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Avaliação neurofarmacológica das atividades tipo ansiolítica e/ou antidepressiva da fração diclorometano, ácido oleanólico e (*E*)-metilisoeugenol das folhas de *Pimenta pseudocaryophyllus* (Gomes) L. R. Landrum (Myrtaceae) quimiotipo (*E*)-metilisoeugenol

JAMES OLUWAGBAMIGBE FAJEMIROYE

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Orientador (a):	Elson Alves Costa						
E-mail:	elson@ufg.br						
Co-orientador (a):*	Jordan K Zjawiony						
E-mail:	jordan@olemiss.edu						

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Co-orientador: Prof. Dr. Jordan K Zjawiony

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Ao Deus poderoso, que restaura minha paz e sanidade no meio do silêncio e da escuridão do mundo, da solidão, da exaustão, da tempestade e deixa as minhas esperanças vivas. As superações vêm através das graças do criador, que fez o melhor das minhas fraquezas e imperfeições. Era o meu sonho, mas a realidade da vitória nunca mais poderá atribuída aos meus saberes, conhecimentos, influências ou esforços pessoais. Todas as honras e glórias pertencem a Ele pela providência divina, oportunidade, saúde e as vidas das pessoas que vem construindo esta história de successo. Obrigado Senhor pela força interior, alegria indizivel, fé inabalável, amor incondicional.

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

ACh	Acetilcolina
ANOVA	Análise de Variância
ATC	Antidepressivo Tricíclico
AO	Ácido Oleanólico
BDNF	*Brain Derived Neurotrophic Factor*
BNST	*Bed Nucleus of the Stria Terminalis*
CA	Campo Aberto
CEUA	Comissão de Ética no Uso de Animais
CPFM	Córtex Pré-Frontal Medial
CORT	Corticotropina
DA	Dopamina
DL	Coluna Dorsolateral
EPM	Erro Padrão da Média
FD	Fração Diclorometano
GABA	Ácido Gama Aminobutírico
IMAO	Inibidores da Monoamina Oxidase
i.p	Intraperitoneal

ISRS	Inibidores Seletivos da Recaptação de Serotonina
ISRN	Inibidores Seletivos da Recaptação de Noradrenalina
LCE	Labirinto em Cruz Elevado
CCE	Caixa Claro-Escuro
MAO	Monoamina Oxidase
MIE	(*E*)-metilisoeugenol
N	Núcleo
NE	Norepinefrina
PAG	Substância Cinzenta Periaquedutal
PVN	Núcleo Paraventricular do Hipotálamo
p.o.	*per os* (oral route)
SNC	Sistema Nervoso Central
TNF	Teste do Natação Forçada
TSC	Teste da suspensão pela cauda
5-HT	Serotonina
UFG	Universidade Federal de Goiás
VL	Coluna Ventrolateral do PAG
VTA	Área Tegmental Ventral

v/v	Volume/Volume
w/v	Peso/Volume

LISTA DE FIGURA

RESUMO

Ansiedade e depressão são transtornos psiquiátricos de interesse global. Estes transtornos estão entre as principais causas da incapacidade laboral das pessoas. Apesar de uma gama de farmacoterapias disponíveis, os resultados clínicos mostram que os fármacos não produziram efeitos terapêuticos desejados e se faz necessário a busca de novos fármacos. As plantas medicinais continuam sendo uma das fontes mais importantes para a descoberta de novos fármacos e entidades químicas. Estudos anteriores mostraram efeito calmante e ansiolítico da fração orgânica do extrato das folhas de *Pimenta pseudocaryophyllus* (Gomes) L. R. Landrum (Myrtaceae). O presente estudo buscou investigar a atividade tipo antidepressiva da fração diclorometano (FD) do extrato etanólico das folhas desta espécie, bem como antidepressiva e ansiolítica do ácido oleanólico (AO), (*E*)-metilisoeugenol (MIE) e os possíveis mecanismos de ações envolvidos. Modelos experimentais como o sono induzido por barbitúricos, caixa claro escuro (CCE), labirinto em cruz elevado (LCE), campo aberto (CA), teste de arame, teste de convulsão induzida por pentilenotetrazol, teste de natação forçada (TNF) e teste de suspensão pela cauda (TSC) foram realizados para avaliar alterações comportamentais induzidas pela administração do veículo, FD, AO, MIE ou fármacos de referência. Na tentativa de elucidar os possíveis mecanismos de ação, foram realizados bioensaios (*ex vivo* e *in vitro*) da monoamina oxidase (MAO) e do fator neurotrófico derivado do cérebro (BDNF do hipocampo). A administração oral da FD 125, 250 ou 500 mg/kg potencializou o efeito hipnótico de pentobarbital sódico. No TNF e TSC, a FD 125 ou 250 mg/kg induziu efeito tipo antidepressivo. Os dados obtidos no campo aberto sugerem efeito sedativo da fração

diclorometano na dose de 500 mg/kg. O pré-tratamento (i.p) com *p* - clorofenilalanina metil éster (PCPA) 100 mg/kg (depletor de serotonina) ou α - metil - p - tirosina (AMPT) 100 mg/kg (depletor de catecolamina) bloqueou o efeito tipo antidepressivo da FD no TNF. O bioensaio da atividade enzimática mostrou que a FD não alterou a atividade da MAO. A administração oral do AO (5-20 mg/kg) aumentou a duração do sono induzido por pentobarbital sódico e demonstrou efeito tipo ansiolítico no CCE e LCE. O AO 5-20 mg/kg demonstrou efeito tipo antidepressivo no TNF e TSC sem alterar a atividade locomotora dos animais. O efeito tipo antidepressivo do AO foi atenuado por pré-tratamento com NAN-190 (antagonista não-seletivo do receptor 5-HT1A), AMPT, PCPA e PRAZ-prazosin (antagonista do receptor α1 adrenérgico). A administração crônica do AO aumentou o nível de BDNF no hipocampo. A administração oral do MIE 250 ou 500 mg/kg potencializou o efeito hipnótico de pentobarbital sódico sem proteger os animais contra a convulsão induzida por PTZ. Os parâmetros avaliados na CCE e LCE sugerem que MIE têm efeito tipo ansiolítico. Este efeito foi bloqueado pelo pré-tratamento com WAY100635 (antagonista seletivo do receptor 5-HT1A). MIE 125 ou 250 mg/kg apresentou efeito tipo antidepressivo no TNF. Não houve alteração na atividade locomotora dos animais no CA após a administração do MIE 125 ou 250 mg/kg. O pré-tratamento com PCPA atenuou o efeito tipo antidepressivo do MIE no TNF. Os resultados demonstraram efeito tipo ansiolítico e/ou antidepressivo da fração diclorometano, ácido oleanólico e (*E*)-metilisoeugenol, sugerindo o envolvimento de vias monoaminérgicas nestes efeitos.

Palavras chaves: *Pimenta pseudocaryophyllus,* ansiedade, depressão, fração diclorometano, ácido oleanólico, (*E*)-metilisoeugenol

ABSTRACT

Depression and anxiety are widely acclaimed as psychiatric disorders of global concern. These disorders are among the leading causes of disability worldwide. Unsatisfactory responses of patients to the available pharmacotherapy make the search for new drugs a necessity. Medicinal plants remain important source of new drugs and new chemical entities. The ethnopharmacological knowledge and previous data have revealed calming and anxiolytic like effects of the organic leaf extract of *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum. The present study sought to investigate antidepressive like effect of dichloromethane fraction (DF) of the ethanolic leaf extract of *Pimenta pseudocaryophyllus* as well as anxiolytic and antidepressive like effects of oleanolic acid (OA), (*E*) methyl isoeugenol (MIE) and possible mechanisms of action that are involved. Animal models like barbiturate-induced sleep, light dark box test (LDB), elevated plus-maze (EPM), open field (OF), wire hanging test, pentylenetetrazol-induced convulsion test, forced swimming test (FST), tail suspension test (TST) were conducted to evaluate behavioural alterations that were elicited by the administrations of vehicle, DF, OA, MIE or reference drugs. Bioassays (*ex vivo* and *in vitro*) of monoamine oxidase (MAO) and quantification of hippocampal level of brain derived neurotrophic factor (BDNF) were conducted in an attempt to elucidate possible mechanisms of action. Oral administration of DF 125, 250 or 500 mg/kg (potentiated the hypnotic effect of sodium pentobarbital). In the TST and FST, DF 125 or 250 mg/kg induced antidepressant-like response. The data obtained in the OF suggest sedative effect of DF at 500 mg/kg. Pretreatment (i.p) with *p*-chlorophenylalanine methyl ester (PCPA) 100 mg/kg (serotonin depletor) or α-methyl-*p*-tyrosine (AMPT) 100 mg/kg (catecholamine depletor) blocked anti-immobility effect of DF

in the FST. The enzymatic activity of MAO remained unaltered by DF. Oral administration of OA (5-20 mg/kg) increased the duration of barbiturate - induced sleep and demonstrated anxiolytic like effect in both LDB and EPM. In the FST and TST, OA 5-20 mg/kg elicited antidepressant like effect without altering locomotion activity of the animals. The antidepressant like effect of OA was attenuated by NAN-190 (non-selective antagonist of 5-HT1A), AMPT, PCPA, WAY and PRAZ. Chronic administration of OA increased hippocampal level of BDNF. Oral administration of MIE 250 or 500 mg/kg potentiated hypnotic effect of sodium pentobarbital without protecting mice against PTZ - induced convulsion. The parameters evaluated in the LDB, EPM and OF demonstrated anxiolytic like property of MIE. This effect was blocked by WAY (selective antagonist of 5-HT1A) pretreatment. MIE 125 or 250 mg/kg showed antidepressant like effect in the FST. Locomotion activity of the animal in the OF remained unaltered by MIE administration at 125 or 250 mg/kg. Pretreatment of mice with PCPA attenuated antidepressant like property of MIE. In conclusion, our findings demonstrated anxiolytic and/or antidepressant like effects of dichloromethane fraction, oleanolic acid and (*E*) methyl isoeugenol, thereby suggesting the involvement of monoaminergic pathway.

