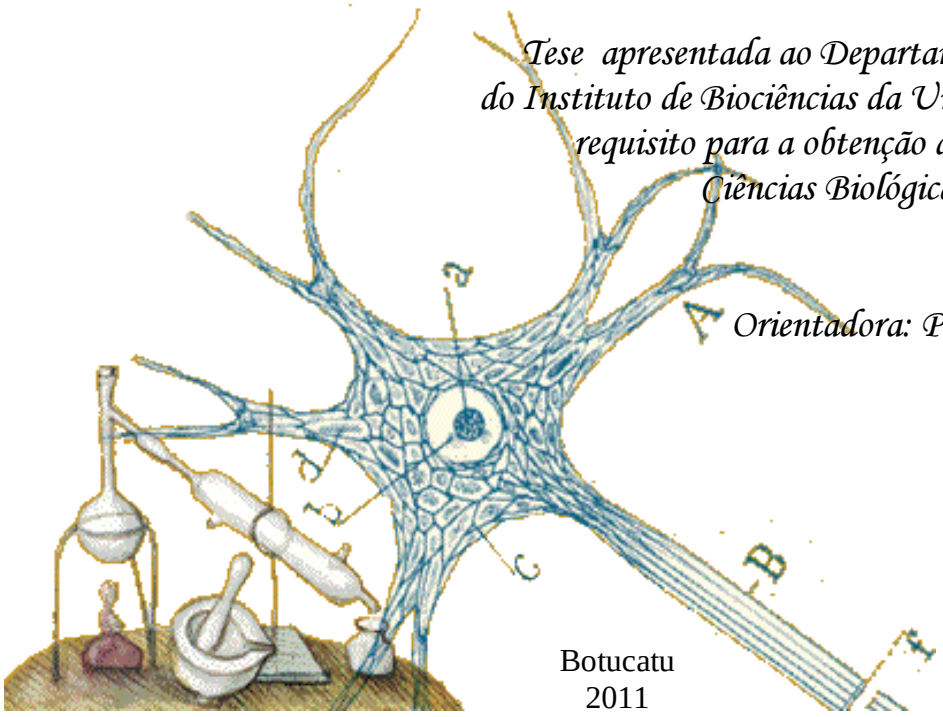
Universidade Estadual Paulista
Instituto de Biociências – Departamento de Farmacologia
Programa de Pós-graduação em Ciências Biológicas

Tese de Doutorado

"Estudo farmacológico e neuroquímico dos óleos essenciais de *Cymbopogon citratus* (D.C.) Stapf e *Citrus aurantium* L."

Celso Acácio Rodrigues de Almeida Costa



Tese apresentada ao Departamento de Farmacologia do Instituto de Biociências da Unesp de Botucatu como requisito para a obtenção do Título de Doutor em Ciências Biológicas (AC: Farmacologia).

Orientadora: Profª. Drª. Mirtes Costa

Botucatu
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Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação
Divisão Técnica de Biblioteca e Documentação - Campus de Botucatu - UNESP
Bibliotecária responsável: *Sulamita Selma Clemente Colnago – CRB 8/4716*

Costa, Celso Acácio Rodrigues de Almeida.
Estudo farmacológico e neuroquímico dos óleos essenciais de *Cymbopogon citratus* (D.C.) Stapf e *Citrus aurantium* L. / Celso Acácio Rodrigues de Almeida Costa. – Botucatu, 2011

Tese (doutorado) - Instituto de Biociências de Botucatu, Universidade Estadual Paulista, 2011
Orientadora: Mirtes Costa
Capes: 21003009

1. Neuropsicofarmacologia. 2. Etnofarmacologia. 3. Essências e óleos essenciais - Uso terapêutico.

Palavras-chave: Ansiedade; Camundongo; Citrus aurantium; Cymbopogon citratus; Depressão; Neuroquímica; Óleo essencial

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2º Titular: Profª. Drª. Thereza Christina Monteiro de Lima (UFSC/Florianópolis)

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1º: Profº. Drº. Reinaldo Nóbrega Almeida (UFPB/João Pessoa)

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3º: Profª. Drª. Clélia Akiko Hiruma-Lima (UNESP/Botucatu)

Botucatu
2011

Auxílio Financeiro: **FAPESP**
(Fundação de Amparo à Pesquisa do Estado de São Paulo)
Processo nº 2006/07195-8

"Que riqueza não é, até entre os pobres, ser filho de bons pais."

(Juan Luis Vives)

Aos meus pais

"E a pergunta que me vem, em tom de agradecimento, é: por que será que você aconteceu na minha vida? Não espero respostas, mas deixo-me aos seus cuidados."

(Fernanda Young)

À Flávia – "Kalose"

"Nenhum indício melhor se pode ter a respeito de um homem do que a companhia que freqüenta: o que tem companheiros decentes e honestos adquire, merecidamente, bom nome, porque é impossível que não tenha alguma semelhança com eles."
(Adam Parfrey)

À Deus!

Aos pequenos e nobres animais pela imprescindível contribuição dada a este trabalho. Antes de qualquer agradecimento, meu respeito!

Aos grandes amigos que tive o prazer de conviver junto: Adriano e Érica, Fábio (Raridade), Gustavo (Babito), Lucas (K rrompido), Thiago (Mingo) e Uilian (Prumo).

À moçada que está longe e ao mesmo tempo sempre perto: Fernando, Gabriel, Júlio, Manu....

À galerinha da Comissão do Workshop da Pós-Graduação!!!

À moçada do "Lab. Clélia", agregados e afins!!! Valeu Ariane (Sponja) pela companhia nos vários almoços e pelas boas risadas!!!

Aos amigos que fiz no laboratório: Carlos (Rosquinha), Filipe (Carninha), Valéria, Thaís (Miss), Leandro (Flamb´s), Bruna (Tuba) e Vinícius. Obrigado por contribuírem no meu crescimento e por mostrarem como é bom (e porque não dizer difícil!) conviver com as diferenças. Muuuito obrigado pelos ótimos momentos que pudemos rir juntos! E claro, Valéria... valeu pelos muitos sábados e domingos passados no laboratório!!!

Aos meus tios de São Paulo e, em especial, ao meu primo Alê que sempre abriu as portas de sua casa quando eu precisava ir pra São Paulo.

À todos do Depto. de Farmacologia: Professores, Funcionários – Paulão, Luis/Purga, Cris e Flávia -, Estagiários, Pós-graduandos, que fizeram parte dessa caminhada.

À moçado do "Lab. André" pelo empréstimo de material quando necessário! Juliana (Pupua), Ricardo (Nulo), Vanessa (Busilis), Enio e Fernanda.

Ao pessoal do LaFit-Botu (“Lab. Di Stasi”) também pelo empréstimo de material e mão-de-obra sempre que preciso (certo, Léo?). Obrigado Aline, Ana, Léo e todos os outros alunos.

À sessão de Pós-graduação: Luciene, Herivaldo, Davi e Luciana. E o cara das antigas: Serginho!

Aos Professores de Música: Daniel (batera) e Hélião (baixo) – pelos momentos de descontração! “*Music is food for the soul.*” ♫

Aos bons amigos que fiz na pós: Léo, Walter, Rafael, Eduardo, Hélio (Ponpon) e família!!!

Ao Profº Luis Fernando Barbisan (Depto. de Morfologia) e seu aluno (e grande amigo!) Lucas T. Bidinotto pelos belos trabalhos em parceria!

À Profª Regina Takahira (Laboratório Clínico Veterinário da FMVZ/UNESP) pela disponibilidade e pelos trabalhos realizados em conjunto!

Às moças que cuidam do Depto. de Farmacologia, em especial a “Janjan” - Valeu Janete!!!

Ao pessoal do CIE (Centro de Isótopos Estáveis) pela colaboração, em especial ao Evandro, à Juliana Denadai e ao Prof. Ducatti (Tatão!).

À Profª Drª Silvia M. Nishida pela amizade e pelos bons ‘papos de corredor’.

Ao Prof. Dr. Jorge Camilo Flório do Depto. de Patologia da FMVZ/USP por estar sempre disposto a adaptar-se, de alguma forma, aos “imprevistos” de minhas visitas à USP. Grato pela dedicação e paciência!

Aos professores da Banca, pelo aceite e disponibilidade: Profª. Thereza, Profº. Ricardo, Profª. Silvia, Profº. Di Stasi, Profº. Reinaldo, Profª Cleopatra e Profª. Clélia.

À FAPESP pelo incentivo científico e suporte financeiro.

À todos aqueles que, por um lapso, não foram aqui citados!!!

Não tenham dúvida: este trabalho leva um pouco de todos vocês!

"O professor medíocre expõe. O bom professor explica.

O professor superior demonstra. O grande professor inspira!"

(William Arthur Ward)

Agradeço, primeira e especialmente, à minha Professora e Orientadora Drª Mirtes Costa, por pouco mais de 8 anos de um contínuo acompanhamento, pela amizade, compreensão e sabedoria.
Meus mais sinceros agradecimentos!

"Até hoje os cientistas discutem como a vida começou. Até hoje não se tem certeza de onde viemos. Os filósofos ainda querem entender quem somos. E existem umas duzentas teorias para onde vamos. Se a orientação sexual é definida pela genética. Porque você boceja quando alguém boceja. Os biólogos querem entender como os pássaros migram. E os nutricionistas se o ovo faz mal à saúde. Os economistas querem explicar a crise. ***E os cientistas como o cérebro funciona.*** *Como você pode ver, não são as respostas que movem o mundo. São as perguntas!"*

(Criação da FMazza Saatchi & Saatchi para o Canal Futura)

"...é coisa de primeiro mundo!

Eu paro pra pensar e falo: - Que primeiro mundo é esse? De onde vem isso?

Ou então quando alguém fala de alguma coisa negativa que acontece na nossa vida cotidiana:

- Isso só acontece no Brasil!

Eu tenho uma boa e uma má notícia! Praqueles que usam essa expressão e gostam do primeiro mundo: ***o primeiro mundo faliu!!!*** *Em todos os sentidos. Faliu financeiramente! Faliu moralmente! Faliu eticamente!*

E agora vem a boa notícia: ***o primeiro mundo agora é aqui!!!*** *"*

(Miguel Nicoletis)

Resumo:

A depressão e a ansiedade representam algumas das mais comuns e proliferativas desordens mentais no mundo. Tradicionalmente se postula que ansiedade e depressão são desordens separadas e distintas. Entretanto, muitos estudos clínicos têm demonstrado uma grande sobreposição entre esses dois estados. Atualmente, a maioria dos transtornos de ansiedade é tratada com drogas que interferem, de modo mais ou menos seletivo, com a neurotransmissão gabaérgica, serotonérgica e/ou noradrenérgica. A depressão, por sua vez, tem seu tratamento fundamentado no aumento da disponibilidade de serotonina e/ou noradrenalina. As drogas disponíveis na clínica não são capazes de controlar estas desordens em boa parte dos pacientes e possuem efeitos colaterais importantes. Dessa forma, os múltiplos efeitos adversos das drogas disponíveis, e a sua eficácia limitada em uma considerável proporção de pacientes, justificam os esforços no sentido de se encontrar novas substâncias com potencial atividade no tratamento destas desordens. As espécies vegetais *Cymbopogon citratus* e *Citrus aurantium* apresentam este potencial, pois são utilizadas na medicina popular como calmante e sedativa. Resultados prévios demonstraram a presença de atividade ansiolítica no óleo essencial (OE) obtido de ambas as espécies. No presente trabalho o OE de *C. citratus* apresentou atividade ansiolítica resultante da interação com o complexo receptor GABA-benzodiazepínico na dose de 10 mg/kg após tratamento agudo (v.o.). Além disso, o tratamento concomitante com doses não efetivas de diazepam e OE demonstrou efeito sinérgico entre as duas preparações. Por sua vez, o OE de *C. aurantium* apresentou atividade ansiolítica na dose de 5 mg/kg (v.o.) mediada pelos receptores serotonérgicos do tipo 5-HT_{1A} e, depois de 14 dias de tratamento, o OE apresentou efeito ansiolítico na dose de 1 mg/kg/dia. Não foram detectadas alterações neuroquímicas no córtex, estriado, hipotálamo e ponte após tratamento agudo, bem como não foram verificadas modificações histopatológicas e bioquímicas, exceto a redução nos níveis de colesterol dos animais tratados repetidamente com as espécies em estudo. O tratamento agudo ou repetido, para todas as doses testadas dos OE, não provocou prejuízo da atividade locomotora. Os resultados obtidos corroboram o uso popular e garantem consideravelmente o uso seguro das espécies estudadas.

Palavras-chave: Ansiedade; Camundongo; *Citrus aurantium*; *Cymbopogon citratus*; Depressão; Neuroquímica; Óleo essencial.

Abstract:

Depression and anxiety represent two of the most common and proliferating mental disorders in the world. Traditionally, anxiety and depression disorders have been considered separate and distinct. However, many clinical studies have shown a significant comorbidity between these two states. Currently, most anxiety disorders are treated with drugs that interfere, more or less selectively, with GABAergic, serotonergic and/or noradrenergic neurotransmission. The treatment of depression, on the other hand, has been based on the increased availability of serotonin and/or norepinephrine. The drugs available in a clinic are not able to control these disorders in many patients and also have significant side effects. Thus, based on the multiple adverse effects of available drugs and their limited efficacy in a considerable proportion of patients, significant efforts are justified for finding new substances that could potentially treat these disorders, particularly, the plant species *Cymbopogon citratus* and *Citrus aurantium* because they are used in folk medicine as tranquilizers and sedatives. Previous results demonstrated the presence of anxiolytic-like activity in the essential oil (EO) obtained from both species. In this work, the *C. citratus* EO showed anxiolytic-like activity resulting from interaction with the GABA-benzodiazepine receptor complex at a dose of 10 mg/kg after acute oral treatment. Moreover, concomitant treatment with ineffective doses of diazepam and EO showed a synergistic effect between the two preparations. In turn, the *C. aurantium* EO showed anxiolytic-like activity at a dose of 5 mg/kg (p.o.) mediated by serotonin receptors of 5-HT_{1A} type, and, after 14 days of treatment, the EO showed anxiolytic-like effects at a dose of 1 mg/kg/day. Neurochemical changes were not detected in the cortex, striatum, hypothalamus, and pons after acute oral treatment, and there were no histopathological or biochemical changes, except for the reduction in cholesterol levels in animals treated repeatedly with these species. At each dose of EO neither the acute nor the repeated treatment caused locomotor impairment. The results corroborate the popular use and help ensure the safe use of the species.

Keywords: Anxiety; *Citrus aurantium*; *Cymbopogon citratus*; Depression; Essential oil; Mice; Neurochemistry.

SUMÁRIO

Prólogo:

Durante todo o período do curso de Doutorado no Programa de Pós-graduação em Ciências Biológicas - Área de Farmacologia houve a possibilidade de participação em diferentes atividades extracurriculares além de outros trabalhos de pesquisa desenvolvidos no laboratório. Estes trabalhos possibilitaram a divulgação de resultados em Congressos Nacionais e Internacionais e envio de artigos para publicações em periódicos científicos internacionais.

No mais, o desenvolvimento das atividades de Professor-bolsista possibilitaram o exercício da relação professor-aluno em todos os níveis: regência de aulas teóricas e teórico-práticas, preparação e correção de provas, além do contato com os aspectos administrativos da docência.

**** Elaboração de artigos científicos:***

Artigos publicados:

1. BLANCO, M. M.; COSTA, C. A. R. A.; FREIRE, A. O.; SANTOS Jr., J. G.; COSTA, M. Neurobehavioral effect of essential oil of *Cymbopogon citratus* in mice. **Phytomedicine** (Stuttgart), v. 16, p. 265-270, 2009.

2. GARGANO, A. C.; COSTA, C. A. R. A.; COSTA, M. Essential Oils from *Citrus latifolia* and *Citrus reticulata* reduce anxiety and prolong ether sleeping time in mice. **Tree and Forestry Science and Biotechnology**, v. 2, p. 121-124, 2008.

Artigo aceito para publicação:

1. BIDINOTTO, L. T.; COSTA, C. A. R. A.; SALVADORI, D. M. F.; COSTA, M.; RODRIGUES, M. A. M.; BARBISAN, L. F. Protective effects of lemongrass (*Cymbopogon citratus* STAPF) essential oil on DNA damage and carcinogenesis in female Balb/C mice. **Journal of Applied Toxicology**, 2010. doi:10.1002/jat.1593.

Artigos submetidos:

1. COSTA, C. A. R. A.; KÖHN, D. O.; LIMA, V. M.; GARGANO, A. C.; FLÓRIO, J. C.; COSTA, M. Gabaergic system contributes to the anxiolytic-like effect of essential oil from *Cymbopogon citratus* (lemongrass). **Journal of Ethnopharmacology.**

2. BIDINOTTO, L. T.; COSTA, C. A. R. A.; COSTA, M.; RODRIGUES, M. A. M.; BARBISAN, L. F. Modifying effects of lemongrass essential oil on tissue response to carcinogen N-methyl-N-nitrosurea (MNU) in female Balb/C mice. **Journal of Medicinal Food.**

3. COSTA, C.A.R.A.; BIDINOTTO, L.T.; TAKAHIRA, R. K.; SALVADORI, D.M.F.; BARBISAN, L.F.; COSTA, M. Safety evaluation of lemongrass (*Cymbopogon citratus* (DC) Stapf) essential oil in male Swiss mice. **Food and Chemical Toxicology.**

4. RIBEIRO, C. A. S.; COSTA, C. A. R. A.; COSTA, M. Sequential procedures battery to assess the anxiolytic- and antidepressant-like activity in mice. **Brazilian Journal of Medical and Biological Research.**

5. COSTA, C. A. R. A.; CURY, T. C.; CASSETTARI, B. O.; FLÓRIO, J. C.; COSTA, M. Behavioral, neurochemical and biochemical evaluation of *Citrus aurantium* L. essential oil. **European Neuropsychopharmacology.**

**** Prêmios e títulos:***

⇒ Menção Honrosa pela apresentação do painel "Envolvimento do complexo receptor GABA-benzodiazepínico na atividade ansiolítica do óleo essencial de *Cymbopogon citratus* (D.C.) Stapf", durante a XXIV Reunião Anual da FeSBE, 2009.

**** Experiência Didática:***

1. Professor Bolsista vinculado ao Departamento de Farmacologia do Instituto de Biociências da Unesp/Botucatu nas disciplinas de Farmacologia nos cursos de Ciências Biológicas (Modalidade Licenciatura), Ciências Biomédicas, Enfermagem e Medicina durante o ano de 2010. (60 horas)

2. Métodos de Pesquisa em Psicofarmacologia, na disciplina de Farmacologia do Curso de Ciências Biológicas – Licenciatura, Unesp/Botucatu, 2010. (4 horas).

3. Métodos Experimentais em Psicofarmacologia, na disciplina optativa de Farmacologia "Metodologia Experimental Farmacológica", Unesp/Botucatu, 2009. (4 horas).

4. Professor-Palestrante da Disciplina de Microbiologia e Imunologia Clínica da FAIT-ACITA (Associação Cultural e Educacional de Itapeva), 2009. (20 horas).

5. Métodos de Pesquisa em Psicofarmacologia, na disciplina optativa de Farmacologia "Metodologia Experimental Farmacológica", Unesp/Botucatu, 2008. (4 horas).

6. Neuropsicofarmacologia de Produtos Naturais, nas disciplinas de Farmacologia dos Cursos de Ciências Biológicas (Bacharelado e Licenciatura) e Ciências Biomédicas, Unesp/Botucatu, 2008 (12 horas).

**** Co-orientações:***

1. Camila Maschieto. *Serjania erecta*: estudo da potencialidade antihipertensiva, 2010. Co-orientação. Curso: Nutrição - UNESP. Fundação de Amparo à Pesquisa do Estado de São Paulo. Orientador: Profª. Drª. Juliana I. F. de Gobby.

2. Leandro Jorge Coelho. Avaliação da atividade antidepressiva do óleo essencial de *Cananga odorata* (Lam.) Hook. f. & Thomson em camundongos, por via oral, 2010. Co-orientação. Curso: Ciências Biológicas - Bach./Licenc. - UNESP. Orientadora: Profª. Drª. Mirtes Costa.

3. Thaís Canto Cury. Estudo da atividade ansiolítica e antidepressiva do óleo essencial de *Citrus aurantium* L. administrado por via inalatória em camundongos, 2010. Co-orientação. Curso: Ciências Biomédicas - UNESP. Orientador: Profª. Drª. Mirtes Costa.

**** Participação em bancas de comissões julgadoras e pareceres:***

1. Parecer de relatório final de estágio curricular (Iniciação Científica) de aluno do Curso de Ciências Biomédicas do Instituto de Biociências de Botucatu, 2010.

2. Membro da Comissão Científica para seleção dos painéis apresentados durante o VIII Workshop da Pós-Graduação do Instituto de Biociências de Botucatu, 2009. Unesp/Botucatu.

3. Avaliador de painéis durante o XX Congresso de Iniciação Científica da Unesp, 2008. Unesp/São José dos Campos.

**** Estágios/Visitas Técnicas:***

1. Faculdade de Medicina Veterinária e Zootecnia (FMVZ/USP). Treinamento técnico realizado junto ao Departamento de Patologia Animal – Laboratório de Farmacologia e Toxicologia - da Faculdade de Medicina Veterinária e Zootecnia da USP, perfazendo um total de 120 horas, 2008-2011.

2. Laboratório de Tecnologia Farmacêutica (LFT). Visita técnica realizada no LTF da Universidade Federal da Paraíba (UFPB) na cidade de João Pessoa/PB, 2010.

3. Departamento de Ciências Experimentais e da Saúde. Visita técnica realizada na Universidade Pompeu Fabra (UPF) na cidade de Barcelona/Espanha, 2010.

4. Instituto de Medicina Molecular de Lisboa (IMM). Visita técnica realizada na unidade de Neurociência do Instituto de Medicina Molecular da Faculdade de Medicina de Lisboa/Portugal, 2009.

5. *Centro de Bioactivos Marinos* (CEBIMAR). Visita técnica realizada no *Centro de Bioactivos Marinos* (CEBIMAR) na cidade de La Habana/Cuba, 2009.

6. Instituto Agronômico de Campinas (IAC). Visita e Treinamento Técnico realizados durante a Disciplina da Pós-Graduação: "Biossíntese, métodos de extração e identificação de metabólitos secundários em plantas medicinais e aromáticas", 2008.

**** Participação em eventos científicos:***

1. XXV Reunião Anual da FeSBE, 2010. Águas de Lindóia/SP.

2. 11th International Society for Ethnopharmacology Congress and I Encuentro Hispano Portugués de Etnobiología, 2010. Albacete/Espanha.

