh) with isocratic elution (Hexane:Acetone 4:1 v/v) to give ursolic acid (87 mg).

2.2. Animals

Male Swiss mice (40-50 g) were maintained at constant room temperature (21±1°C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 24 h before the behavioral procedure. The animals were randomly distributed into specified experimental groups. All experiments were carried out between 11:00 and 17:00 h, with each animal used only once (N=7-10 animals per group). All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.3. Drugs and treatment

The following drugs were used: (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390), sulpiride, apomorphine, 1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SKF38393) and bupropion (all from Sigma Chemical Company, St. Louis, MO, U.S.A.). All drugs were administered by intraperitoneal (i.p) route, except SCH23390 and SKF38393 that were administered by

subcutaneous (s.c.) route. All drugs were administered in a constant volume of 10 ml/kg body weight. Drugs were dissolved in saline, except bupropion that were diluted in distilled water and sulpiride that was diluted in saline with 5% dimethylsulfoxide (DMSO). Control animals received appropriate vehicle.

Ursolic acid (0.001-10 mg/kg) was dissolved in distilled water with 10% Tween 80 and administered acutely by oral route (p.o.). The solution of ursolic acid was freshly done from the liophylized power immediately before its administration by gavage. A control group received distilled water with 10% Tween 80 as vehicle.

2.4. Experimental design

In order to investigate the antidepressant-like effect produced by an oral administration of ursolic acid, it was administered at the dose range 0.001-10 mg/kg (p.o.), 60 min before the TST or open-field test.

In order to investigate the involvement of the dopaminergic system in the antidepressant-like action of ursolic acid in the TST, the following experimental protocols were performed:

Firstly, mice were pretreated with vehicle (control group), SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist) or sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist) (Capra et al., 2010; Machado et al., 2007) and 30 min later they received vehicle or ursolic acid (0.1 mg/kg, p.o.). A further 60 min was allowed to elapse before the animals were tested in the TST or open-field test.

In a second experimental approach, we studied the possible synergistic antidepressant-like effect elicited by the administration of a sub-effective dose of ursolic acid (0.001 mg/ kg, p.o.) with sub-effective doses of SKF38393 (0.1 mg/kg, s.c., a dopamine D_1 receptor agonist) or

apomorphine (0.5 μg/kg, i.p., a dopamine D_2 receptor agonist). Ursolic acid or vehicle were administered 30 min before the administration of the dopamine receptor agonists. In another set of experiments, a sub-effective dose of ursolic acid (0.001 mg/kg, p.o.) was administered immediately before the administration of a sub-effective dose of bupropion (1 mg/kg, p.o., a dopamine reuptake inhibitor). A further 60 min was allowed to elapse before the animals were tested in the TST or open-field test.

All the experimental protocols and drug doses utilized in the present study were previously evaluated in the TST by our group (Binfaré et al., 2009; Capra et al., 2010; Cunha et al., 2008; Machado et al., 2007, 2009; Rodrigues et al., 2002).

2.5. Behavioral tests

2.5.1. Tail suspension test (TST)

The TST has become one of the most widely used tests for assessing antidepressant-like activity in mice. It is based on the fact that animals subjected to the short-term inescapable stress of being suspended by their tail, will develop an immobile posture. The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period. Mice were considered immobile only when they hung passively and completely motionless. Antidepressant treatments reduce the time of immobility and increase active escape behaviors displayed during inescapable situation the in the TST (Cryan et al., 2005). The immobility time was recorded by an observer blind to the drug treatment.

2.5.2. Open-field test

To assess the possible effects of ursolic acid and the other drugs used in the present study on locomotor and exploratory activities, an independent group of mice was evaluated in the open-field paradigm as previously described (Machado et al., 2007; Rodrigues et al., 1996). Mice were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 equal rectangles. The number of crossings defined as the rectangles crossed by the animal with its four paws and the number of rearings defined as the animal standing upright on its hind legs were registered during a period of 6 min. The number of crossings was considered as indicative of locomotor activity and number of rearings was an indicative of exploratory behavior (Felipe et al., 2007; Rodrigues et al., 1996). Crossings and rearings were recorded by an observer blind to the drug treatment. The floor of the open-field apparatus was cleaned with 10% ethanol between tests to remove any olfactory cues.

2.6. Statistical analysis

Comparisons between experimental and control groups were performed by analysis of variance (ANOVA) followed by Tukey HSD test when appropriate. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of acute treatment with ursolic acid on the immobility time in the TST and on the locomotor and exploratory activities in the open-field test

The effects of the oral administration of ursolic acid on the immobility time in the TST are shown in Fig. 1A. Ursolic acid given by oral route at the dose of 0.01 and 0.1mg/kg decreased the immobility time, as compared to the control group (Fig. 1A). The percent of reduction in the immobility time was 19.4% and 27.1%, respectively. The one-way ANOVA revealed a significant effect of ursolic acid in tail suspension test [$F(5,41)= 4.32$, $P<0.01$]. The effects of the oral

administration of ursolic acid (dose range 0.001-10 mg/kg, p.o.) in the open-field test are shown in Table 1. As shown in Table 1, the acute administration this compound did not cause any significant change in the locomotor activity of mice as compared to control group [$F(5,43)=2.11$, $P=0.08$]. Similarly, the results depicted in Table 1 show that ursolic acid (dose range 0.001-10 mg/kg, p.o.) did not significantly alter the number of rearings in the open-field test as compared to the control group [$F(5,43)=1.10$, $P=0.37$].

[Figure 1 and Table 1 near here]

3.2. Investigation of the dopaminergic mechanisms underlying the antidepressant-like effect of ursolic acid in the TST

The results shown in Fig 2A indicate that the pretreatment of mice with SCH23390 (0.05 mg/kg, s.c.) prevented the antidepressant-like effect elicited by ursolic acid (0.1 mg/kg, p.o.) in the TST. A two-way ANOVA showed significant differences for SCH23390 pretreatment [$F(1, 30)=30.42$, $P<0.01$], ursolic acid treatment [$F(1, 30)=14.79$, $P<0.01$] and SCH23390 pretreatment X ursolic acid treatment interaction [$F(1, 30)=18.08$, $P<0.01$]. Moreover, Fig 2B illustrates that the pretreatment with sulpiride (50 mg/kg, i.p.) also prevented the antidepressant-like effect of ursolic acid (0.1 mg/kg, p.o.) in the TST. A two-way ANOVA revealed significant differences for sulpiride pretreatment [$F(1, 29)=41.19$, $P<0.01$], ursolic acid treatment [$F(1, 29)=11.42$, $P<0.01$] and sulpiride pretreatment X ursolic acid treatment interaction [$F(1, 29)=30.57$, $P<0.01$].

Table 2 shows that ursolic acid (0.1 mg/kg, p.o.) administered alone or in combination with SCH23390 (0.05 mg/kg, s.c.) did not significantly cause changes in the locomotor activity of mice in the open-field test. The two-way ANOVA showed no significant effects of SCH23390 pretreatment [$F(1,30)=0.71$, $P=0.40$], ursolic acid treatment [$F(1, 30)=1.35$, $P=0.25$] and

SCH23390 pretreatment X ursolic acid interaction [F(1, 30)=0.96, P=0.33]. Similarly, administration of ursolic acid alone or in combination with SCH23390 did not cause any change in the rearing responses. The two-way ANOVA showed no significant effects of SCH23390 pretreatment [F(1,30)=0.01, P=0.92], ursolic acid treatment [F(1, 30)=1.94, P=0.17] and SCH23390 pretreatment X ursolic acid interaction [F(1, 30)=1.32, P=0.26].

Sulpiride (50 mg/kg, i.p.) alone or in combination with ursolic acid (0.1 mg/kg, p.o.) did not cause any significant alteration in the number of crossings and rearings in the open-field. Regarding crossing responses, two-way ANOVA showed no significant effects of sulpiride pretreatment [F(1, 29)=0.03, P=0.85], ursolic acid treatment [F(1, 29)=0.54, P=0.46] and sulpiride pretreatment X ursolic acid interaction [F(1, 29)=0.32, P=0.57]. Concerning rearings responses, two-way ANOVA showed no significant effects of sulpiride pretreatment [F(1, 29)=0.007, P=0.93], ursolic acid treatment [F(1, 29)=0.94, P=0.33] and sulpiride pretreatment X ursolic acid interaction [F(1, 29)=0.54, P=0.46].

[Figure 2 and Table 2 near here]

Additionally, in order to reinforce the notion that the antidepressant-like effect of ursolic acid in the TST is dependent on the activation of dopamine D_1 and D_2 receptors, in another set of experiments we investigated the effect of the administration of a sub-effective dose of ursolic acid (0.001 mg/kg, p.o.) in combination with sub-effective doses of the dopamine D_1 receptor agonist SKF38393, the dopamine D_2 receptor agonist apomorphine or the antidepressant bupropion on the immobility time in the TST. As shown in Fig. 3A, a sub-effective dose of ursolic acid (0.001 mg/kg, p.o.) produced a synergistic antidepressant-like effect with SKF38393 (0.1 mg/kg, s.c.), since the combined treatment produced a significant reduction (39.6%) in the immobility time as compared to the control group. The two-way ANOVA revealed significant differences of ursolic

acid pretreatment [F(1, 29)=27.87, P<0.01], SKF38393 treatment [F(1, 29)=4.56, P<0.05] and ursolic acid pretreatment X SKF38393 treatment interaction [F(1, 29)=10.60, P<0.01]. The results showed in Fig 3B indicate that the combined administration of apomorphine (0.5 μg/kg, i.p.) with ursolic acid (0.001 mg/kg, p.o.) produced a significant reduction (30.4%) in the immobility time as compared to the control group. The two-way ANOVA revealed significant differences of ursolic acid pretreatment [F(1, 29)=20.83, P<0.01] and ursolic acid pretreatment X apomorphine treatment interaction [F(1, 29)=6.94, P<0.01], but not of apomorphine treatment [F(1, 29)=2.48, P=0.12]. Fig. 3C shows that a sub-effective dose of ursolic acid (0.001 mg/kg, p.o.) combined with a sub-effective dose of bupropion (1 mg/kg, p.o.) also caused an antidepressant-like effect (39.4% reduction in the immobility time) in the TST. The two-way ANOVA revealed significant differences of ursolic acid treatment [F(1, 30)=16.85, P<0.01], bupropion treatment [F(1, 30)=10.55, P<0.01] and ursolic treatment acid X bupropion treatment interaction [F(1, 30)=4.41, P<0.05].

Table 3 shows that ursolic acid (0.001 mg/kg, p.o.) alone or in combination with SKF38393 (0.1 mg/kg, s.c.) did not cause any change in the locomotor activity of mice, as revealed by two-way ANOVA: ursolic acid pretreatment [F(1, 28)=2.69, P=0.11], SKF38393 treatment [F(1, 28)=0.02, P=0.88] and ursolic acid X SKF38393 interaction [F(1, 28)=1.103, P=0.75]. Also, the number of the rearings of mice treated with ursolic acid and/or SKF38393 was not significantly different from the control group. The two-way ANOVA showed no significant differences for ursolic acid treatment [F(1, 28)=2.00, P=0.16], SKF38393 treatment [F(1, 28)=0.20, P=0.65] and ursolic acid X SKF38393 interaction [F(1, 28)=0.13, P=0.72] on rearing responses.

Similarly, Table 3 shows that ursolic acid (0.001 mg/kg, p.o.) alone or in combination with apomorphine (0.5 μg/kg, i.p.) caused no significant effects on the number of crossings and

rearings in the open-field test as shown by two-way ANOVA: Crossings: ursolic acid treatment [F(1, 28)=0.45, P=0.50], apomorphine treatment [F(1, 28)=0.70, P=0.40] and ursolic acid X apomorphine interaction [F(1, 28)=1.07, P=0.30]; Rearings: ursolic acid pretreatment [F(1, 28)=0.00, P=0.96], apomorphine treatment [F(1, 28)=0.16, P=0.28] and ursolic acid X apomorphine interaction [F(1, 28)=1.06, P=0.31].

Table 3 also shows that the pretreatment of ursolic acid (0.001 mg/kg, p.o.) and bupropion (1 mg/kg, p.o.) alone or in combination did not cause any change in the crossing and rearing responses in the open-field test, as revealed by two-way ANOVA. Crossings: ursolic acid treatment [F(1, 28)=0.38, P=0.54], bupropion treatment [F(1, 28)=0.84, P=0.36] and ursolic acid X bupropion interaction [F(1, 28)=1.16, P=0.29]; Rearings: ursolic acid treatment [F(1, 28)=0.29, P=0.59], bupropion treatment [F(1, 28)=0.73, P=0.39] and ursolic acid X bupropion interaction [F(1, 28)=0.01, P=0.93].

[Figure 3 and Table 3 near here]

4. Discussion

This study shows, to our knowledge for the first time, that the acute administration of ursolic acid (0.1 mg/kg) by p.o. route is effective in reducing the immobility time in the mouse TST, which is consistent with an antidepressant-like effect of this compound. The TST is reported to be a predictive test of antidepressant action sensitive to the acute administration of antidepressant drugs (Cryan et al., 2005; Steru et al., 1985). This test induces a state of immobility in animals facing an inescapable situation and the antidepressant-like activity of a compound is expressed by a decrease in the immobility duration (Steru et al., 1985). However, it should be considered that drugs enhancing the locomotor activity may give a false positive effect in this test.

Thus, in order to rule out the possibility that the reduction in the immobility time elicited by a drug is due to an enhancement in the locomotor activity, behavioral tests assessing the locomotor activity of mice, such as the open-field test, are usually employed. In this study, we observed that the reduction in the immobility time elicited by ursolic acid in TST is not due to a psychostimulant action, since the doses (0.01 and 0.1 mg/kg) of this compound that were able to induce a significant decrease in the immobility time in the TST did not increase the locomotor activity in the open-field. Indeed, a tendency to decrease the number of crossings and rearings in this test was observed, which may underestimate the antidepressant-like effect of this compound.

Ursolic acid is one of the main constituent of *Rosmarinus officinalis* (Benincá et al., 2011). It is interesting to note that the extract from this plant exerts antidepressant-like effect in the TST at the dose range of 10-100 mg/kg, p.o. (Machado et al., 2009). Therefore, the results presented here indicate that ursolic acid has greater potency (but similar efficacy) as compared to the extract from *Rosmarinus officinalis* in the TST, since it produces antidepressant-like effect at a dose 100-10,000 fold lower than the active doses of the extract from *Rosmarinus officinalis.* These results indicate that ursolic acid exerts a significant role in the antidepressant-like effect of the extract of this plant in the TST.

A protective role for ursolic acid against the cell death induced by the dopaminergic neurotoxin MPP^+ in PC12 cells (Tsai and Yin, 2008) through anti-oxidative and anti-inflammatory mechanisms was recently reported. However, this result also suggests that this compound might modulate the dopaminergic system. Noteworthy, this system has been implicated in pathophysiology of depression and in the mechanism of action of some antidepressant agents (Dally et al., 2004; Dunlop and Nemeroff, 2007; Papakostas, 2006; Yamada et al., 2004). Several clinical studies, including postmortem investigations, have show that depression may be related to reduced dopamine levels and its metabolite homovanillic acid in the brain in depressed and/or

suicidal patients compared to normal individuals, indicating a diminished dopamine turnover (Dunlop and Nemeroff, 2007; Ebmeier et al., 2006; Papakostas, 2006). Since dopamine is the main neurotransmitter involved in the mesocorticolimbic reward pathway, it has been proposed that an increase in dopaminergic neurotransmission might counteract the anhedonia, which is a core symptom of depression (D'Aquila et al., 2000). Moreover, some substances that modulate the dopaminergic system, such as bupropion, are clinically used as antidepressants (Dhillon et al., 2008).

Animal models predictive of antidepressant action, such as the TST show considerable responsiveness to manipulations of dopaminergic neurotransmission (Dunlop and Nemeroff, 2007). Previous studies of our group literature data have shown that conventional and putative antidepressant agents (Binfaré et al., 2009, 2010; Cunha et al., 2008; Hirano et al., 2007) as well as plant extracts and their bioactive compounds whose mechanisms of action are mediated, at least in part, by the modulation of the dopaminergic system produce positive results in the TST (Capra et al., 2010; Freitas et al., 2010; Machado et al., 2007, 2009; Yi et al., 2011).

The results presented here clearly indicate that the dopaminergic system is implicated in the antidepressant-like action of ursolic acid in the TST. This conclusion derives from some set of evidence. First, the reversal of the antidepressant-like effect of ursolic acid by SCH23390 or sulpiride, selective dopamine D_1 and D_2 receptor antagonists, respectively, is an indicative that the effect of ursolic acid in the TST is dependent on the activation of these receptors. This result is somewhat in agreement with previous studies that show that SCH23390 and sulpiride are able to prevent the antidepressant-like effect of bupropion in the forced swimming test (Yamada et al., 2004) and several putative antidepressant agents in the TST (Binfaré et al., 2010; Capra et al., 2010; Hirano et al., 2007; Machado et al., 2009; Yi et al., 2011). The second evidence is given by the finding that the administration of a sub-effective dose of ursolic acid with SKF38393

(dopamine D_1 receptor agonist) or apomorphine (dopamine D_2 receptor agonist) produced a reduction in the immobility time, as compared with the administration of either drug alone, suggesting a synergistic effect of ursolic acid with these agonists in the TST. This result further reinforces the notion that the antidepressant-like effect of ursolic acid in this test is dependent on an activation of dopamine D_1 and D_2 receptors. Furthermore, the synergistic antidepressant-like effect of ursolic acid with bupropion, a dopamine reuptake inhibitor, demonstrated in this work reinforces this conclusion. Similarly to the result observed here, a previous study has shown the synergistic antidepressant-like effect of zinc with bupropion in the TST (Cunha et al., 2008). However, it is important to emphasize that the current results do not allow us to indicate whether ursolic acid directly interact with dopamine D_1 and D_2 receptors or whether it enhance dopamine levels in the synaptic cleft that in turn activates these receptors. Further studies are necessary to better clarify this issue.

It should be emphasized that all the results presented here cannot be attributed to a locomotor effect, since the administration of ursolic acid alone or in combination with all the dopaminergic agents did not significantly affect the ambulatory or exploratory behavior of mice in the open-field test.

The importance of our results are highlighted by the fact that dopamine receptor agonists have been thought as one of the promising candidates to improve outcomes of patients with treatment-resistant and non remitting depression (Dunlop and Nemeroff, 2007; Rakofsky et al., 2009). Moreover, it is noteworthy that the reduction in the immobility time (27.1%) elicited by ursolic acid (0.1 mg/kg, p.o) in the TST is similar to the effect (27.4% reduction in the immobility time in the TST) produced by bupropion administered at a dose 100 fold higher (10 mg/kg, p.o.) (Cunha et al., 2008). Therefore, it remains to be established if ursolic acid alone or in combination

with bupropion is effective in animals models of depression associated with anhedonia, a core symptom of depression.

5. Conclusion

The present work demonstrates that ursolic acid is effective in producing an antidepressant-like effect in the TST when administered by p.o. route. Moreover, a clear involvement of the dopaminergic system in its antidepressant-like effect was indicated by the reversal of its effect by the pretreatment of mice with dopamine D_1 and D_2 receptor antagonists and also, by the synergistic antidepressant-like effect afforded by the dopamine D_1 and D_2 receptor agonists, as well as the antidepressant bupropion. Altogether, the results suggest that an activation of dopamine D_1 and D_2 receptors is implicated in the antidepressant-like effect of ursolic acid in the TST. Overall, the results reported in the present study indicate the antidepressant potential of ursolic acid in a preclinical test widely recognized as effective to assess antidepressant activity, suggesting that it should be further investigated as a possible agent for the treatment of depression.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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Legends to the Figures

Figure 1. Effect of the acute treatment of mice with ursolic acid (0.001-10 mg/kg, p.o.) on the immobility time in the TST. Each column represents the mean +S.E.M. of 7-10 animals. * P<0.05, ** P<0.01 compared with the vehicle-treated control (C).

Figure 2. Effect of pretreatment of mice with SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist; panel A) or sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist; panel B) on the ursolic acid (0.1 mg/kg, p.o)-induced reduction in immobility time in the TST. Each column represents the mean + S.E.M. of 8-9 animals. **P <0.01 compared with the vehicle-treated control. # P<0.01 as compared with ursolic acid group pretreated with vehicle.

Figure 3. Effect of a sub-effective dose of ursolic acid (0.001 mg/kg, p.o) in combination with SKF38393 (0.1 mg/kg, s.c., a dopamine D_1 receptor agonist; panel A), apomorphine (0.5 μg/kg, i.p., a dopamine D_2 receptor agonist; panel B) or bupropion (1 mg/kg, p.o, a dopamine reuptake inhibitor, panel C) in the TST. Each column represents the mean + S.E.M. of 7-9 animals. *P <0.05, **P <0.01 compared with the vehicle-treated control.

Tables

Table 1. Effect of the acute treatment of mice with ursolic acid (0.001-10 mg/kg, p.o.) in the number of crossings and rearings in the open-field test.

Group	Dose (mg/kg, p.o.)	N	Number of Crossings	Number of Rearings
Vehicle	_	9	58.9± 9.0	19.9 ±4.3
Ursolic acid	0.001	8	27.7±11.9	14.0±5.3
Ursolic acid	0.01	8	32.4±10.9	13.6±5.5
Ursolic acid	0.1	8	28.7±9.3	11.6±4.5
Ursolic acid	1	8	28.7±4.7	4.0±1.5
Ursolic acid	10	8	36.4±14.5	13.0±6.9

Results are expressed as mean ± S.E.M.

Table 2. Effect of the administration of ursolic acid (0.1 mg/kg, p.o.) alone or in combination with SCH23390 (0.05 mg/kg, s.c.) or sulpiride (50 mg/kg, i.p.) in the number of the crossings and rearings in the open-field test.

Experimental Group	N	Number of Crossings	Number of Rearings
Vehicle/Vehicle	9	26.8±14.3	10.1±5.3
SCH23390/Vehicle	8	28.1±8.3	15.1±3.7
Vehicle/Ursolic Acid	8	25.0±9.2	9.1±4.2
SCH23390/Ursolic Acid	9	7.2±3.6	4.9±2.1
Sulpiride/Vehicle	7	30.9±9.2	12.9±4.1
Sulpiride/Ursolic Acid	9	16.9±7.3	5.7±2.4

Results are expressed as mean ± S.E.M.

Table 3. Effect of the administration of ursolic acid (0.001mg/kg, p.o.) alone or in combination with SKF38393 (0.1 mg/kg, s.c.), apomorphine (0.5 μg/kg, i.p.) or bupropion (1 mg/kg, p.o.) in the number of the crossings and rearings in the open-field test.

Experimental Group	N	Number of Crossings	Number of Rearings
Vehicle/Vehicle	9	50.1±12.4	19.4 ±5.1
Vehicle/SKF38396	7	44.8±9.0	23.9±4.8
Vehicle/Ursolic Acid	7	28.0±13.8	13.7±6.1
Ursolic Acid/SKF38396	9	30.0±8.7	14.2±5.4
Vehicle/Apomorphine	7	25.8±10.8	8.1±2.5
Ursolic Acid/apomorphine	9	30.5±13.4	13.4±6.0
Vehicle/Bupropion	7	23.7±16.4	11.7±9.9
Ursolic Acid/Bupropion	9	30.2±13.7	8.9±3.9

Results are expressed as mean ± S.E.M.

Figure 1

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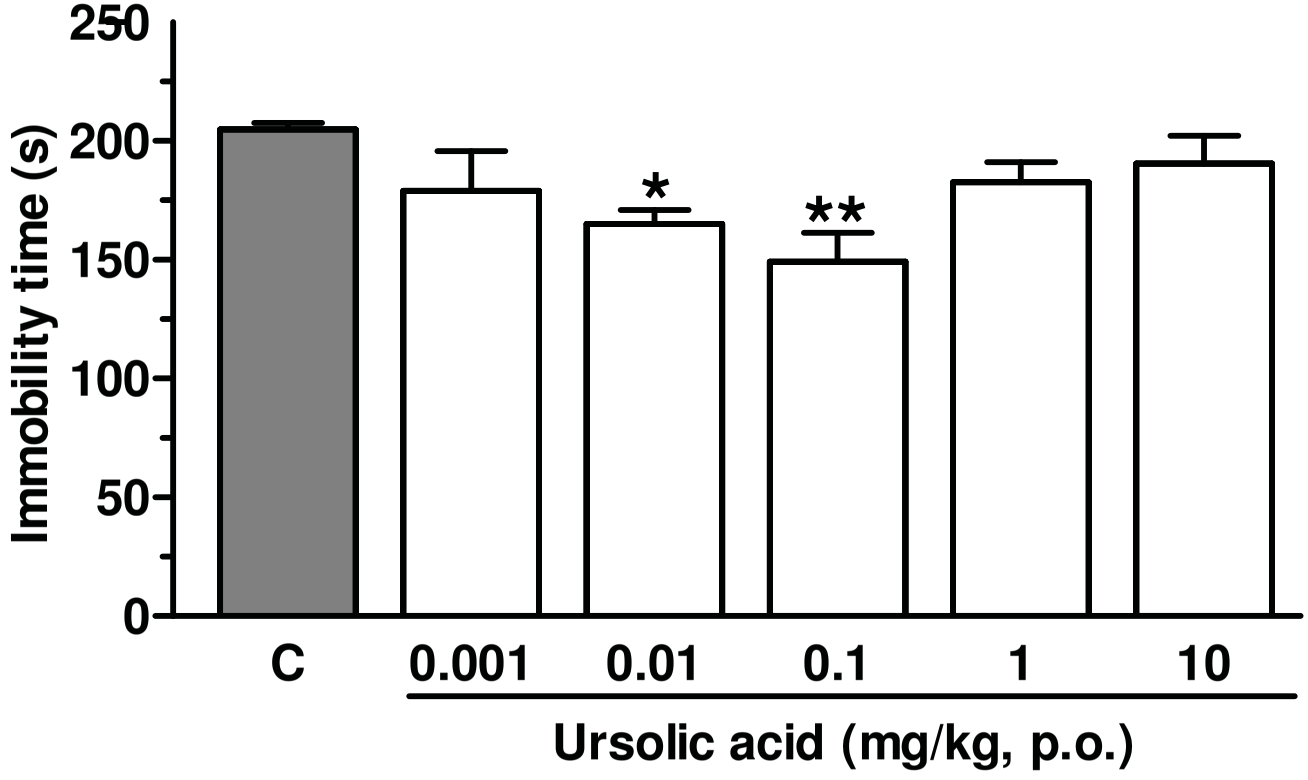

Figure 2

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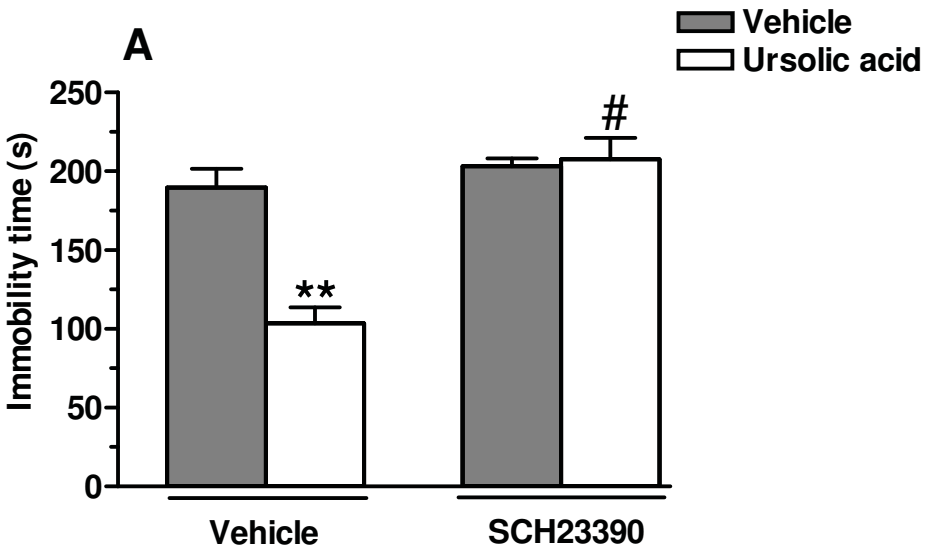

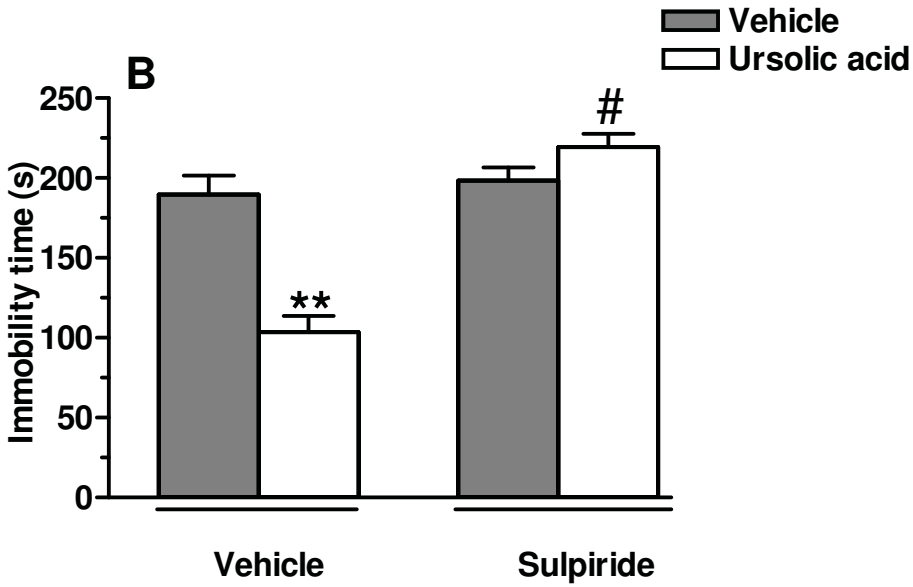

Figure 3

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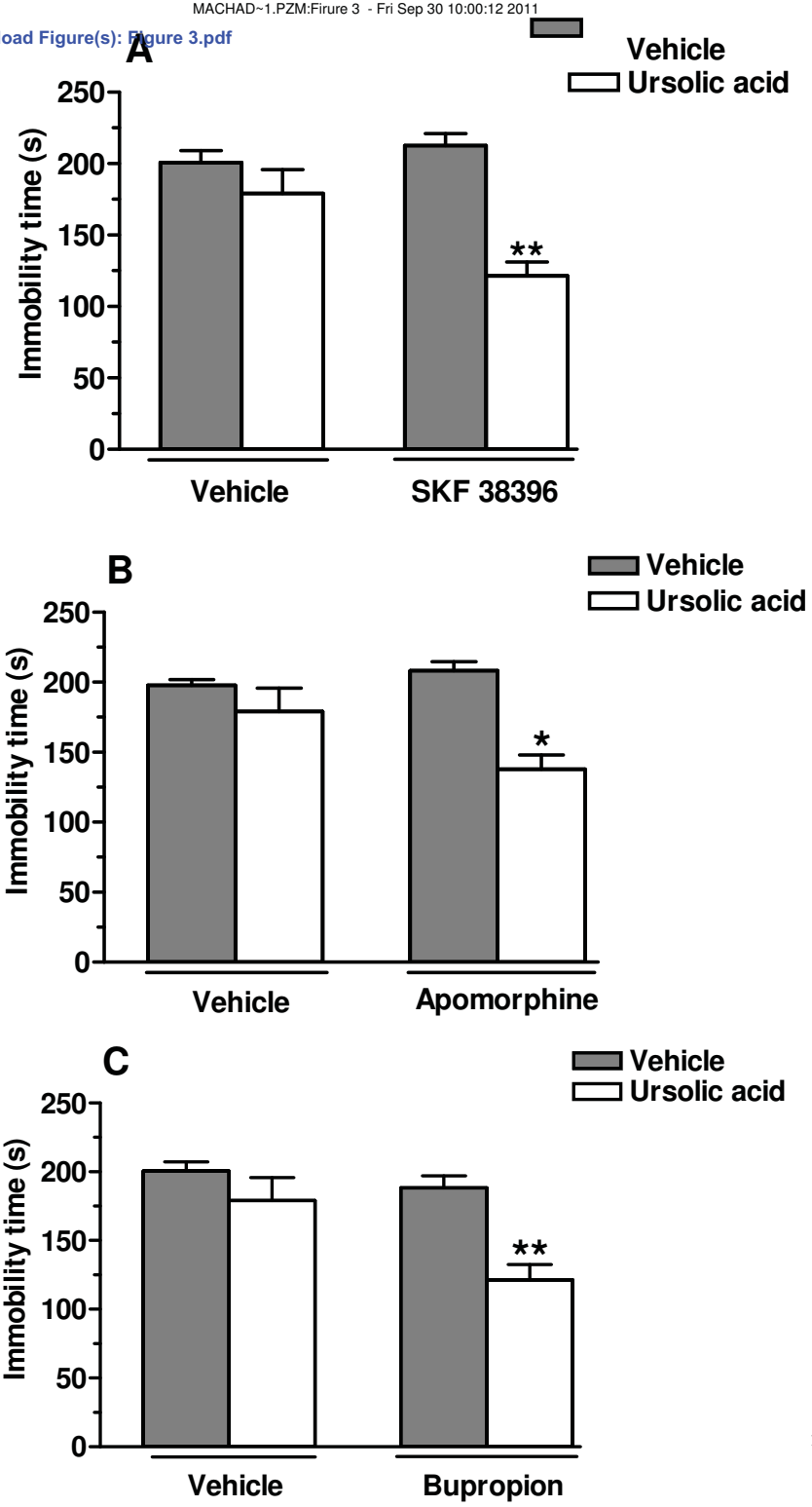

CAPÍTULO 4

Fluoxetine reverses hyperactivity, anhedonic behavior and increased hippocampal acethylcholinesterase activity induced by olfactory bulbectomy

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Fluoxetine reverses hyperactivity, anhedonic behavior and increased hippocampal acethylcholinesterase activity induced by olfactory bulbectomy

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Abstract

The olfactory bulbectomy (OB) is an animal model of depression that results in behavioral, neurochemical and neuroendocrinological changes, features comparable to those seen in depressive patients. This study investigated OB-induced alterations in locomotor activity and exploratory behavior in the open-field test, anhedonic behavior in splash test, hyperactivity in the novel object test and novel cage test, and the influence of chronic treatment with fluoxetine (10 mg/kg, p.o. once daily for 14 days) on these parameters. Fluoxetine reversed OB-induced hyperactivity in the open-field test, the locomotor hyperactivity and the increased of the exploratory behavior induced by novelty in the novel object test and novel cage test, and anhedonic behavior in the splash test. Moreover, OB decreased the number of grooming and fecal boli in the open-field and novel cage test, alterations that were not reversed by fluoxetine. OB caused an increase in hippocampal, but not cerebrocortical AChE activity. Fluoxetine was able to reverse the increased hippocampal AChE activity induced by OB. Serum corticosterone was increased in SHAM and bulbectomized mice treated with fluoxetine. In conclusion, OB mice exhibited hyperactivity and anhedonia associated with an increased hippocampal AChE activity, effects that were reversed by chronic treatment with fluoxetine.