Keywords: *Pimenta pseudocaryophyllus*, anxiety, depression, monoamines, dichlomethane fraction, oleanolic acid, (*E*) methyl isoeugenol

1. Introdução geral

1.1. Neurobiologia da ansiedade e depressão

Ansiedade e depressão são transtornos psiquiátricos de interesse global. Esses transtornos continuam sendo as doenças psiquiátricas mais debilitantes que podem comprometer o bem-estar de seres humanos (ANDREWS et al., 2000). Os transtornos de humor são episódios patológicos do estado emocional associados a anormalidades na cognição e no comportamento (AMERICAN PSYCHIATRIC ASSOCIATION, 1994).

A compreensão da neurobiologia da ansiedade e da depressão é vital para o entendimento do mecanismo do tratamento efetivo. Tem sido demonstrado que a desregulação dos processos neuronais no sistema nervoso central desempenha um papel importante em diversas doenças psiquiátricas (WANG et al., 2009). O papel límbico na emoção foi identificado por James Papez no começo de 1930. Ele descreveu o "sistema de emoção" como uma das principais vias do sistema límbico que conectam grupos de estruturas cerebrais (giro cingulado, hipocampo, hipotálamo e os núcleos do tálamo) ao redor do tronco cerebral (PALAZIDOU, 2012). Drevets et al. (2007) hipotetizou os circuitos anatômicos que envolvem o córtex pré-frontal medial (CPFM) e amígdala dentro do contexto de um modelo em que a disfunção do CPFM resulta na desinibição de transmissão límbica através da amígdala, gerando as manifestações emocionais, cognitivas, endócrinas, autonômicas e neuroquímicas da depressão (Figura 1).

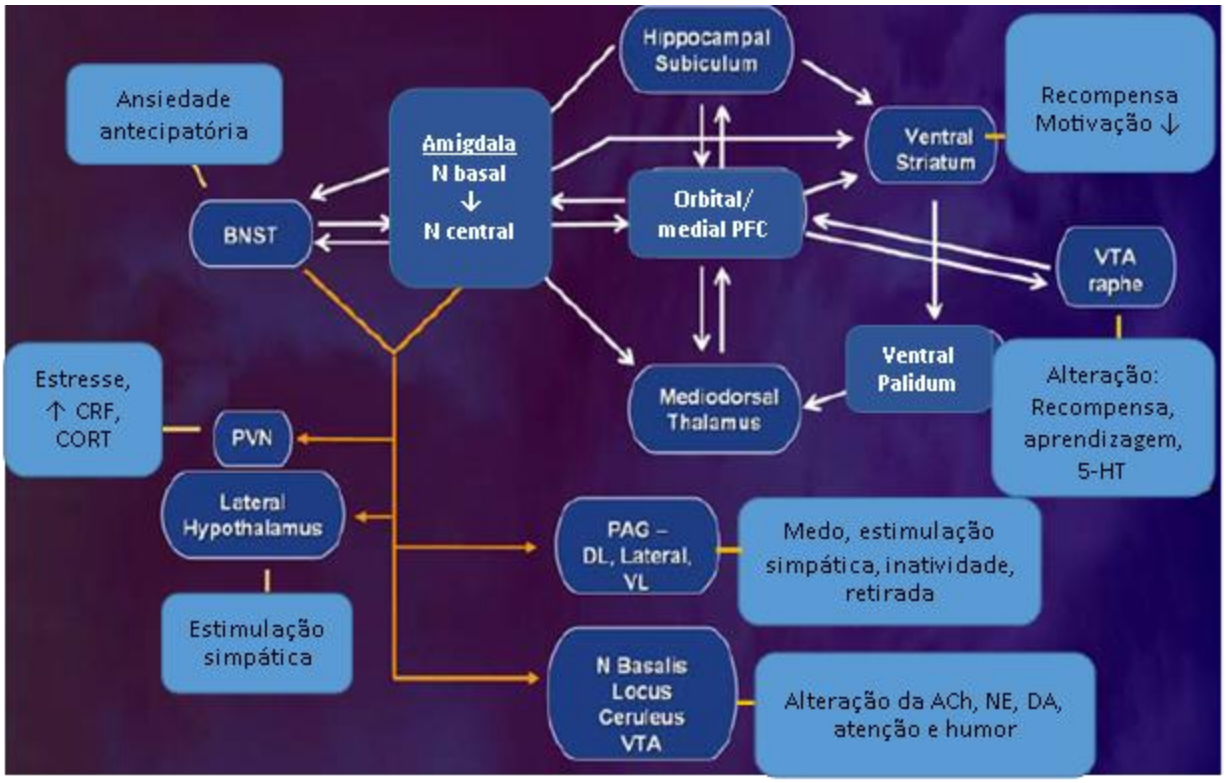
Do ponto de vista da neuropatológia ou da neurofisiológia, anormalidades neuroquímicas existentes dentro da rede visceromotor pode prejudicar a modulação do sistema endócrino, autônomo, respostas emocionais e comportamentais a estímulos aversivos ou contextos relacionados á recompensa (ONGUR et al., 2003), desta

maneira causando transtornos do humor (Figura 1). O aumento do fluxo sanguíneo no cérebro e metabolismo anormal no córtex orbital, ventrolateral do CPF (córtex pré-frontal), amígdala, estriado ventral e tálamo medial em depressão implica o envolvimento do circuito límbico-tálamo-cortical e o circuito do limbico-estriado-palidal-talâmico (DREVETS et al., 1992). Um exemplo de circuito de excitação mostrado na Figura 1,envolve a amigdala basolateral, o CPFM e o núcleo do tálamo mediodorsal que estão interligados por projeções excitatórias (AMARAL E INSAUSTI, 1992; BACON et al., 1996; JACKSON e MOGHADDAM, 2001; KURODA E PREÇO, 1991). Um aumento no metabolismo de glicose nessas estruturas reflete o aumento da transmissão sináptica através do circuito límbico-tálamo-cortical. O circuito límbico-estriado-pallidal-tálamo constitui as projeções desinibitórias entre o CPF ou amígdala e o núcleo do tálamo mediodorsal. A amígdala e o CPF enviam projeções sobrepostas e excitatórias para o corpo estriado ventromediano (RUSSCHEN et al., 1985). O corpo estriado envia uma projeção inibitoria ao pallidum ventral (GRAYBIEL, 1990) que por sua vez envia neurônios gabaergicos ao núcleo do tálamo mediodorsal (Kuroda e preço, 1991). Desde que os neurônios palidais têm altas taxas de desparo espontâneo (DELONG, 1972), a atividade no CPF ou amígdala que ativa o estriado e, por sua vez inibe o pallidum ventral pode liberar o núcleo do tálamo mediodorsal da influência pallidal inibitória, consequentemente desinibindo a transmissão do circuitos limbico-talamo-cortical.

As alterações neuropatológicas evidentes no CPFM em transtornos do humor podem prejudicar o papel modulador dessa estrutura cortical sobre as expressões emocional das respostas límbicas. Regulações recíprocas entre o CPFM e amígdala que medeiam a expressão emocional têm sido demonstradas pelos estudos eletrofisiológicos e análise de lesão. Ratos expostos ao medo-condicionado (estímulo) mostram uma redução na atividade (disparo neuronal) do CPFM. A magnitude desta redução se correlaciona inversamente com o aumento da atividade dos neurônios da amígdala e alteração comportamental (GARCIA et al., 1999). Os farmacos e estimulações profundas do cérebro com efeito antidepressivo reduzem a atividade fisiológica na amígdala (DREVETS et al., 2002a;. MAYBERG et al., 2005;. VAN et al., 2006). Um comprometimento da função do CPFM que desinibir atividade da amígdala

pode contribuir para as anormalidades do sistema neuroendócrino, autonômico, alterando a atenção e comportamentos direcionados pela recompensa (Figura 1). O estresse ativa a amígdala que por sua vez desinibe a liberação do fator liberador de corticotropina (CRF) do núcleo paraventricular do hipotálamo e posteriormente a secreção do glicocorticóide (HERMAN E CULLINAN, 1997). A estimulação do locus coeruleus (LC), hipotálamo lateral e substância cinzenta periaquedutal (PAG) pela amígdala aumenta a excitação autônomo simpático em roedores (LEDOUX, 2003). A disfunção do CPFM e hiperatividade da amígdala podem contribuir para a anedonia, perda de motivação e atenção na depressão (Figura 1). O núcleo basolateral da amígdala (N basal) envia projeções eferentes para o núcleo central (N central) da amígdala e o núcleo leito da estria terminal (BNST). As projeções eferentes dessas estruturas para o hipotálamo, substância cinzenta periaquedutal (PAG), núcleo basal (N basalis), LC, rafe e outros núcleos (Figura 1).

Figura 1. Circuitos anatômicos que envolvem o córtex pré-frontal medial (CPFM) e amígdala dentro do contexto de um modelo em que a disfunção do CPFM resulta da desinibição de transmissão límbica através da amígdala, gerando as manifestações emocionais, cognitivas, endócrinas, autonômicas e neuroquímicas da depressão.



Abreviações: 5-HT serotonina, ACh acetilcolina, DA dopamina, PAG substância cinzenta periaquedutal, DL coluna dorsolateral do PAG, N núcleos, NE norepinefrina, PVN núcleo paraventricular do hipotálamo, VL coluna ventrolateral do PAG, VTA área tegmental ventral, BNST "bed nucleus of the stria terminalis", CRF fator liberador de corticotropina, CORT corticotropina (Reproduzido do DREVETS et al., 2007; DREVETS et al., 2008).

Evidências crescentes têm sido acumuladas nos últimos anos acerca do impacto da ansiedade e depressão sobre as estruturas e os processos funcionais que ocorrem no cérebro. A partir do ponto de vista inicial, esses distúrbios são causados pelo "desequilíbrio químico" no cérebro. Entretanto, esta consideração tem sido baseada em uma teoria complexa que implica o envolvimento das redes e plasticidade neurais (CASTRÉN, 2005).