3. XXI Simpósio de Plantas Medicinais do Brasil, 2010. João Pessoa/PB.

4. IX Workshop da Pós-Graduação & X Workshop de Genética, 2010. Botucatu/SP.

5. XXIV Reunião Anual da FeSBE, 2009. Águas de Lindóia/SP.

6. XVIII Congreso Italo-latinoamericano de Etnomedicina, 2009. La Habana/Cuba.

7. 2º Congresso Iberoamericano de Fitoterapia, 2009. Lisboa/Portugal.

8. VIII Workshop da Pós-Graduação do Instituto de Biociências de Botucatu, 2009. Botucatu/SP.

9. I Ciclo de Palestras sobre Ética na Experimentação Animal: Cuidados e Atenção na Experimentação Animal, 2009. Botucatu/SP.

10. XX Simpósio Brasileiro de Plantas Medicinais e X Congresso Internacional de Etnofarmacologia, 2008. São Paulo/SP.

11. I Congresso de Neurociências da América Latina, Caribe e Península Ibérica, 2008. Búzios/RJ.

12. V Simpósio de Ciências Aplicadas da FAIT, 2008. Itapeva/SP.

13. VIII Workshop de Plantas Medicinais de Botucatu, 2008. Botucatu/SP.

14. VII São Paulo Research Conferences, 2007. São Paulo/SP.

**** Trabalhos apresentados em eventos científicos nacionais:***

1. COSTA, C. A. R. A.; COSTA, M. Investigação do mecanismo de ação ansiolítica do óleo essencial de *Citrus aurantium* L.. In: XXV Reunião Anual da FeSBE, 2010, Águas de Lindóia.

2. LIMA, V. M.; COSTA, C. A. R. A.; COSTA, M. Investigação da atividade antidepressiva e ansiolítica do óleo essencial de *Rosmarinus officinalis* L. (Alecrim). In: XXI Simpósio de Plantas Medicinais do Brasil, 2010, João Pessoa/PB.

3. FERREIRA, F. G.; RIBEIRO, C. A. S.; COSTA, C. A. R. A.; COSTA, M. Avaliação das atividades ansiolítica e antidepressiva dos óleos essenciais de *Mentha piperita* e *Cananga odorata* em camundongos por via inalatória. In: XXI Simpósio de Plantas Medicinais do Brasil, 2010, João Pessoa/PB.

4. MASCHIETO, C.; COSTA, C. A. R. A.; COELHO, R. G.; COSTA, M.; GOBBI, J. I. F. Administração crônica do decocto da raiz de Serjania erecta não modifica parâmetros de ansiedade em ratos SHR. In: IX Workshop da Pós-graduação e X Workshop de Genética, 2010, Botucatu/SP

5. BIDINOTTO, L. T.; COSTA, C. A. R. A.; COSTA, M.; BARBISAN, L. F. Efeitos do óleo essencial de Capim-limão sobre alterações na cinética celular do epitélio mamário de fêmeas BALB/c, induzidas pela N-Metil-N-Nitrosuréia. In: VIII Workshop da Pós-Graduação do Instituto de Biociências de Botucatu, 2009, Botucatu/SP.

6. KÖHN, D. O.; COSTA, C. A. R. A.; COSTA, M. Estudo da atividade anticonvulsivante do extrato hidroalcoólico de *Ruta graveolens* L.. In: VIII Workshop da Pós-Graduação do Instituto de Biociências de Botucatu, 2009, Botucatu/SP.

7. COSTA, C. A. R. A.; COSTA, M. Envolvimento do complexo receptor GABA-benzodiazepínico na atividade ansiolítica do óleo essencial de *Cymbopogon citratus* (DC) Stapf. In: XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental, 2009, Águas de Lindóia.

8. KÖHN, D. O.; COSTA, C. A. R. A.; COSTA, M. Pharmacological activity of *Ruta graveolens* L.: disagreement between ethnopharmacological information and experimental data. In: 41º Congresso Brasileiro de Farmacologia e Terapêutica Experimental, 2009, Ribeirão Preto.

9. COSTA, C. A. R. A.; FERREIRA, F. G.; COSTA, M. Avaliação do Óleo Essencial (OE) de *Cymbopogon citratus* em Modelos de Transtorno Obsessivo Compulsivo (TOC) e Depressivo (TD). In: XX Simpósio Brasileiro de Plantas Medicinais e X Congresso Internacional de Etnofarmacologia, 2008, São Paulo.

10. BIDINOTTO, L. T.; COSTA, C. A. R. A.; COSTA, M; BARBISAN, L. F. Lack of protective effects of lemongrass essential oil against aberrant crypt foci development in females Balb/C mice. In: XX Simpósio Brasileiro de Plantas Medicinais e X Congresso Internacional de Etnofarmacologia, 2008, São Paulo.

11. BIDINOTTO, L. T.; COSTA, C. A. R. A.; COSTA, M.; BARBISAN, L. F. Efeitos do capim-limão sobre as taxas de proliferação celular e apoptose em criptas intestinais do cólon de fêmeas Balb/C expostas à N-metil-N-nitrosuréia. In: 11º Encontro Regional de Biomedicina, 2008, Botucatu.

12. BIDINOTTO, L. T.; COSTA, C. A. R. A.; SALVADORI, D. M. F.; BARBISAN, L. F. Efeitos de capim-limão (*Cymbopogon citratus* STAPF) sobre danos de DNA causados por N-Metil-N-Nitrosuréia em fêmeas Balb/C. In: XXIII Reunião Anual da Federação de Sociedades de Biologia Experimental, 2008, Águas de Lindóia.

**** Trabalhos apresentados em eventos científicos internacionais:***

1. COSTA, C. A. R. A.; COSTA, M. Evaluation of the action mechanism of anxiolytic-like effect of essential oil from *Citrus aurantium* L. In: 11th ISE Congress and I EHPE, 2010, Albacete/Espanha. Revista de Fitoterapia - Book of Abstracts, 2010. v. 10. p. 140-140.

2. COSTA, C. A. R. A.; COSTA, M. Neuropharmacological profile of essential oil and major compounds from *Cymbopogon citratus* (Lemongrass). In: XVIII Congreso Italo-latinoamericano de Etnomedicina, 2009, Ciudade de La Habana. XVIII Congreso Italo-latinoamericano de Etnomedicina "Juan Tomáz Roig", 2009.

3. COSTA, C. A. R. A.; KÖHN, D. O.; LIMA, V. M.; COSTA, M. Tratamento agudo, mas não subcrônico, com óleo essencial de *Cymbopogon citratus* promove efeito ansiolítico por meio de receptores benzodiazepínicos. In: 2º Congresso Iberoamericano de Fitoterapia, 2009, Lisboa. Revista de Fitoterapia - Livro de Resumos Fito 2009. Valencia/España, 2009. v. 9. p. 153-153.

4. COSTA, C. A. R. A.; GARGANO, A. C.; COSTA, M. Influência do Ciclo Estral na Resposta Ansiolítica de Diazepam e Imipramina em Camundongos. In: I Congresso de Neurociências da América Latina, Caribe e Península Ibérica, 2008, Búzios/RJ.

5. KÖHN, D. O.; RIBEIRO, C. A. S.; COSTA, C. A. R. A.; COSTA, M. Avaliação da interferência do ciclo circadiano sobre os comportamentos de camundongos avaliados no labirinto em cruz elevado. In: I Congresso de Neurociências da América Latina, Caribe e Península Ibérica, 2008, Búzios/RJ.

**** Formação complementar:***

1. Farmacodinâmica Molecular, 3 horas. FeSBE, 2010.

2. ***Extensão universitária*** em Detecção de Danos no DNA pelo Ensaio Cometa, 32 horas. Multigene, 2009.

3. ***Extensão universitária*** em Animais de Laboratório - Uma especialidade, 40 horas. Instituto Butantan, 2009.

4. Problemas éticos e científicos relacionados com a invalidez e a ineficiência de trabalhos com animais de laboratório, 3 horas. FeSBE, 2009.

5. Fitoquímica, Farmacologia e Fitoterapia, 6 horas. Instituto de Biociências/Unesp, 2008.

6. Efeitos Farmacológicos de Plantas em Modelos Animais, 3 horas. XX Simpósio Brasileiro de Plantas Medicinais, 2008.

7. Modelos animais em neurociências, 3 horas. Congresso de Neurociências da América Latina, Caribe e Península Ibérica, 2008.

8. Plasticidade e Degeneração no Sistema Nervoso, 8 horas. SBNeC, 2007.

9. Introdução à Neurobiologia, 24 horas. São Paulo *Research Conferences*, 2007.

**** Disciplinas cursadas na Pós-graduação:***

1. Bioinformática (2007/2 créditos/ Conceito: A)
2. Classificação de Receptores Farmacológicos (2007/3 créditos/ Conceito: A)
3. Tópicos de Atualização em Ciências (2007/1 crédito/ Conceito: A)
4. Teoria e Prática de Técnicas Espectroscópicas de UV-VIS, Fluorescência e Quimiluminescência: Aplicações em Ciências Biomédicas (2008/2 créditos/ Conceito: A)
5. Tópicos de Atualização em Ciências (2008a/1 crédito/ Conceito: A)
6. Empreendedorismo (2008/4 créditos/ Conceito: A)
7. Tópicos de Atualização em Ciências (2008b/1 crédito/ Conceito: A)
8. Neurofarmacologia e Comportamento (2009/2 créditos/ Conceito: A)
9. Tópicos de Atualização em Ciências (2009a/1 crédito/ Conceito: A)
10. Tópicos de Atualização em Ciências (2009b/1 crédito/ Conceito: A)

**** Organização de evento:***

1. IX Workshop da Pós-Graduação & X Workshop de Genética, 2010.
2. VIII Workshop da Pós-Graduação do IB de Botucatu, 2009.
3. VII Workshop da Pós-Graduação em Ciências Biológicas, 2008.
4. VI Workshop da Pós-Graduação em Ciências Biológicas, 2007.

**** Trabalhos técnicos:***

1. Desenvolvimento dos Anais do VIII Workshop da Pós-Graduação do IB de Botucatu, 2009.
2. Desenvolvimento dos Anais do VII Workshop da Pós-Graduação em Ciências Biológicas, 2008.

I. Introdução:

A comorbidade entre depressão e ansiedade é assunto ainda bastante controverso. Historicamente se postula que ansiedade e depressão são desordens separadas e distintas, entretanto, muitos estudos pré-clínicos e clínicos têm demonstrado uma grande sobreposição entre esses dois estados (Andreatini e Bacellar, 1999; Cryan e Holmes, 2005; Kessler *et al.*, 2005; Lamers *et al.*, 2011), inclusive pela classificação, sujeita a questionamentos na prática clínica e na pesquisa, na categoria de diagnóstico chamado "transtorno misto ansioso depressivo" - *mixed anxiety-depressive disorder* - MADD (Spijker *et al.*, 2010).

A depressão e as desordens de ansiedade representam algumas das mais comuns e proliferativas doenças de saúde no mundo (Cryan e Holmes, 2005). De acordo com levantamento feito pela OMS por meio do Consórcio Internacional em Epidemiologia Psiquiátrica (ICPE), a ansiedade generalizada atinge, ao longo da vida, de 3 à 9% da população a qual o estudo se refere: Brasil, Canadá, Holanda e Estados Unidos da América (Andrade *et al.*, 2003). Por sua vez, a depressão tem sua prevalência estimada entre 3 à 17% na população estudada de 10 países: Canadá, Estados Unidos, Brasil, Chile, México, República Tcheca, Alemanha, Holanda, Turquia e Japão (Kessler *et al.*, 2002, 2003). No Brasil, um estudo multicêntrico de morbidade psiquiátrica em adultos demonstrou as seguintes estimativas de prevalência para os transtornos ansiosos: 12,1% para Brasília, 6,9% para São Paulo e 5,4% para Porto Alegre (Almeida Filho *et al.*, 1992).

Conceitualmente a ansiedade é caracterizada por sintomas psicológicos e físicos. Os sintomas psicológicos predominantes são a sensação persistente e antecipatória de medo, cuja fonte é incerta ou desconhecida, pensamentos preocupantes repetitivos, inquietação, irritabilidade e falta de concentração. Os sintomas físicos estão associados à tensão muscular (que pode causar dores e tremores musculares e dores de cabeça tensionais) e à hiper atividade autonômica que causa vários sintomas somáticos tais como sensação de aperto e dor no peito, hiperventilação e taquicardia e aumento da pressão arterial (Rickels *et al.*, 2002).

Já a depressão caracteriza-se por alterações do humor, geralmente acompanhadas de sintomas cognitivos, comportamentais e somáticos. Queixas de ansiedade, irritabilidade, preocupação, desinteresse ou perda da capacidade de sentir prazer nas atividades habituais, bem como sentimentos de culpa e baixa auto-estima estão entre os principais sintomas dos transtornos depressivos (Nestler *et al.*, 2002).

Não são recentes as tentativas de desenvolvimento de teorias psicológicas e biológicas para se entender o substrato neuroquímico na etiologia da depressão e da ansiedade (Jesberger e Richardson, 1985). Como em outros distúrbios psiquiátricos a etiologia da depressão e da ansiedade patológica, parece depender de uma interação entre predisposição e fatores ambientais. Estudos fornecem indícios de que estes quadros possuam origens genéticas comuns, porém a predisposição pode também ser resultado de diferentes causas ambientais, como por exemplo: problemas familiares, eventos estressantes da vida (morte de um parente, exposição a um evento traumático), circunstâncias econômicas, além de outros (Cerdá *et al.*, 2010; Hettema, 2008). Além disso, as associações destes transtornos

costumam ser mais graves do que os transtornos isolados em termos de sintomas, evolução, resposta ao tratamento, risco de suicídio e prevalência de transtornos psiquiátricos entre os familiares (Lamers *et al.*, 2011).

É bastante improvável que os transtornos de depressão e ansiedade sejam decorrentes de alterações em um único sistema de neurotransmissão, uma vez que diversos neurotransmissores participam, em maior ou menor grau, da modulação dos comportamentos defensivos, além de interagirem entre si (Charnay e Léger, 2010; Graeff e Hetem, 2004; Neumann *et al.*, 2011). O conhecimento atual da participação de neurotransmissores nos processos de depressão e ansiedade advém, principalmente, do estudo do mecanismo de ação de drogas que atenuam ou agravam estes processos. Estes estudos neurobiológicos investigam principalmente duas características da comorbidade ansiedade-depressão: por um lado buscam semelhanças nas alterações neurofisiológicas que embasam os dois tipos de adoecimento e; por outro lado investem na especificidade dos transtornos e comparam o funcionamento do sistema nervoso central em cada patologia e na sua associação (Kalueff e Tuohimaa, 2004).

Um mecanismo fisiopatológico comum para a ansiedade e a depressão, consistiria de modificações nos sistemas de neurotransmissão relacionados às monoaminas, por meio da desregulação dos sistemas serotonérgico, noradrenérgico e, em menor grau do sistema dopaminérgico (Neumann *et al.*, 2011), além da desregulação do eixo Hipotálamo-Pituitária-Adrenal (Baldwin *et al.*, 2002; Nutt e Stein, 2006; Wardenaar *et al.*, 2011). Atualmente, a maioria dos transtornos de ansiedade é tratada com drogas que interferem, de modo mais ou menos seletivo, com a neurotransmissão

gabaérgica, serotonérgica e noradrenérgica. A depressão, por sua vez, tem seu tratamento fundamentado no aumento da disponibilidade de serotonina ou noradrenalina, mecanismo comum às principais drogas disponíveis para a terapêutica, como os inibidores da monoamina-oxidase e os antidepressivos tricíclicos.

Disponibilizados a partir do final da década de 1970, os inibidores seletivos da recaptação de serotonina atuam com menos intensidade sobre as outras monoaminas, inclusive a noradrenalina (Taylor *et al.*, 2005). Sem dúvida a serotonina foi o neurotransmissor mais extensivamente estudado nas últimas décadas e a sua importância na fisiopatologia destas desordens é reforçada pela eficácia dos inibidores da recaptação de serotonina tanto no tratamento da ansiedade quanto no da depressão (Blier e Ward, 2003; Taylor *et al.*, 2005). Parece que as duas vias serotoninérgicas originárias dos Núcleos da Rafe estariam distintamente relacionadas com estes dois transtornos. A primeira, representada por neurônios que se originam no Núcleo Mediano da Rafe e que se projetam para o lobo temporal e hipocampo, estaria relacionada com a depressão, enquanto a segunda, que faz a conexão entre a rafe dorsal e a amígdala contribuiria para a regulação dos quadros ansiosos (Graeff e Hetem, 2004).

A neurotransmissão mediada por noradrenalina guarda semelhança com a mediada por serotonina, já que várias estruturas cerebrais recebem aferências de ambos os sistemas. A partir do *locus coeruleus* os neurônios que formam o feixe noradrenérgico dorsal atingem várias regiões telencefálicas, entre elas a amígdala, área septal, formação hipocampal e todo o neocórtex, por meio de fibras que compõem o feixe prosencefálico medial, o mesmo que também contém as fibras serotonérgicas

(Graeff e Hetem, 2004). O estudo das interconexões entre estes sistemas tem mostrado que a interação é recíproca, sendo ambos fortemente influenciados pelo sistema gabaérgico (Fink e Göthert, 2008).

Esta relação com o GABA é particularmente interessante, desde que este é o principal aminoácido inibitório do sistema nervoso central, e o alvo da ação dos benzodiazepínicos. A ação ansiolítica destes compostos é devida a sua atividade sobre a porção basolateral da amígdala, onde a concentração de receptores benzodiazepínicos é máxima, assim como à redução do funcionamento do sistema septo-hipocampal, por diminuírem a atividade da aferência serotonérgica e noradrenérgica (Graeff e Hetem, 2004; McGaugh *et al.*, 2002; Truitt *et al.*, 2009).

Desde a década de 1960 os benzodiazepínicos têm sido extensivamente usados para tratar formas severas de ansiedade mostrando clara eficácia e sendo atualmente a classe de fármacos mais prescrita no mundo (López-Muñoz *et al.*, 2011). Os benzodiazepínicos não são capazes de controlar os quadros ansiosos em aproximadamente 25% dos pacientes e possuem efeitos colaterais importantes, incluindo: sedação, relaxamento muscular, potencialização do efeito depressor do etanol, amnésia anterógrada e dependência (Uzun *et al.*, 2010). Outras drogas, posteriormente introduzidas na terapêutica como a buspirona e seus correlatos, que atuam sobre o sistema serotonérgico, apresentam latência de cerca de duas semanas para o início dos efeitos ansiolíticos, e falham no controle dos sintomas de ansiedade em uma parcela importante dos pacientes (Lowry *et al.*, 2005).

Se, por um lado a atividade ansiolítica de drogas que atuam sobre o sistema gabaérgico é muito bem estabelecida, por outro, a atividade deste sistema sobre a depressão não é tão evidente. No entanto, estudos recentes têm associado o receptor tipo $GABA_B$ com os quadros depressivos, sugerindo que antagonistas destes receptores podem ter efeitos antidepressivos por meio de mudanças na função e na expressão de tais receptores (Ghose *et al.*, 2011).

Desta forma, considerando os altos índices de comorbidade entre os quadros de ansiedade e depressão, em torno de 60 a 70 % da população geral (Kalueff *et al.*, 2007; Lamers *et al.*, 2011) e a eficácia do tratamento com antidepressivos, tanto para quadros ansiosos (como é o caso do Transtorno de Pânico, da Fobia Social, do Transtorno Obsessivo-compulsivo e mesmo da Ansiedade Generalizada), quanto para os casos de Depressão com ou sem componente ansioso importante (Argyropoulos, 2000; Ravindran e Stein, 2010), é possível supor que uma mesma substância exógena possa exercer atividade terapêutica em ambos os quadros. No mais, os múltiplos efeitos adversos das drogas disponíveis, e a sua eficácia limitada em uma considerável proporção de pacientes, justificam os esforços no sentido de encontrar novas substâncias com potencial atividade sobre a ansiedade e/ou a depressão (Cryan e Holmes, 2005; Olivier, 2003; Pilc e Nowak, 2005).

Assim, é neste amplo contexto que surgem as plantas medicinais. Os metabólitos secundários da grande diversidade de espécies vegetais representam uma rica fonte de novas moléculas que podem servir como protótipo de substâncias úteis em diversos transtornos do sistema nervoso central (Elisabetsky e Souza, 2004).

Recorrer à natureza como forma de amenizar sintomas ou curar doenças é uma prática comum e bastante antiga. De acordo com a Organização Mundial da Saúde (OMS, 2000) aproximadamente 80% da população mundial dependem de práticas tradicionais, sendo que 85% dessa parcela utilizam plantas ou preparações à base de vegetais. Além disso, o mercado mundial de fitoterápicos movimenta cerca de US$ 22 bilhões por ano. No Brasil, por exemplo, estima-se que o comércio de fitoterápicos seja da ordem de 5% do mercado total de medicamentos, avaliados em mais de US$ 400 milhões (Pinto *et al.*, 2002).

Durante as últimas décadas, o interesse nas terapias naturais e o uso de medicamentos fitoterápicos pela população têm aumentado (WHO, 2004). O desenvolvimento de medicamentos pela indústria farmacêutica a partir de produtos naturais vem ressurgindo e as plantas continuam a ser a maior fonte de busca de novos medicamentos (Pitchai *et al.*, 2010). Além disso, de acordo com Calixto (2005), de 25-30% dos medicamentos disponíveis na terapêutica atual são derivados diretos ou indiretos de produtos naturais.

Estima-se que 43% da população que sofre com desordens de ansiedade e a grande maioria dos acometidos com depressão usam alguma forma de terapia complementar, sendo os medicamentos naturais os tratamentos mais populares, como apontado nos trabalhos feitos por Ernst (2006, 2007).

As espécies *Cymbopogon citratus* (D.C.) Stapf e *Citrus aurantium* L. foram selecionada com base em dados etnofarmacológicos, que as indicam como detentoras de ação sobre o sistema nervoso central. Essa é uma estratégia eficiente para escolha de plantas para pesquisa, visto que seu uso

tradicional pode ser considerado como uma pré-triagem quanto a sua utilidade terapêutica (Elisabetsky e Souza, 2004) e possibilita o desenvolvimento de grupos de drogas inteiramente novos, com mecanismos de ação diferentes das drogas já disponíveis (Andreatini *et al.*, 2001).

No Brasil, de acordo com levantamento feito por Nogueira (1983), o *C. citratus* foi indicado como medicamento para os transtornos do sistema nervoso ou "males psiconeurológicos" por 201 entre 479 (41,96%) mulheres que freqüentaram centros de Saúde de São Paulo. O chá das folhas é largamente utilizado na medicina popular como antiespasmódico, sedativo, calmante, analgésico suave, carminativo, antipirético, emenagogo, diurético, sudorífico, hipotensor e anti-reumático (Di Stasi *et al.*, 1989). Seguramente o emprego como sedativo do sistema nervoso ou calmante é o uso mais comum (Carlini, 1985). Também há registro de uso caseiro para combater diversas afecções das vias respiratórias e digestivas e também inflamação da bexiga. A planta foi incluída na lista da extinta Ceme (Central de Medicamentos) como espécie de estudo prioritária (Akisue, 1996).