Keywords: anhedonic behavior; acethylcholinesterase; fluoxetine; hyperactivity; olfactory bulbectomy.

Abbreviations: AChE, enzyme acetylcholinesterase; ANOVA, analysis of variance; HPA, hypothalamic–pituitary–adrenal (HPA) axis; OB, olfactory bulbectomy; 5-HT, serotonin; SSRI, selective serotonin reuptake inhibitor.

1. Introduction

Animal models are widely used tools for understanding the mechanisms responsible for the etiology and treatment of various diseases, including depression. The olfactory bulbectomy (OB) is an animal model of depression characterized by the bilateral destruction of the olfactory bulbs, which produces behavioral, neurochemical and neuroendocrinological changes that resemble some of the symptoms observed in depressed patients (Kelly et al., 1997; Leonard, 1984; Song and Leonard, 2005). OB causes several modifications in brain regions, as consequence of the disrupted connections between the bulbs and other brain regions, mainly the olfactory - limbic circuitry (Jesberger and Richardson, 1988; Kelly et al., 1997; Song and Leonard, 2005). The lesion caused by OB induces a reorganization processes in the limbic and cortical areas and appears to be responsible for the secondary effect, behavioral abnormalities appearing after 2 weeks in rodents (Jarosik et al., 2007; Van Riezen and Leonard, 1990; Zueger et al., 2005).

A hyperactivity response, the major behavioral change in this model, can be reversed by chronic treatments with antidepressants, mimicking the slow onset of antidepressant action reported in clinical studies (Kelly et al., 1997; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990). Furthermore, OB leads to different signs of anhedonia, combined with cognitive deficits (Harkin et al., 2003; Kelly et al., 1997; Song and Leonard, 2005). Most of the studies evaluate only hyperactivity in bulbectomized rodents (Harkin, et al., 2003; Kelly et al., 1997; Leonard, 1984; Song and Leonard, 2005; Zueger et al., 2005). There are scarce literature results reporting anhedonic behavior associated with hyperactivity in bulbectomized rats (Romeas et al., 2009).

OB has been argued to possess good face validity with human depressive disorder, especially agitated depression (Harkin et al., 2003; Kelly et al., 1997; Lumia et al., 1992; Romeas et al., 2009). Another important behavioral change triggered by OB is the increased vulnerability and responsiveness to stress induced by novelty. These behavioral deficits may result from inappropriate

and enhanced reactivity to a novel environment (Leonard and Tuite, 1981; Song and Leonard, 2005; Van Riezen and Leonard, 1990).

Moreover, OB in rodents has been also associated with biochemical alterations, as the reduced levels of brain monoamine neurotransmitters serotonin and norepinephrine (Kelly et al., 1997; Song and Leonard, 2005; Lumia et al., 1992), dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis with an increased serum corticosterone levels in bulbectomized animals (Cairncross et al., 1977; Marcilhac et al., 1999) and cholinergic dysfunction (Moriguchi et al., 2006; Nakajima et al., 2007).

The majority of the biochemical alterations found in bulbectomized rodents mentioned above are consistent with the well reported hypofunction of monoaminergic systems and dysregulation in the HPA axis that is implicated in the pathophysiology of depression (Krishnan et al., 2008; Nestler et al., 2002). In addition, a higher activity of serum acetylcholinesterase was reported to be found in depressive patients (Tiwari et al., 1982). Although the cholinergic dysfunctions may account for the development of depression (Dagytė et al., 2011) and may be associated with behavioral changes found in bulbectomized rodents, there are scarce data regarding acetylcholinesterase activity in brain structures closely implicated with depression, such as hippocampus and cerebral cortex in bulbectomized rodents (Yamada et al., 2011).

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has been widely used for the treatment of depression (Taylor and Stein, 2006; Wong et al., 2005). Moreover, several pre-clinical studies have shown the antidepressant effect of fluoxetine in predictive tests of antidepressant activity as the forced swimming test and tail suspension test (Brocardo et al., 2008; Cunha et al., 2008; Lobato et al., 2010; Machado et al., 2009; Porsolt et al., 1979). Regarding OB, the effects of chronic treatment with fluoxetine seem to be controversial, since some studies show that this SSRI is not effective in reversing the major behavioral changes triggered by this model (Bellver et al., 1990) and other works show the ability of this antidepressant to reverse the OB-induced hyperactivity in the open-field (Butler and Leonard, 1990; Roche et al., 2007; Rodríguez-Gaztelumendi et al., 2009). Therefore, the

ability of fluoxetine to reverse the main behavioral and biochemical alterations in this model deserves further studies.

This study was aimed at investigating the effects of chronic administration of fluoxetine in three main behavioral changes induced by OB in mice: hyperactivity in open-field test, hyperactivity induced by novelty, and anhedonic behavior. In addition, the present study investigated the effects of OB on serum corticosterone and hippocampal and cerebrocortical acethylcholinesterase (AchE) activity as well as the ability of chronic fluoxetine treatment to reverse the possible OB-induced alterations in these biochemical parameters.

2. Materials and methods

2.1. Animals

Female Swiss mice (50 to 55 days old, weighing 35-40 g) were used for this study and maintained at constant room temperature (21±1°C) with free access to water and food, under a 12:12h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 24 h before the behavioral procedure. All experiments were carried out between 9:00 and 16:00 h, (N= 9-11 animals per group). The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

After 14 post-operative days (recovery period), mice were assigned to the following groups: (I) SHAM-operated/distilled water (SHAM/vehicle) as the control group, (II) SHAM-operated/antidepressant treated mice (SHAM/fluoxetine); (III) bulbectomized mice treated with distilled water for 14 days (bulbectomized/vehicle); (IV) bulbectomized mice treated with fluoxetine for 14 days (bulbectomized/fluoxetine).

2.2. Drugs and treatment

Fluoxetine from Sigma Chemical Company, St. Louis, MO, U.S.A. was used. Fluoxetine was dissolved in distilled water and given once a day per oral route (p.o.) by gavage over a period of 14 days (in a constant volume of 10 ml/kg body weight) at a dose of 10 mg/kg. The solutions were freshly prepared each day. Controls received an identical volume of distilled water (vehicle). The administration schedule and the dose of the drug used were chosen on the basis of experiments previously performed in our laboratory and literature data (Machado et al., 2007, 2009; Rodríguez-Gaztelumendi et al., 2009).

2.3. Bilateral olfactory bulbectomy (OB) surgery

After a 2-week acclimatization period, OB was performed according to Leonard and Tuite, 1981. Briefly, mice were anesthetized with xylazin (20 mg/kg; Virbac©, Brazil) in combination with ketamine (100 mg/kg; Virbac©, Brazil), diluted in saline (0.9% NaCl) administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight. The skull covering the olfactory bulbs was exposed by skin incision and two burr hole were drilled using a dentist drill. The olfactory bulbs were bilaterally aspirated by blunt hypodermic needle (with for 1.0 to 1.2 cm long and with a rounded tip of 0.80 to 1.2 mm of diameter) attached the syringe (10 ml) for the suction, taking care not to cause damage to the frontal cortex. Finally, the burr hole was filled with acrylic resin, in order to avoid bleeding and contamination at the surgical site. SHAM-operations were performed in the same way, but with the olfactory bulbs left intact. After this surgical procedure, all animals recovered in a post-operative cage (24°C) for 3 hours. After this time period, mice were returned to their home cage. The technique was adapted (Leonard, 1984; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990; Zueger et al., 2005) and standardized in our laboratory.

At the end of the experiments, all animals were sacrificed and the lesions were verified. The bulbectomized animals that showed incomplete removal or damage to other brain area were excluded from the subsequent analysis (Jarosik et al., 2007; Kelly et al., 1997) (less than 15% of the total).

The 14 days post-surgery was used as a period sufficient for an adequate recovery of animals. In this period, the development of persistent BO-induced effects, such as increase of locomotor activity and exploratory behavior are observed (Jarosik et al., 2007; Van Riezen and Leonard, 1990; Zueger et al., 2005).

As depicted in Figure 1, fourteen days after surgery (1°-14° Day, recovery period), drug treatment was started and continued for a period of 14 days (15°-28° Day, treatment period).

[Figure 1 near here]

2.4. Behavioral Tests

One day before surgery, the locomotor activity and exploratory behavior was analyzed in open-field test. Behavioral changes after OB and/or chronic treatment with fluoxetine was examined by testing locomotor activity and exploratory behavior in open-field 2 and 4 weeks after OB (2 weeks after the beginning of chronic fluoxetine treatment), as depicted in Figure 1. All tests were carried out during the light phase of the light/dark cycle. On the first test day (day 29 of the experiment, 24 h after the last drug treatment), mice were submitted to the open-field test. After two hours, mice were submitted to splash test in order to investigate anhedonic behavior. On the second test day (day 30 of the experiment, 48 hours after the last drug treatment), mice were tested in the novel object test. On day 31 (72 h after the last drug treatment), mice were tested in the novel cage test. Light intensity was approximately 200 lux.

2.4.1. Open-field test

The open-field test was used to investigate locomotor activity and exploratory behavior, since locomotor hyperactivity is the key behavioral feature of bulbectomized rodents. Mice were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 squares. Number of crossings (number of squares crossed by the animal with the four paws) were used to evaluate

locomotor activity and number of rearings (number of times the mice stood on its hind legs or vertical exploratory activity) to assess the exploratory behavior (Machado et al., 2009; Van Riezen and Leonard, 1990; Zueger et al., 2007). Additionally, the number of grooming (washing of the coat) and fecal boli were registered (Kalueff and Tuohimaa, 2004; Walsh and Cummins, 1976).

These parameters were registered in a 6-min period. The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

2.4.2. Splash test

The splash test was adapted from the one used by Yalcin et al., 2005. This test was carried out to evaluate grooming behavior, defined as cleaning of the fur by licking or scratching, after the vaporization of 10% sucrose solution on the dorsal coat of mice. The viscosity of the sucrose solution dirties the coat and animals initiate grooming behavior, with depressive symptoms characterized by an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010). Latency and time spent grooming were recorded for 5 min.

2.4.3. Novel object test

The novel object test was performed in the same arena and test conditions employed for the open-field test, in order to evaluate the exploratory behavior of mice submitted to an unknown object (50 ml Falcon tube stylized with colorful stripes, placed top down). In this experimental protocol, the novel object was placed in the center of the open-field (wooden box measuring 40 x 60 x 50 cm, with the floor divided into a central area measuring 19.5 x 18.5 cm). The outdoor area that exceeds the central part is considered peripheral area. Latency for entering the central area of the apparatus where the novel object was, time spent exploring the novel object and numbers of rearings in the central area of the apparatus were recorded for 6 minutes (adapted from Zueger et al. 2005). The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

2.4.4. Novel cage test

To investigate the exploratory behavior of the animal in a novel environment, a circular blue plastic arena (d = 44 cm h = 22 cm) with the floor divided into 9 parts was used. The animals were placed in the center of this apparatus in the beginning of the test. The number of crossings and rearings were recorded for 6 minutes (Adapted protocol from Zueger et al., 2005). Additionally, the number of grooming (washing of the coat) and fecal boli were registered (Kalueff and Tuohimaa, 2004; Walsh and Cummins, 1976).

After each test, the apparatus was sprayed with a solution of ethanol 10% and wiped thoroughly to clean and eliminate the residual odor.

2.5. Biochemical analysis

The blood collection was performed by decapitation 6 hours after the last behavioral test of the experimental protocol, as depicted in Figure 1. The blood samples were collected and allowed to coagulate at room temperature for 30 min and were subsequently centrifuged at 3.000g for 10 min. Serum was removed and stored at -80°C until analysis.

For AchE determination, hippocampus and frontal cortex were homogenized in potassium phosphate buffer (0.1 M, pH 8). The homogenates were centrifuged at 2300 X g for 15 min and the supernatant was separated and stored at -80°C until analysis.

2.5.1. Serum corticosterone determination

Serum corticosterone concentration was determined by a commercially available ELISA kit according to the manufacturer's instructions (Assay Designs Inc., Ann Arbor, MI).

2.5.2. Determination of AChE activity

AChE activity was measured by the method of Ellman et al. 1961, using acetylthiocholine iodide as a substrate in homogenates of hippocampus and cerebral cortex. Each sample was taken from

one animal and assayed in triplicate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of the thiol compound which when reacted with DTNB produced the color-forming compound thionitrobenzoic acid.

2.5.3. Protein determination

The protein content in hippocampal and frontal cortex homogenate samples was quantified by the method of Bradford, 1976, using bovine serum albumin as standard.

2.6. Statistical analysis

Comparisons between pre-operative and post-operative period (SHAM X OB groups) were performed by repeated one-way ANOVA. Two-way ANOVA was used for the study of the post-treatment period (SHAM X OB-vehicle treatment and SHAM X OB-fluoxetine treatment groups), followed by Duncan's multiple range post-hoc test when appropriate. All data are expressed as mean ± standard error of the mean (S.E.M.). Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. OB-induced locomotor and exploratory behavior in the open-field test

Two weeks after OB (post-operative period), bulbectomized mice showed increased number of crossings (67.4%) and rearings (74.4%) in the open-field test (Figure 2A and 2B, respectively), which indicates an increased locomotor and exploratory behavior. Moreover, locomotor and exploratory activities of bulbectomized mice were higher than those observed in SHAM-group (73.0% and 107.6%, respectively). The repeated measures one-way ANOVA revealed a significant effect of OB [$F(1,38)= 35.39$, $P<0.01$] and time [$F(1,38)= 34.09$ $P<0.01$] in locomotor activity and exploratory behavior, respectively, when compared to SHAM-operated controls.

[Figure 2 near here]

3.2. Effect of chronic treatment with fluoxetine on OB-induced locomotor and exploratory hyperactivity in the open-field test

The results depicted in Figure 3A and 3B show that bulbectomized mice presented an increased number of crossing (110.9% of increase) and rearing responses (309.5% of increase) in the open-field test, as compared to control group (SHAM-vehicle), indicating and OB-induced enhancement of locomotor and exploratory activities. However, bulbectomized mice submitted to chronic treatment with fluoxetine by oral route, demonstrated a significant decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant main effect of OB [$F(1,36) = 13.14$, $P<0.01$] and treatment X OB interaction [$F(1,36) = 10.03$, $P<0.01$], but no significant effect of the treatment [$F(1,36) = 0.71$, $P=0.40$] in locomotor activity in the open-field test. The two-way ANOVA also revealed a significant effect of OB [$F(1,36) = 11.37$, $P<0.01$], treatment X OB interaction [$F(1,36) = 16.24$, $P<0.01$], but no significant effect of the treatment [$F(1,36) = 0.21$, $P=0.64$] in exploratory activity (rearing responses) in the open-field test.

Additionaly, bulbectomized mice presented a decrease in the number of grooming and fecal boli as compared to the control group (SHAM-vehicle) (Figure 3C and 3D, respectively). Fluoxetine did not reverse the OB-induced alterations on these parameters. The two-way ANOVA revealed a significant main effect of OB: [$F(1,36) = 10.55$, $P<0.01$], but no significant effect of treatment: [$F(1,36) = 2.37$, $P=0.13$] and treatment X OB interaction: [$F(1,36) = 0.25$, $P=0.61$] in number of grooming in the open-field test. The two-way ANOVA also revealed a significant effect of OB: [$F(1,36) = 14.81$, $P<0.01$], but no significant effect of the treatment: [$F(1,36) = 0.52$, $P=0.47$] and treatment X OB interaction: [$F(1,36) = 3.56$, $P=0.067$] in number of fecal boli in the open-field test.

[Figure 3 near here]

3.3. Effect of chronic treatment with fluoxetine on OB- induced hyperactivity induced by novelty in the novel object test

Figure 4 shows that OB caused a significant decrease on the latency for entering the central area of the apparatus (where the novel object was located), an increased time spent exploring the novel object and an increased number of rearings in the central area of the apparatus as compared to the control group (SHAM-vehicle) (Figure 4A, 4B, 4C, respectively), indicating an OB-induced hyperactivity in the novel object test. Furthermore, chronic treatment with fluoxetine in bulbectomized mice caused a reversal of the hyperactivity induced by novelty, since it decreased the time spent exploring the novel object and the number of rearings in the central area when compared with the OB-vehicle group (Figure 4B and 4C), but did not alter the OB-induced reduction in the latency for entering the central area of apparatus (Figure 4A). The two-way ANOVA revealed a significant main effect of OB [$F(1,37)= 22.01$, $P<0.01$], but no significant effect of treatment [$F(1,37)= 0.36$, $P=0.55$] and treatment X OB interaction [$F(1,37)=0.69$, $P=0.41$] in the latency parameter. Moreover, a significant main effect of OB [$F(1,37)= 12.79$, $P<0.01$] and treatment X OB interaction [$F(1,37)=7.35$, $P<0.01$], but not of treatment [$F(1,37)= 3.29$, $P=0.08$] was observed in the time of exploration of the novel object. Regarding the number of rearings, a significant main effect of OB [$F(1,37)= 9.62$, $P<0.01$], treatment [$F(1,37)= 5.93$, $P<0.05$] and treatment X OB interaction [$F(1,37)=7.70$, $P<0.01$] was observed.

[Figure 4 near here]

3.4. Effect of chronic fluoxetine treatment on the hyperactivity induced by novelty in bulbectomized mice in the novel cage test

As shown in Figure 5, bulbectomized mice showed locomotor and exploratory hyperactivity induced by the novel environment when compared to SHAM-vehicle group (141% and 187% of increase, respectively) in the novel cage test (Figure 5A and 5B, respectively). However, the bulbectomized mice submitted to chronic treatment with fluoxetine demonstrated a significant

decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant effect of OB [$F(1,37) = 12.98$, $P<0.01$], treatment X OB interaction [$F(1,37) = 10.76$, $P<0.01$], but no significant main effect of treatment [$F(1,37) = 0.073$, $P=0.78$] in the locomotor activity in the novel cage test. The two-way ANOVA also revealed a significant main effect of OB [$F(1,37) = 17.54$, $P<0.01$], treatment X OB interaction [$F(1,37) = 8.94$, $P<0.01$], but not of the treatment [$F(1,37) = 0.035$, $P=0.85$] in the exploratory activity in the novel cage test.

Moreover, bulbectomized mice showed a decreased number of grooming and fecal boli as compared to the control group (SHAM-vehicle) (Figure 5C and 5D, respectively) in the novel cage test. Bulbectomized mice submitted to chronic treatment with fluoxetine did not demonstrate alteration in these parameters, as compared to the OB-vehicle. However, SHAM-group treated with fluoxetine presented a decrease in the number of groomings, but no fecal boli, as compared the control group (SHAM-vehicle) The two-way ANOVA revealed a significant main effect of OB [$F(1,37) = 6.84$, $P<0.05$], treatment [$F(1,37) = 6.35$, $P<0.05$] and treatment X OB interaction [$F(1,37) = 4.30$, $P<0.05$] in number of grooming in novel cage test. The two-way ANOVA also revealed a significant effect of OB [$F(1,37) = 18.73$, $P<0.01$], but no significant effect of the treatment [$F(1,37) = 0.27$, $P=0.60$] and treatment X OB interaction [$F(1,37) = 1.11$, $P=0.29$] in number of fecal boli in the novel cage test.

[Figure 5 near here]

3.5. Effect of chronic treatment with fluoxetine on OB-induced depressive-like behavior in the splash test

The effects of the chronic fluoxetine treatment in the anhedonic behavior induced by OB was inferred through the latency and time spent grooming in the splash test, as shown in Figure 6A and 6B, respectively. The results show an increased latency (idle time between spray and initiation of

grooming) and decreased time spent grooming (anhedonia-behavior) in bulbectomized mice. Noteworthy, chronic treatment with fluoxetine significantly reversed these behavioural changes elicited by OB (Figure 6A and 6B, respectively). The two-way ANOVA revealed a significant main effect of OB [F(1,35): 9.43, $P<0.01$] and a significant effect of treatment X OB interaction [F(1,35): 4.31, $P<0.05$,], but no significant main effect of treatment [F(1,35): 1.54, $P=0.222$] in the latency to exhibit the grooming behavior in the splash test. The two-way ANOVA also revealed a significant main effect of OB [F(1,35): 7.55, $P<0.01$], treatment [F(1,35): 17.68, $P<0.01$] and treatment X OB interaction [F(1,35): 10.02, $P<0.01$] in the time spent grooming.

[Figure 6 near here]

3.6. Effect of chronic treatment with fluoxetine on serum corticosterone levels in bulbectomized mice

Figure 7 shows that chronic administration of fluoxetine increased serum corticosterone levels in both SHAM and OB groups, as compared with their respective control groups (SHAM-vehicle and OB-vehicle, respectively). Additionally, serum corticosterone levels were not significantly increased in bulbectomized mice, although a 2.5 fold increase was observed in OB group as compared with the control group (SHAM-vehicle). The two-way ANOVA revealed a significant main effect of OB [F (1,31) = 10.27, $P< 0.01$] and treatment [F (1,31) = 101.54, $P< 0.01$], but not of treatment x OB interaction [F (1,31) = 1.83, P= 0.18].

[Figure 7 near here]

3.7. Effect of chronic treatment with fluoxetine on cerebrocortical and hippocampal AChE activity of bulbectomized mice

Figure 8 shows the effect of chronic administration of fluoxetine on the activity of the enzyme

acetylcholinesterase (AChE) in the frontal cortex (Figure 8A) and hippocampus (Figure 8B) of bulbectomized mice. As demonstrated in Figure 8A, the activity of the enzyme AChE in the frontal cortex was lower in the group of bulbectomized mice treated with fluoxetine (10 mg/kg, p.o.) as compared with the BO-vehicle group. The two-way ANOVA revealed a significant effect of treatment x OB interaction [$F(1,25)=7.281$, $P<0.05$], but not of OB [$F(1,25)=1,379$, $P=0.251$] and treatment [$F(1,25)=1.130$, $P=0.297$] on the activity of AChE in the frontal cortex. However, Figure 8B shows a significant increase on hippocampal AChE enzyme activity of bulbectomized mice, as compared with control group (SHAM-vehicle), an effect reversed by fluoxetine (10 mg/kg, p.o.). The two-way ANOVA revealed significant main effect of OB [$F(1,25)=4.819$, $P<0.05$], treatment [$F(1,25)=6.919$, $P<0.05$] and treatment X OB interaction [$F(1,25)=4.316$, $P<0.05$] in the activity of AChE in the hippocampus.

[Figure 8 near here]

4. Discussion

The removal of olfactory bulbs in rodents results in several behavioral, neurochemical, neuroendocrinological alterations, comparable to those seen in patients with major depression (Kelly et al., 1997; Song and Leonard, 2005). The present study sought to investigate the ability of fluoxetine administered chronically to reverse the OB-induced behavioral (mainly hyperactivity and anhedonia) and biochemical (serum corticosterone and hippocampal and cerebrocortical acetylcholinesterase activity) changes in mice. The majority of studies available in the literature report changes induced by OB in male rats and few studies have investigated hyperactivity and anhedonic behavior in female mice. Notably, depression is more prevalent in women than in men - the female:male ratio can be as high as 5:2 (Wong and Licinio, 2001).

A distinguishing feature of OB is the association between hyperactivity with anhedonic behavior. Thus, this model mimics the symptoms of depression associated with psychomotor agitation (Romeas et al., 2009). Our results are in accordance with literature data, since the bulbectomized mice showed a significant increase in locomotor and exploratory activities in the open-field test, indicating a depressive-like behavior (Harkin et al., 2003; Kelly et al., 1997; Zueger et al., 2005). Noteworthy, the open-field test was assessed under a high luminance condition, which was previously shown to be an aversive condition necessary for bulbectomized animals present hyperactivity (Mar et al., 2002).

The effects of SSRI antidepressants have received more attention in OB model as compared to other classes of antidepressants, since OB was reported to cause a hyposerotonergic depression-related phenotype (Kelly et al., 1997; Lumia et al., 1992; Song and Leonad, 2005). However, the ability of chronic treatment with fluoxetine in reversing hyperlocomotion in bulbectomized rodents is controversial (Bellver et al., 1990; Butler et al., 1990; Mar et al., 2002; Possidente et al., 1996; Rodríguez-Gaztelumendi et al., 2009). In the present study the administration of fluoxetine by oral route (10 mg/kg) to female Swiss mice for 14 days was able to mitigate the changes in locomotor activity and exploratory behavior triggered by OB, consistent with an antidepressant-like effect, similarly to the results previously shown with the same dose of fluoxetine administered by i.p or s.c route for 14 days (Butler et al., 1990; Rodríguez-Gaztelumendi et al., 2009) or for 21 days (Mar et al., 2002) to male rats, validating the model for female mice.

In our investigation, bulbectomized mice showed locomotor hyperactivity and an increased exploratory behavior induced by novelty, indicated by an increase in behavioral reactivity when submitted to the novel object test (decreased latency for entering the central arena, increased time spent exploring and increased rearings responses in the central area where the novel object was located) and to the novel cage test (increased number of crossings and rearings in the new environment). These results are consistent with literature data, since it is reported that the hyperactivity induced by OB is directly related to a greater reactivity to novel environments or deficit in habituation to new situations (Mar et al., 2000, 2002; Van Riezen and Leonard, 1990; Zueger et al.,

2005). These findings are similar to the ones reported by Zueger et al. (2005), which demonstrated an increased exploration in the novel object test and exploratory behavior in novel cage test (neophilic behavior) in bulbectomized mice. In addition to these results, they showed a decreased exploration in the center of open-field in bulbectomized mice (anxious-like behavior) before the introduction of the object in the center of the apparatus. In our study, we did not register the exploration of mice in the center of the open-field before the introduction of the object in the center of the apparatus making the comparison with the study by Zueger et al. (2005) difficult. However, the increased activity in novel arenas and increased interaction with novel object might indicate an anxiolytic effect. This hypothesis is apparently reinforced by the OB-induced decreased in the grooming and fecal boli in open-field test and novel cage test. Nevertheless, it is important to mention that the decreased latency for entering the central arena of the apparatus where the novel object was located may be due to an impulsive-like activity a higher reactivity to a novel situation induced by the OB procedure. Enhanced locomotor activity as observed in tests of exploratory behavior in novel environments, although traditionally thought to represent an anxiolytic effect, could represent an increase in behavioral disinhibition, and has also been described as an increase in impulsive behavior (Scearce-Levie et al., 1999; Winstanley et al., 2004). Impulsivity can be broadly defined as action without foresight, and serotonin depletion enhances impulsive action (Winstanley et al., 2004). It is noteworthy to observe that OB causes a reduction in the levels of serotonin and its metabolites (Hellweg et al., 2007), a condition that could account for the impulsive behavior.