O surgimento de técnicas de neuro-imagem, ressonância magnética nuclear, tomografia por emissão de pósitrons, emissão de fóton único (tomografia computadorizada) e outras tecnologias têm avançado o processo de elucidação da participação das diferentes estruturas cerebrais nas experiências e comportamentos humanos (NEWBERG et al., 2012). Estudos das imagens em pacientes com depressão têm revelado a redução do volume da substância cinzenta no córtex pré-frontal (BREMNER et al., 2002; BOTTERON et al., 2002; DREVETS, 2001) e no hipocampo (MACQUEEN et al., 2003; MERVAALA et al., 2000; SHELINE et al., 2003; SHELINE, 2003; FRODL et al., 2002). Alterações morfológicas do hipocampo tem sido associadas com déficits funcionais como o da memória (MACQUEEN et al., 2003). Estas alterações morfológicas parecem ser reversíveis por terapias antidepressivas (DREVETS, 2001; DREVETS et al., 2002).

1.1.1. Ansiedade

Os transtornos de ansiedade estão entre as mais prevalentes classes de transtornos psiquiátricos nos Estados Unidos (KESSLER et al., 2005a) e em muitos outros países (ALONSO; LEPINE, 2007). Um estudo multicêntrico de morbidade psiquiátrica em três áreas urbanas brasileiras (Brasília, São Paulo e Porto Alegre), estimou que a prevalência do transtorno de ansiedade é da ordem de 12,1% para Brasília, 6,9 % para São Paulo e 5,4 % para Porto Alegre (ALMEIDA et al., 1992; ALMEIDA et al., 1997). No mesmo estudo, os autores mostraram que a ansiedade e as fobias constituem os principais problemas de saúde mental da população brasileira, com prevalências variando de 8 % a 18 %.

De acordo com o DSM-IV (AMERICAN PSYCHIATRIC ASSOCIATION, 2000), os transtornos de ansiedade incluem: (A) transtorno de pânico (caracterizado por ataques inesperados e recorrentes), que pode ocorrer com ou sem (B) agorafobia (medo de ter a experiência do pânico em situações com nenhuma oportunidade de escapar), (C) transtorno de estresse pós-traumático (tipificado por memórias intrusivas e angustiantes de um evento traumático com hipervigilância persistente), (D) transtorno de ansiedade generalizada (um padrão crônico de preocupação excessiva e incontrolável), (E) transtorno de ansiedade social (caracterizada pela prevenção de situações sociais devido ao medo da avaliação negativa), (F) transtorno obsessivo-compulsivo (caracterizado pela presença de obsessões intrusivas e comportamentos compulsivos), e (G) fobias específicas (SANJAY et al., 2008).

Os fármacos como hidrato de cloral, meprobamato (BERGER, 1970) e clordiazepóxido (THOMAS, 2006) que foram e ainda podem ser utilizado no tratamento

da ansiedade, apesar de terem sido descobertos por acaso. A eficácia dos inibidores seletivos da recaptação de serotonina - ISRS, inibidores seletivo da recaptação de noradrenalina - ISRN e benzodiazepínicos no tratamento de ansiedade chamaram a atenção sobre o papel da neurotransmissão serotoninérgica, noradrenérgica e gabaérgica (BALDWIN; GARNER, 2008). A importância da neurotransmissão serotoninérgica na resposta ao tratamento em pacientes com transtornos de ansiedade é evidente nos estudos randomizados controlados de depleção de triptofano em pacientes tratados com ISRS (BELL, 2001, BELL et al., 2002 e ARGYROPOULOS et al., 2004). A melatonina (agonista MT1) e agomelatina (agonista MT2 que também antagoniza 5 - HT2C) tem propriedades ansiolíticas em modelos animais (MILLAN et al., 2005) e é útil no alívio da ansiedade em pacientes com depressão (LOO et al., 2002a, b), e em desordem de ansiedade generalizada (STEIN et al., 2008). O antagonista do receptor serotoninérgico 5 - HT2C (SB242084) potencializou o efeito do citalopram e fluoxetina em modelos animais de depressão (CREMERS et al., 2004). Diferentes transtornos de ansiedade podem ser caracterizados por diferentes perturbações do sistema monoaminérgico. A administração da clonidina (agonista do receptor α2 adrenérgico) induziu um efeito ansiolítico em pacientes com transtorno de pânico (CHARNEY et al., 1989; COPLAN et al., 1992), mas não em pacientes com transtorno obsessivo e compulsivo (RASMUSSEN et al., 1987; HEWLETT et al., 1992) ou desordem de ansiedade generalizada (CHARNEY et al., 1989). Os benzodiazepínicos são ansiolíticos potentes, mas podem induzir sedação, amnésia, tolerância dentre outros (GARNER et al., 2009).

Existem vários neurotransmissores que exercem efeitos diretos ou indiretos sobre o receptor $GABA_A$, dentre esses podemos citar: neuroesteróides, fator liberador de corticotropina (CRF), L – arginina, vasopressina, neuropeptídio, colecistoquinina, substância P, neurotensina, glutamato, somatostatina, noradrenalina, dopamina, acetilcolina, serotonina e N - metil - d - aspartato (GARNER et al., 2009). Dentre estes, alguns agentes tem melhorado os efeitos do GABA de uma maneira que não envolve o sítio benzodiazepínico. Segundo Pollack et al. (2005), esses agentes podem aumentar a síntese do GABA, inibir a sua degradação e recaptação. Os análogos do GABA (pregabalina, gabapentina) tem sido utilizados para modular os canais iônicos de cálcio dos neurônios pré-sinápticos e a atividade pós-sináptica de tal forma que a liberação de neurotransmissores excitatórios, como aspartato, substância P e glutamato, é reduzida (STAHL, 2004).

1.1.2. Depressão

Além da ansiedade, estima-se que no Brasil existam aproximadamente 54 milhões de pessoas que em algum momento de suas vidas terão algum tipo de depressão, sendo que 7,5 milhões terão episódios agudos e graves, muitas destas com risco de suicídio (NARDI, 2000). A depressão é uma doença crônica e recorrente que afeta cerca de 20% da população mundial. É comum a ocorrência da comorbidade da depressão com pânico, fobia social, síndrome do estresse pós-traumático e transtorno obsessivo-compulsivo (KESSLER et al., 2005b; DREVETS et al., 2008). Os impactos sócio-econômicos e sofrimentos dos pacientes fazem parte das maiores preocupações globais (NIKOLA et al., 2012).

O diagnóstico da depressão baseia-se nas observações clínicas dos sintomas estabelecidos pelo DSM-IV (AMERICAN PSYCHIATRY ASSOCIATION, 2000). De acordo com este manual um indivíduo é diagnosticado com depressão maior se preencher critérios, tais como: (A) humor deprimido, (B) anedonia, (C) alterações do sono - insônia ou hipersônia, (D) distúrbios da atividade psicomotora - letargia ou agitação psicomotora, (E) fadiga ou falta de energia, (F) falta de atenção ou diminuição da concentração, (G) perda ou ganho de peso ou de apetite, (H) sentimentos de desvalia ou culpa, (I) pensamentos recorrentes de morte ou suicídio. O indivíduo diagnosticado com depressão maior apresenta pelo menos um dentre os dois primeiros sintomas e mais quatro dos sintomas C a I (AMERICAN PSYCHIATRIC ASSOCIATION, 1994). Os sintomas da ansiedade também são importantes durante os episódios depressivos (KESSLER et al., 2005b).

Existem várias classes de antidepressivos usados para o tratamento da depressão. A história dos antidepressivos foi marcada pela "descoberta acidental" de que iproniazida, um fármaco para o tratamento da tuberculose e a imipramina, um anti-histamínico, elevavam o estado de animo do paciente, promovendo assim uma revolução no tratamento de desordens de humor (HEALY, 1997; KUHN, 1996; THOMAS, 2006). Em meados da década de 1950, isoniazida e iproniazida (derivados da hidrazina) foram usados no tratamento da doença pulmonar (tuberculose). Além de curar a tuberculose, este tratamento também melhorou o humor dos pacientes. Muitos dos pacientes que receberam iproniazida tornaram-se eufóricos e exibiram comportamento hiperativo. Isto levou a estudos sobre os efeitos comportamentais da iproniazida em voluntários saudáveis e em pacientes deprimidos e logo em seguida, as propriedades antidepressivas foram confirmadas em estudos controlados. A despeito disso, o fator causador da alteração de humor não foi revelado (JONES, 2010). Os antidepressivos tricíclicos (desipramina, nortriptilina), inibidores da monoamina oxidase (isocarboxazida, tranilcipromina), inibidores seletivos da recaptação de serotonina (fluoxetina, sertralina, paroxetina), aminocetonas (bupropiona), triazolopiridinas (trazodona) estão sendo utilizados no tratamento de depressão (PRESKORN, 1993). Vale ressaltar que alguns desses fármacos têm sido usados para tratar a ansiedade também (ALLAN et al., 2001).

Hipóteses envolvendo as monoaminas e a neurogênese têm sido propostas na tentativa de elucidar os mecanismos de ação de fármacos antidepressivos. A hipótese do envolvimento das monoaminas na depressão produziu várias gerações de agentes antidepressivos, tais como inibidores da monoamina oxidase (IMAO), antidepressivos

tricíclicos - ATC, ISRS, ISRN, antidepressivos atípicos, entre outros (PRESKORN, 1993; BEZCHLIBNYK-BUTLER, 1999). A hipótese da neurogênese é apoiada pela observação recente de que o tratamento crônico com antidepressivos aumenta a neurogênese e o crescimento de novos neurônios no hipocampo de roedores. A neurogênese e o crescimento neuronal se correlacionam com as alterações comportamentais indicativas do efeito antidepressivo (MALBERG et al., 2000; SANTARELLI et al., 2003). Em um nível mais sutil, fármacos antidepressivos podem aumentar as ramificações de axônio (VAIDYA et al., 1999) e dendritos (FUJIOKA et al., 2004) e facilitar a maturação dos neurônios recém-formados (FUJIOKA et al., 2004). Um possível mecanismo através do qual os antidepressivos podem aumentar a plasticidade das conexões neuronais no hipocampo e no córtex cerebral é a ativação da sinalização neurotrófica (ALTAR, 1999; CASTRÉN, 2004). Uma vez que o fator neurotrófico derivado do cérebro (BDNF) é produzido e liberado por neurônios (THOENEN, 1995), os antidepressivos e o estimulo por promovem o aumento da expressão e da sinalização de BDNF no hipocampo e no córtex (NIBUYA et al., 1995; RUSSO - NEUSTADT et al., 2000; SAARELAINEN et al., 2003).