Já a espécie *C. aurantium* é popularmente empregada para curar ou aliviar insônia, tratar nervosismo, ansiedade e histeria, e as flores são utilizadas com fins sedativos (Santos *et al.*, 1988; Lehrner *et al.*, 2000). Um destes estudos foi realizado com homens e mulheres expostos a gotas de óleo essencial de *Citrus sinensis* dispersas na ante-sala de um consultório odontológico. Esta exposição provocou efeito relaxante nos pacientes, especificamente nos casos das mulheres, que apresentaram menor nível de ansiedade, melhora no humor e maior estado de tranquilidade (Lehrner *et al.*, 2000).

Resultados prévios obtidos em nosso laboratório demonstraram a presença de atividade ansiolítica tanto para o óleo essencial obtido de *C. citratus* (Blanco *et al.*, 2009) quanto para o obtido de *C. aurantium* (Carvalho-Freitas, 2002; Pultrini *et al.*, 2006). Estas espécies vegetais, a despeito da grande utilização empírica, foram objetos de poucos estudos controlados no sentido de se caracterizar suas atividades terapêuticas ou tóxicas sobre o sistema nervoso central.

A triagem de novas drogas no campo da psicofarmacologia está intimamente relacionada às ferramentas experimentais (modelos animais adequados) que permitam a correta avaliação/identificação de tais substâncias, o delineamento dos mecanismos de ação e o estudo da neurobiologia dos transtornos mentais (Andreatini *et al.*, 2006; Lapiz-Bluhm *et al.*, 2008).

Se, de um lado os avanços nos métodos da neurobiologia aumentam nosso conhecimento básico, por outro eles também são insuficientes para elucidar a etiologia e a fisiopatologia dos transtornos afetivos bem como não são suficientes para o desenvolvimento de novos tratamentos (Neumann *et al.*, 2011). Assim, independentemente da abordagem utilizada, os critérios básicos para serem cumpridos por um modelo satisfatório são: as validades de face, preditiva e de construto.

A validade de face indica que o fenótipo do modelo é semelhante ao da síndrome humana a qual se supõe imitar; A validade preditiva estipula que uma manipulação que influencia a patologia humana vai ter efeitos semelhantes no modelo animal sendo a abordagem mais utilizada da validade preditiva o isomorfismo farmacológico (capacidade do modelo de detectar drogas potencialmente úteis na clínica) e finalmente; a validade de

construto, na qual a validade de face e a validade preditiva contribuem, pressupõe que uma doença humana e o modelo animal possuem substratos patológicos comuns, que podem explicar a patologia em ambos, permitindo assim a geração de uma teoria mecanicista comum e testes experimentais dos aspectos clínicos mais salientes de uma dada doença (Neumann *et al.*, 2011; Andreatini *et al.*, 2006).

Teoricamente um procedimento experimental deveria cumprir todos os três critérios para ser um bom modelo, entretanto esta situação não é vista com muita freqüência. Por exemplo, os modelos tradicionais de depressão falham na validade de face e de construto, porém são muito bons na validade preditiva (Kalueff e Tuohimaa, 2004), tal como acontece com os principais modelos de ansiedade (Cryan e Holmes, 2005). O fato é que os modelos animais servem para a triagem dos possíveis efeitos ansiolíticos e/ou antidepressivos de novos medicamentos e, quanto mais próximo um modelo se aproximar dos sintomas clínicos significativos da patologia, mais robusto ele será na geração de bons dados (Miczek e de Wit, 2008).

Dentre os diferentes procedimentos experimentais para o estudo da ansiedade, o da Caixa Claro/Escuro - CCE (figura 1) parece ser adequado para detecção de substâncias ativas no tratamento dos Transtornos de Ansiedade Generalizada – TAG (File, 1990), enquanto o Teste de Esconder Esferas – TEE (figura 2) parece ser mais sensível a substâncias utilizadas para o tratamento do Transtorno Obsessivo Compulsivo - TOC (Witkin, 2008).

O procedimento da CCE tem a vantagem de ser rápido e fácil de usar, sem a necessidade de treinamento prévio dos animais e sem submetê-los a estímulos nocivos, como choques elétricos, privação de água ou

alimentos, que geram estados motivacionais (dor, sede, fome) que podem interferir no comportamento do animal e assim obscurecer as análises de ansiedade (Bourin e Hascoët, 2003).

Um conflito natural ocorre quando os animais são expostos a um ambiente não familiar ou a um novo objeto. O conflito ocorre entre a tendência de explorar e a tendência inicial de evitar o desconhecido – neofobia. A atividade exploratória reflete o resultado combinado destas tendências na nova situação. Assim, na CCE, as drogas ansiolíticas induzem um aumento nos comportamentos executados na parte clara. Um aumento na transição entre os ambientes claro e escuro, sem um aumento na locomoção espontânea, é considerado reflexo da atividade ansiolítica (Bourin e Hascoët, 2003).

Por sua vez, o TEE explora o comportamento de roedores de ocultar objetos e drogas que apresentam atividade ansiolítica diminuem o número de objetos escondidos pelos animais (Treit *et al.*, 1981). O modelo está relacionado ao transtorno obsessivo-compulsivo caracterizado por obsessões e/ou compulsões, tendo em vista que a principal classe de droga ativa no modelo são os inibidores seletivos de recaptação de serotonina (ISRS) e os animais previamente expostos às esferas continuam a atividade de enterrar, mesmo na ausência das esferas, o que caracterizaria o comportamento compulsivo (Gyertyan, 1995).

As substâncias ativas nestes procedimentos experimentais também são distintas. Assim, na CCE, os derivados benzodiazepínicos são capazes de aumentar o tempo de permanência no compartimento claro e no TEE, drogas antidepressivas são capazes de reduzir o número de esferas escondidas. Similarmente, estas são as drogas mais ativas nestas desordens

na clínica, ou seja, os benzodiazepínicos são as drogas mais ativas no TAG e os antidepressivos – em especial os ISRS – as drogas mais ativas no TOC (Davidson *et al.*, 2010; Witkin, 2008).

Já no que diz respeito aos procedimentos relacionados à depressão, dois são os principais modelos utilizados na avaliação de drogas com potencial efeito antidepressivo (Cryan *et al.*, 2002; Pollak *et al.*, 2010): Teste do Nado Forçado (TNF) e Teste da Suspensão pela Cauda (TSC).

Baseado no teste originalmente descrito por Porsolt e colaboradores em 1977, o TNF (figura 3) é composto por duas sessões de natação: uma chamada treino, e uma segunda, realizada 24 horas após, chamada teste. Durante a sessão de teste cada animal permanece na água durante 5 minutos e tem seu comportamento registrado para análise posterior, resultando na classificação do comportamento exercido: natação, escalada ou imobilidade.

O comportamento de natação é caracterizado por movimentos, usualmente horizontais, através do recipiente; o comportamento de escalada é caracterizado por movimentos ascendentes das patas dianteiras, geralmente, ao longo das paredes do recipiente; a imobilidade é definida quando nenhuma atividade adicional é realizada, com os animais executando movimentos mínimos para manter suas cabeças acima da água (Cryan *et al.*, 2002). O comportamento de imobilidade reflete o estado de baixo humor relacionado com a impossibilidade de escape durante a situação experimental (Porsolt *et al.*, 1977). O antidepressivo tricíclico imipramina, um inibidor não seletivo de recaptação de noradrenalina e serotonina interfere, em ratos, de acordo com Cryan e colaboradores (2002), nos comportamentos ativos executados no TNF (escalar e nadar).

O TSC (figura 4) descrito por Steru e colaboradores (1985), baseia-se na observação que camundongos quando suspensos pela cauda, sem possibilidade de fuga, adotam um comportamento característico no qual ocorre um período de alta atividade inicial, seguido de períodos de imobilidade, sendo esta imobilidade relacionada com o estado de desistência, semelhante à síndrome depressiva humana. Fármacos antidepressivos diminuem este tempo de imobilidade e muitas vezes aumentam a latência até a imobilidade ocorrer, constituindo um procedimento adequado de triagem para novas drogas antidepressivas (Steru *et al.*, 1985).

Com o intuito de se descartar possíveis resultados falso-positivo, as avaliações foram complementadas com o teste da Barra Giratória (figura 5). Descrita por Dunham e Miya (1957), esta técnica pode detectar prejuízos neurológicos como ataxia, sedação e hiperexcitabilidade ou alterações motoras caracterizadas pela inabilidade do animal em se manter sobre a barra giratória.

Deste modo, o presente trabalho avaliou o efeito dos óleos essenciais de *C. citratus* e *C. aurantium* sobre modelos experimentais de depressão, além de investigar o mecanismo de ação ansiolítica dos óleos essenciais por meio da identificação de alterações nos sistemas de neurotransmissão, utilizando técnicas comportamentais e neuroquímicas, bem como a caracterização de possíveis ações inespecíficas dos óleos essenciais, que poderiam refletir efeitos tóxicos, por meio de análises bioquímicas, genotóxicas e histopatológicas de órgãos-alvo.

Figura 1 – Caixa Claro/Escuro

Figura 2 – Teste de Esconder Esferas

A – Antes do início do teste

B – Depois do teste

Figura 3 – Teste do Nado Forçado

Adaptado de Cryan *et al.*, 2002

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Figura 4 – Teste da Suspensão pela Cauda

Figura 5 – Barra-giratória

THE GABAERGIC SYSTEM CONTRIBUTES TO THE ANXIOLYTIC-LIKE EFFECT OF ESSENTIAL OIL FROM *Cymbopogon citratus* (LEMONGRASS).

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Artigo submetido ao periódico científico: **Journal of Ethnopharmacology**

Abstract

Ethnopharmacological relevance

The essential oil (EO) from *Cymbopogon citratus* (DC) Stapf is reported to have a wide range of biological activities and is widely used in traditional medicine as an infusion or decoction. However, despite this widely use, there are few controlled studies confirming its biological activity in central nervous system.

Material and Methods

The anxiolytic-like activity of the EO was investigated in light/dark box (LDB) and marble-burying test (MBT) and the antidepressant activity was investigated in forced-swimming test (FST) in mice. Flumazenil, a competitive antagonist of benzodiazepine binding and the selective 5-HT_{1A} receptor antagonist WAY100635 was used in experimental procedures to determine the action mechanism of EO. To exclude any false positive results in experimental procedures, mice were submitted to the rota-rod test. We also quantified some neurotransmitters at specific brain regions after EO oral acute treatment.

Results

The present work found anxiolytic-like activity of the EO at the dose of 10 mg/kg in a LDB. Flumazenil, but not WAY100635, was able to reverse the effect of the EO in the LDB, indicating that the EO activity occurs via the $GABA_A$ receptor-benzodiazepine complex. Only at higher doses did the EO potentiate diethyl-ether-induced sleeping time in mice. In the FST and MBT, EO showed no effect. Finally, the increase in time spent in the light chamber, demonstrated by concomitant treatment with ineffective doses of diazepam (DZP) and the EO, revealed a synergistic effect of the two compounds. The lack of activity after long-term treatment in the LDB test might be related to tolerance induction, even in the DZP-treated group. Furthermore, there were no significant differences between groups after either acute or repeated treatments with the EO in the rota-rod test. Neurochemical evaluation showed no amendments in neurotransmitters levels evaluated in cortex, striatum, pons, and hypothalamus.

Conclusions

The results corroborate the use of *C. citratus* in folk medicine and suggest that the anxiolytic-like effect of its EO is mediated by the $GABA_A$ receptor-benzodiazepine complex.

Keywords: Anxiolytic; *Cymbopogon citratus;* Essential oil; GABAergic system; Lemongrass; Sedative.

1. Introduction

Anxiety and depressive disorders are the most common mental illnesses in the world and a prominent health care problem (Herrera-Ruiz et al., 2006; Yu et al., 2007). The World Health Organization (WHO) estimates that the prevalence of these diseases ranges from 0.1 to 16.9% (Demyttenaere et al., 2004). Benzodiazepines are the major class of compounds used to treat generalized anxiety disorder and acute anxiety states (Rickels and Schweizer, 1997; Rupprecht et al., 2006). However, these compounds have a number of undesirable effects such as sedation, muscle relaxation, amnesia, dependence and tolerance (Rickels and Schweizer, 1997; Reynolds, 2008). Therefore, the search for new therapeutic agents continues, and medicinal plants have emerged as a crucial source for the development of drugs to treat neurological disorders and play an important role for patients who respond poorly to conventional treatments (Carlini, 2003; Herrera-Ruiz et al., 2006).

Cymbopogon citratus (DC) Stapf (Poaceae), commonly known as lemongrass, is widely used in traditional medicine as an infusion or decoction for treating nervous disturbances (Carlini, 1986). In Mexico, *C. citratus* is used as a sedative (Tortoriello and Romero, 1992), and in Brazil, an infusion or the cold juice of the leaves has been employed as a sedative and analgesic (Hiruma-Lima et al., 2002). An antinociceptive effect of *C. citratus* has been detected in the rodent hot plate test, an experimental procedure related to central activity (Viana et al., 2000). In previous work (Blanco et al., 2009; Costa et al., 2006), we have demonstrated that acute treatment with the essential oil (EO) from *C. citratus* is effective against generalized anxiety disorder and epilepsy in experimental procedures in mice.

In the current study, we investigated the effect of the EO after long-term treatment and the putative synergistic effect of the EO and diazepam (DZP), a classic anxiolytic drug. We administered repeated dosages, instead of a single dosage, to mimic human drug regimens and to allow for reductions in individual dosages. To better characterize its central nervous system activity, the EO was also evaluated in different experimental procedures related to depressive and obsessive-compulsive disorders, thus complementing the anxiolytic and sedative/hypnotic protocols. Finally, the mechanism of action of the EO was investigated through blockade of the GABAergic or serotonergic neurotransmission systems. In this manner, we sought to elucidate the pharmacological activity of the EO from *C. citratus* in order to validate its popular use as an anxiolytic or adjuvant drug in combination with classic drugs such as benzodiazepines; such a combination could represent a novel approach for the treatment of anxiety.

2. Material and methods

2.1. Plant material

Plant seedlings of specimens from the garden of medicinal plants (Lageado Farm, UNESP) were cultivated at the Institute of Biosciences. The plant was identified in the BOTU Herbarium, Department of Botany, UNESP, where a voucher specimen (# 23031) had been deposited. Leaves of *C. citratus* were collected between May and June of 2008 and immediately processed to obtain the EO.

2.2. Essential oil extraction and phytochemical analysis

Fresh leaves were processed with a Clevenger apparatus, and the EO was obtained by hydrodistillation, protected against light and heat, and stored until behavioral assays. Previously, the EO was analyzed by gas chromatography coupled with mass spectrometry as described (Blanco et al., 2009).

2.3. Animals

All experiments were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Biosciences Institute – Ethics Committee for Animal Research (CEEA). Adult Swiss male mice (30 days old) from the colony at the UNESP Central Animal House facility were used in all experiments after a one-week adaptation period at the Animal House of the Department of Pharmacology. Thus, the animals used were approximately 40 to 45 days old. The animals were maintained under controlled environmental conditions of temperature (21 ± 2 ^{o}C) and light (12/12 light/dark cycle) with food and water *ad libitum* until 2 h prior to the experimental procedures.

2.4. Drugs

Diazepam (DZP, Germed - EMS, Brazil) was used as the standard anxiolytic and hypnotic drug and imipramine hydrochloride (IM, Sigma-Aldrich, USA) as the standard antidepressant drug. Flumazenil (FLU, Flumazil® - Cristália, Brazil) was utilized as a competitive antagonist of benzodiazepine binding, and WAY100635

(WAY, Sigma-Aldrich) was used as a highly selective 5-HT_{1A} antagonist. For intraperitoneal injections (i.p.), DZP and IM were dissolved in isotonic saline (SAL - 0.9% NaCl). For oral administration (p.o.), the EO was dissolved in 0.01 % (v/v) polyoxyethylene sorbitan monooleate (TW - Tween 80®, Sigma-Aldrich) in saline, which was used to treat control groups. All samples were freshly prepared on the test day and administered at 10 ml/kg of body weight, except FLU, which was administered at 20 ml/kg due to the concentration (0.1 mg/ml) of its commercial form.

2.5. Treatments

In a preliminary experiment, the behavioral data did not differ among the animals that received the three vehicle solutions – Tween (TW), saline and water – (data not shown), which justified our choice to include only the TW control group data for comparison. For the single treatment, mice were treated 30 min before experimental procedures, whereas for long-term treatment (WHO, 1993), animals were treated with the EO for 21 days and exposed to the experimental procedure 30 min after the last treatment. For evaluation of antidepressant activity, mice were treated three times within the 24 h before the experimental procedure. To address a possible contribution from the GABAergic system, mice were co-administered DZP (1 mg/kg, i.p.) or the EO (10 mg/kg, p.o.) 30 min before the test and then were given FLU (2 mg/kg, i.p.) 15 min before testing. To evaluate possible interference from the serotonergic system, animals were pretreated with WAY (0.1 mg/kg, i.p.) and 15 minutes afterwards, received the EO (10 mg/kg) or the standard drug DZP (1 mg/kg); the experimental procedure was carried out 30 min after the last drug treatment. To evaluate a putative synergism between treatments, groups of animals were simultaneously treated with non-effective doses of DZP (0.25 mg/kg, i.p.) and the EO (2.5 mg/kg, p.o.).

2.6. Neurochemical evaluation

Animals treated with TW or EO (10 mg/kg) were euthanatized, and their brains were rapidly dissected (not more than 3 min after extraction) on ice. Briefly, the striatum, hypothalamus, pons, and frontal cortices were dissected, weighed, and stored in liquid nitrogen until the neurochemical analyses.

Following sample collections, tissues were homogenized in 0.1M perchloric acid by manual sonication, and centrifuged at 10,000 rpm for 20 minutes to remove supernatant, and stored at – 80 ºC until the determination of monoamine levels.

Neurotransmitters and its metabolites - dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA) - were measured by reverse-phase using an high performance liquid chromatography (HPLC) system (model 6A, Shimadzu, Kyoto, Japan) with an electrochemical detector as described in detail elsewhere (Felício et al. 1996). Briefly, 20 µl samples were loaded into a sample injector and the mobile phase was delivered at a constant rate of 1.2 ml/min. The runtime for each sample was 15 min, and concentrations of all neurotransmitters and its metabolites were expressed as nanograms per gram of tissue (ng/g).

2.6. Behavioral procedures

Mouse behavior in the forced-swimming test and light/dark box test were videotaped under white light illumination using a video camera and a computer. Digital video was subsequently played, and behaviors were scored by a highly trained observer who was blind to treatment condition. In other procedures, the behaviors were registered in real time. All experimental procedures occurred between 9:00am and 5:00pm.

2.6.1. Marble-Burying Test (MBT)

The marble-burying procedure is based on a defensive burying behavior that can be elicited in rodents in response to aversive stimuli such as a shock prod, noxious food or an effective unconditioned stimulus such as glass marbles (Broekkamp et al., 1986; Poling et al., 1981). The mice used in the marble-burying procedure were previously selected to represent those that exhibit a stable burying score (at least 13 out of 25 marbles) for three consecutive days before the test (Pultrini et al., 2006). Thirty minutes after treatments with EO (5, 10 or 50 mg/kg), the animals were placed individually in cages (27 long x 16 wide x 13 cm high) with 25 glass marbles uniformly distributed on a 5-cm layer of sawdust. Each cage was covered with a smooth, acrylic, transparent plate with small punctures for ventilation. The mice were left in the cages with the marbles for 30 minutes, after which they were removed to count the number of marbles that were totally hidden by sawdust.

2.6.2. Forced-Swimming Test (FST)

This test is the most widely used and recognized pharmacological model for assessing antidepressant activity (Petit-Demouliere et al., 2005). This procedure was carried out following a modified version (Cryan et al., 2002) of the conditions proposed by Porsolt et al. (1977, 1978). The apparatus consisted of a clear Plexiglas cylinder (21 cm high x 20.5 cm diameter) filled with water (23 $\pm$ 2 ºC) to a depth of 13 cm. Each animal underwent a pre-test swimming session for 15 min; the pre-tests were carried out 24 h prior to the 5-min swimming tests. At the end of the swimming sessions, the animals were removed from the water and gently dried. Mice were treated with EO at 5, 10 or 50 mg/kg according to the following schedule: the first treatment (T1) was administered 1 h after finishing the pre-test session and subsequently 5 h (T2) and 30 min (T3) before the test session. The digital video recording of each mouse in the water was evaluated using a time-sampling technique (Detke et al. 1995) to classify in 10-s bins the behavioral response into one of these three possible categories: climbing behavior, defined as upward-directed movements of the forepaws along the side of the swimming chamber; swimming behavior, defined as movements throughout the swimming chamber; and immobility time, defined as time during which the mouse remained floating in the water and made only the movements necessary to maintain its head above the water (Cryan et al., 2002).

2.6.3. Sleep induced by diethyl-ether (SID)

The effectiveness of the EO was measured by its capacity to reduce the latency to sleep and/or to increase the duration of sleep without interfering with hepatic metabolism (Tsuji et al., 1996). Thirty minutes after the treatments with EO (500, 1000 or 1500 mg/kg), animals were induced to sleep by inhalation of diethyl-ether in a 3-L flask. Each mouse was placed individually in a cylindrical glass chamber saturated for 20 min with vapor delivered from 5 ml of diethyl-ether absorbed on a piece of cotton bound in the coiled cap of the flask. Each mouse was left there for 60 sec after loss of its postural reflex – sleep latency was defined as time until loss of the postural reflex – and then was withdrawn and placed in an individual cage to record the time to recover the postural reflex (sleep duration) (Héllion-Ibarrola et al., 2006).

2.6.4. Light/Dark Box Test (LDB)

The light/dark box (46 long x 27 wide x 30 cm high) was divided into the typical dimensions of one-third for the dark compartment with black walls, floor and roof and two-thirds for the light compartment with white walls and floor and illuminated by a 20-W fluorescent lamp. The light and dark compartments were connected by an opening (7.5 x 7.5 cm) located at floor-level in the center of the partition (Crawley and Goodwin, 1980). Thirty minutes after treatment with EO (5, 10, 50 or 100 mg/kg), each animal was individually placed in the center of the light compartment, facing the dark one, and observed for 5 minutes after the first entry into the dark compartment. During this period, we recorded the number of shuttle crossings (an activity-exploration index), number of rearings and the time spent in the light compartment, which is the most consistent and useful measure for assessing anxiolytic-like activity. This parameter provides consistent dose-effect results for anxiolytic drugs including benzodiazepines, 5-HT_{1A} receptor agonists or 5-HT_3 receptor antagonists (Bourin and Hascoët, 2003; Young and Johnson, 1991).

