UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Papel de receptores monoaminérgicos, colinérgicos e glutamatérgicos no efeito pro-mnéstico de *Ptychopetalum olacoides* Bentham (Olacaceae).

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“Como foi que os povos pré-modernos descobriram que um chá feito com essa árvore em particular, dentre todas as plantas da floresta, aliviaria os sintomas da malária? Devem ter experimentado toda árvore (...). Isso constitui um sólido conjunto de experimentos científicos continuados ao longo de gerações(...). Deveríamos estar fazendo esforços muito maiores para explorar os tesouros desse conhecimento popular em todo o mundo. “

Carl Sagan

“ ... um cientista deve, acima de tudo, ser como uma criança. Se ele vê algo, deve dizer o que está vendo, independente daquilo ser o que ele imaginava ver ou não.”

Douglas Adams

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Resumo

As raízes de *Ptychopetalum olacoides* Bentham (PO) (Olacaceae), popularmente conhecida como Marapuama, são usadas na Amazônia para tratar sintomas de doenças relacionadas ao sistema nervoso central. Já havíamos demonstrado que o extrato etanólico de PO (EEPO) facilita a evocação da memória de longa duração (MLD) e reverte o déficit de memória em camundongos envelhecidos na tarefa de esquiva inibitória, tem propriedades antioxidantes e anticolinesterásicas. Neste estudo mostramos que EEPO melhora a memória de curta duração (MCD) e MLD tanto quando administrado por via intraperitoneal (50 e 100 mg/kg) quanto por via oral (800 e 1000 mg/kg). Além disso, mostramos que o efeito pró-mnésico de EEPO (100 mg/kg) também se verifica na tarefa não aversiva de reconhecimento de objetos. Através do uso de antagonistas verificou-se que o efeito pró-mnésico de EEPO requer a participação de receptores colinérgicos, β (mas não α_1) adrenérgicos, D_1 (mas não D_2) dopaminérgicos, serotoninérgicos 5-HT_{2A}. Estes resultados estão de acordo com estudos prévios que sugeriram a interação de EEPO com vários sistemas de neurotransmissores. Estes resultados também estão de acordo com as alegações terapêuticas feitas por usuários de Marapuama, usada prevalentemente por idosos, convalescentes de doenças do SNC e/ou períodos de grande stress físico ou mental. Finalmente, considerando os resultados deste trabalho, os correlatos bioquímicos já identificados em EEPO e o uso tradicional em humanos, os resultados sugerem fortemente o potencial desta espécie para o desenvolvimento de droga útil a condições que apresentam déficit cognitivo, tais como envelhecimento e mal de Alzheimer.

Abstract

Ptychopetalum olacoides Bentham (PO) (Olacaceae) roots, commonly known as Marapuama, are used throughout the Amazon to treat symptoms related to central nervous system disturbances. We had previously shown that an ethanol extract of PO (POEE) facilitates long term memory (LTM) retrieval, and reverses memory deficits in ageing mice using the inhibitory avoidance task, possesses antioxidant and anticholinesterase properties. In this study we show that POEE facilitates short term memory (STM) and LTM when administered intraperitoneally (50 e 100 mg/kg) or orally (800 e 1000 mg/kg). Moreover, we showed that the promnesic effect of POEE (100mg/kg) is also verified with the non aversive task of object recognition. By using specific antagonists we also verified that the promnesic effect of POEE requires the participation of cholinergic, β (but not α_1) adrenergic, D_1 (but not D_2) dopaminergic, 5-HT_{2A} serotoninergic receptors. These results are in agreement with previous data suggesting the interaction of POEE with various neurotransmitter systems. The present results are also in accordance with the therapeutic properties alleged by Marapuama traditional users, which is predominantly used by the elder, by those convalescents from CNS disorders and/or those undergoing periods of high physical or mental stress. Finally, considering the results of this study, the neurochemical correlates already identified for POEE and the traditional use in human beings, these results markedly point a great potential of this species for drug development for conditions presenting cognitive deficit, including ageing and Alzheimer disease.

Lista de abreviaturas

AMPA	**ÁCIDO α-AMINO-3-HIDROXI-5-METIL-4-ISOXAZOLE-PROPIÔNICO**
5-HT	**SEROTONINA (5-hidroxitriptamina)**
α	**alfa**
EGb 761	**EXTRATO PADRONIZADO DE *Ginkgo biloba* 761**
APP	**PROTEÍNA PRECURSORA DO β-AMILÓIDE**
ACh	**ACETILCOLINA**
OMS	**ORGANIZAÇÃO MUNDIAL DA SAÚDE**
OPAS	**ORGANIZAÇÃO PAN-AMERICANA DE SAÚDE**
β	**BETA**
AChE	**ACETILCOLINESTERASE**
ADREN	**ADRENÉRGICO**
AVE	ACIDENTE VASCULAR ENCEFÁLICO
D	**DOPAMINA**
DA	**DOENÇA DE ALZHEIMER**
DP	**DOENÇA DE PARKINSON**
DPOC	**DOENÇA PULMONAR OBSTRUTIVA CRÔNICA**
EEPO	**EXTRATO ETANÓLICO DE *Ptychopetalum olacoides***
GABA	ÁCIDO GAMA-AMINOBUTÍRICO
GB	**Gingko biloba**
INAMPS	**INSTITUTO NACIONAL DE ASSISTÊNCIA MÉDICA DA PREVIDÊNCIA SOCIAL**

IP	**INTRAPERITONEAL**
LC	***locus ceruleus***
MCD	**MEMÓRIA DE CURTA DURAÇÃO**
MLD	MEMÓRIA DE LONGA DURAÇÃO
MPTP	**1-METIL-4-FENIL-1,2,3,6-TETRA-HIDROPIRIDINA**
NA	**NORADRENALINA**
NMDA	**N-METIL-D-ASPARTATO**
PG	***Panax ginseng***
PO	***Ptychopetalum olacoides***
POEE	***Ptychopetalum olacoides* ETHANOL EXTRACT**
SNC	**SISTEMA NERVOSO CENTRAL**
SUS	SERVIÇO ÚNICO DE SAÚDE
VO	**VIA ORAL**

1. INTRODUÇÃO

1.1.Envelhecimento: indivíduo e sociedade

Definir um indivíduo como idoso é extremamente difícil. A definição de um indivíduo como idoso pode, por exemplo, basear-se em argumentos de caráter biológico: paralelamente à evolução cronológica, podemos definir o envelhecimento como um conjunto de alterações físicas e biológicas que ocorrem em um indivíduo com o passar do tempo. Tais alterações ocorrem de forma diferenciada entre indivíduos de uma mesma sociedade, já que nesse segmento conhecido como terceira idade se incluem indivíduos diferenciados entre si, tanto do ponto de vista sócio-econômico como demográfico e epidemiológico. Enquanto o indivíduo envelhece com o aumento da idade, a população como coletivo envelhece na medida em que aumenta a idade média das pessoas que a compõem. Considerando os aspectos demográficos, a Organização Mundial da Saúde (OMS) define a população idosa como aquela a partir dos 60 anos de idade. Esse limite é válido para os países em desenvolvimento, mas admite-se um ponto de corte de 65 anos para países desenvolvidos, que usam esse índice há várias décadas (PEREIRA *et al.*, 2003). É sabido que durante a maior parte da História da humanidade somente uma em cada 10 pessoas teria chegado ao 65º aniversário; hoje, nos países desenvolvidos oito em cada 10 pessoas ultrapassam os 65 anos e no Brasil isto ocorre entre seis de cada 10 homens e sete de cada 10 mulheres (CAMARANO, 2002). A expectativa média de vida da população americana passou de 47 anos ao início do século XX para cerca 77 anos ao seu final. Estatística realizada pelas Nações Unidas e o Banco Mundial estimam que em 2010 aproximadamente 7,3% da população mundial terá mais de 65 anos, enquanto que para 2030 esta porcentagem atingirá 20%

(MENDLEWICZ,1998). Assim como no resto do mundo, a população brasileira sofre um processo evolutivo caracterizado por uma progressiva queda da mortalidade em todas as faixas etárias, e um conseqüente aumento da expectativa de vida. Atualmente, a expectativa média de vida da população brasileira ao nascer é de 69 anos para os homens e 72 para as mulheres. A análise do crescimento populacional de diferentes faixas etárias mostra que o grupo de idosos (65 anos ou mais) é o que mais está crescendo no país: de 1980 a 2000, a proporção da população brasileira entre 0-14 passou de 38,24% para 29,60%; enquanto que o grupo de idosos cresceu de 4,01 para 5,85 % (IBGE, 2001). A rapidez com que se processam as mudanças na estrutura etária da população brasileira leva a uma estimativa de que até 2025 o país cairá da 16ª para a 6ª posição mundial em termos de número absoluto de indivíduos com 60 anos ou mais, com a proporção de idosos duplicando até 2050, alcançando 15% do total da população (CHAIMOWICZ, 1998).

O progressivo incremento absoluto e relativo do contingente populacional de idosos impõe ao sistema de saúde arcar com a elevação dos custos demandados pela atenção às doenças crônico-degenerativas comuns à terceira idade, e ainda adequar sua organização às necessidades próprias desse grupo etário (OPAS/OMS, 1998), uma preocupação até há pouco restrita aos países industrializados. Reconhece-se que o envelhecimento populacional traz novos desafios; um deles diz respeito às pressões políticas e sociais para a transferência de recursos na sociedade. Por exemplo, a pressão sobre o sistema previdenciário aumenta significativamente e as demandas de saúde se modificam com maior peso nas doenças crônico-degenerativas, o que implica maior custo de internamento e tratamento, equipamentos e medicamentos mais dispendiosos. Para a Organização Pan-Americana de Saúde (OPAS) o “bom

envelhecimento" depende em grande parte de medidas de prevenção de enfermidades, da manutenção da atividade física e de funções cognitivas, além da participação ininterrupta em atividades sociais e produtivas. Em uma pesquisa realizada pela OMS em doze países da América Latina, concluiu-se que a situação econômica e a falta de acesso aos serviços de saúde são os principais problemas enfrentados pelos idosos (CHAIMOWICZ, 1998).

O envelhecimento também traz uma sobrecarga para a família, sobrecarga que aumenta em proporção à idade (CAMARANO *et al*., 1999), já que o envelhecimento da população traz como uma de suas conseqüências o aumento na prevalência de problemas de saúde característicos que incluem: doenças cardiovasculares, neoplasias, diabetes, doenças reumatológicas, e alguns transtornos mentais. Apesar de a imensa maioria dos idosos nos países desenvolvidos ser razoavelmente saudável e viver de forma independente em suas comunidades, 80% deles experimentam pelo menos uma forma de doença crônica (VERAS *et al*., 1987). A demência, por exemplo, afeta aproximadamente 5% dos idosos aos 65 anos de idade e 20% daqueles com 80 anos ou mais (VERAS; MURPHY, 1994). Depressão é outro transtorno mental freqüente entre idosos, com taxas de prevalência variando entre 5% e 35% de acordo com o nível de gravidade da depressão (BLAY *et al*., 1989; ADDONIZIO; ALEXOPOULOS, 1993; GAZALLE et al., 2004). Os distúrbios psiquiátricos dos idosos interferem de forma negativa na vida daqueles envolvidos com seus cuidados, e já representam uma das principais áreas de gasto com a saúde da população em países desenvolvidos (GRAFSTRÖOM; WINBLAD, 1995).

Estudos recentes têm demonstrado uma participação desproporcional dos idosos na demanda por serviços de saúde, principalmente hospitalares. No Brasil em 1985, os

custos com as internações de câncer, doenças cardiovasculares e diabetes na rede hospitalar contratada do INAMPS atingiram 135 milhões de dólares, mais de 1/3 dos custos totais de internações (VERAS *et al*., 1987). Levantamentos estatísticos mais atuais constatam que a população de idosos, que representava 8,5% da população, consumiu R$ 1,217 bilhão em hospitalizações pelo SUS, contra R$ 922 milhões consumidos com a população de 0 a 14 anos que representava 29,6% da população brasileira total (SUS, 2001).

Idade e sexo são características naturalmente associadas ao uso de serviços hospitalares, em razão da sua influência biológica na morbi-mortalidade (HULKA; WHEAT, 1985; SAWYER et al., 2002); por isso análises de utilização de serviços hospitalares devem ser ajustadas por faixa etária e gênero. Segundo dados levantados pelo IBGE, em 1999, dos 86,5 milhões de pessoas que declararam terem consultado um médico nos últimos 12 meses, 73,2% tinham mais de 65 anos. As pessoas de pior condição socioeconômica têm mais necessidade de assistência à saúde e, consequentemente, de fazer uso dos serviços de saúde (HULKA; WHEAT, 1985; TRAVASSOS, 1997). Porém, um maior uso de serviços de saúde por pessoas de pior situação socioeconômica também depende do funcionamento do sistema de saúde. Nos EUA, a pobreza e o desemprego apresentaram associação positiva com as taxas de internação, enquanto que educação mostra associação negativa (MCLAUGHLIN *et al.*, 1989; MCMAHON *et al*., 1993); o efeito das características socioeconômicas nas taxas de hospitalização foi maior nos adultos jovens e diminuía com a idade (HOFER *et al*., 1998). No Canadá, as pessoas de menor renda usaram mais serviços hospitalares (NEWBOLD *et al*., 1995; GLAZIER *et al*., 2000), mas maiores taxas de cirurgia foram encontradas em áreas de maior escolaridade e maior proporção de indivíduos de

origem canadense, americana ou inglesa (ROOS; ROOS, 1982). Na Espanha, não foram encontradas desigualdades nas internações hospitalares entre classes sociais, medidas por uma adaptação da "Classificação de Classes Sociais" desenvolvida na Inglaterra que é baseada nas ocupações (BORRELL *et al*., 2000). No Rio de Janeiro, o fator explicativo mais importante da utilização de serviços de saúde por idosos foi a necessidade medida pela morbidade auto-referida, e o uso de serviços em áreas de pior condição econômica dependeu mais da renda individual do que em áreas de melhor condição econômica (PINHEIRO; TRAVASSOS, 1999). Nos Estados Unidos estudos indicam que os usuários persistentes têm mais idade, referem pior estado de saúde, apresentam maior número de sintomas físicos e um grau maior de estresse psicológico, especialmente depressão (MCFARLAND *et al*., 1985; FREEBORN *et al*., 1990). Um estudo realizado no Brasil demonstrou que as pessoas que se internaram referiram maior número de doenças crônicas, pior estado de saúde, pior estado funcional, mais restrição das atividades habituais e ter estado acamado por motivo de saúde; o aspecto favorável à internação foi dispor de cobertura por plano de saúde e ter um serviço de saúde do qual se faz uso regular (CASTRO *et al*., 2002).

Relevante à pesquisa objeto desta tese, alguns estudos mostram que as internações hospitalares em idosos podem ocorrer como um sintoma secundário à demência. Entre os diversos fatores de risco para quedas, seguido ou não de fratura, têm-se apontado o déficit cognitivo e a demência (SALGADO *et al.*, 1994; GUO *et al*., 1998). CARVALHO E COUTINHO (2002) demonstraram a associação entre demência e ocorrência de quedas e fraturas entre idosos na cidade do Rio de Janeiro, verificando que acidentes dentro de casa vitimaram 78% dos idosos com demência contra 55% daqueles sem. As quedas nesta faixa etária da população podem ainda estar

associadas ao uso de medicamentos, tais como, benzodiazepínicos, bloqueadores de cálcio, neurolépticos, antidepressivos, antiarritmicos (LEIPZIG *et al*., 1999a,b; COUTINHO; DA SILVA, 2002).

A percentagem de idosos com demência pode ser elevada: um estudo verificou que quase um quarto dos pacientes idosos internados na clínica médica do Hospital Universitário-UFSC apresentou demência. O perfil deste grupo foi de brancos, com baixa escolaridade e com alta freqüência de doenças crônicas, tais como: 50% com história de hipertensão arterial sistêmica, 30% de diabetes melitus, 21% com história prévia ou atual de acidente vascular encefálico (AVE) e 20% com doença pulmonar obstrutiva crônica (DPOC). Estes estudos não apontam as causas ou os tipos de demência, mas realçam a importância da detecção de quadros demenciais em idosos internados, para melhor traçar o perfil sócio-demográfico da população brasileira (CORDINI *et al*., 2002; GARRIDO; MENEZES, 2002), visto que são escassos os estudos no Brasil sobre a prevalência de transtornos mentais no idoso.

Com relação ao consumo de medicamentos, ANDERSON *et al.* (1998) entrevistaram 93 idosos da Universidade Aberta da Terceira Idade no Rio de Janeiro e relataram que 85% da amostra utilizava pelo menos um medicamento regularmente. Nessa mesma universidade, SAYD *et al*. (2000) entrevistaram 140 idosos que afirmaram ter ingerido 2,3 medicamentos em média na véspera da entrevista. ALMEIDA *et al.* (1999) descreveram que 41,3% de 184 idosos atendidos em um ambulatório de psicogeriatria de São Paulo consumiam três ou mais medicamentos por dia. Das drogas não psicotrópicas, os anti-hipertensivos foram os mais freqüentemente utilizados (32,6%). Em suma, os idosos no Brasil são portadores de, pelo menos, uma doença crônica e utilizam um medicamento regularmente. Um em cada três idosos pode

apresentar sintomas psiquiátricos, e seus cuidadores informais sofrem um impacto decorrente desse papel. Os serviços de saúde disponíveis não são suficientes para as necessidades de cuidado dessas pessoas (GARRIDO; MENEZES, 2002). Portanto, é urgente o desenvolvimento de estratégias e drogas que possam retardar ou minimizar as doenças decorrentes da idade avançada.

1.2. Desenvolvimento de drogas para o tratamento de deficits cognitivos.

A prevalência de demência duplica a cada cinco anos após os 60 anos, resultando em aumento exponencial com a idade. Deste grupo, a Doença de Parkinson afeta 1/ 500 indivíduos (JENNER *et al.,* 1992), enquanto 50 a 60 % sofrem de Doença de Alzheimer (BOWLING; BEAL, 1995; LENDON *et al.,* 1997).

Os sintomas característicos da Doença de Parkinson (DP) são: tremor em repouso, bradicinesia ou hipocinesia, rigidez muscular e alterações posturais e da marcha, freqüentemente provocando quedas. Alterações na motricidade ocular extrínseca, demência e ataxia cerebelar surgem numa fase mais avançada da doença. Sem tratamento a DP progride em 5 a 10 anos até um estágio rígido, acinético, no qual o paciente não consegue cuidar de si mesmo. A doença de Parkinson se caracteriza pela degeneração progressiva e irreversível de neurônios dopaminérgicos da via nigroestriatal, pela qual as informações são enviadas da substância negra ao corpo estriado (COOPER *et al.*,1996; SHIMOHAMA *et al*., 2003). Em 1982 um grupo de jovens acidentalmente produziu uma droga, que induziu um distúrbio semelhante ao Parkinson, criando o chamado “dependente congelado”. Esse composto denominado 1-

metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP), provoca a destruição irreversível dos neurônios dopaminérgicos nigroestriatais (COOMES, 1997; SHIMOHAMA *et al*., 2003).

Estudos de PET (tomografia com emissão de pósitrons) revelam que o aparecimento dos primeiros sintomas do Parkinson surgem quando o conteúdo de dopamina do estriado se encontra 20-40% abaixo do normal (RIEDERER; WUKETICH, 1976; RANG; DALE, 2004). Sabe-se que incidência da DP aumenta com a idade; assim, um processo acelerado do envelhecimento, pode diminuir o número de neurônios até um nível a partir do qual, se iniciaria a doença (MCGEER *et al.,* 1989). O tratamento com L-DOPA ou agonistas dopaminérgicos pode repor a dopamina e minimizar os sintomas da doença, porém, não evita a degeneração celular. A DP, em geral, não é considerada uma doença genética primária, embora 10% dos pacientes podem ter algum componente genético (DUVOISIN, 1986; GOLBE *et al.,* 1990; JONHSON *et al.,* 1990; MARAGANORE *et al.,* 1991). Sugere-se que a influência ambiental (industrialização e uso de pesticidas) possa ser uma das causas do seu desenvolvimento (TANNER, 1989; VANACORE *et al.,* 2002). O estresse oxidativo poderia também contribuir tanto para a progressão da doença quanto para a morte neuronal (JENNER, 1996; 2003), sendo que o aumento da lipoperoxidação e de danos oxidativos ao ADN têm sido reportados especialmente para substância negra de portadores da doença (ALAM *et al.,* 1997; DE IULIIS *et al*., 2002).

A doença de Alzheimer ou também chamada de demência degenerativa primária do tipo Alzheimer, é a forma mais comum de demência que aparece tanto na idade pré-senil (antes dos 65 anos) quanto na senil, e existe tanto na forma familiar, quanto esporádica (LENDON *et al.,* 1997). O termo “demência do tipo Alzheimer” engloba todas as demências que não sejam de origem orgânica óbvia, como acidente vascular,

lesão cerebral ou abuso do álcool (RANG; DALE, 2004). Assim como na DP, na Doença de Alzheimer (DA) várias hipóteses têm sido apontadas para as causas do desenvolvimento da doença. A causa do desenvolvimento da DA pode apresentar um componente genético. Estudos em DA familiar relataram mutações no gene que codifica o APP (proteína precursora do β-amilóide), e mutações nos genes que codificam a proteína denominada presenilina, localizados nos cromossomos 21 , 1 e 14 (VIANA, 1999; ALBERT *et al.*,1999; PRITCHARD *et al*., 2005). Além disto outras causas têm sido sugeridas para o aparecimento de DA como: a exposição a agentes ambientais danosos (intoxicação por alumínio ou infeção virótica), desenvolvimento de processos de auto-imunidade, alterações nos níveis de neurotransmissores e seus metabólitos, receptores, e a atividade enzimática dos sistemas colinérgicos, dopaminérgicos, noradrenérgicos e serotoninégicos (TOMAZ, 1996; FOSTER, 2002; LANDRIGAN *et al*., 2005).

O início da DA é gradual e pode evoluir em três estágios, onde o primeiro (2 a 4 anos) caracteriza-se por alterações na capacidade de recordar informações, dificuldade no manuseio de dinheiro, pouca capacidade de decisão, perda de iniciativa e ansiedade. O segundo estágio (2 a 10 anos) caracteriza-se por uma maior perda de memória, confusão, inquietude, problemas motores e perceptuais, desintegração social, problemas de linguagem e nos pensamentos lógicos. O terceiro estágio (1 a 3 anos) inclui a incapacidade de reconhecer a si mesmo, incontinência, afasia, alterações de humor, dificuldade de deglutição e convulsões (VIANA, 1999).

Os alvos terapêuticos incluiriam o aumento da transmissão colinérgica; redução do estresse oxidativo, diminuição dos processos inflamatórios, prevenção da formação da

proteína β-amilóide ou de sua toxicidade, e aumento da circulação de agentes neurotróficos (PERRY *et al.*,1999; RANG; DALE, 2004). Nos últimos cinco anos medicamentos efetivos para o tratamento de DA tornaram-se mais disponíveis, embora tragam apenas modestos benefícios na sintomatologia desta doença (FLICKER, 2002). Com a introdução do primeiro inibitor de cholinesterase (ChEI) em 1997, as drogas colinérgicas (donepezil, galantamina e rivastigmina) são consideradas a primeira escolha para o tratamento de DA leve a moderada. Estas substâncias possuem propriedades farmacológicas ligeiramente diferentes, mas todas aumentam a oferta de acetilcolina, um neurotransmissor central em processos de memória, através do bloqueio da enzima acetilcolinesterase. Há só quatro medicamentos comerciais aprovados para tratamento de DA, e produtos naturais têm papel importante na pesquisa de novos inibidores de acetilcolinesterase, como exemplificado pela descoberta de galantamina (VIEGAS *et al*., 2005) e pelo protótipo deste grupo que é a fisiostigmina.

Além destes agentes, há no mercado a presença marcante de suplementos alimentares e/ou medicamentos fitoterápicos usados neste contexto terapêutico. As mais conhecidas são preparações contendo *Ginkgo biloba* (Linnaeus) (GB), considerada sagrada pelos budistas que a têm cultivado junto a seus templos. Esta espécie faz parte do milenar arsenal terâpeutico chinês, sendo seu uso citado por Chen Noung (2767-2687 A.C) naquela que é considerada a primeira farmacopéia chinesa (DEFEUDIS, 1991).

Na Europa, GB foi a droga mais comercializada entre 1985-95, atingindo em 1993 cerca de 1,2 milhões de prescrições por mês, gerando lucro anual de U$ 200

milhões (STICHER, 1993). O extrato padronizado, “EGb 761” (extrato de *Ginkgo biloba* 761), foi desenvolvido e registrado por Dr. Willimar SCHWABE em 1965 na Alemanha. Esta espécie é conhecida no ocidente como ornamental em numerosos países de clima variado, começou a ser estudada há cerca de quatro décadas (TESKE; TRENTINI, 1994). Preparações de EGb 761 são atualmente comercializadas em mais de 30 países (DEFEUDIS, 1991; ERNST, 2002).

Estudos realizados em indivíduos jovens com diminuição da capacidade cognitiva ou disposição física associadas com perda de memória, dor de cabeça e vertigem, mostraram que EGb 761 melhorou a capacidade cognitiva (DEFEUDIS, 1991). A administração aguda de 600 mg de GB levou a uma melhora da memória em mulheres jovens e saudáveis (CURTIS-PRIOR *et al.*, 1999). Além disso, uma das indicações de EGb 761 está relacionada com distúrbios do SNC que ocorrem preferencialmente em pacientes idosos, incluindo demências que podem estar associadas com insuficiência na circulação cerebral ou neurodegeneração progressiva. A demência em idosos freqüentemente envolve amnésia anterógrada (memória de curta-duração) e diminuição do estado de alerta, atenção e motivação. O EGb 761 é comercializado também como terapia adjuvante para DP e DA. Como a DP pode estar relacionada com o estresse oxidativo, verificou-se em ensaio *in vitro* que o EGb 761 preveniu a oxidação da glutationa e o aumento da geração de peróxidos na mitocôndria (SASTRE *et al.*, 1998), além de inibir a produção de óxido nítrico (NO) (KOBUCHI *et al*., 1997). Muitos estudos, tanto em animais quanto clínicos, vem sendo realizados com EGb 761 verificando sua eficácia na prevenção e/ou retardo da progressão da DA, na qual o hipocampo é uma estrutura seriamente comprometida. Em um número substancial desses estudos, o EGb 761 foi capaz de estabilizar e/ou melhorar a função cognitiva e, no caso dos estudos

clínicos, melhorar também o desempenho social de indivíduos portadores da doença (LE BARS *et al*., 1997; LE BARS *et al*., 2000).

Outra espécie bem conhecida e estudada quanto ao seu possível efeito neuroprotetor é o *Panax ginseng* Meyer (PG), conhecida popularmente por Ginseng. Conhecida há pelo menos 4.000 anos na China, tradicionalmente indicada nos distúrbios psiquiátricos e neurológicos associados à terceira idade, também é indicada para melhorar a capacidade psicomotora em indivíduos saudáveis (ZHONG *et al.*, 2000). Vários estudos demonstram o efeito anti-estresse (temperatura variável, imobilização) e anti-fadiga (esteira rolante, roda giratória), bem como uma melhora do aprendizado em ratos tratados com o extrato ou compostos isolados de PG (CARLINI, 1991). Evidências sugerem que PG pode promover a estimulação e/ou inibição da atividade cortical, podendo agir como modulador da neurotransmissão no sistema nervoso central (KIM *et al.*, 1999), aumentar a atividade colinérgica em modelos animais e atuar como agente neuroprotetor (PERRY *et al.*, 1999; RADAD et al., 2006).

1.3 Contribuição da Flora para a Farmacologia

A OMS estima que 65-80% da população mundial utiliza, de algum modo, plantas tradicionais para suprir as necessidades de assistência médica primária. Igualmente, observa-se a intensa utilização de terapias alternativas nos países desenvolvidos, incluindo produtos fitoterápicos como preventivos ou auxiliares no tratamento de várias doenças (YAMADA, 1998). Além disso, há inúmeros exemplos de drogas alopáticas desenvolvidas a partir de espécies vegetais. No contexto de déficits cognitivos, um produto de origem natural descoberto através da abordagem etnobotânica na Rússia, a galantamina (Reminyl®), foi isolado de *Galanthus woronowii*

Losinsk. (Amaryllidaceae) no início da década de 1950 (HEINRICH; TEOH, 2004). Galantamina está aprovada para o tratamento de DA, retardando o processo que leva à degeneração neuronal através da inibição da AChE, bem como da modulação de receptores colinérgicos nicotínicos (nAChR) (PIRTTILA *et al*., 2004). O resultado de vários estudos confirmando o efeito benéfico de GB e PG em ensaios relacionados a doenças neurodegenerativas ou déficit cognitivos, e a importância de produtos naturais enquanto anticolinesterásicos, renovou o interesse no estudo de outras plantas com perfil de uso tradicional semelhante. Podemos encontrar estudos de extratos ou compostos isolados de plantas usadas tradicionalmente em regiões como Índia, China, Japão e Brasil, verificando o efeito sobre o aprendizado/memória em roedores ou sobre fatores relacionados a doenças degenerativas. Cita-se, como por exemplo, *Celastrus paniculatus* Willdenow (NALINI *et al*., 1995; KUMAR; GUPTA, 2002), *Hypericum perforatum* Linnaeus (KUMAR *et al*., 2000; KHALIFA, 2001), *Bacopa monniera* Wettst (BHATTACHARYA *et al.*, 2000; KISHORE; SINGH, 2005) *Paullinia cupana* H.B. & K (ESPINOLA *et al*., 1997), *Panax quinquefolium* Linnaeus (LI *et al*., 1999) entre outras.

A flora é reconhecida como manancial de novas substâncias com potencial terapêutico, já que o reino vegetal é muito rico em compostos químicos, sub-produtos de rotas biossintéticas. Estes compostos chamados de metabólitos secundários, como taninos, alcalóides, flavonóides, etc, muitas vezes possuem função defensiva contra predadores (herbívoros, fungos, insetos, vírus), por serem indigestos ou mesmo tóxicos. Metabólitos secundários podem, igualmente, auxiliar na competição de espécies por ambientes comuns ou também ajudar na reprodução vegetal como atrativo de polinizadores (ELISABETSKY; SHANLEY,1994). A biogênese destes compostos está sob controle genético, mas deve-se considerar que fatores

ambientais, como o tipo de solo, época de ano e estágio de desenvolvimento do vegetal influenciam na produção e acumulação dos metabólitos secundários (BEGON *et al.*, 1987; HARBORNE, 1990). Os primeiros isolamentos de princípios ativos datam do século XIX, e atualmente existem inúmeros medicamentos derivados de plantas (HAMBURGER; HOSTETTMANN,1991; CURTIS-PRIOR *et al*.,1999), compostos simplesmente extraídos ou derivados da molécula natural com estrutura remodelada, ou ainda medicamentos semi-sintéticos. Compostos de origem natural têm tido papel proeminente na identificação de novos mecanismos de ação, tais como, vimblastina, vincristina, mescalina, os canabinóides, ioimbina, forskolina entre muitos outros (FARNSWORTH, 1990).

O papel de produtos naturais na terapêutica contemporânea é freqüentemente subestimado: das 20 substâncias mais vendidas no mundo em 1995, 11 continham um ingrediente ativo com estrutura baseada em produto natural (O´NEILL, 1998), num valor de U$ 16,53 bilhões. Mesmo assim, o potencial das plantas superiores como fonte de novos medicamentos ainda é pouco explorado mesmo em países, como o Brasil, ricos em biodiversidade e culturas populares que fazem uso destas espécies e, portanto, conhecem suas propriedades. Com base no uso e no papel inestimável que estas drogas têm no alívio do sofrimento humano, justifica-se a continuidade de pesquisas que busquem novas e melhores drogas derivadas de plantas.

1.4. *Ptychopetalum olacoides* Bentham como “Tônico dos nervos”.

Desde os primórdios da civilização, das práticas nômades e posteriormente com o desenvolvimento da agricultura permitindo a fixação de grupos populacionais em determinadas regiões, o manejo do meio ambiente permitiu ao homem adquirir uma relação estreita e intensa com a flora e fauna. Estas não apenas proviam fonte constante de alimento mas também supriam as necessidades básicas da população como material para abrigo temporário; além disso usando intuição e inteligência as comunidades passaram a diferenciar as diversas espécies vegetais que eram tóxicas, medicinais ou alimentícias (ENGELKE, 2003). Além da identificação, desenvolveram técnicas de extração e conservação de frutos, cascas, folhas e raízes. A utilização de plantas medicinais como fonte de alívio para doenças pode ser percebida em documentos de civilizações muito antigas na China, Índia e Europa. Para ilustrar como a utilização de plantas medicinais pode ser remota, foram encontrados amostras de pólen, de diversas espécies vegetais, em um sítio arqueológico (um cemitério Neanderthal), datado do Paleolítico Médio. Este sítio arqueológico está localizado na província de Shanidar, no Iraque, possui aproximadamente 60.000 anos, os pólens encontrados foram identificados em 6 gêneros que surpreendentemente continuam sendo usados como medicamentos na população rurais no Iraque (MOERMAN, 2005). Acredita-se que as farmacopéias tradicionais foram elaboradas ao longo de muitos séculos de experimentação (ELISABETSKY; SETZER,1985) e o conhecimento acumulado é preservado e transmitido entre gerações.

O uso de plantas medicinais brasileiras por populações indígenas foi descrito por descobridores portugueses em 1500 (PETROVICK *et al.*, 1999), sendo ainda amplamente comum no Brasil, tanto no meio rural quanto urbano. Este fato é bastante

propício tendo em vista que o Brasil é rico em recursos genéticos e, devido a diferenças climáticas, desenvolvem-se aqui diversas formações vegetais (Floresta Amazônica, Mata Atlântica, Cerrado entre outras). Igualmente, do ponto de vista cultural, o variado desenvolvimento do conhecimento a respeito destas plantas medicinais deve-se ao conjunto da sabedoria tradicional das populações indígenas, como por exemplo, o guaraná (*Paullinia cupana* H.B.K., Sapindaceae) ou contribuições trazidas por imigrantes da Europa (por exemplo, camomila - *Matricaria chamomilla* L., Asteraceae), Ásia (confrei - *Symphytum officinale* L., Boroginaceae) e África (erva-guiné - *Petiveria alliacea* L., Phytolaccaceae; SIMÕES *et al.*, 1995).

A descoberta de drogas a partir de plantas medicinais tem evoluído para incluir numerosos campos de investigação e vários métodos de análise. O processo começa tipicamente com botânicos, etnobotânicos, e etnofarmacólogos, que coletam e identificam a(s) planta(s) de interesse. A coleta pode envolver espécies com atividade biológica conhecida ou propriedade terapêutica alegada para as quais o(s) componente(s) ativo(s) não está(ão) ainda isolado(s), ou pode envolver um Taxon coletado fortuitamente para seleção posterior (triagem).