Embora existam muitos medicamentos psicotrópicos disponíveis para o tratamento de ansiedade e depressão, os resultados clínicos dos pacientes que ainda não respondem aos tratamentos mostram as necessidades da buscar de novos fármacos ansiolíticos e antidepressivos (GARNER et al, 2009; PHIL, 1999). As modificações químicas desses fármacos nas últimas décadas ainda não atingiram os objetivos desejados quanto ao perfil farmacológico dos mesmos. A maioria destes fármacos requer mais de duas semanas de tratamento para produzir efeitos

terapêuticos significativos. Este fenômeno tem sido denominado como "demora terapêutica" (PHIL, 1999). Além disso, estudos indicam que 30 % da população não responde às terapias atuais (PHIL, 1999). Sendo assim, o acesso aos produtos naturais capazes de controlar distúrbios no sistema nervoso central (RAVINDRAN et al., 2009) poderia fornecer opções terapêuticas viáveis.

1.2 Plantas medicinais

Por muitas décadas, a cultura humana, religião e etnia têm influenciado na percepção folclórica para a exploração global de produtos naturais. A história da medicina com uso de ervas está ligada à da medicina moderna. No Brasil, as práticas médicinais tradicionais são caracterizadas pela mistura cultural dos africanos (ioruba e banto), europeus (principalmente Portugueses) e nativos (IVONE; ELAINE, 2012).

As plantas têm sido utilizadas como medicamentos há milhares de anos (SAMUELSSON, 2004) na forma de preparações brutas, tais como: tintura, chá e pó (BALICK, COX, 1997; SAMUELSSON, 2004). As plantas medicinais utilizadas e os métodos de aplicação para uma determinada doença foram passados de geração a geração através da história (BALUNAS; KINGHORN, 2005). Vários metabólitos secundários de origem vegetal (salicina, digoxina, vincristina, efedrina, morfina, codeína) foram úteis como modelos estruturais para a modificação química. A triagem de extratos de plantas é de grande interesse para os cientistas na descoberta de novos fármacos para o tratamento de várias doenças (DIMAYUGA; GARCIA, 1991). A extração de compostos bioativos de plantas medicinais permite estudo farmacológico que pode levar à descoberta de moléculas novas, além de proporcionar a descoberta de compostos que facilitem a síntese de fármacos com perfil de atividade desejável (PAMPLONA- ROGER, 1999).

Os seres humanos consomem uma grande variedade de alimentos, medicamentos e suplementos que podem ser oriundos de plantas psicoativas. As propriedades psicoativas destas plantas estão associadas à presença de metabólitos secundários, produtos químicos que podem não ser necessários para a sobrevivência

imediata das plantas (KENNEDY; WIGHTMAN, 2011). O repertório químico das plantas é extremamente vasto, provavelmente bem acima de 100.000 substâncias potencialmente ativas, das quais apenas uma porcentagem ínfima foi investigada por laboratórios farmacêuticos (BRUNETON, 1997). No Brasil, várias espécies vegetais (fonte de diversos compostos psicoativos) são popularmente usadas no tratamento de várias doenças, dentre elas, as que pertencem à família Myrtaceae (CRUZ; KAPLAN, 2004).

A família Myrtaceae teve provável origem na Gondwana (WILSON et al., 2001; SYSTMA et al., 2004) e, atualmente, ocorre nas regiões tropicais e subtropicais do mundo, tendo a Austrália, Sudeste da Ásia e América do Sul como centros de diversidade (WILSON et al. 2001). A família Myrtaceae apresenta cerca de 4000 espécies, distribuídas em 130 gêneros (em todo o mundo). Baseado numa análise filogenética de plastídeos de DNA, a classificação dessa família reconhece 17 tribos e duas subfamílias, Myrtoideae e Psiloxyloideae (WILSON et al., 2005). No Brasil, Myrtaceae está entre as dez famílias com maior riqueza de espécies (aproximadamente 1000 espécies) com 23 gêneros (PAULA, 2011; SOBRAL et al., 2012). As espécies pertencentes a essa família apresentam características, tais como: arbóreas, arbustos ou subarbustos, pilosas com indumento simples ou com tricomas dibraquiados, caducos ou persistentes; suas folhas são simples ou opostas com glândulas translúcidas sem estípulas; as flores são geralmente bissexuadas, brancas, odoríferas, tetrâmeras, pentâmeras ou hexâmeras; os frutos são comumente na forma de baga, drupa, capsula, nucula (LANDRUM; KAWASAKI, 1997; SOUZA; LORENZI, 2005). A tribo Myrteae está atualmente dividida nos seguintes grupos, baseado nas

características dos principais gêneros, tais como: *Plinia, Myrcia, Myrceugenia, Myrteola, Eugenia* e *Pimenta*.

Além do uso popular das espécies pertencentes à familia Myrtaceae, algumas espécies tem as avaliações farmacológicas sendo realizadas e relatadas na literatura. O óleo essencial da *Eugenia caryophyllata* Thunb elicitou um efeito anticonvulsivante no modelo de convulsão induzida por pentilenetetrazol (POURGHOLAMI et al., 1999). *Pimenta dioica* (L.) Merrill possui atividade analgésica e antipirética (LÓPEZ, 1998). Extratos orgânicos obtidos a partir das folhas de *Pimenta dioica* mostraram atividade hipotensora e anti-hipertensiva em ratas albinas normotensas anestesiadas (SUÁREZ et al., 2000). *Pimenta racemosa* (P. Miller) J.W. Moore é uma planta nativa das ilhas do Caribe, que é utilizada na medicina popular para o tratamento de diferentes doenças, como dor de dente, dor abdominal, febre, gripe, reumatismo e pneumonia. García et al. (2004) e Fernández et al. (2001) relataram atividades analgésica e anti-inflamatória no extrato aquoso das folhas desta espécie. Lima et al. (2006) mostraram a atividade antimicrobiana do óleo essencial das folhas de *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum para *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) e *Staphylococcus aureus* (ATCC 6538) e relataram o uso popular de suas folhas na forma de chá como calmante e diurético. Vale ressaltar que os usos populares das preparações das folhas de *Pimenta pseudocaryophyllus* indicam a presença de compostos psicoativos capazes de melhorar o humor dos seres humanos (LANDRUM, 1986; LANDRUM, KAWASAKI, 1997; NAKAOKA-SAKITA et al., 1994; LIMA et al., 2006; PAULA et al., 2008; SANTOS et al., 2009).

1.3 *Pimenta pseudocaryophyllus*

Pimenta pseudocaryophyllus (Gomes) L.R. Landrum da família Myrtaceae é popularmente conhecida como “craveiro-do-mato”, “craveiro”, “louro-cravo”, “cataia”, “chá-de-bugre”, “louro” e “pau-cravo” (LANDRUM, 1986; LANDRUM, KAWASAKI, 1997). O gênero *Pimenta* é composto por cerca de 15 espécies conhecidas, das quais apenas *Pimenta pseudocaryophyllus* é nativa da flora brasileira (PAULA et al., 2010).

Pimenta pseudocaryophyllus é uma espécie vegetal aromática que apresenta variabilidade infraespecífica quanto aos constituintes de seus óleos essenciais (PAULA et al, 2011). A coleta desta espécie em duas localidades geográficas do Cerrado brasileiro tem monstrado diferenças na percepção olfativa da composição química do óleo essencial (PAULA, 2006). As diferencas qualitativas e quantitativas nos compostos majoritários chamaram a atenção para a possibilidade de existência de polimorfismo químico. A investigação da ocorrência de variabilidade infraespecífica nessa espécie vegetal destacou o químiotipo (*E*)-metilisoeugenol entre os outros devido ao alto conteúdo dessse fenilpropanoide no óleo essencial extraído das folhas de *Pimenta pseudocaryophyllus* (PAULA et al., 2011).

As folhas desta espécie são usadas na forma de chá, como calmante no município de Campos do Jordão, São Paulo, Brasil (LANDRUM, 1986; LANDRUM, KAWASAKI, 1997; NAKAOKA-SAKITA, 1994; LIMA et al., 2006; PAULA et al., 2008; DOS SANTOS et al., 2009). Estudos anteriores com o óleo essencial, extrato etanólico e frações (fração aquosa, acetato de etila, diclorometano e hexânica) das folhas demonstraram alterações comportamentais no campo aberto, labirinto em cruz elevado, caixa claro escuro e sono induzido por barbitúrico (FAJEMIROYE et al., 2012,

FAJEMIROYE et al., 2011). Os resultados anteriores mostraram efeito tipo ansiolítico da fração diclorometano (FD) obtido do extrato etanólico das folhas de *Pimenta pseudocaryophyllus* e sugeriram a participação do receptor 5-HT1A (FAJEMIROYE et al., 2012). Com base na aplicação terapêutica dos antidepressivos como ansiolíticos, existe a hipótese de que um agente que possua propriedades ansiolíticas poderia induzir o efeito antidepressivo. O fato da ansiedade e da depressão compartilharem alguns sintomas nos pacientes sugere a possibilidade da eficácia de um agente ansiolítico no modelo antidepressivo. Diversos estudos demonstraram os efeitos promissores antidepressivos e ansiolíticos de extratos orgânicos ou de fitoconstituintes (HATTESOHL et al., 2008; BRAIDA et al., 2009). O estudo fitoquímico da FD mostrou a presença do acido oleanolico - AO (FAJEMIROYE et al., 2013). Esse triterpeno pentacíclico tem sido consumido amplamente, por muitos séculos, sem relato de risco para a saúde humana (NEWMAN; CRAGG, 2007; MICHAEL et al., 2007). Vários estudos relatam atividades tipo ansiolítica e antidepressiva de triterpenóides como α - amirina e β - amirina (CHEN et al., 2005; WOODE et al., 2001).