Chronic treatment with fluoxetine (10 mg/kg, p.o.) was able to reverse OB-induced locomotor hyperactivity and exploratory behavior induced by novelty in the novel object test as well as the OB-induced increase in the number of crossings and rearings induced by novelty in the novel cage test. These results are in accordance with other studies that indicate that chronic treatment with antidepressants including fluoxetine (10 mg/kg, i.p., 21 days) are effective to restore normal responding by permitting more effective adaptation to novel stimuli in the bulbectomized rodents. Thus, these antidepressants increase the habituation induced by novelty in bulbectomized animals,

without altering this parameter in SHAM-group (Mar et al., 2000, 2002). Interestingly, a result that extends literature data is that fluoxetine treatment was not able to alter the OB-induced decrease in the latency for entering the central arena of the apparatus where the novel object was located. We can speculate that this parameter is not associated with a depressive-like behavior, reinforcing the notion that it may be related to the initial reactivity to the apparatus, suggesting an impulsive-like activity. This hypothesis is somewhat in agreement with the one raised by Mars et al (2002) which proposes that fluoxetine increases the rate of habituation in olfactory bulbectomized rats. It remains to be established whether other antidepressant agents would be able to alter the OB-induced decrease in this parameter.

Anhedonia, or hyposensitivity to pleasure, is one of the key symptoms of depression. This behavior can be inferred through a reduction in sucrose intake or decreased time spent grooming that is an indicative of self-cleaning behavior evoked by spraying a solution of sucrose in the back of the animals in the splash test (Jancsar and Leonard, 1981; Song and Leonard, 2005; Yalcin et al., 2005). In this test, the majority of studies use only the total time spent grooming or the frequency of grooming as parameters to infer the anhedonic behavior induced by a model of depression (David et al., 2009; Detanico et al., 2009; Yalcin et al., 2008). However, our study also assessed the latency in the splash test as a complementary parameter to the assessment of anhedonia (d'Audiffret et al., 2010). Our study show that OB produced an anhedonic behavior in the splash test, since bulbectomized mice demonstrated both a slower and less frequent grooming response compared with that exhibited by control mice (SHAM). This result is somewhat in agreement with a previous study that showed a blunted response to the rewarding properties of amphetamine and a reduction in sucrose intake in bulbectomized rats, also suggesting that OB causes anhedonia (Romeas et al., 2009; Stock et al., 2000). Interestingly, in our study, fluoxetine was able to reverse the anhedonic-behavior induced by OB in the splash test, indicating that OB is suitable to investigate the effects of compounds endowed with antidepressant/antianhedonic-like properties.

In a second experimental phase of this study, some biochemical parameters that could be changed by OB procedure, as serum corticosterone levels, and activity of the enzyme AChE in the hippocampus and frontal cortex were investigated considering the relationship between hypofunction of monoaminergic system, reported in bulbectomized rodents (Hellweg et al., 2007; Lumia et al., 1992), with HPA and cholinergic dysfunctions (Krishnan and Nestler, 2008; Nakajima et al., 2007; Nestler et al., 2002).

The concentration of corticosterone circulating in the serum was measured in bulbectomized mice treated or not with fluoxetine as an indicator of the activation of the HPA axis. We found that serum corticosterone level was not significantly increased in bulbectomized mice, although a 2.5 fold increase was observed in animals submitted to OB as compared to sham control group. Indeed, either an increase (Cairncross et al 1977; Marcilhac et al., 1999) or no changes (Broekkamp et al., 1986; Van Hoomissen et al., 2011) in serum or plasma corticosterone levels were reported in bulbectomized rodents. The nature relationship of cortisol with human depression remains uncertain, since HPA axis activation is neither a necessary nor sufficient condition for being depressed, as many hypercortisolemic individuals are not depressed, nor many depressed individuals are not hypercortisolemic (Wolkowitz et al., 2009). In line with this, hypocortisolemia was reported to be more frequent in atypical depression, which is characterized by symptoms of hypoarousal, hypersomnia, hyperphagia, lethargy, pain, fatigue, and relative apathy, whereas hypercortisolemia is observed in melancholic depression, in which hyperarousal, anxiety, insomnia, and loss of appetite are commonly found (Gold et al., 2002; Wolkowitz et al., 2009).

Interestingly, chronic administration of fluoxetine increased serum corticosterone levels both in SHAM and OB groups. These results are somewhat in agreement with those reported by Weber et al. (2006) that showed an increased plasma and brain corticosterone concentration following acute and chronic (14 days) administration of fluoxetine (10 mg/kg, p.o.) in mice. Moreover, fluoxetine induces an increase in serum corticosterone levels following acute administration (Serra et al., 2001), an effect that was associated with the HPA axis activation, since this effect was blocked by the

glucocorticoid dexamethasone (Duncan et al., 1998). Fluoxetine was also able to increase the neuroactive steroids allopregnanolone, pregnenolone, progesterone, and deoxycorticosterone in plasma or serum and hippocampus and cerebral cortex, probably through an enhancement of the activity of neurosteroidogenic enzymes (Marx et al., 2006; Serra et al., 2001; Uzunova et al., 2004). Overall considering our results, we can speculate that the antidepressant-like effect of fluoxetine in bulbectomized mice, including its anti-anhedonic property, is not correlated with serum corticosterone levels.

The OB procedure produces changes in many brain regions as a consequence of the disrupted connections between the bulbs and other brain regions which regulate emotion, including the hippocampus and cerebral cortex (Kelly et al., 1997; Wrynn et al., 2000). The dysfunction in brain areas of the limbic system have been implicated in the depression and antidepressant action (Drevets et al., 2008; Krishnan and Nestler, 2008). Furthermore, studies reported that the neurochemical and behavioral alterations induced by OB are in part due to neurodegeneration of specific brain structures, such as hippocampus and frontal cortex (Jarosik et al., 2007; Kelly et al., 1997; Wrynn et al., 2000). Taking this information into account, and the well know implication of the cholinergic system with the behavioral alterations elicited by OB (Moriguchi et al., 2006; Nakajima et al., 2007) and in the pathophysiology of depression (Dagytė et al., 2011), the present study also deals with the determination of AChE activity in the hippocampus and frontal cortex. AChE is an important constituent of cholinergic neurotransmission that catalyzes the hydrolysis of acetylcholine in the synaptic cleft, thus terminating its action. In the present study we demonstrated an increased activity of AChE in the hippocampus, but not in the frontal cortex, in bulbectomized mice, an effect that was reversed by fluoxetine. This finding suggests that an increased hippocampal activity of this enzyme, with the consequent reduced acetylcholine levels may be related to depressive-like behavior observed in the bulbectomized animals. In line with this, the SSRI citalopram causes acetylcholine release in the hippocampus (Dagytė et al., 2011; Egashira et al., 2006). Also, the administration of ZSET1446, which acts directly on the release of acetylcholine, causes a reduction in depressive-like behavior in

bulbectomized mice (Shioda et al., 2010). Therefore, this effect observed in the hippocampus may be related with the abundant cholinergic innervations received by this structure. Furthermore, the cholinergic dysfunctions may account for the diminished hippocampal functioning and the increased vulnerability to development of cognitive deficits associated with depression (Dagytė et al., 2011; Kelly et al., 1997). On the other hand, reduction in AChE expression in the hippocampus of bulbectomized mice was reported (Moriguchi et al., 2006; Nakajima et al., 2007), which may indicate a compensatory effect.

Noteworthy, in our study, fluoxetine was able not only to reverse the OB-induced increase in hippocampal AchE, but also to reduce AChE activity in the frontal cortex of bulbectomized mice, corroborating the notion that this SSRI modulates the cholinergic system (Dagytė et al., 2011). In line with this, treatment with fluoxetine, an antidepressant that increases synaptic levels of serotonin, decreased the activity of AChE in human serum and erythrocyte membrane (Müller et al., 2002). Moreover, it is interesting to mention that donepezil, a classical AChE inhibitor, was able to reduce cerebrocortical AchE in bulbectomized mice (Yamada et al., 2011), a result similar to the one shown in the present study with fluoxetine.

Regarding the ability of fluoxetine to reduce the activity of AchE, consequently increasing acethylcholine levels, we may raise the hypothesis that this could lead to a desensitization of nicotinic acethylcholine receptors. In line with this, it has been proposed that a fine balance between the activation and desensitization of nicotinic receptors is required to yield relevant antidepressant-like effects (Mineur and Picciotto, 2010). Overall our results with fluoxetine are consistent with the notion of an interaction between monoaminergic and cholinergic neurotransmission in its antidepressant effect.

In conclusion, the present study shows that OB mice exhibited hyperactivity and anhedonia associated with an increased hippocampal AChE activity, parameters that were reversed by chronic treatment with fluoxetine, indicating that it may be an effective tool to study agitated depression

associated with anhedonia and also for the treatment of this sub-type of depression. Moreover, our study, also contributes with the behavioral characterization of OB model in mice.

Acknowledgements

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LEGENDS TO THE FIGURES

Figure 1. Schematic representation of the experimental protocol with the treatment period and behavioral tests period (OFT: open-field test, Splash test, NOT: novel object test and NCT: novel cage test). The animals were sacrificed by decapitation 6 hours after completion of the behavioral testing on day 31, blood samples were collected and hippocampus and frontal cortex dissected, then stored in a freezer at - 80° C for subsequent biochemical analysis.

Figure 2. Effect of OB on the number of crossings (panel A) and rearings (panel B) in the open-field test in the pre-operative and post-operative (2 weeks after OB) periods. Each column represents the mean + S.E.M. of 9-11 animals. ***P*<0.01 as compared to control group (SHAM); ++*P*<0.01 when compared with pre-operative period. Results were analyzed by repeated measures ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 3. Effects of the chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o.) on the number of crossings (panel A), rearings (panel B), groomings (panel C) and fecal boli (panel D) in bulbectomized mice in the open-field test. Each column represents the mean + S.E.M. of 9-11 animals. **P*<0.05, ***P*<0.01 as compared to control group (SHAM-vehicle); +*P*<0.05, ++*P*<0.01 when compared with OB - vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 4. Effect of chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o.) on the exploratory activity of bulbectomized mice in the novel object test. The exploratory behavior was assessed monitoring the latency for entering the central arena of the apparatus (area where is located the novel object; panel A), time spent exploring the novel object (panel B) and the number of rearing responses in the central area of the open-field (panel C). Each column represents the mean + S.E.M. of

8-11 animals. **P<0.01 compared with the control group (SHAM-vehicle) and ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 5. Effect of chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o) in the number of crossings (panel A), rearings (panel B), groomings (panel C) and fecal boli (panel D) in bulbectomized mice in the novel cage test. Each column represents the mean + S.E.M. of 8-11 animals. *P<0.05, **P<0.01 compared with the control group (SHAM-vehicle) and +P<0.05 when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 6. Effect of chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o.) in bulbectomized mice submitted to the splash test. The anhedonic behavior of bulbectomized mice was assessed through latency for initiation of grooming behavior (panel A) and time spent grooming (panel B). Each column represents the mean + S.E.M. (n = 8-11). **P<0.01 compared with the control group (SHAM-vehicle); +P<0.05 and ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 7- Effect of the chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o.) on serum corticosterone levels in bulbectomized mice. Each column represents the mean + S.E.M. of 6-9 animals. **P<0.01 compared with the control group (SHAM-vehicle) and ++P<0.01 when compared with OB - vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 8- Effect of the chronic treatment of mice (14 days) with fluoxetine (10 mg/kg, p.o.) on AChE activity in frontal cortex and hippocampus of bulbectomized mice. The enzyme activity was expressed

as nmol/min/mg protein. Each column represents the mean + S.E.M. of 7-8 animals. $^{**}P<0.01$ compared with the control group (SHAM-vehicle) and $^{+}P<0.05$, $^{++}P<0.01$ when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 1
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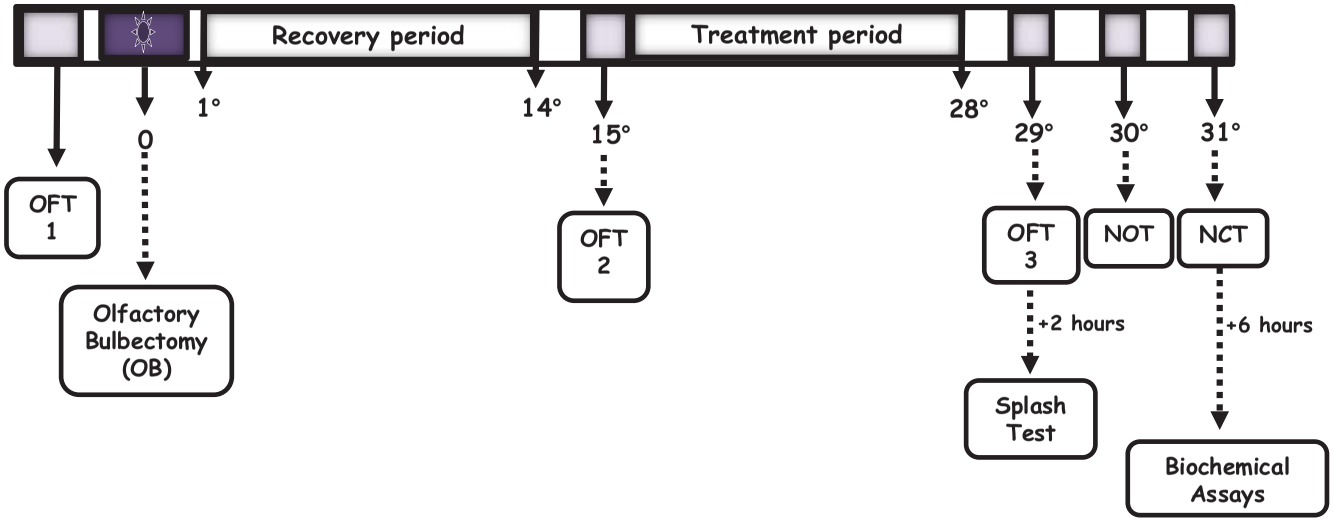

Figure 2
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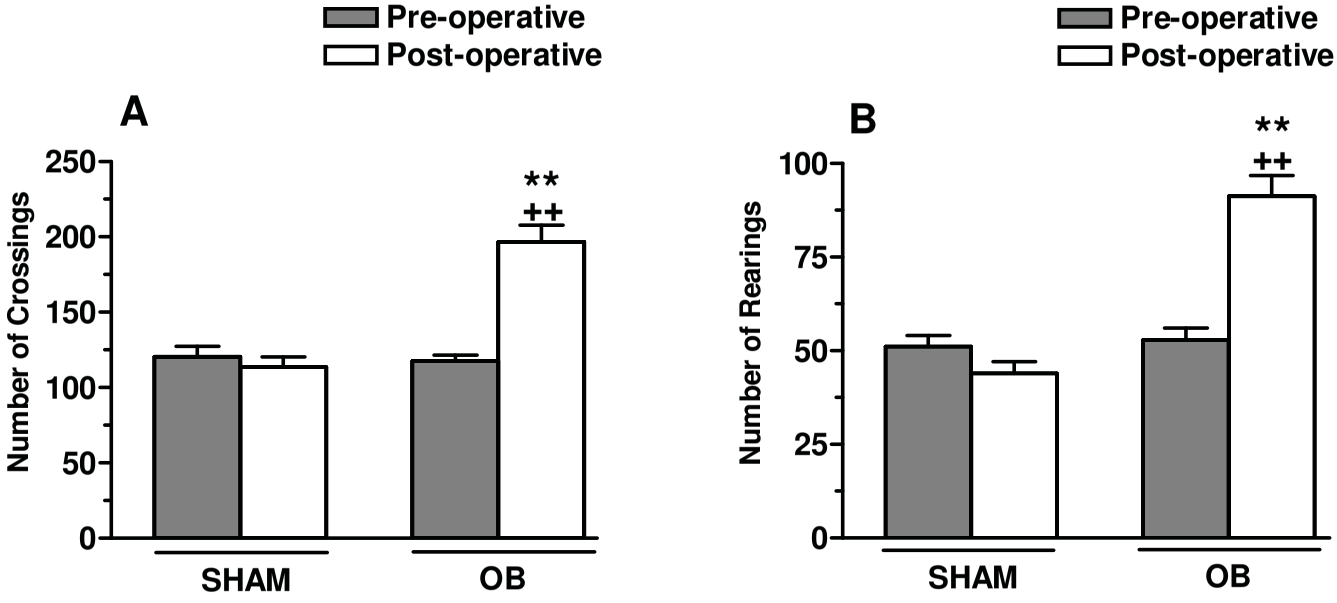

Figure 3
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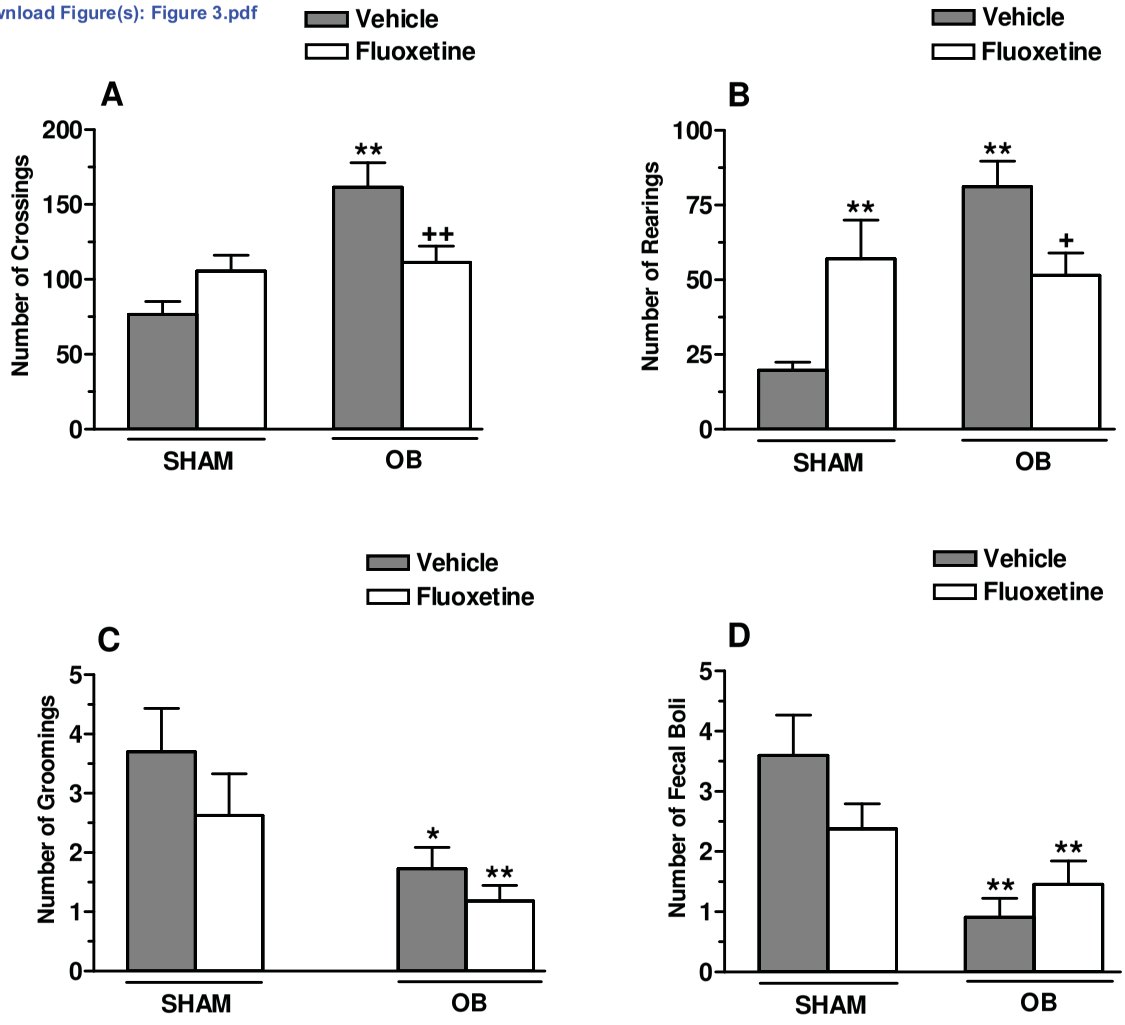

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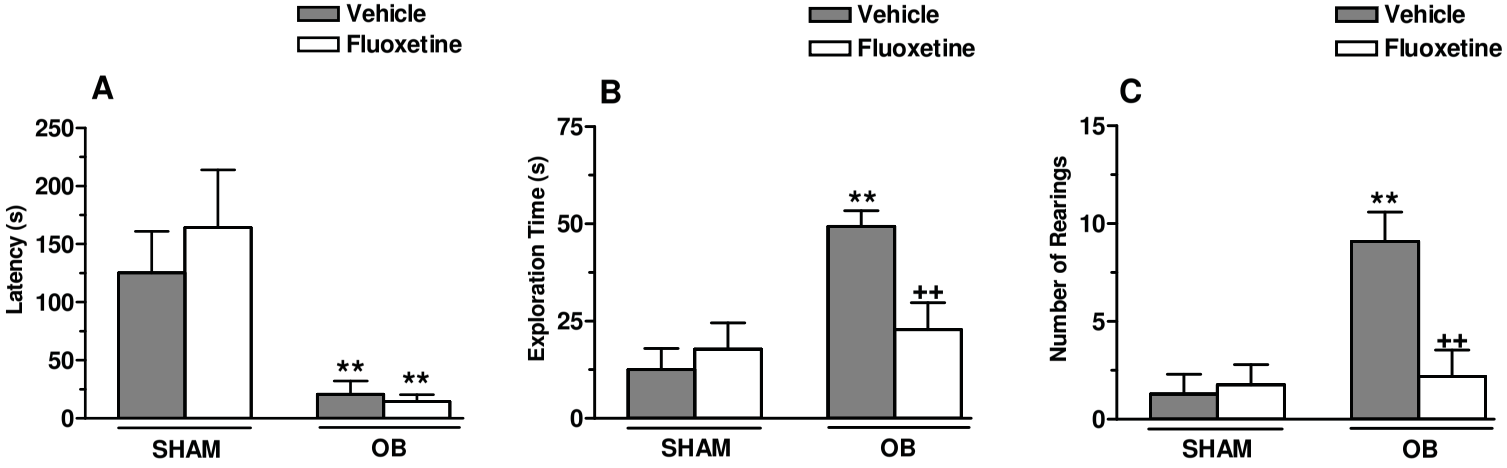

Figure 5

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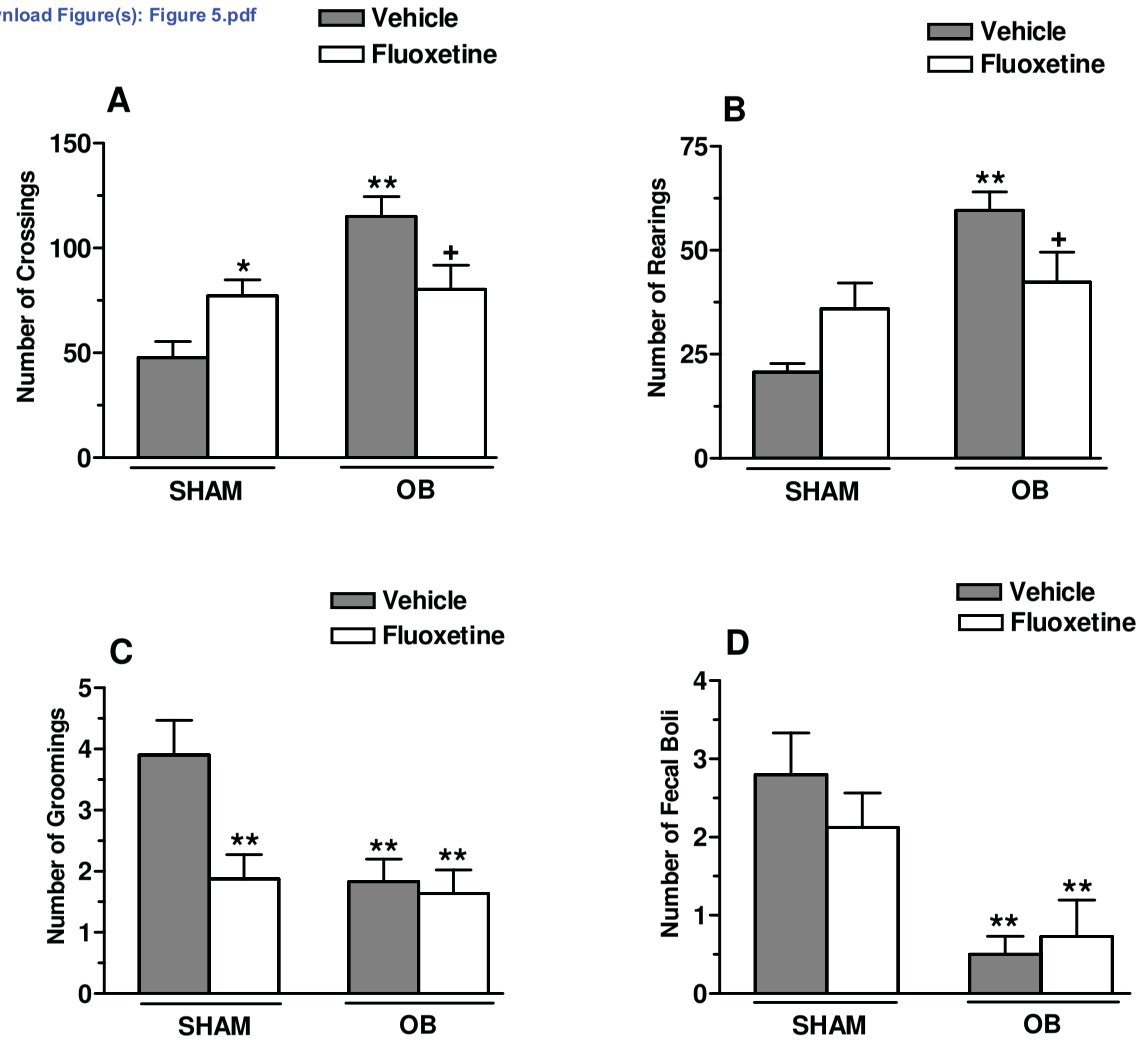

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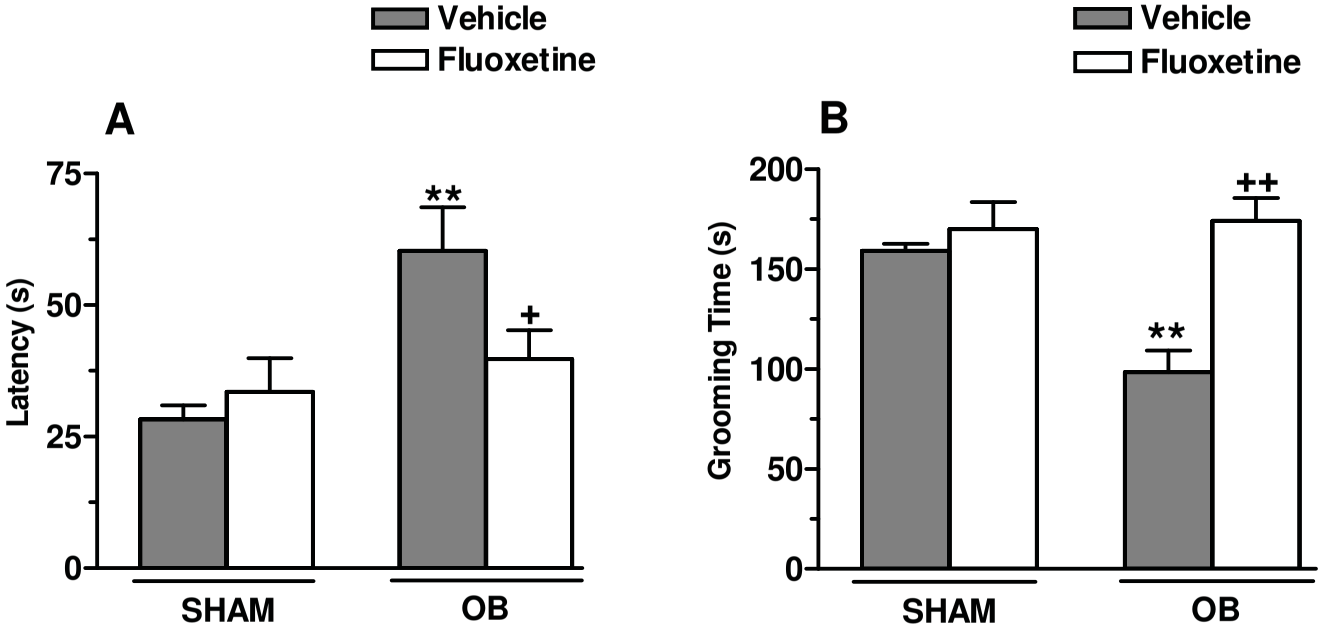

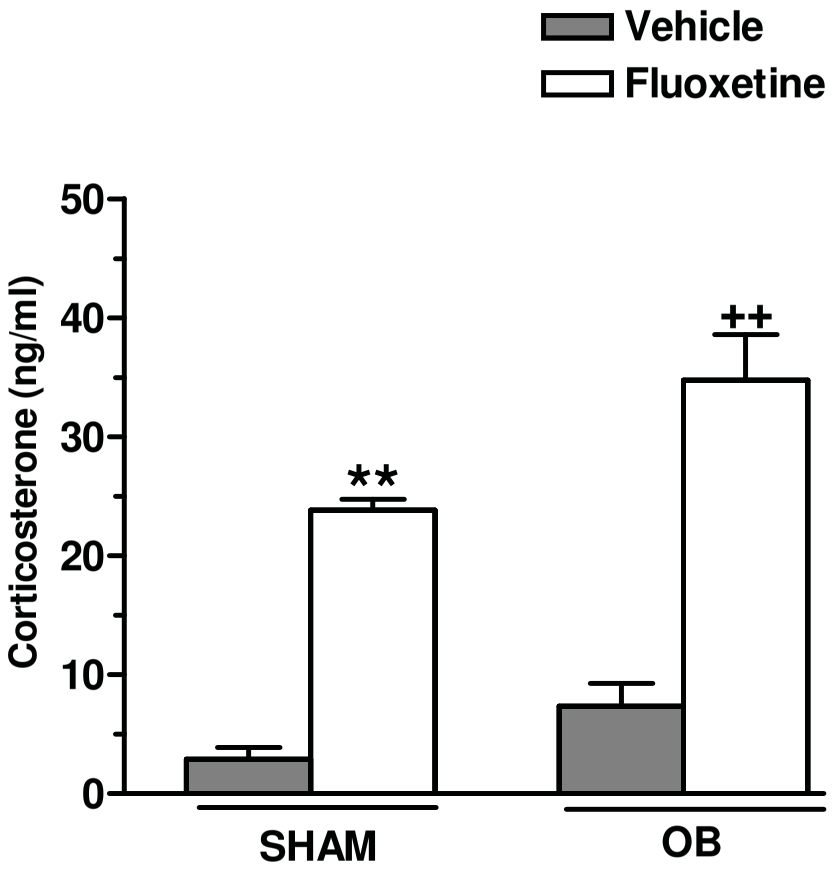
Vehicle
Fluoxetine
Corticosterone (ng/ml)
50
40
30
20
10
0
**
++
SHAM
OB

Figure 8
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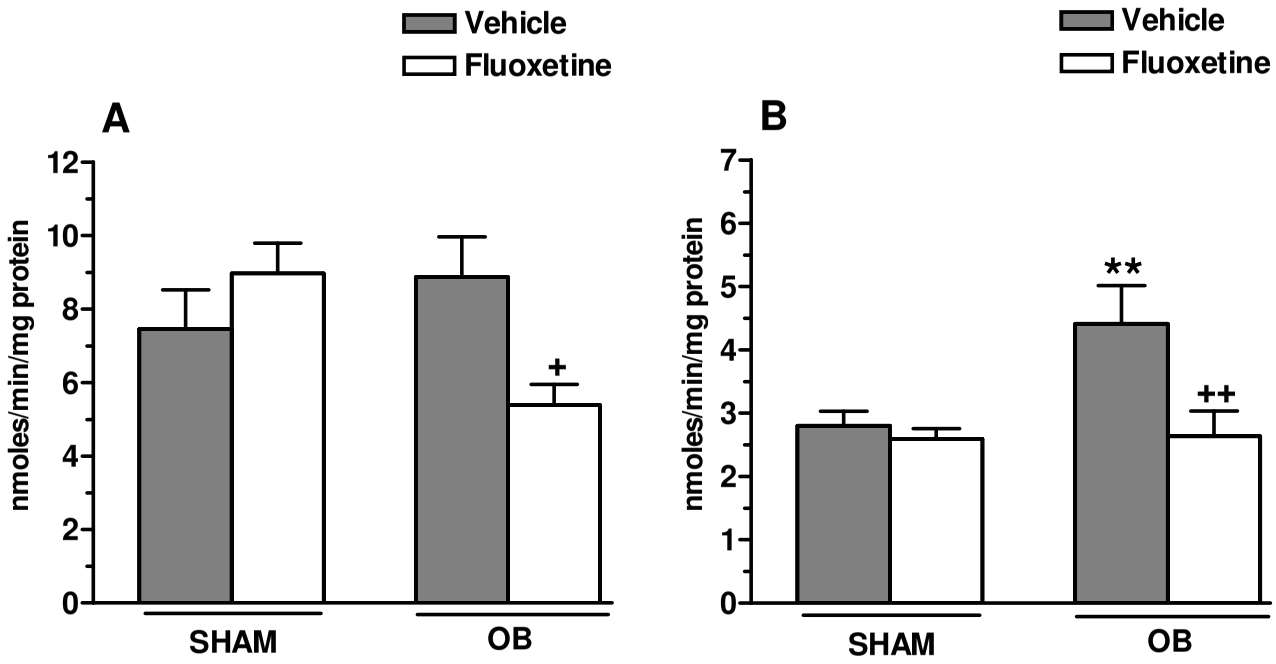

CAPÍTULO 5

***Rosmarinus officinalis* L. hydroalcoholic extract, similarly to fluoxetine, reverses depressive-like behavior without altering learning deficit in olfactory bulbectomized mice**

Manuscrito submetido à revista Journal of Ethnopharmacology

Rosmarinus officinalis L. hydroalcoholic extract, similarly to fluoxetine, reverses depressive-like behavior without altering learning deficit in olfactory bulbectomized mice

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Abstract

Ethnopharmacological relevance: Rosemary, *Rosmarinus officinalis* L. has several therapeutic applications in folk medicine for the treatment of a wide range of diseases, including depression.