Estudos etnofarmacológicos entre populações cablocas do estado do Pará levaram à identificação de uma síndrome denominada “fraqueza dos nervo”, que inclui vários sintomas, sendo enfatizados: lassitude, impotência sexual e tremores (ELISABETSKY; SETZER, 1985; ELISABETSKY, 1987; ELISABETSKY *et al.*, 1992). A “fraqueza dos nervo” pode incluir sintomas de sedação ou hiperexcitação do sistema nervoso central, acomete especialmente pessoas de idade mais avançada, mas sintomas semelhantes podem ocorrer em jovens que estejam passando por períodos de estresse intenso, psicológico ou físico. A “fraqueza dos nervo” é combatida com “tônicos

nervosos" ou "tônicos nervinos". O conceito de "Tônico nervino é comum a muitos sistemas tradicionais de medicina (ELISABETSKY; SETZER, 1985, ELISABETSKY *et al.*, 1992). Espécies de muitas famílias são usadas como fonte de "tônico dos nervo", principalmente das famílias Apocynaceae, Convolvulaceae, Euphorbiaceae, Loganaceae, Malphiguiaceae e Rubiaceae. Dos 73 gêneros usados como "tônico dos nervos" e afrodisíacos, mais de 35% são conhecidas por conter alcalóides que poderiam ter atividade no SNC (ELISABETSKY *et al.*, 1992).

Em Belém do Pará, o Mercado "Ver-o-Peso" é o principal mercado da cidade e inclui uma parte inteiramente dedicada à chamada "medicina da terra", isto é, dedicada à venda de produtos medicinais obtidos a partir de recursos naturais (ELISABETSKY; SETZER, 1985). Informações coletadas entre raizeiros do "Ver-o-Peso" mostraram que dentre as principais plantas indicadas como tônico, para os nervos e tratar a "fraqueza dos nervos" estão as raízes de Marapuama, *Ptychopetalum olacoides* Bentham. Marapuama é uma palavra de origem tupi que significa "pau duro", e de fato a raiz desta espécie é muito resistente ao corte (PACHECO, 1980), inclusive de difícil manipulação por indústrias. A espécie é também famosa como afrodisíaca, e o nome contribui para esta fama. Em realidade, várias das espécies usadas para tratar "fraqueza dos nervos" pelos caboclos são mais populares como afrodisíacos do que por outras alegadas propriedades; algumas das espécies usadas como afrodisíacos são também indicadas para promover o apetite, como estimulantes dos nervos ou estimulantes gerais. Preparações a base de Marapuama são ainda indicadas no Pará para convalescentes de doenças em geral e, especificamente, das que afetam o SNC (como "derrames", lapsos de memória, dificuldades de concentração), ou durante períodos de estresse físico ou mental (ELISABETSKY; SIQUEIRA, 1998).

Embora *P. olacoides* (PO) seja usada pelos caboclos amazônicos como "tônico dos nervos", afrodisíaca, moduladora de apetite e de tremores (ELISABETSKY, 1987; ELISABETSKY *et al*., 1992), é mais conhecida pelo mercado de fitoterápicos como afrodisíaco (YOUNGKEN, 1921; STEINMETZ, 1962; SANGIRARDI JR., 1981; FONSECA, 1981; CABRAL; AGRA, 1996). Usualmente, suas raízes são preparadas em infusão alcoólica em cachaça ou em vinho, mas é encontrada também em várias outras formulações comerciais (pó seco, liofilizado seco por "*spray-dryer",* tinturas, misturas de extratos). O uso de PO é também amplamente documentado em outros lugares da Amazônia, inclusive a não-brasileira (SIQUEIRA, 1997). Marapuama é encontrada ainda em fitoterápicos no Brasil, Europa e Estados Unidos da América (por exemplo, Catuama®, Energil®, Catuaba Selvagem®, Power-up, Vigorax, etc) sendo comercializadas inclusive via internet (Herbal vY for Men, Composto Alimentar Forte Tônico, etc), freqüentemente em formulações de marapuama em associação com outras plantas (DA SILVA, 2001).

1.5. Farmacologia de *Ptychopetalum olacoides* Bentham

As propriedades afrodisíacas desta planta atraem não apenas o mercado farmacêutico como a comunidade científica. Num estudo clínico preliminar realizado no Instituto de Sexologia em Paris (França), 262 pacientes que apresentavam impotência e diminuição da libido foram tratados com 1 a 1,5 g de extrato hidroalcoólico (4:1) de PO durante 2 semanas (4 a 6 x dia), sendo que 62% dos pacientes apresentaram melhoras no desempenho sexual e 51% relataram ação benéfica sobre a ereção (WAYNBERG, 1990). Há evidências da relação de dopamina com o comportamento

sexual (BOWERS *et al.*, 1971; SAIBENE, 1997), assim como a transmissão noradrenérgica também está relacionada com o comportamento sexual (PFAUS; EVERITT, 1994).

Uma boa parte dos dados disponíveis sobre a farmacologia de *P. olacoides* foi obtida com um extrato específico (EEPO) objeto de patente do nosso grupo de pesquisa (PI0205432-9/ PI0307647-4, INPI/Br, 2000). A descrição da síndrome caracterizada como “fraqueza dos nervos” pela população nativa da Amazônia pode ser também vista como uma descrição de depressão. EEPO foi ativo (50-200 mg/kg) em testes com valor preditivo para antidepressivos, como proteção da letalidade induzida por ioimbina e reversão de ptose induzida por reserpina em camundongos (SIQUEIRA *et al.* 1998; ELISABETSKY; SIQUEIRA, 1998), sugerindo uma possível ação no sistema noradrenérgico e/ou serotoninérgico. EEPO também mostra atividade antidepressiva nos modelos de nado forçado e cauda suspensa em camundongos, nos quais os receptores β-adrenérgico and D_1 dopaminérgico parecem ter papel importante (PIATO *et al*., 2006).

EEPO (60, 100 e 200 mg/kg) preveniu a expressão de estereotipia induzida por apormorfina (agonista dopaminérgico), podendo indicar uma interação indireta com o sistema dopaminérgico, ou ainda com o sistema colinérgico, uma vez que este não se mostrou efetivo em outros testes diretamente relacionados com o sistema dopaminérgico (SIQUEIRA *et al.* 1998; ELISABETSKY; SIQUEIRA, 1998). EEPO (200 e 300 mg/kg) ainda diminuiu a temperatura corporal e nas doses de 100 e 200 mg/kg potencializou o sono barbitúrico em camundongos (SIQUEIRA, 1997).

Os efeitos de EEPO (30-300 mg/kg) em camundongos submetidos ao teste da placa perfurada demonstraram efeito levemente ansiogênico, com redução significativa

do número de espreitamentos e levantamentos, além de aumento da latência para o primeiro espreitamento (DA SILVA *et al*., 2002). O teste de rota-rod tem sido usado para avaliar efeitos não-específicos de drogas ativas no SNC tais como relaxamento muscular, sedação ou prejuízo na coordenação motora (DALLMEIR; CARLINI, 1981; TANG *et al*., 1995; HELTON *et al*., 1998). Animais tratados com EEPO (30-300 mg/kg) foram submetidos ao teste de rota-rod 30 e 60 minutos após a administração do extrato; em nenhuma dose os animais apresentaram alteração de coordenação motora (DA SILVA, 2001).

Drogas que aumentam a memória são chamados de pró-mnésicas ou ampliadores cognitivos (STAHL, 2002). Usando-se o paradigma de esquiva inibitória em camundongos adultos (2,5 meses) com 24 horas de intervalo entre treino e teste, constatou-se que EEPO quando administrado intraperitonialmente, nas doses de 50 e 100 mg/kg, aumentou significativamente (efeito pró-mnésico), e de maneira dose-dependente, a evocação, sem afetar a aquisição e a consolidação da memória (da Silva, 2001).

Os usos tradicionais e dados obtidos com extrato etanólico de *Ptychopetalum olacoides* (EEPO) sugerem a presença de compostos bioativos com possíveis interações com o sistema dopaminérgico (afrodisíaco, antidepressivo, antitremor, modulador do apetite, adaptógeno); com o sistema noradrenérgico e/ou serotoninérgico (afrodisíaco, antidepressivo, modulador do apetite); ou ainda com o eixo hipotálamo-hipófise-adrenal (adaptógeno e antidepressivo) (ELISABETSKY; SIQUEIRA, 1998).

2. OBJETIVOs

2.1. OBJETIVO Geral

O objetivo geral deste estudo foi dar início ao esclarecimento do mecanismo de ação do extrato etanólico de *Ptychopetalum olacoides* denominado de EEPO (PI0205432-9/ PI0307647-4, INPI/Br, 2000).

2.2. OBJETIVOS ESPECÍFICOS:

I) Investigar os efeitos do extrato EEPO, administrado agudamente pela via oral, sobre a evocação da memória aversiva de longa duração, em camundongos adultos (2,5 meses), utilizando a tarefa de esquiva inibitória;

II) Investigar os efeitos do extrato EEPO, administrado agudamente pela via oral, sobre a evocação da memória aversiva de longa duração, em camundongos com 14 meses, utilizando a tarefa de esquiva inibitória;

III) Investigar os efeitos do extrato EEPO, administrado agudamente pela via oral e pela via intraperitoneal, sobre a memória aversiva de curta duração, em camundongos adultos, utilizando a tarefa de esquiva inibitória;

IV) Investigar os efeitos do extrato EEPO, administrado agudamente pela via intraperitoneal, sobre a evocação da memória de curta duração em camundongos com 14 meses, utilizando a tarefa de esquiva inibitória;

V) Investigar os efeitos do extrato EEPO administrado pela via oral sub-crônicamente (21 dias) sobre a memória de longa duração em camundongos adultos, utilizando a tarefa de esquiva inibitória;

VI) Investigar o efeito da administração de EEPO, administrado agudamente pela via intraperitoneal, sobre a memória não aversiva em camundongos adultos, utilizando a tarefa de reconhecimento de objetos;

VII) Investigar os efeitos de antagonistas dopaminérgicos, serotoninérgicos e noradrenérgicos sobre a ação pró-mnésica de EEPO em camundongos adultos, utilizando a tarefa de esquiva inibitória;

VIII) Investigar os efeitos do extrato EEPO, agudamente pela via intraperitoneal, nas amnésias induzidas por escopolamina e MK-801 em camundongos adultos, utilizando a tarefa de esquiva inibitória.

PARTE II

Artigos Científicos

Capítulo 1. DA SILVA AL, PIATO AL, BARDINI S, NETTO CA, NUNES DS, ELISABETSKY E. Memory retrieval improvement by *Ptychopetalum olacoides* in young and aging mice. J Ethnopharmacol., 95(2-3):199-203, 2004.

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Memory retrieval improvement by *Ptychopetalum olacoides* in young and aging mice

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Abstract

Amazonian peoples use traditional remedies prepared with *Ptychopetalum olacoides* (PO) roots for treating various age-related conditions. This study shows that a single intraperitoneally (i.p.) administration of *Ptychopetalum olacoides* ethanol extract (POEE, 50 and 100 mg/kg) improved memory retrieval in step-down inhibitory avoidance ($P \leq 0.05$ and $P \leq 0.01$, test session latency 102 [19.38–300] and 192 [91.3–300] s, respectively versus control 24.7 [12.9–89.6]), without interfering with acquisition or consolidation in adult (2.5-month-old) mice. Comparable results were obtained with POEE given p.o. at 800 and 1000 mg/kg ($P \leq 0.05$ and $P \leq 0.01$, 52.7 [19.5–297.2] and 85.7 [44.4–260.4] versus control 20.5 [8–92.6]). Moreover, memory amelioration was also observed ($P \leq 0.01$) in aging (14 months) mice presenting memory deficit (14.95 [10.8–41]) as compared to adult (2.5 months) mice (57 [15.7–141.2]), with the extract given acutely i.p. 100 mg/kg (300 [133.1–300] versus control 14.95 [10.8–41]) or p.o. 800 mg/kg (28.4 [15.1–84.6] versus control 11.5 [7.8–23.3]). Indeed, aging mice treated with POEE (800 mg/kg, p.o.) performed as well as adult mice. Consistently with its traditional use, the data suggest that POEE facilitates memory retrieval. Although the antioxidant and acetylcholinesterase inhibitory properties previously described for this extract may be of relevance, the molecular mechanism(s) underlying the improvement in memory retrieval here reported merit further scrutiny.

Keywords: *Ptychopetalum olacoides*; Marapuama; Memory retrieval; Step down; Aging; Neurodegenerative disorders

1. Introduction

Cognitive deficits are often observed in old humans, as well as in various neurological conditions. It has been previously proposed (Kubanis and Zornetzer, 1981) that memory retrieval in the elderly appears to be more impaired than acquisition or storage. Moreover, the first symptoms in Alzheimer's disease include impairment of new information storage or retrieval (Dringenberg, 2000). With the increase of life expectancy and the consequent increase in the number of patients suffering from brain degenerative disorders, the search for products able to reduce or minimize cognitive deficits associated with aging has become even more attractive. Plant species traditionally used in non-western medical systems for enhancing cerebral function, like *Gingko biloba* and *Panax ginseng*, have proven to be effective in animal memory tests and useful in cognitively impaired humans (LeBars et al., 1997; Yamaguchi et al., 1997; Curtis-Prior et al., 1999; Zhong et al., 2000).

Abbreviations: PO, *Ptychopetalum olacoides*; POEE, *Ptychopetalum olacoides* ethanol extract; DMSO, dimethyl sulphoxide; ACTH, adrenocorticotrophic hormone

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Ptychopetalum olacoides (PO) Bentham (Olacaceae), known as marapuama, muirapuama or miriantã, is traditionally used in the Brazilian Amazon as a "brain tonic", specially by those recovering from central nervous systems (CNS) illnesses, by the elderly, and in general to cope with stressful situations; the pharmacological meaning and specific properties of such tonics have yet to be elucidated (Elisabetsky et al., 1992; Elisabetsky and Siqueira, 1998a). Therapeutic outcomes expected from the use of "brain tonics" include facilitating the recovery of cognitive and motor deficits after brain injuries (such as stroke), as well as improvement of cognitive functions, like alertness and memory, in the elderly.

Ptychopetalum olacoides is currently found in dozens of herbal products and multivitamin supplements in several American and European countries (Elisabetsky, 1987; Elisabetsky and Siqueira, 1998b; Paiva et al., 1998), with a diverse range of alleged effects. Previous pharmacological studies demonstrated that an ethanol extract of PO roots potentiated yohimbine-induced lethality, reversed reserpine-induced ptosis and prevented apormophine-induced stereotypy in mice (Siqueira et al., 1998), favorably influenced performance in the forced swimming test (Paiva et al., 1998) and acted as an anxiogenic in the hole board model (da Silva et al., 2001), supporting the hypothesis that *Ptychopetalum olacoides* has central nervous system effects likely to affect the dopaminergic and noradrenergic systems.

The purpose of this study was to investigate the effects of *Ptychopetalum olacoides* ethanol extract (POEE) on memory modulation (acquisition, consolidation and retrieval) in adult (2.5-month-old) and aging mice (14-month-old), using the step-down inhibitory avoidance test.

2. Material and methods

2.1. Animals

Experiments were performed using male adult mice, CF1 strain, received from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) immediately after weaning (21 days) or at 14 months of age (35–50 g, aging mice). Animals were maintained in our own animal facility room under controlled environmental conditions (22 ± 1 °C, 12 h-light/12 h-dark cycle, free access to food [Nuvilab CR1] and water); animals were maintained up to 10 weeks of age (25–40 g, adult mice), or in the case of aging mice for at least two weeks before the experiments. All procedures were carried out in accordance with institutional policies on experimental animal handling.

2.2. Plant material

Roots of *Ptychopetalum olacoides* Bentham (Olacaceae) were collected in Pará State (Brazil) and identified by Nelson Rosa. Voucher specimens were deposited at the herbarium of the Goeldi Museum (MPEG 108.036).

2.3. Preparation of extract

Ptychopetalum olacoides ethanol extract (POEE) was prepared as detailed elsewhere (Elisabetsky and Siqueira, 1998a). Briefly, the dried roots were peeled and the ground bark (2.5 kg) was extracted with ethanol (12 L) using a Soxhlet apparatus (40 h). The extract was evaporated under reduced pressure resulting in the POEE (150 g, 6% yield).

2.4. Drugs

Saline (NaCl 0.9%) and dimethyl sulphoxide (DMSO) were acquired from Delaware. POEE (10, 30, 50 and 100 mg/kg i.p. and 500, 800, and 1000 mg/kg p.o.) was dissolved in a 20% DMSO solution (in water).

2.5. Step-down inhibitory avoidance

The test used was adapted from Netto and Izquierdo (1985) and from Maurice et al. (1994). Mice were habituated in a dimly-lit room for at least 30 min before the experiments. The inhibitory avoidance training apparatus was a plastic box (30 cm × 30 cm × 40 cm), with a platform (5 cm × 5 cm × 4 cm) fixed in the center of the grid floor. Each mouse was placed on the platform and the latency to step down (four paws on the grid), was automatically recorded in training and test sessions. In the training session, the mouse received a 0.3 mA scrambled foot shock for 15 s, upon stepping down. Animals exhibiting step-down latencies greater than 30 s in training were excluded from experiments; less than 5% of the animals met this exclusion criterion. The test session was performed 24 h later, with the same procedure except that no shock was administered after stepping down; an upper cut-off time of 300 s was set.

Drugs (saline, DMSO or POEE) were administered intraperitoneally (i.p.) as follows: 30 min before training, to evaluate effects on task acquisition (in this case, no exclusion criterion was applied); immediately after training, to evaluate effects on memory consolidation, and 30 min before testing, to assess memory retrieval.

In order to assess memory retrieval with orally (p.o.) administered treatments, the drugs (saline, DMSO or POEE) were administered 90 (adult mice) or 120 min (aging mice) before the test session.

2.6. Statistical analysis

The step-down latencies are expressed as medians (interquartile ranges). Data were analyzed by Kruskal–Wallis non-parametric analysis of variance; comparisons between groups were run using the Mann–Whitney *U* test (two-tailed). Comparisons between training and test sessions within each group were made by the Wilcoxon test. The Spearman-rank correlation coefficient was used to check dose-effect associations.

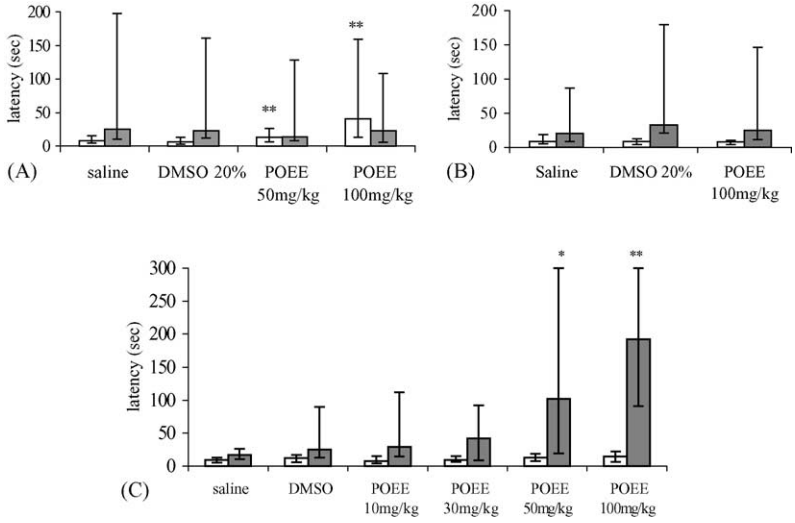

Fig. 1. Effect of *Ptychopetalum olacoides* ethanol extract (POEE) given i.p. 30 min prior to training (A), immediately post-training (B), and 30 min prior to testing (C) on retention test performance of adult mice trained in step-down inhibitory avoidance (0.3 mA footshock, 24 h training-test interval). DMSO, dimethyl sulphoxide 20%. $N = 20$ per group. Each column represents the median (interquartile ranges) of training (light columns) or test (dark columns) session latencies $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference compared with controls (saline and DMSO) in Mann–Whitney U test, following Kruskal–Wallis.

3. Results

Confirming that learning and memory took place with the training paradigm used in this study, there were significant and consistent differences ($P < 0.05$) between training and test session latencies, in both saline-treated and DMSO-treated adult (2.5 month-old) and aging (14-month-old) groups. No differences in latencies were found in the various groups of training sessions, except for POEE (50 and 100 mg/kg) injected pre-training, when significant ($P < 0.01$) increases in step-down latencies were observed. Due to the marked increase in training session, latency induced with POEE 100 mg/kg administered pre-training, this is the only group where there is no significant difference between the training and test sessions (Wilcoxon, $P = 0.22$), although its test latency does not differ from those observed in all of the other similarly treated groups (Kruskal–Wallis, $P = 0.42$).

The doses found to be effective in facilitating retrieval were tested for potential effects in memory acquisition and consolidation. When tested for effects in memory acquisition, it was found that the higher POEE dose (100 mg/kg) significantly increased the latency in the training session; therefore, only the lowest dose effective in facilitating retrieval (50 mg/kg) could be properly analyzed for its effects on memory acquisition. No differences in test session performance were found after either pre- (Fig. 1A) or post-training (Fig. 1B) DMSO and POEE administrations. Most importantly, POEE (50 and 100 mg/kg i.p. or 800 and 1000 mg/kg p.o.) significantly improved retrieval ($P < 0.05$ and $P < 0.01$, respectively) in adult mice that received pre-test injections (Figs. 1C and 2). Correlation analysis using the Spearman test showed that this effect is dose-dependent ($r = 1$, $P < 0.001$).

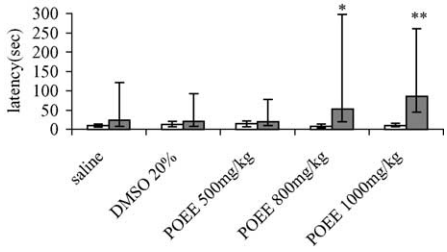

Fig. 2. Effect of *Ptychopetalum olacoides* ethanol extract (POEE) given p.o. 90 min prior to retention test performance of adult mice trained in step-down inhibitory avoidance (0.3 mA footshock, 24 h training-test interval). DMSO, dimethyl sulphoxide 20%. $N = 20$ per group. Each column represents the median (interquartile ranges) of training (light columns) or test (dark columns) session latencies $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls (saline and DMSO), in Mann–Whitney following Kruskal–Wallis.

With aging (14 months) saline-treated mice there were no significant latency differences between training and test sessions, evidencing learning and memory deficits for this task; these aging mice also presented a retrieval deficit ($P < 0.05$) when compared to adult (10-week-old) animals. POEE (100 mg/kg i.p. and 800 mg/kg p.o.) administered pre-test improved memory retrieval (Figs. 3 and 4) in aging mice when compared to their controls ($P < 0.01$).

4. Discussion

This study showed that *Ptychopetalum olacoides* ethanol extract (POEE) improves retrieval in the step-down inhibitory avoidance test in a dose-dependent way, affecting neither memory consolidation nor task acquisition. With reference to the influences of locomotor activity (Zarrindast et al.,

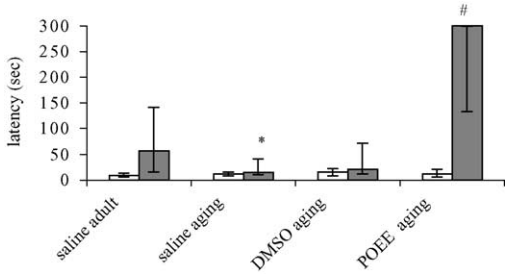

Fig. 3. Effect of *Ptychopetalum olacoides* ethanol extract (POEE) given i.p. 30 min prior to retention test performance of aging mice trained in step-down inhibitory avoidance (0.3 mA footshock, 24 h training-test interval). DMSO, dimethyl sulphoxide 20%; POEE, POEE 100 mg/kg. Each column represents the median and interquartile ranges of training (light columns) or test (dark columns) session latencies. $N = 20$ per group. $^{*}P < 0.05$, as compared to saline-treated adult mice, $^{\#}P < 0.01$ as compared to saline- and DMSO-treated aging and saline-treated adult mice, in Mann–Whitney following Kruskal–Wallis.

1996), although the step-down inhibitory avoidance test is more reliable than other methods for memory assessment, false positives related to diminished motor activity leading to increased test session latencies should, nevertheless, be considered. In this case, influence on motor activity by the extract can be ruled out since previous studies demonstrated that POEE (30–100 mg/kg) affected neither locomotor activity in the hole-board test nor motor coordination as evaluated in the rota-rod test (da Silva et al., 2001). However, POEE decreases exploratory behavior (head dipping) in the hole-board test, a result compatible with the profile of anxiogenic drugs (Takeda et al., 1998; da Silva et al., 2001).

The step-down inhibitory avoidance task is a classic paradigm to assess memory with a strong aversive component (Cahill et al., 1986). An anxiogenic effect could account for the increased latencies observed in training sessions when POEE was injected pre-training (with no significant effect in test session latencies with this particular drug administration paradigm). It has been reported that stress hormones, when administrated pre-test, may enhance memory retrieval (Izquierdo et al., 2002). Catecholamines and glucocorticoids have been reported to improve memory consolidation and impair memory retrieval (Cahill and McGaugh, 1998; Roozental, 2002). ACTH, adrenaline, vasopressin and β-endorphin enhanced step-down memory retrieval at low doses in 3 month-old rats (Izquierdo et al., 2002). Furthermore, low to moderate doses of neuromodulators and peripheral hormones can be associated with memory retrieval enhancement (Izquierdo et al., 2000). Further studies are, therefore, necessary to distinguish POEE effects on anxiety and memory retrieval. It is noteworthy that POEE did not present different effects depending on the route of administration, since administration by the intraperitoneal or oral routes improved memory retrieval dose-dependently in adequate dose ranges. In the aging mice oral gavage was given 120 min before session tests (instead of 90 min in adult mice) due to age-related changes in drug absorption, including increased gastric pH, reduced gastrointestinal motility and decreased absorption surface (Gareri et al., 2000).

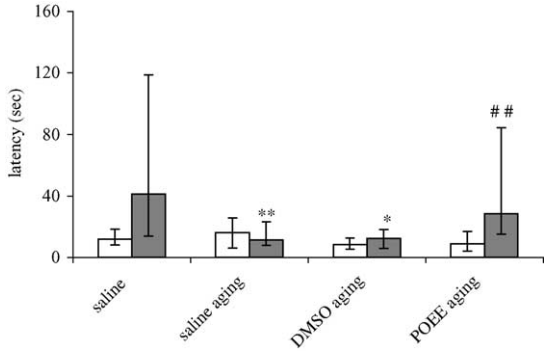

Fig. 4. Effect of *Ptychopetalum olacoides* ethanol extract (POEE) given p.o. 120 min prior to retention test performance of aging mice trained in step-down inhibitory avoidance (0.3 mA footshock, 24 h training-test interval). DMSO, dimethyl sulphoxide 20%; POEE, 800 mg/kg. Each column represents the median and interquartile ranges of training (light columns) or test (dark columns) session latencies. $N = 20$ per group. $^{*}P < 0.05$ as compared to saline adult mice, $^{**}P < 0.01$ as compared to saline adult mice, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared to saline aging mice, in Mann–Whitney following Kruskal–Wallis.

The molecular mechanism by which POEE facilitates memory retrieval remains to be elucidated. Its behavioral profile (Siqueira et al., 1998; da Silva et al., 2001) suggests interactions with a diversity of neurotransmitters (including noradrenaline, serotonin and dopamine), a pattern consistent with the current understanding of the modulation of memory processes (Izquierdo et al., 2000). Moreover, we have recently reported that POEE has the ability to inhibit AChE as evaluated by in vitro and ex vivo assays (Siqueira et al., 2003), indicating improvement of cholinergic function as a potential neurochemical correlate of POEE effects on memory retrieval deficit in aging mice.

It is estimated that within the next 50 years, approximately 30% of the world population will be aged 65 years or older (Youdim and Joseph, 2000); more importantly, by the year 2025 70% of world's older population will be living in developing countries (Kalache et al., 2002). According to Chaimowicz (1997), the Brazilian population has been aging quickly (aging population index of 6.4 in 1960 reaching 13.9 in 1991), an increase of more than 100% in only three decades. It is, therefore, crucial to develop appropriate health care means for age-related neurodegenerative disorders, including cognitive deficits. It is worth noting that the results of this study are remarkably in agreement with therapeutic claims by traditional communities for *Ptychopetalum olacoides*-based home-made remedies. Particularly because experimental results are consistent with anecdotal reports of therapeutic effects in humans taking such remedies orally, the data revealed in this study are of relevance for the development of drugs for managing memory deficits associated with age and with a number of neurological disorders.

Considering both the lack and the need of drugs proven to be effective in improving memory retrieval (Espinola et al.,

1997; Abe and Saito, 2000; Bhattacharya et al., 2000; Vohora et al., 2000), the specific facilitating effect on retrieval reported here is of particular interest and deserves further scrutiny, including POEE effects in memory paradigms devoid of aversive components.

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Capítulo 2. DA SILVA AL, PIATO AL, FERREIRA JG, MARTINS BS, NUNES DS., ELISABETSKY E. Promnesic effects of *Ptychopetalum olacoides* in aversive and non aversive learning paradigms. (Submetido ao Journal of Ethnopharmacology).

Promnesic effects of *Ptychopetalum olacoides* in aversive and non aversive learning paradigms.

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Abstract

Home made remedies with *Ptychopetalum olacoides* (PO) roots are used by Amazonian peoples for treating various age-related conditions. We previously reported that *P. olacoides* ethanol extract significantly improved step-down inhibitory avoidance long-term memory in adult and reversed memory deficits in aging mice. Adding to previous data, this study shows that a single ip administration of *P. olacoides* ethanol extract (POEE 50 and 100 mg/kg) improved step-down inhibitory avoidance short-term memory (STM) 3 h after training in adult (2.5 month) mice; comparable results were obtained with POEE given po at 800 mg/kg. Moreover, memory improvement was also observed in aging (14 months) mice presenting memory deficit as compared to adult mice. Furthermore, POEE (100 mg/kg) improved non aversive memory systems in adult mice in an object recognition paradigm. Consistently with its traditional use this study ad to previously reported data and reinforces that POEE facilitates memory processes. Although the acetylcholinesterase inhibitory properties described for this extract may be of relevance for improving memory processes, the molecular mechanism(s) underlying the memory improvement here reported needs further scrutiny.

Key words: *Ptychopetalum olacoides,* Marapuama, long-term memory, short-term memory, step-down inhibitory avoidance, object recognition, aging.

1. Introduction

The aging processes cause a large number of detrimental changes in the organism at all levels of biological organization, ultimately resulting in limited functionality and decreased homeostasis; in the brain, aging leads to synaptic damage and neuronal death, and associated neurodegenerative diseases (Barja, 2002; Jelic and Winbland, 2003). Progressive memory loss, dementia, and cognitive deficits are currently seen as medical and social problems of disastrous dimension (Kidd, 1999). While it is estimated that within the next 50 years approximately 30% of the world population will be aged 65 years or older (Youdim and Joseph, 2001), it is even more worrying the presumption that, by the year 2025, 70% of world older population will be living in developing countries (Kalache et al., 2002). In Brazil alone, the 60+ years old population is estimated to be 14.5 million people (Chaimowicz, 1997; Barreto et al., 2004). Therefore, developing appropriate health care practices for age-related neurodegenerative disorders, including cognitive deficits, is of paramount importance worldwide.

A number of different forms of learning and memory involving diverse neural systems are currently recognized (Milner et al., 1998; Zyzak et al., 1995). Rather than a single entity, there are at least two major memory forms: a capacity for conscious recollection of facts and events (declarative memory), and a collection of nonconscious learning capacities (non declarative memory) (Squire and Zola, 1996; Milner et al., 1998; Beggs et al., 1999). Three (acquisition, consolidation, and retrieval) or four (encoding, consolidation, storing, and retrieving) distinguished components or phases have been recognized in the field (Lechner et al., 1999; McGaugh and Izquierdo, 2000; Amadio et al., 2004) and the memory for a specific episode may fail due to errors occurring during one of these phases or components.

Additionally, memories are also classified according to their duration, as short- (STM) and long-term memory (LTM) (Vianna et al., 2000b). Assisting in establishing these

memories as separate entities the main structures involved in STM and LTM were identified (Izquierdo et al., 1998a, 1999), as well as treatments that block STM without affecting LTM (Izquierdo et al., 1998b). While long-term memory (LTM) is being consolidated it is believed that a short-term memory (STM) system holds information for 1-6 hours (Milner et al., 1998; Izquierdo et al., 1999; Albright et al., 2000; Barros et al, 2005).

Studies of learning and memory in animals involve a variety of behavior experimental models. The method most commonly used for aversively motivated tasks is the step-down inhibitory avoidance, based on learning to resist from stepping down from a platform in order to avoid a mild foot shock; this assay is considered a model of human declarative (episodic) memory. Another model of human declarative memory involves a natural preference displayed by rodents for novel objects: the object recognition tasks is considered a non-aversive, non-spatial type of memory, and have been shown to be very useful for assessing changes in neuronal function induced by drugs, aging, and cholinergic dysfunction (Deschaux et al., 1997; Puma et al., 1999; Lebrun et al., 2000).

We have previously shown that an ethanol extract of *Ptychopetalum olacoides* Bentham (Olacoides), traditionally used by Amazonian communities as a "nerve tonic" (Elisabetsky and Siqueira, 1998), posses antioxidant properties *in vitro* (Siqueira et al., 2002) and *in vivo* (Siqueira et al., 2004b), act as neuroprotector in hippocampal slices submitted to oxygen and glucose deprivation (Siqueira et al., 2004a), and has anticholinesterase activity *in vitro* and *in vivo* at various relevant brain areas (Siqueira et al., 2003). Relevant to this study, it was shown that the extract specifically facilitates the retrieval of long-term memory (inhibitory avoidance) in mice; it is also noteworthy that the memory deficit observed in aging (14 month old) mice was reversed by orally given extract (da Silva et al., 2004).

Considering the different nature and composition of LTM and STM, aversive and non aversive motivated memories, the purpose of this study was to advance the knowledge of the

memory facilitating properties of POEE. For that matter, the effects of POEE were analyzed on the step-down STM (acquisition, consolidation and retrieval in adult, and retrieval in aging mice) and on the object recognition test (3 and 24 h training-test intervals, acquisition, consolidation and retrieval).

2. Material and Methods

2.1. Animals

Experiments were performed using male (CF1) adult albino mice, received from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) with 2.5 or 14 months of age (35-50g). Animals were maintained in our own animal facility under controlled environmental conditions (22 ±1^{o}C, 12 hr-light/dark cycle, free access to food [Nuvilab CR1] and water), for at least two weeks before the experiments. All procedures were carried out in accordance with our institutional policies on experimental animals handling, which follows the NIH guidelines (NIH Guide for Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985).