Os efeitos psicotrópicos dos óleos essenciais e seus compostos isolados tem sido relatados na literatura (HAMID et al., 2011). Reinaldo et al. (2011) documentou a atividade anticonvulsivante dos óleos essenciais e seus constituintes. A análise qualitativa e quantitativa dos óleos essenciais de *P. pseudocaryophyllus* mostraram a presença e predominância de derivados fenilpropanóides (*E*)-metilisoeugenol (MIE) (93,9 %) entre os componentes voláteis (PAULA et al., 2011). Os estudos preliminares sugerem a participação do MIE nas atividades neurofarmacológicas do óleo essencial das folhas de *P. pseudocaryophyllus*. MIE, um dos compostos isolados da FD, foi

investigado para as atividades tipo ansiolítica e antidepressiva, adicionalmente, o efeito anticonvulsivante desse composto foi avaliado devido ao fato de ser um derivado do eugenol, que é um fitoconstituente com propriedade anticonvulsivante (REINALDO et al., 2011).

1.4 Objetivos

1.4.1 Objetivo geral

Estudar as alterações comportamentais induzidas pela fração diclorometano (FD) do extrato etanólico das folhas de *Pimenta pseudocaryophyllus* quimiotipo, (*E*)-metilisoeugenol e investigar os efeitos neurofarmacológicos do ácido oleanólico (AO) e (*E*)-metilisoeugenol (MIE).

1.4.2 Objetivos específicos

- Obter a material botânico
- Avaliar o efeito tipo antidepressivo da FD
- Investigar o mecanismo neural do efeito tipo antidepressivo da FD
- Investigar o efeito tipo ansiolítico e antidepressivo do AO
- Investigar os efeitos farmacológicos gerais do MIE
- Avaliar os efeitos tipo ansiolítico, antidepressivo e anticonvulsivante do MIE
- Elucidar os possíveis mecanismos dos efeitos tipo ansiolítico e antidepressivo do AO e MIE

2. Material e Métodos

2.1. Animais

Foram utilizados camundongos Swiss machos fornecidos pelo biotério central da Universidade Federal de Goiás e mantidos à temperatura de 23 ± 1 ° C, 12 h ciclo claro-escuro com livre acesso à água e comida. Os animais foram climatizados no laboratório de Farmacologia de Produtos Naturais uma semana antes das observações comportamentais. Os camundongos foram distribuídos de forma aleatória nos grupos experimentais. Os procedimentos experimentais foram aprovados pelo Comitê de Ética no Uso de Animais (CEUA) da Universidade Federal de Goiás (UFG 104/08 – Anexo 1). Todo procedimento experimental buscou minimizar o sofrimento dos animais. Todos os estudos envolvendo animais foram desenvolvidos como recomendado em KILKENNY et al. (2010).

2.2. Material botânico e fármacos

***Material botânico*:** Folhas de *Pimenta pseudocaryophyllus* foram coletadas na cidade de São Gonçalo do Abaeté, MG, Brasil (Longitude - 180 20' 58,4" S e a Latitude - 450 55' 23,4" W), com uma altitude de 864 m. O material vegetal foi identificado pela Professora Carolyn Elinore Barnes Proença (PhD) e Professor José Realino de Paula (PhD). Uma exsicata (voucher n º 27.159 - UFG) foi depositada no herbário da Universidade Federal de Goiás. As folhas foram dessecadas a 40 ^{0}C em estufa com ventilação forçada e triturada em moinho de facas (PAULA et al., 2012). As folhas pulverizadas foram maceradas em etanol a 95 % (v/v, 1:5, w/v) à temperatura ambiente, seguidos por filtração e concentração no rotaevaporador a 40 ^{0}C. O extrato foi concentrado até um peso constante. O extrato foi dissolvido em 250 mL de

metanol/água (7:3) e submetido à partição líquido/líquido com solvente de polaridade crescente (hexano, diclorometano e acetato de etila) de acordo com FERRI (1996). O rendimento da fração diclorometano (13,20%) obtida neste processo é a razão de massa da fração e massa do extrato em porcentagem (FAJEMIROYE et al., 2012; PAULA et al., 2012). A fração diclorometano foi armazenada a -10 ^{0}C para os usos experimentais.

***Fármacos*:** Ácido oleanolico, AO (Sigma-Aldrich, St-Quentin- Fallavier, França); (*E*)-metilisoeugenol, MIE (Sigma-Aldrich, St. Louis, MO, EUA); diazepam, DZP (Cristália, Itapira, SP, Brasil); buspirona, BUS (Cristália, Itapira, SP, Brasil); pentilenetetrazol, PTZ (Sigma-Aldrich, St. Louis, MO, EUA); fluoxetina (Sigma-Aldrich, St. Louis, MO, EUA); imipramina, IMI (Cristália, Itapira, SP, Brasil); *p*-clorofenilalanina metil éster, PCPA (Sigma-Aldrich, St. Louis, MO, EUA); α-metil-*p*-tirosina, AMPT (Sigma - Aldrich, St. Louis, MO, EUA); prazosina, PRAZ (Cristália, Itapira, SP, Brasil); ioimbina, YOH (Sigma - Aldrich, St. Louis, MO, EUA); (hidrobrometo de 1-(2-metoxifenil)-4-[4-(2-ftalimido)butil]piperazina, NAN-190 (Sigma-Aldrich , St. Louis , MO , EUA); N-{2-[4-(2-metoxifenil)-1-piperazinil]etil}-*N*-2-piridinilciclo-hexanocarboxamida, WAY (Sigma-Aldrich, St. Louis, MO, EUA); Tween 80 (Sigma-Aldrich, St. Louis, MO, EUA).

2.3. Métodos neurofarmacológicos

2.3.1. Teste geral da atividade farmacológica

O teste geral da atividade farmacológica foi realizado usando uma modificação do método adotado por MALONE (1983). Este teste preliminar permite observar as alterações comportamentais gerais e relatar qualquer sinal de toxicidade induzida, faixa das doses tóxicas ou sub - efetivas e a via de administração adequada. Os animais

foram tratados e observados periodicamente durante 7 dias. Os comportamentos dos animais foram relatados na ficha padrão modificada da descrita por Malone **(**Anexo 2**)** para uma avalição posterior.

2.3.2. Indução do sono por barbitúrico

A indução do sono barbitúrico foi realizado como previamente descrito por Carlini e Burgos (1979). Os animais foram tratados por via oral com veículo, DZP ou substância teste uma hora antes de injeção do pentobarbital sódico (i.p). O tempo para a perda do reflexo postural (latência do sono) e a recuperação voluntária do reflexo postural (duração do sono) foram registrados e analisados para detectar o efeito depressor ou estimulante no sistema nervoso central.

2.3.3 Teste de labirinto em cruz elevado (LCE)

O teste de labirinto em cruz elevado é um modelo comportamental amplamente utilizado para avaliar o efeito ansiolítico de um composto (PELLOW et al., 1985). O aparelho de LCE (Scientific Equipment Insight, SP, Brasil) possui dois braços abertos (30 x 5 x 0,5 cm) e dois braços fechados (30 x 5 x 15 cm) ligados por uma plataforma central (5 x 5 cm) a uma altura de 30 cm do chão. Os animais foram tratados oralmente com veículo, DZP ou substância teste. Sessenta minutos após o tratamento por via oral, os animais foram colocados individualmente no centro do labirinto em cruz com a sua cabeça voltada para o braço fechado. O teste de LCE foi gravado por 5 min. O aparelho foi limpo com álcool (10 %, v/v) após a retirada de cada animal. O tempo despendido e o número de entradas dos animais com todas as quatro patas nos braços abertos e fechados foram registrados para a análise estatística.

2.3.4 Teste de caixa claro escuro (CCE)

O teste da caixa claro escuro (CCE) utilizado neste estudo consiste de compartimento escuro (20 × 30 cm) e um compartimento iluminado (40 × 30 cm). Os dois compartimentos são delimitados por uma parede com uma abertura (4 x 4 cm) através da qual o animal pode transitar entre os dois compartimentos. Os animais foram tratados por via oral com veículo, DZP ou substância teste. Os animais experimentais foram colocados no centro da área do compartimento iluminado com sua cabeça virada para a abertura da área escura, após 1 hora de tratamento. O aparelho foi limpo com álcool (10 %, v/v) após a retirada de cada animal. O número de transições entre os dois compartimentos e o tempo despendido no compartimento escuro ou claro foram registrados por um período de 5 min (CRAWLEY; GOODWIN, 1980).

2.3.5 Teste de suspensão pela cauda (TST)

O teste de suspensão pela cauda (TST) compartilha o princípio básico com o teste de natação forçada, em que os animais desenvolvem uma postura imóvel quando colocados em uma situação estressante inescapável após os movimentos iniciais orientados para a fuga. Foi realizada versão modificada do teste de suspensão pela cauda validada por Steru et al. (1985). Os animais foram distribuídos aletoriamente em grupos experimentais diferentes. A administração aguda de um medicamento antidepressivo antes da exposição do animal ao TST, deve prolongar comportamentos direcionados à fuga ativa (CRYAN et al., 2005). Os animais foram tratados oralmente com veículo, IMI ou substância teste. O tempo de imobilidade é avaliado durante 4 minutos em camundongos suspensos pelo menos 50 cm acima do chão e presos pela

extremidade da cauda com fita adesiva (MANTOVANI et al., 2003; BINFARÉ et al., 2009).

2.3.6 Teste de natação forçada (TNF)

Os animais foram submetidos ao teste de natação forçada modificada do descrito por Porsolt et al. (1977). Os animais foram tratados com uma dose oral única (aguda) ou dose oral diária (crônica) do veículo, IMI ou substância teste. Os animais foram colocados individualmente em um recipiente cilíndrico de água (42 cm de altura, 18 cm de diâmetro) com água até 30 cm a 24 ± 2 °C. Inicialmente, o animal tenta escapar, mas acaba adotando uma postura de imobilidade, caracterizada pela falta de movimento ativo, exceto aquele que é necessário para manter o animal flutuando. Após a exposição do animal (durante 6 minutos), o recipiente foi limpo com solução de álcool (10 %, v/v).