Aim of the study: To evaluate the ability of *Rosmarinus officinalis* hydroalcoholic extract (ROHE), as compared to the positive control fluoxetine, to reverse behavioral (hyperactivity, anhedonic behavior and learning deficit in water maze) and biochemical alterations (serum glucose level and acethylcholinesterase, AChE, activity) induced by an animal model of depression, the olfactory bulbectomy (OB) in mice.

Materials and methods: Locomotor and exploratory behavior was assessed in the open-field, novel object and novel cage tests, anhedonic behavior was assessed in the splash test, cognitive deficits were evaluated in the water maze task. For the first set of experiments, ROHE (10-300 mg/kg) or fluoxetine (10 mg/kg) was administered once daily (p.o.) for 14 days after OB and the behavioral tests were performed. For the second set of experiments, serum glucose and hippocampal and cerebrocortical AChE activity were determined in OB and SHAM-operated mice treated orally with ROHE (10 mg/kg), fluoxetine (10 mg/kg) or vehicle.

Results: ROHE (10-300 mg/kg), similarly to fluoxetine, reversed OB-induced hyperactivity, increased exploratory and anhedonic behavior. OB needed significantly more trials in the training session to acquire the spatial information, but they displayed a similar profile to that of SHAM mice in the test session (24 h later), demonstrating a selective deficit in spatial learning, which was not reversed by ROHE or fluoxetine. A reduced serum glucose level and an increased hippocampal AChE activity were observed in bulbectomized mice, only the latter effect was reversed by fluoxetine, while both effects were reversed by ROHE.

Conclusions: ROHE exerted an antidepressant-like effect in bulbectomized mice and was able to abolish AchE alterations and hypoglycemia, but not spatial learning deficit induced by OB. Overall,

results suggest the potential of *Rosmarinus officinalis* for the treatment of depression, validating the traditional use of this plant.

Keywords: anhedonic behavior; acethylcholinesterase; *Rosmarinusofficinalis*; hyperactivity; olfactory bulbectomy spatial learning.

Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; OB, olfactory bulbectomy; SSRI, selective serotonin reuptake inhibitor.

1. Introduction

According to the World Health Organization about 80% of the world's population in developing countries depend essentially on plants for their primary health care (WHO, 2002).

Moreover, the knowledge of medicinal plants often represents the only therapeutic option for many communities and ethnic groups in poor countries. However, few plants have been scientifically studied for the assessment of their quality, safety and efficacy (Calixto, 2005).

Rosemary, *Rosmarinus officinalis* L. (Labiatae), is among a variety of plants used by folk medicine worldwide for the first health care. It is an evergreen perennial shrub native native to Asia Minor and southern Europe; today it has been cultivated in many parts of the world (Al-Sereiti et al., 1999; Heinrich et al., 2006). Several reports in the literature have shown the ethnopharmacological uses of *Rosmarinus officinalis* for the treatment of inflammatory diseases, physical and mental fatigue, improvement of memory and treatment of nervous agitation, hysteria and depression, among other applications (Duke, 2000; Heinrich et al., 2006).

Preclinical studies have demonstrated that the extract of this plant exerts a number of pharmacological activities, such as hepatoprotective (Sotelo-Félix et al., 2002), antibacterial (Del Campo et al., 2000), diuretic (Haloui et al., 2000), antidiabetic (Bakirel et al., 2008), antioxidant (Bakirelet al., 2008), antinociceptive (González-Trujano et al., 2007) and anti-inflammatory (Benincá et al., 2011). We have shown that *Rosmarinus officinalis* hydroalcoholic extract (ROHE) produces an antidepressant-like effect in the in forced swimming test and tail suspension test, predictive tests of antidepressant activity, by a mechanism dependent on the interaction with the monoaminergic systems (Machado et al., 2009). However, the ability of *Rosmarinus officinalis* to reverse depressive-like behavior induced by a model of depression that mimics several symptoms observed in depressed patients was not reported in the literature.

The olfactory bulbectomy (OB) is an animal model of depression characterized by the bilateral destruction of the olfactory bulbs, which produces behavioral, neurochemical and neuroendocrinological changes that resemble some of the symptoms observed in depressed individuals (Kelly et al., 1997; Leonard, 1984; Song and Leonard, 2005). Therefore, OB provides a good model for studying antidepressant drugs and also may provide neurochemical and neuroanatomical data that are relevant to understand the biological substrates of emotion and the causes of depression in humans (Jesberger and Richardson, 1988). It is also important to mention that OB is one of the few models of depression that mimics the slow onset of antidepressant action reported in clinical studies, since the efficacy of antidepressants in this model is evident almost exclusively after 14 days of treatment (O'Neil and Moore, 2003). Hyperactivity response, the major behavioral change observed in this model, can be reversed by chronic treatments with antidepressants (Kelly et al., 1997; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990). Additionally, OB causes different signs of anhedonia, as well as cognitive deficits (Harkin et al., 2003; Kelly et al., 1997; Song and Leonard, 2005).

OB in rodents has been also associated with biochemical alterations, including a reduction in the brain levels of monoamine neurotransmitters serotonin and norepinephrine (Kelly et al., 1997; Lumia et al., 1992; Song and Leonard, 2005), serum glucose level alterations (Montilla et al. 1984; Perassi et al., 1975), and cholinergic dysfunction (Moriguchi et al., 2006; Nakajima et al., 2007).

Therefore, this study was aimed at investigating the effects of chronic administration of ROHE in behavioral and biochemical alterations induced by OB in mice.

2. Materials and methods

2.1. Plant material and preparation of ROHE

Stems and leaves of *Rosmarinus officinalis* (Labiatae) were collected in Santo Amaro da Imperatriz, Santa Catarina, Brazil, and identified by Dr. Daniel Falkenberg, from Department of Botany, Federal University of Santa Catarina. A voucher specimen (Excicata number 34918) was deposited in the Herbarium of the Department of Botany, Federal University of Santa Catarina, Brazil.

The preparation of extract, dried aerial parts of *Rosmarinus officinalis* (600 g) was submitted to maceration in ethanol (96%) during fifteen days at room temperature (25±2 °C). Thereafter, the extract was filtered and then concentrated under reduced pressure (at approximately 60°C). The maceration was repeated three times. After removing the solvent by liophylization, this procedure gave 61 g of a green solid and dry hydroalcoholic crude extract (10.2% w/w yield).The ROHE was obtained according to the methodology described by Machado et al. (2009).The extract was kept in closed bottle at 4◦C in a refrigerator for further use.

2.2. HPLC profile of ROHE

The liquid chromatography (HPLC) profile of ROHE was performed according to Benincá et al. (2011). Carnosol used as standard for quantification was obtained according to Benincá et al. (2011). The triterpenes betulinic acid, oleanolic acid, ursolic acid and rosmarinic acid (Sigma–Aldrich, Steinheim, Germany) were also used as standards.

2.3. Animals

Female Swiss mice (50 to 55 days old, weighing 35-40 g) were used for this study and maintained at constant room temperature (21±1°C) with free access to water and food, under a 12:12h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 24 h before the behavioral procedure (N= 9-11 animals per group). All experiments were carried out between 9:00 and 16:00 h. The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.4. Drugs and treatment

ROHE (10-300 mg/kg, p.o.) and the antidepressant fluoxetine (10 mg/kg, p.o., Sigma Chemical Company, St. Louis, MO, U.S.A.) were dissolved in distillated water and given once a day per oral route (p.o.) by gavage over a period of 14 days (in a constant volume of 10 ml/kg body weight) to mice. Fluoxetine, used here as a positive control, was administered at a dose previously shown to cause antidepressant-like effects (Machado et al., 2009). The dissolution of ROHE was freshly done from the liophylized power immediately before its administration by gavage. Controls received an identical volume of distilled water (vehicle).The administration schedule and the doses of ROHE were chosen on the basis of experiments previously performed in our laboratory (Machado et al., 2009).

After 14 post-operative days (recovery period), mice were assigned to the following groups: (I) SHAM-operated/ treated with distilled water for 14 days (SHAM/vehicle) as the control group, (II) SHAM-operated/treated with extract for 14 days (SHAM/extract); (III) SHAM-operated/treated with fluoxetine for 14 days (SHAM/fluoxetine); (IV) bulbectomized mice treated with distilled water for 14 days (bulbectomized/vehicle); (V) bulbectomized mice treated with extract for 14 days (bulbectomized/extract); (VI) bulbectomized mice treated with fluoxetine for 14 days (bulbectomized/ fluoxetine).

2.5. Bilateral olfactory bulbectomy (OB) surgery

After a 2-week acclimatization period, OB was performed according to the procedure described by Leonard and Tuite (1981). Briefly, mice were anesthetized with xylazin (20 mg/kg; Virbac©, Brazil) in combination with ketamine (100 mg/kg; Virbac©, Brazil), diluted in saline (0.9% NaCl) administered intraperitoneally (i.p.); 10 ml/kg body weight. The skull covering the olfactory bulbs was exposed by skin incision and two burr holes were drilled using a dentist drill. The olfactory bulbs were bilaterally aspirated using a blunt hypodermic needle (1.0 to 1.2 cm long and with a rounded tip of 0.80 to 1.2 mm of diameter) attached to a 10 ml syringe (taking care not to cause any damage to the frontal cortex).

Finally, the burr hole was filled with acrylic resin, in order to avoid bleeding and contamination at the surgical site. SHAM-operations were performed in the same way, but the olfactory bulbs were left intact. After being submitted to the surgical procedure, all animals were allowed to recover in a post-operative cage (maintained at 24°C) for 3 hours. After this time period, mice were returned to their home cage. This technique was adapted from previous studies (Leonard, 1984; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990; Zueger et al., 2005).

At the end of the experiments, all animals were sacrificed and the presence of the lesions was verified. The bulbectomized animals that showed incomplete removal of the olfactory bulbs or damage to other brain areas (less than 15% of the total) were excluded from subsequent analysis following the criteria previously described (Jarozik et al., 2007; Kelly et al., 1997).

A 14 days post-surgery period time interval was considered to be sufficient to guarantee an appropriate recovery of the animals, as indicated in literature studies (Jarozik et al., 2007; van Riezen and Leonard, 1990, Zueger et al., 2005).

As depicted in Figure 1, fourteen days after surgery (1°-14° Day, recovery period), drug treatment was started and continued for a period of 14 days (15°-28° Day, treatment period).

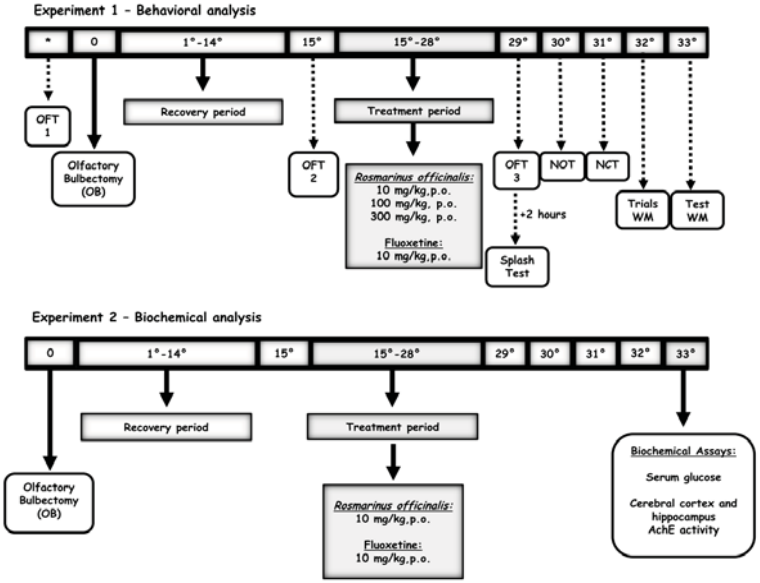

Figure 1. Schematic representation of the experimental protocol with the treatment period with ROHE and behavioral tests period (Panel A). The animals were killed by decapitation 6 hours after the end of the behavioral testing on day 33. Blood samples were collected and hippocampus and frontal cortex dissected, then stored in a freezer at - 80° C for subsequent biochemical analysis (Panel B). (OFT: open-field test, NOT: novel object test, NCT: novel cage test, Trials WM: training session of the water maze and Test WM: test session of the water maze).

2.6. Behavioral Tests

One day before surgery, locomotor activity and exploratory behavior was analyzed using the open-field. Behavioral changes after OB and/or chronic treatment with ROHE were examined by testing locomotor activity and exploratory behavior in the open-field 4 weeks after OB and 2 weeks after the beginning of chronic drug treatment (Figure 1). All tests were carried out during the light phase of the light/dark cycle. Light intensity was approximately 200 lux. On the first test day (day 29 of the experiment), 24 h after the last drug treatment, mice were submitted to the open-field. After two

hours, mice were submitted to the splash test in order to investigate anhedonic behavior. On the second test day (day 30 of the experiment), 48 hours after the last drug treatment, mice underwent the novel object test. On day 31, 72h after the last drug treatment, mice were submitted to the novel cage test. On day 32, 96 h after the last drug treatment, mice were subject to training sessions of the water maze task and on day 33, 120 h after the last drug treatment, mice were subject to a test session of the water maze task.

2.6.1. Open-field test

The open-field test was used to investigate locomotor activity and exploratory behavior, since locomotor hyperactivity is the key behavioral feature of bulbectomized rodents. Mice were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 squares. Number of crossings (number of squares crossed by the animal with the four paws) was used to evaluate locomotor activity whereas number of rearings (number of times the mice stood on its hind legs or vertical exploratory activity) to assess the exploratory behavior (Machado et al., 2009; van Riezen and Leonard, 1990; Zueger et al., 2005). All the parameters were registered in a 6-min period.

The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

2.6.2. Splash test

The splash test was adapted from Yalcin et al. (2005). This test evaluates grooming behavior, defined as cleaning of the fur by licking or scratching, after the vaporization of 10% sucrose solution on the dorsal coat of mice. The viscosity of the sucrose solution dirties the coat and animals initiate grooming behavior, with depressive symptoms characterized by an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010).The grooming behavior included nose/face grooming (along the snout), head washing

(semicircular movements over the top of the head and behind the ears), and body grooming (body fur licking) (Kalueff and Tuohimaa, 2004). Latency and time spent grooming were recorded for 5 min.

2.6.3. Novel object test

The novel object test was performed in the same arena and test conditions employed for the open-field test, in order to evaluate the exploratory behavior of mice when exposed to an unknown object (50 ml Falcon tube stylized with colorful stripes, placed top down). In this experimental protocol, the novel object was placed in the center of the open-field (wooden box measuring 40 x 60 x 50 cm, with a central area measuring 19.5 x 18.5 cm). The area that surrounds this central part is referred to as the peripheral area. The time spent exploring the novel object, as well as number of rearings in the central area of the apparatus was recorded for 6 minutes (adapted from Zueger et al., 2005). The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors.

2.6.4. Novel cage test

To investigate the exploratory behavior of the animal in a novel environment, a circular blue plastic arena (d = 44 cm h = 22 cm) with the floor divided into 9 parts was used. The animals were placed in the center of this apparatus in the beginning of the test. The number of crossings and rearings was registered for 6 minutes (Adapted protocol from Zueger et al., 2005). After each test, the apparatus was sprayed with a solution of ethanol 10% and wiped thoroughly to clean and eliminate the residual odor.

2.6.5. Water maze test-memory reference task

The water maze task was performed in a circular swimming pool similar to that described by Morris et al. (1982). The pool was made of black painted fibreglass, 97 cm in diameter and 60 cm in

height. For the tests, the tank was filled with water maintained at 23 ± 2°C. The target platform (10 x10 cm) was made of transparent Plexiglas and it was submerged 1-1.5 cm beneath the surface of the water. Starting points for animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues (55 x 55 cm) were placed on the walls of the water maze room. They were all positioned with the lower edge 30 cm above the upper edge of the water tank and in the standard setting, the position of each symbol marked the midpoint of the perimeter of a quadrant (circle = NE quadrant, square = SE quadrant, cross = SW quadrant, and diamond = NW quadrant). The apparatus was located in a room with indirect incandescent illumination. A monitor and a video-recording system were installed in an adjacent room. The experiments were video-taped and the scores for latency of escape from the starting point to the platform during the training sessions and the time spent in the correct quadrant during the probe test session were later measured through the ANY-maze™ video tracking system (Stoelting Co., Wood Dale IL, USA).

Mice were submitted to a spatial reference memory version of the water maze using a protocol that was similar to one described previously (Prediger et al., 2007).The training session consisted of ten consecutive trials during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform. The platform was located in a constant position (middle of the southwest quadrant), equidistant from the centre and the wall of the pool. If the animal did not find the platform during a period of 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 10 s after escaping to it and was then removed from the tank for 20 s before being placed at the next starting point in the tank. This procedure was repeated ten times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. The test session was carried out 24 h later and consisted of a single probe trial where the platform was removed from the pool and each mouse was allowed to swim for 60 s in the maze. The time spent in the correct quadrant (i.e. where the platform was located on the training session) was recorded and the percentage of the total time was analyzed.

2.7. Biochemical analysis

Blood collection was performed by decapitation 6 hours after the last behavioral test. Animals fasted for 8 hours before blood collection in order to cause no interference in the analysis of serum glucose. The blood samples were collected and allowed to coagulate at room temperature for 30 min and were subsequently centrifuged at 3,000g for 10 min. Serum was removed and stored at -80°C until analysis.

For AChE determination, hippocampus and frontal cortex were homogenized in potassium phosphate buffer (0.1 M, pH 8). The homogenates were centrifuged at 2,300g for 15 min and the supernatant was separated and stored at -80°C until analysis.

2.7.1. Serum glucose determination

The serum glucose levels were measured by commercial kit Kovalent, by enzymatic colorimetric method Glucose GOD-PAP. The principle of this method is based on the determination of glucose after enzymatic oxidation by glucose oxidase. The colorimetric indicator is quinonimine, which is generated from 4-aminoantipipirina and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction).

2.7.2. Determination of AChE activity

AChE activity was measured by the method described by Ellman et al. (1961), using acetylthiocholine iodide as a substrate in homogenates of hippocampus and cerebral cortex. Each sample was assayed in triplicate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of the thiol compound, which reacts with DTNB producing the colored product thionitrobenzoic acid.

2.7.3. Protein determination

The protein content in hippocampal and frontal cortex homogenate samples was determined using the method of Bradford (1976), using bovine serum albumin as a standard.

2.8. Statistical analysis

Comparisons between the pre-operative and post-operative periods (SHAM X OB groups) and training session of water maze task were performed by repeated one-way-measures analysis of variance (ANOVA), one-way-ANOVA for test session of water maze task and two-way ANOVA for study of the post-treatment period (SHAM X OB-vehicle treatment and SHAM X OB- extract or fluoxetine treatment groups) followed by Duncan test when appropriate. All data are expressed as mean ± standard error of the mean (S.E.M.). Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. Phytochemical analysis and High- performance liquid chromatographic profile (HPLC)

As shown in Figure 2 the major compounds identified in ROHE were carnosol, ursolic acid, oleanolic acid, betulinic acid and rosmarinic acid. Ursolic acid (15.71%) and carnosol (10.03%) are the compounds present at higher concentrations in ROHE. Although at lower concentrations, the terpenes betulinic acid (6.21%) and oleanolic acid (5.73%), as well as the phenolic acid, rosmarinic acid (5.99%) were also found in ROHE.

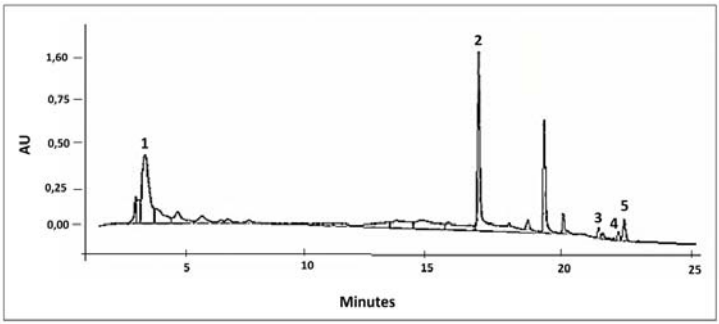

Figure 2. Chromatographic profiles (HPLC) of crude extract from *Rosmarinus officinalis* L. Peaks represent rosmarinic acid (1), carnosol (2), betulinic acid (3), oleanolic acid (4), and ursolic acid (5).

3.2. Effect of chronic treatment with ROHE on OB-induced locomotor and exploratory hyperactivity in the open-field test

The results depicted in Figure 3A and 3B show that bulbectomized mice presented an increased number of crossing and rearings in the open-field test, as compared to control group (SHAM-vehicle), indicating that OB induced an enhancement of locomotor and exploratory activities. However, the bulbectomized mice submitted to chronic p.o. treatment with extract (10-300 mg/kg) and fluoxetine (10 mg/kg) demonstrated a significant decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant main effect of OB [$F(1,91) = 37.53$, $P<0.01$] and treatment X OB interaction [$F(4,91) = 3.99$, $P<0.01$],but no significant effect of the treatment [$F(4,91) = 1.08$, $P=0.37$] in locomotor activity in the open-field test. The two-way ANOVA also revealed a significant effect of OB [$F(1,91) = 33.96$, $P<0.01$], treatment X OB interaction: [$F(4,91)=6.82$, $P<0.01$], but no significant effect of the treatment [$F(4,91)=1.36$, $P=0.25$] in exploratory activity in the open-field test.

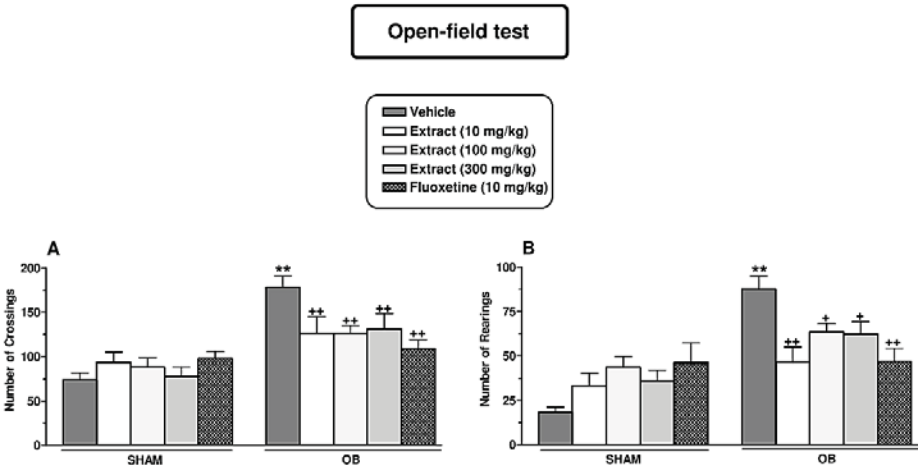

Figure 3. Effect of the chronic treatment of mice (14 days) with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on the number of crossings (panel A) and rearings (panel B) in bulbectomized mice in the open-field test. Each column represents the mean + S.E.M. of 9-11 animals. ***P*<0.01 as compared to control group (SHAM-vehicle); +*P*<0.05, ++*P*<0.01 when compared with OB - vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

3.3. Effect of chronic treatment with ROHE on OB-induced hyperactivity as assessed with the novel object and novel cage tests

Figure 4 shows that OB caused a significant increased time spent exploring the novel object and an increased number of rearings in the central area of the apparatus as compared to the control group (SHAM-vehicle) (Figure 4A, 4B, respectively). These results indicate an OB-induced hyperactivity in the novel object test. Furthermore, chronic treatment with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) in bulbectomized mice caused a reversal of the hyperactivity induced by novelty, since it decreased the time spent exploring the novel object and the number of rearings in

the central area when compared with the OB-vehicle group (Figure 4A and 4B). The two-way ANOVA revealed a significant effect of OB [F(1,90)=11.73, *P*<0.01] and treatment X OB interaction [F(4,90)=3.06, *P*<0.05], but not of treatment [F(4,90)=0.90, *P*=0.46] in the time exploring the novel object. Regarding the number of rearings around the object, a significant effect of OB [F(1,90)=9.65, *P*<0.01] and treatment X OB interaction [F(4,90)=3.21, *P*<0.05], but not of treatment [F(4,90)=1.64, *P*=0.17] was observed.

As shown also in Figure 4C-D, bulbectomized mice showed locomotor and exploratory hyperactivity induced by the novel environment when compared to SHAM-vehicle group (147.53% and 187.03% of increase, respectively) in the novel cage test. However, the bulbectomized mice submitted to chronic treatment with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) demonstrated a significant decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant effect of OB [F(1,90)=26.61, *P*<0.01], treatment X OB interaction:[F(4,90)=4.60, *P*<0.01], but no significant main effect of treatment [F(4,90)=0.61, *P*=0.65] in the locomotor activity in the novel cage test. The two-way ANOVA also revealed a significant main effect of OB [F(1,90)=32.03, *P*<0.01], treatment X OB interaction [F(4,90)=3.71, *P*<0.01], but no significant effect of treatment [F(4,90)=1.14, *P*=0.343] in the exploratory activity in the novel cage test.

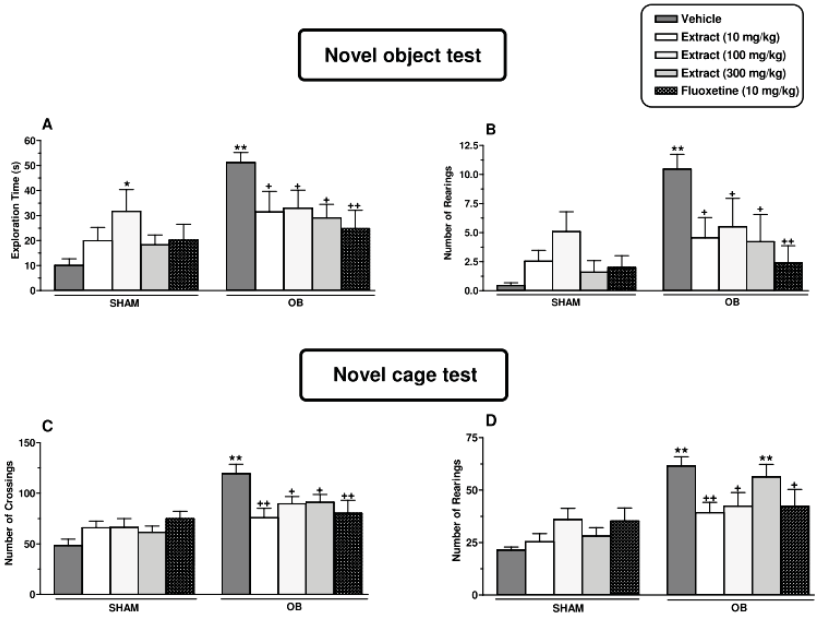

Figure 4. Effect of chronic treatment of mice (14 days) with hydroalcoholic extract of *Rosmarinus officinalis* (10-300 mg/kg, p.o.) and Fluoxetine (10 mg/kg, p.o.), on the exploratory activity of bulbectomized mice in the novel object test and novel cage test. The exploratory behavior was assessed monitoring the time spent exploring the novel object (panel A) and the number of rearing responses in the central area of the open-field (panel B) in the novel object test; and the number of crossings (panel C) and rearings (panel D) of bulbectomized mice in the novel cage test. Each column represents the mean + S.E.M. of 9-11 animals. **P*<0.05, ***P*<0.01 compared with the control group (SHAM-vehicle) and +*P*<0.05, ++*P*<0.01 when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

3.4. Effect of chronic treatment with ROHE on OB-induced anhedonic-like behavior assessed by the splash test

The effects of chronic treatment with ROHE in the anhedonic-like behavior induced by OB was inferred through the latency and time spent grooming in the splash test, as shown in Figure 5A and 5B, respectively. The results show an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (anhedonic-like behavior) in bulbectomized mice. Noteworthy, chronic p.o. treatment with extract (10-300 mg/kg) or fluoxetine (10 mg/kg) significantly reversed the increased latency (Figure 5A) and the decreased time spent grooming (Figure 5B) elicited by OB. These results indicated that ROHE and fluoxetine were able to abolish the anhedonic-like behavior induced by OB. A two-way ANOVA revealed a significant main effect of OB [$F(1,94)=20.48$, $P<0.01$] and significant treatment X OB interaction [$F(4,94)=2.96$, $P<0.05$], but no significant main effect of treatment [$F(4,94)=2.00$, $P=0.10$] in the latency to exhibit grooming behavior. A two-way ANOVA also revealed a significant main effect of OB [$F(1,94)=4.23$, $P<0.05$] and treatment [$F(4,94)=6.64$, $P<0.01$] as well as treatment X OB interaction [$F(4,94)=4.10$, $P<0.01$] in the time spent grooming.