2.2. Preparation of Extract

Roots of *Ptychopetalum olacoides* Bentham (Olacaceae) were collected in Pará (Brazil), and identified by Nelson Rosa (MPEG 108.036 voucher deposited at the Goeldi Museum Herbarium). *Ptychopetalum olacoides* ethanol extract (POEE) was prepared as detailed elsewhere (Elisabetsky and Siqueira, 1998). Briefly, dried ground plant material (2.5 kg) was extracted with ethanol (12 L), using a Soxhlet apparatus (40 h). The extract was evaporated under reduced pressure resulting in the POEE (150 g, 6% yield).

2.3. Step-down inhibitory avoidance

The test used was adapted from Netto and Izquierdo (1985), Maurice et al. (1994), and Khalilzadeh et al. (2005). Mice were habituated in the dim lighted room for at least 30 minutes before the experiments. The inhibitory avoidance training apparatus was a plastic box with 30 x 30 x 40 cm, with a platform (5 x 5 x 4 cm) fixed in the center of the grid floor. Each mouse was placed on the platform and the latency to step down (four paws on the grid), was automatically recorded in training and test sessions. In the training session, the mouse received a 0.3 mA scrambled foot shock for 15 sec, upon stepping down. Animals exhibiting step down latencies greater than 30 sec in training were excluded from experiments; less than 5% of the animals met this exclusion criterion. The test session was performed 3 h later (short-term, STM), with the same procedure except that no shock was administered after stepping down; an upper cut-off time of 300 sec was set. N=20 per treatment group.

2.4. Object Recognition

We followed the method described by Deschaux et al. (1997), Slane McQuade et al. (2002), King et al. (2004), de Lima et al. (2005): the task took place in a 60 cm diameter open field surrounded by 30 cm high acrylic walls (Fig. 1). All animals were submitted to a habituation session, freely exploring the object free open field for 5 min. Twenty-four hours latter, training took place by placing individual mice for 5 min at the field in which two identical objects (A1 and A2; identical Lego toys) were placed in a symmetrical position about 10 cm away from the wall; exploration was defined as the time spent sniffing or touching the object with the nose and/or forepaws. Animals failing to explore objects for at least 10 sec were discarded from the study (roughly 10 % of all animals). Test sessions were performed either 3 h or 24 h after training, when mice were allowed to explore the open field for 5 min in the presence of one familiar (A) and one novel (B or C for 3 and 24 h, respectively) object. All

objects presented similar textures, colors, and sizes, but distinctive shapes; after each trial objects were washed with 10% ethanol to discard smells or residues. According to Sik et al. (2003) and Lima et al. (2005) a recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$ [T_A = time spent in exploring the familiar object A; T_B = time spent in exploring the novel object B (or C)]. N=8-13 per group.

2.5. Drug administration

Saline (NaCl 0.9 %) and dimethyl sulphoxide (DMSO) were acquired from Delaware. POEE (50, 100 and 800 mg/kg) was dissolved in DMSO 20 % (v/v). Saline, DMSO 20%, POEE 50 or 100 mg/kg were administered intraperitoneally (ip) as follows: 30 min before training to evaluate effects on task acquisition (in treated groups no exclusion criteria was applied); immediately after training to evaluate effects on memory consolidation, and 30 min before testing to assess memory retrieval, in both behavioral paradigms. Oral treatments (saline, DMSO or POEE 800 mg/kg) were administered 90 min (adult mice) before the test session for assessing the effects of POEE in step-down retrieval.

2.6. Statistical analysis

Step-down latencies are expressed as medians [interquartile ranges]. Data were analyzed by Kruskal-Wallis non-parametric analysis of variance; comparisons among treatment groups were completed through Mann-Whitney U-test (two-tailed), and within treatment groups by the Wilcoxon test. Recognition index are expressed as mean (standard error of mean); comparison among groups were done by one way ANOVA followed by Duncan post hoc test.

3. Results

3.1. Step-down inhibitory avoidance

Confirming that learning and memory took place with the training paradigm used in this study, there were significant ($p< 0.05$ for saline and $p<0.01$ for DMSO) and consistent differences between training (saline: 13.6 [3.2-19.0]sec; DMSO: 4.8 [3.3-9.4]sec) and test session latencies (saline:11.5 [7.1-35.2]sec; DMSO: 15.2 [7.6-32.7] sec), in both saline- and DMSO-treated adult (2.5 months old) mice (Figure 2a). No meaningful differences in training latencies were found among the various groups, except for the group treated pre-training with POEE 100 mg/kg that shows a slight but significant ($p<0.05$) increase in training latency (15 [9.6/86.7] sec) when compared to saline group (13.6 [3.2/19.0] sec) (Figure 2A); the difference between the training (15.0[9.6/86.7] sec) and test (120.7 [25.6/300] sec) latencies for this POEE (100 mg/kg) treated group remained significant (Wilcoxon, $p<0.01$).

The effect of POEE in memory acquisition can be seen in Figure 2 (A, B). With POEE administered ip (Fig. 2A), Kruskal-Wallis revealed a significant drug effect ($H (3) = 20.6$, $p<0.01$); Mann-Whitney U-test showed that POEE 50 (41.6[20.6/55.6] sec) and 100 mg/kg (120.7[25.6/300] sec) caused significantly and comparable increases in test session latencies ($p<0.05$ and $p<0.01$, respectively) compared to controls (saline and DMSO). In addition, with oral administration, there were significant and consistent differences ($p< 0.05$) between training (saline: 9.7 [4.8-15.6] sec; DMSO: 9.4[3.9-15.9] sec; POEE 800 mg/kg: 7.9[5.5-13.2] sec) and test session latencies (saline:17.7[5.1-36.8] sec; DMSO: 15.6[9.9-88.8] sec; POEE 800 mg/kg: 35.2[18.3-113.9] sec), in both saline- and DMSO-treated adult (2.5 months old) mice (Figure 2B). A significant drug effect ($H(2)$ 6.38, $p<0.05$) was also observed when POEE (800 mg/kg) was administrated orally (Fig. 2B), inducing a significantly higher test session latency (35.2 [18.3/113.9] sec) (Mann-Whitney U-test, $p< 0.05$, compared with saline (17.7[5.1/ 36.8] sec) and DMSO (15.6[9.9/88.8] sec).

The effect of POEE in memory consolidation can be seen in Figure 3 (A, B). For ip administration (Fig. 3A), comparisons within treatment groups showed a significant differences ($p< 0.01$) between training (saline: 10.9 [4.3-20.7] sec; DMSO: 10.6 [5.7-18.0] sec; POEE 50 mg/kg: 12.2 [6.3- 18.0] sec; POEE 100 mg/kg: 14.9[10.1-18.1] sec) and test session latencies (saline:22.6 [12.7-38.1] sec; DMSO: 26.2[15.2-59.7] sec; POEE 50 mg/kg: 63.2 [31.0- 94.7] sec; POEE 100 mg/kg: 287 [34.3-300] sec), in adult (2.5 months old) mice. Kruskal-Wallis revealed a significant drug effect ($H (3) = 17.349$ $P<0.05$); POEE 50 (63.2[31.0/94.7] sec) and 100 mg/kg (287[34.3/300] sec) caused a significantly increase in test session latencies (Mann-Whitney U-test, $p<0.05$ and $p<0.01$, respectively), compared to controls (saline 22.6[12.7/38.1 sec] and DMSO 26.2[15.2/59.7] sec).

Wilcoxon showed significant differences ($p< 0.01$) between training (saline: 15.2 [6.1-19.3] sec; DMSO: 10.1 [5.9-17.9] sec; POEE 800 mg/kg: 11.8[8.4-16.1] sec) and test session latencies (saline: 27[8.8/43.1] sec; DMSO: 20.2[13.3/29.8] sec; POEE 800 mg/kg: 48.3[24.5/115. 8] sec), in adult (2.5 months old) mice (Figure 3B). The same effect was observed with oral administration (Fig. 3B), when POEE 800 mg/kg increased the test latency (48.3[24.5/115.8] sec) ($p <0.05$) compared with controls (saline 27[8.8/43.1] sec and DMSO 20.2[13.3/29.8] sec).

Figure 4 illustrates the effect of POEE in memory retrieval. For ip treatment (Fig. 4A) Wilcoxon shows significant differences between training (saline: 7.7 [5.2-11.43] sec; DMSO: 7.2 [4.7-10.0] sec, $p< 0.05$; POEE 50 mg/kg: 7.1 [5.0- 11.5] sec; POEE 100 mg/kg: 9.0 [4.3-12.8] sec, $p < 0.01$) and test session latencies (saline: 12.2[4.3/20.7] sec; DMSO: 9.6[6.9/21.9] sec; POEE 50 mg/kg: 16.7[13.4/23.9] sec; POEE 100 mg/kg: 49.6[12.4/234.8] sec) in adult mice. Kruskal-Wallis revealed a significant drug effect ($H(3)= 15.691$, $p<0.01$). Mann-Whitney U-test indicate that POEE 50 (16.7[13.4/23.9] sec) and 100 mg/kg (49.6[12.4/234.8] sec) causes significantly increases in test latencies ($p<0.05$ and $p<0.01$, respectively)

compared to controls (saline 12.2[4.3/20.7 sec] and DMSO 9.6[6.9/21.9 sec]). With orally administered treatments (Figure 4B) Wilcoxon showed significant ($p< 0.01$) differences between training (saline: 7.8 [5.4-16.3] sec; DMSO: 6.7 [3.4-14.3] sec; POEE 800 mg/kg: 10.4[5.6-19.5] sec) and test session latencies (saline: 12.5[6.1/71.6] sec; DMSO: 12.3[7.2/68.9] sec; POEE 800 mg/kg: 46 [28.3/101.6] sec). POEE 800 mg/kg administered po (Fig 4B) improved retrieval, expressed by increased test latency (46[28.3/101.6 sec]) (Mann-Whitney U-test, $p < 0.05$) in comparison with controls (saline 12.5[6.1/71.6] sec and DMSO 12.3[7.2/68.9] sec).

Figure 5 illustrates the effect of POEE in memory retrieval in aging mice. The results clearly show that aging mice (saline and DMSO) present memory deficit for this task (Figure 5), established by the lack of significant differences between saline aging training (8.8 [5.8/18.1] sec) and test (9.9 [5.9/17.1] sec) latencies (Wilcoxon, $p = 0.498$), or DMSO aging training (11.8 [6.5/17.7] sec) and test (9 [4.6/12.6] sec) sessions (Wilcoxon, $p = 0.976$) (Fig 5); the memory deficit is also indicated by comparing test session latencies from adult (2.5 months old) and aging (14 months old) mice (Mann-Whitney U-test, $p< 0.05$). Except for non treated aging mice, Wilcoxon shows significant differences ($p<0.01$) between training (saline adult: 7.8 [3.8-15.6] sec; POEE 50 mg/kg: 9.9 [3.9- 13.4] sec; POEE 100 mg/kg: 8.3 [5.1-14.0] sec) and test session latencies (saline adult: 28.4 [14.0-43.2] sec; POEE 50 mg/kg: 26.7 [13.7- 40.3] sec; POEE 100 mg/kg: 22.6 [9.0-80.8] sec). Regarding POEE treatment, Kruskal-Wallis revealed a significant drug effect ($H(4)= 17.925$, $p<0.01$): POEE 50 (26.7[13.7/40.3] sec) and 100 mg/kg (22.6[9.0/80.8] sec) administered ip pre-test to aging mice improved memory retrieval (test latencies) when compared to controls [saline aging (9.9 [5.9/17.1] sec) and DMSO aging (9 [4.6/12.6] sec); $p< 0.01$ and $p< 0.05$, respectively]; the test performance of POEE-treated aging mice is comparable (Mann-Whitney U-test compared to controls $p= 0.84$ for POEE 50 mg/kg, and $p=0.94$ for POEE 100 mg/kg) to that of adult mice

(28.4[14/43.2] sec). Unfortunately, due to difficulties in obtaining 14 moth old mice only the effect of ip POEE in retrieval was assessed.

3.2. Object recognition test:

The study was conducted with the same POEE doses that enhanced step-down ST and LT memories, using the same training-test intervals. Total exploration time (e.g., exploring familiar and new objects) were similar in all groups and experiments (data not shown), except for when POEE 100 mg/kg was given before training (in the training session, $p<0.05$), or before test (in test sessions, $p<0.05$); in these groups mice showed a slight decreased in total exploration time, although the inferior cut-off time of 10 seconds was never reached.

Figure 6 presents recognition index results (RI) with 3h or 24h of training-test interval. With the 3h training-test interval, no significant differences (one way ANOVA), between groups were observed when treatments were given prior to training ($F(3,44)=0.65$, $p=0.59$, Figure 6A), post training ($F(3,35)=0.93$, $p=0.43$, Figure 6B) or pre test ($F(3,39)=2.62$, $p=0.065$, Figure 6C). Nevertheless, for the 24 hour training-test interval ANOVA showed a significant drug effect for RI with the extract given pre-training ($F(3,44)=9.74$, $p<0.001$, Figure 6A), post training ($F(3,35)=5.99$, $p=0.002$, Figure 6B), or pre-test ($F(3,35)=5.99$, $p=0.002$, Figure 6C). Duncan analysis showed that POEE 50 and 100 mg/kg cause statistically significant and comparable increases in recognition index as compared to controls (saline and DMSO), when administered pre-training ($p<0.01$, Figure 6A), post training ($p<0.05$, Figure 6B) , or pre test ($p<0.05$ and $p<0.01$, 50 and 100 mg/kg respectively, Figure 6C).

4. Discussion and conclusion

The first purpose of this study was to complement our previously reported POEE facilitating effects on long-term memory retrieval (step-down inhibitory avoidance paradigm) (da Silva et al., 2004), by now examining the effects of the same extract on short-term memory in adult and aging mice. Results clearly show that POEE improves acquisition, consolidation, and retrieval of step down STM in adult mice, and reverses the retrieval deficit in aging mice. Since the pharmacokinetic of the extract is not known, an alternative interpretation of the data is that POEE treatment before training could have an effect on the early STM consolidation processes, whereas POEE treatment after training could affect consolidation but also STM retrieval processes.

These results add to, and are consistent with, those previously reported (da Silva et al., 2004); however, while POEE equally influences the three memory phases in STM, it only affects LTM retrieval. While some drugs equally influence STM and LTM (e.g., scopolamine, CNQX, AP5, MCPG, muscimol), others either affect STM but not LTM (e.g., intrahippocampal SCH23390, SKF 38393, 8-OH-DPAT) or LTM but not STM (e.g., norepinephrine) (Izquierdo et al., 1998b; Vianna et al., 2000b; Schroder et al., 2003). Differential effects are consistent with the idea that STM and LTM pertain to (and are regulated by) separate subsystems in the brain, some but not all times sharing a given brain structure (Izquierdo et al., 1999; Rosenzweig et al., 1993). Age related memory deficits differently affect specific types of memories, including short-term memory (Baxter, 2003; Yokota et al., 2000; Nilsson, 2003; Ramaswami, 2003). The relative importance of intervention in one or another would be expected to vary with the nature of the task, and with the relation of each task to others (Izquierdo, 1989, Medina et al., 1999).

A second purpose of the study was to verify if the POEE facilitating memory effect was specific for aversively motivated learning. We examined the effects of POEE with the object

recognition task, one that requires no learning rule other than "familiarity judgment" (Abe et al., 2004). It has been previously reported that adult rodents are able to discriminate between a familiar and a new object up to 1.5 h or less (Deschaux et al., 1997; de Lima et al., 2005), but not after 24 h (Ennaceur et al., 1989; Bartolini et al., 1996; Puma and Bizot, 1998; Prickaerts et al., 2002). Therefore, in this learning paradigm amnesic effects of drugs can be detected with trials interval up to 1.5 h, whereas promnesic effects with inter trial intervals longer than 1.5 h (Puma et al., 1998). In order to compare the effects of the extract in the two learning paradigms, experiments were done with 3 h and 24 h training-test intervals. The results show that acute administration of POEE improves object recognition memory after 24, but not 3, hours after training. Other treatments, such as diphenyl diselenide and deprenyl, were also reported to facilitate object recognition with longer (24 h) but not short (1.5 h) training-test intervals (Rosa et al., 2003; de Lima et al., 2005).

Abe et al. (2004) showed that a decrease in exploratory behavior (spontaneous locomotor activity) could impair exploration time of novel objects; the extract doses used in this study do not affect spontaneous locomotion (data not shown). Total exploration time were similar in most instances, but decreased when POEE 100 mg/kg was given before training or test sessions; nevertheless, both 50 and 100 mg/kg of POEE resulted in consistent differences in times exploring familiar and new objects (hence higher recognition indexes) regardless of changes in total exploration time. In fact, RI is considered a more reliable measure of discrimination because it corrects for the total exploratory activity (Sik et al., 2003). The differences in training performance found in this study are somewhat comparable to the ones seen with pre training POEE administration in step down (da Silva et al., 2004); in both cases a possible explanation is the moderate anxiogenic activity of this dose of POEE identified through the hole-board model (da Silva et al., 2002). Two factors can be contributing to the observed decrease in total exploration time with pre test administration: an improved memory

(decreasing familiar object exploration), and anxiety (delaying initial exploration of the new object due to its novelty). Actually, studies show that animals displaying decreased anxiety also show an impairment of object recognition memory (Contarino et al., 1999; Piper and Meyer, 2004), and it has been repeatedly shown that adequate levels of anxiety are necessary for good memory performance (Cahill and McGaugh, 1998; Micheau and Van Marrewijk, 1999**;** Izquierdo et al., 2000, 2002; Quevedo et al., 2003).

POEE improved inhibitory avoidance but not object recognition memory when tests were performed 3 h after training. Although associative and non associative memories are dependent on hippocampus integrity (Thiel et al., 1998, 1999; Clark et al., 2000), the definition of short and long term nonassociative memory is not as clearly defined as with inhibitory avoidance because the mechanisms for memory formation in both tasks recruit different pharmacological and biochemical processes (Vianna et al., 2000a). The difference in results obtained with POEE in the two learning paradigms may also suggest that emotionally and non emotionally motivated memories are differentially regulated by POEE, possibly by affecting specific brain structures and/or regulatory systems.

Consistent with the notion that acetylcholine is critical in the processes underlying attention, learning and memory, the aging brain is characterized by cognitive deficits and a central cholinergic hypofunction (Vannucchi et al., 1997). Thiel et al. (1998) reported elevated hippocampal extracellular acetylcholine levels associated with novelty as well as in open field habituation, nonassociative learning tasks. Object recognition is impaired by anticholinergic drugs in rats (Ennaceur and Meliani, 1992), as well as cholinergic neuronal lesions in rats (Bartolini et al., 1996) and monkeys (Aigner et al., 1987, 1991). Conversely, improved performance in object recognition was observed with drugs that enhance cholinergic activity, inhibit acethylcolinesterase (Scali et al., 1997a, b; Lamirault et al.,

2003a,b; Rispoli et al., 2004; Prickaerts et al, 2005), stimulate nicotine receptors (Scali et al, 1997b; Luine et al., 2002), or increase ACh release (Vannucchi et al., 1997).

Nootropic drugs, such as piracetam and pramiracetam, were shown to improve object recognition memory (Ennaceur et al., 1989); likewise, the analogs oxidacetam and aniracetam improved discrimination between novel and familiar objects, and restored object recognition impaired by aging, scopolamine and nbM lesions, suggesting a cholinergic mechanism (Bartolini et al., 1996). However, it has been suggested that oxiracetam also modulates glutamatergic transmission and protein kinase C (PKC) (Raiteri et al., 1992; Fordyce et al., 1995), while aniracetam and piracetam potentiate AMPA receptor mediated ion conductance and metabotropic glutamate receptor activity (Gouliaev and Senning, 1994; Johansen et al., 1995; Noorbala et al., 1999; Pittaluga et al., 1999; Ruthrich et al., 1999). Therefore, the mechanism of action responsible for memory facilitation by nootropic drugs in not fully known, and may involve various neurotransmitters.

Thus, the promnesic effect of POEE found in this study is coherent with its inhibitory properties for acetylcholinesterase (Siqueira et al., 2003), but also with the behavior profile so far established for this extract (Siqueira et al., 1998; da Silva et al., 2002) suggests interactions with various neurotransmitters (including noradrenaline, serotonin and dopamine).

It is noteworthy that POEE is effective when administered orally to young and aged mice, concurrent with the traditional use with its alleged effects in elderly people. Especially because experimental results are consistent with the traditional use of this medicinal plant species, the data here reported justify further studies with this plant extract in the context of treating attention and cognitive deficits associated with aging and/or specific neurodegenerative diseases (such as Alzheimer, dementia with Lewy bodies, vascular dementia, Huntington).

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Figure 1. Open field and training/test paradigm. Two identical objects at the open circular arena during the first trial; in a second trial (retention interval of 3 or 24 h) the familiar and a novel objects are placed at the arena.

Figure 2. Effect of POEE (**2A** ip, **2B** *po*) administrated pre-training in adult mice on step-down inhibitory avoidance task (STM, 3 h training-test interval). DMSO = dimethyl sulphoxide 20%. N=19-21. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies. § $p<0.05$ and $^{\S\S}p<0.01$ test x training latencies for each treatment, Wilcoxon. * $p<0.05$ x controls training latencies and POEE 50 mg/kg; $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ x controls test latencies; Mann-Whitney/Kruskal-Wallis.

Figure 3. Effect of POEE (**3A** ip, **3B** *po*) administrated post-training in adult mice on step-down inhibitory avoidance task (STM, 3h training-test interval). DMSO = dimethyl sulphoxide 20%. N=18-21. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies. § $p<0.05$ and $^{\S\S}p<0.01$ test x training latencies for each treatment, Wilcoxon. $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ x controls test; Mann-Whitney/Kruskal-Wallis.

Figure 4. Effect of POEE (**4A** ip, **4B** *po*) administrated pre-test in adult mice on step-down inhibitory avoidance task (STM, 3h training-test interval). DMSO = dimethyl sulphoxide 20%. N=18-22. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies. § $p<0.05$ and $^{\S\S}p<0.01$ test x training latencies for each treatment, Wilcoxon. $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ x controls test; Mann-Whitney/Kruskal-Wallis.

Figure 5. Effects POEE (*ip* 30 min prior test) administrated pre-test in aging mice on step-down inhibitory avoidance task (STM, 3 h training-test interval). DMSO = dimethyl sulphoxide 20%, POEE50 = POEE 50mg/kg, POEE100 = POEE 100mg/kg. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies N=20. § $p<0.05$ and $^{\S\S}p<0.01$ test x training for each treatment, Wilcoxon. $^{\#}$ $p<0.05$ x aging controls test; Mann-Whitney/Kruskal-Wallis.

Figure 6. Effects of POEE on object recognition index, with 3h and 24 training-test interval. RI expressed as mean±SEM. POEE administered 30 min pre-training (**A**), immediately post-training (**B**) and 30 min pre-test (**C**). $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ X control, ANOVA/Duncan.

Figure 1

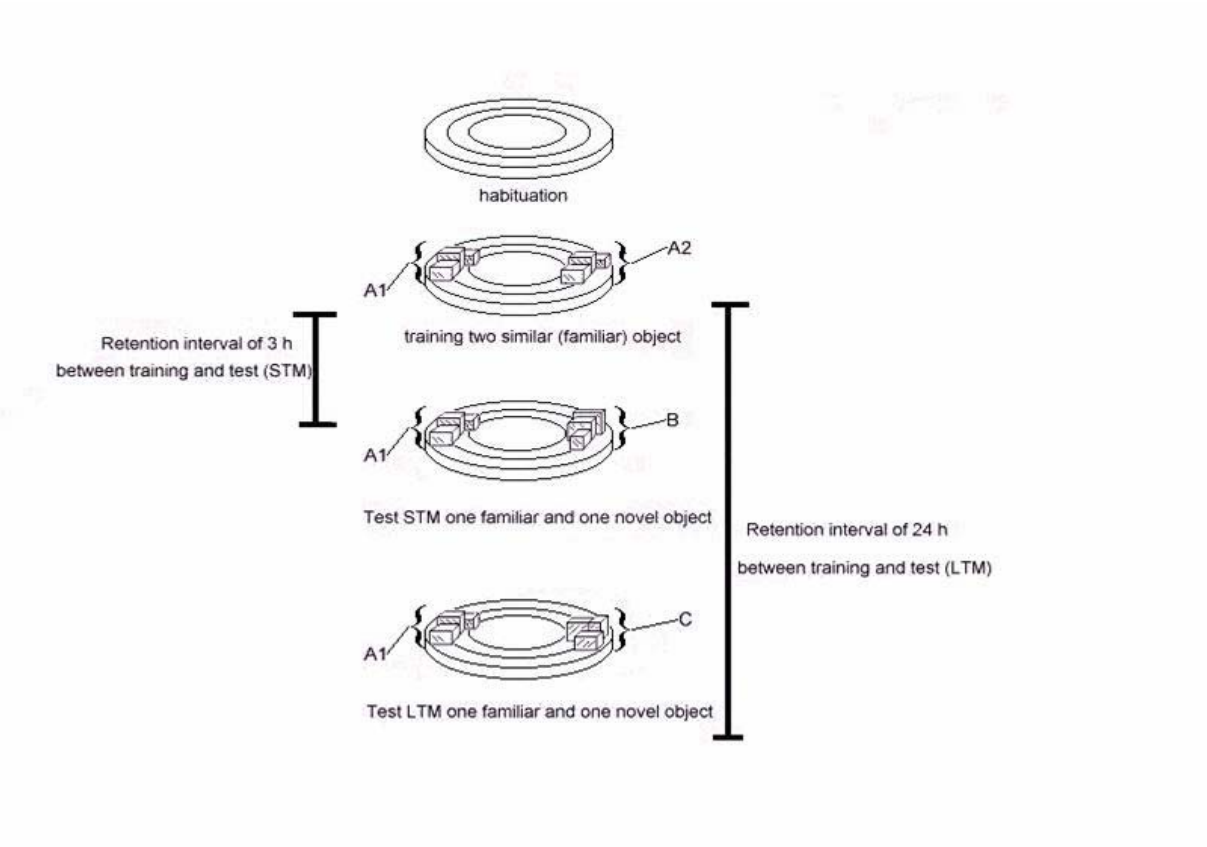

habituation
A1
A2
training two similar (familiar) object
Retention interval of 3 h
between training and test (STM)
A1
B
Test STM one familiar and one novel object
Retention interval of 24 h
between training and test (LTM)
A1
C
Test LTM one familiar and one novel object

Figure 2A

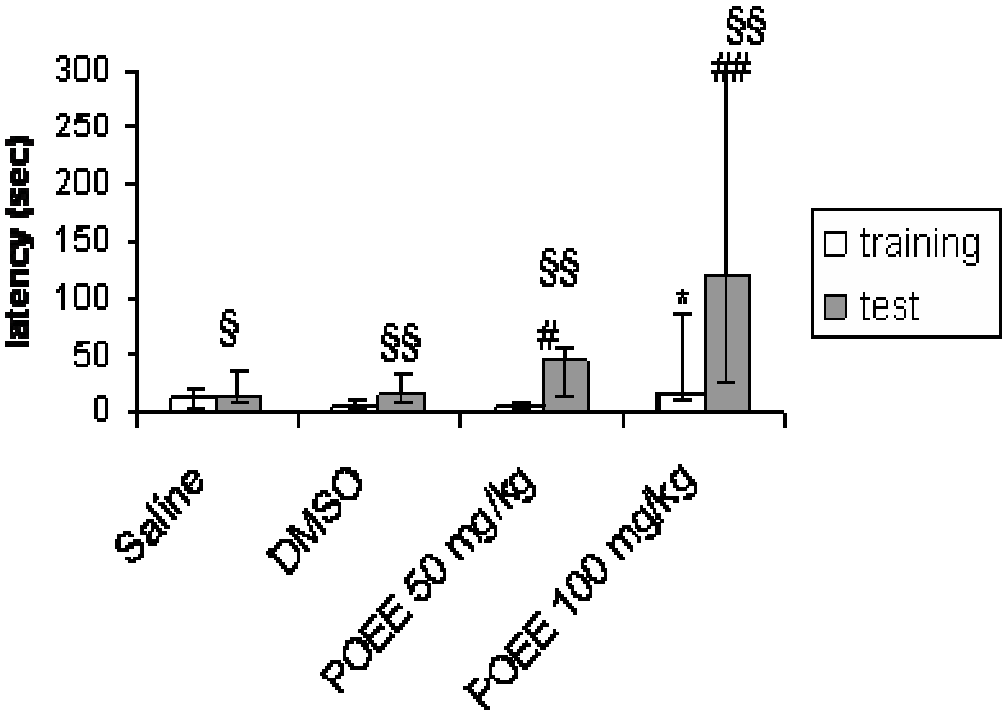

latency (sec)
300
250
200
150
100
50
0
§
§§
§§
#
*
§§
##
training
test
Saline
DMSO
POEE 50 mg/kg
POEE 100 mg/kg

Figure 2B

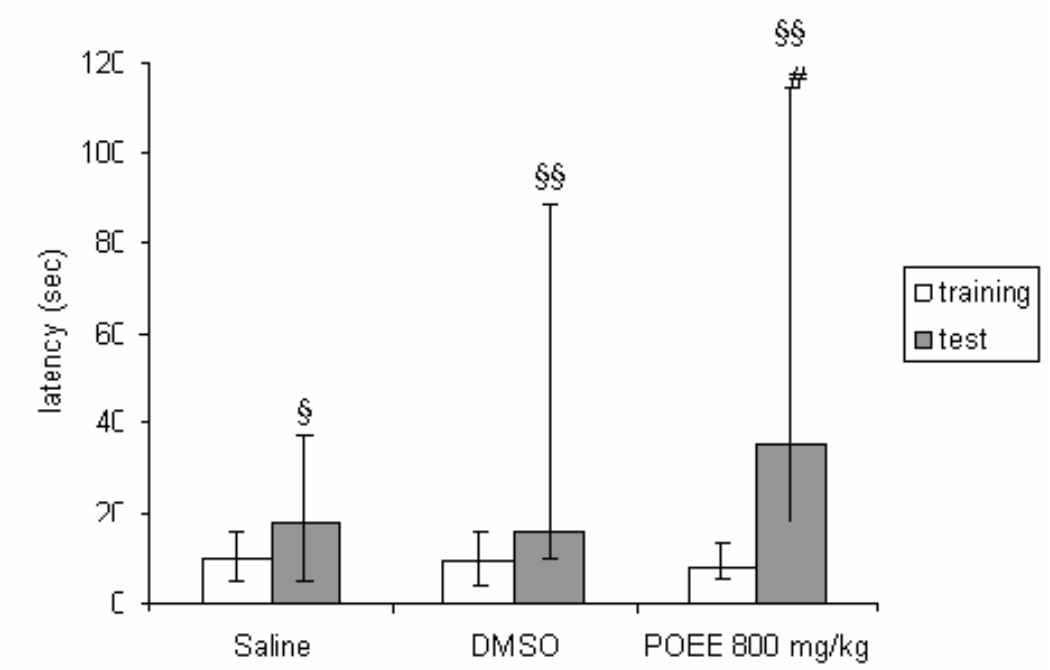
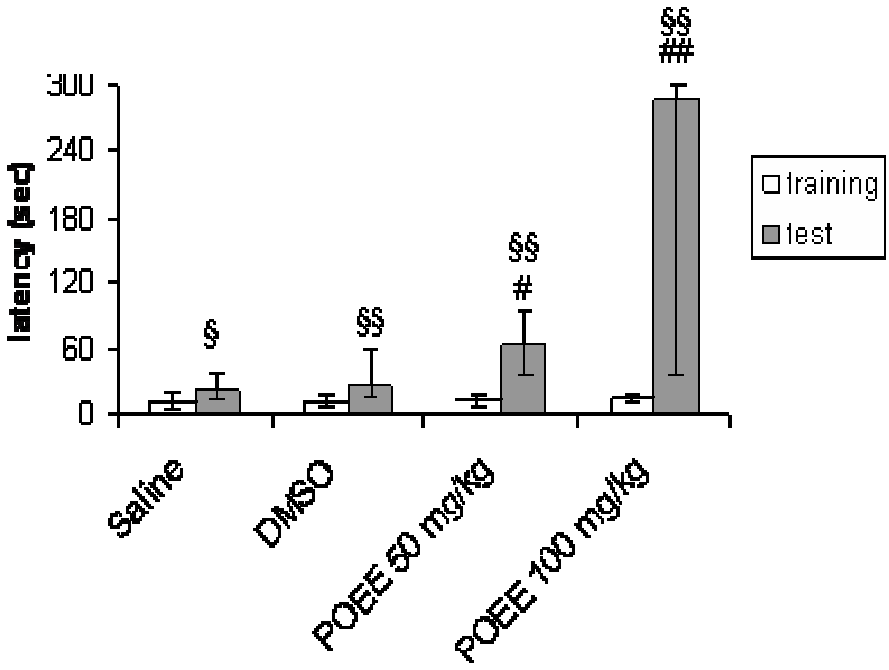


Figure 3A


latency (sec)
300
240
180
120
60
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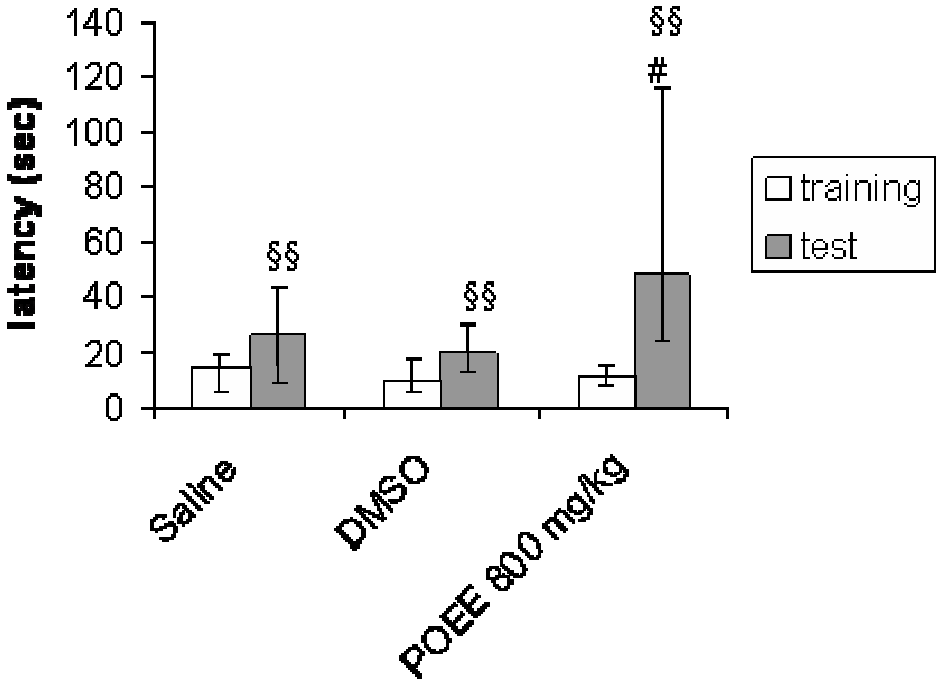
training
test
Saline
DMSO
POEE 50 mg/kg
POEE 100 mg/kg