2.3.7 Teste de campo aberto (CA)

Os animais foram tratados com veículo, DZP ou substância teste por via oral como descrito em trabalhos anteriores e após 60 minutos foram colocados num campo aberto circular [πr^2 (área de base) = 62,80 cm^2 com 50 cm de altura]. A superfície do campo aberto é dividida em oito setores iguais. Os animais foram expostos ao campo aberto e filmados durante 5 minutos. Os parâmetros, tais como, número de cruzamentos, levantamentos, autolimpezas e tempo despendido no centro foram avaliados.

2.3.8 Teste de convulsão - induzida por pentilenotetrazol

A atividade anticonvulsivante de substância teste foi avaliada usando o modelo de convulsão induzida por pentilenotetrazol. Os animais foram divididos aleatoriamente

em grupos. Após 1 hora de administração oral de veículo, DZP ou substância teste, o pentilenotetrazol (PTZ) foi administrado pela via intraperitoneal em cada animal. Os comportamentos dos animais foram filmados por 30 minutos. Parâmetros tais como a latência para a primeira mioclonia, duração e a gravidade da crise, e índice de sobrevivência (% de proteção) foram registrados. A gravidade da crise é uma medida de alterações coletivas no comportamento de animais [mioclonia, vocalização, Straub, acinesia, tremor, salto, paralisia, convulsões clônicas, rigidez e extensão tônica dos membros posteriores e morte (Anexo 3)].

2.3.9 Teste de arame

O teste de arame é um modelo pré-clínico *in vivo* para avaliar o efeito farmacológico na função motora do animal experimental. Os animais foram divididos aleatoriamente em grupos e submetidos à administração oral. Uma hora após à administração do veículo, DZP ou substância teste, colocou-se o animal pendurado em um arame (2 mm de diâmetro; 20 cm de comprimento) com suas patas dianteiras a uma altura de 20 cm acima do chão. O tempo que decorreu até primeira queda do animal (a latência) foi registrado.

2.3.10 Investigação do mecanismo da (s) ação (ões) tipo ansiolítico e/ou antidepressivo

Ferramenta farmacológica: Para elucidar o mecanismo pelos quais o substância teste induziu os efeitos tipo ansiolítico e/ou antidepressivo, foram usados os animais pré-tratados (i.p) com antagonistas ou inibidores farmacológicos (*p*-clorofenilalanina metil éster, PCPA - depletor de serotonina; α-metil-*p*-tirosina, AMPT - depletor de catecolaminas; prazosina, PRAZ - antagonista do receptor α1 adrenérgico; ioimbina,

YOH - antagonista do receptor α2 adrenérgico, pentilenetetrazol, PTZ - antagonista competitiva do receptor $GABA_A$); NAN-190, antagonista farmacológico não seletivo do receptor 5-HT1A; WAY - antagonista farmacológico do receptor 5-HT1A) antes da administração oral da substância teste. O intervalo entre o pré-tratamento e tratamento ou duração de pré-tratamento ou tratamento foram baseados nos estudos preliminares ou na literatura. Uma hora após o tratamento, os animais foram submetidos ao labirinto em cruz elevado ou teste de caixa claro escuro como foi descrito nos itens 2.3.3 ou 2.3.4, respectivamente, para elucidar o mecanismo do efeito tipo ansiolítico ou teste de suspensão pela cauda ou teste de natação forçada como foi descrito nos itens 2.3.5 ou 2.3.6, respectivamente, para elucidar o mecanismo do efeito tipo antidepressivo***.***

Determinação da atividade da monoamina oxidase (MAO) pelo método espectrofotométrico: Para realizar o ensaio *ex vivo*, grupos de animais foram submetidos a um tratamento agudo do substância teste por via oral. Os animais foram sacrificados por decapitação após 1 h. Para o ensaio *in vitro,* tecidos cerebrais dos animais sem tratamento prévio foram retirados e homogeneizados em tampão de fosfato de potássio 1:20 (w/v) usando homogeneizador mecânico (Turrax). O homogeneizado foi centrifugado a 1200 x g e 4 ^{o}C por 7 min. Este procedimento foi repetido com o sobrenadante a 12500 x g e 4 °C durante 15 min. O pellet resultante foi suspenso em 1,5 mL de tampão de homogeneização e centrifugado a 12500 x g e 4 °C durante 15 min, Em seguido, o pellet resultante foi ressuspenso em 1,0 mL de tampão de homogeneização, armazenado a -20 °C em alíquotas e usado como a fonte da MAO. A concentração de proteína total foi estimada pelo método de Bradford (BRADFORD, 1976). A medição da atividade da MAO *in vitro* e *ex vivo* foram

realizados utilizando um método espectrofotométrico modificado (HOLT et al., 1997; STAFFORD et al., 2007).

Quantificação do fator neurotrófico derivado do cérebro (BDNF): Os animais receberam a dose oral única (administração aguda) ou diária durante 14 dias (administração crônica) da substância teste, fluoxetina ou veículo. Após o sacrifício dos animais, os hipocampos foram recolhidos. Tecidos de hipocampo foram homogeneizados em tampão de lise (NaCl 1 mM; EDTA 4 mM, Tris - HCl 100 mM; albumina a 2%, Triton X-100 a 2 %, timerosal 0,01 %, pH 7,0, glicerol a 10 %, cocktail inibidor de protease - GE) em proporção 1:40 w/v. Após a centrifugação (16800 x g, a 4 °C, 35 min), o sobrenadante foi armazenado a -80 °C. O nível do BDNF hippocampal foi medido por kit de ELISA (kit de imunoensaio Sistema BDNF Emax®, Promega, Madison, WI, EUA) de acordo com as instruções do fabricante. A normalização do nível de proteína total das amostras foi feita, através do método de Bradford (1976), usando, albumina sérica bovina como padrão.

2.3.11 A análise estatística

De acordo com as hipóteses experimentais, análise de variância (ANOVA) de uma via foi utilizada para detectar o efeito do tratamento (variável independente) sobre o comportamento do animal (variável dependente) e seguido por Dunnett como pós - teste para comparar grupo tratado com veículo e os grupos tratados com fármacos de referências ou substância teste. A análise de variância (ANOVA) de duas vias, conforme detalhado por NETER et al. (1990) foi utilizada para detectar o efeito de período e/ou tratamento (variáveis independentes) ou pré-tratamento/tratamento (variáveis independentes) sobre o tempo de imobilidade (variável dependente). O teste

de Bonferroni foi utilizado como pós - teste para realizar as comparações múltiplas. Os dados não-paramétricos foram analisados com teste de Kruskal–Wallis seguido por teste de Dunns. Os dados experimentais foram expressos como média do grupo ± EPM usando o programa GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, EUA). Os resultados foram considerados significativos para $p < 0,05$.

3. Síntese dos resultados

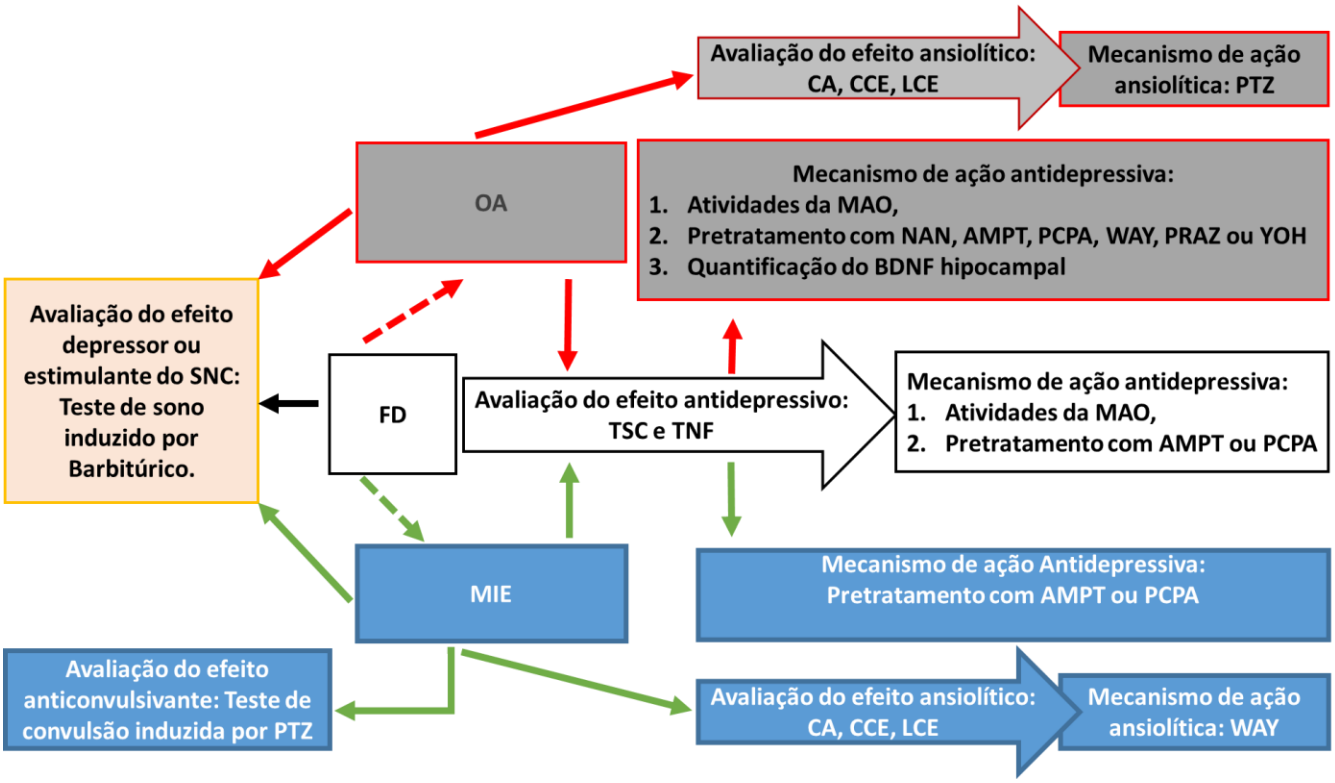


Fig. 2 Fluxograma das avaliações biológicas e mecanismos de acão ansiolítica e/ou antidepressiva da fração diclorometano (FD) e seus isolados [acido oleanolicos (AO) e (*E*)-metilisoeugenol (MIE)]. A seta traçada indica isolamento do AO (cor vermelha) ou MIE (cor verde) a partir da FD – uma fração orgânica obtida através da partição líquido/líquido do extrato etanólico bruto das folhas de *Pimenta pseudocaryophyllus* quimiotipo (*E*)-metilisoeugenol.