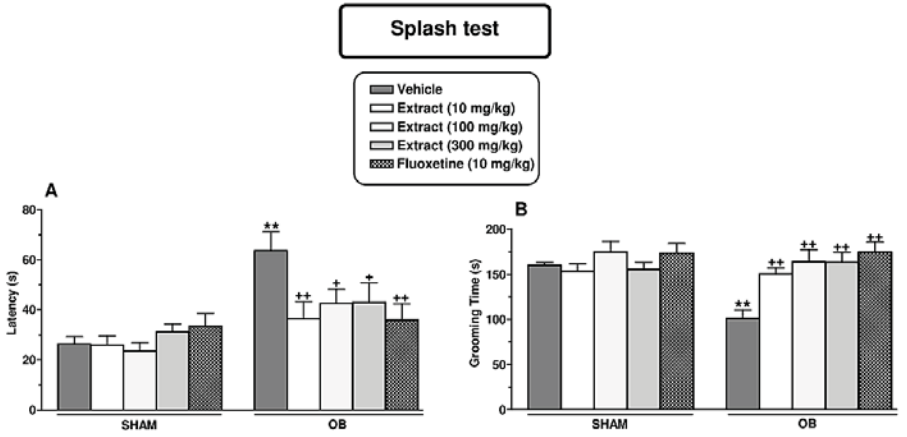

Figure 5. Effect of chronic treatment mice (14 days) with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.), in bulbectomized mice in the splash test. The anhedonic behavior of bulbectomized mice was analyzed through latency for initiation of grooming behavior (panel A) and time spent grooming (panel B). Each column represents the mean + S.E.M. (n = 9-11). ***P*<0.01 compared with

the control group (SHAM-vehicle); +P<0.05, ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

3.5. Effect of chronic treatment with ROHE in the cognitive performance of SHAM and bulbectomized mice in the water maze task

We tested the ability of SHAM-operated and bulbectomized mice to acquire (training session) and retrieve (test session) spatial information in the water maze paradigm as indicative of learning and memory functions. Firstly, to rule out a possible *per se* effect of ROHE tested in the spatial learning and memory of mice, additional groups of mice were evaluated in the water maze 33°days after surgery and after the repeated administration (14 days) of ROHE (10, 100 and 300 mg/kg, p.o.) or fluoxetine (10 mg/kg, p.o.) . The results illustrated in Figure 6A and 6B suggest that the treatment with ROHE did not interfere, at least at the present doses, with the spatial learning and memory of the animals, since no alterations in the escape latency (training session) were observed. A one-way ANOVA with repeated measures revealed no significant effect of treatment in the escape latencies during the training trials [$F(4,45) = 2.75$, P=0.39] (Figure 6A). Moreover, a one-way ANOVA revealed no significant effect of treatment in time spent in the correct quadrant in test session [$F(4,45) = 1.89$, P=0.13] (Figure 6B).

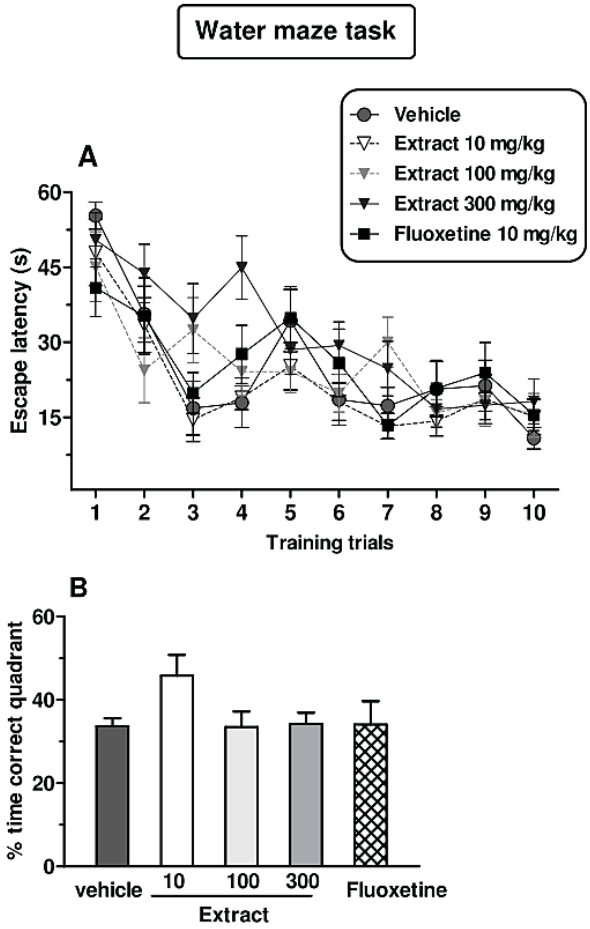

Figure 6. The effects of chronic administration (14 days) with ROHE (10-300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of SHAM-operated mice evaluated in the water maze task (Panel 6A and 6B). Data are presented as means ± S.E.M. latency, in seconds, for escape to a submerged platform (6A) (n = 9-11 animals in each group) and % time in the correct quadrant (6B). The probe test session was performed 24 h after the training trials. Data are presented as means ± S.E.M. of the time spent in the correct quadrant.

As can be seen from Figure 7A, OB resulted in a significant decline in spatial learning, as indicated by longer latencies to find the platform [$F(1,18) = 8.33$, $P<0.05$]. Subsequent Duncan post-hoc tests indicated that SHAM/vehicle mice learned quicker than bulbectomized mice, since although both groups displayed similar final escape latencies to find the platform, the learning curve of bulbectomized mice was clearly offset to the right, i.e. they needed a greater number of trials to satisfactorily acquire the spatial information (Figure 7A).The one-way ANOVA revealed no significant effect of OB in time spent in the correct quadrant (test session) [$F(1,18)=0.06$, $P=0.81$], as observed in the water maze (Figure 7B).

Moreover, repeated administration (14 days) by p.o. route of ROHE (10, 100 and 300 mg/kg) or fluoxetine (10 mg/kg) did not promote any significant effect on the spatial learning [$F(4,48) = 0.36$; $p=0.83$] and memory [$F(4,48) = 0.38$; $p=0.81$] of bulbectomized mice (Figure 7C and 7D).

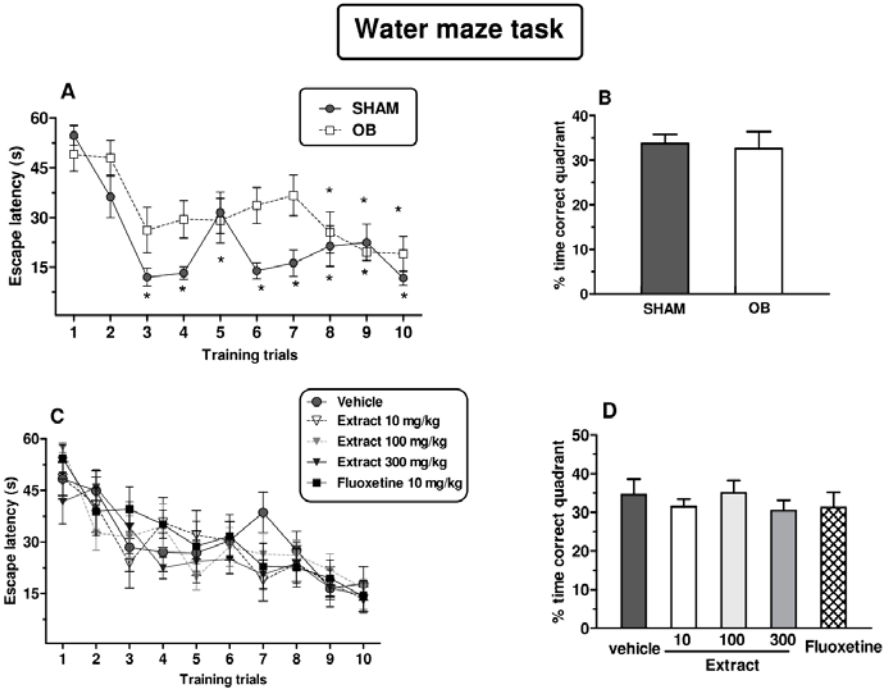

Figure 7. The effects of chronic administration (14 days) with ROHE (10- 300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of olfactory bulbectomized mice evaluated in the water maze task. Training trials were carried out on day 32 after OB. Data are presented as means ± S.E.M. latency, in seconds, for escape to a submerged platform (A,C) (n = 9-11 animals in each group). The probe test session was performed 24 h after the training trials. Data are presented as means ± S.E.M. of the time spent in the correct quadrant (B,D). *$P \leq 0.05$ compared to the first trial of the same group (Duncan post-hoc test).

3.6. Effect of chronic treatment with ROHE on the serum glucose level in bulbectomized animals

The results depicted in Figure 8A show a decreased serum glucose level in bulbectomized mice as compared with the control group (SHAM-vehicle). This effect was abolished by p.o. treatment with ROHE (10 mg/kg), but not by fluoxetine (10 mg/kg). A two-way ANOVA indicated a significant effect of treatment x OB interaction [$F(2,46)=4.11$, $P<0.05$], but not of OB[$F(1,46)=1.57$, $P=0.21$] and treatment [$F(2,46)=0.20$,$P=0.81$].

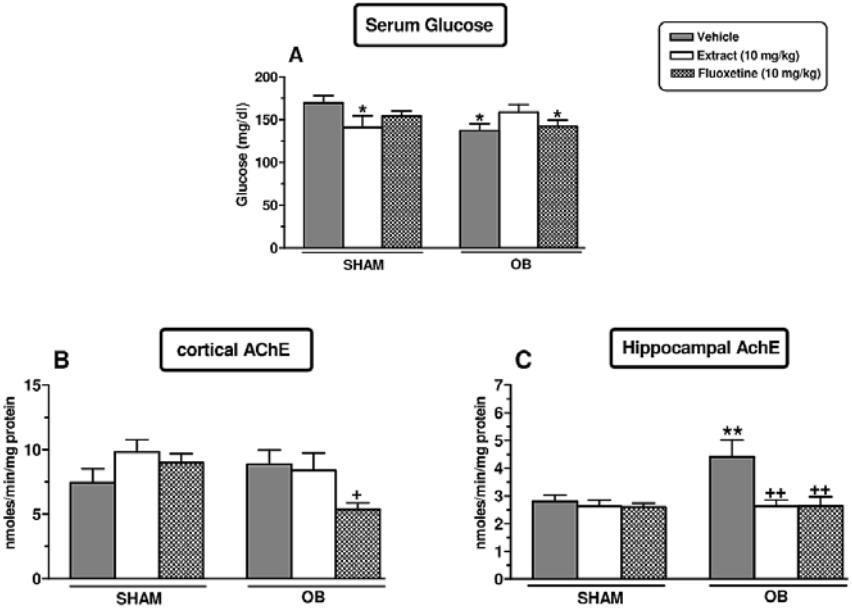

Figure 8. Effect of the chronic treatment of mice (14 days) with ROHE (10 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on serum glucose level (expressed as mg/dl) (panel A) and on AChE activity in frontal cortex (panel B) and hippocampus (panel C) of bulbectomized mice. Enzyme activity was expressed as nmol/min/mg protein. Each column represents the mean + S.E.M. of 7-9 animals. **P*<0.05, ***P*<0.01 compared with the control group (SHAM-vehicle) and +*P*<0.05, ++*P*<0.01 when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

3.7. Effect of chronic treatment with ROHE on cerebrocortical and hippocampal AChE activity in bulbectomized mice

Figure 8 also shows the effect of chronic p.o. administration of ROHE (10 mg/kg) and fluoxetine (10 mg/kg) on the activity of the enzyme AChE in the cerebral cortex (Figure 8B) and hippocampus (Figure 8C) of bulbectomized animals. As demonstrated in Figure 8B, the activity of AChE in the frontal cortex was not changed in the group of bulbectomized mice treated with ROHE as compared with the control (SHAM-vehicle) and with BO-vehicle groups. However, as shown in Figure 8B, the activity of AChE in the frontal cortex was lower in the group of bulbectomized mice treated with fluoxetine (10 mg/kg, p.o.) as compared with the bulbectomized/vehicle group. A two-way ANOVA revealed a significant effect of treatment x OB interaction [$F(2,38)=3.49$, $P<0.05$], but not of OB [$F(1,38)=2.29$, $P=0.13$] and treatment [$F(2,38)=1.91$, $P=0.16$] in the activity of AChE in the frontal cortex. However, Figure 8C shows a significant increase on hippocampal AChE activity in bulbectomized/vehicle group, as compared with control group (SHAM-vehicle), an effect reversed by ROHE (10 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.). A two-way ANOVA revealed a significant main effect of OB [$F(1,38)=4.28$, $P<0.05$], treatment [$F(2,38)=6.19$, $P<0.01$] and treatment X OB interaction [$F(2,38)=3.96$, $P<0.05$] on the activity of AChE in the hippocampus.

4. Discussion

To gain a better understanding of the potential antidepressant of *Rosmarinus officinalis*, this study evaluated the effects of the extract of this plant in the OB model, since the removal of olfactory bulbs in rodents results in several behavioral, neurochemical, neuroendocrinological alterations, comparable to those seen in depressed patients (Kelly et al., 1997; Song and Leonard, 2005). Indeed, the antidepressant-like effect of ROHE was firstly investigated by our group in two behavioral models predictive of antidepressant activity, the forced swimming test (FST) and tail suspension test (TST) in mice. ROHE produced an antidepressant-like effect, since the acute treatment of mice with the extract by p.o. route significantly reduced the immobility time in the FST (100 mg/kg) and TST (10-100 mg/kg). Moreover, the repeated administration (14 days) of the ROHE by p.o. route also produced an antidepressant-like effect in the TST (100–300 mg/kg). However, the behavioral tests with predictive validity are designated with this terminology because they are based exclusively on the behavioral effects of drugs used clinically, but do not mimic the symptoms of disease. In addition, another drawback of these tests is the fact that they are sensitive to the acute administration of antidepressants, but the action of classical antidepressants in the existing therapies can take up to several weeks to show their full therapeutic effect (Cryan et al., 2002, 2005). Interestingly, the OB model used in the present study has been suggested to possess a good face validity with human depressive disorder, especially agitated depression (Harkin et al., 2003; Kelly et al., 1997; Romeas et al., 2009).

The behavioral abnormalities induced by OB appear after 2 weeks in rodents; probably because the lesion caused by OB induces reorganization processes in the limbic and cortical areas (Jarozik et al., 2007; van Riezen and Leonard, 1990; Zueger et al., 2005). In the present study bulbectomized mice showed a significant increase in locomotor and exploratory activities in the open-field, novel object and novel cage tests and also an anhedonic-like behavior. These behavioral alterations are indicative of a depressive-like profile of these animals (Harkin et al., 2003; Kelly et al., 1997; Zueger

et al., 2005). Noteworthy, ROHE, similarly to the antidepressant fluoxetine, used here as a positive control, was able to abolish these behavioral alterations.

The hyperactivity in bulbectomized animals is the main alteration reported in literature. Various classes of clinically active antidepressants abolish the hyperactivity of bulbectomized rodents in the open-field, such as: serotonin reuptake inhibitor -SSRI (citalopram, fluoxetine, paroxetine, sertraline, venlafaxine), noradrenaline reuptake inhibitor-NE (reboxetine), tricyclic antidepressant (amitriptyline, desipramine, imipramine) (Butler and Leonard, 1990; Connor et al. 2000; Jarozik et al., 2007; Kelly et al., 1997; Possidente et al., 1996; Rodríguez-Gaztelumendi, et al., 2009; Song and Leonard, 2005). Additionally, it was reported that curcumin, the active component of *Curcuma longa*, was able to reverse hyperactivity induced by OB in rats (Xu et al., 2005).

Furthermore, another relevant behavioral change triggered by the OB is the increased vulnerability and responsiveness to environmental stress (Mar et al., 2000; van Riezen e Leonard, 1990). In our investigation, the bulbectomized mice showed locomotor and exploratory hyperactivity induced by novelty, indicated by an increase in behavioral reactivity when mice were submitted to the novel object test (decreased latency for entering the central arena, increased time spent exploring and increased rearings responses in the central area where the novel object was located) and to the novel cage test (increased number of crossings and rearings in the new environment).These results are in agreement with literature data that report that the hyperactivity induced by OB is directly related to a greater reactivity to novel environments or deficit in habituation to new situations (Mar et al., 2000; van Riezen e Leonard, 1990; Zueger et al, 2005). The chronic treatments with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10mg/kg) were able to reverse all the OB-induced behavioral alterations in the novel cage test. These results are in accordance with the fact that antidepressants such as fluoxetine and amitriptyline are effective to restore normal responding by permitting more effective adaptation to novel stimuli in the bulbectomized rodents (Mar et al., 2000, 2002), reinforcing the notion that ROHE has an antidepressant -like action.

Anhedonia, or hyposensitivity to pleasure, is one of the key symptoms for a diagnosis of depression (WHO, 1992). In the present study this behavior was inferred by the decreased grooming time and increased latency to grooming in the splash test in bulbectomized mice as compared to those exhibited by control mice (SHAM). This result is in accordance with some studies that have shown an anhedonic-like behavior in bulbectomized rats (Romeas et al., 2009, Stock et al., 2000). Noteworthy, in our study, the anhedonic-like behavior induced by OB was abolished by ROHE and fluoxetine. This result is in line with the ability the compounds with antidepressant properties, such as the classical antidepressants fluoxetine, imipramine and desipramine (David et al., 2009; Detanico et al., 2010;Yalcin et al., 2005) as well as *Ptychopetalum olacoides* Bentham (marapuama) extract (Piato et al., 2008), which were capable of reversing anhedonic behavior induced by stress models of depression. Moreover, this result also reinforces the notion the ROHE has antidepressant properties.

In accordance with previous studies reporting a reduced performance of OB rodents in different paradigms used to investigate learning and memory processes (Harkin et al., 2003; Kelly et al. 1997; Mucignat-Caretta et al. 2006) our results show a poor performance of OB compared to SHAM-mice in the spatial version of the Morris water maze. Interestingly, it was shown in our study that OB mice needed significantly more trials in the training session to acquire the spatial information, but they displayed a similar profile to that of SHAM-groups in the test session (24 h later), demonstrating a selective deficit in spatial learning in water maze task. Important to note, this results is opposed to some reports that have demonstrated impairment of OB in both spatial learning and memory in the water maze. This discrepancy with early data may be explained by differences between the protocols utilized to evaluate the spatial learning and memory in the water maze. In these previous studies, each mice was given 4 trials per day for 4-5 consecutive days to find the platform (Mucignat-Caretta et al. 2006), while in the current study each mice was given 10 consecutive trials during the training session (only 1 day) and the test session occurred 24 h later, similar to study reported by Prediger et al. (2005). Thus, it is possible that a training schedule with a higher number of consecutive trials instead of

repeatedly training over a number of days promotes equivalence in the learning performance for both strains, which can be observed in the similar pattern of the escape latencies of the latter training trials.

The Morris water maze is a test of hippocampal function (Morris, 1982) that does not depend upon olfactory cues, but more on visual cues. A study reported by Van Rijzingen et al. (1995) showed that two weeks after OB, the Morris maze performance was severely impaired. However this alteration is a transient cognitive deficit since the recovery occurs spontaneously approximately 6 weeks following surgery. Thus, the Morris maze performance of OB animals and SHAM- controls 6 weeks after surgery did not show a difference in escape latency neither during acquisition nor during the probe trial.

In the present study the chronic treatment with ROHE (10-300 mg/kg, p.o.) or fluoxetine did not abolish the deficit in spatial learning induced by OB. This model of depression induced an impairment of learning and memory in the three-panel runway task in rats (Yamamoto et al., 1997) and in passive avoidance task in mice on the 7th and 14th day after the surgery (Hozumi et al., 2003). The impairment of learning and memory induced by OB in these tests, on the 14th day was improved by administration of the cholinesterase inhibitor physostigmine. Additionally, the ability of several compounds, including curcumin (Xu et al., 2005), nobiletin (Nakajima et al., 2007) and an active ginseng metabolite (20(S)-protopanaxadiol) (Xu et al., 2010), to reverse cognitive deficit in olfactory bulbectomized rodents in the step down was reported. However, there are few data dealing with the effects of *Rosmarinus officinalis* on cognitive performance, but it has a history of usage as cognitive enhancer (Kennedy and Scholey, 2006). A study by Hosseinzadeh et al. (2004) reported that the essential oil of *Rosmarinus officinalis* injected intraperitoneally to rats 0.5 h before training for 5 consecutive days improved the intact memory and scopolamine-induced learning deficits in rats performing the Morris water maze task. A recent study in an elderly population has reported that this plant when administered at a low dose caused an improvement on cognitive function but at a high dose caused an impairing effect (Pengelly et al., 2012). Moreover, in one study of 144 healthy individuals, airborne *Rosmarinus officinalis* essential oil significantly enhanced cognitive performance and mood

(Moss et al., 2003). These studies reported that the *Rosmarinus officinalis* is a promising candidate for the improvement of memory in healthy people or for the treatment diseases associated with cognitive deficit, an effect that may be due to, at least in part to its anticholinesterase properties (Duke, 2007; Ingole et al., 2008; Kennedy and Scholey, 2006; Singh et al., 2011). Regarding the present study, it remains to be established if a more prolonged treatment with ROHE, as well as fluoxetine, would be able to abolish the OB-induced cognitive deficit.

In a second experimental phase of this study, we evaluated some biochemical parameters that could be changed by OB procedure, as serum glucose level, and activity of the enzyme AChE in the hippocampus and frontal cortex. The ability of the ROHE and fluoxetine (positive control) to blunt some of the alterations induced by OB on these parameters was also evaluated.

This study showed a decrease in the serum glucose level in bulbectomized animals, as compared with the control-SHAM, similarly to results previously reported in the literature (Belló and Rummler, 1979; Perassi et al., 1975). Moreover, a recent study showed that acute hypoglycemia causes depressive-like behaviors (increased immobility in the forced swim test and reduced saccharin preference, an indicative of anhedonic-like behavior) in mice which were prevented by the antidepressants fluoxetine and desipramine (Park et al., 2012). However, in present study the chronic treatment with fluoxetine was not able to alter the reduction of serum glucose level induced by OB. Interestingly, treatment with ROHE decreased serum glucose level in SHAM mice, but abolished the decrease on glucose levels induced by OB, since it was able to restore serum glucose level to normal. Thus, the effects of ROHE and fluoxetine on serum glucose level in SHAM and OB mice are quite different. Indeed literature data have reported that *Rosmarinus officinalis* exerts notable hypoglycemic or anti-hyperglycemic activity (Abu-Al-Basal, 2010; Bakirel et al, 2008). Although interesting the effects of ROHE and fluoxetine on serum glucose level do not seem to be associated with the behavioral alterations described in our study.

Taking into account the well known implication of the cholinergic system with the behavioral alterations elicited by OB (Moriguchi et al., 2006; Nakajima et al. 2007) and in the pathophysiology of

depression (Dagyte et al., 2011), the present study also dealt with the determination of AChE activity in the hippocampus and frontal cortex. AChE is an important constituent of cholinergic neurotransmission that catalyzes the hydrolysis of acetylcholine in the synaptic cleft and neuromuscular junctions (Soreq and Seidman, 2001). Interestingly, in the present study an increase on AChE activity in the hippocampus, but not in the frontal cortex, in the bulbectomized mice was reversed by chronic treatment with ROHE (10 mg/kg) and fluoxetine (10 mg/kg). In line with this, the *in vitro* anticholinesterase effect of *Rosmarinus officinalis* was reported (Adsersen et al., 2006). Moreover, treatment with the antidepressant fluoxetine decreased the activity of AChE in human serum and erythrocyte membrane (Müller et al, 2002). Our results suggest that an increased hippocampal activity of this enzyme may be associated with the depressive-like behavior observed in the bulbectomized mice. Regarding the ability of ROHE and fluoxetine to reduce the activity of AchE, consequently increasing acethylcholine levels, we may raise the hypothesis that this could lead to a desensitization of nicotinic acethylcholine receptors. Indeed, it has been proposed that a fine balance between the activation and desensitization of nicotinic receptors is required to yield relevant antidepressant-like effects (Mineur and Picciotto, 2010). However, in our study an absence of alteration of AChE in cerebral cortex was observed in olfactory bulbectomized mice, a result that is similar to a finding reported by Yamada et al. (2011) in bulbectomized mice.

5. Conclusion

The present study shows that OB mice exhibited hyperactivity and anhedonic-like behavior associated with an increased hippocampal AChE activity, parameters that were abolished by chronic treatment with ROHE, similarly to the effects produced by fluoxetine. These results suggest that *Rosmarinus officinalis* may be further investigated as an effective therapeutic alternative for the treatment of agitated depression associated with anhedonia.

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

Acknowledgements

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LEGENDS TO THE FIGURES

Figure 1. Schematic representation of the experimental protocol with the treatment period with ROHE and behavioral tests period (Panel A). The animals were killed by decapitation 6 hours after the end of the behavioral testing on day 33. Blood samples were collected and hippocampus and frontal cortex dissected, then stored in a freezer at - 80° C for subsequent biochemical analysis (Panel B). (OFT: open-field test, NOT: novel object test, NCT: novel cage test, Trials WM: training session of the water maze and Test WM: test session of the water maze).

Figure 2. Chromatographic profiles (HPLC) of crude extract from *Rosmarinus officinalis* L. Peaks represent rosmarinic acid (1), carnosol (2), betulinic acid (3), oleanolic acid (4), and ursolic acid (5).

Figure 3. Effect of the chronic treatment of mice (14 days) with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on the number of crossings (panel A) and rearings (panel B) in bulbectomized mice in the open-field test. Each column represents the mean + S.E.M. of 9-11 animals. ***P*<0.01 as compared to control group (SHAM-vehicle); +*P*<0.05, ++*P*<0.01 when compared with OB - vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 4. Effect of chronic treatment of mice (14 days) with hydroalcoholic extract of *Rosmarinus officinalis* (10-300 mg/kg, p.o.) and Fluoxetine (10 mg/kg, p.o.), on the exploratory activity of bulbectomized mice in the novel object test and novel cage test. The exploratory behavior was assessed monitoring the time spent exploring the novel object (panel A) and the number of rearing responses in the central area of the open-field (panel B) in the novel object test; and the number of crossings (panel C) and rearings (panel D) of bulbectomized mice in the novel cage test. Each column represents the mean + S.E.M. of 9-11 animals. **P*<0.05, ***P*<0.01 compared with the control group

(SHAM-vehicle) and +P<0.05, ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

Figure 5. Effect of chronic treatment mice (14 days) with ROHE (10-300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.), in bulbectomized mice in the splash test. The anhedonic behavior of bulbectomized mice was analyzed through latency for initiation of grooming behavior (panel A) and time spent grooming (panel B). Each column represents the mean + S.E.M. (n = 9-11). **P<0.01 compared with the control group (SHAM-vehicle); +P<0.05, ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

Figure 6. The effects of chronic administration (14 days) with ROHE (10-300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of SHAM-operated mice evaluated in the water maze task (Panel 6A and 6B).Data are presented as means ± S.E.M. latency, in seconds, for escape to a submerged platform (6A) (n = 9-11 animals in each group) and % time in the correct quadrant (6B). The probe test session was performed 24 h after the training trials. Data are presented as means ± S.E.M. of the time spent in the correct quadrant.

Figure 7. The effects of chronic administration (14 days) with ROHE (10- 300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of olfactory bulbectomized mice evaluated in the water maze task. Training trials were carried out on day 32 after OB. Data are presented as means ± S.E.M. latency, in seconds, for escape to a submerged platform (A,C) (n = 9-11 animals in each group). The probe test session was performed 24 h after the training trials. Data are presented as means ± S.E.M. of the time spent in the correct quadrant (B,D). *$P\leq$ 0.05 compared to the first trial of the same group (Duncan post-hoc test).