Figure 3B


latency (sec)
140
120
100
80
60
40
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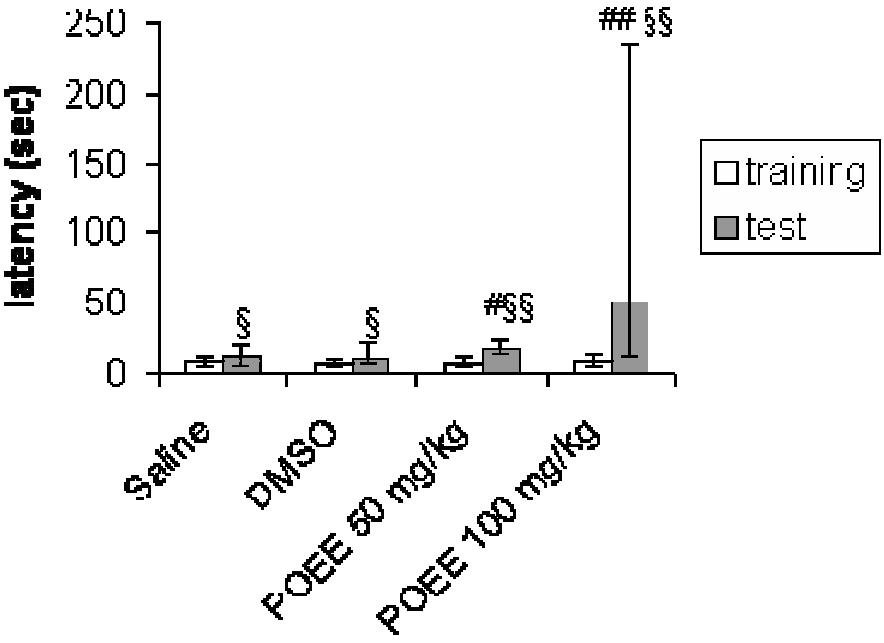
training
test
Saline
DMSO
POEE 800 mg/kg

Figure 4A


latency (sec)
250
200
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100
50
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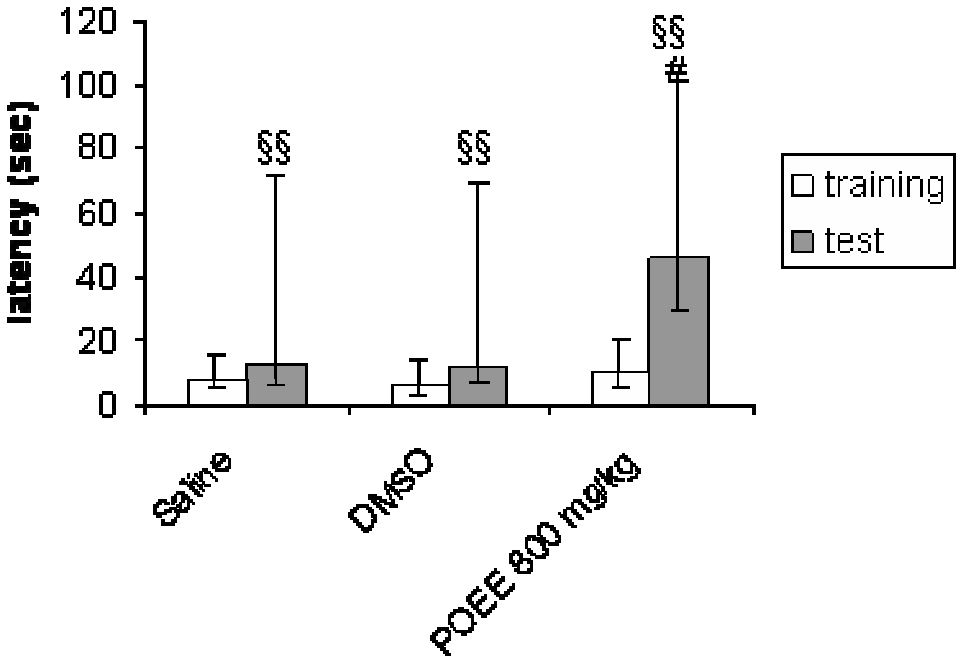
Saline
DMSO
POEE 50 mg/kg
POEE 100 mg/kg
training
test

Figure 4B


latency (sec)
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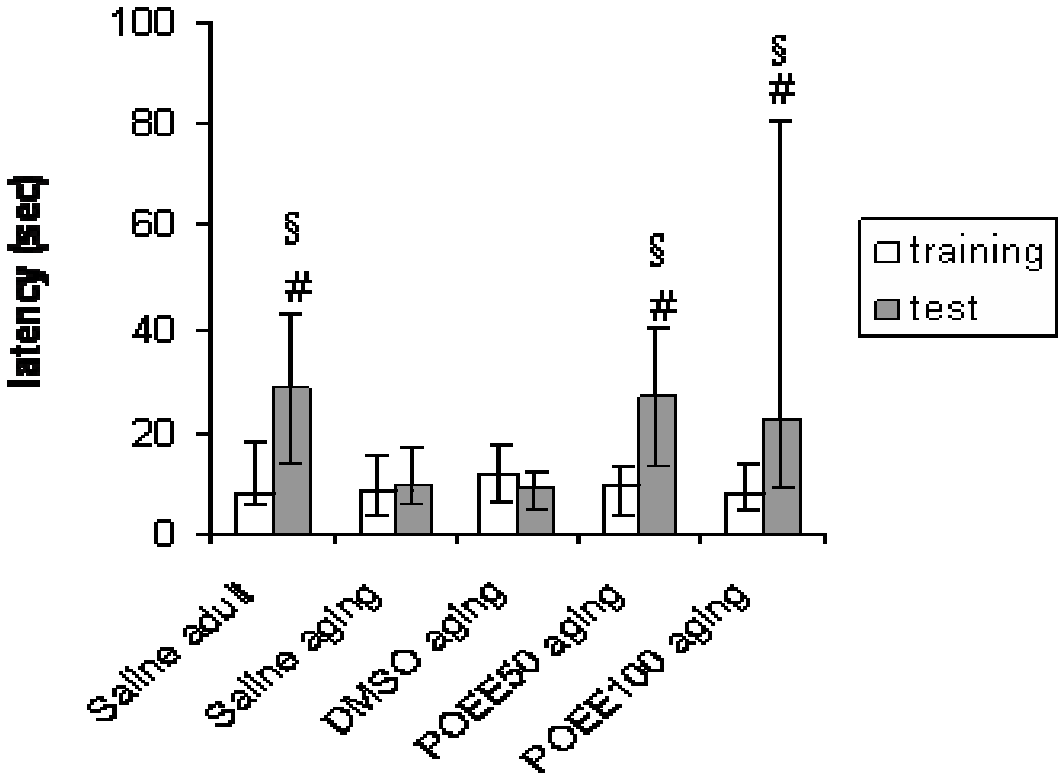
training
test
Saline
DMSO
POEE 800 mg/kg

Figure 5


latency (sec)
100
80
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#
training
test
Saline adult
Saline aging
DMSO aging
POEE50 aging
POEE100 aging

Figure 6A

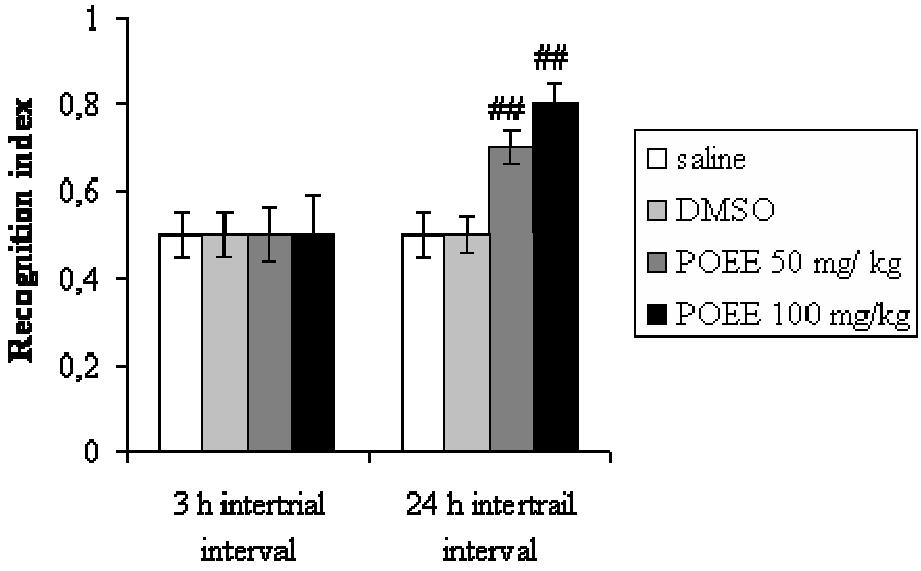
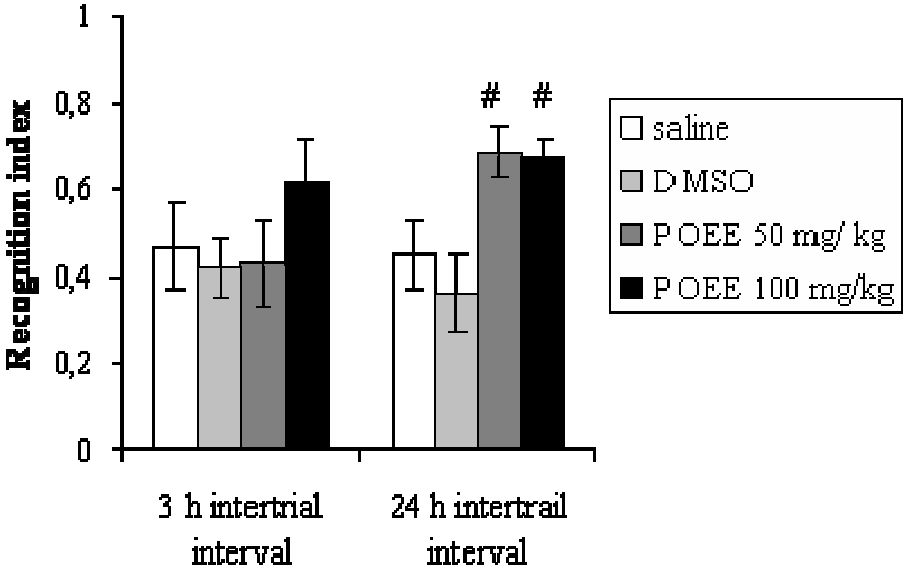

Recognition index
1
0,8
0,6
0,4
0,2
0
##
##
saline
DMSO
POEE 50 mg/ kg
POEE 100 mg/kg
3 h intertrial interval
24 h intertrail interval

Figure 6B


Recognition index
1
0,8
0,6
0,4
0,2
0
# #
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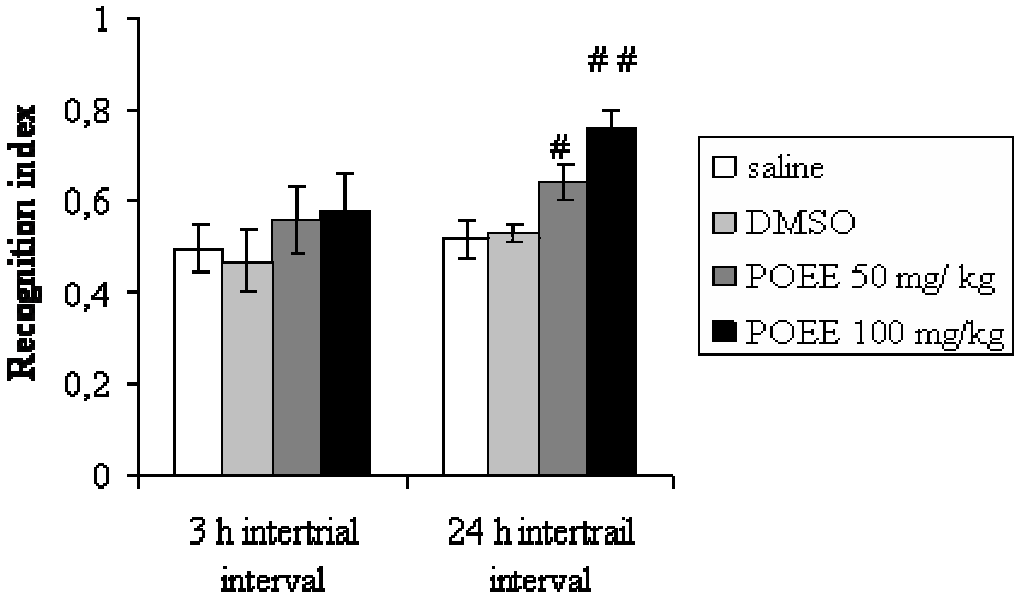
DMSO
POEE 50 mg/ kg
POEE 100 mg/kg
3 h intertrial interval
24 h intertrail interval

Figure 6C


Recognition index
1
0,8
0,6
0,4
0,2
0
##
#
saline
DMSO
POEE 50 mg/ kg
POEE 100 mg/kg
3 h intertrial interval
24 h intertrail interval

Capítulo 3. DA SILVA AL, PIATO AL, FERREIRA JG, MARTINS BS, OLIVEIRA S, MAI N, NUNES DS, ELISABETSKY E. Promnesic properties of *Ptychopetalum olacoides* Bentham extract: role of adrenergic and dopaminergic receptors**.**

(Submetido ao Fitoterapia).

Promnesic properties of *Ptychopetalum olacoides* Bentham extract: role of adrenergic and dopaminergic receptors

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Abstract

Age related central nervous system disorders are treated among Amazonian indigenous communities with home made remedies prepared from *Ptychopetalum olacoides* (PO) roots. A standardized PO ethanol extract (POEE) was shown to improve memory using the step-down inhibitory avoidance and object recognition task in mice. This study aimed to investigate the participation of adrenergic and dopaminergic mechanisms in the promnesic effects of POEE. Confirming previously reported results, the acute administration of POEE (50.0 and 100.0 mg/kg i.p.) increased step-down latencies at test sessions (short term memory [STM] and long term memory [LTM], 3 and 24h training-test interval respectively). Pretreatment with the alpha adrenoceptor antagonist phentolamine (3.0 mg/kg), the beta adrenoceptor antagonist propranolol (4.0 mg/kg), the dopamine D_1 receptor antagonist SCH 23390 (0.1 mg/kg), and the dopamine D_2 receptor antagonist sulpiride (10.0 mg/kg) revealed that beta adrenergic and D_1 dopamine receptors play significant roles in the facilitatory effects of POEE on STM acquisition, consolidation and retrieval, as well as in LTM retrieval. These results add to the antioxidant and anticholinesterase properties previously described for POEE as the pharmacodynamic basis of its nootropic profile.

Key words: *Ptychopetalum olacoides,* Marapuama, memory, step-down, dopamine, adrenoceptors, neurodegenerative disorders.

Introduction

Neurotransmitter systems have been implicated in memory processes, being postulated that monoaminergic systems facilitate unconscious (implicit) memory, while muscarin cholinergic receptors facilitate conscious (declarative) memory [1]. Accordingly, anticholinergic drugs and/or conditions that reduce the cholinergic function have been associated with deficits declarative memory. Nevertheless, in line with the current understanding on age-related catecholaminergic alterations, a number of studies showed that noradrenaline (NA) and dopamine (DA) concentrations were found to be reduced in striatum of aged rats [2-7]. Birthelmer et al. [7] suggested that in fact cholinergic, noradrenergic, and serotonergic reuptake and/or release functions may affect cognitive process in aging mice.

The concept of adrenergic receptors participating in memory processes is appealing because the adrenergic system is activated during arousing emotional experiences. There is an extensive literature on the influence of stress and stress hormones on memory retrieval of various tasks [8-11], while the influence of these hormones in memory consolidation have been convincingly shown to rely on mechanisms involving β-adrenoceptors in the basolateral amygdala [9,12,13]. The activation of the adrenergic system has been reported to enhance memory of aversively trained animals [14,15], and it has been proposed that NA release in the hippocampus alters information processing via β_1 receptors to promote memory retrieval [16].

DA is one of the transmitters mostly involved in modulating neural mechanisms underlying states of fear and anxiety [17,18]; in fact, there is strong support for an important role of the stress-responsive mesolimbic dopaminergic system in the control of mood [19, 20]. There is also convincing evidence for the participation of the

dopaminergic system in the mechanisms of formation, expression, retrieval and extinction of conditioned fear [21]. It is therefore not surprising that dopamine receptors should play an important role in learning and memory [22,23].

We have previously shown that a standardized ethanol extract of *Ptychopetalum olacoides* Bentham (POEE) facilitates short-term (STM, acquisition, consolidation and retrieval) and long-term (LTM, retrieval) memories in mice trained in the step-down inhibitory avoidance paradigm; it is also noteworthy that the memory deficit observed in aging (14 month old) mice was reversed by orally given extract [24]. Moreover, we established that POEE posses anticholinesterase activity *in vitro* and *in vivo* at various relevant brain areas [25], antioxidant properties *in vitro* [26] and *in vivo* [27], and protects hippocampal slices submitted to oxygen and glucose deprivation [28]. This neuropharmacological profile is remarkably coherent with the traditional use of this plant by the elder population in Amazonian communities.

The observation that POEE potentiates yohimbine–induced lethality and reverses reserpine-induced ptosis [29] suggests its interaction with the noradrenergic and dopaminergic systems. Hence, we here examined the participation of adrenergic and dopaminergic receptors in the facilitatory effect of POEE on STM (acquisition, consolidation, retrieval) and LTM (retrieval) using appropriate antagonists.

Material and Methods

Animals: Male (CF1) adult albino mice, received from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) with 2.5 months of age (35-45g) were used. Animals were maintained in our own animal facility under controlled environmental conditions (22 $\pm 1^{o}$C, 12 hr-light/dark cycle, free access to food [Nuvilab CR1] and water), for at

least two weeks before experiments. All procedures were carried out in accordance with institutional policies on experimental animals handling, which follows the NIH guidelines (NIH Guide for Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985).

Preparation of Extract: Roots of *Ptychopetalum olacoides* Bentham (Olacaceae) were collected in Pará (Brazil), and identified by Nelson Rosa (MPEG 108.036 voucher at the Goeldi Museum Herbarium). *P. olacoides* standardized ethanol extract (POEE) was prepared and characterized as detailed elsewhere [27]; briefly, dried ground plant material (2.5 kg) was extracted with ethanol (12 L), using a Soxhlet apparatus (40 h); the extract was evaporated under reduced pressure resulting in the POEE (150 g, 6% yield). Preliminary phytochemical screening gave positive test for saponin, phenolic compounds and terpenic compounds [27,29,30].

Drugs: Dimethyl sulphoxide (DMSO) was acquired from Delaware (Brazil). Phentolamine hydrochloride (phent), DL-propranolol HCl (prop), R (+)-SCH 23390 HCl (sch), and S (-)-Sulpiride (sulp) were obtained from Sigma (USA).

Phentolamine and propranolol were dissolved in distilled water; SCH23390 was diluted in DMSO 10%; POEE (30, 50 and 100 mg/kg) was dissolved in DMSO 20 % (v/v). Sulpiride was dissolved in a minimal volume of 0.1 N HCl, and due volume completed with distilled water; pH was adjusted to 5–6 with 1 N NaOH. Saline (NaCl 0,9%, sal) was used as blank control. All chemicals were of analytical grade.

Doses and administration schedules for the antagonists were adopted from [31,32]. Propranolol (4.0 mg/kg) was administered 15 min, phentolamine (3.0 mg/kg) 30 min, SCH 23390 (0.1 mg/kg) 30 min, and sulpiride (10.0 mg/kg) 60 min before POEE (30.0, 50.0 or 100.0 mg/kg) controls injection. Drugs and vehicles were administered

intraperitoneally always as 0.1 ml/10 g of body weight. Control groups received saline or respective solvents, and were run concurrently with drug-treated groups; for this reason (and statistical accuracy) graphics display the groups pertaining to experiments run at a given time.

Behavioral procedures

Step-down inhibitory avoidance: the test used was adapted from Netto and Izquierdo [33], and Maurice et al. [34]. Mice were habituated in the dim lighted room for at least 30 minutes before the experiments. The inhibitory avoidance training apparatus was a plastic box (30 x 30 x 40 cm), with a platform (5 x 5 x 4 cm) fixed in the center of the grid floor. Each mouse was placed on the platform, and the latency to step down (four paws on the grid) was automatically recorded in training and test sessions. In the training session, the mouse received a 0.3 mA scrambled foot shock for 15 sec upon stepping down. Animals exhibiting step down latencies greater than 30 sec in training were excluded from experiments; less than 5% of the animals met this exclusion criterion. The test session was performed 3 h (STM) or 24 h(LTM) later, with the same procedure except that no shock was administered after stepping down; an upper cut-off time of 300 sec was set. N=18-21 per treatment group.

Results

Confirming that learning and memory took place with the training paradigm used in this study, there were significant and consistent differences ($p< 0.05$) between training and test session latencies in controls groups. In agreement with previous results [24], POEE (50.0 and 100.0 mg/kg, but not 30 mg/kg) improved acquisition,

consolidation, and retrieval for short-term memory (Figs. 3 and 5), as well as long-term memory retrieval (Figs. 1, 2, and 4), expressed by increased ($p<0.05$) test sessions latencies in comparison with control groups.

Figure 1 indicates the effects of phentolamine on POEE-induced enhancement of LTM retrieval. No differences were found between phentolamine and controls (sal+sal 18[12.4-52.3]sec, phent+sal 22.9 [9.8-41.42] sec, phent+dmso 15 [8.1-88.9], sec). Regarding Fig 1A , Kruskal-Wallis revealed a significant drug effect ($H(4) = 23.37$, $p<0.001$); Mann-Whitney showed that sal+POEE 100 mg/kg (199.9[23.5/300] sec) and phent+ POEE 100 mg/kg (300 [22.8/300] sec) caused significant ($p<0.05$ and $p<0.01$ respectively), and comparable ($p=0.74$), increases in test session latencies compared to controls. Additionally (Fig. 1B): Kruskal-Wallis revealed a significant drug effect ($H(6) = 17.15$, $p=0.009$), while Mann-Whitney showed that sal+POEE 50 mg/kg (74[30.6/300] sec) and phent+POEE 50 mg/kg (99.4 [40.8/220.8]sec] caused significant ($p<0.05$), and comparable ($p=0.98$), increases in test session latencies compared to controls. No differences ($p=0.194$) were found between sal+ POEE30mg/kg 17.2 [6.8-210.1] sec and phen+POEE 30mg/kg 31.2 [22.8-192.3] sec.

Fig. 2 indicates the effects of propranolol on POEE-induced LTM retrieval improvement. No differences were found between propranolol and controls (sal-sal 22.6[6.9-101.6]sec, prop+sal 16.7 [7.9-37.5]sec and prop+dmso 12.9[9.4-79.9]sec). Kruskal-Wallis revealed a significant drug effect ($H(4) = 14.88$, $p<0.01$); Mann-Whitney showed that sal+POEE 100 mg/kg (79.2[22.0/300.0] sec) caused a significant ($p<0.05$) increase in test session latencies compared to controls; pre administration of propranolol reversed the promnesic effect of POEE (prop+POEE 100 mg/kg 24.4 [9.4-73.5], $p = 0.086$ compared controls).

Fig 3, illustrates the effects of propranolol on POEE-induced STM improvement. No differences were found between propranolol and controls (sal+sal 11.3[7.4-33.6]sec, prop+sal 10.4 [7.8-30.2]sec and prop+dmso 14.1 [8.3-29.1]sec). Regarding acquisition (3A), Kruskal-Wallis revealed a significant drug effect ($H(4) = 12.65$, $p=0.013$); Mann-Whitney showed that POEE (100 mg/kg) caused a significant ($p<0.01$) increase (33.6[19.1/106.9] sec) in test session latencies as compared to controls;this effect was reversed by propranolol (prop+POEE 11.7[5.1- 37.7]sec, $p=0.005$ x sal+POEE). Regarding consolidation (3B), Kruskal-Wallis revealed a significant drug effect ($H(4) = 22.06$, $p<0.01$); Mann-Whitney showed that POEE (100 mg/kg) caused a significant ($p<0.01$) increase (31.9[19.8/77.4] sec) in test session latencies as compared to controls; this effect was also reversed by propranolol (prop+POEE 16.6[9.7-25.7] sec, $p=0.009$ x sal+POEE). Regarding retrieval (3C), Kruskal-Wallis revealed a significant drug effect ($H(4) = 16.73$, $p<0.01$); Mann-Whitney showed that POEE (100 mg/kg) caused a significant ($p<0.01$) increase (47.3[21.3/157.2] sec) in test session latencies as compared to controls; this effect was also reversed by propranolol (prop+POEE 14.8 [7.2-55.4]sec, $p=0.014$ x sal+POEE).

Figure 4 shows the effects of dopamine antagonists on POEE-induced LTM retrieval improvement. Fig. 4A indicates the effect of the D_1 antagonist SCH 23390. No differences were found between SCH 23390 and controls (sal+sal 24.5 [12.6-37.3]sec, sch+sal 22.3 [11.4-48.1]sec and sch+dmso 27.2 [8.5-94.2]sec). Kruskal-Wallis revealed a significant drug effect ($H(4) = 14.19$, $p=0.007$); Mann-Whitney showed that POEE 100 mg/kg (58.2[35.3-224.5] sec) caused a significant ($p< 0.05$) increase in test session latencies compared to controls; pre administration of SCH reversed this promnesic effect (sch+POEE 33.1[14.1-69.1], $p=0.025$ x sal+POEE). Fig. 4B indicates the effect

of the D_2 antagonist sulpiride. No differences were found between sulpiride and controls (sal+sal 24.1[10.1-54.4]sec, sulp+sal 14.2 [8.6-163.0] sec and sulp-dmso 18.0[7.3-29.8]sec). Kruskal-Wallis revealed a significant drug effect (H (4) = 12.55, p=0.006); Mann-Whitney showed that POEE 100 mg/kg (58.2[35.3/224.5] sec) and sulp+POEE (41.7[17.9/300]sec) caused significant and comparable (p=0.62), increases in test session latencies as compared to controls.

Fig. 5 indicates the effect of SCH 23390 on POEE-induced STM improvement. No differences were found between SCH and controls (sal+sal 21.3[5.1-43.7]sec, sch+sal 15.15[8.1-27.5]sec and sch+dmso 18.8[8.0-52.2]sec). Regarding acquisition (5A), Kruskal-Wallis revealed a significant drug effect (H (4) = 11.46, p=0.02); Mann-Whitney showed that POEE 100 mg/kg (51.1[24.4/76.6] sec) caused a significant increase in test session latencies ($p<0.05$) compared to controls; this effect was reversed by SCH (sch+POEE (14.7[7.5/58.5], p=0.025 x sal+POEE). Regarding consolidation (5B), Kruskal-Wallis revealed a significant drug effect (H (4) = 14.44, p=0.006); Mann-Whitney showed that POEE 100 mg/kg (40.6[24.9/112.1] sec) caused a significant increase in test session latencies ($p<0.05$) compared to controls; this effect was reversed by SCH (sch+POEE 22.9[13.1/84.7], p= 0.048 x sal+POEE). Regarding retrieval (5C), Kruskal-Wallis revealed a significant drug effect (H (4) = 15.6, p=0.004); Mann-Whitney showed that POEE 100 mg/kg (66.1[22.5/292.5] sec) caused a significant increase in test session latencies ($p<0.05$) compared to controls; this effect was likewise reversed by SCH (sch+POEE 28.4[21.8-33.5], p=0.026 x sal+POEE).

Discussion:

Adding to previously reported data [24] the present study reveals that POEE promnesic effects on short-tem (acquisition, consolidation and retrieval) and long-term (retrieval) memories is likely to be mediated by β adrenoreceptors, and D_1 (but not D_2) dopamine receptors. Because antagonist alone were devoid of effects, antagonist-induced changes in POEE promnesic effects can not be attributed to either antagonist-induced impairment of learning, nor its effects on locomotion. Although neural mechanisms underlying relevant for STM and LTM may differ [35, 36], since phentolamine and sulpiride apparently did not alter POEE promnesic effects on LTM retrieval, these antagonists were not studied in regard to short-term memory.

Dopamine D_1, β-noradrenergic, serotonin $5HT_{1A}$ and cholinergic muscarine receptors have been consistently shown to be crucially involved in the consolidation of short- and long-term memories [8, 9, 14, 36, 37] as well as in long-term memory retrieval [38,39], of the one-trial inhibitory avoidance task. Additionally, retrieval memory has been shown to depend on the PKA and MAPK enzymes [8, 14, 38, 40, 41,42], which are modulated by dopamine D_1, β-noradrenergic, $5HT_{1A}$ and muscarine receptors [43,44].

A significant number of animal and human studies demonstrate that memories for new experiences are more effectively encoded under environmental conditions which elevate NA concentrations. Hypotheses focused on memory consolidation maintain that adrenergic signaling is important for an enhanced consolidation of memories associated with emotionally laden events [14,45]. Moreover, experimental evidence suggests that NA has an important role in regulating plasticity in the CNS [46,47].

The need for an activated adrenergic system for optimal memory processing is coherent with studies demonstrating that of blockade post-synaptic β-adrenoceptors leads to impaired memory consolidation [48,49], in various learning paradigms. In rats, α_2-adrenergic blockade (increasing NA release) improved performances in tasks assessing attention, learning, and memory [50, 51, 52]; in contrast, α_2-agonists (reducing NA release) had deleterious effects on attention [53].

It has been suggested that deficiencies in the LC is a decisive factor in the progression of neurodegenerative disorders, such as PD and AD [55, 56]. Post-mortem studies of AD patients revealed degeneration of the locus coeruleus (LC, ≅ 60% compared to age-matched controls), and a reduction of noradrenergic markers at Meynert nucleus, cortex and hippocampus [54]. Among others [58,59], Mavrids et al. [57] have postulated that the LC may hold the key to govern compensatory and neuroprotective mechanisms that may counteract neurodegeneration and support recovery [57-59]. Accordingly, the activation of noradrenergic transmission (through blockade of α_2-adrenergic auto-adrenoreceptors) has been proposed as an approach to provide both symptomatic and trophic benefits in AD [60,61].

An adrenergic basis for the promnesic properties of POEE, is consistent with its ability to increased yohimbine–induced lethality and reverse reserpine-induced ptosis [30,62]. Moreover, it could be relevant for the reversal of memory deficit in ageing mice observed with this extract [24]. Because, α_2-, but not α_1,-NA receptors were shown to modulate cortical DA release [63], further experiments to clarify the interaction of POEE with specific α adrenergic receptors are warranted.

The results also indicate that POEE facilitation of long-term memory retrieval is mediated by dopamine D_1 receptor. The role of dopamine in the CNS has been broadly

described as “a modulator of cognitive, motivational, neuroendocrine, and motor activities” [64, 65]. Several authors have examined the role of D_1 and D_2 receptors in emotional learning [66- 69]: Inoue et al. [70] reported a relative impairment in the acquisition of conditioned freezing after administration SCH 23390; however, D_1 receptors have been thought to be predominantly involved in working memory and executive function [71-74]. Human studies have also provided support for a relationship between dopamine and cognition: while young subjects’ performances on a spatial task improved with the $D_{1/2}$ agonist pergolide [75], the agonist bromocriptine improved working memory [76], and the nonspecific DA antagonist sulpiride caused behavioral deficits on spatial working memory tasks [77]. Many studies in monkeys [78] and rats [79] reported that suitable doses of D_1 agents improve working memory, but higher doses disrupt it [80]. The principal part of the working memory system is thought to be the dorsolateral prefrontal cortex, whose circuitry (prefrontal cortical circuit, PFC) is controlled by the mesocortical dopaminergic system [81]. Early studies demonstrated that depletion of PFC dopamine caused performance deficits; moreover, the amount of dopamine released in the PFC is positively correlated with accurate memory performance [82-84].

While a reasonable body of evidence point to the catecholamines (DA and NA) as major players in memory processes in the healthy brain [73], a current challenge is to understand the various functions of the ascending monoamine and cholinergic systems in cognition. Generic terms as “arousal” or “stress” are still being used to capture how the activity of these systems fluctuates in different circumstances, and the Yerkes-Dodson principle postulates optimal levels of arousal or stress for optimal performance of behavior and cognition [86, 87]. Methylphenidate (used for treating of attention-

deficit/hyperactivity disorder [ADHD], which blocks DA and NA transporters, improves spatial working memory performance in normal volunteers [88,89]. It has been proposed that ADHD cardinal symptoms (poor attention regulation, impulsivity, and hyperactivity) may all arise from a weakened prefrontal cortex (PFC) regulation of behavior and thought [73]; therefore, it is of great interest that catecholamines are critical to proper PFC function [73].

Relevant to the pharmacodynamic basis of POEE promnesic effects, is the cross talk between the acetylcholine (ACh) and the DA, and the Ach and the NA systems, all apparently crucial for the maintenance of accurate cognitive performance. In the radial-arm maze paradigm, the deficit induced by nicotinic blockade with mecamylamine is potentiated by haloperidol [90]; this effect is also seen with the D_2 antagonist raclopride, but not with the D_1 antagonist SCH 23390, implying that it is the D_2 receptor which is important for the potentiation of the mecamylamine effect [90]. Tzavara et al [91] showed that atomoxetin, a selective inhibitor of NA transporters, increases cholinergic neurotransmission in cortical regions (PCF in particular). Both NA (eg, by reboxetine) and DA (eg., by methylphenidate) increase were shown to stimulate ACh release in the cortical region [91], while amphetamine increases ACh efflux in the striatal complex (including the nucleus accumbens [92]. Furthermore, previous studies reported a synchronous activation of $\alpha 1$ and D_1 receptors as the mechanism implicated in cortical ACh release [63, 93]. It is also noteworthy the report of mechanisms involving the simultaneous action of D_1, β, and muscarine receptors favoring LTM retrieval [39].

In conclusion, adding to the described promnesic effects of POEE [24,94], we here report the involvement of β adrenergic and D_1 dopamine receptors in POEE enhancement of STM and LTM; consistently, the same receptors were found to be

essential for POEE antidepressive profile in various rodent models [29, 95]. These results are in agreement with the current understanding of memory processes modulation, as well as traditional uses of this plant species. Studies are necessary to further clarify POEE mechanisms of action, and determine the relevance of using this herbal drug or its active components in treating age related cognitive deficits, and/or specific neurodegenerative diseases.

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Figure 1. Effect of phentolamine (α-adrenergic antagonist) on POEE-induced improvement of step-down LTM retrieval. Phent= phentolamine 3.0mg/kg; dmso = dimethyl sulphoxide 20%; **1A** POEE 100= POEE 100.0 mg/kg and **1B** POEE30/50= POEE 30.0, 50.0 mg/kg. N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * $p<0.05$ and ** $p<0.01$ x controls test latencies; Mann-Whitney/Kruskal-Wallis.