2.6.5. Rota-rod Test (RRT)

The rota-rod procedure was described by Duhan and Miya (1957) and is suitable for detecting motor impairment due to pharmacological agents such as skeletal muscle relaxants or central nervous system depressants. The apparatus consisted of a non-slippery plastic rod, 3.0 cm in diameter and 50 cm in length, rotating at 5 rpm at a height of 25 cm. Immediately after the LDB, each mouse was placed on the apparatus to register the total time it performed on the rotating bar in three successive trials totaling 1 min. To avoid a bias on account of inability not related to drug treatment, one day prior to testing animals were evaluated, using the same conditions as those in the test, to select those that had the ability to walk on the bar.

2.7. Statistical analysis

Quantitative data from behavioral tests were subjected to Kruskal-Wallis non-parametric variance analysis, followed by the Mann-Whitney test when appropriate. Proportions in the RRT were compared by Fisher's exact test. Neurochemical data were analysed by unpaired t-test. All statistical analysis was made using the GraphPad InStat®

version 3.02. Contrasts were made between treated and TW (vehicle) group, and differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Essential oil composition

The main EO compounds were identified by the retention time and retention index as monoterpene citral (71.29%), a mixture of the stereoisomers geranial and neral, or beta-myrcene (16.5%). Other compounds detected in the EO include geraniol (1.28%), 6-methyl-5-hepten-2-ona (0.64%), and undecan-2-one (0.29%), as detailed in Bidinotto et al. (2010).

3.2. Neurochemical evaluation

The acute treatment with lemongrass EO did not result in neurotransmitters or their metabolites producing changes (DA, DOPAC, HVA, 5HT, and 5HIAA) as evaluated in cortex, striatum, pons, and hypothalamus (Table 1).

3.3. Effect of the EO on the Forced-swimming test (FST) and Marble-burying test (MBT)

The EO administered at 5, 10 or 50 mg/kg was unable to modify parameters evaluated in the FST or MBT, as shown in Table 1. Imipramine, the standard drug in both experimental procedures, significantly decreased the number of immobility events and concomitantly augmented the number of climbing and swimming events during the FST while also reducing the number of marbles buried in the MBT.

3.4. Effect of the EO on diethyl ether-induced sleep

The EO significantly decreased sleep latency after treatment at 1000 or 1500 mg/kg and increased sleep duration at the same doses, as did the standard drug DZP (5 mg/kg). Data are presented in Table 2.

3.5. Anxiolytic-like effect of the EO in the LDB

The results obtained in the LDB test after acute treatment with the EO (Fig. 1, left panel) reveal a significant increase in time spent in the LDB light chamber after acute administration of the EO at 10 mg/kg when compared to the TW group. A discrete increase in exploratory parameters (number of transitions between compartments and, especially, rearing) could be observed after treatment with either DZP or the EO. However, neither DZP nor the EO, administered for 21 days, was able to modify the time spent on the illuminated side of the LDB. Additionally, the group that was repeatedly treated with saline and that received one treatment with DZP 30 minutes before the experimental procedure showed an anxiolytic-like effect analogous to acute DZP treatment (Fig. 1, right panel). This result validates the experimental conditions used in the LDB session and confirms the absence of an anxiolytic-like effect in mice repeatedly treated with DZP. Reductions in the number of rearings and transitions between compartments were observed after long-term treatment with 100 mg/kg of the EO, which was the same trend observed after long-term treatment with DZP (Fig. 1 B and C).

3.6. The role of GABA or 5-HT neurotransmission on the anxiolytic-like effect of the EO in the LDB

Experimental sessions to evaluate the interference of flumazenil (FLU) or WAY100635 (WAY) in the acute effect of the EO (10 mg/kg) or DZP (1 mg/kg) were carried out independently; the results are presented in Fig. 3. Results from FLU or WAY alone did not differ from those obtained from the TW group, showing no *per se* effect of these blockers on evaluated parameters. Mice treated with DZP or the EO spent more time in the light chamber, when compared with the TW group – an effect not observed in mice treated concomitantly with FLU, a benzodiazepine antagonist (Fig. 2A, left panel). However, concomitant treatment with WAY not only was unable to block the effect of DZP, as expected, but also could not block the effect of the EO (Fig. 2A, right panel). Neither FLU nor WAY could reverse the effect of DZP or the EO on the exploratory or motivational parameters such as the numbers of rearings and transitions between chambers (Fig. 2 B and C).

3.7. Effect of combined administration of DZP and the EO in the LDB

The upper left corner of Fig. 3 displays the observed effect of DZP treatment at 1.0 mg/kg, the effective dose in the LDB. Thus, mice treated with 0.25 mg/kg of DZP or with 2.5 mg/kg of the EO did not spend more time

than the TW group on the illuminated side of the apparatus, the main anxiety parameter in the LDB. The EO at 5.0 mg/kg showed a significant but discrete augmentation in this parameter. Furthermore, when mice were treated with a combination of DZP and the EO, at these non-effective doses, the anxiolytic-like effect was evident, showing the mutual potentiation of action between the EO and DZP (Fig. 3).

3.8. Effect of the EO and DZP on the RRT

The rota-rod test was carried out to measure locomotor impairment in mice treated acutely or repeatedly with DZP or the EO. The results, presented in Table 3, include the number of disabled mice in relation to the total number of treated animals in each experimental group. The disabled mice, defined as those that could not stay on the rotating bar in a maximum of three attempts at 5 rpm for 1 min, did not differ statistically from the TW group, suggesting an absence of treatment-induced motor impairment.

4. Discussion

The already considerable attention directed at the use of natural essential oils from plants is continuing to increase. The widespread uses of these medicinal oils for bactericidal, antiparasitic, insecticidal and medicinal applications are examples of their pharmaceutical use (Bakkali et al., 2008; Fandohan et al., 2008).

Despite the frequent use of *C. citratus* (lemongrass) in traditional medicine, there are few controlled studies confirming its biological activity. According to a review of herbal remedies for anxiety (Ernst, 2006), there has been only one placebo-controlled study on Brazilian lemongrass tea, conducted by Leite et al. (1986). Recently, our laboratory demonstrated the anxiolytic-like activity of the EO from *C. citratus* in experimental models of generalized anxiety disorder and epilepsy (Costa et al., 2006; Blanco et al., 2009) at 500 and 1000 mg/kg.

The observation in mice of antinociceptive activity of the EO from *C. citratus* at the dose of 25 mg/kg (Viana et al., 2000) and an anticonvulsant effect of the EO from *C. winterianus* (Quintans-Júnior et al., 2008) at doses below 400 mg/kg encouraged us to evaluate the anxiolytic activity of lower doses of the EO from *C. citratus*. Thus, we decided to study essential oil effects in a dose range lower than previously tested (Blanco et al., 2009). This approach showed positive results in anxiety-related parameters in the LDB, in which EO was active at 10 mg/kg.

The anxiolytic effect of benzodiazepines is due to their binding to $GABA_A$ receptor at a site distinct from the GABA binding site (Whiting, 2003). To determine whether the anxiolytic-like effect of the EO in the LDB

occurs through the GABAergic system, mice treated with the EO or DZP were co-administered FLU, a benzodiazepine $GABA_A$ site antagonist. This procedure reversed the anxiolytic-like activity of not only the standard drug DZP but also the EO (Fig. 2). The ability of FLU, a specific $GABA_A$ receptor antagonist, to antagonize the anxiolytic-like activity of the EO strongly suggests, for the first time, a role of this receptor complex in mediating EO activity. Furthermore, according to Silva et al. (2010) the EO from *C. citratus* administered i.p. had anticonvulsant effect, by intraperitoneal route, which was blocked by the pretreatment with flumazenil, suggesting and enhancing a possible effect on the GABAergic neurotransmission system. The authors did not exclude a possible interference of other systems (e.g., glycinergic) in such activity.

The neurochemical assessment revealed no alterations in neurotransmission mediated by dopamine and serotonin after acute oral treatment with EO. It has been suggested that serotonin could be a central neurotransmitter involved in the modulation of anxiety and anti-anxiety effects of benzodiazepines, however neurochemical studies on the effects of that class of drugs on brain serotonin metabolism/or turnover are not very consistent (Batool and Haleem, 1997). On the other hand, the diazepam seems to alter levels of dopamine in the nigrostriatum of rats only at doses greater than those with anxiolytic and anticonvulsant effects (Invernizzi et al., 1991). Further, the role of mesolimbic DA projections in the effects of benzodiazepine-site ligands is still unclear (Heikkinen et al., 2009).

Even over a large range of doses, the EO produced no effect in the experimental models of obsessive-compulsive disorder (MBT) and depression (FST). These experimental models are not sensitive to treatment with benzodiazepines but are sensitive to drugs like imipramine, which is clinically useful for treating obsessive-compulsive disorder and depression. Therefore, it was not surprising that the EO activity was not disrupted by previous exposure to the selective 5-HT_{1A} antagonist WAY100635. In our experience, WAY at 0.1 mg/kg is able to block the activity of imipramine (30 mg/kg) in the FST (data not shown). In this manner, the WAY and EO results corroborate the hypothesis that the anxiolytic-like activity of the EO is mediated by the GABAergic system.

When doses of DZP (0.25 mg/kg) and the EO (2.5 mg/kg) that were ineffective *per se* were co-administered, they had a synergistic effect on each other. In other words, mice treated with DZP and the EO at their respective ineffective doses showed a significant increase in time spent in the LDB light chamber. In addition, mice treated with effective doses of the EO (5.0 mg/kg) and DZP showed a significantly elevated light-chamber duration, when compared to the EO 5.0 mg/kg alone or with DZP (Fig. 3).

After a single treatment with the EO, mice showed a significant increase in LDB parameters, in contrast to long-term treatment, either with the EO or DZP, which produced no such increases (Fig. 1). In this procedure, only the animals repeatedly treated with saline that received DZP 30 minutes before the experimental procedure showed

significant differences on the LDB anxiety parameters. Long-term treatment with the EO at 100 mg/kg produced a behavioral profile similar to that observed with DZP in the same conditions, with statistical significance in reducing the number of rearings and transitions between black and white compartments, without observable effects on motor coordination in the rota-rod test (Table 3). The reduction in efficacy after long-term treatment with DZP, and perhaps with the EO, could be due to the development of tolerance (Reynolds, 2008), as already described in mice after two weeks of continuous DZP use (Flaishon et al., 2003) and which is an EO hypothesis also assessed in other species (Pultrini et al., 2006).

The EO was effective over a wide dose range. Its sedative effects reflected in the sleep induction studies were achieved at doses that are 100-fold higher than those found effective in the LDB. In the experimental procedure involving ether-induced sleeping time, only at 1500 mg/kg did the EO decrease the latency to sleep and increase the sleeping time, which proved to be a specific central nervous system effect. According to Tsuji et al. (1996), this effect is not related to a pharmacokinetic interaction with hepatic enzymes, a recognized limitation of barbiturate-induced sleeping time. The decrease in spontaneous motor activity (after long-term treatment) and potentiation of ether-induced sleeping time (after acute treatment at higher doses) strongly suggest central depressant activity of the EO.

To exclude any false positive results in experimental procedures related to anxiety disorders, mice underwent the rota-rod test. All animals treated with different EO doses after acute or long-term treatment showed no impairment of the locomotor system (Table 3), thus confirming that the EO from *C. citratus* is anxiolytic in rodents without any motor impairment.

The absence of toxicity signals or symptoms in mice, whether treated acutely or repeatedly, at the doses used in the present study shows the relative safety of the EO. The absence of a deleterious effect on the morphological structure of the stomach or liver in rats treated sub-acutely with doses up to 1500 mg/kg has been described previously (Fandohan et al., 2008). However, the major compound in the EO from *C. citratus* induced maternal toxicity in pregnant rats at doses higher than 125 mg/kg (Nogueira et al., 1995). This observation confirms that acute toxicity data limit the pre-clinical applications of the EO because a cumulative toxic effect does occur even at very low doses (Mabeku et al., 2007), reinforcing the importance of complementary toxicological studies to evaluate the safety of the EO as a therapeutic alternative in folk medicine.

Finally, essential oils are complex mixtures of numerous molecules. The major compounds of the EO from *C. citratus* were identified by GC/MS as follows: citral (a mixture of the cis and trans isomers – neral and geranial; followed by myrcene and geraniol. According to Vale et al. (2002), citral and myrcene were not able to modify

parameters of anxiolytic activity in mice, as evaluated by the elevated plus maze. These data reinforce the idea that the major compounds are not always responsible for biological activity and that such activity cannot always be attributed to only one constituent from the herbal preparation, according to the concept of a multitargeted approach as a therapeutic strategy (Wagner and Ulrich-Merzenich, 2009). Moreover, for biological purposes, it is more informative to study whole oil rather than some of its components because the concept of synergism appears to be more meaningful (Bakkali et al., 2008).

Acknowledgements

We are grateful to MSc. Filipe Galvão Ferreira, MSc. Flávia Bonamin and Dr. Leonardo Noboru Seito for technical assistance. This work was part of the Ph.D. dissertation and was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Celso A R A Costa – Proc. nº. 2006/07195-8) and CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (André C Gargano).

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Conflict of interest

The authors declare that they have no conflicts of interest.

Role of the funding source

This work was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Celso A R A Costa – Proc. nº. 2006/07195-8) and CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (André C Gargano). The FAPESP and CAPES had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Figure captions

Fig. 1. Median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated chamber (A), number of transitions (B) and number of rearings (C) in the light/dark box after acute or long-term treatment with essential oil (EO) from *C. citratus* (p.o.); TW - vehicle, 10 ml/kg (p.o.); SAL– saline, 0.9% (p.o.); DPZ - diazepam, 1 mg/kg (i.p.). n = 6-8 animals per group. * $p \leq 0.05$, ** $p \leq 0.01$ in relation to TW group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Fig. 2. Influence of flumazenil (FLU, 2.0 mg/kg, i.p.) or WAY100635 (WAY 0.1, mg/kg, i.p.) on the anxiolytic effect of diazepam (DZP, 1 mg/kg, i.p.) or essential oil (EO, 10 mg/kg, p.o.), expressed as the median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated chamber (A), number of transitions (B) and number of rearings (C) in the light/dark box; TW - vehicle, 10 ml/kg (p.o.). n = 6-9 animals per group. * $p \leq 0.05$, ** $p \leq 0.01$ in relation to TW group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Fig. 3. Effect of co-administration of essential oil (EO – 2.5 or 5.0 mg/kg, p.o.) 30 min after an ineffective dose of diazepam (DZP – 0.25 mg/kg, i.p.) treatment, expressed as the median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated chamber of the light/dark box. TW - vehicle, 10 ml/kg (p.o.). The small figure embedded, upper left corner, represents the observed effect after DZP treatment at 1 mg/kg under the same experimental conditions. n = 6-9 animals per group. ** $p \leq 0.01$ in relation to TW group; # $p \leq 0.05$ in relation to EO 5.0 mg/kg group; Δ $p \leq 0.05$ in relation to EO 2.5 mg/kg group; + $p \leq 0.05$ in relation to DZP 0.25 mg/kg group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Graphical abstract

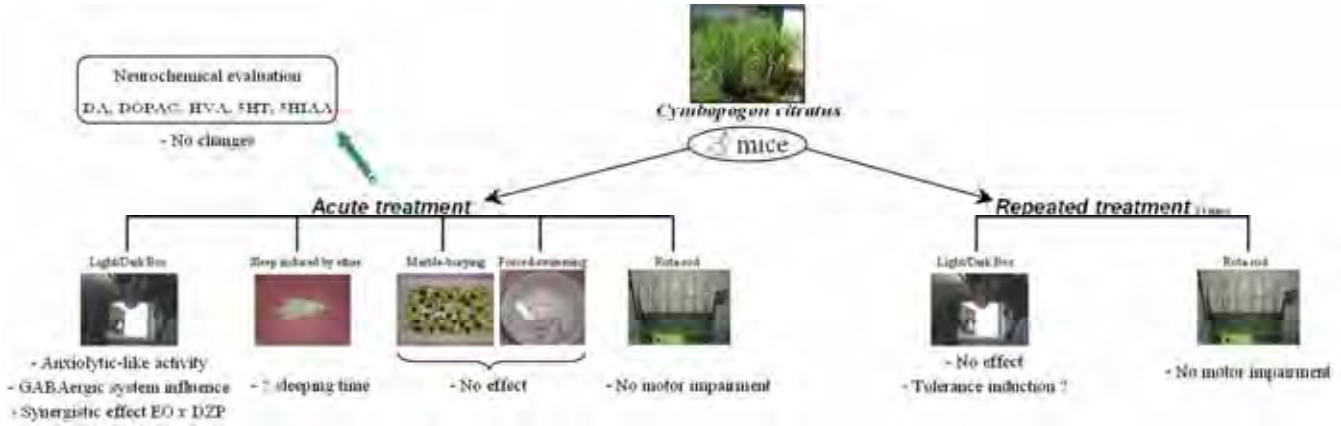
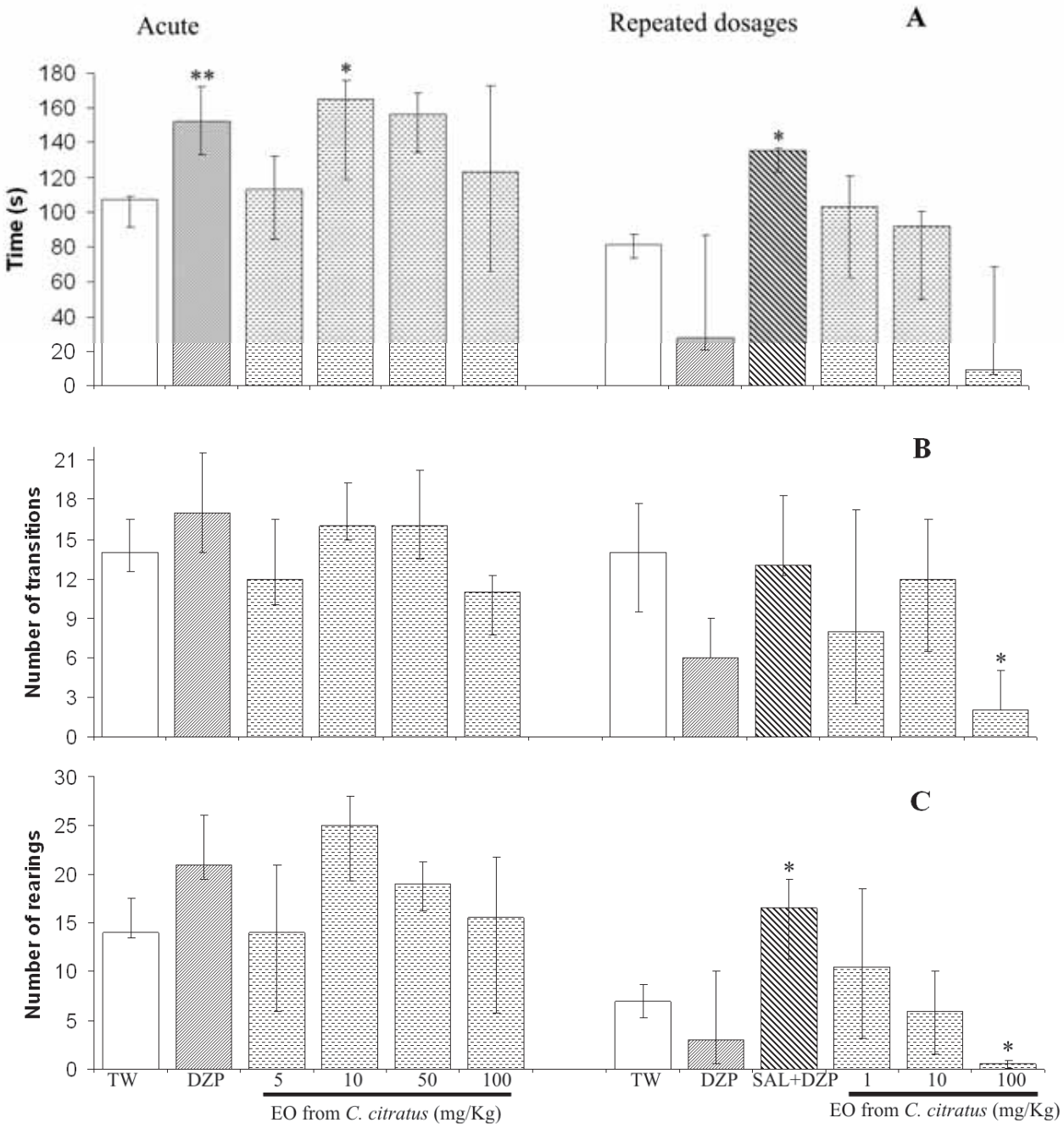