Figure 8. Effect of the chronic treatment of mice (14 days) with ROHE (10 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on serum glucose level (expressed as mg/dl) (panel A) and on AChE activity in frontal cortex (panel B) and hippocampus (panel C) of bulbectomized mice. Enzyme activity was expressed as nmol/min/mg protein. Each column represents the mean + S.E.M. of 7-9 animals. *P<0.05, **P<0.01 compared with the control group (SHAM-vehicle) and +P<0.05, ++P<0.01 when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

Figure 1

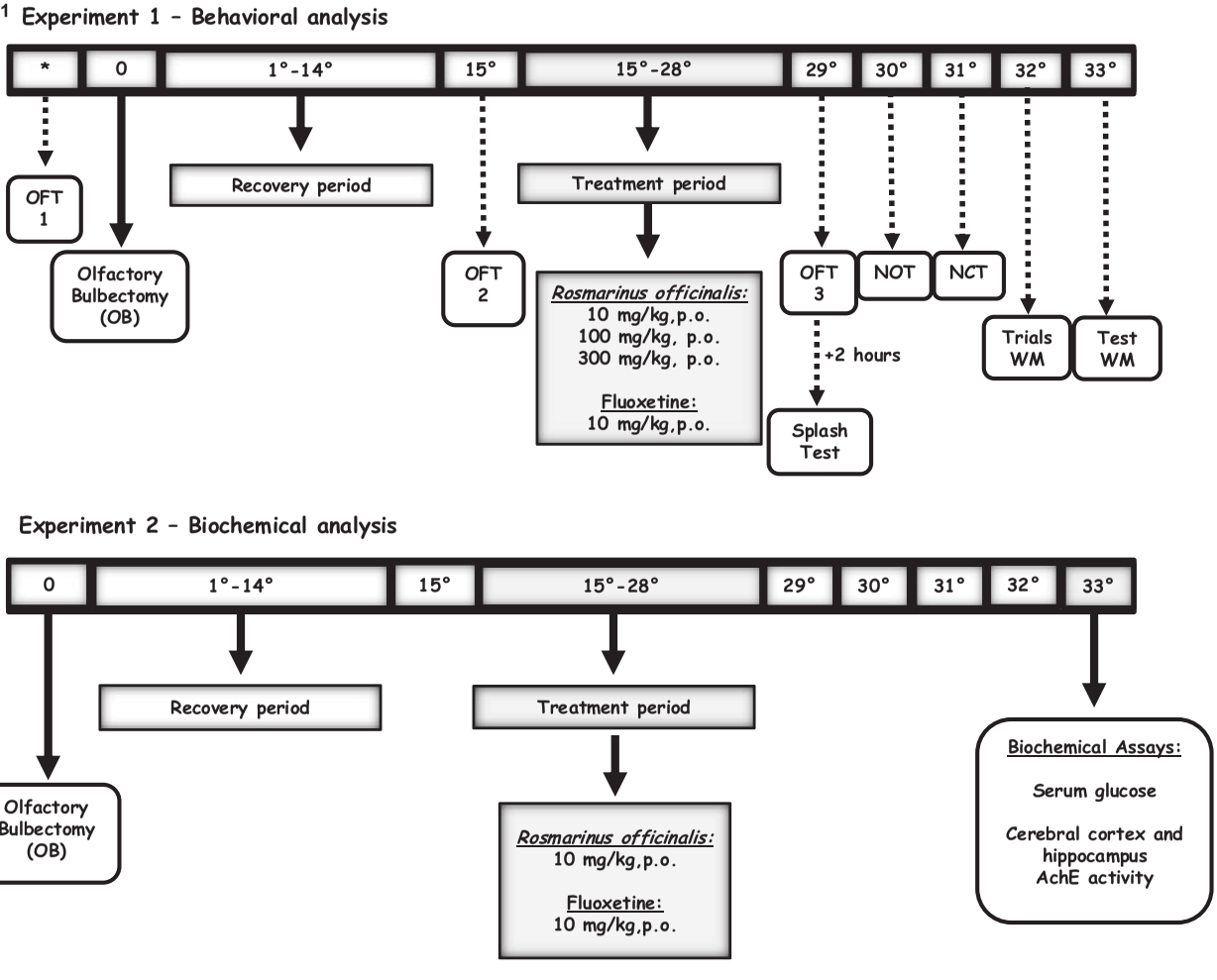
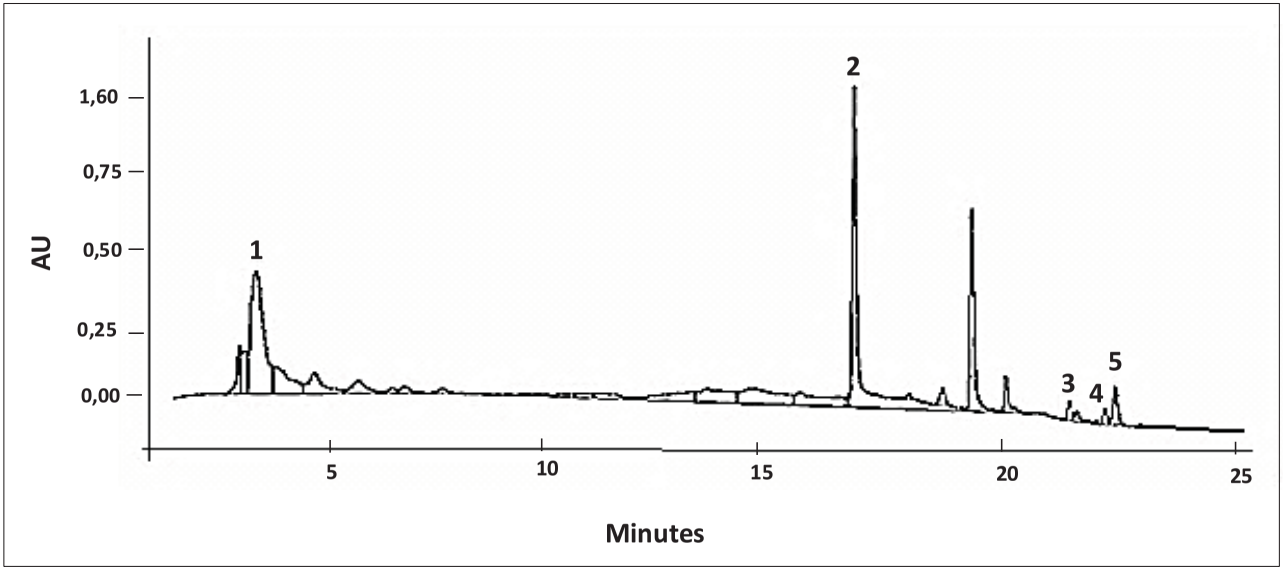
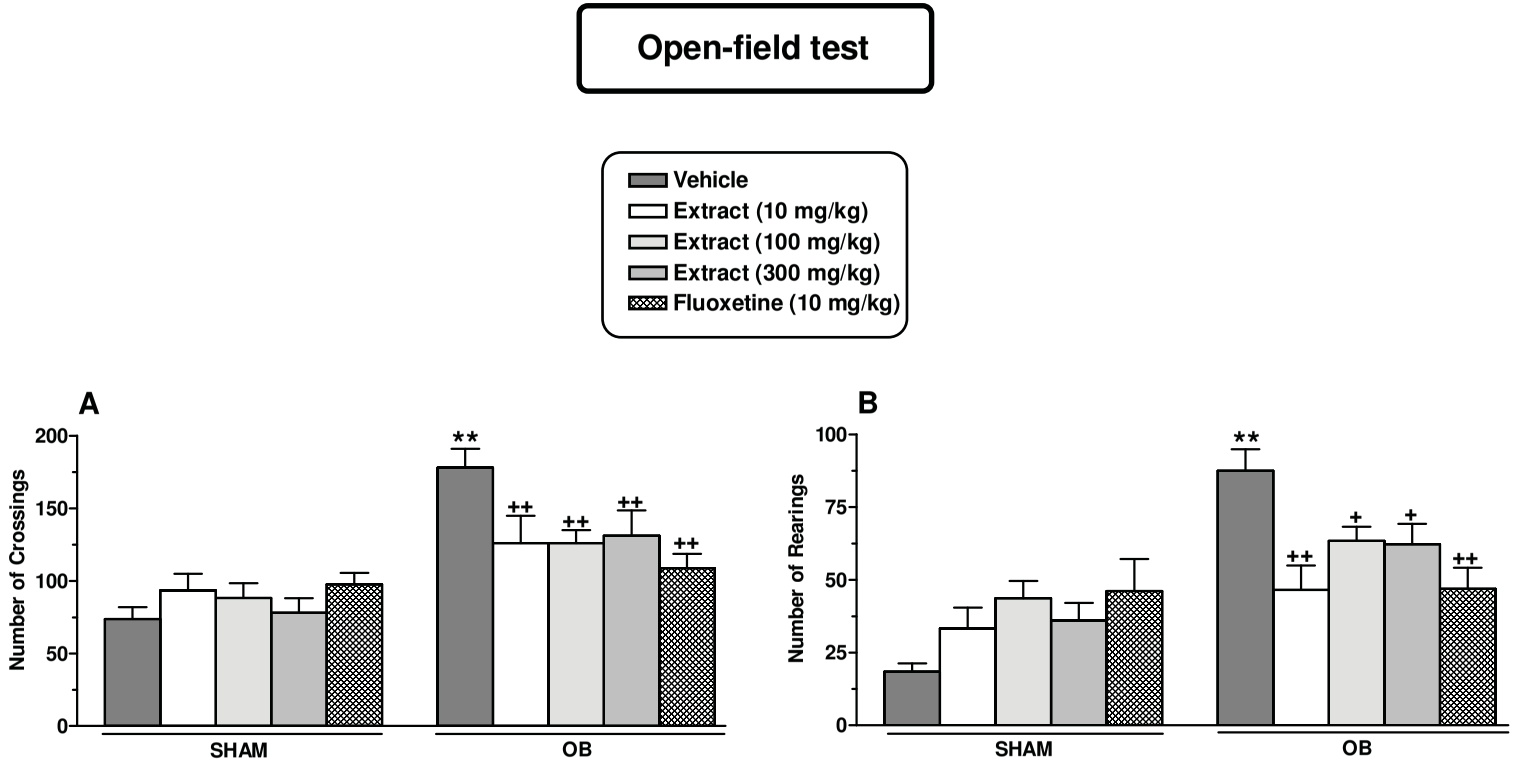

Experiment 1 – Behavioral analysis
*
0
1°-14°
15°
15°-28°
29°
30°
31°
32°
33°
OFT 1
Olfactory Bulbectomy (OB)
Recovery period
OFT 2
Treatment period
Rosmarinus officinalis:
10 mg/kg,p.o.
100 mg/kg, p.o.
300 mg/kg, p.o.
Fluoxetine:
10 mg/kg,p.o.
OFT 3
+2 hours
Splash Test
NOT
NCT
Trials WM
Test WM
Experiment 2 – Biochemical analysis
0
1°-14°
15°
15°-28°
29°
30°
31°
32°
33°
Olfactory Bulbectomy (OB)
Recovery period
Treatment period
Rosmarinus officinalis:
10 mg/kg,p.o.
Fluoxetine:
10 mg/kg,p.o.
Biochemical Assays:
Serum glucose
Cerebral cortex and hippocampus
AchE activity

Figure 2




Open-field test
Vehicle
Extract (10 mg/kg)
Extract (100 mg/kg)
Extract (300 mg/kg)
Fluoxetine (10 mg/kg)
A
Number of Crossings
200
150
100
50
0
**
++
++
++
++
SHAM
OB
B
Number of Rearings
100
75
50
25
0
**
++
+
+
++
SHAM
OB

ARTIGO~2.PZM:Figure 4 - Sun Feb 19 22:42:10 2012

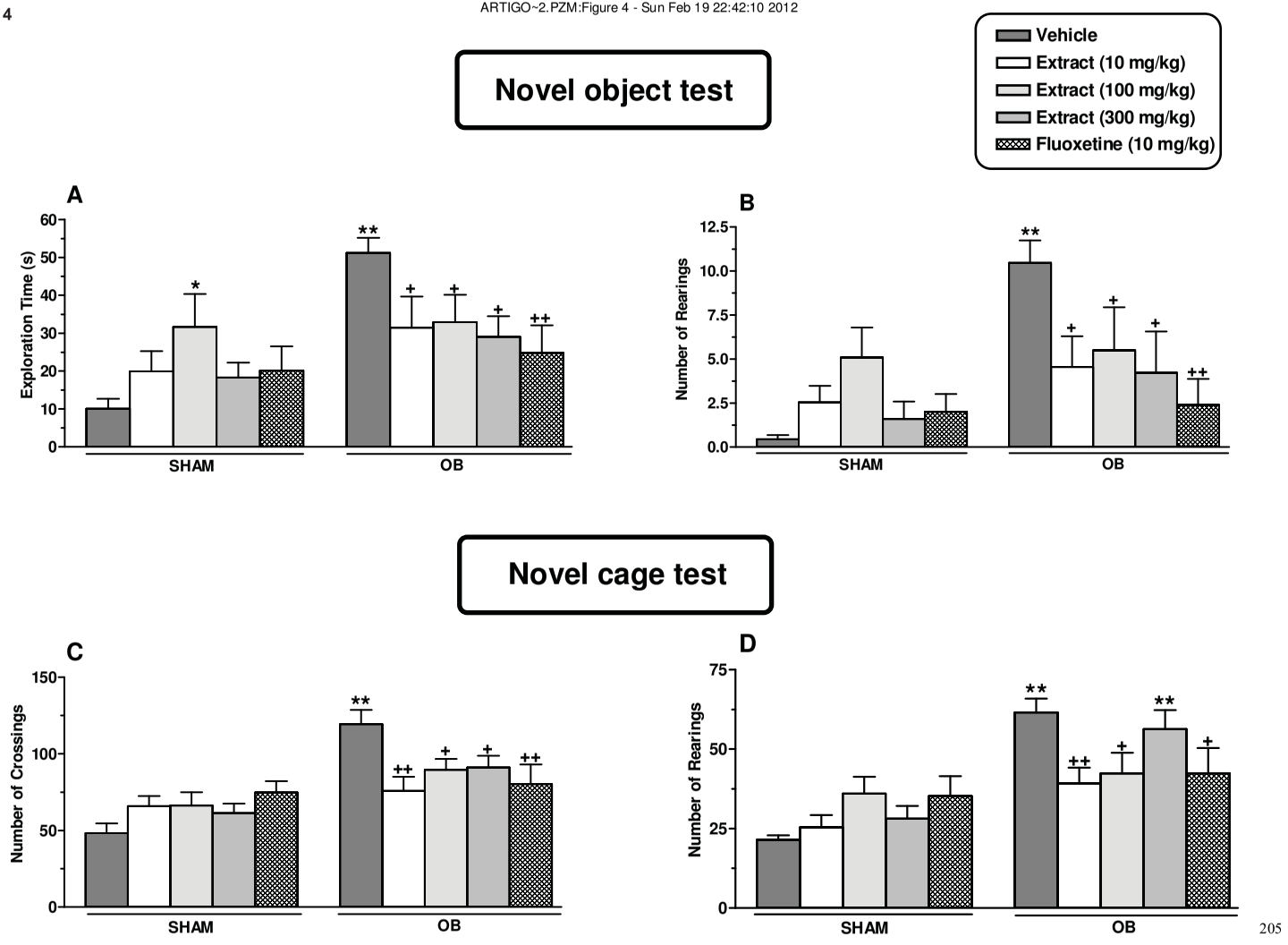
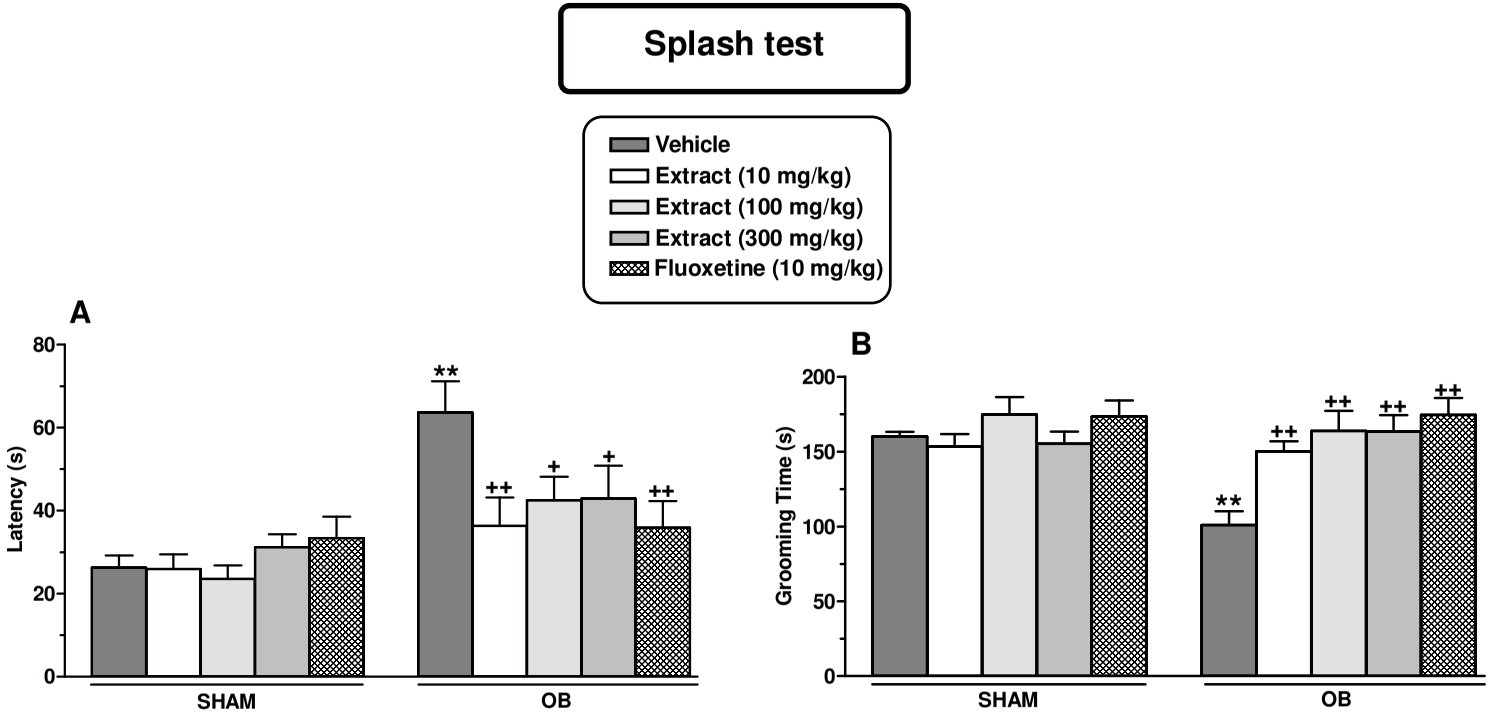
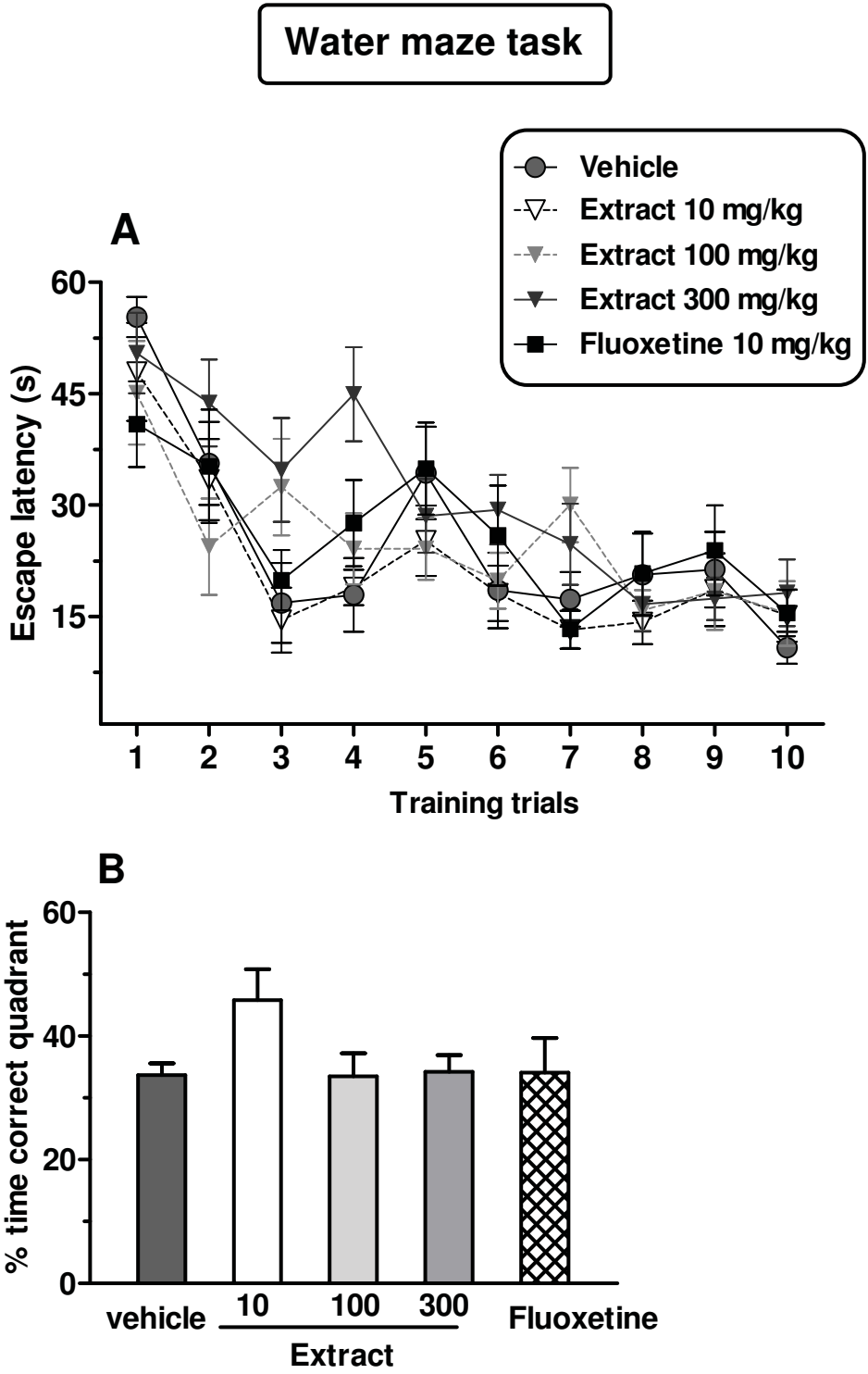
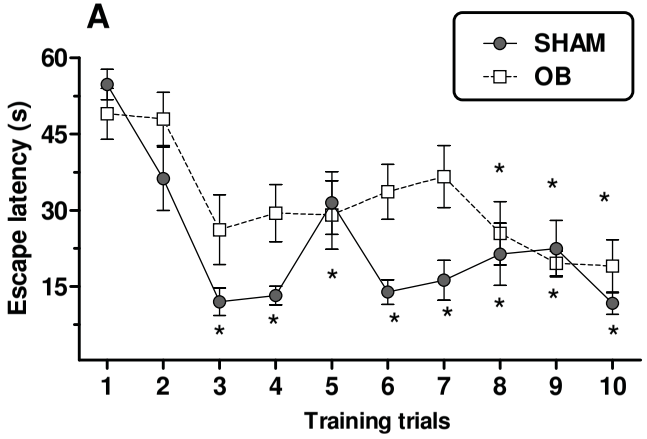


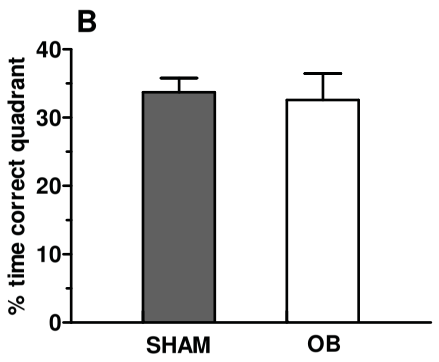
Figure 5

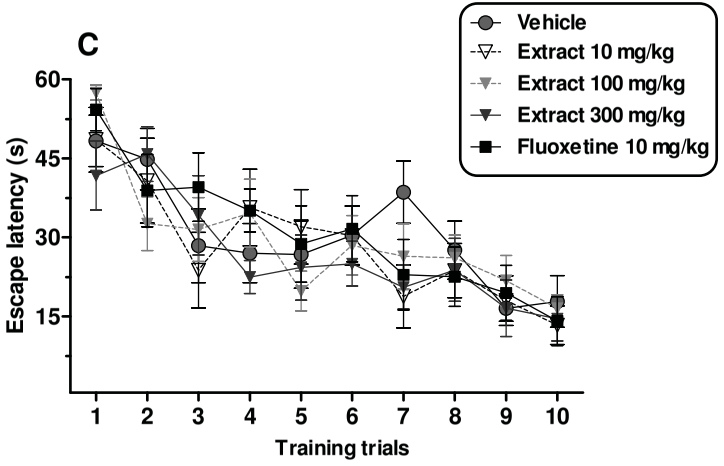

Splash test
Vehicle
Extract (10 mg/kg)
Extract (100 mg/kg)
Extract (300 mg/kg)
Fluoxetine (10 mg/kg)
A
Latency (s)
80
60
40
20
0
**
++
+
+
++
SHAM
OB
B
Grooming Time (s)
200
150
100
50
0
**
++
++
++
++
SHAM
OB


Water maze task
A
Vehicle
Extract 10 mg/kg
Extract 100 mg/kg
Extract 300 mg/kg
Fluoxetine 10 mg/kg
Escape latency (s)
60
45
30
15
1
2
3
4
5
6
7
8
9
10
Training trials
B
% time correct quadrant
60
40
20
0
vehicle
10
100
300
Fluoxetine
Extract

Water maze task







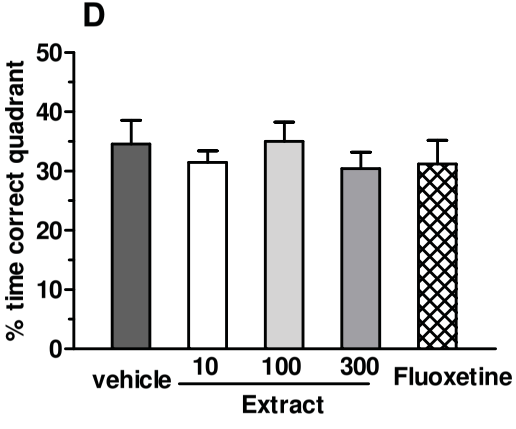
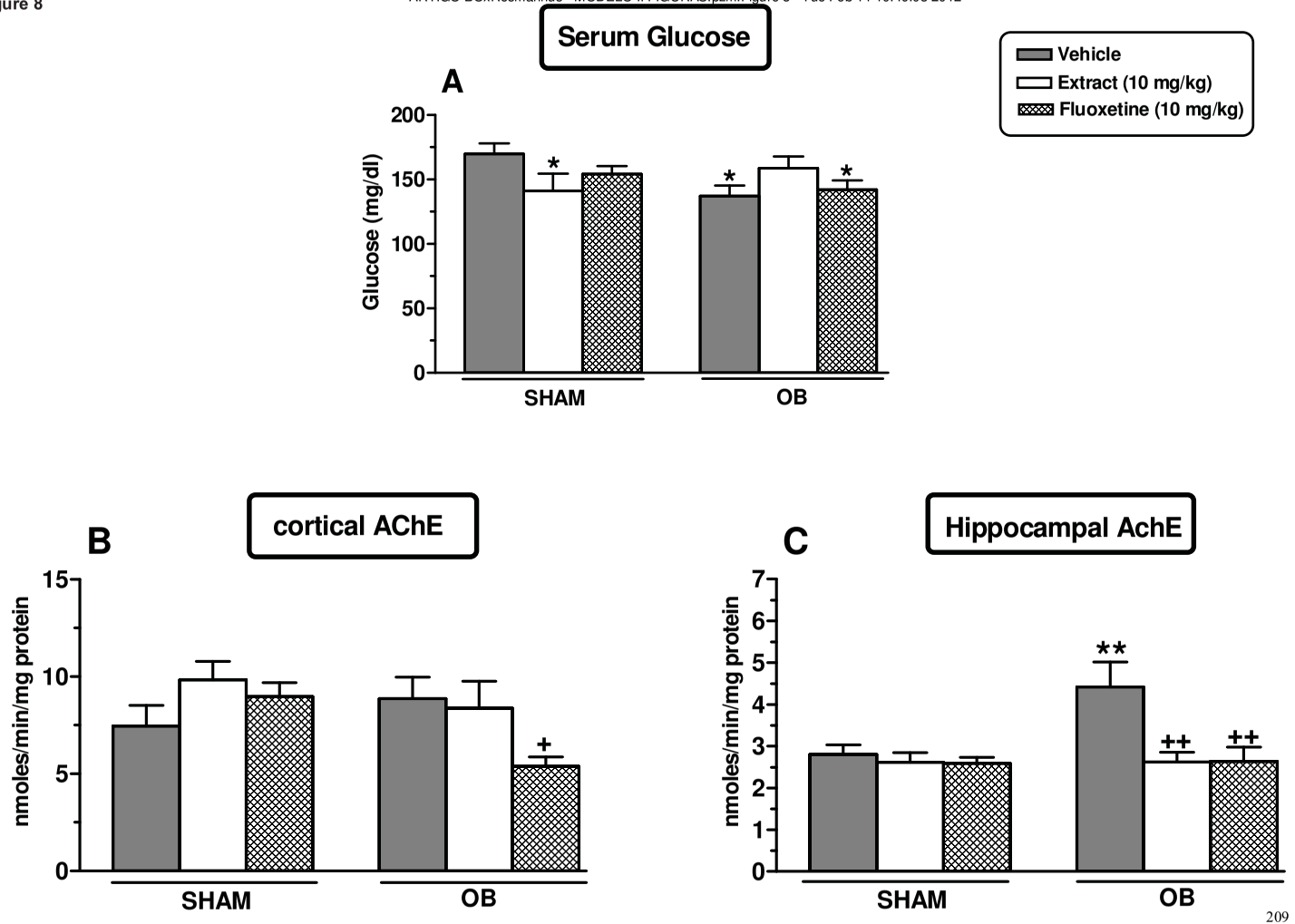
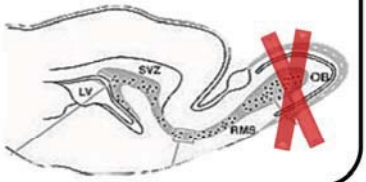


Figure 8


Serum Glucose
A
Vehicle
Extract (10 mg/kg)
Fluoxetine (10 mg/kg)
Glucose (mg/dl)
200
150
100
50
0
*
*
*
SHAM
OB
B
cortical AChE
nmoles/min/mg protein
15
10
5
0
+
SHAM
OB
C
Hippocampal AchE
nmoles/min/mg protein
7
6
5
4
3
2
1
0
**
++
++
SHAM
OB

Olfactory Bulbectomy (OB)



OB-induced behavioral alterations

*Hyperactivity in open-field test,

*Hyperactivity induced by novelty in novel object test and novel cage test,
*Anhedonic behavior in Splash test,

*Spatial learning deficit in Water maze task.

OB-induced biochemical alterations

*Hypoglycemia,
* Increased hippocampal AChE activity.

Antidepressant-like effect

Behavioral alterations

*ROHE (10-300 mg/kg):

-Abolished OB-induced hyperactivity, increased exploratory and anhedonic behavior.

-Did not reverse OB-induced spatial learning deficit.

-Results similar to fluoxetine.

Biochemical alterations:
*ROHE (10 mg/kg):

-Reversed OB-induced hypoglycemia, and the increased hippocampal AChE activity.

3. DISCUSSÃO GERAL

3.1. Potencial antidepressivo de *Rosmarinus officinalis* em testes preditivos de atividade antidepressiva

O estudo do efeito antidepressivo das plantas medicinais tem despertado um crescente interesse, devido ao aumento na incidência da depressão e do uso dessa forma de terapia nos transtornos psiquiátricos. Soma-se à essa realidade, o fato do tratamento da depressão com os antidepressivos convencionais (inibidores da monoamina oxidase, antidepressivos tricíclicos e inibidores seletivos da recaptação de serotonina, seletivos da recaptação de noradrenalina) proporcionar uma remissão completa apenas para cerca de 50% dos indivíduos (Nestler et al., 2002a).

Vários estudos científicos mostraram que os extratos de plantas medicinais e seus constituintes, incluindo os extratos de plantas tais como *Hyperforatum pericum, Curcuma longa, Ginkgo biloba, Schinus molle, Origanum vulgare* e compostos isolados como berberina, curcumina, rutina, entre outros, exercem efeito antidepressivo em modelos animais de depressão (Rodrigues et al., 2002; Zhang, 2004; Sakakibara et al., 2006; Machado et al., 2007, 2008; McGarry et al., 2007; Peng et al. 2007; Zhang et al., 2007; Kulkarni et al., 2008; Wang et al. 2008; Huang et al., 2011; Mechan et al., 2011).

O TNF e TSC são amplamente utilizados para a investigação de compostos com potencial antidepressivo. Antidepressivos reduzem o tempo de imobilidade em ambos os testes, o TNF e TSC. O comportamento de imobilidade exibido em roedores quando submetidos à uma situação de estresse inescapável, reflete um estado de desespero comportamental, que é comparado e relacionado ao estado depressivo em humanos (Porsolt et al., 1977; Steru et al., 1985; Cryan et al., 2002). No presente estudo, nossos resultados mostraram que o tratamento agudo com o extrato de *Rosmarinus officinalis* produziu uma resposta tipo-antidepressiva em ambos os testes comportamentais, TNF e TSC. Além disso, nosso estudo mostrou que a administração repetida (14 dias) do extrato das folhas de *Rosmarinus officinalis* também foi capaz de produzir um efeito antidepressivo no TSC. Estes resultados estão em conformidade com a evidência do efeito etnofarmacológico desta planta (Ducke, 2000; Negraes, 2003; Heinrich et al., 2006; Franco e Fontana, 2007).