Figure 2. Effect of propranolol (β-adrenergic antagonist) on POEE (100.0 mg/kg)-induced improvement of step-down LTM retrieval. prop= propranolol 4.0 mg/kg; dmso = dimethyl sulphoxide 20%. N=19-21. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * $p<0.05$ x controls test latencies; Mann-Whitney/Kruskal-Wallis.

Figure 3. Effect of propranolol (β-adrenergic antagonist) on POEE (100 mg/kg)-induced improvement of step-down STM acquisition (**3A**), consolidation (**3B**), retrieval (**3C**). prop = propranolol 4.0 mg/kg; dmso = dimethyl sulphoxide 20%. N=19-21. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). ** $p<0.01$ x controls test latencies; Mann-Whitney/Kruskal-Wallis.

Figure 4. Effect of dopaminergic antagonists on POEE-induced improvement in step-down LTM retrieval. dmso = dimethyl sulphoxide 20%; POEE= POEE 100mg/kg; sch 0.1= SCH 23390 (D_1 antagonist) 0.1 mg/kg (**4A);** Sulp 10= Sulpiride (D_2 antagonist) 10 mg/kg **(4B).** N=19-21. Each column represents latencies (sec) median (interquartile

ranges) of training (light columns) or test (dark columns). * p<0.05 x controls test latencies; Mann-Whitney/Kruskal-Wallis.

Figure 5. Effect of SCH 23390 on POEE (100 mg/kg)-induced improvement of step-down STM acquisition (**5A**), consolidation (**5B**), retrieval (**5C**). sch 0.1= SCH 23390 0.1 mg/kg; dmso = dimethyl sulphoxide 20%. N=19-21. Each column represents latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * p<0.05 x controls test latencies; Mann-Whitney/Kruskal-Wallis.

1A

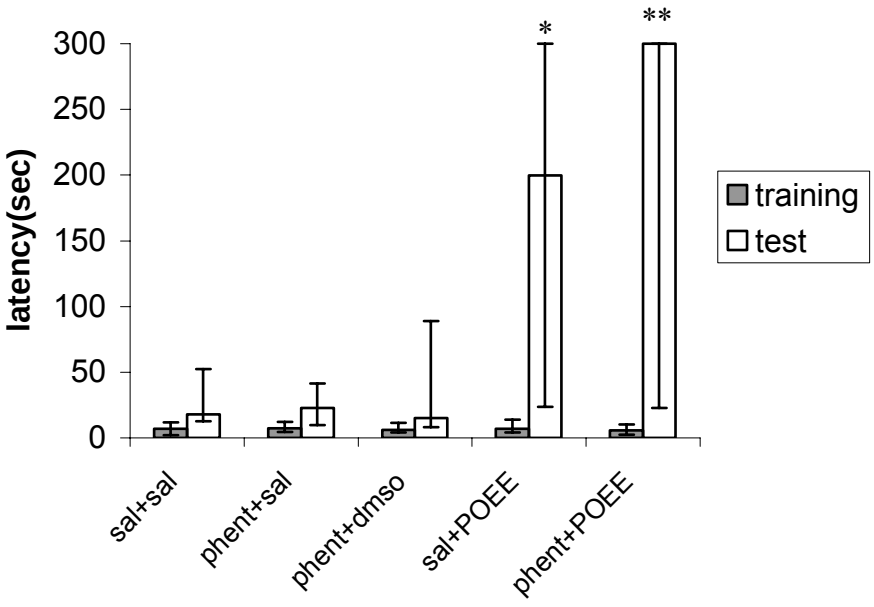
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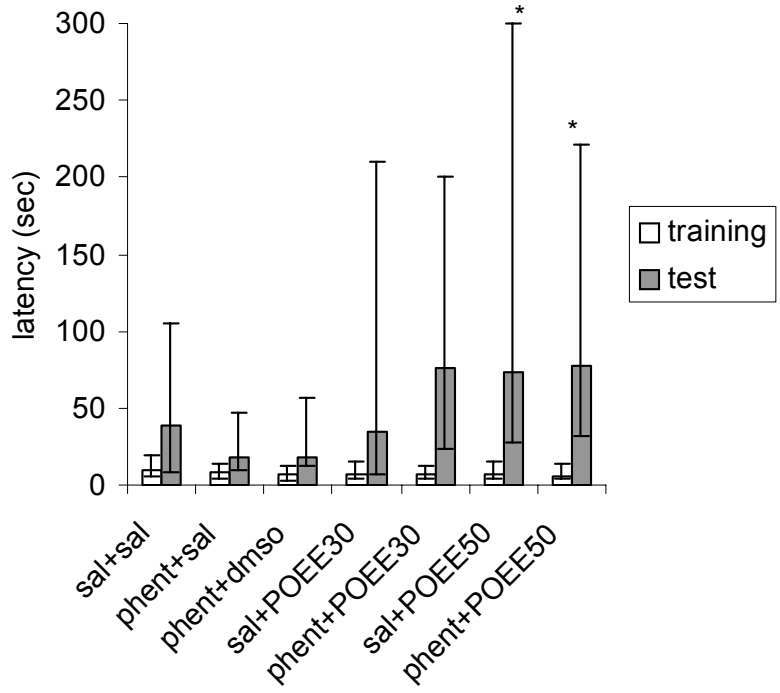
Fig 1B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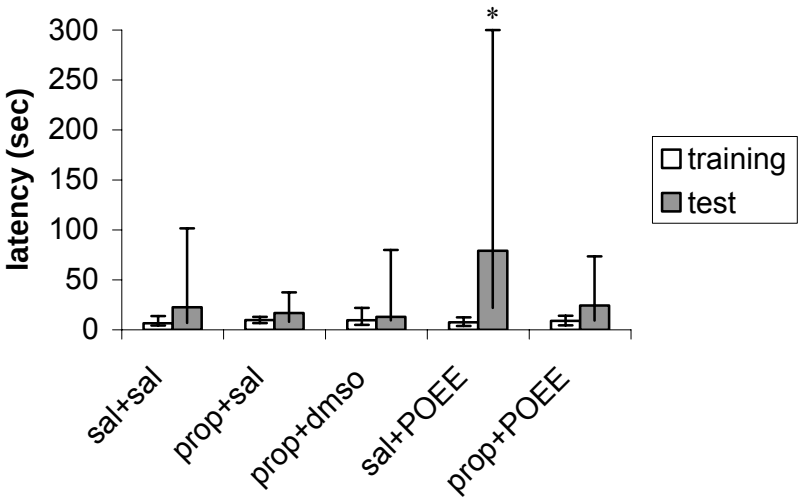
phent+POEE30
sal+POEE50
phent+POEE50

Fig 2.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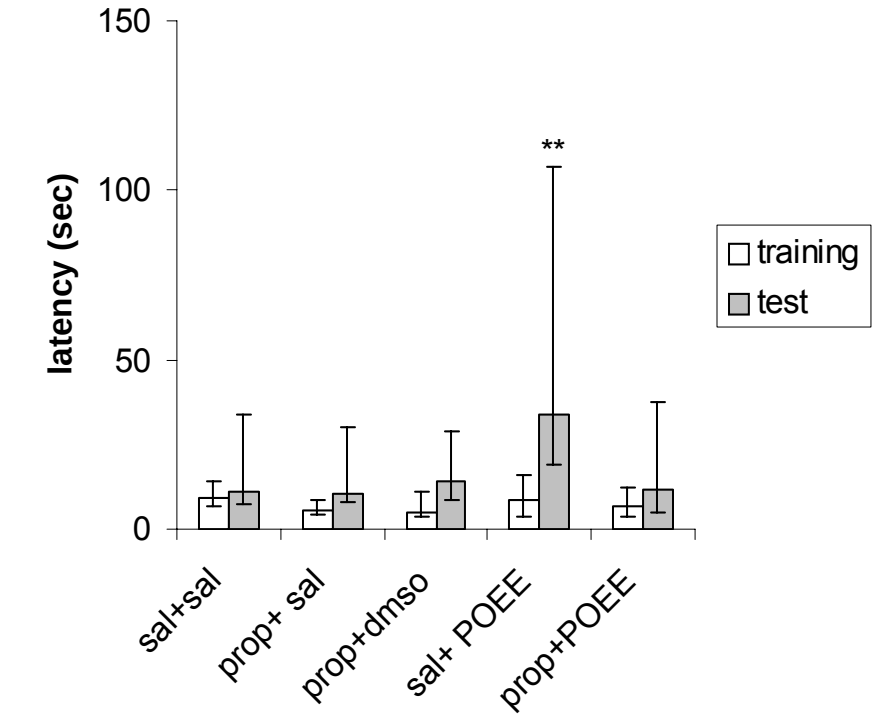
3A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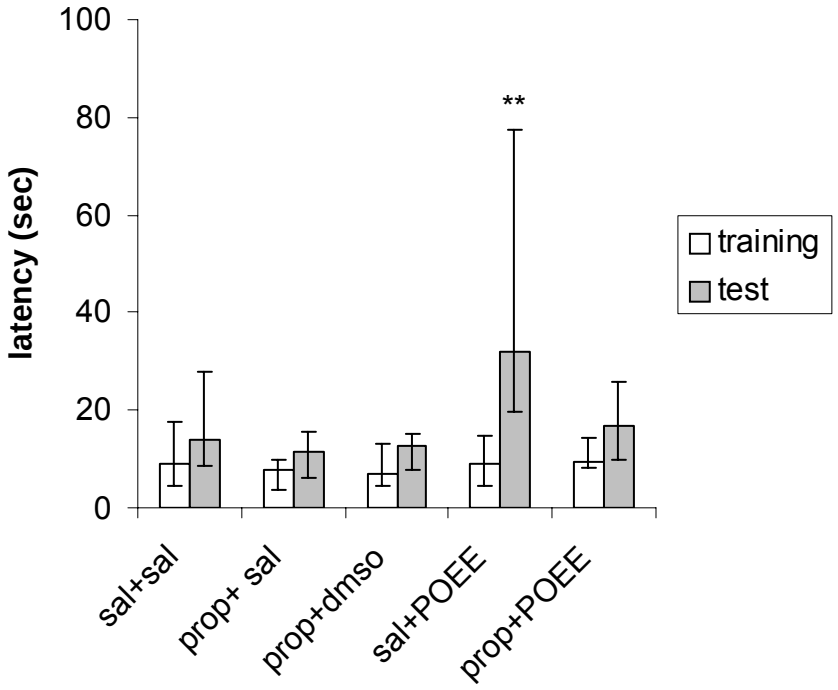
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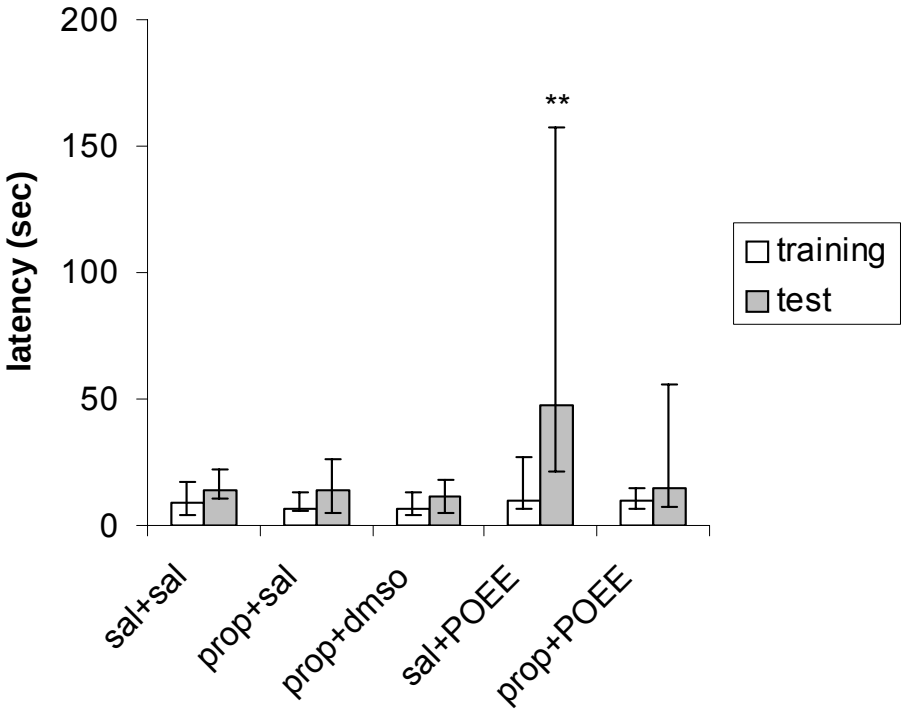
3C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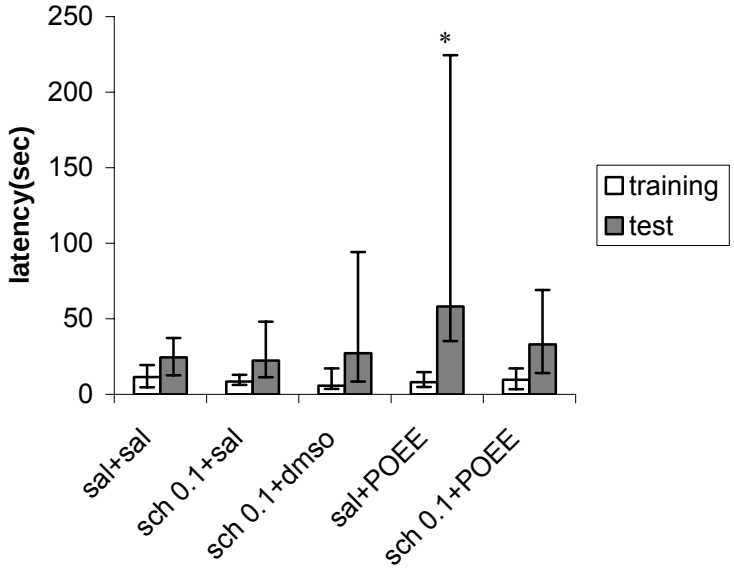
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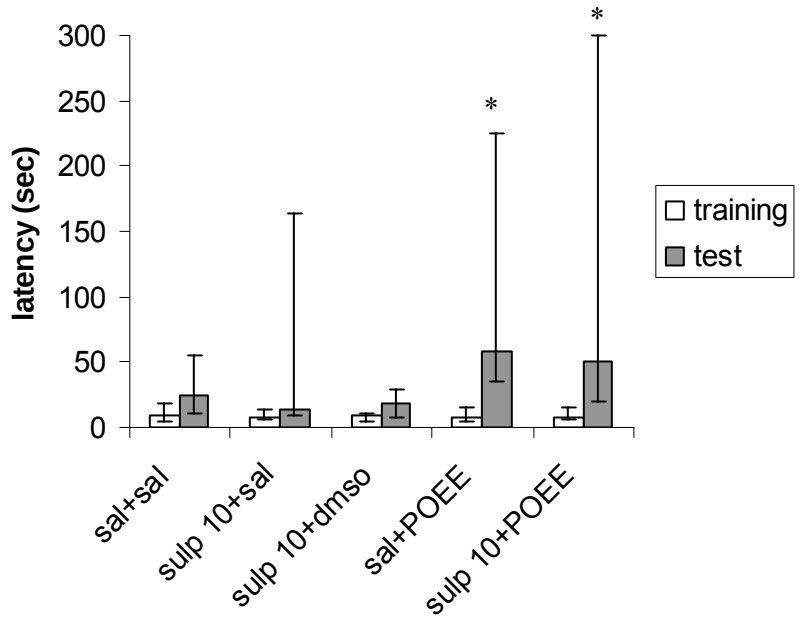
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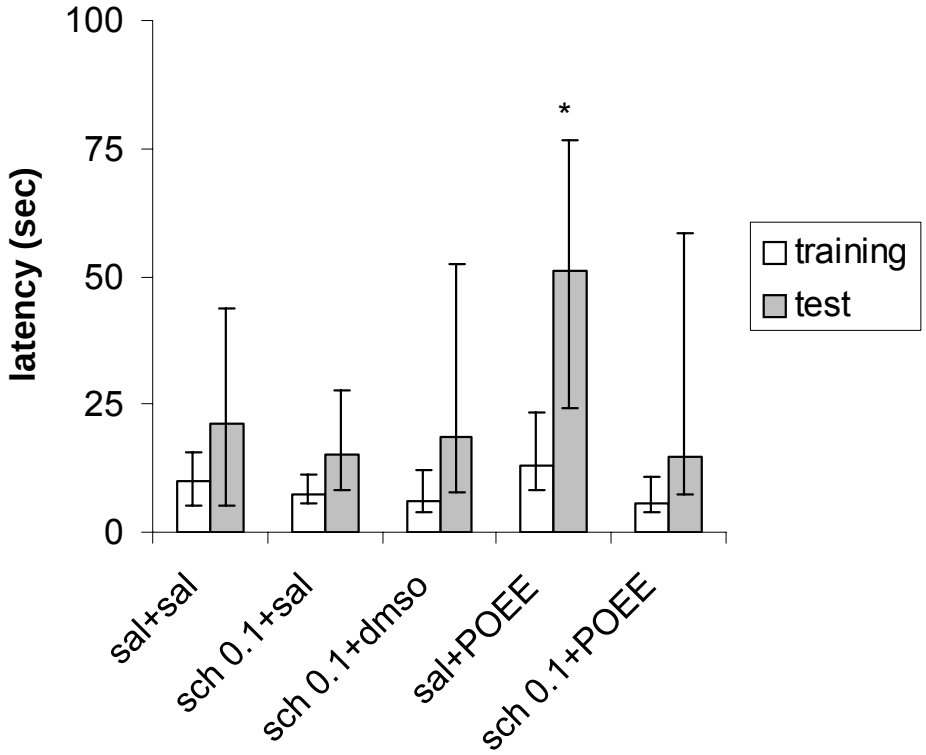
5A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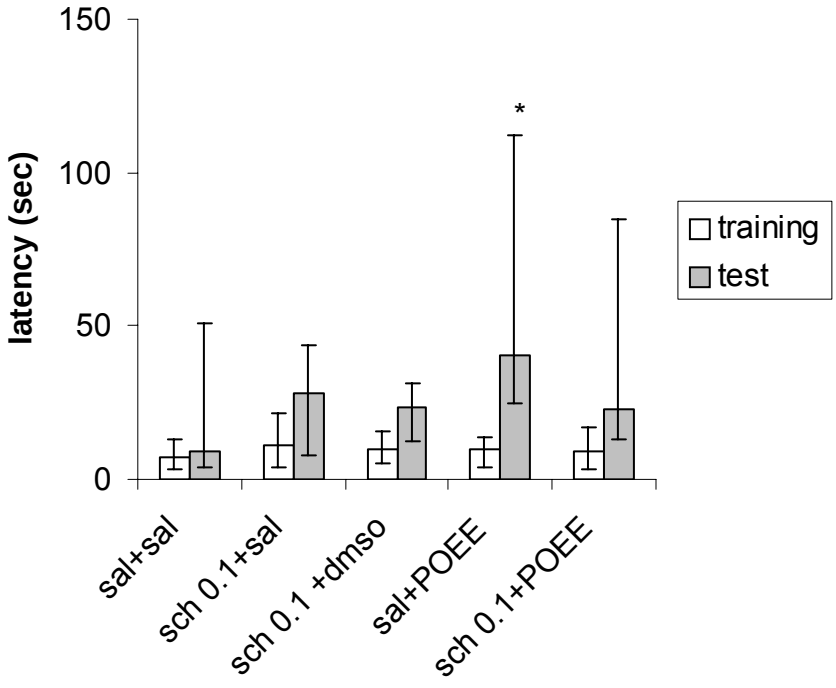
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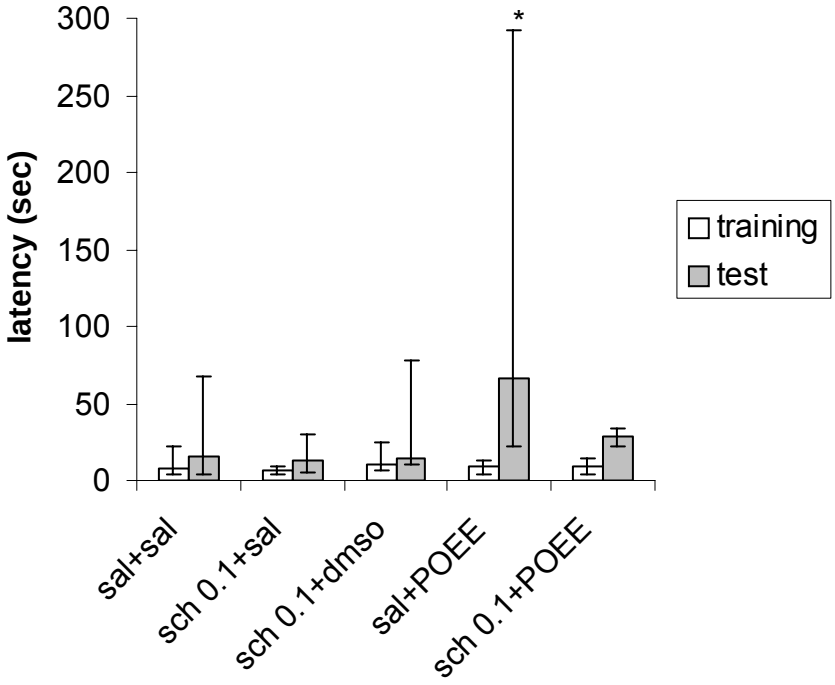
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Capítulo 4. DA SILVA AL, PIATO AL, FERREIRA JG, MARTINS BS, OLIVEIRA S, MAI N, NUNES DS, ELISABETSKY E. Role of serotonin 5-HT_{2A} receptor in the promnesic effects of *Ptychopetalum olacoides* Bentham.

(Submetido ao Pharmacological Research).

Role of serotonin 5-HT_{2A} receptor in the promnesic effects of *Ptychopetalum olacoides* Bentham.

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Total number of pages: 20

Total number of figures: 5

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Abstract

Nootropic, antioxidant, and neuroprotector properties have been shown with a standardized ethanol extract obtained from *Ptychopetalum olacoides* (POEE) roots, a medicinal plant traditionally used by Amazonian elderly population. It has been revealed that POEE mechanisms of action include anticholinesterase properties, the involvement of β adrenergic, and dopamine D_1 receptors. The purpose of this study was to verify the role of serotonin receptors in the promnesic effects of the extract, by using the step down task in mice and specific anatagonists. The study reveals that POEE promnesic effects on short-term (acquisition, consolidation and retrieval) and long-term (retrieval) memories is increased by $5HT_{2A,}$ but not $5HT_{1A,}$ serotonin antagonists (spiperone and pindolol, respectively). The effects of an ineffective dose POEE when combined with spiperone can be interpreted as the combined effects of two sub effective dosages of either two 5HT antagonists or an acetylcholinesterase inhibitory agent and a 5HT antagonist. It is suggested that $5HT_{2A}$ serotonin receptors are relevant for the promnesic effects of this extract.

Key words: *Ptychopetalum olacoides,* Marapuama, memory, step-down, serotoninergic, neurodegenerative disorders.

Introduction

Since the discovery of serotonin (5-hydroxytriptamine, 5-HT) over 50 years ago, it stands as a major research target; such continued interest is not surprising considering the nature of the 5-HT neuronal system, the diversity of 5-HT receptors, and its relevance for a variety of physiological actions [1]. Moreover, 5-HT is known to cross-talk with various neurotransmitter systems, the literature being particularly rich on the interaction of 5-HT and dopamine, and 5-HT and glutamate [1, 2]. The realization that heterogeneous family of at least 14 receptor subtypes mediates various 5-HT effects in the central nervous system (CNS) [3], lead to the expectation that selective and potent ligands could create better treatments for a variety of CNS disorders [4].

Recently, attention has been given to the role of serotonin in mood, the processing of emotional events, and cognition [5]. There are increasing experimental evidence indicating that 5HT plays an important modulatory role in learning and memory through $5HT_{1A/B/D}$, $5HT_{2A/2C}$, $5HT_3$, and $5HT_4$ receptors. 5-HT_1 and 5-HT_2 receptors are present in areas associated with learning and memory processes; data suggest that drugs that stimulate or block the 5-HT_{1B} and/or 5-$HT_{2A/2C}$ receptors impair or enhance learning, respectively [6,7]. Electrophysiological and autoradiography studies have shown that 5HT receptors are localized in the CA1 region hippocampus, vital for spatial learning and memory [8, 9]. The consolidation of short- and long-term memories is strongly modulated by dopamine, β-adrenergic, cholinergic muscarine, and $5HT_{1A}$ serotonin receptors [10].

It has been suggested that serotonergic alterations may also be relevant for behavioral disturbances commonly observed in the elderly [11]. $5HT_{2A}$ antagonists (ketanserin and DOI) have been shown to be promnesic, and 5-$HT_{2A/2B/2C}$ receptor blockade may be useful to restore memory deficits associated with decreased cholinergic and glutamatergic neurotransmission [12, 13]. A synergistic interaction between achetylcholine and serotonin seems to be relevant to memory, since the mixed 5-HT agonist/antagonist m-chlorophenylpiperazine (m-CPP) when co-administered with scopolamine reduces scopolamine-induced memory deficits in healthy elderly subjects [14], while ketanserin and physostigmine facilitates memory in aged rats [15].

A standardized ethanol extract obtained from *Ptychopetalum olacoides* Bentham (POEE) roots have been shown nootropic [16], antioxidant [17, 18], and neuroprotector [19] properties. This medicinal species, known locally as Marapuama, is a traditional herbal drug commonly used by the Amazonian elderly population. The pharmacodynamic basis of POEE

facilitatory effects on diverse memory types [16] includes anticholinesterase properties [20], and the involvement of dopamine D_1 and β adrenergic receptors (da Silva AL *et al.*, SBFTE 2004, FESBE 2005, respectively).

Considering that POEE enhances memory, reverses memory deficits in ageing mice, and possess anticholinesterase activity, the purpose of this study was to verify the role of serotonin receptors in the promnesic effects of the extract. For that matter, the effects 5-HT_{2A} (spiperone) and 5-HT_{1A} (pindolol) antagonists were studied in POEE-induced facilitation effects of short term (STM, acquisition, consolidation, and retrieval) and long-term memory (LTM, retrieval) in step down task in mice.

Material and Methods

Animals: male (CF1) adult albino mice, were used, received from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) with 2.0 months of age (35-45g). Animals were maintained in our own animal facility under controlled environmental conditions (22 ±1^{o}C, 12 hr-light/dark cycle, free access to food [Nuvilab CR1] and water), for at least two weeks before the experiments. All procedures were carried out in accordance with institutional policies on experimental animals handling, which follows the NIH guidelines (*NIH Guide for Care and Use of Laboratory Animals*, NIH publication no. 85-23, 1985)

Standardized Extract: Roots of *Ptychopetalum olacoides* Bentham (Olacaceae) were collected in Pará (Brazil) and identified by Nelson Rosa (voucher at the Goeldi Museum Herbarium, MPEG 108.036). *P. olacoides* ethanol extract (POEE) was prepared as detailed elsewhere [21]; briefly, dried ground plant material (2.5 kg) was extracted with ethanol (12 L), using a Soxhlet apparatus (40 h). The extract was evaporated under reduced pressure resulting in the POEE (150 g, 6% yield). Preliminary phytochemical screening gave positive test for saponin, phenolic compounds and terpenic compounds [18, 20].

Drugs: Saline (NaCl 0.9 %) and dimethyl sulphoxide (DMSO) were acquired from Delaware (Brazil); pindolol and spiperone from Sigma (USA). Pindolol was dissolved in distilled water and Spiperone were diluted in ethanol 0.03% (v/v). POEE (30, 50 and 100 mg/kg) was dissolved in DMSO 20 % (v/v).

Doses and administration schedules for antagonists were adopted from Zarrindast et al. [22] and Khalifa [7]. Pindolol (5HT_{1A} antagonist, 0.5 mg/kg) was administered 15 min, and spiperone (5HT $_{2A}$ antagonist, 0.03 mg/kg) 30 min before POEE (30 and 100 mg/kg) or controls injections. Drugs and vehicles were administered intraperitoneally, always as 0.1 ml/10 g of body weight. Control animals received respective solvent injections, and were run concurrently with drug-treated groups in randomized fashion.

Behavioral procedure

Step-down inhibitory avoidance: the method was adapted from Netto and Izquierdo [23], Maurice *et al.* [24] and Khalifa [7]. The inhibitory avoidance training apparatus was a plastic box with 30 x 30 x 40 cm, with a platform (5 x 5 x 4 cm) fixed in the center of the grid floor. Mice were habituated in the dim lighted room for at least 30 minutes before the experiments. Each mouse was placed on the platform and the latency to step down (four paws on the grid) was automatically recorded in training and test sessions. In the training session, upon stepping down, the mouse received a 0.3 mA scrambled foot shock for 15 sec. Animals training latencies greater than 30 sec were excluded from experiments; less than 5% of the animals met this exclusion criterion. The test session was performed 3 h or 24 h later, with the same procedure except that no shock was administered; an upper cut-off time of 300 sec was set. N=18-20 per treatment.

Results

Confirming that learning and memory took place with the training paradigm used in this study, there were significant and consistent differences ($P< 0.05$) between training and test sessions in all control groups. In agreement with previously reported data [16] POEE (100 mg/kg) improved ($P<0.05$) short-term memory (STM) acquisition (Fig. 2A), consolidation (Fig. 2B), and retrieval (Fig. 2C), as well as long-term memory (LTM) retrieval (Fig. 1A and 1B).

Figure 1 display the effects of serotonin antagonists on POEE-induced enhancement of LTM retrieval. **Figure 1A** refers to pindolol: no differences ($P=0.90$) were found between pindolol per se and controls (sal+sal 10.2[7.8/35.0] sec, pin+sal 14.8 [7.4/30] sec, pin+dmso 7.9 [5.9/12.9] sec). Kruskal-Wallis revealed a significant drug effect ($H (4) = 24.25$, $P<0.001$); Mann-Whitney showed that POEE 100mg/mg caused a significant ($P<0.01$) increase in test session latencies compared to controls (sal+POEE 100 mg/kg 49.6[19.6/300] sec), an effect not altered by pre treatment with pindolol (pin+POEE 58.8 [20.2/255.0] sec). Because pindolol did not have any effects on POEE-induced facilitation of LTM retrieval, it was not tested in STM. **Figure 1B** shows the effect of spiperone. No differences were found between spiperone per se and controls (sal+sal 11.5 [5.5/43.4] sec, spi+sal 13.5 [8.9/33.3]sec, spi+dmso 14.5 [7.2/33.9]sec, ETOH+sal 14.5[7.5/27.3] sec). Kruskal-Wallis revealed a significant drug effect ($H (7) = 40.2$, $P<0.001$); Mann-Whitney showed that POEE 100, but not 30 mg/kg, caused a significant ($P<0.05$) increase in test session latencies compared to

controls (sal+POEE 30mg/kg 27.2[7.3/44.9] sec, sal+POEE 100 mg/kg 38.4[21.4/138.5] sec). Nevertheless, both POEE doses significantly (P<0.01) facilitated memory when combined with spiperone (spi+POEE 30 mg/kg (147.2[25.5/300] sec, P=0.035; spi+POEE 100 mg/kg (211.6[56.1/300] sec) compared to controls. Additionally, the activity of POEE 100mg/kg is significantly (P=0.02) increased when combined with spiperone as compared to POEE 100mg/kg alone.

Figure 2 illustrates the effects of spiperone on POEE-induced STM improvement. Regarding acquisition (**2A**), no differences were found between spiperone per se and controls (sal+sal 16.6[6.8-74.0] sec, ETOH +sal 24.4 [11.9/60.3] sec, spi+sal 17.4 [9.2-77.6] sec, spi+dmso 27.3 [19.9-49.8] sec). Kruskal-Wallis revealed a significant drug effect (H (5) = 23.55, P<0.001); Mann-Whitney U-test showed that POEE alone or combined with spiperone caused significant (P<0.01 and P<0.05, respectively) and comparable (P=0.73) increases in test session latencies (sal+POEE 100 mg/kg (74.7[44.1/148.4] sec, and spi+POEE (54 [33.8/300] sec). The same pattern was seen regarding STM consolidation (**Figure 2B**) and retrieval (**Figure 2C**). Figure 2B: spiperone alone does not cause significant effects (sal+sal 13.3[6.6/22.0] sec, ETOH +sal 3.5[6.8/31.7]sec, spi+sal 17.3 [10.4/73.8]sec, spi+dmso 12.7 [6.6/26.8] sec). Kruskal-Wallis revealed a significant drug effect (H (5) = 16.62, P=0.005); Mann-Whitney showed that POEE alone or combined with spiperone caused significant (P<0.01 and P<0.05, respectively) and comparable (P=0.16) increases in test session latencies (sal+POEE 100 mg/kg (47.7[18.7/165.8] sec, spi+POEE 18.6 [14.0/127.8] sec). Figure 2C: no differences were found between spiperone and controls (sal+sal 19.1[8.8/76.4] sec, ETOH +sal 11.6[6.4/30.3] sec, spi+sal 18.0 [5.4/80.6] sec, spi+dmso 25.7 [5.0/56.7] sec). Kruskal-Wallis revealed a significant drug effect (H (5) = 18.37, P=0.003); Mann-Whitney showed that POEE 100mg/kg alone or combined with spiperone caused significant (P<0.05) and comparable (P=0.84) increases in test session latencies (sal+POEE 100 mg/kg 99.1[16.63/270.2] sec, and spi+ POEE 62.5 [26.2/253.7] sec).

Discussion

The present study reveals that POEE promnesic effects on and long-term memory retrieval, but not short-term memory, is increased by $5HT_{2A}$ (spiperone), but not $5HT_{1A}$ (pindolol) antagonist. Since antagonists alone were devoid of effects, changes caused by spiperone on POEE-induced memory improvement can not be attributed to its own potential impairment or improvement in memory or locomotion.

In vitro studies showed that spiperone has high affinity for 5-HT_{2A}, and low affinity for 5-HT_{1A} receptor, while pindolol has high affinity for 5-HT_{1A} and low affinity for 5-HT_{1B} receptor [5]. Spiperone has been shown to possess affinity for serotonin (5-HT_{2A}, 5-HT_7, 5-HT_{1A}), but also dopamine (D_2 and D_3), and α_1-adrenergic receptors. The dose of spiperone used in this study has been previously used to examine the role of 5-HT_2 receptors in 5HT-mediated behavior [5, 7]. Although the potential contribution of non serotonergic receptors modulated by spiperone to the data here reported has not been examined, such results would be in opposition to the usually detrimental role of these receptors in learning and memory [10, 25, 26].

5-HT_{2A} receptors are highly concentrated in the cortical layer V pyramidal neurons [27], where they may be involved in integrating cognitive and perceptual information from many diverse cortical and subcortical regions [28,29]. 5-HT_{1A} receptors are present in high density in the hippocampus, septum, amygdala, hypothalamus, and neocortex of human brain. 5-HT_{2A} receptors are present in all neocortical regions, with lower densities in hippocampus, basal ganglia, and thalamus [30]. 5-HT is involved in modulating ACh release in various cerebral structures, such as cholinergic pathways from the medial septum/diagonal band Broca (MS/DBB) to the hippocampus, or from the nucleus basalis magnocellularis (NBM) to cerebral cortex and amygdala [31-34]. Immunocytochemical studies have also indicated that NMDA receptors subunit NR1 co-localizes with the 5-HT_{2A} receptor in the ventral tegmental and other areas [35], with direct relevance to neuronal plasticity.