3.1 Propriedade tipo antidepressiva da fração diclorometano

Estudo fitoquimico

O rendimento do extrato etanólico bruto das folhas de *Pimenta pseudocaryophyllus* quimiotipo (*E*)-metilisoeugenol foi 28,45%. Os rendimentos das frações obtidas por partição líquido/líquido do extrato etanólico bruto com solventes de polaridade crescentes foram 8,16%, 13,20%, 21,34% e 32,28% para a fração hexânica (FH), diclorometano (FD), acetato de etila (FAE) e aquosa (FAQ), respectivamente. As Figuras 1a e b do Anexo 4 mostram os cromatogramas da FD e AO, respectivamente. A estimativa da composição relativa do AO na FD foi 7,82 %.

Teste de natação forçada (TNF) e suspensão pela cauda (TSC)

Nos testes de suspensão pela cauda e natação forçada (Figuras 2a e b do Anexo 4, respectivamente), o tratamento oral com imipramina 30 mg/kg, ou com a FD 125 ou 250 mg/kg, reduziu o tempo de imobilidade.

Teste de campo aberto

As Figuras 3a e b do Anexo 4 mostram uma redução nos números de cruzamentos e levantamentos no campo aberto, respectivamente, pelos tratamentos com FD 500 mg/kg e diazepam DZP 5 mg/kg. A redução desses parâmetros (cruzamentos e levantamentos) pelo tratamento com FD 500 mg/kg ou DZP 5 mg/kg sugere alteração nas atividades exploratorias.

Investigação do mecanismo de ação da propriedade tipo antidepressiva

Os pré-tratamentos com PCPA ou AMPT bloquearam o efeito tipo antidepressivo da FD (Figura 4a e b do Anexo 4). Entretanto, a FD não alterou a atividade da monoamina oxidase (MAO).

3.2 Pluralidade dos mecanismos de alteração da ansiedade e depressão pelo AO

Teste de sono induzido

Neste estudo, o tratamento com AO (5-20 mg/kg) não provocou alteração na latência do sono (o tempo que leva os animais para perder o reflexo postural) (Fig. 1a do Anexo 5). No entanto, a análise estatística da duração do sono mostrou um efeito significativo do AO (Fig. 1b do Anexo 5).

Teste CCE e LCE

Os resultados ilustrados na Figura 2a e Figura 2b do Anexo 5 demonstraram alterações significativas no número de transições e tempo despendido no compartimento claro da CCE, respectivamente. O tempo despendido no compartimento claro foi aumentado pelo tratamento com AO 10 ou 20 mg/kg. AO 40 mg/kg reduziu o número de transições. No LCE, o AO provocou alteração significativa do tempo despendido e do número de entradas nos braços abertos (Fig. 2c e Fig. 2d do Anexo 5, respectivamente). O teste de Dunnett revelou que AO aumentou o tempo despendido nos braços abertos de LCE nas doses de 20 e 40 mg/kg. O porcentagem de entradas nos braços abertos (Fig. 2d do Anexo 5) não foram alterados até a dose de 20 mg/kg do AO.

TNF e TSC

A administração oral do AO (5 - 20 mg/kg) elicitou efeito significativo no tempo de imobilidade do animal no TNF (Fig. 3a do Anexo 5). O tratamento crônico com AO não alterou o efeito antidepressivo que foi visto com o tratamento agudo na mesma dose (Fig. 3b do Anexo 5). No teste de suspensão pela cauda, ANOVA de uma via revelou efeito significativo da AO na redução tempo de imobilidade (Fig. 3c do Anexo 5)

Teste de campo aberto

A Figura 4a do Anexo 5 demonstrou os efeitos do AO no número de cruzamentos e levantamentos no campo aberto. O teste de Dunnett revelou que os tratamentos com AO 20 mg/kg ou DZP 1 mg/kg não alteraram o número de cruzamentos. Nas doses de 20 e 40 mg/kg, AO induziu uma redução no número de levantamentos (Fig. 4b do Anexo 5).

Investigação do mecanismo de ação do efeito tipo ansiolítica

As Figuras 5a e b do Anexo 5 demonstraram o efeito do pré-tratamento (SAL ou PTZ - variável independente) e tratamento (veículo, AO 20 mg/kg, DZP 1 mg/kg - variável independente) no tempo despendido no compartimento claro e no número de transições (variáveis dependenteS) na CCE. Como foi mostrado na figura 5b do Anexo 5, ANOVA de duas vias seguido por teste de Bonferroni mostrou aumento no tempo despendido no compartimento claro nos grupos tratados com SAL + AO e SAL + DZP em comparação com o grupo que recebeu SAL + Veículo. Na figura 5a do Anexo 5, ANOVA de duas vias seguido por teste de Bonferroni não apresentou alteração no número de transições no grupo tratado com AO (SAL + AO vs SAL + Veículo, $p>0,05$). O diazepam elicitou um aumento no número de transições (SAL + DZP vs SAL + veículo, $p<0,05$). O efeito de DZP no número de transições foi atenuado com o pré-tratamento de PTZ (SAL + DZP vs PTZ + DZP, $p<0,05$).

Investigação do mecanismo de ação da propriedade tipo antidepressiva

Os efeitos dos pré-tratamentos com NAN-190 0,5 mg/kg, PCPA 100 mg/kg ou AMPT 100 mg/kg no comportamento dos animais no TNF foram mostrados na Figura 6 do Anexo 5. ANOVA de duas vias seguida pelo teste de Bonferroni mostrou uma redução no tempo de imobilidade no grupo tratado com AO (SAL + AO vs SAL + Veículo, $p<0,01$). No primeiro experimento (EXP 1), o efeito anti-imobilidade do AO foi bloqueado por NAN-190 [SAL + AO vs NAN-190 + AO, ($p<0,05$) e SAL + Veículo vs NAN-190 + OA ($p<0,05$)]. No segundo experimento (EXP 2), o efeito do AO foi atenuado pelo pré-tratamento com PCPA (SAL + Veículo vs PCPA + AO, $p>0,05$). No terceiro experimento (EXP 3), o pré-tratamento com AMPT atenuou o efeito do AO [SAL + AO vs AMPT + AO ($p<0,05$) e SAL + Veículo vs AMPT + AO ($p<0,05$)].

Efeitos dos pré-tratamentos com WAY, prazosina ou ioimbina

A Figura 7 do Anexo 5 mostrou o efeito do pré-tratamento (salina, prazosin ou ioimbina, variável independente) e tratamento (veículo ou AO 20 mg/kg - variável independente) no tempo de imobilidade (variável dependente) no TNF. O pré-tratamento com ioimbina não atenuou o efeito anti-imobilidade do AO [SAL + AO vs YOH + AO ($p>0,05$), ANOVA de duas vias seguido por teste de Bonferroni]. O efeito do AO foi atenuado por WAY [SAL + Veículo vs WAY + AO ($p>0,05$), ANOVA de duas vias seguido por teste de Bonferroni] e PRAZ [SAL + Veículo contra PRAZ + AO ($p>0,05$), ANOVA de duas vias seguido por teste de Bonferroni].

Quantificação do BDNF hipocampal

A Figura 8 do Anexo 5 demonstrou o efeito do período de tratamento (administração aguda ou crônica - variável independente) e tratamentos (Veículo, AO 20 mg/kg ou

Fluoxetina 20 mg/kg - variável independente) no nível de BDNF hipocampal (variável dependente). O tratamento crônico com AO ou fluoxetina aumentou o nível de BDNF no hipocampo (nível de significância para AO, $p<0,001$ ou fluoxetina, $p<0,05$, Fig. 8 do Anexo 5).

Atividades da monoamina oxidase (MAO)

O ensaio *in vitro* da atividade da MAO mostrou que a AO não alterou a atividade catabólica desta enzima (Fig. 9a do Anexo 5). Uma queda acentuada na atividade da enzima à concentração mais elevada de 1 mM de AO pode ser uma precipitação de proteína. A Figura 9b apresenta o gráfico de barras do ensaio *ex vivo* de MAO. Neste estudo, a atividade da MAO não foi alterada pelo AO (teste de Dunnett como teste post hoc, $p>0,05$).

3.3 Efeito tipo ansiolítico e antidepressivo do (*E*)-metilisoeugenol (MIE)

Teste geral da atividade farmacológica

No teste geral da atividade farmacológica, os efeitos induzidos (contorção abdominal, alienação ambiental, ataxia, sedação, analgesia, perda de preensão da pata, aumento ou diminuição na atividade exploratória) foram dependente da dose, tempo após a administração e via de administração (sc, ip ou po). Contudo, todas as manifestações comportamentais desapareceram após 4 horas de observação (Tabela 1 do Anexo 6).

Teste de sono induzido por pentobarbital sódico

A administração de diazepam 1 mg/kg ou MIE 500 mg/kg provocou uma diminuição na latência do sono ($p<0,001$, ANOVA de uma via seguido por teste de Dunnett, Fig. 1a do Anexo 6]. A Figura 1b revelou um aumento significativo (ANOVA de uma via seguido por teste de Dunnett) na duração do sono pelo tratamento oral do MIE 250 mg/kg ($p<0,05$) e MIE 500 mg/kg ($p <0,001$).

Teste de convulsão induzida por pentilenetetrazol (PTZ)

Na Figura 2a do Anexo 6, a ANOVA de uma via seguida por teste de Dunnett não mostrou alteração significativa na latência para a convulsão mioclónica no grupo tratado com MIE 125, 250 ou 500 mg/kg ($p>0,05$). A duração da convulsão não foi alterada significativamente pelos tratamentos com MIE ($p>0,05$, Fig. 2b do Anexo 6). A gravidade da convulsão [Fig. 2c do Anexo 6] induzida por PTZ não foi significativamente influenciada pela administração do MIE. Além disso, a porcentagem de animal protegido pela administração do MIE nas doses 125, 250 ou 500 mg/kg foi

diminuída (60, 40 e 30%, respectivamente) de forma dose dependente (Fig. 2d do Anexo 6).