Vale ressaltar que o efeito produzido pelo extrato desta planta foi semelhante ao produzido pelo antidepressivo clássico fluoxetina (10

mg/kg, p.o.). O efeito antidepressivo da administração aguda do extrato foi observada em uma dose mais baixa no TSC (10 mg /kg, p.o.) do que no TNF (100 mg/ kg, po). A princípio o parâmetro de avaliação é idêntico no TNF e TSC, pois ambos avaliam o tempo de imobilidade dos animais frente à situações de estresse inescapável, mas a variabilidade de resposta a certos antidepressivos indica que cada teste atua em substratos neurais e vias neuroquímicas distintas (Bai et al., 2001). Isso se deve, pelo menos em parte, ao fato da natureza do estresse inescapável ser distinto em cada teste. Estes fatores mencionados podem ser a base das diferenças comportamentais encontradas (Bai et al., 2001). Além disso, o TNF em camundongos não tem sido tradicionalmente considerado como um modelo sensível para a detecção dos inibidores seletivos de recaptação de serotonina, enquanto esses antidepressivos são geralmente descritos como ativos no TSC (Cryan et al., 2005). Ainda, o TSC foi proposto como o teste de maior sensibilidade farmacológica em comparação ao TNF (Thierry et al., 1986; Cryan et al., 2005).

A fim de evitar falsos resultados positivos no TNF e TSC, é importante descartar a possibilidade de que as reduções no tempo de imobilidade não foram devido a um efeito psico- estimulante do extrato. Em nosso estudo, tanto o tratamento agudo ou repetido com o extrato de Rosmarinus officinalis não aumentou a atividade locomotora e exploratória em doses que produziram um efeito antidepressivo, indicando um efeito específico desta planta sobre estes modelos comportamentais preditivos de ação antidepressiva.

Um estudo pré-clinico mostrou que o tratamento com o extrato aquoso de *Rosmarinus officinalis* por via oral (nas doses de 291,2 e 582,4 mg/kg) por cinco dias em ratos Wistar não causou sinais físicos de toxicidade (piloereção ou alterações locomotoras) ou alteração significativa de peso corporal ou de órgãos vitais, como: fígado, rins, pulmões, cérebro e glândula pituitária (Silveira e Sá, 2006).

3.2. Envolvimento do sistema monoaminérgico no potencial antidepressivo de *Rosmarinus officinalis*

Considerando que o sistema monoaminérgico está implicado na fisiopatologia e tratamento da depressão humana (Elhwuegi, 2004; Nestler et al., 2002a,b), o presente estudo teve como objetivo investigar a influência de agentes farmacológicos que modulam o sistema monoaminérgico na atividade antidepressiva do extrato de *Rosmarinus officinalis* (10 mg/kg, po) no TSC.

Em nosso estudo, o efeito antidepressivo induzido pelo extrato de *Rosmarinus officinalis* foi completamente prevenido pelo pré-tratamento dos camundongos com o um inibidor da enzima triptofano hidroxilase (depletor neuronal dos estoques de serotonina), PCPA, bem como os antagonistas de receptores 5-HT_{1A}, 5-HT_{2A} , NAN-190 e cetanserina, respectivamente. Além disso, o pré-tratamento dos camundongos com o agonista de receptores 5-HT_3, mCPBG (biguanida) foi capaz de reverter o efeito antidepressivo do extrato, enquanto que a MDL72222, antagonista do receptor 5-HT_3, administrado em combinação com o extrato, produziu efeito antidepressivo sinérgico no TSC. Desta forma, os resultados fornecem evidência de que o efeito antidepressivo do extrato de *Rosmarinus officinalis* é dependente da interação com o sistema serotoninérgico.

PCPA, um inibidor da enzima triptofano hidroxilase, administrado em camundongos na dose empregada no presente estudo por quatro dias consecutivos, é eficaz em depletar cerca de 60% os estoques de serotonina endógena sem afetar os níveis de noradrenalina e dopamina (Eckeli et al., 2000; Zomkowski et al., 2004). A reversão do efeito antidepressivo do *Rosmarinus officinalis* no TSC pelo pré-tratamento dos camundongos com PCPA sugere que a integridade do sistema serotoninérgico desempenha um papel fundamental no efeito antidepressivo do extrato no TSC. O PCPA também foi previamente demonstrado pelo nosso grupo, como ferramenta farmacológica eficaz em prevenir completamente o efeito antidepressivo fluoxetina, utilizada como controle positivo (Machado et al., 2007; Rodrigues et al., 2002), mas não da imiprimina no TSC (Rodrigues et al., 2002). Além disso, no estudo de O'Leary et al. (2007) foi demonstrado que o pré-tratamento PCPA em camundongos altera a resposta de fluoxetina e citalopram no TSC, mas não de inibidores da recaptação de noradrenalina.

A deficiência na função e expressão dos receptores 5-HT_{1A} é um importante fator que predispõe o desenvolvimento de quadros depressivos (Leitch et al., 2003). Além disso, o mecanismo de ação de várias classes de drogas antidepressivas como os tricíclicos, ISRS e iMAO (Hensler, 2002), bem como de algumas plantas medicinais (Kim et al., 2007; Machado et al., 2007) ocorre com participação dos receptores 5-HT_{1A}. Os ISRSs fluoxetina e paroxetina falham em diminuir o tempo de imobilidade no TSC em camundongos mutantes para o receptor 5-HT_{1A}, em dose que foi ativa em camundongos selvagem e mutantes para receptores 5-HT_{1B}. No entanto, o camundongo mutante para o receptor 5-HT_{1A}, ainda demonstrou uma resposta antidepressiva ao inibidor da recaptação de noradrenalina,

desipramina. Estes dados sugerem que a presença de receptores 5-HT_{1A} pode ser crítico para a expressão das respostas comportamentais antidepressivas dos ISRS no TSC (Mayorga et al., 2001). Em nosso estudo, o pré-tratamento dos camundongos com o antagonista de receptor 5-HT_{1A}, NAN-190 aboliu a redução do tempo de imobilidade induzido pelo extrato de Rosmarinus officinalis no TSC, sugerindo o envolvimento de receptores 5-HT_{1A} no efeito antidepressivo desta planta.

Estudos clínicos e pré-clínicos têm relatado o papel fundamental dos receptores 5-HT_2 na fisiopatologia da depressão, bem como na ação de muitos antidepressivos (Boothman et al., 2006; Wang et al., 2008). No entanto, há relatos de que o agonista parcial de receptores 5-HT_{2A}, DOI aumenta o efeito antidepressivo de alguns compostos (Khisti e Chopde, 2000; Zomkowski et al., 2004). Em nosso estudo, o pré-tratamento com cetanserina preveniu a redução do tempo de imobilidade do extrato de *Rosmarinus officinalis*, sugerindo que seu efeito no TSC é mediada através de uma interação com o receptor 5-HT_{2A}, uma vez que a cetanserina tem uma alta afinidade para estes receptores (Baxter et al., 1995). Dados do nosso grupo têm demonstrado que a cetanserina foi capaz de reverter os efeitos antidepressivos de alguns compostos e extratos vegetais, tais como: agmatina (Zomkowski et al., 2004), extratos de *Schinus molle* (Machado et al., 2007) e de *Tabebuia avellanedae* (Freitas et al., 2010), ácido fólico (Brocardo et al., 2008), cloreto de magnésio (Cardoso et al., 2009) e escopoletina (Capra et al., 2010).

A participação de 5-HT_3 na fisiopatologia da depressão é pouco reportada na literatura, mas alguns estudos indicam que as diferentes classes de antidepressivos agem como antagonistas funcionais no 5-HT_3, indicando que a supressão da atividade do receptor 5-HT_3 pode contribuir para a ação dos antidepressivos (Eisensamer et al., 2003). Os antagonistas de receptor 5-HT_3 administrados agudamente reduziram o tempo de imobilidade no TNF (Bravo e Maswood, 2006).

Enquanto que agonista dos receptores 5-HT_3 atenua a diminuição da imobilidade produzida pela desipramina, imipramina e mianserina, e o antagonista dos receptores 5-HT_3 potencializa os efeitos antidepressivos de vários ISRS no TNF (Nakagawa et al., 1998). No presente estudo, mCPBG, um agonista do receptor 5-HT_3, atenuou a diminuição do tempo de imobilidade induzida por *Rosmarinus officinalis*, embora mCPBG não afetou a duração da imobilidade quando foi administrada isoladamente. Este resultado sugere que o efeito

antidepressivo do extrato é dependente de uma redução na receptor de ativação de 5-HT_3.

Esses resultados estão de acordo com o estudo de Kos et al. (2006) que mostrou que MDL72222, um antagonista dos receptores 5-HT_3, administrado em uma dose mais elevada (3 mg/kg, i.p.) do que a empregada neste trabalho, produziu um efeito antidepressivo no TSC. Além disso, a hipótese de que a supressão da atividade do receptor 5-HT_3 contribui para o efeito antidepressivo do extrato de *Rosmarinus officinalis* foi reforçada pela atividade antidepressiva sinérgica observada quando os camundongos foram tratados com MDL72222 (0,1 mg/kg, i.p.) em combinação com a dose sub-ativa do extrato de Rosmarinus officinalis (1 mg/kg, p.o.).

A depressão também parece ser associada a uma hipofunção do sistema noradrenérgico, e alguns antidepressivos atuam aumentando a disponibilidade sináptica de noradrenalina (Elhwuegi, 2004, Taylor et al., 2005). Em nosso estudo, o pré-tratamento de camundongos com prazosina (antagonista dos receptores adrenérgicos α_1) foi capaz de reverter o efeito antidepressivo do extrato de *Rosmarinus officinalis*, ao passo que a ioimbina (antagonista α_2 adrenérgicos) foi ineficaz em reverter o tempo de imobilidade nos camundongos. Este resultado indica que o extrato pode exercer o seu efeito no TSC, interagindo com receptores α1, mas não com α_2 - adrenérgicos.

Em estudos com animais, a implicação do sistema dopaminérgico em depressão tem sido estudada essencialmente através da utilização de agonistas que aumentam a atividade dopaminérgica, resultando em efeitos antidepressivos em modelos animais de depressão (Renard et al., 2001, Binfaré et al., 2010). Estudos, incluindo investigações *post mortem*, particularmente em indivíduos com depressão grave, mostraram redução nas concentrações de metabólitos de dopamina, no líquido cefalorraquidiano (líquor) e em regiões do cérebro que medeiam humor e motivação (Papakostas, 2006).

Além disso, estudos de neuroimagem suportam a hipótese de que a depressão está associada a um estado de redução na transmissão dopaminérgica, possivelmente refletidos por uma *up-regulation* compensatória dos receptores dopaminérgicos D_2. A deficiência de dopamina mesolímbica é um dos principais candidatos a etiologia de alguns sintomas da depressão (por exemplo, anedonia e perda de motivação) (Dunlop e Nemeroff, 2007). Em nosso estudo, o antagonista seletivo dos receptores de dopamina D_1, SCH23390 e o antagonista dos receptores de dopamina D_2, sulpirida, foram capazes de reverter o efeito antidepressivo do extrato de *Rosmarinus officinalis.* Estes resultados

estão de acordo com dados da literatura indicando que ambos receptores de dopamina D_1 e D_2, podem desempenhar um papel na depressão (Yamada et al., 2004; Papakostas de 2006; Machado et al., 2007).

Em conjunto, nossos resultados indicam pela primeira vez que o extrato de *Rosmarinus officinalis* produz um efeito antidepressivo, sendo que este efeito parece ser mediada por uma interação com o sistema monoaminérgico.

3.3. Potencial antidepressivo de frações, compostos isolados e óleo essencial de *Rosmarinus officinalis*

A análise dos compostos isolados de *Rosmarinus officinalis* foi efetuado através do método de isolamento biomonitorado, em que a investigação do potencial antidepressivo foi realizado à medida que os compostos foram isolados e identificados.

O presente estudo mostrou que todas as frações derivadas a partir do extrato bruto de Rosmarinus officinalis demonstraram efeito antidepressivo efeito semelhante no TSC, bem como os compostos isolados e identificados a partir desta planta, ácido carnosol e ácido betulínico, podem ser responsáveis, pelo menos em parte, pela sua atividade antidepressiva. O óleo essencial também demonstrou um efeito anti-imobilidade neste teste preditivo em camundongos. Convém destacar que o efeito do extrato de Rosmarinus officinalis no TSC foi semelhante ao efeito produzido pela administração oral de fluoxetina, utilizada como um controle positivo.

É importante notar que este estudo fornece evidências convincentes de que as frações, compostos isolados (carnosol e ácido betulínico) e o óleo essencial de Rosmarinus officinalis administrados por via oral produz um efeito antidepressivo específico no TSC, uma vez que a redução do tempo de imobilidade provocada por estas administrações não pode ser atribuída a um efeito psicoestimulante (aumento na locomoção quando avaliada em teste de campo aberto).

Estes resultados são consistentes com a utilização etnofarmacológica desta planta para o tratamento de depressão (Duke, 2000; Heinrich et al., 2006), reforçando a evidência anterior do nosso grupo que demonstrou o potencial efeito antidepressivo do extrato bruto hidroalcoólico neste mesmo protocolo experimental e apresentado no presente trabalho no capítulo I (Machado et al., 2009). Além disso, o presente estudo é o primeiro trabalho que demonstrou o efeito antidepressivo do carnosol e ácido betulínico, além do óleo essencial de

Rosmarinus officinalis, quando administrado por via oral para camundongos.

Em nosso estudo, as frações isoladas de *Rosmarinus officinalis* L. AcOEt 1, ET e IOE, mostram efeito antidepressivo similar na faixa de dose de 0,1-100 mg/kg, p.o. (com percentual de redução de 18-34% do tempo de imobilidade no TSC). Entretanto, também se observou uma diferença na faixa de dose e no percentual de redução nas frações AcOEt 2 (0,1-1 mg/kg, p.o.; 27-43%) e HEX (0,1-10 mg/kg, p.o.; 28-45%) no TSC. Assim, estas frações mostraram um efeito em concentrações mais baixas, e apresentaram uma maior percentagem de redução, em comparação com as outras frações testadas.

Importante notar, a análise fitoquímica das frações desta planta utilizando HPLC revelou a presença de carnosol, ácido betulínico e ácido ursólico, como alguns dos compostos principais. Os compostos que predominam em cada fração pode ser decisivo para o potencial antidepressivo das frações. Portanto, o carnosol é o constituinte principal das frações AcOEt 1, HEX e IOE; enquanto o ácido ursólico é o composto principal na AcOEt 2 e EtOH. Deve ser considerado que outros compostos também foram detectados nestas fracões tais como o ácido betulínico, encontrado principalmente em AcOEt 2, EtOH e IOE; ácido rosmarínico na fração IOE e ácido oleanólico em AcOEt fração 2.

O efeito antidepressivo do carnosol agudamente administrado (0,01-0,1 mg/kg, p.o.) e ácido betulínico (10 mg/kg, p.o.) foi observado no TSC. No entanto, apesar de no presente estudo indicar que o ácido betulínico e carnosol podem ser responsáveis pela ação antidepressiva das frações a partir de Rosmarinus officinalis, não podemos excluir a participação de outros compostos fitoquímicos neste efeito biológico.

Esses resultados indicam inicialmente, que estes constituintes fitoquímicos parecem ser os responsáveis, pelo menos em parte, pelo potencial antidepressivo de *Rosmarinus officinalis.*

De forma similar, um estudo recente demonstrou que o carnosol, ácido ursólico e ácido betulínico, foram responsáveis pelo potencial antiinflamatório de *Rosmarinus officinalis* (Benincá et al., 2010).

O presente estudo mostrou que o óleo essencial de *Rosmarinus officinalis* também apresentou efeito antidepressivo (0,1-100 mg/kg, p.o.) no TSC, sem alterar a locomoção no teste do campo aberto. Curiosamente, os nossos resultados estão de acordo com um estudo recente que informou que o óleo essencial de *Rosmarinus officinalis* produziu um efeito antidepressivo no TNF, um outro teste preditivo do potencial antidepressivo, em ratos (Seol et al., 2010). A análise química revelou que o óleo essencial desta planta contém como principais

compostos: 1,8-cineol (45,14%), cânfora (21,75%), borneol (4,77%), α-pineno (4,62%) e α-terpineol (4,57%). Em geral, o óleo essencial de Rosmarinus officinalis pode ser classificado em três quimiotipos, com base na análise química dos seus compostos predominantes, tais como, *cineoliferum* (teor elevado em 1,8-cineol), *camphoriferum* (cânfora%> 20) e *verbenoniferum* (verbenona> 15%). Destaca-se que a análise química do óleo essencial desta planta é complexa e muitos outros quimiotipos têm sido reconhecidos de acordo com a abundância relativa de outros compostos relevantes, tais como α-pineno e outros constituintes (Napoli, et al., 2010). Além disso, a diversidade de quimiotipos pode ser explicado pelo fato de que esta planta é cultivada em regiões diferentes, com uma variedade de condições de solo e clima que interferem na composição fitoquímica do óleo essencial, bem como no seu quimiotipo.

No presente estudo, considerando-se o conteúdo elevado de 1,8-cineol (45,14%) no óleo essencial, este pode ser caracterizada como quimiotipo *cineoliferum*. Desta forma, o quimiotipo do óleo essencial desta planta foi estabelecido pela presença elevada de 1,8-cineol, indicando que este pode ser o composto responsável pelo efeito antidepressivo do óleo essencial. Estudos futuros são necessários para testar esta hipótese.

É interessante notar que o ácido ursólico isolado de *Rosmarinus officinalis* também produziu um efeito antidepressivo em TSC em camundongos e esta ação pode ser devido ao envolvimento do sistema dopaminérgico, como demonstrado no capítulo III do presente trabalho (Machado et al., Submetido). Além disso, foi relatado que os ácidos rosmarínico e cafeico produzem um efeito antidepressivo no TNF (Takeda, et al., 2002).

Este conjunto de resultados indica que o potencial antidepressivo de várias frações de *Rosmarinus officinalis* pode ser atribuído, pelo menos em parte, à presença de carnosol e ácido betulínico. Além disso, o óleo essencial desta planta também produziu efeito antidepressivo e seu composto principal, 1,8-cineol podem estar envolvidos nesta ação, como demonstrado no capítulo II do presente trabalho. No entanto, outros compostos podem desempenhar um papel para o efeito antidepressivo de *Rosmarinus officinalis* e mais estudos são necessários, a fim de esclarecer os princípios bioativos responsáveis por estas atividades, bem como os mecanismos subjacentes a sua ação.

O presente estudo claramente reforça a hipótese que *Rosmarinus officinalis* tem um potencial terapêutico como um antidepressivo, uma vez que os efeitos de todas as frações, compostos isolados e óleo

essencial no TSC são semelhantes ao efeito produzido pelo antidepressivo clássico fluoxetina. Considerando a ampla utilização desta planta como condimento, sua propriedade antidepressiva pode ser de interesse farmacológico e nutracêutico e deve ser confirmada em futuros estudos clínicos.

3.4. Potencial antidepressivo do ácido ursólico, composto isolado de *Rosmarinus officinalis* L. em camundongos: Evidência do envolvimento do sistema dopaminérgico

Dos compostos isolados deste estudo, o ácido ursólico destacou-se como o composto de melhor atividade antidepressiva no que se refere ao percentual de redução do tempo de imobilidade (cerca de 27%), em relação aos demais compostos testados (14-16%). Além disso, o fato de que o ácido ursólico produz efeito antidepressivo em uma dose 100-1000 vezes menores que as doses de ativos do extrato de Rosmarinus officinalis indica que ele exerce um papel importante no efeito antidepressivo do extrato desta planta no TSC.

Tendo em vista o fato de que moduladores dopaminérgicos são um dos alvos terapêuticos importantes para o tratamento da depressão resistente (Dunlop e Nemerof, 2007; Rakofsky et al. 2009) e a carência de novas alternativas terapêuticas para o tratamento da depressão que agem através deste mecanismo de ação, foi realizado em etapa experimental subseqüente, o estudo do mecanismo de ação dopaminérgico no potencial antidepressivo do ácido ursólico.

Rakofsky et al. (2009) descreveu que as principais abordagens na busca de novas intervenções terapêuticas são: a) compostos que atuem na optimização da modulação da neurotransmissão monoaminérgica, b) o desenvolvimento de medicamentos que atuam sobre outros neurotransmissores além do sistema monoaminérgico e c) o uso de inibidores triplos de recaptação de monoamina, antipsicóticos atípicos e agonistas de receptores dopaminérgicos.

Modelos animais de depressão mostram resposta considerável a manipulações na neurotransmissão dopaminérgica (Dunlop e Nemeroff, 2007), inclusive são capazes de detectar compostos naturais dotados de potencial antidepressivo no TSC, que atuam no sistema dopaminérgico (Machado et al., 2007, 2008; Cunha et al., 2008; Binfaré et al., 2010).

No presente estudo, vale ressaltar que o efeito produzido pelo ácido ursólico (0,1 mg/kg, p.o.), que produziu uma redução de 26,9 % no tempo de imobilidade foi comparável e semelhante ao resultado demonstrado em um estudo anterior do nosso grupo, no qual a

percentagem de redução do tempo de imobilidade produzido pelo antidepressivo bupropiona (10 mg/kg,p.o.) no TSC foi 27,4% (Cunha et al., 2008).

Considerando que a deficiência de dopamina mesolímbica é um dos principais candidatos a etiologia de alguns sintomas da depressão, como anedonia e perda de motivação (Dunlop e Nemeroff, 2007) e as implicações da ativação dopaminérgica nas respostas de alguns antidepressivos (Papakostas, 2006), o nosso estudo investigou o possível envolvimento do sistema dopaminérgico no efeito antidepressivo do ácido ursólico no TSC.

Nossos resultados mostraram que o sistema dopaminérgico está claramente envolvido no potencial antidepressivo do ácido ursólico, visto que o pré-tratamento dos camundongos com SCH23390 ou sulpirida (antagonistas dos receptores dopaminérgicos D_1 e D_2, respectivamente) preveniu o efeito antidepressivo induzido pelo ácido ursólico, sem alterar a atividade locomotora ou exploratória dos animais. Estes resultados corroboram com dados da literatura que mostram que os antagonistas dopaminérgicos, SCH23390 e sulpirida, revertem efeito antidepressivo de alguns antidepressivos no TNF e TSC (Yamada et al., 2004; Machado et al., 2009; Binfaré et al., 2010; Capra et al., 2010).

Yamada et al. (2004) mostraram que o efeito anti-imobilidade da bupropiona, um inibidor da recaptação de dopamina, foi previnido por sulpirida no TNF, sugerindo que o receptor dopaminérgico D_2 está envolvido no efeito anti-imobilidade da bupropiona. Estes dados são consistentes com a hipótese de que a sensibilização do sub-tipo de receptores D_2 pode ser fundamental para a ação clínica de inibidores da recaptação de dopamina (IRD). Nossos resultados estão de acordo com dados da literatura que indicam que ambos os receptores de dopamina D_1 e D_2 podem desempenhar um papel na depressão (Yamada et al., 2004) e no potencial antidepressivo de vários compostos naturais (Zhang et al., 2004; Machado et al., 2007; Capra et al., 2010).

O efeito sinérgico do tratamento combinado de ácido ursólico com agonistas dos receptores dopaminérgicos D_1 e D_2, SKF38393 e apomorfina, respectivamente, reforça a hipótese do envolvimento do sistema dopaminérgico na ação antidepressiva do ácido ursólico. Estes resultados estão de acordo com a evidência na literatura de que agonistas de receptores dopaminérgicos têm sido considerados como candidatos promissores para melhorar os resultados obtidos no tratamento dos pacientes com depressão resistente e/ou não remissão dos sintomas com a terapêutica convencional (Dunlop e Nemeroff, 2007) e com a constatação de que os agonistas DA, como a apomorfina

e SKF38393 têm sido utilizados como ferramentas farmacológicas para avaliar a capacidade de resposta dos receptores DA no mecanismo de ação dos compostos dopaminérgicos com potencial antidepressivo (Binfaré et al., 2010). Além disso, foi relatado na literatura que o agonista do receptor dopaminérgico D_1, SKF38393, aumentou o efeito anti-imobilidade de antidepressivos (ISRS) no TNF (Renard et al., 2001).

Estudos clínicos evidenciam que os agonistas dos receptores dopaminérgicos D_2 são eficazes no tratamento de pacientes depressivos (Waehrens e Gerlach, 1981) e também tem uma quantidade considerável de investigações farmacológicas em relação à eficácia de antidepressivos com efeitos dopaminérgicos no tratamento da depressão (Papakostas, 2006).

Adicionalmente mostramos o efeito sinérgico do ácido ursólico com o antidepressivo bupropiona, um inibidor da recaptação de dopamina. Este resultado é similar a resultados prévios de nosso grupo, de que a co-administração de melatonina ou de zinco com a bupropiona reduziu o tempo de imobilidade, produzindo desta forma, efeito antidepressivo no TSC (Cunha et al., 2008; Binfaré et al., 2010). Esses resultados observados de que o ácido ursólico potencializou o efeito de doses sub-efetivas da bupropiona e dos agonistas do receptor dopaminérgicos no TSC, ocorreram sem modificar significativamente o comportamento locomotor ou exploratório dos camundongos quando submetidos as respectivas intervenções, indicam que um efeito locomotor não pode ser responsável pelas alterações comportamentais observadas no TSC.

Vale destacar que este conjunto de resultados indica que o ácido ursólico pode contribuir para o efeito antidepressivo do *Rosmarinus officinalis* e que seu mecanismo de ação parece ser semelhante ao do extrato, conforme demonstrado em estudo anterior do nosso grupo (Machado et al., 2009).

3.5. Potencial antidepressivo de Ro*smarinus officinalis* no modelo de depressão, Bulbectomia Olfatória Bilateral (BO):

Considerando que os testes preditivos de atividade antidepressiva, TNF e TSC, possuem a limitação de responderem a tratamentos agudos com antidepressivos e não reproduzirem a sintomatologia da depressão (Cryan et al., 2005), foi selecionado o modelo de depressão induzida pela BO para investigar o potencial antidepressivo do extrato de

Rosmarinus officinalis, visto que este modelo acarreta alterações comportament

ais, neuroquímicas e morfológicas compatíveis e comparáveis ao que é observado em pacientes com depressão (Kelly et al., 1997; Song e Leonard, 2005).

Neste contexto, em uma fase preliminar, foi padronizado a técnica da BO em nosso laboratório para avaliar o efeito do tratamento repetido com o antidepressivo clássico fluoxetina (10 mg/kg, p.o.; ISRS) em possíveis alterações comportamentais (hiperatividade e comportamento anhedônico) e alterações bioquímicas (atividadede da enzima acetilcolinesterase hipocampal e corticocerebral e coticosterona sérica) induzidas pela BO camundongos fêmeas. Esta fase foi fundamental, pois possibilitou avaliar os efeitos deste antidepressivo sobre os danos causados pela BO, fundamentando desta forma uma melhor execução das etapas subseqüentes referentes à avaliação do potencial antidepressivo do extrato de *Rosmarinus officinalis*, foco do presente estudo.

3.5.1. Fluoxetina reverte a hiperatividade, comportamento anedônico e o aumento da atividade da acetilcolinesterase hipocampal induzida pela BO em camundongos

O presente estudo investigou a habilidade da fluoxetina administrada cronicamente (14 dias) em reverter as alterações comportamentais (principalmente a hiperatividade e anedonia) e bioquímicas (corticosterona sérica e atividade da acetilcolinesterase hipocampal e cerebrocortical) induzidas pela BO em camundongos.

A maioria dos estudos na literatura reporta as alterações induzidas pela BO em ratos machos e poucos estudos têm investigado a hiperatividade e comportamento anêdonico em camundongos fêmeas. Notavelmente, a depressão é mais prevalente em mulheres do que em homens, a proporção de fêmea:macho é de de 5:2 (Wong and Licinio, 2001).

A característica que distingue a BO de outros modelos de depressão é a associação da hiperatividade com o comportamento anedônico. Desta forma, este modelo mimetiza os sintomas da depressão com a agitação psicomotora, ou seja, da depressão agitada (Romeas et al., 2009).

Nossos resultados estão de acordo com os dados da literatura, visto que os camundongos bulbectomizados apresentaram um aumento nas atividades locomotora e exploratória no teste do campo aberto,

indicando um comportamento tipo-depressivo (Harkin et al., 2003; Kelly et al., 1997; Zueger et al., 2005). É importante destacar que o teste do campo aberto foi realizado propositadamente em um ambiente de alta iluminação, pois foi previamente demonstrado que esta condição ambiental é fundamental para os animais bulbectomizados apresentarem hiperatividade (Mar et al., 2002).

É digno de nota que um estudo reportou que a hiperatividade não se manifesta em ratos bulbectomizados expostos ao teste do campo aberto com baixa iluminação, ou seja, em um ambiente menos aversivo. Por exemplo, quando da execução do teste do campo aberto sob luz vermelha, os animais bulbectomizados não apresentam esta hiperatividade (Stockert et al., 1988).

Os efeitos dos antidepressivos ISRS têm recebido mais atenção na BO, em comparação a outras classes de antidepressivos, visto que este modelo causa depressão associada a hipofunção serotoninérgica (Lumia et al., 1992; Kelly et al., 1997; Song e Leonard, 2005). Entretanto, a capacidade do tratamento crônico com fluoxetina em reverter a hiperlocomoção em roedores bulbectomizados é controversa (Bellver et al., 1990; Butler et al., 1990; Possidente et al., 1996; Mar et al., 2002; Rodríguez-Gaztelumendi et al., 2009).

No presente estudo a administração de fluoxetina por via oral (10 mg/kg) em camundongos Swiss fêmeas por 14 dias foi capaz de reverter a hiperatividade locomotora e exploratória desencadeada pela BO, consistente com um efeito tipo-antidepressivo, semelhante aos resultados anteriormente apresentados com a mesma dose de fluoxetina administrada por via i.p. ou s.c. por 14 dias (Butler et al., 1990; Rodríguez-Gaztelumendi et al., 2009) ou por 21 dias (Mar et al., 2002) em ratos machos, validando desta forma o modelo em camundongos fêmeas.

Em nossa investigação, camundongos bulbectomizados mostraram hiperatividade locomotora e aumento no comportamento exploratório induzidos pela novidade, dados que indicam um aumento na reatividade comportamental dos animais submetidos ao teste do objeto novo. Entre as alterações observadas neste teste destacam-se a diminuição da latência para entrar na área central, aumento do tempo dispendido para explorar o objeto e aumento no número de levantamentos verticais (rearings) na área central onde o objeto novo estava localizado. No teste da caixa nova observa-se o aumento do número de cruzamentos e rearings no novo ambiente. Estes resultados são consistentes com dados da literatura, uma vez que foi relatado que a hiperatividade induzida pela BO está diretamente relacionada a uma

maior reatividade a novos ambientes ou déficit na habituação a novas situações (Van Riezen e Leonard, 1990; Mar et al., 2000, 2002; Zueger et al., 2005). Estes resultados foram similares ao reportado por Zueger et al. (2005), que demonstrou um aumento do comportamento exploratório no teste de objeto novo e no teste da caixa nova (comportamento neofílico) em camundongos bulbectomizados. Além desses resultados, foi relatada uma diminuição da exploração no centro do campo aberto (comportamento tipo-ansioso) em camundongos bulbectomizados antes da introdução do objeto no centro do aparato. Em nosso estudo, não foi registrado a exploração dos camundongos no centro do campo aberto antes da introdução do objeto no aparato.