The role of serotonin in learning and memory is not clear cut. Destruction or pharmacological blockade of serotonin pathways have been reported to impair [36], be ineffective [37, 38], or facilitate rat performance in different memory tasks [39, 40]. Short- and long-term memory consolidation, as well as long-term memory retrieval, can be harmed by serotonin $5HT_{1A}$ agonist (OH-DPAT), dopaminergic D_1 (SCH23390), β-noradrenergic (timolol), and cholinergic muscarine (scopolamine) antagonists [10, 25, 41]. This scenario suggests that specificity of memory tasks may be of relevance for outcome. In humans, Alzheimer's disease (AD) has been associated with decline in 5-HT markers (uptake/transporter, and number of 5-$HT_{1A, 1B, 1D, 2A, 2C,}$ and $_4$ receptor subtypes in the raphe), and different 5-HT mechanisms are under investigation as potential treatments for amnesia and AD [42-44]. Age related decline in cortical 5-HT_{2A} binding in healthy subjects is supported by PET imaging with [11C]N-methylspiperone, a ligand with affinity for both 5-HT_{2A} and dopamine D_2 receptors [45,46]. Additionally, modulation of cholinergic activity by 5-HT may be significant for cognitive processes [36, 38, 40], as well as for AD and age-

related memory deficits. 5-HT_2 receptor blockade may be useful to reverse conditions characterized by poor memory consolidation, associated with decreased cholinergic, glutamatergic, and/or serotonergic neurotransmission [13]. Clinical trials data suggest that blockade of 5-HT_{2A} receptors may improve cognition in schizophrenia, and 5-HT_{2A} antagonists are likely to be well tolerated with minimal peripheral side-effects [47, 48]. Because serotonergic pathways are known to interact extensively with the cholinergic, noradrenergic, GABAergic and dopaminergic systems, it would not be surprising if serotonergic therapies could be used to manipulate other neurotransmitters systems to alleviate behavioral and psychological syndromes of dementia (BPSD) [49].

The role of serotonin receptors seems to be rather complex even when its participation on specific memory phases are scrutinized. While the 5-HT_{2A} antagonist EMD 28014 improved working memory acquisition in monkeys [50], ritanserin retarded the acquisition of conditioned responses during classical conditioning of rabbit's nictitating membrane, and blocked conditioned olfactory learning in neonate rat pups [51, 52]. The 5-HT_{2C} selective antagonists SB 200646A and SDZ SER 082, the 5-HT_{2A} selective antagonist SR 46349B, and the 5-$HT_{2A/2C}$ antagonist ritanserin, impaired acquisition in T-maze test, but no effects are observed with the selective 5-HT_{2A} antagonist RP 62203 in the same test [53]. Pharmacological modulation of 5-HT_{1-7} receptors or 5-HT reuptake sites affects consolidation [54]. Administration of the 5-HT_{1A} agonist 8-OH-DPAT, and the 5-HT_2 agonist DOI impaired passive avoidance acquisition and consolidation; nevertheless, the 5-HT_2 antagonist ketanserin blocked the impairing effect of DOI on passive avoidance consolidation [55]. While $5HT_{2A/2C}$ receptor antagonists (such as MDL 11939, pizotifen and cyproheptadine) impaired consolidation, others (like ketanserin, pirenperon and ritanserin) improved it [56, 57]. Increased 5-HT brain activity (e.g., through agonists RU-24969 and *m*CPP) has been shown to be detrimental to spatial discrimination, while decreased activity (e.g., antagonists ketanserin and ritanserin) improve its consolidation [6]. Ketanserin improved consolidation in senescent SAMP8 mice showing memory deficits [58]. Breier [59], and Nowakowska *et al.* [60] proposed that reversal of serotonin dysfunction and prefrontal hypoactivity (for instance by risperidone) is related to improved memory retrieval.

Serotonin activity may modulate learning and memory directly or indirectly by interfering with emotional aspects of behavior [8]. It has been shown that high levels of anxiety in rats is related to decreased levels of serotonin in the ventral striatum [61], as well as with deficits in performance and memory [62]. However, reduced fear (increased serotoninergic neurotransmition) is also expected to cause impairment of passive avoidance [63]. Ritanserin

and mianserin were prejudicial for conditioned eye blink behavior in rabbits [64], while ritanserin impaired elevated T-maze inhibitory avoidance in rats [65], reinforcing differential roles of serotonin receptors in diverse learning paradigms.

Two lines of reasoning can account for the results obtained in this study. Spiperone is known to induce ACh release, which would increasing cholinergic transmission add POEE acetylcholinesterase inhibition properties. Promnesic effects of acetylcholinesterase inhibitors are well documented, and similar positive interactions (or synergic) effects were observed by Normile and Altman [15] with ketanserin and physostigmine. Alternatively, since $5HT_2$ antagonism (ritanserin, ketanserin) were shown to facilitate inhibitory avoidance memory in mice, the effects ineffective dose POEE (30.0 mg/kg) when combined with spiperone could be interpreted as the combined effects of two sub effective dosages of either two 5HT antagonists or an acetylcholinesterase inhibitory agent and a 5HT antagonist. Promnesic effects of serotonin antagonists may be explained by the fact that 5-HT seems to act as an inhibitory neurotransmitter on cholinergic neurons. Therefore, decreases in serotonergic tone would help to maintain cholinergic input in cholinoceptive deficient target areas [66]. As discussed by Normile and Altman [15] there are significant complexities and discrepancies in the precise role of serotonin in mediating ACh, as well as the receptor subtype that mediate such responses [67,68]. In fact, a dual response characterized by a rapid and transient enhancement of ACh release mediated by $5\text{-}HT_2$ receptor, followed by a prolonged inhibition of efflux sensitive to both $5\text{-}HT_1$ and $5\text{-}HT_2$ antagonists have been reported [11,69]. This pattern could account for contradictory results from experiments using different pretreatment drug schedules.

Our data suggests that $5HT_{2A}$ serotonin receptors may be relevant for the promnesic effects of this plant extract. Further research is needed to identify the active constituent of the extract responsible for its nootropic activity, and binding studies with such compounds will better clarify how exactly POEE interacts with serotonin receptors.

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Figure 1. Effect of serotonergic antagonists (**1A** pindolol, 5-$HT_{1A;}$ **1B** spiperone, 5-HT_{2A}) on POEE-induced improvement of step-down LTM retrieval. ETOH= etanol 0.03%; dmso = dimethyl sulphoxide 20%; POEE 30= POEE 30.0 mg/kg POEE100=POEE 100.0 mg/kg; spi=spiperone 0.03mg/kg; pin= pindolol 0.5 mg/kg. N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies. * P<0.05 and ** P<0.01 x controls test latencies; # P<0.05 x Spi + POEE, Mann-Whitney/Kruskal-Wallis.

Figure 2. Effect of spiperone on POEE-induced improvement of step-down STM (**2A** acquisition, **2B** consolidation, and **2C** retrieval). ETOH= etanol 0.03%; dmso = dimethyl sulphoxide 20%; POEE = POEE 100.0 mg/kg; spi=spiperone 0.03mg/kg. N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns) latencies. * P<0.05 and ** P<0.01 x controls test latencies, Mann-Whitney/Kruskal-Wallis.

Figure

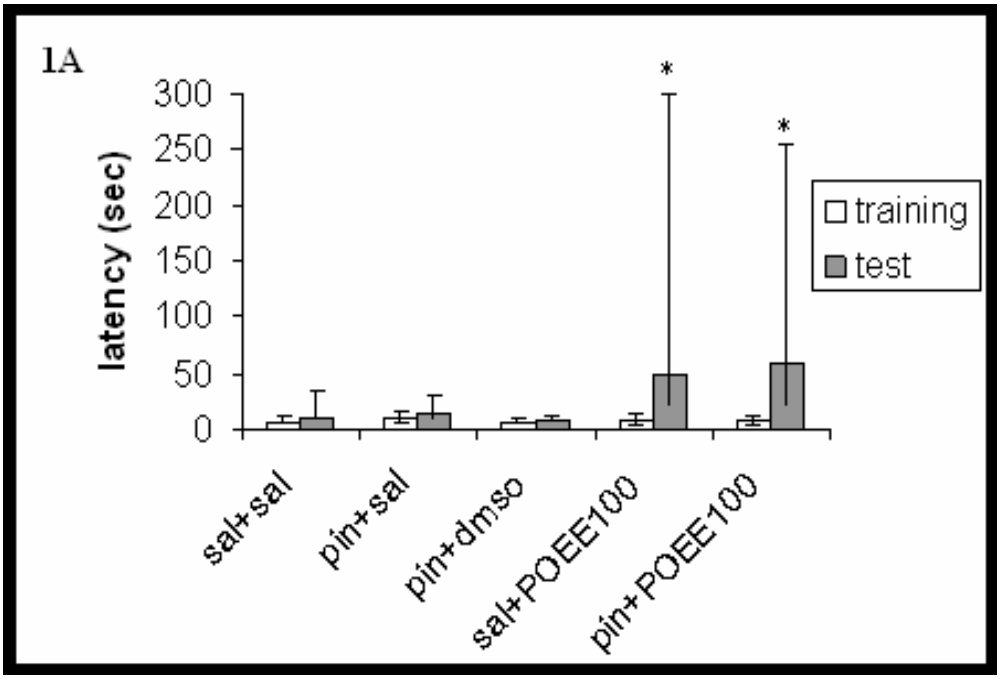
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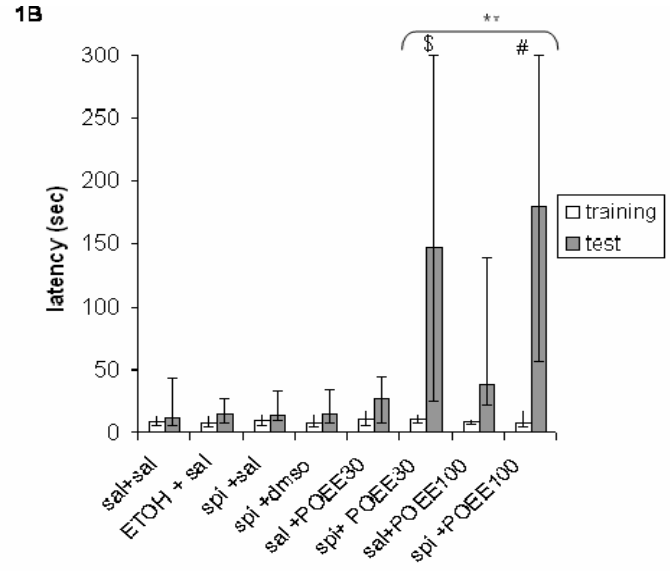
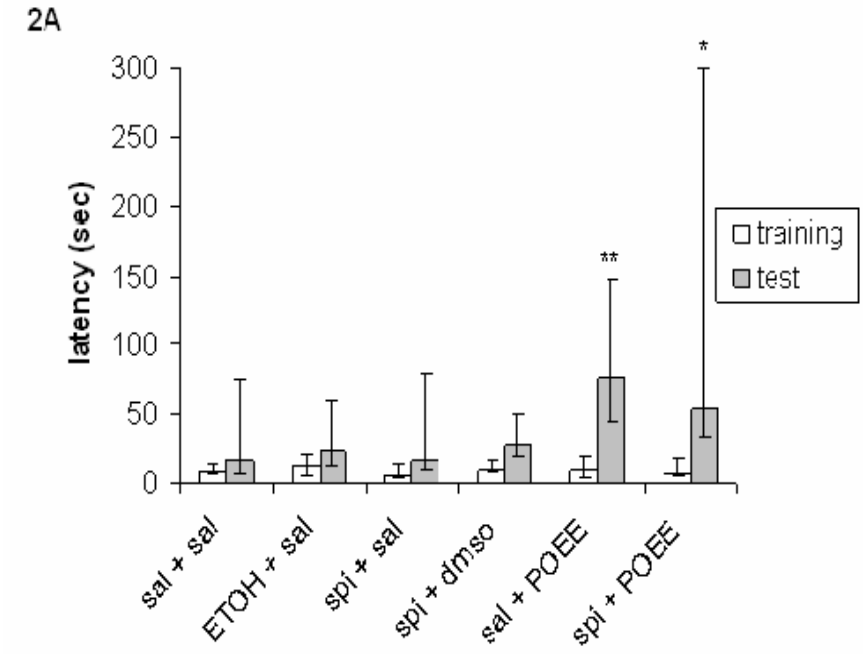
Figure



Figure



Figure

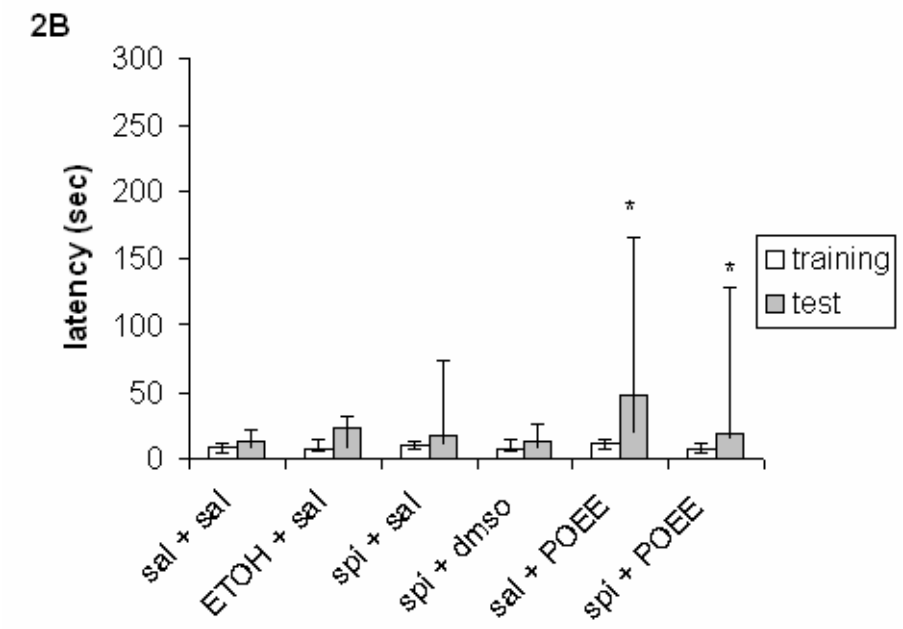

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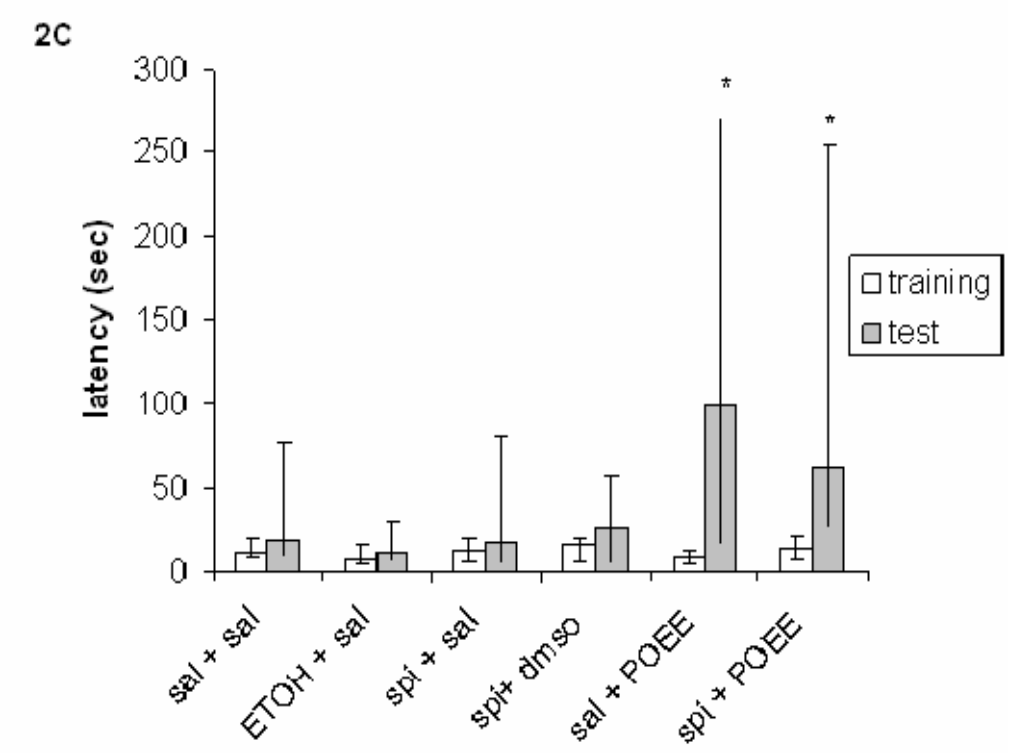
Figure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spi+ dmso
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spi + POEE

Capítulo 5. DA SILVA AL, PIATO AL, MARTINS BS, MAI N, NUNES DS, ELISABETSKY E. *Ptychopetalum olacoides*, a traditional brain tonic, reverses MK-801 and scopolamine induced amnesias in mice.

(A ser submetido ao Phytomedicine).

Ptychopetalum olacoides, a traditional brain tonic, reverses MK-801 and scopolamine induced amnesias in mice.

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Abstract

Traditional remedies prepared from *Ptychopetalum olacoides* (PO) is used to alleviate age-related decline function, which may be associated with anticholinesterase properties previously described for an ethanol extract of this species. In the present study, the effects of POEE on scopolamine-induced amnesia in mice were investigated. In addition, the participation of NMDA mechanisms in the memory facilitatory effect of POEE was examined. Scopolamine (3.0 mg/kg i.p.), a muscarinic cholinergic antagonist, significantly impaired memory in the a step-down inhibitory avoidance test. Administration of POEE (50 and 100 mg/kg/kg i.p.) reversed the memory impairment induced by scopolamine, as well as the memory consolidation deficit induced by MK-801 (0.1 mg/kg) NMDA antagonist. These results suggest that POEE may be relevant for treating cognitive deficits mainly associated with cholinergic function.

Key words: *Ptychopetalum olacoides,* Marapuama, memory, cholinergic, glutamatergic, neurodegenerative disorders.

Introduction

Reduction in cholinergic function, particularly at the level of muscarinic receptors, is a characteristic of patients suffering from dementia; this deficit has been used to model the condition in animals (Fadda et al., 2006). Brain cholinergic neurons degenerate in Alzheimer's disease (AD) and senile dementia of the Alzheimer's type, and the degree of cholinergic degeneration parallels functional loss in these disorders (Davies and Maloney, 1976; Perry et al., 1978; Coyle et al., 1983). This clinical evidence, as well as experimental observations in animals, strongly supports the hypothesis that cholinergic activity plays a crucial role in various forms of cognitive function (Bartus et al., 1982; Hepler et al., 1985; Matsuoka et al., 1991).

Studies have shown that acetylcholine release increases in the hippocampus during performance of a learned spatial memory task (Ragozzino et al., 1999; Stancampiano et al., 1999). The increase in acetylcholine (ACh) is positively correlated to the improvement in performance during task learning (Fadda et al., 2000), showing that cholinergic neurons are functionally modified during learning, becoming progressively more active. The inhibitory avoidance response is a learning task that depends upon activation of the cholinergic system, as shown by its impairment with pre- (Izquierdo et al.; 1998; Giovannini et al., 1999) or post-training administration of muscarine antagonists (Izquierdo et al., 1998; Giovannini et al.; 1999; McGaugh and Izquierdo, 2000), and enhancement by muscarine agonists (Baratti et al.; 1979; Barros et al., 2002). Scopolamine, a nonselective muscarine antagonist, has been used as a pharmacologic tool to induce amnesia and evaluate the effects of nootropic drugs on memory deficits in experimental animals (Ebert and Kirch, 1998; Smith, 1988).

A most important neurotransmitter in the central nervous system is glutamate, playing a central role in neuroplasticity, learning and memory, as well as neurodegenerative diseases

(Kawabe et al., 1998; Moser and Moser, 1998; Zhang et al., 2000). While rapid transmission is mediated via ionotropic glutamate receptors (such AMPA, kainate, and NMDA), transmission of the G-protein-coupled metabotropic glutamate (mGlu) receptors is more modulatory in nature (Parsons et al., 1998; Pin and Acher, 2002). Although NMDA receptors are ubiquitously distributed in the brain, it presents higher densities in the basolateral amygdala nuclei (Monaghan and Cotman, 1985), and the highest binding sites concentrations are found in the hippocampus CA1 area, with substantial concentrations also localized within the dentate gyrus (Monaghan et al., 1983). In hippocampus CA1, NMDA receptors are extremely important in regulating synaptic plasticity, long term-potentiation (LTP), as well as learning and memory processes, including short- and long-term memories (Kensner and Dakis , 1995; Nguyen and Kandel, 1996; Kawabe et al. 1998; Moser and Moser, 1998; Zhang et al., 2000; Riedel et al., 2003). Competitive and noncompetitive NMDA receptor antagonists have been shown to impair learning and memory processes in various behavioral tasks (Zanatta et al., 1996; Roesler et al., 1998; Jafari-Sabet et al., 2005) as do lesions of certain glutamatergic pathways (Myhrer, 2000).

Alcoholic infusions of *Ptychopetalum olacoides* Bentham (PO, OLACACEAE), known as "Marapuama", are consumed in the Amazon for the treatment of central nervous systems (CNS) related conditions, and/or during highly stressful periods (Elisabetsky, 1987; Siqueira et al., 1998). In the context of the regional traditional medicine, a rich mixture of Amerindian, European and African components (Elisabetsky and Setzer, 1985), this species is considered a "brain tonic". We have previously reported that standardized extract of PO (POEE) possesses various CNS relevant proprieties, including antioxidant (*in vitro* [Siqueira et al., 2002] and (*in vivo*) [Siqueira et al., 2006], and neuroprotective (Siqueira et al., 2004). Regarding cognition, we showed that POEE facilitates acquisition, consolidation and retrieval of short-term and retrieval of long-term memories (inhibitory avoidance) in adult and aged mice (Siqueira et al.,

1998; da Silva et al., 2004), and that it inhibits AChE (*in vitro* and *ex vivo* assays), suggesting that improvement in cholinergic function might be a neurochemical correlate of the extract behavioral effects (Siqueira et al., 2003).

The purpose of this study was to investigate the effects of POEE administration on MK-801 and scopolamine-induced amnesias in mice, using the step-down inhibitory avoidance tasks.

Material and Methods

Animals: experiments were performed using male (CF1) adult albino mice, received from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) with 2.0 months of age (35-40g). Animals were maintained in our own animal facility under controlled environmental conditions (22 ±1^{o}C, 12 hr-light/dark cycle, free access to food [Nuvilab CR1] and water), for at least two weeks before the experiments. All procedures were carried out in accordance with institutional policies on experimental animals handling, which follows the NIH guidelines (NIH Guide for Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985).

Preparation of Extract: Roots of *Ptychopetalum olacoides* Bentham (Olacaceae) were collected in Pará (Brazil), and identified by Nelson Rosa (MPEG 108.036 voucher at the Goeldi Museum Herbarium). *P. olacoides* standardized ethanol extract (POEE) was prepared and characterized as detailed elsewhere (Elisabetsky and Siqueira, 1998); briefly, dried ground plant material (2.5 kg) was extracted with ethanol (12 L), using a Soxhlet apparatus (40 h); the extract was evaporated under reduced pressure resulting in the POEE (150 g, 6% yield). Preliminary phytochemical screening gave positive test for saponin, phenolic compounds and terpenic compounds (Elisabetsky and Siqueira, 1998; Siqueira et al., 1998; Siqueira et al., 2006).

Drugs: Dimethyl sulphoxide (DMSO) was acquired from Delaware (Brazil). Scopolamine (scop), eserine (ese) were obtained from Sigma (USA) MK-801 was obtained from RBI (USA).

Scopolamine, eserine and MK-801 were dissolved in distilled water. Saline (NaCl 0.9%, sal) was used as blank control. All chemicals were of analytical grade.

Doses and administration schedules for the antagonists were adopted from Zarrindast et al. (1996) and Khalifa (2001). Saline, DMSO 20%, eserine (0.075 mg/kg) POEE (50.0 or 100.0 mg/kg) was administered 45 min and scopolamine (3.0 mg/kg) 15 min before training (acquisition), test (retrieval) or immediately posttraining (consolidation). POEE (100.0 mg/kg) was administered 60 min and MK-801 (0.1 mg/kg) 30 min before training (acquisition), test (retrieval) or immediately posttraining (consolidation). Drugs and vehicles were administered intraperitoneally, as 0.1 ml/10 g of body weight. Control groups received saline or respective solvents, and were run concurrently with drug-treated groups; for this reason and statistical accuracy graphics display the groups pertaining to experiments run at a given time.

Behavioral procedures

Step-down inhibitory avoidance: the test used was adapted from Netto and Izquierdo (1985), Maurice *et al.* (1994), Bernaerts et al. (2004). Mice were habituated in the dim lighted room for at least 30 minutes before the experiments. The inhibitory avoidance training apparatus was a plastic box of 30 x 30 x 40 cm, with a platform (5 x 5 x 4 cm) fixed in the center of the grid floor. Each mouse was placed on the platform and the latency to step down (four paws on the grid), was automatically recorded in training and test sessions. In the training session, the mouse received a 0.4 mA scrambled foot shock for 15 sec, upon stepping down. Animals exhibiting step down latencies greater than 30 sec in training were excluded from experiments; less than 5% of the animals met this exclusion criterion. The test session was performed 3 h (STM) or 24 h (LTM) later, with the same procedure except that no shock was administered after stepping down; an upper cut-off time of 300 sec was set. N=18-21 per treatment group.

In order to determine whether the effects of the drugs on step-down inhibitory avoidance could be explained in terms of drug-induced learned aversion or other inespecífica drug effects the following experiment was included: mice were divided in four groups, (1) saline non-shocked, (2) POEE non-shocked, (3) saline shocked, (4) POEE shocked. The non-shocked animals were treated just as described above, except that no foot shock was administered following stepping down. In test sessions (performed 24 h, LTM) later, animals of groups 2 and 4 received POEE 100 mg/kg, while groups 1 and 3 received saline 30 minutes before returning to the inhibitory avoidance apparatus.

Results

Significant and consistent differences ($p< 0.05$) between training and test session latencies in controls groups were observed, confirming that learning and memory took place with the

training paradigm used. The observed increases in step-down in test sessions are unlikely to be the result of non specific drug effects on behavior since non shocked (NS) animals either treated with saline or POEE (100 mg/kg) do not present increased latencies on test sessions ($p=0.11$), significantly ($p<0.05$) differing from control or POEE treated animals that received foot shock **(Figure 1)**.

Consistent with previously reported data (da Silva et al., 2004), POEE (50.0 and 100.0 mg/kg) improved STM acquisition (Figs. 2A and 4A), STM consolidation (Figs. 2B and 4B), and ST (Figs.3A and 5A), and LT (Figs.3B and 5B) retrieval, as expressed by increased ($p<0.05$) test sessions latencies in comparison with control groups.

Figure 2 shows the effects of POEE on scopolamine-induced amnesia in acquisition (Fig. 2A) and consolidation (Fig. 2B). Referring to STM acquisition **(Fig 2A)**, Kruskal-Wallis revealed a significant drug effect ($H(4) = 33.0$, $p<0.001$); Mann-Whitney showed that groups that receive scopolamine show lower ($p<0.01$) STM test latencies (sal+scop 7.5[2.8/9.5] sec, dmso+scop 6.1[2.4/18.6]sec) when compared to controls (sal+sal 27.1[16.7-75.1] sec). Scopolamine-induced amnesia was reversed by pre-treatment with either POEE or eserine (POEE100 mg/kg + scop (32.3[14.9/77.4] sec), ese+ scop (20.9 [12.0/85.0] sec), with significant ($p<0.01$) and comparable ($p=0.61$) increases in test session latencies in comparison with amnesic controls. Regarding LTM aquisition, Kruskal-Wallis revealed a significant drug effect ($H(4) = 31.2$, $p<0.001$); Mann-Whitney showed that groups that received scopolamine (sal+scop 5.8[2.8/14.6] sec, dmso+ scop 8.5[4.5/30.8]sec) showed lower ($p<0.01$) latencies when compared to controls (sal+sal 79.5[11.8/206.4]). This amnesia was also reversed by POEE (100 mg/kg + scop 32.3[14.9/77.4] sec), or eserine (ese+ scop 29.6 [14.8/77.4] sec), which caused significant ($p<0.01$) and comparable ($p=0.77$) increases in test session latencies as compared to amnesic controls. Referring to STM consolidation (Fig. **2B**), Kruskal-Wallis revealed a significant drug effect ($H(4) = 27.4$, $p<0.001$); Mann-Whitney showed that groups

that received scopolamine have amnesia as expressed by lower ($p<0.05$) STM test latencies (sal+scop 8.6[3.6/14.3] sec, dmso+ scop 8.4[5.0/16.4] sec) as compared to controls (sal+sal 19.6[14.9-44.7] sec). The amnesia is likewise reversed by pre treatment with POEE (POEE100 mg/kg + scop, 19.0[10.6/57.6] sec), and eserine (ese+scop 16.7 [11.5/24.0] sec), which caused significant ($p<0.01$) and comparable ($p=0.29$) increases in test session latencies as compared to amnesic controls. Regarding LTM consolidation, Kruskal-Wallis revealed a significant drug effect ($H(4) = 25.3$, $p<0.001$); Mann-Whitney showed that groups that receive scopolamine (sal+scop 10.8[3.5/18.3] sec, dmso+scop 8.1[5.6/18.5]sec) were amnesic ($p<0.05$) when compared to controls (sal+sal 21.3[10.9/46.6] sec). POEE (100 mg/kg+scop, 32.4[17.0/168.1] sec), and eserine (ese+scop, 16.0 [11.4/106.4] sec) were able to reverse the scopolamine–induced amnesia, causing significant ($p<0.01$ and $p<0.05$ respectively) and comparable ($p=0.093$) increases in test session latencies compared to amnesic controls.

Figure 3 shows the effects of POEE on scopolamine-induced amnesia in STM. (Fig. 3A) and LTM (Fig. 3B) retrieval. Referring to STM **(Fig 3A)**, Kruskal-Wallis revealed a significant drug effect in ($H(4) = 30.27$, $p<0.001$); Mann-Whitney showed that groups that receive scopolamine (sal+scop 16.6[5.1/29.7] sec, dmso+ scop 15.2[7.9/27.4] sec) shown lower ($p<0.05$) latencies when compared to control (sal+sal 23.9[16.6-58.2] sec). POEE100 mg/kg (57.4[33.9/300] sec), and eserine (25.2 [17.8/47.1] sec) reversed scopolamine–induced amnesia, causing significant ($p< 0.01$, and $p<0.02$ respectively) increases in test session latencies compared to amnesic controls. Referring to LTM retrieval **(Fig 3B),** Kruskal-Wallis revealed a significant drug effect ($H(5) = 14.92$, $p<0.011$); Mann-Whitney showed that groups that receive scopolamine (sal+scop 22.9[13.1/37.0] sec, dmso+ scop 21.7[6.4/52.9]sec) show lower ($p<0.05$) latency LTM retrieval when compare to control (sal+sal 83.3[19.5-300] sec). POEE 100 mg/kg (61.1[24.5/300] sec), and eserine (40.9 [20.6/300] sec), but not POEE 50 mg/kg (66.7[13.4/128.3] sec), reversed scopolamine–

induced amnesia causing significant ($p<0.01$ and $p<0.05$ respectively) and comparable ($p=0.39$) increases in test session latencies compared to amnesic controls.

Figure 4 shows the effects of POEE (100mg/kg) on MK-801-induced amnesia in acquisition (**Fig. 4A**) and consolidation (**Fig. 4B**). Referring to STM acquisition (**Fig. 4A**), Kruskal-Wallis revealed a significant drug effect in STM ($H(4) = 54.9$, $p<0.001$); Mann-Whitney showed that groups that receive MK-801 (sal+MK 2.8[1.6/7.4] sec, dmso+MK 3.7[1.9/6.6]sec) show lower ($p<0.05$) latencies when compared to control (sal+sal 23.9[14.3/48.6] sec). When POEE and MK801 (POEE+MK 8.4[4.6/12.5] sec) were given before the test session, POEE did not attenuate MK801-induced amnesia. POEE (POEE+sal 67.0[22.3/150.4]sec) caused significant increases in test session latencies compared to saline (sal+sal, $p=0.032$) and amnesic controls ($p<0.01$). Regarding LTM retrieval, Kruskal-Wallis revealed a significant drug effect in LTM ($H(4) = 59.5$, $p<0.001$); Mann-Whitney showed that groups that receive MK-801 (sal+MK 3.0[2.4/5.7] sec, dmso+MK 4.3[2.5/7.0]sec) show lower ($p<0.01$) latencies when compared to control (sal+sal 14.4[8.0/119.9] sec). When POEE and MK-801 (POEE-MK 9.0[4.8/15.5] sec) were given before the test session, the extract did not attenuate MK-induced amnesia. (POEE+sal 44.4[25.2/100.3]sec) caused significant ($p<0.01$) increases in test session latencies compared to amnesic controls (but not to saline, $p=0.064$). Referring to STM consolidation **(Fig 4 B)**, Kruskal-Wallis revealed a significant drug effect in STM ($H(4) = 37.6$, $p<0.001$); Mann-Whitney showed that groups that received MK-801 (sal+MK 10.4[4.3/16.7] sec, dmso+MK 9.8[6.0/15.7]sec) show lower ($p<0.05$) latencies when compared to control (sal+sal 12.8[10.0/26.8] sec). When POEE and MK801 (POEE+MK 25.5[17.0/42.4] sec) were given after the training session, POEE attenuated MK-induced amnesia ($p<0.01$, compared to sal+MK and DMSO+MK). POEE (POEE+sal 30.4[19.3/86.8]sec) caused significant increases in test session latencies compared to sal+sal ($p=0.005$) and amnesic controls ($p<0.001$). In LTM session, Kruskal-Wallis revealed a

significant drug effect (H (4) = 18.5, p<0.001); Mann-Whitney showed that groups that receive MK-801 (sal+MK 8.4[5.7/26.6] sec, dmso+MK 14.7[12.7/15.9]sec) show lower (p<0.01) latencies when compared to control (sal+sal 53.4[21.4/113.2] sec). When POEE and MK-801 (POEE+MK 21.3[17.0/55.7] sec), were given after training sessions, POEE attenuated MK-induced amnesia (p<0.05, compared to sal+MK and DMSO+MK). POEE (POEE+sal 21.1[13.3/90.4]sec) caused increases (p<0.01) in test session latencies compared to amnesic controls, but not to sal+sal group (p=0.239).