Figure 01


Acute
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12
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6
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C
Number of rearings
30
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*
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TW
DZP
5
10
50
100
EO from C. citratus (mg/Kg)
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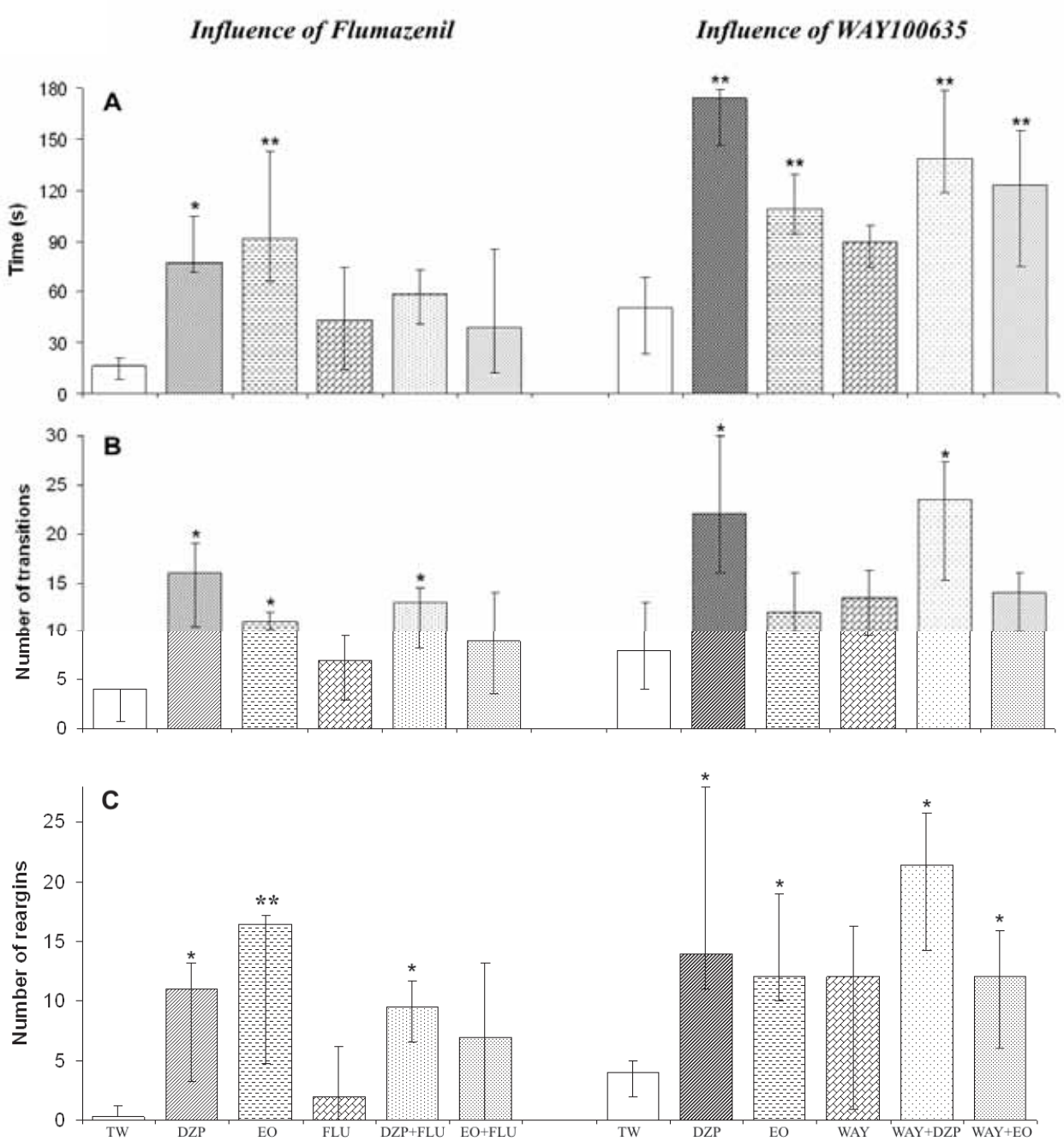
SAL+DZP
1
10
100
EO from C. citratus (mg/Kg)

Figure 02


Influence of Flumazenil
Influence of WAY100635
A
B
C
Time (s)
Number of transitions
Number of reargins
TW
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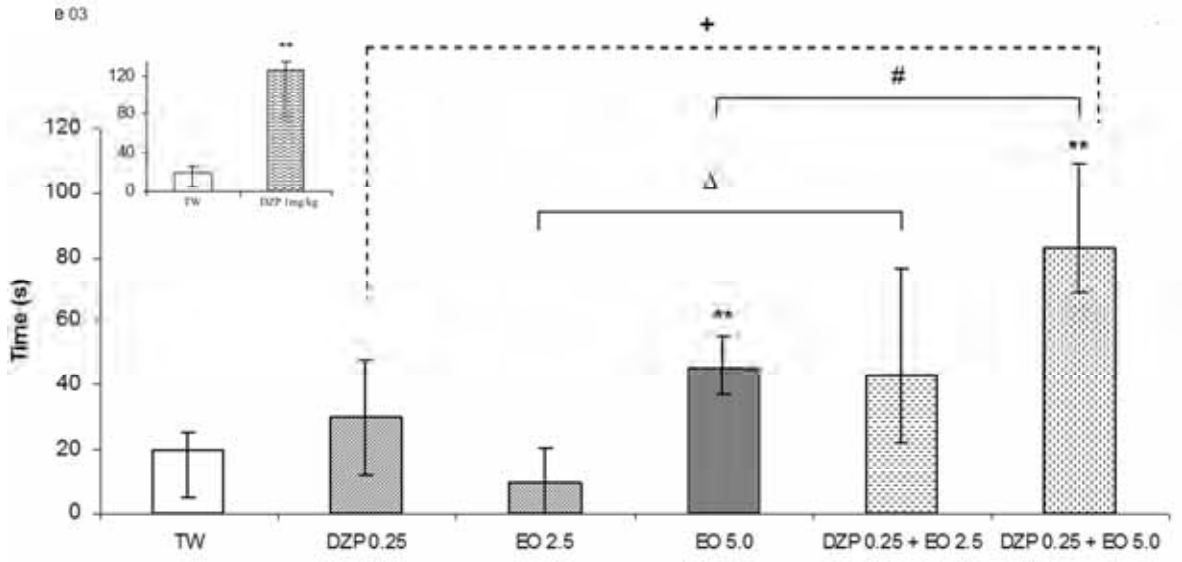
EO+FLU
TW
DZP
EO
WAY
WAY+DZP
WAY+EO

Figure 03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DZP 0.25 + EO 5.0

Table 1. Neurotransmitter levels and their metabolites (ng/g) after acute oral treatment with *Cymbopogon citratus* EO (10 mg/kg).

	Structures and treatments							
	Cortex		Striatum		Pons		Hypothalamus	
	TW (n=12)	EO (n=8)	TW (n=11)	EO (n=7)	TW (n=11)	EO (n=8)	TW (n=12)	EO (n=8)
DA	722.35±744.32	965.83±1188.11	8324.29±1715.02	8712.33±3507.29	26.62±17.60	36.00±23.71	53.08±12.14	62.72±14.97
DOPAC	94.63±55.36	77.27±57.60	437.36±147.60	435.87±213.41	22.44±8.73	24.33±13.99	58.64±19.48	53.96±31.37
HVA	427.32±278.46	332.11±218.76	1121.94±320.96	892.25±324.48	300.94±281.20	305.98±437.55	236.17±127.67	390.57±348.13
5HT	1246.99±297.33	1246.72±257.36	1686.34±339.54	1739.64±495.20	2601.34±793.69	2549.14±1005.78	1660.08±437.24	1487.79±290.16
5HIAA	192.72±60.71	153.07±33.83	317.12±102.38	306.52±156.38	869.54±226.91	745.84±271.76	341.73±72.53	283.28±47.89

TW (tween). Data are presented as mean±SD. Unpaired t-test.

N is different in each group as some structures could not be evaluated due to technical reasons.

Table 2. Effects of essential oil (EO) from *Cymbopogon citratus* in the Forced-Swimming Test, Marble-Burying Test and Sleep induced by diethyl-ether inhalation

Forced-Swimming Test					**Marble-burying Test**			**Sleep induced by diethyl-ether inhalation**			
Treatment	n	Immobility	Climbing	Swimming	Treatment	n	Marbles buried	Treatment	n	Latency (s)	Sleeping time (s)
TW	7	25 (24-27)	2 (1-2)	4 (3-4)	TW	6	23 (19-24)	TW	7	47 (44-54)	102 (97-107)
IM	8	18 (16-19)***	6 (3-7)*	8 (7-11)**	IM	6	9 (6-12) *	DZP	7	34 (29-49)	177 (169-207)**
EO 5	8	26 (23-28)	2 (1-4)	2 (1-4)	EO 5	6	24 (23-24)	EO 500	8	38 (34-43)	115 (106-156)
EO 10	8	25 (24-27)	2 (1-2)	3 (1-5)	EO 10	6	20 (15-21)	EO 1000	7	35 (33-35) **	131 (116-156)*
EO 50	8	26 (25-27)	2 (1-3)	3 (2-3)	EO 50	6	21 (14-23)	EO 1500	8	29 (25-33) **	146 (123-191)**

TW (tween); IM (imipramine hydrochloride); DZP (diazepam)

Data are expressed as median and interquartile range (Q1-Q3).

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ in relation to TW group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Table 3. Effects of treatments of essential oil (EO) on motor coordination evaluated by the Rota-rod Test

	Motor Impairment	
Treatment	Acute	Repeated dosages
TW	1/6	1/6
DZP 1 mg/kg	0/5	0/6
EO 1 mg/kg	Not evaluated	0/6
EO 10 mg/kg	0/5	0/7
EO 100 mg/kg	Not evaluated	1/7

TW (tween); DZP (diazepam).

Results are expressed as the fraction of total animals in each experimental group that were unable to perform the test. Fisher´s exact test.

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Dear Mr. Costa,

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Safety evaluation of lemongrass (*Cymbopogon citratus* (DC) Stapf) essential oil in male Swiss mice.

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Artigo submetido ao periódico científico: **Food and Chemical Toxicology**

Abstract

Cymbopogon citratus (lemongrass) is currently used in traditional folk medicine. Although this species presents widespread use, there are no scientific data on its efficacy or safety after repeated treatments. Thus, this work investigated the toxicity and genotoxicity of the lemongrass essential oil (EO) in male Swiss mice. The single LD_{50} based on a 24h acute oral toxicity study was found to be around 3500 mg/kg. In a repeated-dose 21-day oral toxicity study, mice were randomly assigned to two control groups, saline- or Tween 80 0.01%-treated groups, or one of the three experimental groups receiving lemongrass EO (1, 10 or 100 mg/kg). No significant changes in gross pathology, body weight, absolute or relative organ weights, histology (brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder), urinalysis or clinical biochemistry were observed in EO-treated mice when compared to the control groups. Additionally, a reduction of blood cholesterol was observed after EO-treatment at the highest dose tested. Similarly, data from the comet assay in peripheral blood cells showed no genotoxic effect of the EO. In conclusion, our findings verified the safety of lemongrass intake at the doses used in folk medicine and indicated the beneficial effect of reducing the blood cholesterol level.

Keywords: Biochemistry; *Cymbopogon citratus*; lemongrass; essential oil; genotoxicity; acute toxicity.

Introduction

The use of medicinal plants in bactericidal, antiparasitical, insecticidal, medicinal and cosmetic applications has increased extensively, especially by pharmaceutical companies, since most of the essential oils obtained from such plants are known to be non-toxic (Bakkali et al., 2008; Fandohan et al., 2008). Essential oils are very complex natural mixtures formed by aromatic plants as secondary metabolites. Usually, these oils are devoid of potential genotoxic activity and some of them present antimutagenic action that may be related to anticarcinogenic activity (Bakkali et al., 2008).

Cymbopogon citratus (DC) Stapf (Poaceae), commonly known as lemongrass, is a widely cultivated aromatic plant used as infusion or decoct in traditional medicine. In Brazil, this plant is mostly used for treating nervous excitement and gastrointestinal disturbances (Blanco et al., 2009; Carlini, 1986; Negrelle and Gomes, 2007). Moreover, lemongrass has been used worldwide in the treatment of different diseases. The Cuban population has employed the specie as an antihypertensive and anti-inflammatory drug (Carbajal et al., 1989). In eastern Nigeria, this plant has been utilized for treating diabetes, obesity and coronary disease (Adeneye and Agbaje, 2007). Additionally, various studies have shown antimutagenic/anticarcinogenic and antioxidant properties of lemongrass extracts or their majority compounds (citral, β-myrcene and geraniol) in different *in vitro* and *in vivo* systems (Cheel et al., 2005; Choi et al., 2000; Mitić-Ćulafić et al., 2009; Melo et al., 2001; Pereira et al., 2008; Rabbani et al., 2006; Suaeyun, 1997; Tapia et al., 2007).

Recently, we demonstrated that lemongrass essential oil protected DNA against chemically-induced damage and also exhibited anticarcinogenic activity against chemically induced mammary carcinogenesis in female Balb/C mice (Bidinotto et al., 2010). Furthermore, our research group also demonstrated an anxiolytic-like effect mediated by the GABAergic system at low doses of *C. citratus* essential oil (Costa et al., 2009). In fact, it has been speculated that secondary metabolites of herbal medicines may serve as alternatives to synthetic chemical products (Bakkali et al., 2008). Despite the widespread folk use of lemongrass, there are few controlled toxicological studies confirming its efficacy and safety in a long-term treatment, since sub-acute toxicity data are required to verify these two objectives (WHO, 2000). Therefore, the aim of the current study was to investigate the toxicological profile of *C. citratus* essential oil after acute or repeated oral treatment in male Swiss mice, focusing on biochemical and histopathological endpoints, and its genotoxicity by means of the comet assay.

Material and Methods

Plant material, essential oil (EO) extraction and phytochemical analysis

Plant seedlings of specimens, from the garden of medicinal plants (Lageado Farm, UNESP), were cultivated at the Botucatu Biosciences Institute. The plant was identified in the BOTU Herbarium, Department of Botany, UNESP, where a voucher specimen (# 23031) was deposited. Leaves of *C. citratus* were collected between May and June 2008, and immediately processed to obtain the essential oil. Fresh leaves were submitted to a Clevenger apparatus and the EO was obtained by hydrodistillation (yield: 0.46% v/w), protected against light and heat, and stored at 4^{o}C up to the moment of use. The profile of EO compounds was analyzed by gas chromatography coupled with mass spectrometry as previously described (Blanco et al., 2009). EO was dissolved in Tween (polyoxyethylenesorbitan monooleate - Tween 80® - 0.01% v/v in saline, Sigma-Aldrich, USA). All samples were freshly prepared at the moment of use.

Animals

All experiments were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee for Animal Research of the Botucatu Biosciences Institute. Adult male Swiss mice (30 days old) from the colony of the UNESP Central Animal House were used in all experiments after a one week-adaptation period at the Animal House of the Department of Pharmacology. The animals were maintained under controlled environmental conditions of temperature (21$\pm$2 ^{o}C) and light (12/12 light/dark cycle) with food and water *ad libitum* until 2 h before experimental procedures.

Toxicity evaluation of acute and repeated dosages

In our first approach to evaluating signs of acute toxicity, single doses of EO (5 - 4000 mg/kg b.w.) were given to groups of mice (7 to 8 animals per group) by gavage (p.o.). Clinical signals of toxicity, motor activity and posture were checked during a 24-hour period. The deaths that had occurred were registered and these data were used to determine the LD50, using the probit test (Litchfield and Wilcoxon, 1949).

Additionally, in a repeated dose 21-day acute toxicity study, mice were treated with EO (1, 10 or 100 mg/kg b.w., p.o.), diazepam (1 mg/kg, i.p.), saline 0.9% or Tween 0.01% (5 to 7 animals per group) for 21 days, between 01:00 p.m. and 03:00 p.m. At the 21st day, the animals were euthanized by decapitation and blood samples were collected for serum biochemical analyses. The brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder were removed, rinsed in saline 0.9%, and weighed. Animal body weight was measured every 5 days. Relative body weight was calculated as: [absolute body weight after a time interval divided by initial body weight of each mouse] x 100. The relative organ weights were calculated based on organ-to-body weight ratio (Michael et al., 2007).

Tissue fragments of brain, heart, kidneys, liver, lungs, stomach, spleen, and urinary bladder were fixed in 10% buffered formalin, dehydrated with successive concentrations of ethanol (70% to 100%), cleared in xylene, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological analysis. The urinary bladder was inflated with fixative and processed. Degenerative or proliferative lesions associated with drug toxicity were evaluated using criteria published by the Society of Toxicologic Pathology (SSNDC, 2006)

Serum and biochemical analyses

Peripheral blood samples were collected in tubes without anticoagulant, kept at room temperature for 30 minutes and centrifuged at 4000rpm for 20 min. Serum samples were aspirated off and stored at -80 ºC until analysis in a Cobas Mira Plus® Chemistry Analyzer (Roche Diagnostic Systems, USA). The following clinical biochemistry parameters were measured: aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase (GGT), urea, albumin, creatinine, total protein, cholesterol and triglycerides.

Comet assay

In order to test the genotoxicity produced by long-term EO administration, animals were treated with EO at dose of 100 mg/kg. Negative control animals received only saline solution or TW (vehicle control). Additionally, to evaluate the antigenotoxic potential of EO at low doses, animals were treated with 1 or 10 mg/kg of EO for 21 days; prior to blood collection the genotoxic agent MNU (30 mg/kg, i.p.) was administered. Blood samples were collected 4 hours after MNU treatment for the comet assay. Comet assay was performed under alkaline conditions according to a previously described protocol (Tice et al., 2000). Briefly, 5 µl of blood was mixed with 120 µl of 0.5% low-melting-point agarose at 37^{0}C and layered onto

conventional microscope slides, precoated with 1.5% normal-melting-point agarose (Invitrogen). The slides were left overnight in a cold freshly-prepared lysing solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na_2EDTA, 10 mM Tris with 10% dimethylsufoxide, pH 10.0) and then inserted into a horizontal electrophoresis apparatus containing alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) at 4 ^{0}C for 20 min. Using the same buffer, electrophoresis was performed at 25 V and 300 mA for 20 min. After electrophoresis, the slides were washed twice in neutralizing buffer (0.4 M Tris–HCl, pH 7.5), fixed in absolute alcohol, air-dried, and stored at room temperature. The slides were stained with 50 μl of ethidium bromide (20 μg/ml) and immediately examined at 400 x magnification in an epi-illumination fluorescence microscope connected to an image analysis system (Comet II; Perspective Instruments, Suffolk, UK). Coded slides were scored blindly and 50 nucleoids were randomly analyzed for each animal (25 cells per slide from two slides per animal). Tail intensity (the quantity of DNA in the tail of the comet) was used to measure the extent of DNA damage. Comet images with a ‘‘cloudy” appearance or with a very small head and tail like a balloon were excluded from the analysis (Hartmann and Speit, 1997).

Statistical analysis

Body weight evolution was analyzed by a repeated-measures analysis of variance with treatment as the main factor and days of treatment as the within-subject variable; contrasts were made by the Fisher LSD test. Data on relative organ weights after 21-day treatment as well as the biochemical and comet assays were analyzed by the one-way ANOVA followed by the Dunnett's Multiple Comparision test, when applicable. For all tests, the level of significance was set at $p \leq 0.05$.

Results

EO composition

The chromatogram with the composition of *C. citratus* EO, obtained by gas chromatography coupled with mass spectrometry, previously described (Bidinotto et al., 2010) indicated monoterpene citral, a mixture of the stereoisomers geranial (51.46%) and neral (19.83%), beta-myrcene (16.5%) and geraniol (1.28%) as the main compounds present in the EO.

Acute toxicity study

Male mice exposed to a single dose (5 to 1500 mg/kg b.w.) of *C. citratus* EO presented no alterations in general behavior when compared to the controls. There was only one death in the group treated with lemongrass EO at 2000 mg/kg dose. The animals that received doses higher than 3000 mg/kg b.w. showed abnormalities such as torpor and cyanosis, and animals exposed to 4000 mg/kg b.w. died within 24h after the oil administration. Therefore, the lethal dose, calculated by the probit test, was around 3500 mg/kg.

21-day acute toxicity

Relative body and organ weights

Figure 1 shows the evolution of the body weight in relation to day 0 prior treatment. Animals treated with *C. citratus* EO did not differ from controls ($F_{(5,33)} = 1.03$, $p = 0.418$), and body weight increased ($F_{(3,99)} = 97.08$, $p < 0.001$) throughout the duration of treatment without interaction between factors ($F_{(15,99)} = 1.27$, $p = 0.24$). There were no significant changes in the absolute (data not shown) or relative organ weight of the EO-treated mice in relation to control groups (Table 1).

Histological examination of the brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder revealed no changes that might suggest *C. citratus* EO toxicity (1, 10 or 100 mg/kg b.w.).

Biochemical analysis

The 21-day treatment with lemongrass EO did not induce abnormalities in serum levels of aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase (GGT), urea, albumin, creatinine, total protein or triglycerides (Table 2). There was a significant reduction in serum total cholesterol ($F_{(4,27)} = 3.061$, $p < 0.05$) after the administration of the highest EO dose (100 mg/kg) when compared to the saline control group.

Comet assay

Data from the lemongrass EO genotoxicity and antigenotoxicity tests are shown in Figure 2. EO at 100 mg/kg did not increase ($F_{(2,15)} = 0.05813$, $p = 0.9437$) DNA damage in mouse blood cells. Similarly, low EO doses (1 and 10 mg/kg) did not protect ($F_{(4,27)} = 15.172$, $p < 0.0001$) against the MNU-induced DNA damage in peripheral leukocytes.

Discussion

The majority of preclinical toxicity studies assess the effect of plant extracts or essential oils after a single acute treatment. However, the utilization of medicinal plants by traditional medicine has rested largely on long-term clinical experience with little or no scientific data on their efficacy or safety after long-term treatment (Ernst, 2004; Zhu et al., 2002). As is the case in relation to many other medicinal plant species, there are few toxicological/toxicogenetic studies about the safety of *Cymbopogon citratus*.

Our data showed that EO did not present an extensive toxic effect in rodents, since the LD_{50} in mice was around 3.5 g/kg, which is a much higher dose than those usually taken as infusion by people. Moreover, it is 350 times greater than the dose that showed anxiolytic-like effect (Costa et al., 2009). Our result was similar to that obtained by Fandohan and colleagues (2008) which demonstrated that LD_{50} in rats was 3.25 g/kg b.w. Administration of citral and β-myrcene, the major compounds of EO, has been reported to cause an embryofetal toxic effect only at high doses in pregnant Wistar rats (Delgado et al., 1993; Nogueira et al., 1995).The LD_{50} of geraniol (the other major constituent) was shown to be around 4.8 g/kg (Lapczynski et al., 2008). These results confirm and partially explain the low toxicity of the acute EO treatment. We did not observe toxic signs such as death occurrence, piloerection, abdominal contortions, locomotion, muscle tone or convulsions in the animals treated with doses from 5 to 1500 mg/kg b.w. Furthermore, these animals were submitted to behavioral procedures for the assessment of anxiolytic- and antidepressant-like and sedative activity of EO in the dose range of 5 to 1500 mg/kg. Previously, our group found anxiolytic-like activity of the EO at the dose of 10 mg/kg in a light/dark box procedure (Costa et al., 2009); the absence of toxicity signals or symptoms in mice after acute or repeated dosages, as described in this experiment, shows the safety of administering the EO at this dose.

During the 21-day acute toxicity treatment, a slight weight loss was observed among the mice treated with EO at 1 or 100 mg/kg, starting after 16 days of treatment. The results

were similar to those obtained by Adeneye and Agbaje (2007). The amount of weight lost by the animals may be directly related to a reduction in food ingestion (not measured in this study). This fact could be due to the existence of an endogenous ligand of central-type benzodiazepine receptors known as endozepine octadecaneuropeptide (ODN), which are inhibitors of food intake in rodents (Do Rego et al., 2007). Such an association makes sense in light of *C. citratus* EO acting on benzodiazepine receptors, as we described previously (Costa et al., 2009).

Histopathological examination of the tissues showed no difference and no apparent abnormalities. Microscopical analysis of the organs revealed that their architectural and cellular appearances were normal and there was no significant difference in the body or organ weights between experimental groups. In Figure 1 we compare the evolution of body weight of animals treated with the classical drug diazepam, the positive control of behavioral experimental models (Costa et al., 2009) and the experimental groups of the present work.