No entanto, o aumento de atividade em ambientes novos e maior interação com o objeto novo pode indicar um efeito ansiolítico. Em nosso estudo, esta hipótese seria consistente com o fato de que a BO induziu uma diminuição no comportamento de auto-limpeza (grooming) e no número de bolo fecal no campo aberto e no teste da caixa nova. Contudo, é importante mencionar que a diminuição na latência para entrar na arena central do aparato onde estava localizado o objeto novo pode ser devido a uma atividade tipo-impulsiva, como um aumento da reatividade a uma nova situação induzida pela BO. O aumento da atividade locomotora e exploratória em ambientes novos, embora tradicionalmente possa representar um efeito ansiolítico, pode também representar um aumento na desinibição comportamental ou um aumento no comportamento impulsivo (Scearce-Levie et al. 1999; Winstanley et al., 2004). A impulsividade pode ser definida como uma ação imprudente ou desprovida de avaliação de risco que se mostra aumentada em situações nas quais há uma depleção de serotonina (Winstanley et al., 2004). É notável observar que a BO provoca uma redução nos níveis de serotonina e de seus metabólitos (Hellweg et al., 2007), uma condição que poderia explicar o comportamento impulsivo.

O tratamento crônico com fluoxetina (10 mg/kg, p.o.) foi capaz de reverter a hiperatividade locomotora e o aumento do comportamento exploratório induzida pela novidade no teste de objeto novo, bem como o aumento no número de cruzamentos e levantamentos verticais (rearings) induzida pela novidade no teste da caixa nova em camundongos bulbectomizados. Estes resultados estão de acordo com outros estudos que indicam que o tratamento crônico com antidepressivos, incluindo a fluoxetina (10 mg/kg, i.p., 21dias) são eficazes em restaurar o comportamento normal frente à situações novas, permitindo uma adaptação mais eficaz a estímulos novos em roedores bulbectomizados.

Assim, esses antidepressivos aumentam a habituação induzida pela novidade em animais bulbectomizados, sem alterar este parâmetro no grupo-SHAM (Mar et al., 2000, 2002). Curiosamente, um resultado que estende aos dados da literatura é que o tratamento com fluoxetina não foi capaz de alterar a diminuição na latência para entrar na arena central do aparato (onde o objeto novo estava localizado) induzida pela BO. Podemos especular que este parâmetro não está associado ao comportamento tipo-depressivo, reforçando a noção de que pode estar relacionado com a reatividade inicial ao aparato, sugerindo uma atividade tipo-impulsiva. Esta hipótese está de acordo com o reportado por Mar et al. (2002), que propõe que a fluoxetina aumenta a taxa de habituação em ratos bulbectomizados. Seria interessante um futuro estudo para averiguar se outros agentes antidepressivos seriam capazes de alterar a diminuição induzida pela BO neste parâmetro.

A anedonia, ou hipossensibilidade ao prazer, é um dos principais sintomas da depressão. Este comportamento pode ser inferido através de uma redução na ingestão de sacarose ou diminuição do tempo gasto no comportamento de auto-limpeza no "Splash Test" (Jancsar e Leonard, 1981; Yalcin et al., 2005). Neste teste, a maioria dos estudos utiliza apenas o tempo total gasto com a auto-limpeza ou a freqüência de auto-limpeza como parâmetros para inferir o comportamento anedônico induzidas por um modelo de depressão (Yalcin et al., 2008; David et al., 2009; Detanico et al., 2009). No entanto, o nosso estudo também avaliou a latência para o início da resposta comportamental como um parâmetro complementar para a avaliação de anedonia (d'Audiffret et al., 2010). Nosso estudo mostra que a BO produziu um comportamento anedônico no splash test, uma vez que camundongos bulbectomizados apresentaram uma resposta de auto-limpeza mais lenta e menos freqüente, em comparação com o comportamento exibido por camundongos controle (SHAM). Este resultado está de acordo com estudos que mostraram uma resposta atenuada ao efeito recompensador das anfetaminas e uma redução na ingesta de sacarose em ratos bulbectomizados, sugerindo também que BO causa anedonia (Stock et al., 2000; Romeas et al., 2009).

A fluoxetina foi capaz de reverter o comportamento anedônico induzido pela BO no splash teste, o que indica que a BO é adequada para investigar os efeitos de compostos dotados de propriedades antidepressivas e antianedônicas.

Na segunda fase experimental deste estudo, alguns parâmetros bioquímicos que poderiam ser alterados pelo procedimento da BO, como os níveis séricos de corticosterona e atividade da AChE no

hipocampo e no córtex frontal foram investigados, considerando a relação existente entre a hipofunção do sistema monoaminérgico, reportado em roedores bulbectomizados (Lumia et al., 1992; Hellweg et al., 2007), com o eixo HPA e disfunções colinérgicas (Nestler et al., 2002; Nakajima et al., 2007; Krishnan e Nestler, 2008).

A concentração de corticosterona circulante no soro foi medida em camundongos bulbectomizados, tratados ou não com fluoxetina, como um indicador da ativação do eixo HPA. Observamos que os níveis séricos de corticosterona não foram significativamente maiores em camundongos bulbectomizados, apesar de que foi observado um aumento de 2,5 vezes neste parâmetro nos animais submetidos à BO, em comparação com grupo controle SHAM. De fato, foram reportados na literatura tanto um aumento (Cairncross et al., 1977; Marcilhac et al., 1999) quanto nenhuma alteração (Broekkamp et al., 1986; Van Hoomissen et al., 2011) nos níveis de corticosterona no soro ou plasma de roedores bulbectomizados. A relação do cortisol com a depressão humana permanece incerta, uma vez que a ativação do eixo HPA não é uma condição necessária, nem suficiente para estar deprimido, uma vez que muitos indivíduos que tem hipercortisolemia não apresentam depressão e nem todos os pacientes acometidos por esta doença apresentam hipercortisolemia (Wolkowitz et al., 2009). É importante observar que a hipocortisolemia é mais freqüentemente observada na depressão atípica, que se caracteriza por sintomas de hipersonia, hiperfagia, letargia, dor, fadiga e apatia relativa, enquanto que hipercortisolemia é observado em depressão melancólica, em que hiperexcitação, ansiedade, insônia e perda de apetite são comumente encontrados (Gold et al., 2002; Wolkowitz et al., 2009).

Curiosamente, a administração crônica de fluoxetina aumentou os níveis séricos de corticosterona, tanto nos grupos SHAM e BO. Estes resultados estão de acordo com os reportados por Weber et al. (2006) que mostraram um aumento na concentração plasmática de corticosterona cerebral após a administração aguda e crônica (14 dias) da fluoxetina (10 mg / kg, po) em camundongos. Além disso, a fluoxetina induz um aumento nos níveis séricos de corticosterona após a administração aguda (Serra et al., 2001), um efeito que foi associado com a ativação do eixo HPA, uma vez que este efeito foi bloqueado pelo glicocorticóide dexametasona (Duncan et al., 1998).

A fluoxetina também foi capaz de aumentar os esteróides neuroativos alopregnanolona, pregnenolona, progesterona e deoxicorticosterona no plasma ou soro, e no hipocampo e no córtex cerebral, provavelmente através de um aumento da atividade de enzimas

neurosteroidogênicas (Serra et al., 2001; Uzunova et al., 2004; Marx et al., 2006). No geral, considerando nossos resultados, podemos especular que o efeito antidepressivo da fluoxetina em camundongos bulbectomizados, inclusive sua propriedade anti-anedônica, não esteja correlacionada com os níveis séricos de corticosterona.

O procedimento da BO produz mudanças em muitas regiões do cérebro como conseqüência das conexões interrompidas entre os bulbos olfatórios e outras regiões do cérebro que regulam as emoções, incluindo o hipocampo e o córtex cerebral (Kelly et al., 1997; Wrynn et al., 2000). A disfunção em áreas do cérebro do sistema límbico tem sido implicada com a depressão e ação antidepressiva (Drevets et al., 2008; Krishnan e Nestler, 2008).

Além disso, estudos relatam que as alterações neuroquímicas e comportamentais induzidas pela BO são, em parte devido à neurodegeneração de estruturas específicas do cérebro, como hipocampo e do córtex frontal (Kelly et al., 1997; Wrynn et al., 2000; Jarosik et al., 2007). Considerando essa informação e a implicação do sistema colinérgico nas alterações comportamentais induzidas pela BO (Moriguchi et al., 2006; Nakajima et al., 2007) e na fisiopatologia da depressão (Dagytė et al., 2011), o presente estudo também determinou a atividade da AChE no hipocampo e no córtex frontal. A AChE é uma enzima importante na neurotransmissão colinérgica que catalisa a hidrólise da acetilcolina na fenda sináptica terminando, desta forma, a ação deste neurotransmissor. No presente estudo demonstramos um aumento da atividade da AChE no hipocampo, mas não no córtex frontal, em camundongos bulbectomizados, um efeito que foi revertido pela fluoxetina. Este achado sugere que um aumento da atividade desta enzima no hipocampo, com a conseqüente redução os níveis de acetilcolina pode estar relacionado com o comportamento tipo-depressivo observado nos animais bulbectomizados. De acordo com estes dados, o citalopram provoca a liberação de acetilcolina no hipocampo (Egashira et al., 2006; Dagytė et al., 2011). Além disso, a administração de ZSET1446, que age diretamente sobre a liberação de acetilcolina, provoca uma redução no comportamento tipo-depressivo em camundongos bulbectomizados (Shioda et al., 2010). Portanto, este efeito observado no hipocampo pode estar relacionado com a abundância de inervações colinérgicas recebidos por esta estrutura. Além disso, as disfunções colinérgicas podem acarretar uma diminuição no funcionamento do hipocampo e uma maior vulnerabilidade para o desenvolvimento de déficits cognitivos associados com a depressão (Kelly et al., 1997; Dagytė et al., 2011). Por outro lado, a redução na

expressão de AChE no hipocampo de camundongos bulbectomizados foi reportado (Moriguchi et al., 2006; Nakajima et al., 2007), o que pode indicar um efeito compensatório.

Em nosso estudo a fluoxetina foi capaz de reverter o aumento da atividade de AchE hipocampal induzida pela BO, e também de reduzir a atividade da AChE no córtex frontal de camundongos bulbectomizados, corroborando com a idéia de que este ISRS modula o sistema colinérgico (Dagytė et al., 2011). De acordo com esta evidência, o tratamento com fluoxetina, um antidepressivo que aumenta os níveis de serotonina na fenda sináptica, diminuiu a atividade da AChE na membrana de eritrócitos humanos e de soro (Müller et al., 2002). Além disso, é interessante mencionar que o donepezil, um inibidor clássico de AChE, foi capaz de reduzir a atividade da AchE no córtex cerebral em camundongos bulbectomizados (Yamada et al., 2011), semelhante ao resultado mostrado no presente estudo com fluoxetina.

Quanto à capacidade da fluoxetina em reduzir a atividade da AChE, conseqüentemente, aumentando os níveis de acetilcolina, podemos levantar a hipótese de que isso poderia levar a uma dessensibilização dos receptores nicotínicos acetilcolina. Em consonância com estes dados, tem sido proposto que um adequado equilíbrio entre a ativação e a dessensibilização dos receptores nicotínicos é necessário para o efeito antidepressivo (Mineur e Picciotto, 2010). Os resultados deste estudo com fluoxetina sugerem que uma interação entre a neurotransmissão colinérgica e monoaminérgica parece ser importante para seu efeito antidepressivo.

Em conclusão desta etapa experimental, o presente estudo mostra que os camundongos bulbectomizados apresentaram hiperatividade e anedonia associada a um aumento na atividade da AchE hipocampal, parâmetros que foram revertidos pelo tratamento crônico com fluoxetina, indicando que este pode ser uma ferramenta eficaz para estudar depressão agitada associada com anedonia e também para o tratamento deste sub-tipo de depressão. Além disso, nosso estudo, também contribui com a caracterização comportamental do modelo da BO em camundongos.

3.5.2. O extrato hidroalcóolico de *Rosmarinus officinalis* L., de forma similar à fluoxetina, reverte o comportamento tipo-depressivo sem alterar o déficit de aprendizado em camundongos bulbectomizados

Para obter uma melhor compreensão do potencial antidepressivo de *Rosmarinus officinalis*, este estudo avaliou os efeitos do extrato desta planta no modelo da BO, uma vez que a remoção de bulbos olfatórios acarreta várias alterações comportamentais, neuroquímicas, neuroendócrinas em roedores, comparáveis às observadas em pacientes com depressão (Kelly et al., 1997; Song e Leonard, 2005). O efeito antidepressivo do extrato hidroalcoólico dos talos e folhas desta planta foi inicialmente investigado pelo nosso grupo em dois modelos comportamentais preditivos de atividade antidepressiva, o teste do nado forçado (TNF) e teste de suspensão da cauda (TSC) em camundongos.

As alterações comportamentais induzidas pela BO aparecem depois de 2 semanas em roedores, provavelmente porque a lesão causada por este procedimento, induz um processo de reorganização nas áreas límbicas e corticais, e parece ser responsável por esses efeitos secundários (Jarozik et al., 2007; Van Riezen e Leonard, 1990; Zueger et al., 2005).

No presente estudo os camundongos bulbectomizados apresentaram um aumento significativo na atividade locomotora e exploratória no teste de campo aberto, no teste de objeto novo e no teste da caixa nova e também comportamento anedônico. Estas alterações comportamentais são indicativas de um perfil tipo-depressivo destes animais (Harkin et al., 2003; Kelly et al., 1997; Zueger et al., 2005). Convém destacar que o extrato de Rosmarinus officinalis, de forma semelhante ao antidepressivo fluoxetina, aqui utilizado como um controle positivo, foi capaz de reverter estas alterações comportamentais.

A hiperatividade em animais bulbectomizados é a principal alteração reportada na literatura. Várias classes de antidepressivos clinicamente ativos revertem a hiperatividade dos roedores bulbectomizados no teste do campo aberto, tais como: inibidores seletivos da recaptação de serotonina-ISRS (citalopram, fluoxetina, paroxetina, sertralina e venlafaxina), inibidor da recaptação de noradrenalina-NE (reboxetina), antidepressivos tricíclicos (amitriptilina, imipramina desipramina,) (Butler e Leonard, 1990; Connor et al., 2000; Jarozik et al., 2007; Kelly et al., 1997; Possidente et al., 1996; Rodriguez-Gaztelumendi et al., 2009; Song e Leonard, 2005). Além

disso, também tem sido relatado que a curcumina exerce potencial antidepressivo similar em roedores bulbectomizados (Xu et al., 2005).

Além disso, outra mudança comportamental relevante desencadeada pelo modelo da BO é o aumento da vulnerabilidade e hiperatividade em reposta ao estresse ambiental (Mar et al., 2000; Van Riezen e Leonard, 1990). Em nosso estudo, os camundongos bulbectomizados mostraram uma hiperatividade locomotora e exploratória induzida pela novidade, indicada por um aumento na reatividade comportamental quando os camundongos foram submetidos ao teste do objeto novo (aumento do tempo despendido na exploraração do objeto e aumento no número de levantamentos verticais (rearing) na área central, onde o objeto novo estava situado) e no teste da caixa nova (aumento do número de cruzamentos e levantamentos no novo ambiente). Estes resultados estão de acordo com dados da literatura, visto que mostram que a hiperatividade induzida pela BO está diretamente relacionada a uma maior reatividade a novos ambientes ou déficit na habituação a novas situações (Mar et al., 2000; Van Riezen e Leonard., 1990; Zueger et al., 2005).

Os tratamentos crônicos com o extrato (10-300 mg/kg, p.o.) e fluoxetina (10 mg/kg, p.o.) foram capazes de reverter o aumento do tempo de exploração do objeto e o aumento do número de levantamentos verticais (comportamento exploratório) induzida pela novidade em camundongos bulbectomizados no teste do objeto novo, bem como o aumento do número de cruzamentos e levantamentos induzidas pela novidade no teste da caixa nova em camundongos submetidos à BO.

Estes resultados estão de acordo com o fato de que os antidepressivos, tais como a fluoxetina, amitriptilina e a buspirona são eficazes em restaurar a resposta normal, por permitir uma adaptação mais eficaz aos novos estímulos nos roedores bulbectomizados (Mar et al., 2000, 2002).

Anedonia, ou hipossensibilidade ao prazer, é um dos sintomas chave para um diagnóstico de depressão (DSM-IV, 1992) e é um componente importante no desenvolvimento de um modelo animal de depressão (Stock et al., 2000). No presente estudo este comportamento foi inferido através de um aumento da latência para exibir o comportamento de auto-limpeza (grooming) e uma diminuição no tempo de permanência neste comportamento em camundongos bulbectomizados, em comparação ao grupo-controle (SHAM) no Splash teste.

Este resultado está de acordo com alguns estudos que têm demonstrado o comportamento anedônico em ratos bulbectomizados (Romeas et al., 2009, Stock et al., 2000). Notavelmente, em nosso estudo, o comportamento anedônico induzido pela BO foi revertido por Rosmarinus officinalis e fluoxetina. Este resultado está de acordo com a habilidade de compostos com propriedades antidepressivas, tais como a antidepressivos clássicos (fluoxetina, imipramina e desipramina) (David et al., 2009; Detanico et al., 2009; Yalcin et al., 2005), bem como Ptychopetalum olacoides Bentham extrato (marapuama) (Piato et al., 2008), que foram capazes de reverter o comportamento anêdonico causado por modelos de depressão induzidos por estresse.

De acordo com estudos prévios, reportando um déficit no desempenho cognitivo de roedores bulbectomizados em diferentes paradigmas utilizados para investigar os processos de aprendizagem e memória (Harkin et al., 2003; Kelly et al. 1997; Mucignat-Caretta et al., 2006), nossos resultados demonstram um baixo desempenho dos animais bulbectomizados em comparação ao grupo-SHAM na tarefa espacial do labirinto aquático de Morris (water maze).

Interessantemente, foi mostrado em nosso estudo, que os camundongos bulbectomizados necessitam mais repetições ou treinos (na sessão de treinamento do water maze) para adquirir a informação espacial necessária para o aprendizado, porém estes animais exibiram um perfil semelhante aos animais do grupo-SHAM na sessão de teste (24 h mais tarde), demonstrando um seletivo déficit no aprendizado espacial na tarefa do labirinto aquático de Morris.

Importante notar que este resultado está de acordo com alguns resultados da literatura, que animais bulbectomizados apresentam um comprometimento na aprendizagem e memória espacial no labirinto aquático (Van Rijzingen et al., 1995). Esta discrepância com os dados prévios na literatura pode ser explicada pelas diferenças entre os protocolos utilizados para avaliar a aprendizagem espacial e a memória no labirinto aquático. Nestes estudos anteriores, cada rato foi submetido a 4 treinos por dia, durante 4-5 dias consecutivos para encontrar a plataforma (Mucignat-Caretta et al. 2006), enquanto no presente estudo cada camundongo foi submetido a 10 treinos consecutivos durante uma única sessão de treinamento (apenas 1 dia) e a sessão de teste ocorreu 24 h depois, semelhante ao estudo reportado por Prediger et al. (2005).

Desta forma, é possível que um protocolo de treinamento com um maior número de treinos consecutivos, em vez de várias vezes a formação ao longo de vários dias promove um desempenho equivalente ou semelhante de aprendizado para ambas as linhagens, o que pode ser

observado no padrão similar das latências de escape para a plataforma, nos últimos treinos da sessão de treinamento.

O labirinto aquático de Morris é um teste cognitivo que abrange a função do hipocampo (Morris, 1982) que não depende de estímulos olfativos, mas mais dependente de pistas visuais. Portanto, nossos resultados indicam que a BO está associada a um déficit de aprendizagem que pode estar associado a um prejuízo da função hipocampal. Um estudo realizado por Van Rijzingen et al. (1995) mostrou que duas semanas depois da BO o desempenho no labirinto aquático de Morris é severamente prejudicado. No entanto, esta alteração é um déficit cognitivo transitório, visto que a recuperação ocorre espontaneamente cerca de 6 semanas após a cirurgia. Assim, o desempenho dos animais bulbectomizados no labirinto aquático de Morris e SHAM-controles, seis semanas após a cirurgia não mostrou diferença na latência de escape durante a aquisição, nem qualquer diferença durante a sessão teste (retenção da memória).

No presente estudo, o tratamento crônico com extrato de *Rosmarinus officinalis* (10-300 mg/kg, p.o.) ou fluoxetina não reverteu esse déficit no aprendizado espacial induzido pela BO. Estes resultados estão em contraste com as evidências da literatura, que o tratamento crônico com antidepressivos, como ISRS, reverte este déficit (Kelly et al., 1997). Estudos mostraram que a BO induziu uma diminuição da aprendizagem e da memória na tarefa da pista de três painéis em ratos bulbectomizados (Yamamoto et al., 1997) e na tarefa de esquiva passiva em ratos bulbectomizados no 7° e 14° dias após a cirurgia (Hozumi et al., 2003). O comprometimento da aprendizagem e da memória induzida pela BO nestes testes, avaliados no 14° dia pós-operatório, foi revertida pela administração do inibidor de colinesterase, fisostigmina. Além disso, vários compostos isolados de plantas tem se mostrado em reverter o déficit cognitivo em roedores bulbectomizados no teste de esquiva inibitória, tais como a curcumina (Xu et al., 2005), nobiletina (Nakajima et ai., 2007) e um metabólito ativo do ginseng (20 (S)-protopanaxadiol) (Xu et al., 2010). No entanto, existem poucos dados que mostram os efeitos de Rosmarinus officinalis sobre o desempenho cognitivo, apesar de ter relatos do seu uso como estimulante cognitivo (Kennedy e Scholey, 2006). Além disso, Hosseinzadeh et al. (2004) relataram que o óleo essencial de *Rosmarinus officinalis* injectados intraperitonealmente em ratos, 30 minutos antes do treino durante 5 dias consecutivos, melhorou a memória intacta e o déficit de aprendizagem induzido por escopolamina em ratos que executaram a tarefa do labirinto aquático de Morris.

Recentemente, um estudo clínico com população idosa mostrou que esta planta quando administrada com uma dose baixa causou uma melhoria sobre a função cognitiva, mas com uma dose elevada causou um prejuízo no desempenho cognitivo (Pengelly et al., 2012). Além disso, em um estudo com 144 indivíduos saudáveis, mostrou que o óleo essencial de alecrim no ar (como um aromatizador de ambiente) melhorou significativamente o desempenho cognitivo e humor (Moss et al., 2003). Alguns estudos tem proposto que o *Rosmarinus officinalis* pode ser um promissor candidato para a melhoria da memória em pessoas saudáveis ou para tratamento de doenças associadas com déficit cognitivo, devido em parte, as suas propriedades anticolinesterásicas (Duke, 2007; Ingole et al., 2008; Kennedy e Scholey, 2006; Singh et al., 2011). No que se referee ao presente estudo, não podemos descartar a possibilidade de que se um tratamento mais prolongado com o extrato da planta, bem como a fluoxetina, seria capaz de reverter o déficit cognitivo induzido pela BO.

Na segunda fase experimental deste estudo, foi avaliado alguns parâmetros bioquímicos que poderiam ser alterados pelo procedimento da BO, como nível de glicose no soro e atividade da enzima AChE no hipocampo e no córtex frontal, bem como a habilidade de *Rosmarinus officinalis* e fluoxetina (controle positivo) em reverter algumas das possivéis alterações induzidas pela BO nestes parâmentos analisados.

Este estudo mostrou uma diminuição na glicemia nos camundongos bulbectomizados, em comparação com o grupo controle (SHAM). Este resultado é semelhante a alguns estudos que relatam uma diminuição dos níveis séricos de glicose em animais bulbectomizados (Belló e Rummler, 1979; Perassi et al., 1975). Um estudo recente mostrou que a hipoglicemia aguda induz a um comportamento tipo-depressivo em camundongos, mensurado pelo aumento no tempo de imobilidade no TNF e pela redução na preferência a sacarina (indicativo de comportamento anedônico) em camundongos, sendo que o aumento do tempo de imobilidade no TNF, foi prevenido pelos antidepressivos fluoxetina e desipramina (Park et al., 2012). Entretanto, no presente estudo o tratamento crônico com fluoxetina não foi capaz de alterar a redução dos níveis de glicose sérica induzida pela BO.

Interessantemente, o tratamento crônico com o extrato de *Rosmarinus officinalis* diminui os níveis séricos de glicose em camundongos SHAM, mas reverteu a diminuição nos níveis séricos de glicose induzido pela BO, uma vez que foi capaz de restaurar os níveis de glicose similar aos nivéis séricos do controle. Desta forma, os efeitos

do extrato de Rosmarinus officinalis e fluoxetina sobre os níveis de glicose no soro em camundongos SHAM e BO são bastante diferentes.

Estes dados corroboram com estudos que mostram que o extrato de *Rosmarinus officinalis* exerce atividade hipoglicêmica notável e anti-hiperglicêmico (Bakirel et al., 2008; Abu-Al-Basal, 2010). Os efeitos sobre os níveis séricos de glicose encontrado no nosso estudo, embora interessantes não parecem estar associadas com as alterações comportamentais descritas neste trabalho.

Tendo em vista que o sistema colinérgico está implicado nas alterações comportamentais induzida pela BO (Moriguchi et al., 2006; Nakajima et al., 2007) e na patofisiologia da depressão (Dagyte et al., 2011), o presente estudo também avaliou a atividade da enzima AChE no hipocampo e córtex frontal. A AChE é um constituinte importante da neurotransmissão colinérgica que catalisa a hidrólise da acetilcolina nas junções fenda sináptica e neuromusculares (Soreq e Seidman, 2001). No presente estudo também foi mostrado um aumento da atividade da enzima AChE no hipocampo, mas não no córtex frontal, nos camundongos bulbectomizados, um efeito que foi revertido pelo tratamento crônico com o extrato de Rosmarinus officinalis (10 mg/kg, p.o.) e pelo antidepressivo clássico fluoxetina (10 mg/kg, p.o.). Reforçando essa evidência, estudos na literatura relataram o efeito anticolinesterase in vitro do extrato e óleo essencial de Rosmarinus officinalis (Adsersen et al., 2006). Além disso, o tratamento com o antidepressivo fluoxetina diminuiu a atividade da AChE em membrana de soro e de eritrócitos humanos (Müller et al., 2002). Os nossos resultados sugerem que um aumento da atividade do hipocampo desta enzima pode estar associada ao comportamento tipo-depressivo observado nos camundongos bulbectomizados. Quanto à capacidade de *Rosmarinus officinalis* e fluoxetina em diminuir ou reestabelecer os valores basais da atividade da AChE, conseqüentemente, aumentando os níveis de acetilcolina, podemos levantar a hipótese de que isso poderia levar a uma dessensibilização dos receptores de acetilcolina nicotínicos. Em consonância com isso, tem sido proposto que um bom equilíbrio entre a ativação e dessensibilização dos receptores nicotínicos é necessária para o efeito antidepressivo eficiente (Mineur e Picciotto, 2010).

Adicionalmente, também foi observado em nosso estudo uma ausência de alteração da AChE em córtex cerebral em camundongos bulbectomizados, um resultado que é semelhante ao mostrado por Yamada et al. (2011) em camundongos bulbectomizados. Entretanto, o

tratamento com fluoxetina, mas não com o extrato reduziu a atividade desta enzima no córtex cerebral de Camundongos bulbectomizados.

Em conclusão, o presente estudo mostra que os camundongos bulbectomizados apresentaram hiperatividade e anedonia associada a um aumento da atividade da AChE hipocampal, e estes parâmetros foram revertidos pelo tratamento crônico com o extrato de Rosmarinus officinalis, indicando que este modelo de depressão pode ser uma ferramenta eficaz para estudar depressão agitada associado com anedonia, e que o extrato desta planta pode ser um composto fitoterápico promissor para o tratamento deste sub-tipo de depressão.

4. CONCLUSÃO GERAL

O presente estudo, comprovou à nível pré-clinico, que o extrato de *Rosmarinus officinalis* produz um efeito antidepressivo, e este efeito parece ser mediada por uma interação com o sistema monoaminérgico.

Em etapa prévia ao isolamento dos compostos, constatou-se que tanto as frações (Acetato de Etila 1 e 2, Hexânica, Etanólica e isenta de óleo essencial) quanto o óleo essencial de *Rosmarinus officinalis* também apresentaram potencial antidepressivo no TSC, sem afetar o desempenho locomotor. Desta forma, tanto compostos apolares quanto polares, parecem contribuir para este efeito biológico.

Subsequentemente, os compostos fitoquímicos, carnosol, ácido betulínico e ácido ursólico, apresentaram potencial antidepressivo e desta forma, contribuem, pelo menos em parte para o potencial antidepressivo desta planta.

Dentre estes, o ácido ursólico apresentou maior potencial antidepressivo, no que se refere ao percentual do efeito anti-imobilidade no TSC, em relação aos demais compostos testados e este efeito parece ser mediado pelo envolvimento do sistema dopaminérgico, semelhante ao mecanismo de ação do extrato de *Rosmarinus officinalis.*

No que se refere ao modelo da BO, primeiramente foi demonstrado que os camundongos submetidos à BO apresentaram hiperatividade locomotora e exploratória, validando a reprodutibilidade e padronização da técnica, em relação aos dados reportados na literatura.

As principais alterações comportamentais (hiperatividade e comportamento anedônico) e bioquímicas (aumento da atividade AchE hipocampal) foram revertidas pelo tratamento crônico com o antidepressivo clássico fluoxetina, indicando que este modelo pode ser uma ferramenta eficaz para estudar depressão agitada associada com

anedonia, e também que o tratamento com este antidepressivo pode ser eficaz para o tratamento deste sub-tipo de depressão. Desta forma, nosso estudo, contribuiu com a caracterização comportamental do modelo da BO em camundongos.

Reforçando a evidência inicial do potencial antidepressivo em testes preditivos de atividade antidepressiva (TNF e TSC), o tratamento crônico com extrato de Rosmarinus officinalis foi eficaz em reverter as alterações comportamentais (hiperatividade e comportamento anedônico) e alterações bioquímicas (aumento da atividade da AchE hipocampal), indicando que o extrato desta planta pode ser um composto fitoterápico promissor para o tratamento deste sub-tipo de depressão, bem como para o tratamento do comportamento anedônico, sintoma relevante neste trantorno de humor.

Em suma, o presente estudo fundamentou o uso etnofarmacológico de *Rosmarinus officinalis*, visto que o efeito tipo-antidepressivo do extrato desta planta foi eficaz tanto em testes preditivos de atividade antidepressiva, quanto no modelo de depressão da BO e este efeito parece ser mediado pela participação do sistema monoaminérgico e colinérgico, tornando-se uma possível ferramenta terapêutica eficaz para o tratamento de depressão, especialmente a depressão agitada associada a anedonia.

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