Figure 5 shows the effects of MK-801 on the promnesic effect POEE in STM (**Fig. 5A**) and LTM (**Fig. 5B**) retrieval. Referring to STM **(Fig. 5A)**, no differences were found between MK-801 and controls (sal+MK 26.7 [12.7-94.6] sec, dmso+MK 16.0[8.3-33.1]sec, and sal+sal 15.6[8.0-29.1]sec). Kruskal-Wallis revealed a significant drug effect (H (4) = 27.0, p<0.001); Mann-Whitney showed that POEE (sal+POEE 136.6[54.1/300.0] sec and POEE+MK (252.5[22.6/300.0]sec) caused significantly (p< 0.05) and comparable (p=0.75) increases in test session latencies as compared to controls. Referring to LTM **(Fig. 5B)**, no differences were found between MK-801 and controls (sal+MK 25.6 [13.2-68.0] sec, dmso+MK 17.1[7.0-31.5] sec, and sal+sal 15.6[8.0-29.1] sec). Kruskal-Wallis revealed a significant drug effect (H (4) = 25.6, p<0.001); Mann-Whitney showed that POEE alone (sal+POEE 140.6[54.3/300.0]sec) or combined with MK-801 (POEE+MK 250.5[22.4/300]sec) caused significantly (p< 0.05) and comparable (p=0.75) increases in test session latencies as compared to controls.

In all the above cases, amnesia reversal is total (test latencies do not differ from saline treated groups, p≥ 0.05), except for STM retrieval where test latencies are actually greater (p<0.01) than saline, and MK-induced amnesia where as expected STM latencies are lower (p<0.05) than saline.

Discussion

The chief finding of this study is that POEE reverses scopolamine-induced amnesia, for both STM and LTM and all memory phases (acquisition, consolidation, and retrieval). Regarding MK801-induced amnesia, POEE is capable of reversing consolidation but not acquisition, again for both STM and LTM. The non shocked control group demonstrates that POEE promnesic effect is not related to non specific drug effect on behavior, since POEE or saline treated non shocked animals failed to exhibit increased test latencies in test sessions.

A large number of studies have reported that muscarinic agonists (Castellano et al., 1996; Eidi et al., 2003), and/or acetylcholinesterase inhibitors improve memory (Degroot and Parent , 2001; Zarrindast et al., 2002), while anticholinergic drugs impair learning and memory in a variety of tasks (Bacciottini et al., 2001; Zhang et al., 2002). Regarding glutamate, it has been suggested that memory improvement can be obtained with NMDA agonists or partial agonists (Flood et al., 1990; Parada-Turska et al., 1990), while both competitive and noncompetitive NMDA antagonists have been shown to impair learning and memory processes in various behavioral tasks (Francis et al., 1993; Roesler et al., 1998; Jafari-Sabet et al., 2005).

Cooperativity (cross-talk) between the glutamate, dopamine and cholinergic systems has been well documented (Hernandez et al., 2003; Cepeda and Levine, 2006), and found to be relevant to learning and memory (Aigner, 1995; Castellano et al., 1996; Li et al., 1997). Both muscarine and nicotine receptors activate glutamatergic pyramidal neurons, increasing glutamate release (Chessell and Humphrey, 1995; Djik et al., 1995). Post-training co-administration of low doses of NMDA and physostigmine, ineffective when given alone, significantly improves retention (Jafari-Sabet, 2006). Moreover, the NMDA antagonists DL-AP5 and MK-801 decrease the effects of physostigmine, and atropine (which by itself

decreases memory retention) potentiates the response to DL-AP5 and MK-801 (Jafari-Sabet, 2006). Similar results were reported with hippocampal slices (Markram and Segal, 1990).

Several pathological conditions usually related to glutamate excitotocity, such as hypoxia (Djuricic et al, 1991; Klein et al., 1993), ischemia (Belay et al, 1991; Scremin and Jenden, 1989), seizures (Flynn and Wedeer, 1987; Jope and Gu, 1991), and brain trauma, also result in choline release into the extracellular space via the activation of NMDA receptors (Zapata et al, 1998). Consequently, it has been suggested that a link between NMDA and muscarinic receptor mediated neurotransmission is involved in the regulation of memory processes (Ohno and Watanabe, 1996). Conversely, diseases more often associated with cholinergic deficit, such as AD, are known to significantly affect glutamate transmission. It is known that the glutamatergic system is down-regulated in the brain of AD patients (Greenamyre et al., 1987; Palmer and Gershon, 1990; Francis et al., 1993; Fonnum et al., 1995, Palmer, 1996). Wang et al. (1999) showed that donepezil (an anticholinesterase drug used in AD) inhibited the [^{3}H]dizocilpine binding to synaptic membrane of rat cerebral cortex. Recently memantine, a drug capable of modulating NMDA activity, has become available for the clinical management of patients with various types of dementia (Parsons et al., 1999; Danysz and Parsons, 2003).

In dementia, patients appear to have a dysfunctional central cholinergic system (Bartus et al, 1982). Many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the use of acetylcholinesterase inhibitors (Brinkman and Gershon, 1983; Rogers et al., 1998). However, these compounds have shown modest efficacy and frequent unfavorable side-effects due to parasympathetic over-stimulation, thereby limiting their long-term treatment (Benzi and Moretti, 1998; Nordberg and Svensson, 1998).

Nootropic drugs improve cognitive function by increasing the activity of nACh and/or NMDA receptors in AD, as well as patients with other forms of dementia (Narahashi et al,

2004). It has been recently reported that galantamine, known to inhibit cholinesterases and potentiate the activity of nAChR (Schrattenholz et al., 1996; Maelicke and Albuquerque, 2000; Samochocki et al., 2003, also potentiates NMDA-evoked currents in rat cortical neurons (Moriguchi et al., 2004). Moriguchi et al (2005) showed that donepezil potentiate NMDA activity, and suggested that this potentiation associated with cholinesterase inhibition would contribute to the improvement of cognition in AD patients.

The present study shows that memory impairment induced in mice by scopolamine and MK-801 can be reversed by POEE, suggesting that its nootropic properties may include glutamatergic along with its previously shown cholinergic properties. As discussed above, such pharmacological profile may have considerable advantages over classic cholinesterase inhibitors. These results reinforces the potential therapeutic value of POEE in conditions presenting cognitive deficits, such as normal ageing, Alzheimer's disease or senile dementia of the Alzheimer's type.

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Figure 1. Influence of POEE (100 mg/kg) and saline on LTM retrieval in step-down inhibitory avoidance of shocked and non-shocked animals. Each column represents the median (interquartile ranges) of training (light columns) or test (dark columns) session latencies *p<0.05, significant difference compared with controls (saline shock) in Mann-Whitnney U test, following Kruskal-Wallis. N=20.

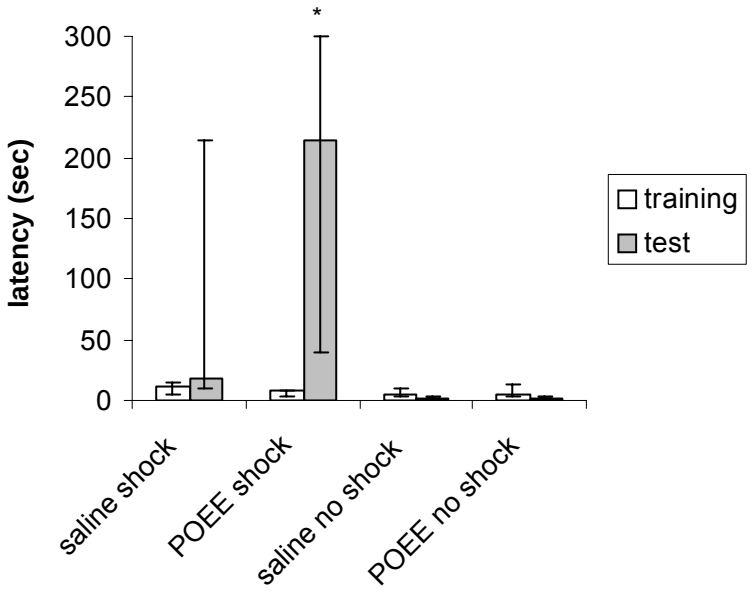
Figure 2. Effects of POEE on scopolamine-induced amnesia in acquisition (**Figure 2A**) and consolidation (**Figure 2B**) STM/LTM. Scop = scopolamine 3.0 mg/kg; ese = eserine 0.075 mg/kg; dmso= dimethyl sulphoxide 20%; POEE = POEE 100.0 mg/kg . N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * p<0.05 and ** p<0.01 x controls test latencies, # p<0.05 and ## p<0.01 x controls amnesic latencies; Mann-Whitney/Kruskal-Wallis.

Figure 3. Effects of POEE on scopolamine-induced amnesia in STM (**Figure 3A**) and LTM (**Figure 3B**) retrieval. Scop = scopolamine 3.0 mg/kg; ese = eserine 0.075 mg/kg; dmso= dimethyl sulphoxide 20%; POEE 50= POEE 50.0 mg/kg POEE 100= POEE 100.0 mg/kg . N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * p<0.05 and ** p<0.01 x controls test latencies, # p<0.05 x controls amnesic latencies; Mann-Whitney/Kruskal-Wallis.

Figure 4. Effects of POEE on MK-801 induced amnesia in acquisition (**Figure 4A**) and consolidation (**Figure 4B**) STM/LTM. MK = MK-801 0.1 mg/kg; dmso= dimethyl sulphoxide 20%; POEE = POEE 100.0 mg/kg . N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). * $p<0.05$ x controls test latencies, ## $p<0.01$ x controls amnesic latencies; Mann-Whitney/Kruskal-Wallis.

Figure 5. Effect of MK-801 on POEE-induced improvement of step-down STM (**Fig 5A**) LTM (**Fig 5B**) retrieval. MK = MK-801 0.1 mg/kg; dmso= dimethyl sulphoxide 20%; POEE = POEE 100.0 mg/kg . N=19-21. Columns represent latencies (sec) median (interquartile ranges) of training (light columns) or test (dark columns). ** $p<0.01$ x controls test latencies, Mann-Whitney/Kruskal-Wallis.

Figure 1


*
300
250
200
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latency (sec)
training
test
saline shock
POEE shock
saline no shock
POEE no shock

Fig 2A Acquisition

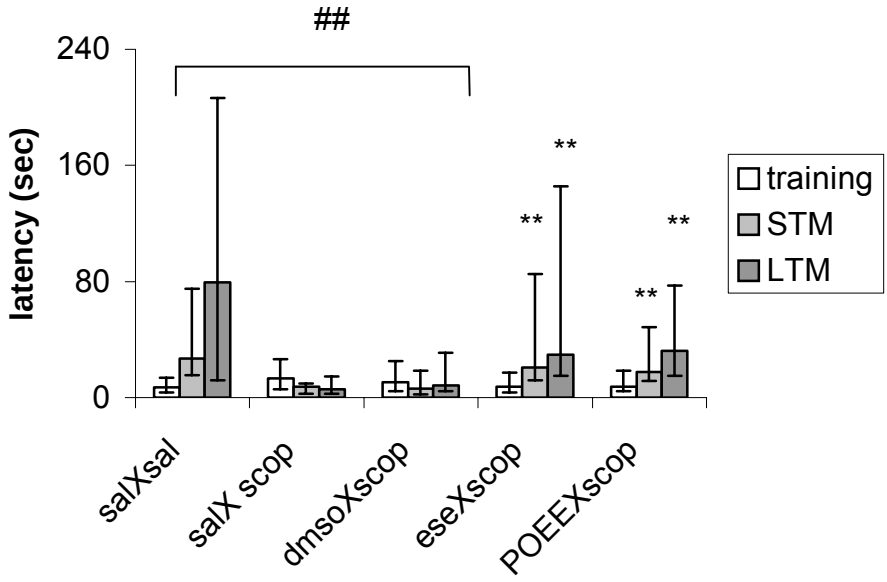


Fig 2B Consolidation

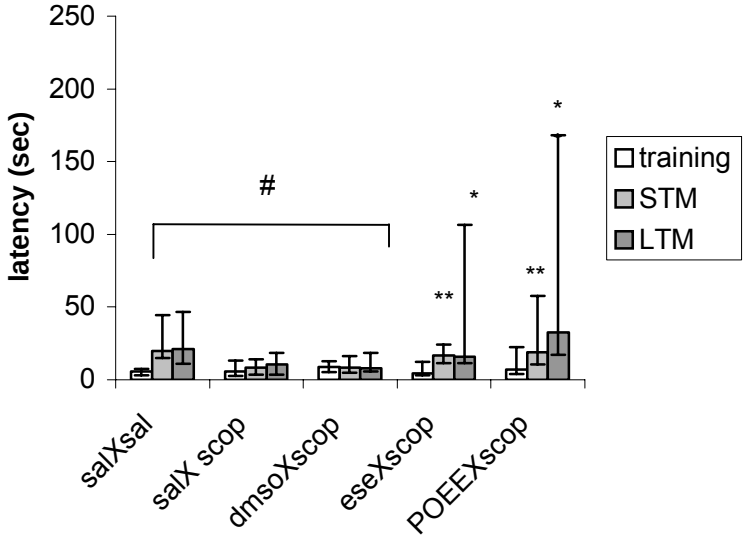


Fig 3A STM Retrieval

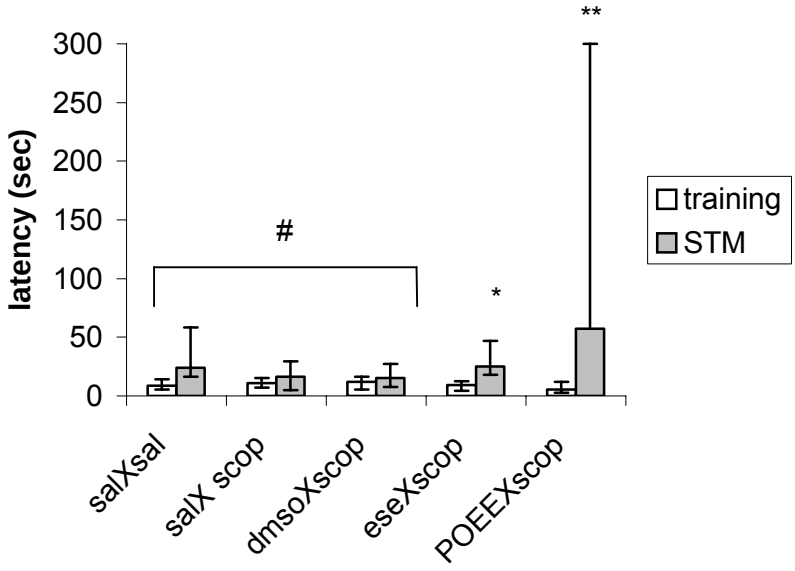


Fig 3B LTM Retrieval

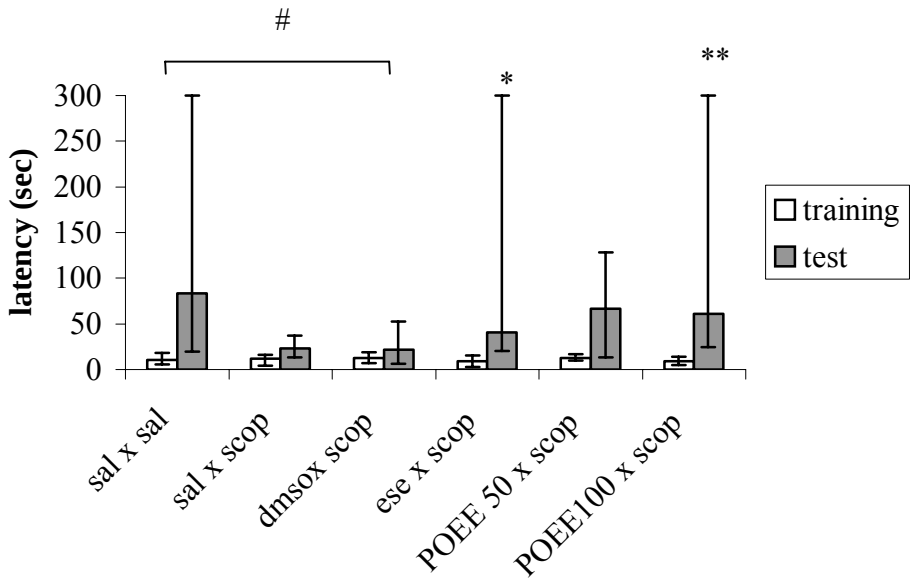


Fig 4A Acquisition

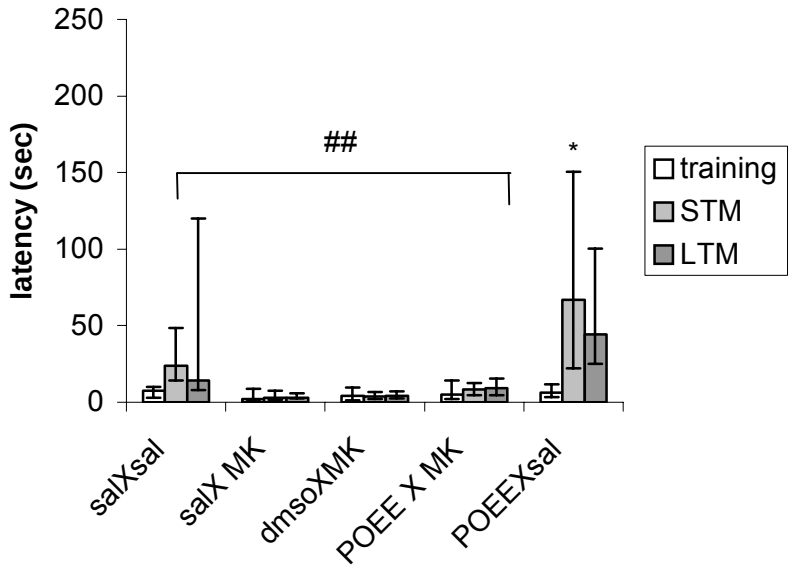


Fig 4B Consolidation

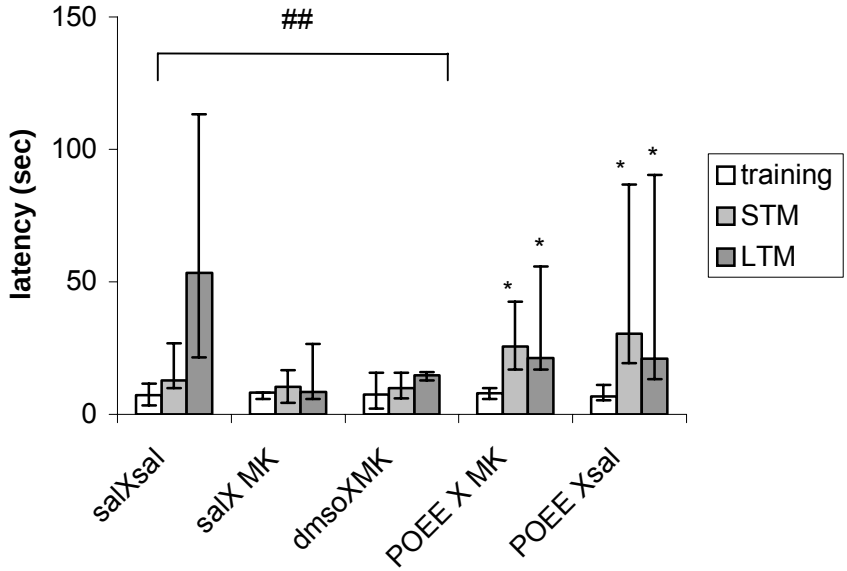


Fig 5A STM Retrieval

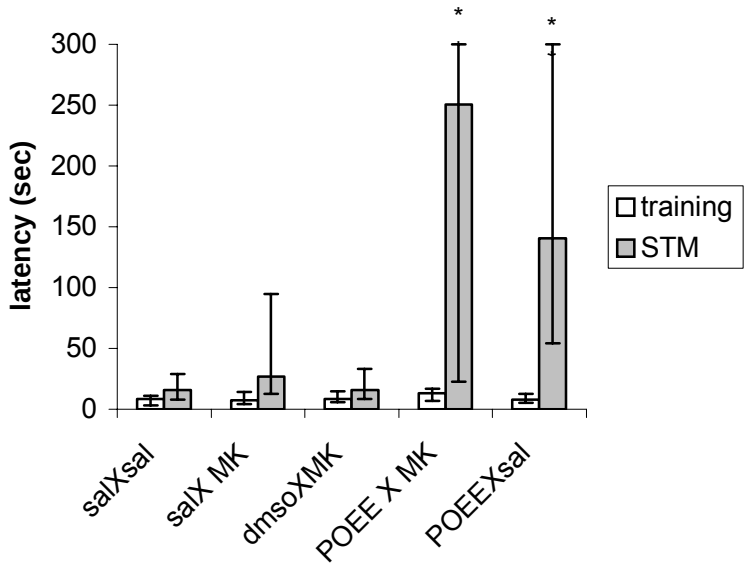
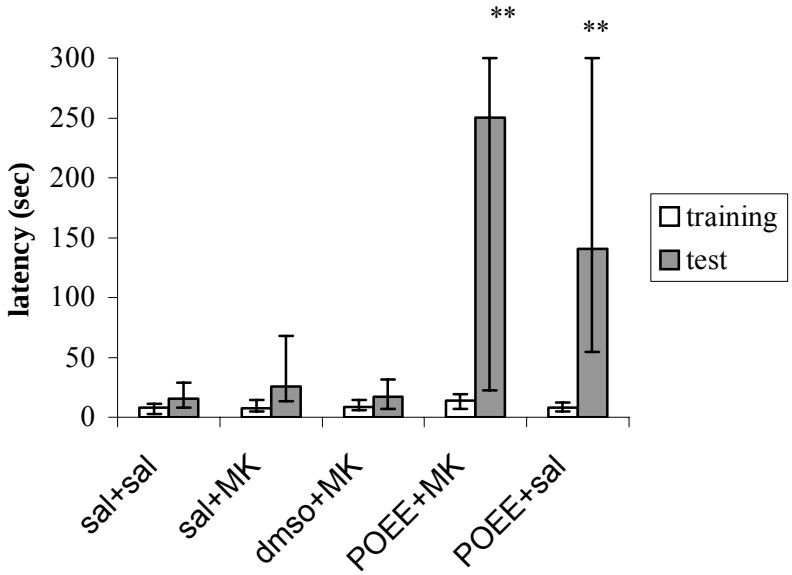


Fig 5B LTM Retrieval



PARTE III

1. Discussão

Situando melhor os resultados obtidos neste estudo, devemos buscar os conceitos para melhor definir a memória. Para tal, podemos definir a memória como um mecanismo pelo qual ocorre a aquisição, retenção e recuperação das informações obtidas pelas experiências diárias (LENT, 2001). A memória pode ser classificada de acordo com sua função, com o tempo de duração e com seu conteúdo. De acordo com o conteúdo, pode ser subdividida em declarativa (registro de fatos, eventos ou conhecimentos) e procedural ou memória de procedimento (registra capacidade ou habilidade motora ou sensorial, como os hábitos). A memória declarativa ainda pode ser do tipo episódica (referente a eventos que assistimos ou participamos) ou semântica (conhecimentos gerais). Autores mais modernos denominam a memória declarativa e a memória procedural em explícita e implícita, respectivamente (DANION *et al*., 2001). A explícita é adquirida com plena intervenção da consciência, enquanto que a implícita refere-se à memória adquirida de maneira inconsciente. As vias neuronais encarregadas de cada um desses dois grandes grupos de memória são diferentes. Além disso, a memória declarativa é mais suscetível à modulação pelas emoções, pela ansiedade e pelo estado de ânimo.

Como descrito na Introdução, as propriedades terapêuticas de preparações à base de Marapuama alegadas pelos usuários, incluem melhora de pacientes que apresentam prejuízo nas tarefas físicas e intelectuais, sejam estes derivados de períodos prolongados de estresse ou devido a problemas de saúde como “derrames”. Tais alegações deram origem ao primeiro estudo onde se avaliou o efeito do extrato etanólico de *Ptychopetalum olacoides* (EEPO) na aquisição,

consolidação e evocação da memória aversiva de longa duração. Utilizando-se a tarefa de esquiva inibitória, verificou-se que EEPO (50 e 100 mg/kg) administrado intraperitonealmente facilitou a evocação da memória de longa duração (MLD), de maneira dose-dependente, em camundongos adultos (2,5 meses) e os de 14 meses com déficit de memória, sem afetar outras fases da memória (DA SILVA, 2001). Dando continuidade, verificamos se o EEPO mostrava atividade semelhante quando administrado por via oral. Isto é importante para o desenvolvimento da droga porque o uso popular das garrafadas de Marapuama se dá por via oral. Como podemos verificar, o EEPO mostra-se ativo (800 mg/kg e 1000 mg/kg) também quando administrado oralmente (**Artigo I**); tanto em camundongos adultos como em animais de 14 meses com déficit de memória. Somente a dose oral de EEPO 800 mg/kg foi testada por via oral nos animais envelhecidos, devido à pouca disponibilidade de animais de 14 meses de idade que não apresentassem problemas de saúde que pudessem interferir com o experimento. A biodisponibilidade por via oral é consistente com o uso tradicional através das garrafadas utilizado pelas populações locais da Amazônia. É também importante saber se o EEPO poderia manter sua ação quando administrado por longos períodos, já que doenças neurodegenerativas são condições crônicas. Deste modo, dando início à investigação dos efeitos da administração sub-crônica, administramos EEPO (800 mg/kg, v.o.) durante 21 dias e, para evitar que o efeito agudo da substância interferisse na análise do efeito crônico, após um dia de pausa na administração, realizamos o experimento de esquiva inibitória. Verificamos que a facilitação sobre a memória se mantém com este regime de tratamento **(Anexo I, Figura 01)**. Formulações fitoterápicas contendo Marapuama

também são vendidas e divulgadas por fabricantes como emagrecedores; com base nesta informação observamos durante a administração sub-crônica o ganho de peso neste período e não observamos qualquer alteração **(Anexo I, Figura 02)**.

Além de a memória ser classificada de acordo com seu conteúdo (declarativas ou procedurais), pode ser também classificada pelo tempo de duração (IZQUIERDO, 2002). De acordo com o tempo, as memórias são basicamente classificadas em memória de curta duração (MCD) e de longa duração (MLD). As memórias declarativas de curta duração existem por poucas horas, justamente o tempo necessário para que a memória de longa duração seja consolidada. A formação da memória de curta e a de longa duração envolvem processos paralelos e até certo ponto independentes. A memória de curta duração requer as mesmas estruturas nervosas que a de longa duração, mas envolve mecanismos próprios e distintos (IZQUIERDO *et al*., 1998). Já as memórias declarativas de longa duração levam tempo para serem consolidadas e são lábeis e suscetíveis à interferência por numerosos fatores, desde traumatismos cranianos ou eletrochoques convulsivos, até uma variedade enorme de drogas ou mesmo a ocorrência de outras memórias (MC GAUGH, 2000; IZQUIERDO, 2002).

Considerando que a MCD envolve mecanismos próprios e distintos em relação à MLD, e que o EEPO atua sobre a MLD, verificamos o seu efeito sobre a MCD nas fases de aquisição, consolidação e evocação, quando administrado por via intraperitoneal e oral (**Artigo II**). O EEPO induziu um significativo aumento na evocação da memória de longa duração nas doses de 800 mg/kg e 1000 mg/kg.

Devido à limitada quantidade de extrato disponível, apenas a menor dose efetiva em MCD (3 horas após o treino) foi testada. Com relação à MCD, uma melhora significativa foi observada na aquisição, consolidação e evocação da memória.

O papel da MCD é, basicamente, manter o indivíduo em condições de responder através de uma "cópia" da memória principal, enquanto esta ainda não tenha sido formada. Este resultado pode ser muito interessante do ponto de vista clínico, já que em idosos a memória de curta duração é a primeira a ser perdida (Izquierdo, 2002).

A tarefa de esquiva inibitória envolve a formação de uma memória declarativa, na qual o animal aprende a inibir uma resposta (descer da plataforma), para não receber um estímulo aversivo (choque elétrico). A esquiva inibitória é ainda uma memória episódica (lembra o fato ocorrido) e também semântica (aprende a evitar a situação de perigo) (Izquierdo, 2002). Sabe-se que vários fatores (como motivação, medo, atenção, alerta, sedação ou ataxia) podem influenciar na interpretação dos resultados na tarefa de esquiva inibitória, principalmente quando analisamos a evocação, onde o tratamento é dado pouco antes da realização da sessão de teste (Zarrindast et al., 1996). A sedação ou ataxia pode ser descartada, já que eepo não interferiu na atividade locomotora medida no ensaio da placa perfurada ou no desempenho no aparelho do rota-rod (da Silva et al., 2002) nas doses em que facilitou a evocação. Verificou-se ainda que EEPO tem ação levemente ansiogênica. Estudos demonstraram que um certo nível de ansiedade ou estresse tende a melhorar o desempenho em tarefas intelectuais (McEwen; Sapolsky, 1995; Graeff, 1999a,b). Além disso, estimulantes do SNC, como a nicotina, demonstraram facilitar a evocação da memória aversiva

de longa duração (Zarrindast et al., 1996). Contudo, vários estudos demonstram que catecolaminas ou glicocorticóides aumentam a consolidação da memória e diminuem a evocação da mesma (Cahill; McGaugh, 1998; Roozentaal., 2002). Tendo em vista que um bom desempenho na tarefa de esquiva inibitória requer que animal iniba seu natural comportamento exploratório associando-o à punição, era importante verificar se a melhora da memória induzida por EEPO não estaria associada (ou parcialmente induzida) à ação ansiogênica provocada pelo extrato. Para esclarecer se o efeito facilitatório de EEPO é específico sobre a memória realizou-se um experimento complementar usando o mesmo protocolo da tarefa de esquiva inibitória, à exceção de que diferentes grupos de animais receberam ou não o choque durante o treino. Como podemos observar no Artigo V, no grupo sem choque não há aumento na latência de descida da plataforma, quer sejam tratados com salina ou EEPO. Com isto, podemos reafirmar a sugestão de que a ação de EEPO é sobre a memória, uma vez que a simples exposição (ou re-exposição) do animal à plataforma do aparelho, com ou sem tratamento com o extrato, não impede a capacidade exploratória natural desta espécie. Complementando ainda estes dados, buscamos verificar o efeito de EEPO sobre outro modelo de memória declarativa que não contivesse componente aversivo. A tarefa de "reconhecimento de objetos" é considerado um modelo de memória não aversivo, não espacial (Deschaux et al., 1997; Puma et al., 1999; Lebrun et al., 2000) e baseia-se na natural preferencia dos roedores por objetos novos. A memória de reconhecimento visual parece depender da integridade do córtex perirrinal em humanos (Buffalo et al., 1998), macacos (Meunier et al., 1993; Suzuki et al., 1993; Gaffan, 1994) e roedores (Mumby; Pinel, 1994; Ennaceur et

al., 1996; Bussey et al., 1999; Winters et al., 2004a). Parece ainda depender da ativação do sistema colinérgico visto que antagonistas colinérgicos muscarínicos como, escopolamina e atropina, podem impedir o reconhecimento visual em humanos (Robbins et al., 1997), macacos (Penetar; McDonough, 1983; Aigner; Mishkin, 1986; Aigner et al., 1991) e ratos (Bartolini et al., 1996; Vannucchi et al., 1997; Pitsikas et al., 2001). Além disto, tratamentos com inibidores da acetilcolinesterase como fisiostigmina, metrifonato, tetraidroaminoacridina pode facilitar o reconhecimento visual em macacos (Aigner; Mishkin, 1986), humanos (Furey et al., 2000a) e ratos envelhecidos (Scali et al., 1997a,b). Nós verificamos que o EEPO parece atuar de modo diferente neste tipo de memória, facilitando a aquisição, consolidação e evocação da memória de longa duração, sem mostrar qualquer efeito na memória de curta duração (Artigo II). Através deste resultado, propomos que o EEPO module diferentemente as memórias declarativas aversivas e não aversivas; mas ainda não podemos precisar como a modulação se processa nos diferentes tipos de memória.

Dando continuidade ao estudo de EEPO, nos pareceu necessário estudar o(s) possível(eis) mecanismo e ação deste extrato sobre a memória. Neste primeiro momento procuramos dar preferência ao mecanismo de ação de EEPO na memória aversiva. Utilizamos antagonistas de receptores α- (fentolamina) e β- (propranolol) adrenérgico (sem especificidade por subtipos de receptores), antagonistas dopaminérgicos D_1 (SCH 233990) e D_2 (sulpirida) **(Artigo III)**, serotoninérgios 5-HT_{1A} (pindolol) e 5-HT_{2A} (espiperona) **(Artigo IV).** Além disto, induzimos amnésia com escopolamina (antagonista colinérgico muscarínico) e MK-801 (antagonista glutamatérgico NMDA) para verificar os efeitos de EEPO

nestas condições **(Artigo V)**. A tabela que se segue resume os resultados obtidos.

Quadro 1. Resumo sobre a interferência dos diversos antagonistas sobre a facilitação da memória induzida por EEPO.

Antagonista	MCD			MLD		
	Aquisição	Consolidação	Evocação	Aquisição	Consolidação	Evocação
α-adren	NT	**NT**	NT	**NT**	**NT**	**=**
β-adren	⇓	⇓	⇓	**NT**	NT	⇓
D_1	⇓	⇓	⇓	**NT**	**NT**	⇓
D_2	**NT**	**NT**	**NT**	**NT**	**NT**	**=**
5-HT_{1A}	**NT**	**NT**	**NT**	**NT**	**NT**	**=**
5-HT_{2A}	**=**	**=**	**=**	**NT**	**NT**	⇑
ACh	↻	↻	↻	↻	↻	↻
NMDA	**=**	↻	**=**	**=**	↻	**=**

NT não testado; ⇓ Inibe a ação de EEPO; ⇑ potencializa a ação de EEPO; **=** sem interferência; ↻ EEPO reverte amnésia.

Estes sistemas de neurotransmissores foram escolhidos por duas razões. Em primeiro lugar, porque os levantamentos etnofarmacológicos indicam usos que sugerem a possível interação de extratos/preparações desta espécie com o sistema dopaminérgico (afrodisíaco, antidepressivo, antitremor, modulador do apetite, adaptógeno), sistema noradrenérgico e/ou serotoninérgico (afrodisíaco, antidepressivo, modulador do apetite), ou ainda com o eixo hipotálamo-hipófise-adrenal (adaptógeno e antidepressivo) (ELISABETSKY; SIQUEIRA, 1998).

Segundo, porque estes neurotransmissores são relevantes para a memória. Além disto, estudos anteriores de nosso grupo indicaram: 1) uma possível ação no sistema noradrenérgico e/ou serotoninérgico, visto que EEPO foi ativo em testes com valor preditivo para antidepressivos, como proteção da letalidade induzida por ioimbina e reversão de ptose induzida por reserpina (ELISABETSKY; SIQUEIRA, 1998; SIQUEIRA *et al.,* 1998); 2) interação com o sistema dopaminérgico, já que o extrato preveniu a expressão de estereotipia induzida por apormorfina (agonista dopaminérgico); 3) ou ainda com o sistema colinérgico, por ser inibidor da enzima acetilcolinesterase (SIQUEIRA *et al.,* 1998; ELISABETSKY ; SIQUEIRA, 1998).