There were no signs of important changes in biochemical parameters evaluated in serum after 21 days of EO treatment. The only modified parameter was the cholesterol level, which was reduced in the group treated with 100 mg/kg/day. This effect was previously described in both rats (Adeneye and Agbaje, 2007) and humans (Elson et al., 1989). In a study conducted with 22 hypercholesterolemic subjects who took a daily capsule containing 140 mg of lemongrass EO, Elson and colleagues (1989) showed evidence that the constituents of lemongrass oil effectively lowered the cholesterol levels of a subset of hypercholesterolemic human subjects. Moreover, Adeneye and Agbaje (2007) showed dose-dependent effects of weight loss, hypoglycemia and hypolipidemia in normal Wistar rats after single, daily oral dosing (125-500 mg/kg) of fresh aqueous leaf extract of *C. citratus* for 42 days. These findings also suggest putative beneficial effects in type 2 diabetes patients, corroborating its folkloric use in Nigeria. But according to the authors, the mechanisms that underlie such activity must be investigated more thoroughly (Adeneye and Agbaje, 2007).

In a previous study (Bidinotto et al., 2010), we found that a long-term administration of EO at a high dose mitigated the genotoxic effects of MNU. Therefore, we decided to evaluate whether this effect is present even at small doses. Our data showed that the administration of low EO doses did not ease these effects. Considering that our relevant findings in the serum (cholesterol reduction) were produced by the highest dose of EO (100 mg/kg), we decided to assess its genotoxicity. The highest EO dose did not increase the DNA damage in peripheral blood leukocytes.

It is recognized that essential oils contain various compounds and that the synergistic or additive effect of multiple constituents as assessed by toxicological tests remains unknown. Therefore, the present study provides new information on the use of the complete oil. In a review of the literature, some authors have presented effects of the two main compounds of the *C. citratus* EO. According to Carnesecchi et al. (2004) geraniol inhibits colon cancer cell proliferation by inducing membrane depolarization and interfering with ionic canals and signaling pathways. Furthermore, the group demonstrated that geraniol inhibits DNA synthesis and reduces the volume of colon tumors. The major compound citral was evaluated in *in vitro* and *in vivo* tests for genotoxicity. The group found, in a two-year feed study, no evidence of toxicological or carcinogenic activity of citral (500 – 4,000 ppm) in F344/N rats and B6C3F1 mice (Nation Toxicology Program, USA, 2003).

The present evaluation of toxicological biomarkers and genotoxicity shows that *C. citratus* EO presents low toxicity and can be considered relatively safe in a long-term treatment at doses up to 100 mg/kg. Therefore, we did not detect any deleterious effect on the liver and kidney functions which remained normal throughout the experiment at all doses tested. Moreover, we showed that the 21-day acute toxicity treatment with lemongrass EO reduced the cholesterol levels. These results corroborate its long-term medicinal use by different traditional populations over the years. However, other pharmacological studies are required to elucidate its action mechanism in reducing serum cholesterol and body weight.

Acknowledgements

We are grateful to MSc. Carlos Alberto da Silva Ribeiro, MSc. Filipe Ferreira Galvão, MSc. Flávia Bonamin, Dr. Leonardo Noboru Seito, and Daniele Oliveira Köhn for technical assistance. This work was part of the Ph.D dissertation of Celso A R A Costa and was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Celso A R A Costa – Proc. nº. 2006/07195-8 and Lucas T Bidinotto – Proc. nº. 2006/58174-0).

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Legends

Figure 1. Body weight gain, in percentage, of mice treated with *C. citratus* essential oil (EO) at 1, 10 or 100 mg/kg (p.o.) for 21 days. DZP (diazepam, 1 mg/kg, p.o.). Each point represents the mean ± SEM of each group.

Figure 2. DNA damage (tail intensity) in peripheral blood leukocytes four hours after the last treatment. (n=5-7 animals per group).
** $p \leq 0.01$ in relation to control group (one-way ANOVA followed by Dunnett Multiple Comparisons Test).

Figure 01

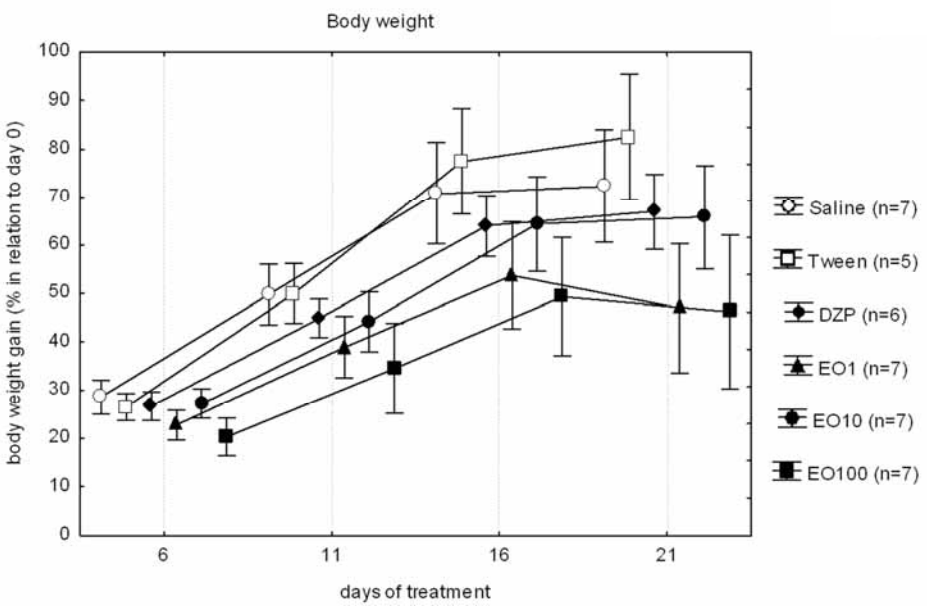
Body weight
body weight gain (% in relation to day 0)
days of treatment
Saline (n=7)
Tween (n=5)
DZP (n=6)
EO1 (n=7)
EO10 (n=7)
EO100 (n=7)

Figure 02

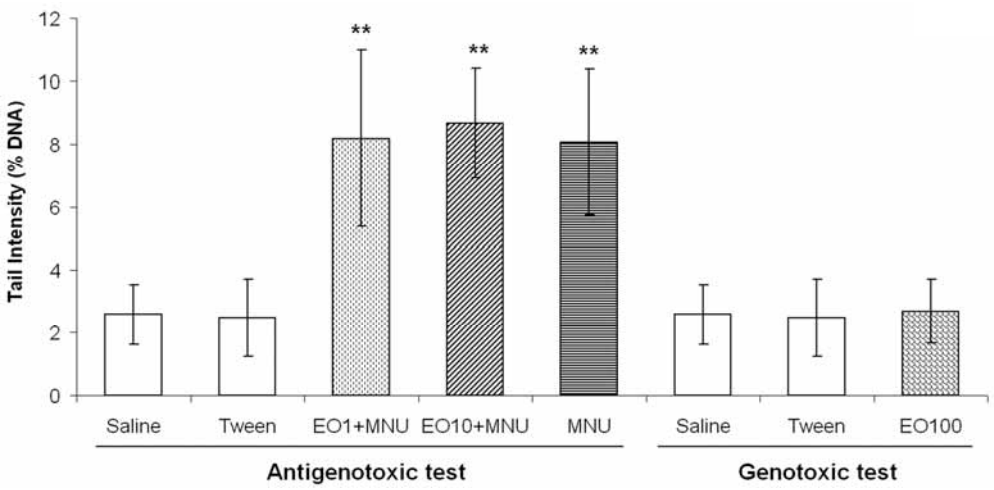
Tail Intensity (% DNA)
12
10
8
6
4
2
0
**
**
**
Saline
Tween
EO1+MNU
EO10+MNU
MNU
Saline
Tween
EO100
Antigenotoxic test
Genotoxic test

Table 1. Relative organ weight (mean±SD) of mice after 21 days treatment with *Cymbopogon citratus* essential oil (EO).

Treatment	n	Relative organ weight (g %)							
		Heart	Lung	Spleen	Kidney R	Kidney L	Liver	Brain	Stomach
Saline	7	0.41±0.07	0.60±0.15	0.38±0.11	0.60±0.07	0.61±0.11	5.33±0.76	1.11±0.11	0.80±0.05
Tween 0.01%	5	0.44±0.06	0.64±0.08	0.35±0.05	0.57±0.08	0.58±0.13	5.24±0.60	1.12±0.11	0.80±0.07
MNU	7	0.43±0.06	0.60±0.15	0.30±0.11	0.59±0.04	0.56±0.07	5.22±0.66	1.06±0.11	0.75±0.13
EO 1 mg/kg	7	0.50±0.08	0.67±0.08	0.31±0.07	0.61±0.08	0.60±0.06	5.18±0.47	1.26±0.28	0.84±0.10
EO 10 mg/kg	7	0.46±0.08	0.63±0.07	0.34±0.05	0.68±0.13	0.65±0.13	5.41±0.46	1.14±0.12	0.86±0.11
EO 100 mg/kg	7	0.51±0.23	0.78±0.22	0.41±0.16	0.61±0.11	0.63±0.14	5.18±0.80	1.29±0.40	0.90±0.20

MNU = N-methyl-N-nitrosourea; R and L = Right and Left, respectively. One-way ANOVA followed by the Dunnett Multiple Comparisons Test.

Table 2. Biochemical parameters (mean±SD) evaluated after 21 days treatment with *Cymbopogon citratus* essential oil (EO).

Treatment	n	Biochemical parameters								
		UR (mg/dL)	CR (mg/dL)	TPRO (g/dL)	ALB (g/dL)	AST (UI/L)	AP (UI/L)	GGT (UI/L)	CHOL (mg/dL)	TRGL (mg/dL)
Saline	7	55.3±9.2	0.33±0.05	6.68±0.56	3.03±0.40	246.57±66.31	270.57±74.48	2.64±1.25	167.43±22.55	247.43±88.42
Tween 0,01%	5	47.5±4.7	0.30±0.00	6.46±0.62	2.78±0.13	235.40±73.61	175.60±100.57	2.20±1.30	152.40±30.70	215.60±95.00
EO 1 mg/kg	7	45.8±12.9	0.31±0.04	6.37±0.48	2.74±0.26	225.43±63.12	214.43±89.30	1.86±0.38	143.43±29.02	213.43±77.11
EO 10 mg/kg	7	51.0±5.7	0.31±0.04	6.21±0.33	2.83±0.11	262.14±30.83	296.86±91.98	3.14±1.34	145.71±31.51	201.43±83.96
EO 100 mg/kg	7	49.0±5.9	0.30±0.00	6.38±0.54	2.88±0.07	284.00±99.45	299.43±76.74	2.14±0.70	125.71±24.57 *****	234.86±62.67

UR (urea), CR (creatinine), TPRO (total protein), ALB (albumin), AST (aspartate aminotransferase), AP (alkaline phosphatase), GGT (gamma-glutamyl transferase), CHOL (cholesterol), TRGL (triglycerides). * $p \leq 0.05$ in relation to control group saline (one-way ANOVA followed by the Dunnett Multiple Comparisons Test).

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Manuscript number (if applicable):	Author name: Celso A R A Costa, Lucas T Bidinotto, Regina K Takahira, Daisy M F Salvadori, Luís F Barbisan, Mirtes Costa
Article Title: Safety evaluation of lemongrass (*Cymbopogon citratus* (DC) Stapf) essential oil in male Swiss mice.	

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This work was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (COSTA, C.A.R.A. – Proc. nº. 2006/07195-8 and BIDINOTTO, L.T. – Proc. nº. 2006/58174-0). The FAPESP has no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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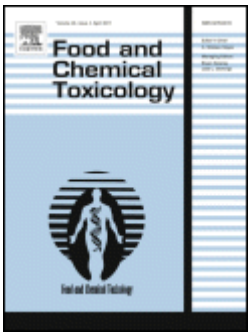

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Behavioral, neurochemical, and biochemical evaluation of *Citrus aurantium* L. essential oil

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Abstract:

Currently available treatments for anxiety disorders and depression have multiple adverse effects, apart from the delayed onset of action, justifying the efforts to find new substances with potential activity in these disorders. *Citrus aurantium* was chosen based on ethnopharmacological data. Traditional medicine refers to the *Citrus* genus as useful in diminishing symptoms of anxiety or insomnia. In a previous work, our group demonstrated sedative and anxiolytic-like effects from different *Citrus* species. In the present work, we investigate the mechanism of the anxiolytic-like effect from *C. aurantium* essential oil (EO) and putative neurochemical changes in specific brain structures of mice after acute treatment, as well as the anxiolytic-like effect and possible serum biochemical changes after a 14-day treatment. The data suggest that the anxiolytic-like activity evaluated in the light/dark box, and after acute oral treatment with a dose of 5 mg/kg, was mediated by the serotonergic system (5-HT_{1A} receptors). After the 14-day repeated oral treatment, the effect remained in the smaller EO dose (1 mg/kg). In the forced swimming test, tail suspension test and marble burying test, which are sensitive to antidepressants, the EO showed no effect after acute treatment. Neurochemical evaluation showed no alterations in neurotransmitter levels evaluated in the cortex, striatum, pons, and hypothalamus. Furthermore, no locomotor impairment was observed after either acute or repeated treatments. This work contributes to a better understanding of the central activity of the *C. aurantium* species, in view of the characterization of the action mechanism for anxiolytic-like activity. Additionally, no toxic effects or biochemical changes, except a reduction in cholesterol levels, were observed in the serum of animals due to a 14-day repeated EO treatment.

Keywords: Anxiolytic; *Citrus aurantium*; Essential oil; Mice; Neurochemistry; Serotonin.

1. Introduction

Since the 1960s, benzodiazepines have been extensively used to treat anxiety disorders and have shown a clear efficacy, leading to their becoming the most widely prescribed drugs worldwide (López-Muñoz et al., 2011). However, benzodiazepines are not able to control anxiety states, and many patients fail to adequately respond to existing pharmacological treatments (Ravindran and Stein, 2010). Furthermore, these drugs have significant side effects, including sedation, muscle relaxation, potentiation of ethanol depressant effect, anterograde amnesia, and addiction (Uzun et al., 2010).

Buspirone, a non-benzodiazepine azapirone agent that was introduced in the late 1980s as a novel therapeutic agent for the treatment of anxiety, acts through an interaction with the 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor subtype (Barrett and Vanover, 1993). Although the azapirones interact with other neurotransmitter systems, they preferentially block the presynaptic D_2 dopamine (DA) receptors (Jadhav et al., 2008). However, the delayed onset of efficacy (1 – 4 weeks), in contrast to the rapid effects observed with benzodiazepines (Lowry et al., 2005), has encouraged researchers to find a robust anxiolytic compound that has fewer side effects than benzodiazepines and a more immediate onset of action than azapirones (Grundmann et al., 2007).

Thus, the multiple adverse effects resulting from available drugs for a considerable proportion of patients, as well as a delayed onset of effects, justifies the efforts to find new substances with potential activity in the treatment of anxiety and/or depression (Cryan and Holmes, 2005; Olivier, 2003; Pilc and Nowak, 2005).

Traditionally, populations in several countries usually refer to species of *Citrus* as useful in diminishing the symptoms of anxiety or insomnia. Sedative and anxiolytic effects were previously described, in mice, for the essential oil (EO) obtained from the peel of *Citrus*

aurantium L. (Carvalho-Freitas and Costa, 2002; Pultrini et al., 2006). There are both experimental and clinical studies regarding the activity of *Citrus* when inhaled. One of them is a study that suggests that *C. aurantium* EO induced a decrease in the level of emotionality evaluated in two experimental models of anxiety in rats (Leite et al., 2008). Another study was conducted with men and women exposed to drops of *Citrus sinensis* EO scattered in the lobby of a dental office. This exposure caused a relaxing effect on patients, specifically in cases of women who had their anxiety levels reduced, mood improved and state of calm enhanced (Lehner et al., 2000).

In light of these considerations, the aim of the present work was to investigate the putative mechanism of the anxiolytic-like effect from *C. aurantium* EO after the blockage of GABAergic or serotonergic neurotransmission systems, as well as to identify possible neurochemical changes resulting from acute treatment with the plant. To better characterize the central nervous system activity, the EO was also evaluated in different experimental procedures related to depressive and obsessive-compulsive disorders. Furthermore, to replicate human drug regimens and to allow for reductions in individual dosages, EO was evaluated in mice after a repeated 14-day oral treatment in anxiety tests, monitoring the evolution of body weight and serum biochemical parameters.

2. Material and Methods

2.1. Plant material

Fruits were harvested from adult plants in an orchard at the Department of Botany, Institute of Biosciences/UNESP/Botucatu. The plant was identified in the BOTU Herbarium, Department of Botany, UNESP, where a voucher specimen (#23123) had been deposited.

Fruits of *C. aurantium* (Rutaceae) were collected between April and June of 2009 and were immediately processed to obtain the EO.

2.2. Essential oil (EO) extraction and phytochemical analysis

Fresh peels were processed with a Clevenger apparatus, and the EO was obtained by hydrodistillation, protected against light and heat, and stored until behavioral assays. Afterwards, an aliquot was separated, and the EO was analyzed by gas chromatography coupled with mass spectrometry as previously described (Pultrini et al., 2006).

2.3. Animals

All experiments were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Biosciences Institute – Ethics Committee for Animal Research (CEEA). Adult Swiss male mice (30 days old) from a colony at the UNESP Central Animal House facility were used in all experiments after a one-week acclimation period in the Animal House of the Department of Pharmacology. Thus, the animals used were approximately 40 to 45 days old. The animals were maintained under controlled environmental conditions of temperature (21±2 ^{o}C) and light (12/12 light/dark cycle), with food and water *ad libitum* until 2 h prior to the experimental procedures. The animals were used only once in this test.

2.4. Drugs

Diazepam (DZP, Germed - EMS, Brazil) was used as the standard anxiolytic drug and imipramine hydrochloride (IM, Sigma-Aldrich, USA) as the standard antidepressant drug. Flumazenil (FLU, Flumazil® - Cristália, Brazil) was utilized as a competitive antagonist of benzodiazepine binding, buspirone (BUSP, Sigma-Aldrich, USA) as a partial agonist of 5-HT_{1A} receptors, and WAY100635 (WAY, Sigma-Aldrich) was used as a highly selective 5-HT_{1A} antagonist. For intraperitoneal injections (i.p.), DZP, IM, and BUSP were dissolved in isotonic saline (SAL, 0.9%). For oral administration (p.o.), the EO or limonene (d-limonene, 98%, LIM, Sigma-Aldrich, USA), a major compound of the EO, were dissolved in 0.01 % (v/v) polyoxyethylene sorbitan monooleate (TW - Tween 80®, Sigma-Aldrich) in saline, which was used to treat control groups. All samples were freshly prepared on the test day and were administered at 10 ml/kg of body weight, except FLU, which was administered at 20 ml/kg due to the concentration (0.1 mg/ml) of its commercial form.

2.5. Behavioral procedures

Mouse behavior in the forced-swimming test, tail suspension test, light/dark box and elevated plus-maze test were videotaped under white light illumination using a video camera and a computer. Digital video was subsequently reviewed, and behaviors were scored by a highly trained observer who had been blinded to treatment conditions. In other procedures (marble-burying test and rota-rod test), the behaviors were registered in real time. All experimental procedures occurred between 9:00 am and 5:00 pm.

2.5.1. Forced Swimming Test (FST)

The FST is the most widely used and recognized pharmacological model for assessing antidepressant activity (Petit-Demouliere et al., 2005). This procedure was carried out following a modified version (Detke et al., 1995; Lucki et al., 1994) of the conditions proposed by Porsolt et al. (1977, 1978). The apparatus consisted of a clear Plexiglas cylinder (21 cm high x 20.5 cm diameter) filled with water (23 $\pm$ 2 ºC) to a depth of 13 cm. Each animal underwent a pre-test swimming session for 15 min; the pre-tests were conducted 24 h prior to the 5-min swimming tests. At the end of the swimming sessions, the animals were removed from the water and gently dried.

Mice were treated with EO at 1, 5, 10 or 50 mg/kg according to the following schedule: the first treatment (T1) was administered 1 h after finishing the pre-test session and subsequently 5 h (T2) and 30 min (T3) before the test session. The digital video recording of each mouse in the water was evaluated to classify the behavioral response into one of the following three possible categories: climbing behavior, swimming behavior, and immobility time (Cryan et al., 2002). All behaviors were measured in seconds using the *Etholog 2.2* software (Ottoni, 2000).

Inhalation treatments were performed in an acrylic box with 2 ml of EO at 0.5%, 1.0%, and 2.5% soaked in cotton swabs. Mice received T1, T2 and T3 on the same schedule described above. Each mouse was placed into the box for 7 min immediately before the tests, as described by Almeida et al. (2004). The positive control group was exposed to the inhalation box with cotton swabs soaked in saline and treated with IM 30 mg/kg (i.p.), and the negative control group was exposed to and treated with saline (i.p.).

2.5.2. Tail Suspension Test (TST)

The TST was conducted as previously described (Steru et al., 1985). The test is conducted based on the rationale that animals subjected to the inescapable stress of being suspended by their tail will develop an immobile posture. Briefly, thirty minutes after treatments (EO 5, 10 or 50 mg/kg, v.o.), mice were individually suspended by the tail with a clamp (1 cm from the tip of the end) for a total of 5 min, and the duration of immobility was observed and measured. Mice were considered immobile only when they hung passively and completely motionless. The duration of immobility was the main parameter measured.

2.5.3. Marble-Burying Test (MBT)

The MBT is based on a defensive burying behavior that can be elicited in rodents in response to aversive stimuli, such as a shock prod and noxious food, or an effective unconditioned stimulus, such as glass marbles (Broekkamp et al., 1986; Poling et al., 1981). The mice used in the MBT were previously selected to represent those that exhibit a stable burying score (at least 13 out of 25 marbles) for three consecutive days before the test (Pultrini et al., 2006). Thirty minutes after treatments with EO (1, 5, 10 or 50 mg/kg, v.o.), the animals were individually placed in cages (27 long x 16 wide x 13 cm high) with 25 glass marbles uniformly distributed on a 5-cm layer of sawdust. Each cage was covered with a smooth, acrylic, transparent plate with small punctures for ventilation. The mice were left in the cages with the marbles for 30 minutes, after which they were removed to count the number of marbles that were totally hidden by sawdust.