Os resultados obtidos na esquiva inibitória estão de acordo com as sugestões anteriores (SIQUEIRA *et al.,* 1998; ELISABETSKY; SIQUEIRA., 1998), reiterando a participação dos sistemas adrenérgicos, dopaminérgicos, serotoninérgicos e colinérgicos nos efeitos comportamentais de EEPO em roedores. Além disto, estes resultados estão de acordo com o entendimento atual sobre sistemas de neurotransmissores relevantes para a memória. A MCD e MLD são dependentes da ativação de receptores glutamatérgicos AMPA, NMDA e metabotrópicos cuja ação é modulada positivamente (facilitada) por receptores colinérgicos muscarínicos, dopaminérgicos D_1 e β-noradrenérgicos e, negativamente (inibida) por receptores $5HT_{1A}$ e GABA (BARROS *et al*., 2001; IZQUIERDO *et al.*, 2000; MCGAUCH *et al*., 2000; VIANNA *et al*., 2000; IZQUIERDO, 2002). Além disto, muitos destes neurotransmissores monoaminérgicos têm demonstrado redução com o envelhecimento normal ou com estados patológicos como, por exemplo, noradrenérgico (BURNETT *et al*.,

1990; HUGUET; TARRADE, 1992; SZOT *et al.*, 2006; BIRTHELMER *et al*., 2003a), dopaminérgico (JOSEPH *et al*., 1978), colinérgico (JOSEPH *et al*., 1990; AUBERT *et al*., 1995; NARANG *et al*., 1996; EGASHIRA *et al*., 1996; BIRTHELMER *et al*., 2003b) e serotoninérgico (GOZLAN *et al*., 1990; NYAKAS *et al*., 1997; BIRTHELMER *et al*., 2003a).

Os grupos celulares dos neurônios noradrenérgicos são encontrados na ponte, na medula oblonga, com prolongamentos axôniais para várias parte do cérebro e medula espinhal. O grupo celular noradrenérgico mais proeminente encontra-se no *locus ceruleus* (LC), que é encontrado na substancia cinzenta da ponte (RANG; DALE, 2004); sua principal função é determinar se a atenção está dirigida ao ambiente externo ou interno. Disfunções no *locus ceruleus* estão relacionadas aos transtornos onde há sobreposição de alteração de humor e cognição como depressão, ansiedade, distúrbios de atenção e do processamento de informações (STAHL, 2002). Os receptores noradrenérgicos são subdivididos em alfa (α_1 e α_2) e beta (β_1 e β_2), sendo que α_2-adrenérgico pode ser tanto pré- como pós-sináptico. Os auto-receptores α_2-adrenérgico podem se localizar no terminal axônico, no corpo celular, e nos dendritos e inibem a liberação de noradrenalina. Assim a inibição de auto-receptores α_2-adrenérgico aumentam a liberação de noradrenalina enquanto que inibição de α_2 heteroreceptores pode levar a um aumento na liberação de serotonina e dopamina (JACKISCH *et al*., 1999; IHALAINEN; TANILA, 2002), mostrando uma intercomunicação entre os sistemas adrenérgicos e serotoninérgicos. A intercomunicação entre os sistemas adrenérgico e colinérgicos foi demonstrada quando as alterações no labirinto radial produzidas por lesões colinérgicas são revertidas pela administração do

bloqueador α_2-adrenérgico (HARREL *et al*., 1990). O funcionamento adequado do sistema noradrenérgico é importante para vários processos cognitivos, como a atenção e a preservação da distração na presença de estímulos irrelevantes (ROBINS, 2002). Vários estudos têm demonstrado que memórias para novas experiências vivenciais são codificadas mais eficientemente sob condições nas quais as concentrações de noradrenalina encontram-se elevadas, induzidas pelo estado de alerta (IZQUIERDO; MEDINA, 1997; MCGAUGH, 2000). Além disto, evidências experimentais têm sugerido que a noradrenalina possui um papel importante na regulação da plasticidade sináptica (THOENEN, 1995; DREYFUS, 1998; DEBEIR *et al*., 2002). Estudos anteriores têm sugerido que a noradrenalina possui um papel crítico não só no aprendizado e memória (IZUMI; ZORUMSKI, 1999; MURCHINSON *et al*., 2004), como também na recuperação funcional após a injúria cerebral (BOYENSON; FEENY,1990). A diminuição da atividade noradrenérgica (BUCCAFUSCO; TERRY, 2000; GESI *et al.*, 2000) e deficiência no sistema noradrenérgico cerebral, em destaque no *locus ceruleus* têm um papel decisivo (atuando como neuroprotetor) na progressão de várias doenças neurodegenerativas, incluindo Doença de Parkinson e Doença de Alzheimer (MARIEN *et al*., 2004; GESI *et al*., 2000). Vários estudos têm sugerido que o sistema noradrenérgico funcional (intacto) parece ser necessário para que drogas que aumentam a acetilcolina sejam eficazes em reduzir o déficit cognitivo (RIEKKINEN *et al.*, 1992; HAROUTUNIAN *et al*., 1990; DECKER; GALLAGHER,1987).

Quanto aos subtipos de receptores envolvidos na modulação da memória, tem-se sugerido que o bloqueio pós-sinaptico de receptores β-adrenérgicos

impede a consolidação (MCGAUGH, 1989) e a evocação (DEVAUGES; SARA, 1991), bem como que a ativação de β_1-adrenérgicos melhora a evocação da memória (MURCHINSON *et al*., 2004). Alterações no labirinto radial produzidas por lesões colinérgicas são potencializadas pela administração do antagonista β-adrenérgico propranolol ainda que quando administrado sozinho não interfira no desempenho dos animais nesta tarefa (HARREL *et al*., 1990). Nossos resultados mostram que a facilitação da memória em qualquer fase é bloqueada por propranolol. Contudo, quando administramos o antagonista fentolamina, verificamos um aumento não estatisticamente significativo do efeito de EEPO **(Figura 1A, Artigo III)**. Considerando os resultados anteriores obtidos com este extrato, tais como, proteção da letalidade induzida por ioimbina (Siqueira et al., 1998), não se pode descartar que o extrato possa atuar como um antagonista α2-adrenérgico, aumentando por este mecanismo a eficiência da sinapse noradrenérgica. Vários fatores podem influenciar estas análises, como por exemplo a ação preferencial pré ou pós-sináptica dos agonistas e antagonistas α2, a seletividade farmacológica, a atividade intrínseca, e a dose utilizada (Bunsey; strupp, 1995). No caso da fentolamina, que inibe receptores adrenérgicos α1- e α2-, pré e pós-sinápticos a influência de receptores α2 no efeito de EEPO pode ter sido mascarada. Deste modo, futuros estudos com antagonistas mais específicos para auto-receptores α2-adrenérgicos são necessários para esclarecer este aspecto.

A dopamina está envolvida em vários distúrbios da função cerebral, como a doença de Parkinson, esquizofrenia, dependência de drogas, distúrbios neuroendócrinos, além do distúrbio de déficit de atenção mencionado acima. Os

subtipos de receptores de dopamina se dividem em família de receptores do tipo D1 (D1e D5) que ativam a adenilciclase, e família de receptores D2 (D2, D3, D4) que inibem a adenilciclase. A maioria dos estudos demonstram que o subtipo de receptor D1 tem um papel importante na modulação da memória, sendo que agonistas D1 aumentariam e antagonistas D1 diminuiriam a memória (Barros et al., 2001). Foi possível verificar que o antagonista D1 (SCH 233390) bloqueou o efeito facilitatório induzido por EEPO sobre a memória de curta e longa duração, sugerindo que a ativação dos receptores D1 um dos mecanismos de ação do EEPO sobre a memória.

A interação entre os sistemas colinérgico e dopaminérgico, bem como entre o sistema colinérgico e noradrenérgico, são bastante conhecidos e descritos. Drogas como reboxitine (aumenta a disponibilidade de NA), metilfenidado e anfetamina (aumenta a disponibilidade de DA) estimulam a liberação de acetilcolina nas regiões corticais e estriatais (Nelson et al., 2000; Tzavara et al., 2006). Por outro lado, estudos in vivo (microdiálise) sugeriram que agonistas muscarínicos aumentariam o disparo de neurônios dopaminérgicos da substância negra e área tegmental ventral aumentando os níveis extracelulares de dopamina no mesencéfalo e estriato (Gronier; Rasmussen, 1998; Gronier et al., 2000). A ativação de receptores nicotínicos também acarreta em aumento dos níveis de dopamina, bem como de noradrenalina em muitas áreas cerebrais envolvidas em processos cognitivos (Singer et al., 2004). Receptores nicotínicos são expressos pré-sinapticamente em neurônios glutamatérgicos aferentes para áreas do mesencáfalo; estes receptores aumentam a transmissão glutamatérgica, aumentando assim a probabilidade da potenciação sináptica de longa duração em

neurônios dopaminérgicos (Mansvelder; McGehee 2000; Dani et al., 2001; Mansvelder et al., 2002). No que diz respeito à interação entre nicotina e serotonina, foi demonstrado previamente que altos níveis de nicotina estimulam, enquanto baixos níveis inibem, a liberação de serotonina (Singer et al, 2004; Rossi et al., 2005). Contudo, estudos in vivo demonstraram que a inibição da acetilcholinesterase (AChE) pode causar disfunção motora tipo parkinsoniana (Salamone et al., 2001). Tem sido relatado que tacrina, um inibidor de AChE, pode exacerbar estes sintomas motores (Ott; Lannon, 1992). Estes efeitos adversos podem ser causados pela diminuição na liberação de dopamina, por exposição prolongada de acetilcolina, causando uma desensibilização nos receptores nicotínicos do estriato (Zhou et al., 2001). Porém, os resultados obtidos com experimentação animal são conflitantes, tendo sido descrito que nicotina pode proteger contra degeneração nigro-estriatal, através da indução de fatores tróficos (Maggio et al., 1998; Belluardo et al., 2000). Assim, os receptores nicotínicos podem ser neuroprotetores ou facilitar a ação excitotóxica de NMDA ou conforme o subtipo de receptor envolvido (Laudenbach et al., 2002). Várias hipóteses têm sido sugeridas para a interação entre acetilcolina e dopamina, algumas sugerem que quando o SNC funciona normalmente, a liberação de dopamina no estriato é regulada pela ativação de receptores nicotínicos (Zhou et al., 2001). Quando os receptores nicotínicos são inibidos ou desensibilizados, a liberação de dopamina diminui (Zhou et al., 2001). Por isto torna-se interessante no futuro verificar se o EEPO exerce uma ação direta sobre o receptor D1 (agindo como agonista), ou se a inibição da AChE, aumentando a acetilcolina na fenda,

leva a um aumento da liberação de dopamina, que por sua vez é inibida pelo antagonista D1 (Artigo III).

É consenso entre alguns pesquisadores que a função dopaminérgica mesencefálica é crítica para um funcionamento normal do cérebro e está bem estabelecido o controle que o sistema serotonérgico exerce sobre a atividade de neurônios dopaminérgicos. Os dados da literatura demonstram que a influência de serotonina em neurônios dopaminérgicos parece ocorrer através de diferentes vias. Foi demonstrado que agonistas inversos de receptores 5-HT_{2C} aumentam a transmissão dopaminérgica nigroestriatal (ALEX *et al*. 2005) e no córtex pré-frontal (GOBERT *et al.*, 2000; PEHEK *et al*., 2001; MELTZER, 2003); antagonistas 5-HT_{2C} aumentam a transmissão dopaminérgica na via mesocorticolímbica (DI MATTEO *et al.* 2002). Em compensação, o antagonista $5HT_{2A}$ M100907 parece reverter tanto o aumento da locomoção induzido por anfetamina (SORENSEN *et al*., 1993), quanto em camundongos "knockout" para o transportador de dopamina (BARR *et al*., 2004), situações onde níveis sinápticos de dopamina se encontram elevados. Demonstrou-se também que a administração de agonistas D_1 em ratos lesionados com 6-OHDA induz hiperlocomoção (BISHOP *et al*., 2004; BISHOP *et al*., 2005), que pode ser revertida pela administração de antagonista 5-HT_{2A}, mas não 5-HT_{2C} (BISHOP *et al*., 2005). Clozapina, um antagonista 5-HT_2, reduz a discinesia induzida por levodopa em pacientes com Parkinson (PIERELLI *et al*., 1998; MELTZER, 1999; DURIF *et al*., 2004). Parece, portanto, que antagonistas 5-HT_{2C} aumentariam a liberação de dopamina, mas os receptores 5-HT_{2A} teriam o papel de estabilizar a neurotransmissão dopaminérgica (ROTH *et al.,* 2004; BISHOP *et al*., 2005).

Considerando ainda interação a entre os sistemas dopaminérgico e serotoninérgico, verificamos a ação do extrato sobre os sistema serotoninérgico **(Artigo IV).** O papel da serotonina sobre a modulação da memória não parece muito claro, os resultados obtidos variam conforme a droga escolhida, o animal, o modelo comportamental de memória utilizado (MENESES,1998). Generalizando, acredita-se que agonistas 5-HT_{2A} prejudicam e que antagonistas 5-HT_{2A} melhorem a memória (MENESES; HONG, 1997, ROTH *et al*., 2004). Sabe-se que a espiperona (antagonista 5-$HT_{2A/1A}$) melhora a evocação na memória de longa duração. Já que espiperona potencializou o efeito de EEPO pode-se sugerir que que EEPO possa atuar como antagonista 5-HT_{2A}, e ainda que receptores 5-HT_{1A} não sejam importantes para a atividade do extrato, uma vez que pindolol não afetou o efeito promnésico de EEPO.

Foi demonstrado que inibidores da enzima acetilcholinesterase administrados conjuntamente com antagonistas que 5-HT_{2A} melhoram o desempenho em memória aversiva (ALTMAN; NORMILE, 1988; NORMILE; ALTMAN, 1992). Este efeito deve-se ao aumento da liberação de acetilcolina via o bloqueio de 5-HT_{2A}, que somado ao bloqueio da acetilcolinesterase aumenta ainda mais da disponibilidade de acetilcolina. Portanto, através de duas vias distintas, ocorre um aumento da acetilcolina disponível na fenda sináptica. Com isto não podemos descartar a hipótese de que a potencialização do efeito de EEPO observado com espiperona, ao invés de ser o resultado da atuação direta de EEPO em receptores 5-HT_{2A}, seja em realidade resultante do efeito da liberação de acetilcolina por espiperona (via antagonismo de 5-HT_{2A}) somado à inibição da acetilcolinesterase (por EEPO).

Além de esclarecer os mecanismos de ação de substâncias pró-mnésicas, o uso de modelos experimentais de amnésia colaboram para a compreensão dos déficits de memória presentes em algumas doenças neurodegenerativas. Ao utilizarmos antagonista colinérgico muscarínico (escopolamina), podemos perceber que EEPO reverte déficit cognitivo induzido por escopolamina em todas as fases de formação da memória, tanto de curta como de longa duração **(Artigo V)**. Como esperado, por apresentar atividade inibidora da acetilcolinesterase e facilitadora da memória, o EEPO reverte a amnésia na aquisição, consolidação e evocação da memória de curta e evocação da memória de longa duração. Surpreendente foi a reversão da amnésia nas fases onde anteriormente o EEPO não apresentou atividade promnésica *per se*, como na aquisição e consolidação da memória de longa duração. Com relação ao sistema glutamatérgico NMDA, verificou-se a reversão da amnésia induzida por MK-801 (antagonista NMDA) somente na fase de consolidação da memória, sendo que a reversão não foi total. É provável que o sistema NMDA não seja o principal alvo de ação do EEPO sobre a modulação da memória, contudo não podemos descartar futuros ensaios com outros subtipos de receptores glutamatérgicos importantes na formação e evocação da memória de longa duração como o receptor AMPA e receptores glutamatérgicos metabotrópicos antes de chegar a uma compreensão total do papel dos receptores glutamatérgicos nos efeitos de EEPO.

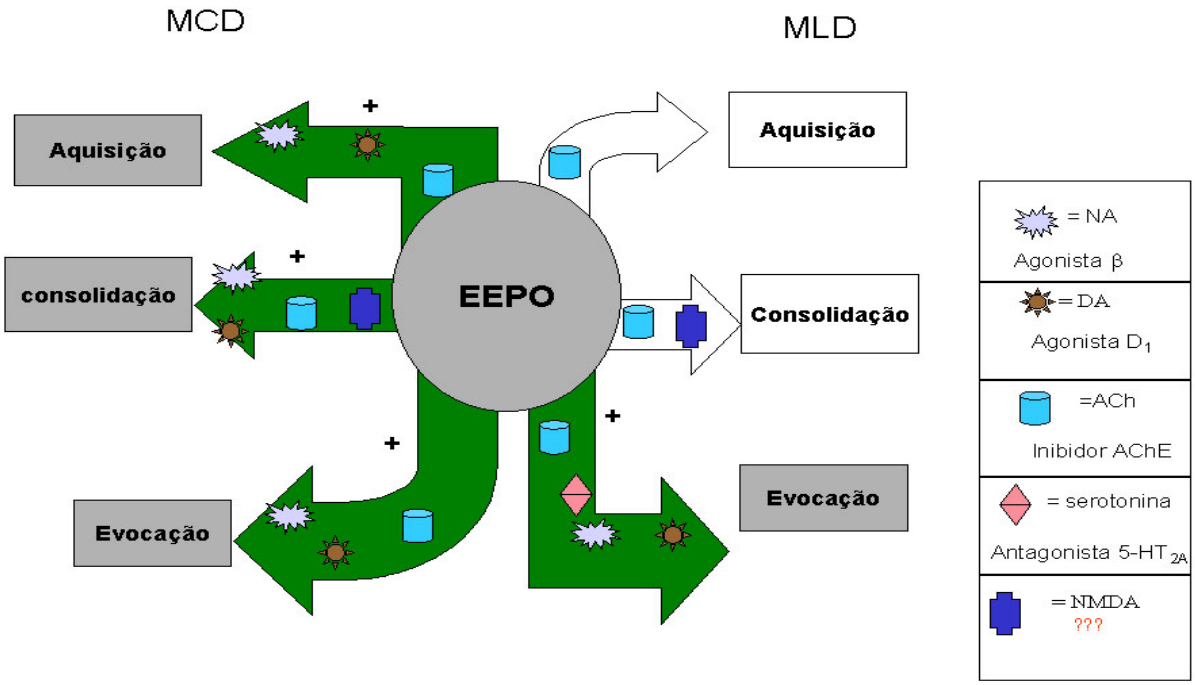
Ao analisarmos os resultados obtidos com EEPO neste estudo, deve-se lembrar que a interação de diferentes neurotransmissores na modulação é complexa. SHEARMAN *et al*.(2006) demonstraram recentemente que memantina produz um aumento extracelular de dopamina e noradrenalina no córtex, e de

acetilcolina no núcleo acumbens e área tegmental ventral, além da redução de dopamina no hipocampo. Alguns estudos têm sugerido que o sistema noradrenérgico facilita a ativação de receptores NMDA levando a uma potenciação de longa duração hipocampal (DAHL; SARVEY**,** 1989; 1990). O uso de um β bloqueador (propranolol), mas não um α-bloqueador (fentolamina) agrava o déficit de memória induzido pelo bloqueio do receptor NMDA (OHNO *et al*., 1996). Vários estudos sugerem que ativação de receptores D_1 e NMDA são críticos para processos cognitivos que envolvem o córtex pré-frontal (AURA; RIEKKINEN, 1999; GOLDMAN-RAKIC *et al.*, 2000; MARCUS, 2005), sendo que agonistas D_1 revertem o déficit de memória induzido por MK-801 (MELE *et al*., 1996) e antagonistas D_1 potencializam o efeito amnésico de MK-801 (CESTARI; CASTELLANO, 1997). Tem-se sugerido que dopamina, via receptores D_1, pode potencializar a despolarização produzida por ativação dos receptores NMDA (WIRKNER *et al*., 2004). Com relação à interação entre NMDA e serotonina, altas densidades de receptores 5-HT_{2A} foram verificadas no neocortex, sugerindo co-localização com receptores NMDA (HIGGINS *et al*., 2003) e que antagonistas 5-HT_{2A} (M100907 e AC-90179) revertem a amnésia e a hiperlocomoção induzida por MK-801 (CARLSSON *et al*., 1999; VARTY *et al*., 1999; WEINER *et al.*, 2001). Donepezil (inibidor da AChE) produz um aumento extracelular de dopamina no córtex e hipocampo, e um aumento de noradrenalina no hipocampo dorsal e no córtex temporal medial; mas diminui no hipocampo ventral. Ambas as drogas (memantina e donepezil) induziram uma redução extracelular de serotonina (SHEARMAN *et al.*, 2006). Contudo, rivastigmina, outro inibidor de AChE, diminuiu a liberação de noradrenalina mas não afetou as concentrações de

dopamina (TRABACE *et al.,* 2000). CHF2819 I, inibidor AChE, diminui os níveis de dopamina, aumenta os de serotonina e não afeta os níveis de noradrenalina (TRABACE *et al*., 2000). Galantamina e tacrina, além de inibirem a AChE, atuam modulando alostéricamente os receptores nicotínicos, esta sensibilização alostérica do receptor nicotínico pré-sinaptico afeta a liberação de glutamato e serotonina (MAELICKE *et al*., 2001). RODRIGUEZ-MORENO *et al.* (2006) recentemente demonstrou que o déficit de memória produzido por MK-801 foi completamente abolido com a administração de um agonista específico de receptor nicotínico RJR-2403. Os heteroreceptores colinérgicos e glutamatérgicos estão presentes em neurônios dopaminérgicos, noradrenérgicos e serotoninérgicos e se distribuem de forma heterogênea; portanto é esperado que a modulação dos receptores colinérgicos e glutamatérgicos possam afetar de maneiras diferentes estes neurônios conforme a região em que se encontram (SHEARMAN *et al*., 2006).

Em resumo é de extrema importância reconhecer que o efeito sobre a memória de um sistema de neurotransmissor pode ser mediado por outro sistema e/ou a manipulação de mais de um sistema de neurotransmissores pode produzir efeito sinérgico sobre a memória (DECKER; MCGAUGH, 1991).

A figura 1 ilustra o que se sabe no momento sobre as bases farmacodinâmicas do efeito pró-mnésico de EEPO.



1.2 Considerações finais

Concluindo, a análise conjunta dos resultados indica que este extrato padronizado de raízes de *P. olacoides* parece agir preferencialmente sobre os sistemas: colinérgicos (por inibir a acetilcolinesterase), dopaminérgicos (via receptores D_1) e noradrenérgicos (via receptores β-adrenérgicos). Além disso, este estudo não permite descartar uma ação do extrato como antagonista α_2-adrenérgico e/ou antagonista 5-HT_{2A}, sendo necessários novos estudos para confirmar estas hipóteses. A modulação de EEPO sobre os receptores NMDA parece ser indireta (através dos sistemas colinérgico, noradrenérgico ou dopaminérgico) visto que

houve uma reversão apenas parcial da amnésia induzida por MK-801 na consolidação.

A memória humana é atualmente considerada um complexo subsistema envolvendo diferentes redes anatomofuncionais, com provável uso de diferentes sistemas de neurotransmissão (DUJARDIN; LAURENT, 2003). Qualquer mudança na integridade funcional pode contribuir para redução na sinalização neuronal e subseqüentemente distúrbios comportamentais (YOUDIM; JOSEPH, 2001). Durante as últimas três décadas foram descobertas muitas informações sobre neurotransmissão química, mas a investigação separada sobre os sistemas de neurotransmissores pode dar a impressão que os neurotransmissores agem independentemente possuindo papeis distintos e cruciais nos processos de memória (BLOKLAND, 1995; DECKER; MCGAUGH, 1991). Contudo, mais evidências apontam que uma interação do sistema colinérgico com noradrenalina, dopamina, serotonina, glutamato, entre outros, pode ser importante para o aprendizado e memória (NORMILE; ALTMAN, 1992; NELSON *et al.*, 2000; MAELICKE *et al*., 2001; TZAVARA *et al*., 2006). Os efeitos amnésicos produzidos por lesões colinérgicas podem ser potencializados ou inibidas por manipulações em outros sistemas de neurotransmissores, muitos dos quais não apresentam efeito *per se*, sugerindo a necessidade de se considerar estratégias de tratamentos envolvendo múltiplos alvos moleculares. Portanto, é importante considerar não somente a contribuição dos neurotransmissores agindo independentemente, mas a interação entre estes sistemas para o déficit no aprendizado e memória associados ao envelhecimento e aos distúrbios cognitivos. É forçoso ainda reconhecer que os processos mais importantes, isto é,

a complexa natureza do circuito neuronal, a interação entre os múltiplos sistemas de neurotransmissores, e a diversidade de efeitos neuroquímicos sobre os mais diferentes receptores, ainda está por ser adequadamente elucidada.

Drogas mais seletivas e mais específicas tem sido pré-requisitos para considerar um substância benéfica para saúde. Contudo, em certas doenças crônicas, estes mesmos compostos com alta seletividade trazem consigo muitos efeitos adversos quando usados clinicamente (KIMURA, 2006). Existem muitos casos em que produtos naturais e componentes sintéticos exercem múltiplas ações farmacológicas podendo auxiliar no tratamento dos multifatoriais sintomas que acompanham as doenças crônicas (YOUDIM; JOSEPH, 2001). Mais recentemente, tem-se afirmado que as doenças neurodegenerativas são processos patogênicos únicos, e as diferenças encontradas se devem às distintas regiões cerebrais em que os processos degenerativos ocorrem (MARIEN *et al*., 2004). Drogas com múltiplos mecanismos de ação podem ter papel importante no tratamento de distúrbios assim caracterizados.

O Brasil vem sofrendo mudanças significativas na sua pirâmide populacional, caracterizadas por um aumento progressivo e acentuado das populações adulta e idosa. Dentro deste contexto, tem-se desenvolvido uma rápida transição nos perfis de saúde/doença que se caracterizam pelo predomínio das enfermidades crônicas não transmissíveis (BRASIL. MS, 1999), problemas de saúde que vão perdurar 15, 20 ou mais anos e que exigem a utilização de um grande número de medicamentos (BEZERRA *et al.*, 1998). Sabe-se que 80% dos idosos apresentam pelo menos uma doença crônica, ao passo que 40% chegam a apresentar duas ou mais (GHOLAMI; SHALVIRI, 1999). DUARTE (1998) chama

atenção para o fato de que as várias drogas utilizadas concomitantemente por idosos podem provocar efeitos nocivos e que reações adversas por uso de fármacos são um dos principais problemas de saúde nos idosos. Alguns dos fatores significantes para esta problemática são as prescrições múltiplas, a presença de doenças crônicas que exigem terapia com vários medicamentos a longo prazo, as dosagens inadequadas e as alterações próprias do envelhecimento (BRASIL. MS, 1999). A premissa de que a alta seletividade e alta potência são as propriedades mais desejáveis para um novo agente terapêutico pode não ser o caso para muitas drogas projetadas para o tratamento de desordens cerebrais (BUCCAFUSCO; TERRY, 2000). Alguns fitoterápicos apresentam mais de um componente ativo atuando de forma complementar ou ainda componentes ativos com múltiplos mecanismos de ação (HOWES; HOUGHTON, 2003; ELISABETSKY, 2002; KHALIFA, 2001); desvantagens da combinação de diferentes classes de drogas, com distintas propriedades farmacodinâmicas e farmacocinéticas, podem ser eventualmente minimizadas e/ou eliminadas com o desenvolvimento de compostos com múltiplos mecanismo de ação (BUCCAFUSCO; TERRY, 2000).

O Brasil possui a maior biodiversidade do mundo, estimada em cerca de 20% do número total de espécies do planeta (LEWINSOHN; PRADO, 2002). Esse imenso patrimônio genético, já escasso nos países desenvolvidos, tem um valor econômico inestimável para a farmacologia. A utilização da abordagem etnofarmacologica pode levar à identificação de produtos com mecanismos de ação ainda desconhecidos, dando suporte científico a projetos que permitam a aplicação racional e sustentada dessa riqueza. Além disto, os

saberes tradicionais podem nos revelar particularidades terapêuticas mais condizentes com o atual entendimento das doenças degenerativas, onde a patofisiologia não é bem conhecida e devido à sua natureza multifatorial pressupõe-se o benefício de atuar em múltiplos alvos farmacológicos (ELISABETSKY, 2002).

Este estudo reforça a idéia de que o EEPO, além de possuir propriedades pró-mnésicas, também possa ser benéfico para tratamento de déficit cognitivos, principalmente daqueles relacionados ao sistema colinérgico. Contudo não se pode descartar uma possível ação em outros sistemas igualmente essenciais para memória como os noradrenérgico e dopaminérgico. Para complementar o estudo psicofarmacológico de EEPO seria importante recorrer a outros antagonistas mais específicos (como α_2-adrenérgico, $5HT_{2A}$ serotoninérgico, AMPA, glutamatérgico metabotópicos). Estudos neuroquímicos seriam também úteis para esclarecer a natureza da ação (direta ou indireta) do EEPO nos diferentes sistemas/receptores observados neste estudo.

Conclusão

- O extrato padronizado de raízes de *P. olacoides* (EEPO) possui propriedades pró-mnésicas, em camundongos adultos e senis, independente da via de administração. Contudo, os resultados sugerem que o EEPO module diferentemente as memória declarativas aversivas e não aversivas;

- A análise conjunta dos resultados indica que EEPO parece atuar preferencialmente sobre os sistemas: colinérgico (por inibir a acetilcolinesterase), dopaminérgico (via receptores D_1), noradrenérgico (via receptores β-adrenérgicos) e glutamatérgico (direta ou indiretamente via receptor NMDA);

- A análise da interação do EEPO com os receptores serotoninérgicos testados não produziu dados conclusivos, necessitando futuros esclarecimentos;

- Os resultados obtidos com EEPO no modelo de memória podem sugerir que os sistemas envolvidos na modulação da memória atuam de modo cooperativo, por isto foram observados efeitos tão diversos utilizando os vários antagonistas neste estudo. Mas o mais provável é que, além disto, o EEPO possa atuar sobre mais de um sistema neurotransmissor cujo

resultado final se traduz na facilitação da memória e reversão do déficit cognitivo, principalmente daquele relacionado ao sistema colinérgico.

- Este estudo demonstra a importância dos estudos etnofarmacológicos não só na identificação de produtos com mecanismos de ação ainda desconhecidos, como nos permite novo pensar sobre os aspectos fisiológicos das doenças crônicas que afetam o sistema nervoso central.

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ANEXO I

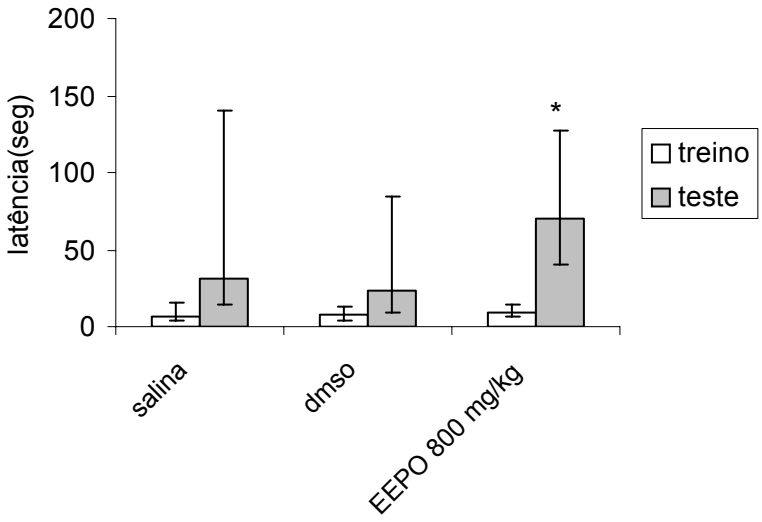

Figura 1. Efeito de do extrato etanólico de *Ptychopetalum olacoides* (EEPO 800 mg/kg, vo) administrado durante 21 dias. DMSO= dimetil sulfoxido 20% (v/v). Resultados expressos em mediana e intervalo interquartil. Mann-Whitnney, * *P*<0,05 comparado aos controles salina e DMSO; N=20.

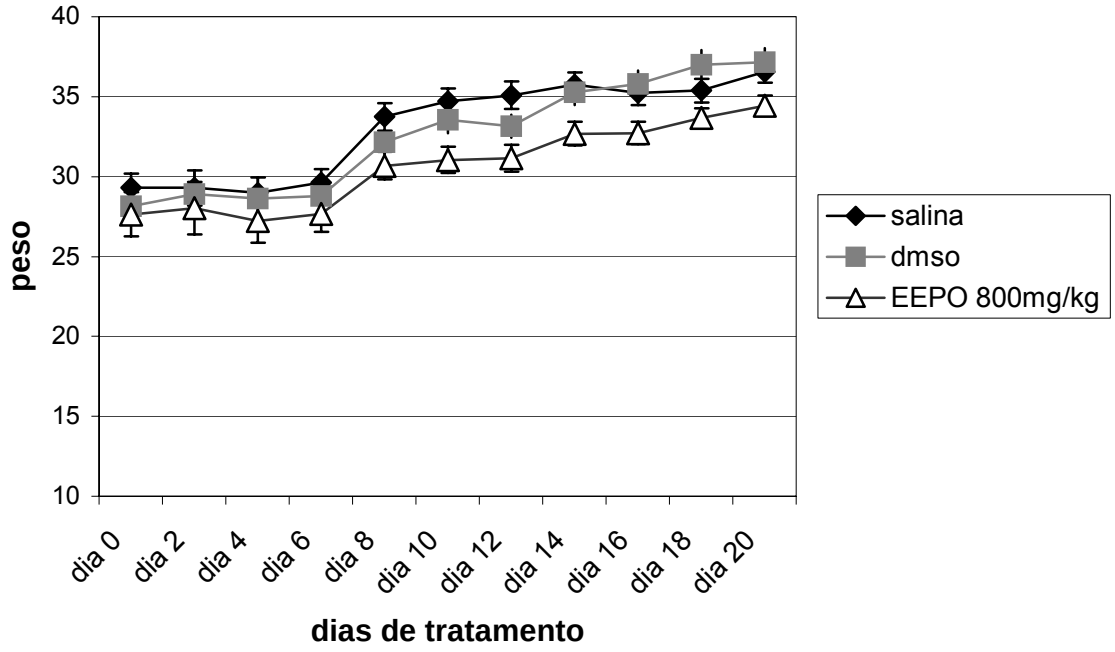

Figura 2. Efeito da administração crônica do extrato etanólico de *Ptychopetalum olacoides* (EEPO 800mg/kg, vo) sobre o peso corporal. A medida de peso foi obtida a cada dois dias. GLM/SNK, N=20.

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