2.5.4. Elevated plus-maze (EPM)

For the EPM, the apparatus for mice consisted of a wooden cross painted black and placed 50 cm above the floor. The four arms (dimension of each arm: 30 cm long x 5 cm wide) were interconnected by a central platform. Two opposite arms were surrounded by walls (closed arms) while the two others were devoid of enclosing walls (open arms). Thirty minutes after treatments, each mouse was placed at the center of the maze, head facing a closed arm. The mice were able to explore the entire apparatus, and traditional aspects of the animals' behavior were recorded during a 5-min period: the time spent in open arms and frequency of entries into open arms. The aversion to the open arms is interpreted as being generated by fear of novelty, which would induce disgust and curiosity, and also by the height of the open arms (Rodgers et al., 1997). Mice submitted to EPM were treated by inhalation route 30 min before procedure, in the same doses and apparatus described for FST. The positive control group was exposed to the inhalation box with cotton swabs soaked in saline and treated with DZP 1.0 mg/kg (i.p.), and negative control group was exposed and treated with saline (i.p.).

2.5.5. Light/Dark Box Test (LDB)

The light/dark box (46 long x 27 wide x 30 cm high) was divided into the typical dimensions of one-third for the dark compartment with black walls, floor and roof and two-thirds for the light compartment with white walls and floor, and it was illuminated by a 20-W fluorescent lamp. The light and dark compartments were connected by an opening (7.5 x 7.5 cm) located at floor-level in the center of the partition (Crawley and Goodwin, 1980).

Mice were treated with EO at 1, 5, 10 or 50 mg/kg, v.o., and after thirty minutes, each animal was individually placed in the center of the light compartment, facing the dark compartment, and then they were observed for 5 minutes after the first entry into the dark compartment. During this period, we recorded the number of shuttle crossings (an activity-exploration index), number of rearings and the time spent in the light compartment, which is the most consistent and useful measure for assessing anxiolytic-like activity (Bourin and Hascoët, 2003; Young and Johnson, 1991).

For long-term treatment (WHO, 1993), groups received 1, 5 or 10 mg/kg/day for 14 days and were submitted to the same procedure 30 min after the last treatment.

To address a possible contribution from the GABAergic system, mice were co-administered DZP (1 mg/kg, i.p.) or the EO (5 mg/kg, p.o.) 30 min before the test, and then they were given FLU (2 mg/kg, i.p.) 15 min before testing. To evaluate possible interference from the serotonergic system, animals were pretreated with WAY (0.1 mg/kg, i.p.); 15 min afterwards, then, mice received the EO (5 mg/kg) or the standard drug DZP (1 mg/kg); the experimental procedure was performed 30 min after the last drug treatment.

2.5.6. Rota-rod Test (RRT)

Mice treated by oral route were submitted to LDB and then immediately placed onto the apparatus to register the total time that it took to perform on the rotating bar in three successive trials, totaling 1 min. RRT was previously described by Duhan and Miya (1957) and is suitable for detecting motor impairment due to pharmacological agents, such as skeletal muscle relaxants or central nervous system depressants. The apparatus consisted of a 5-rpm rotating, non-slippery plastic rod (3.0 cm in diameter and 50 cm in length) that had been lifted to a height of 25 cm. To avoid a bias on account of inability not related to drug treatment,

only animals that performed satisfactorily under the same testing conditions one day prior to testing were selected to walk on the bar under testing conditions.

2.6. Toxicity and biochemical analyses

Mice treated for 14 days with EO at 1, 5 or 10 mg/kg were daily observed for signs and symptoms indicative of toxicity and were periodically weighed to evaluate the evolution of body weight. After the last treatment, blood samples were collected in tubes without anticoagulant that had been kept at room temperature for 30 min and centrifuged at 4,000 rpm for 20 min. Serum samples were aspirated off and stored at -80 ºC until analysis in a Cobas Mira Plus® Chemistry Analyser (Roche Diagnostic Systems, USA). The clinical biochemistry parameters measured were aspartate aminotransferase, alkaline phosphatase, urea, albumin, creatinine, total protein, cholesterol and triglycerides.

2.7. Neurochemical evaluation

Animals acutely treated with TW or EO (5 mg/kg) were euthanatized, and the brains were rapidly dissected (not more than 3 min after extraction) on ice. Briefly, the striatum, hypothalamus, pons, and frontal cortices was dissected, weighed, and stored in liquid nitrogen until the neurochemical analyses.

Following sample collection, tissues were homogenized in 0.1M perchloric acid by manual sonication, centrifuged at 10,000 rpm for 20 minutes to remove supernatant, and stored at –80 ºC until the determination of monoamine levels.

Neurotransmitters and their metabolites-dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5HT) and 5-hydroxyindoleacetic

acid (5HIAA) - were measured using reverse-phase using an high performance liquid chromatography (HPLC) system (model 6A, Shimadzu, Kyoto, Japan) with an electrochemical detector as described in detail elsewhere (Felício et al. 1996). Briefly, 20-µl samples were loaded into a sample injector and the mobile phase was delivered at a constant rate of 1.2 ml/min. The runtime for each sample was 15 min, and concentrations of all neurotransmitters and their metabolites were expressed as nanograms per gram of tissue (ng/g).

2.8. Statistical analysis

Quantitative data from the behavioral procedures were subjected to Kruskal-Wallis non-parametric variance analysis, followed by the Mann-Whitney test when appropriate. Proportions in the RRT were compared by Fisher's exact test. Biochemical data were analyzed by a one-way ANOVA followed by Dunnett's Multiple Comparison test and neurochemical data by unpaired t-test. All statistical analysis was made using GraphPad InStat® version 3.02. Contrasts were made between the treated and TW groups, and differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Essential oils composition

Table 1 shows the results obtained by gas chromatography coupled with mass spectrometry of the *C. aurantium* EO (yield: 0.50% v/w). The main EO compound was identified by the retention time and Kovats retention index (Adams, 2007) as monoterpene

limonene (98.66%). Other compounds detected in the EO include β-pinene (0.41%) and β-myrcene (0.53%).

3.2. Effects of the EO by oral route on behavioral procedures

The acute EO administration was unable to modify parameters evaluated in the FST, TST and MBT tests (Table 2). IM (30 mg/kg, i.p.), the standard drug in these experimental procedures, significantly decreased the immobility time and enhanced the active behaviors in the FST (climbing and swimming), enhanced the latency and reduced the immobility time in the TST and decreased the number of marbles buried in the MBT.

The results obtained in the LDB test after acute treatment with the EO (Fig. 1, left panel) reveal a significant increase in time spent in the light chamber after administration of the EO at 5 mg/kg when compared to the TW group. An increase in exploratory parameters (number of rearings) could be observed after either DZP or the EO (5 or 50 mg/kg) treatment. Moreover the EO, administered for 14 days, was able to modify the time spent on the illuminated side and the number of transitions and rearings in the LDB at 1 mg/kg. The same behavior pattern was observed for the partial agonist of 5-HT_{1A} receptors BUSP (Fig. 1, right panel). Additionally, the group that was repeatedly treated with saline and that received one treatment with DZP 30 min before the experimental procedure showed an anxiolytic-like effect that was analogous to acute DZP treatment (Fig. 1). In our previous experiments, repeated treatment with DZP was ineffective in modifying the parameters evaluated in the LDB (Costa et al., 2009). The major compound of the EO (limonene, 5 or 10 mg/kg, p.o.) was also evaluated after acute treatment and did not modify the parameters evaluated in the LDB (data not show).

3.3. Effects of the EO by inhalation route on behavioral procedures

The treatment with EO using the inhalation route was ineffective in modifying parameters evaluated in the EPM or in the FST procedures (Table 3).

3.4. The role of GABA or 5-HT neurotransmission on the anxiolytic-like effect of the EO in the LDB

Results of the experimental protocols to evaluate the interference of FLU or WAY on the acute effect of the EO (5 mg/kg) or DZP (1 mg/kg) are presented in Fig. 2. Results from FLU or WAY alone did not differ from those obtained from the TW group, showing no effect *per se* of these blockers on the evaluated parameters.

Mice treated with DZP or the EO spent more time in the light chamber, when compared with the TW group. The animals treated with DZP and FLU also had an increase in time spent in the light chamber, but in lower magnitude due to blockage of FLU. However, the FLU was unable to promote reduction in the magnitude of the EO anxiolytic-like effect (Fig. 2 – left panel). As expected, the treatment with DZP and WAY was unable to modify the effect of DZP on time spent in the light chamber. However, treatment with WAY prevents the effect of the EO in all parameters assessed in LDB (Fig. 2, right panel).

3.5. Effect of the EO on motor coordination

The RRT was carried out to measure locomotor impairment in mice treated acutely or repeatedly. The results, presented in Table 4, include the number of disabled mice in relation to the total number of treated animals in each experimental group. The disabled mice, defined

as those that could not stay on the rotating bar in a maximum of three attempts at 5 rpm for 1 min, did not differ statistically from the TW group.

3.6. Toxicity and biochemical analyses

Treatment for 14 days with EO did not induce abnormalities in the serum levels of aspartate aminotransferase, alkaline phosphatase, urea, albumin, creatinine, total protein or triglycerides. The single altered parameter was the serum total cholesterol, significantly reduced by EO 10 mg/kg treatment (Table 5).

3.7. Neurochemical evaluation

The acute treatment with *C. aurantium* EO did not result in neurotransmitters or their metabolites producing changes (DA, DOPAC, HVA, 5HT, and 5HIAA) as evaluated in cortex, striatum, pons and hypothalamus (Table 6).

4. Discussion

Drugs derived from traditional medicines may have therapeutic relevance in the treatment of anxiety (Grundmann et al., 2007). Traditional populations usually reference *Citrus* species as useful as a decoction (Girón et al., 1991; Longuefosse and Nossin, 1996) or infusion (Tortoriello and Romero, 1992; Vásquez et al., 1997) of flowers and/or leaves against nervous system disturbances.

In a previous work, our group demonstrated sedative and anxiolytic-like effects from *Citrus aurantium*, *Citrus latifolia*, and *Citrus reticulata* EO in mice (Carvalho-Freitas and Costa 2002; Gargano et al., 2008; Pultrini et al., 2006).

Therefore, considering that the rate of comorbidity between anxiety and depression is about 60 to 70% in the general population (Kalueff et al., 2007), with indications that these conditions have common genetic origins (Kendler, 1987), it is possible to propose that the same substance can exert therapeutic activity in both disorders. The present work not only investigated the action mechanism of the *C. aurantium* EO anxiolytic-like effect but also the achievable antidepressant-like activity.

The major compound present in the EO was limonene followed by β-myrcene. Both compounds have biological activity related with the central nervous system, since mice treated with these compounds have shown decreases in spontaneous activity, rearing, and grooming in an open field test, and the compounds were able to increase the barbiturate sleeping time (Vale et al., 2002). In the present work, limonene did not modify the parameters evaluated in the LDB at the lower dose (5 mg/kg) or the twofold dose (10 mg/kg) which raised the substance levels to the amount present in the effective dose of EO (5 mg/kg). The results are in accordance with the findings obtained by Vale et al. (2002), which demonstrated that limonene was not able to modify the parameters of anxiolytic-like activity evaluated in EPM.

The 5HT system has been implicated in the modulation of anxiety levels, and some components of this system promote anxiety whereas others reduce its symptoms (Toth, 2007). Since the introduction of buspirone as a novel therapeutic agent for the treatment of anxiety, considerable research activity about its therapeutic role in clinical anxiety and mood disorders has been generated (Ravindran and Stein, 2010). Buspirone is a partial agonist at 5-HT_{1A} autoreceptors which act as an antagonist at certain postsynaptic 5-HT_{1A} receptor sites, and

buspirone also preferentially blocks the presynaptic rather than the postsynaptic D_2 dopamine (DA) receptors (Jadhav et al., 2008). Reports about the effect of the acute administration of buspirone are inconclusive, while chronic treatment results in an anxiolytic-like effect (Barret and Vanover, 1993; Lowry et al., 2005).

As seen in our results, the EO showed anxiolytic effects after acute (5 mg/kg) or 14-day repeated (1 mg/kg/day) treatments, increasing all evaluated parameters in the LDB. After acute treatments, such effects were not antagonized by FLU, an antagonist of benzodiazepine binding, but they were antagonized by the specific 5-HT_{1A} antagonist WAY. Considering that benzodiazepines are not effective in LDB after chronic treatment (Costa et al., 2009) and that the reduction of the anxiolytic-like effect due to WAY administration, we can infer that the EO effect is mediated by the serotonergic system without discarding the possibility of involvement of EO or its metabolites with other neurotransmissions systems. An increase in the synthesis of 5HT and DA in the prefrontal cortex and hippocampus was observed in mice after inhalation of lemon oil vapor (a species from the *Citrus* genus), with the suggestion that lemon oil vapor possibly affects the response to the DAnergic activity via modulation of the 5HTnergic systems (Komiya et al., 2006). In our assessment of DA, 5HT and its metabolites changes, no alteration in the cerebral areas evaluated was observed after acute treatment. In this way, higher EO doses and/or repeated treatment could reveal this effect upon neurotransmitter systems.

In experimental models related to obsessive-compulsive disorder (MBT) and depression (FST and TST), and therefore sensitive to the activity of antidepressant drugs (Cryan et al., 2005; Njung´e and Handley, 1991), EO was unable to modify the assessed behaviors. 5-HT_{1A} ligands are not active in the treatment of obsessive-compulsive disorder and their use for the treatment of depressive disorders seems inconsistent (Lucki et al., 1994).

This inconsistency may be related to differences in the intrinsic activity and potency of 5-HT_{1A} drugs that appear to depend upon the brain region (Barret and Vanover, 1993).

Contradicting our expectation, we could not find an anxiolytic-like effect after exposition to EO inhalation, although it has been observed in a study with rats in the same concentration used in our procedure (Leite et al., 2008). Even thus, no antidepressant-like effect was found in the FST after inhalation of EO.

The evaluation of signs and symptoms related to toxicity due to 14-day repeated EO treatment showed no deleterious consequences. Daily inspection and periodic weighing showed no difference in general aspect, in evolution of body weight and in biochemical parameters among experimental groups, except for a decrease in total cholesterol. This is not an unexpected effect, since Kurowska and Manthey (2004) reported that diets with methoxylated flavones (PMFs) induce a hypolipidemic response in hamsters with experimental hypercholesterolemia, and they refer to studies suggesting that high dietary intake of orange, grapefruit, tangerine juices and orange peel of *Citrus aurantium* might reduce hypercholesterolemia, mediated by PMFs. Already, in a study by Santiago et al. (2010), supplementation with 2% d-limonene, a major compound of *C. aurantium*, to a high fat diet in Wistar rats reversed the changes in lipids and lipid peroxidation, possibly due to the enhancement of lipoprotein lipase. Moreover, Arbo et al. (2008) conducted a study to evaluate the subchronic toxicity and oxidative stress of extracts obtained from *C. aurantium* after 28 consecutive days of treatment in mice. The authors suggest using the low subchronic toxicity based on the fact that the different doses of the extract did not show any sign of toxicity.

In conclusion, *C. aurantium* EO possesses a significant anxiolytic-like activity, and the present results strongly suggest an involvement of the 5-HT_{1A}-receptors. We believe that these results are promising since EO might be considered as an alternative for the treatment of anxiety disorders. However, further investigations are necessary to explore the detailed EO

action mechanism on binding sites. Moreover, EO appears to be well tolerated, considering that the different doses did not cause alterations or signs of toxicity.

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Figure captions

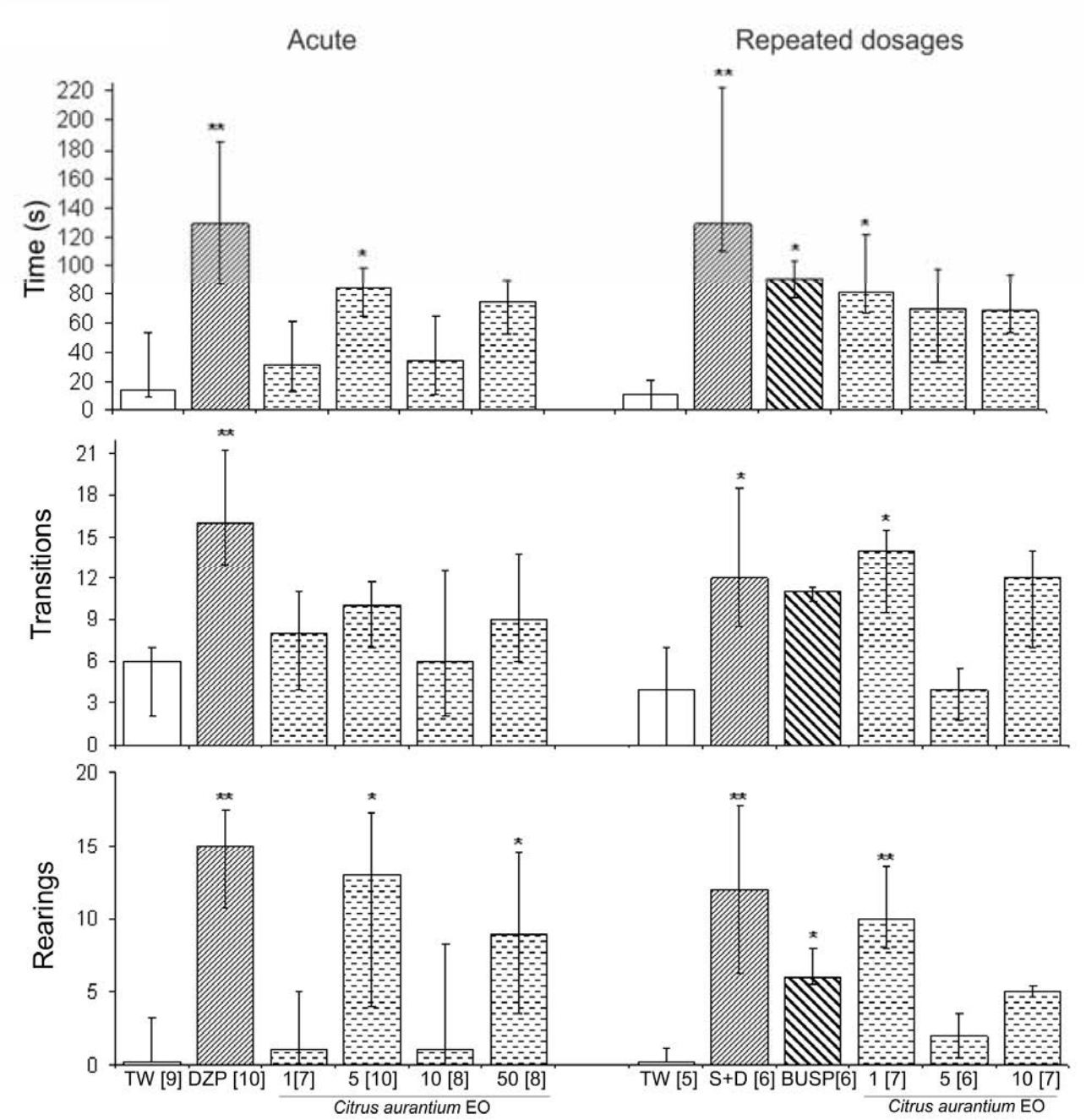
Fig. 1. Median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated chamber, number of transitions, and number of rearings in the light/dark box after acute or repeated treatment with *Citrus aurantium* essential oil (EO) by oral route; TW - vehicle, 10 ml/kg (p.o.); S+D - saline (i.p.) + diazepam (1 mg/kg, i.p.). The numbers in brackets represent the n.

*$p \leq 0.05$, **$p \leq 0.01$ in relation to TW group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Fig. 2. Influence of flumazenil (FLU, 2.0 mg/kg, i.p.) or WAY100635 (WAY 0.5, mg/kg, i.p.) on the anxiolytic-like effect of diazepam (DZP, 1 mg/kg, i.p.) or essential oil (EO, 5 mg/kg, p.o.), expressed as the median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated chamber, number of transitions, and number of rearings in the light/dark box; TW - vehicle, 10 ml/kg (p.o.). The numbers in brackets represent the n.

*$p \leq 0.05$, **$p \leq 0.01$ in relation to TW group; # or Δ $p \leq 0.05$ (Kruskal-Wallis followed by Mann-Whitney U-Test).

Figure 01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Time (s)
Transitions
Rearings
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BUSP[6]
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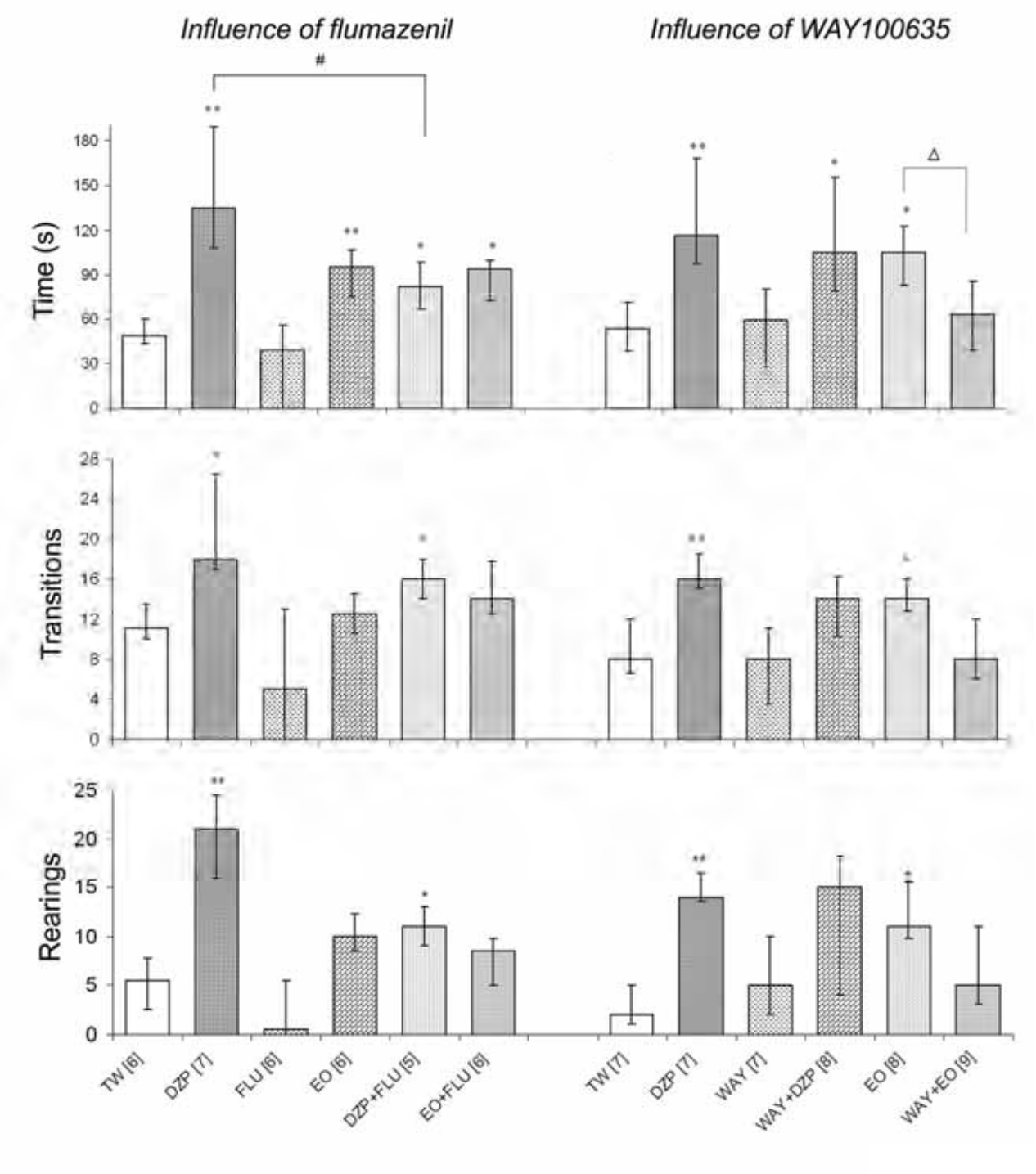
Citrus aurantium EO
Citrus aurantium EO

Figure 02


Influence of flumazenil
Influence of WAY100635
Time (s)
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Rearings
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WAY [7]
WAY+DZP [8]
EO [8]
WAY+EO [9]

Table 1. Chemical composition obtained by gas chromatography coupled with mass spectrometry from *Citrus aurantium* L. essential oil

Constituent	RT	RI	%
β-pinene	6.869	977	0.41
β -myrcene	7.261	991	0.53
Limonene	8.539	1037	98.66
ni	11.072	1100	0.41

RT, retention time; RI, retention index (experimental); ni, not identified

Table 2. Effects of essential oil (EO) from *Citrus aurantium* in the Forced-Swimming Test, Tail Suspension Test, and Marble-Burying Test after acute oral treatment

	Forced-Swimming Test				**Tail Suspension Test**			**Marble-burying test**	
Treatment	n	Immobility (s)	Climbing (s)	Swimming (s)	n	Latency (s)	Immobility (s)	n	Marbles buried
TW	5	226 (225-231)	2 (1-3)	67 (65-74)	8	45 (42-49)	162 (130-179)	5	24 (21-25)
IM	6	156 (120-165) *****	36 (27-50) *****	110 (96-116) *****	8	89 (86-148) ******	42 (24-71) ******	5	1 (0-10) *****
EO 1 mg/kg	6	222 (215-238)	2 (0-12)	61 (56-79)	-	not evaluated	not evaluated	6	24 (19-25)
EO 5 mg/kg	7	234 (217-241)	6 (5-13)	63 (53-72)	7	58 (49-72)	62 (47-101)	6	16 (10-22)
EO 10 mg/kg	8	238(224-255)	2 (1-6)	51 (32-63)	6	43 (39-51)	99 (57-134)	6	20 (18-23)
EO 50 mg/kg	6	217 (206-222)	6 (4-11)	71 (63-79)	6	47 (44-53)	87 (66-127)	5	20 (16-25)

TW (tween); IM (imipramine hydrochloride, 30 mg/kg).

Data are expressed as median and interquartile range (Q1-Q3).

* $p \leq 0.05$, ** $p \leq 0.01$ in relation to TW group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Table 3. Effects of Citrus aurantium essential oil (EO) in the Elevated plus-maze and Forced-Swimming Test after inhalation treatment.

Treatment	Elevated plus-maze			Forced-Swimming Test	
	n	Open arms entries	Time (s) in open arms	n	Immobility (s)
DZP	14	10 (5-13) *	86 (69-101) **	-	not evaluated
IM	-	not evaluated	not evaluated	6	167 (164-171) *
Saline	9	2 (1-4)	30 (20-52)	5	207 (203-219)
EO 0.5%	12	2 (1-3)	34 (15-39)	8	215 (207-221)
EO 1.0%	9	2 (1-4)	37 (30-55)	8	194 (179-208)
EO 2.5%	12	3 (2-4)	33 (7-47)	8	220 (175-234)

DZP (diazepam, 1.0 mg/kg); IM (imipramine hydrochloride, 30 mg/kg).

Data are expressed as median and interquartile range (Q1-Q3).

* $p \leq 0.05$, ** $p \leq 0.01$ in relation to saline group (Kruskal-Wallis followed by Mann-Whitney U-Test).

Table 4. Effects of treatments of essential oil (EO) on motor coordination evaluated by the Rota-rod Test.

Motor Impairment					
Acute treatment				Repeated treatment	
TW	1/7	FLU	0/5	TW	0/5
DZP	0/8	WAY	1/5	SAL+DZP	0/6
EO 1 mg/kg	0/5	DZP+FLU	0/4	BUSP	0/6
EO 5 mg/kg	0/8	WAY+DZP	1/5	EO 1 mg/kg	0/5
EO 10 mg/kg	1/6	EO+FLU	0/4	EO 5 mg/kg	0/5
EO 50 mg/kg	0/6	WAY+EO	0/5	EO 10 mg/kg	0/7

TW (tween); DZP (diazepam); SAL (saline); BUSP (buspirone); FLU (flumazenil); WAY (WAY100635). Results are expressed as the fraction of total animals in each experimental group that were unable to perform the test after acute or repeated treatments or after the protocol for studying the action mechanism. Fisher´s exact test.

Table 5. Biochemical parameters (mean±SD) evaluated after 14 days of treatment with *Citrus aurantium* essential oil (EO).

Treatment	n	Biochemical parameters							
		UR (mg/dL)	CR (mg/dL)	TPRO (g/dL)	ALB (g/dL)	AST (UI/L)	AP (UI/L)	CHOL (mg/dL)	TRGL (mg/dL)
Tween	6	64.50±10.13	0.28±0.05	7.02±0.96	3.95±0.30	320.43±85.46	164.8±74.16	158.83±35.27	173.83±42.53
EO 1 mg/kg	7	64.43±13.66	0.28±0.17	7.91±1.92	4.52±0.99	334.19±52.71	131.42±44.68	131.43±27.18	143.57±50.78
EO 5 mg/kg	6	65.33±13.68	0.25±0.07	6.95±0.83	4.17±0.80	377.45±83.59	161.90±64.01	135.17±15.64	130.83±37.55
EO 10 mg/kg	7	64.14±11.87	0.25±0.03	6.86±0.63	3.87±0.41	309.51±45.24	123.34±34.86	121.86±20.39 *****	132.71±33.39

UR (urea), CR (creatinine), TPRO (total protein), ALB (albumin), AST (aspartate aminotransferase), AP (alkaline phosphatase), CHOL (cholesterol), TRGL (triglycerides).

* $p \leq 0.05$ in relation to control group (one-way ANOVA followed by the Dunnett Multiple Comparisons Test).

Table 6. Neurotransmitter levels and their metabolites (ng/g) after acute oral treatment with *Citrus aurantium* EO (5 mg/kg).

	Structures and treatments							
	Cortex		Striatum		Pons		Hypothalamus	
	TW (*N*=12)	EO (*N* =8)	TW (*N* =11)	EO (*N* =7)	TW (*N* =11)	EO (*N* =8)	TW (*N* =12)	EO (*N* =8)
DA	722.35±744.32	1215.83±1199.85	8324.29±1715.02	8331.24±2751.84	26.62±17.60	28.67±25.74	53.08±12.14	58.29±14.17
DOPAC	94.63±55.36	85.65±49.20	437.36±147.60	415.46±132.00	22.44±8.73	16.87±6.07	58.64±19.48	64.16±13.41
HVA	427.32±278.46	567.86±318.16	1121.94±320.96	1384.97±512.03	300.94±281.20	380.18±375.45	236.17±127.67	388.82±210.59
5HT	1246.99±297.33	1270.13±229.26	1686.34±339.54	1665.87±542.13	2601.34±793.69	2036.21±842.98	1660.08±437.24	1599.22±322.07
5HIAA	192.72±60.71	229.34±95.20	317.12±102.38	320.35±105.88	869.54±226.91	660.45±207.38	341.73±72.53	310.96±54.94

TW (tween). Data are presented as mean ± SD. Unpaired t-test.

N is different in each group as some structures could not be evaluated due to technical reasons.

Acknowledgements

We are grateful to Dr. Márcia Ortiz M. Marques and Dr. Roselaine Facanali (Laboratory of Natural Products – Instituto Agronômico (IAC), Campinas, Brazil) for the GC-MS analysis of essential oil and to Vinícius Bertotti Ribeiro for technical assistance. This work was supported by grants from FAPESP (Celso A R A Costa – Proc. nº. 2006/07195-8).

Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

All authors significantly contributed to and have approved the final manuscript. The work was part of the Ph.D. dissertation of Celso A R A Costa and Scientific Initiation of Thais C Cury and Bruna O Cassettari. The senior author, Mirtes Costa, supervised the entire project.

Role of the funding source

This work was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Celso A R A Costa – Proc. nº. 2006/07195-8). The FAPESP had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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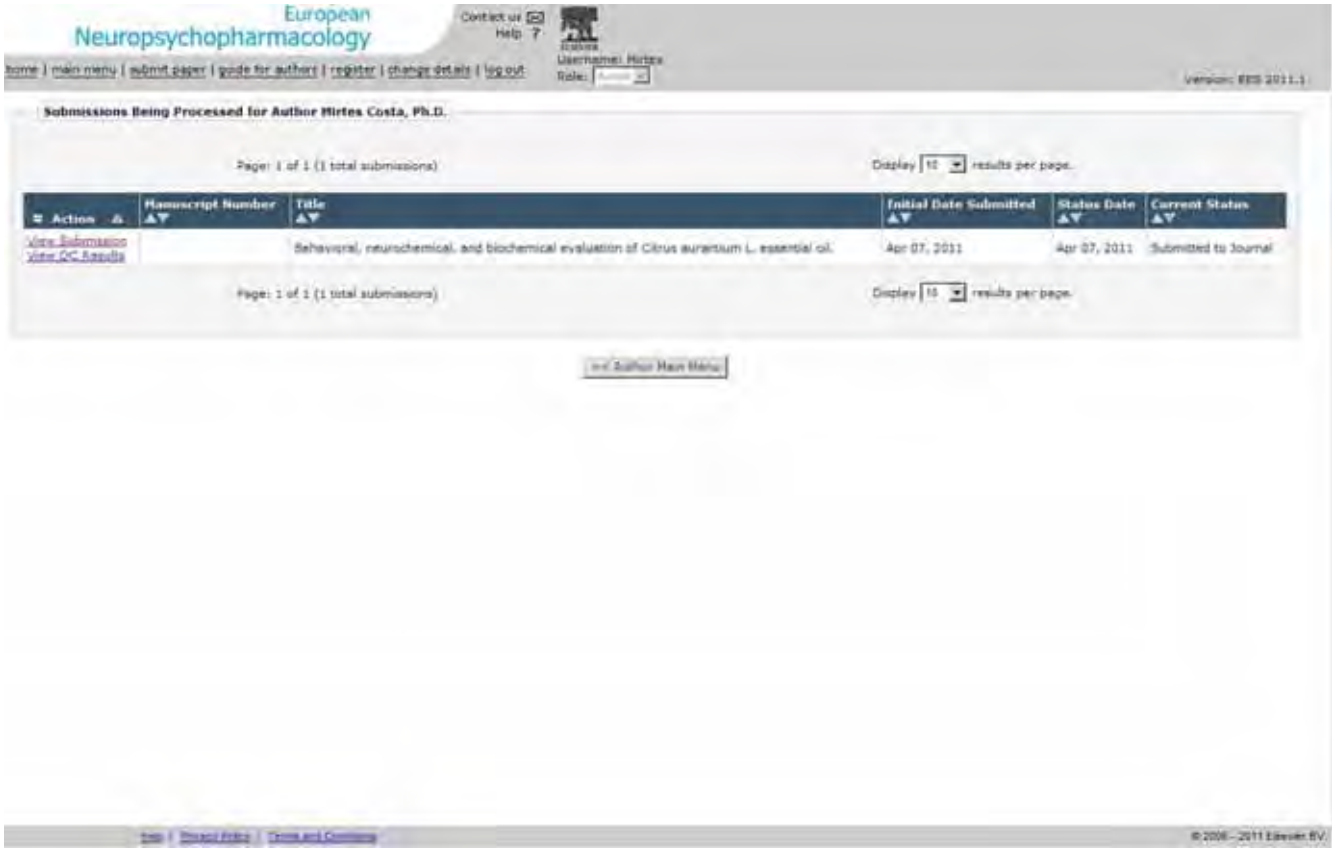

V. Conclusão:

➔ *Cymbopogon citratus*:

- A reversão da atividade ansiolítica do óleo essencial (OE) na Caixa Claro/Escuro (CCE) decorrente do tratamento com o antagonista de receptores GABAérgicos (flumazenil), mas não com o antagonista de receptores serotonérgicos do tipo 5-HT_{1A} (WAY100635), indicou que, a exemplo do que ocorreu com o diazepam (DZP), a atividade do OE resulta da interação com o complexo receptor GABA-benzodiazepínico.

- O tratamento repetido alterou a atividade do OE, assim como do DZP, indicando que tal fenômeno pode estar relacionado com a indução de tolerância.

- Não foi observada atividade do OE no Teste do Nado Forçado (TNF) e no Teste de Esconder Esferas (TEE), modelos experimentais que não são sensíveis ao tratamento com benzodiazepínicos, mas sim à drogas como a imipramina, útil na clínica para o tratamento do transtorno depressivo ou obsessivo-compulsivo.

- Não foi observado comprometimento motor após tratamento agudo ou repetido nos grupos controle e experimental.

- Não foram encontradas diferenças significativas na avaliação neuroquímica realizada no córtex, estriado, hipotálamo e ponte do neurotransmissor dopamina (DA) e seus metabólitos ácido 3,4-dihidroxifenilacético (DOPAC) e ácido homovanílico (HVA) e do neurotransmissor serotonina (5HT) e seu metabólito ácido 5-hidroxiindolilacético (5HIAA) após tratamento agudo com OE.

- Não houve sinais de mudanças importantes nos parâmetros bioquímicos avaliados no soro após 21 dias de tratamento com OE à exceção dos níveis de colesterol, os quais foram reduzidos no grupo tratado com 100 mg/kg/dia.

- O exame histopatológico dos tecidos não mostrou nenhuma anormalidade. A análise microscópica dos órgãos revelou que sua arquitetura e aparência celulares eram normais e não houve diferença significativa no peso corporal e peso relativo dos órgãos entre os diferentes grupos experimentais.

- O tratamento repetido com baixas doses do OE não foi capaz de reverter o efeito genotóxico provocado pelo MNU (N-metil-N-nitrosuréia), enquanto que a alta dose (100 mg/kg/dia) não induziu danos no DNA, mostrando a relativa segurança do OE.

➔ *Citrus aurantium*:

- O tratamento com o antagonista de receptores GABAérgicos (flumazenil) não foi capaz de reverter o efeito ansiolítico do OE, porém o pré-tratamento com antagonista de receptores serotonérgicos do tipo 5-HT_{1A} (WAY100635) reverteu tal efeito indicando que, a atividade do OE resulta da interação com os receptores 5-HT_{1A}.

- Após tratamento repetido (14 dias) a menor dose do OE testada (1 mg/kg/dia) apresentou efeito ansiolítico na CCE, a exemplo do que ocorre, na clínica, com a classe de drogas ansiolíticas do tipo azapironas (buspirona) que demoram de 1 a 3 semanas para apresentar seu início de ação.

- O OE não foi ativo no TNF, Teste da Suspensão pela Cauda e no TEE, modelos experimentais sensíveis à drogas como a imipramina.

- Não foi observado comprometimento motor após tratamento agudo ou repetido nos grupos controle e experimental.

- Após o tratamento agudo com OE não foram verificadas alterações neuroquímicas no córtex, estriado, hipotálamo e ponte do neurotransmissor DA e seus metabólitos DOPAC e HVA e do neurotransmissor 5HT e seu metabólito 5HIAA.

- Por fim, a avaliação dos parâmetros bioquímicos realizada no soro após 14 dias de tratamento com OE demonstrou uma significativa redução dos níveis de colesterol. Não foram constatadas alterações na evolução do peso corporal dos animais.

Assim, o presente trabalho contribui para o aumento no conjunto de informações a respeito das atividades sobre o sistema nervoso central das espécies *C. citratus* e *C. aurantium*, corroborando seus usos populares e garantindo, consideravelmente, o uso seguro de tais espécies. Outros trabalhos devem ser realizados para melhor assegurar o mecanismo de ação, a segurança e a eficácia dos OE, tais como estudos de *binding* e de parâmetros farmacocinéticos.

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A.) Padronização do mecanismo de ação de drogas no Teste do Nado Forçado (TNF)

Os receptores serotonérgicos e noradrenérgicos desempenham um importante papel na patofisiologia da ansiedade e depressão, daí o fato de continuarem sendo um importante alvo para a produção e desenvolvimento de novas drogas que atuem sobre tais receptores (Kim *et al.*, 2007; Lesch e Mossner, 1999).

Os experimentos preliminares aqui descritos foram realizados para a padronização das doses dos antagonistas de receptores serotonérgicos e noradrenérgicos utilizadas no procedimento do TNF. O antidepressivo tricíclico imipramina (IM - 30 mg/kg, i.p.) foi utilizada como droga de referência e as drogas WAY100635 (WAY, Sigma-Aldrich®, EUA) e ioimbina (YO, Sigma-Aldrich®, EUA) foram utilizadas como antagonistas de receptores 5-HT_{1A} e α-2 adrenérgico, respectivamente (Brocardo *et al.*, 2008; Komiya *et al.*, 2006).

Ao procedimento experimental foram submetidos animais pré-tratados com IM ou Tween 0,01%, 24 h, 5 h e 30 min antes da sessão de teste. Cada um destes tratamentos foi precedido (30 min) pela administração dos antagonistas WAY (serotonérgico) ou YO (noradrenérgico) seguindo os protocolos previamente estabelecidos (Ago *et al.*, 2003; Kim *et al.*, 2007; Brocardo *et al.*, 2008).

A figura 1 mostra os resultados obtidos com o pré-tratamento dos animais com WAY ou YO na redução do comportamento de imobilidade no TNF. O bloqueio de receptores 5-HT_{1A} com WAY na dose de 0,1 mg/kg (i.p.) e dos receptores de noradrenalina com YO na dose de 2 mg/kg (i.p.)

foram efetivos em reverter o efeito da IM, indicando a ação da serotonina e da noradrenalina na capacidade do antidepressivo reduzir o tempo de imobilidade no TNF.

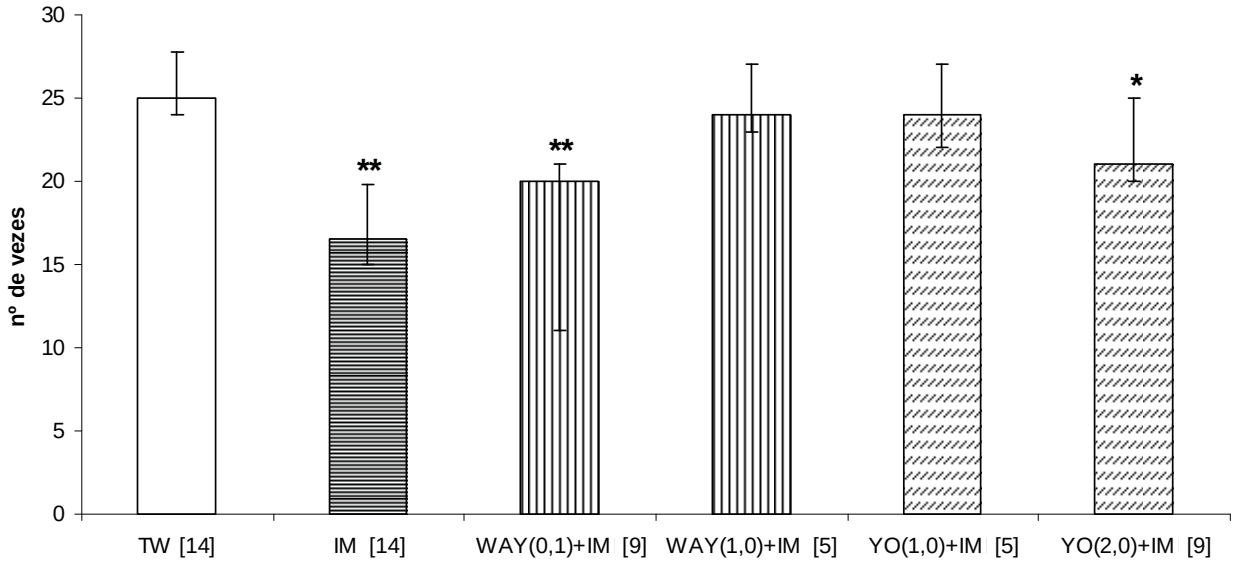

Figura 1. Mediana e intervalo interquartil da contagem do Comportamento de Imobilidade registrado a cada 10 segundos, durante 5 minutos, no teste do nado forçado em camundongos machos tratados 24h, 5h e 30 min com 30 mg/kg de imipramina - IM (i.p.) ou após o pré-tratamento com os antagonistas WAY100635 (WAY - 0,1 ou 1,0 mg/kg) ou ioimbina (YO - 1,0 ou 2,0 mg/kg). Algarismos entre colchetes representam o "n" de cada grupo.
* $p \leq 0,05$, ** $p \leq 0,01$ em relação ao respectivo grupo controle (Teste de Kruskal-Wallis seguido de Mann-Whitney).

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CERTIFICADO

Certificamos que o Protocolo nº 009/06-CEEA, sobre *"Modelos experimentais em ansiedade em roedores"*, sob a responsabilidade de **MIRTES COSTA**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 06/04/06.

Botucatu, 07 de abril de 2006.

Prof. Dr. FRANCISCO DE ASSIS GANEO DE MELLO
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CERTIFICADO

Certificamos que o Protocolo nº 011/06-CEEA, sobre *"Modelos experimentais de sedação/hipnose em roedores"*, sob a responsabilidade de **MIRTES COSTA**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 06/04/06.

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CERTIFICADO

Certificamos que o Protocolo nº 008/06-CEEA, sobre *"Modelos experimentais para avaliação de atividade geral em roedores"*, sob a responsabilidade de **MIRTES COSTA**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 06/04/06.

Botucatu, 07 de abril de 2006.

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CERTIFICADO

Certificamos que o Protocolo nº **155-CEEA**, sobre "Modelos experimentais de depressão em roedores", sob a responsabilidade de **Mirtes Costa**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado *"Ad referendum"* da ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), nesta data.

Botucatu, 19 de janeiro de 2010.

Profª Drª PATRÍCIA FERNANDA F. PINHEIRO
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