Teste de caixa claro escuro

O tratamento com MIE nas diferentes doses aumentou o número de transições [F (4, 35) = 6,67, p<0,001, ANOVA de uma via seguido por teste de Dunnett, Fig. 3a do Anexo 6] e o tempo despendido no compartimento claro [F (4, 35) = 6,19, p<0,001, ANOVA de uma via seguido por teste de Dunnett, Fig. 3b do Anexo 6].

Teste de labirinto em cruz elevado

MIE 500 mg/kg reduziu número total de entradas nos braços abertos e fechados [p<0,05, F (4, 45) = 3,86, ANOVA de uma via seguido por teste de Dunnett, Fig. 4a do Anexo 6]. O número de entradas nos braços abertos foi aumentado por MIE 250 mg/kg [F (4, 45) = 3,48, p<0,05, ANOVA de uma via seguido por teste de Dunnett, Fig. 4b do Anexo 6]. Além disso, o tempo despendido nos braços abertos foi aumentado por MIE 125 mg/kg (p<0,05) e MIE 250 mg/kg (p<0,01).

Teste de arame

Administração oral do MIE não induziu alterações significativas nos valores de latência para a queda do animal do arame [p>0,05, teste de Kruskal–Wallis seguido por Dunns, Fig. 5 do Anexo 6).

Teste de Campo aberto

Os parâmetros avaliados no campo aberto foram significativamente alterados pelos tratamentos com MIE ou diazepam; número de cruzamento total no campo aberto [F (4, 45) = 8,07, p<0,001, ANOVA de uma via, Fig. 6a do Anexo 6], tempo de imobilidade [F (4, 45) = 5,14, p<0,01, ANOVA de uma via, Fig. 6b do Anexo 6],

atividade de autolimpeza [F (4, 45) = 3,17, p<0,05, ANOVA de uma via, Fig. 6c do Anexo 6], o número de levantamentos [F (4, 45) = 4,37, p<0,05, ANOVA de uma via, Fig. 6d do Anexo 6], tempo despendido no centro do campo aberto [F (4, 45) = 4,18, p<0,01, ANOVA de uma via, Fig. 6e do Anexo 6], e cruzamento no centro do campo aberto [F (4, 45) = 4,81, p<0,01, ANOVA de uma via, Fig. 6f do Anexo 6]. O teste de Dunnett mostrou que MIE 500 mg/kg reduziu cruzamento total (p<0,05) e o número de autolimpeza (p<0,01), enquanto que o tempo de imobilidade foi aumentada (p<0,01); MIE 250 mg/kg reduziu o número de autolimpeza (p<0,05). MIE 125 ou 250 mg/kg aumentou o número de cruzamentos no centro do campo aberto (p<0,05). O tempo despendido no centro do campo aberto foi aumentado por MIE 250 mg/kg (p<0,05).

Teste de natação forçada

A administração do MIE 250 mg/kg reduziu o tempo de imobilidade (p<0,05, ANOVA de uma via seguido por teste de Dunnett, Fig. 7 do Anexo 6).

Mecanismo de ação da propriedade tipo ansiolítica

Na Figura 8a do Anexo 6, MIE demostrou aumento no tempo despendido no compartimento claro (SAL + MIE vs SAL + Veículo, ANOVA de duas vias seguido por teste de Bonferonni, p<0,05). O efeito do MIE foi bloqueado por pré-tratamento com WAY (SAL + MIE vs WAY + MIE, ANOVA de duas vias seguido por teste de Bonferonni, p<0,05). Na figura 8b do Anexo 6, MIE aumentou a porcentagem de entradas nos braços abertos (SAL + MIE, ANOVA de duas vias seguido por teste de Bonferonni, p<0,05).

Mecanismo de ação da propriedade tipo antidepressiva

A Figura 9a do Anexo 6 mostrou o efeito do pré-tratamento (SAL ou AMPT - variável independente) e tratamento (veículo ou MIE 250 mg/kg - variável independente) no tempo de imobilidade (variáveis dependentes) no TNF. O tratamento com MIE (SAL + MIE) diminuiu o tempo de imobilidade (ANOVA de duas vias seguido por teste de Bonferonni, $p<0,05$). O pré-tratamento com AMPT não reverteu o efeito anti-imobilidade da MIE (ie SAL + MIE contra AMPT + MIE, ANOVA de duas vias seguido por teste de Bonferonni, $p>0,05$). A Figura 9b do Anexo 6 mostrou o bloqueou do efeito do MIE por pré-tratamento com PCPA.

4. Discussão e conclusão geral

O presente trabalho investigou os efeitos psicoativos da fração diclorometano do extrato etanólico das folhas de *P. pseudocaryophyllus* (FD) e de seus constituentes - ácido oleanólico (AO) e (*E*)-metilisoeugenol (MIE). Trabalhos anteriores realizados por nosso grupo de pequisa demonstraram a atividade tipo ansiolítica da FD (FAJEMIROYE et al., 2012), sendo que presente investigação buscou-se observar a atividade tipo antidepressiva desta e os mecanismos neurais envolvidos.

A proposta de elucidação do mecanismo neural é desafiadora baseada nas complexidades de alterações biológicas que ocorrem tanto na depressão como na ansiedade. Mais ainda são as dificuldades na busca dos mecanismos dos efeitos ansiolíticos e/ou antidepressivos de um extrato orgânico, com inúmeros fitoconstituintes ou mesmo um unico composto com interações múltiplas no SNC. Os métodos farmacológicos que envolvem o bloqueio do (s) receptor (es) por antagonistas, a inibição do transportador ou da recaptação das monoaminas, inibição das enzimas metabólicas, o bloqueio de canal iônicos, lesão, depleção de monoaminas (indolamina e catecolamina), quantificação de fatores neurotróficos entre outros permitem a elucidação de alguns possíveis mecanismos de ação de substância teste com propriedade ansiolítico e/ou antidepressivo (s).

A atividade da monoamina-oxidase é considerado importante em depressão, uma vez que regula o nível das principais monoaminas (serotonina, norepinefrina e dopamina) no cérebro (MEYER et al., 2006). O pré-tratamento de animais com *p*-clorofenilalanina (PCPA - depletor de serotonina), α - metil - *p* - tirosina (AMPT - depletor de catecolamina de) e o registro da atividade da MAO podem ser usadas para

coorelatar o nivel de monoamina disponível ás alterações comportamentais induzidas pela substância teste. Além disso, a transmissão monoaminérgica poderia ser aumentada pela substância teste, por meio da ativação dos receptores monoaminérgicos (serotoninérgicos, adrenérgicos, e dopaminérgicos). Assim, o pretratamento com NAN-190 (antagonista competitivo não seletivo do receptor 5-HT1A), Prazosina (PRAZ - antagonista do receptor α1 adrenérgico), WAY100635 (WAY - antagonista competitivo do receptor 5-HT1A), Ioimbina (YOH - antagonista do receptor α2 adrenérgico), Pentylenetetrazol (PTZ - antagonista competitiva do receptor $GABA_A$), flumazenil (antagonista competitiva do sitio da ligação dos benzodiazepínicos) foram utilizado neste estudo. Além da hipótese de monoaminas na depressão, a diminuição do volume hipocampal ou de outra região cérebral em alguns pacientes deprimidos tem levado o surgimento da hipótese neurotrófica nesta patologia. Apesar de estudos pré-clínicos que demonstraram o efeito antidepressivo da infusão hipocampal do fator neurotrófico derivado do cérebro (BDNF) (SHIRAYAMA et al., 2002), existem resultados conflitantes que mostram efeito pró-depressivo desta proteína em outra região cerebral (KRISHNAN et al., 2007; EISCH et al., 2003). Desta forma, a quantificação dos fatores neurotróficos como BDNF é uma avaliação útil para elucidar os efeitos da substância teste. É importante ressaltar que os alvos neurais para a substância teste como ansiolítica e/ou antidepressiva (s) são mais do que os que foram abordados neste estudo.

A investigação do efeito tipo antidepressivo da FD é apoiado pela hipótese de que o efeito ansiolítico da FD poderia estar relacionado com mecanismos serotoninérgicos. Os dados experimentais dos testes da suspensão pela cauda e de

natação forçada mostraram efeito tipo antidepressivo da FD (FAJEMIROYE et al., 2013). O pré-tratamento dos animais com α - metil - *p* - tirosina e *p*-clorofenilalanina antes da administração oral da FD bloqueou o seu efeito tipo antidepressivo. A atividade metabólica da MAO (monoamina oxidase) permaneceu inalterada nos animais tratados com a FD. A presença de ácido oleanólico (8 % de concentração relativa) entre outros constituintes, foi demonstrada na FD (FAJEMIROYE et al., 2013).

Já que a FD possui atividade tipo ansiolítica e antidepressiva, decidimos investigar os efeitos com os compostos identificados nesta fração. A dose equivalente do AO foi extrapolada, a fim de investigar os seus efeitos ansiolítico e antidepressivo, com base na sua concentração relativa na FD (FAJEMIROYE et al., 2013). A FD foi administrada oralmente na dose efetiva de 250 mg/kg. Assim, considerou-se a dose de 20 mg/kg (8 % de 250 mg/kg) como dose equivalente de AO que pudesse causar as atividades neurofarmacológicas da FD. Neste estudo, optamos por trabalhar com doses inferiores, intermediárias e superiores de AO. A administração oral do AO induziu efeito tipo ansiolítico na CCE e LCE. Para investigar o efeito tipo antidepressivo do AO, os camundongos foram submetidos ao teste de TNF e TSC. A administração oral (crônica ou aguda) do AO mostrou a evidência do seu efeito antidepressivo tanto no TNF como no TSC. Numa tentativa de extrapolar a dose do AO que pode ser administrada na pratica clínica, foi usada a fórmula descrita na seção 2.2; a dose equivalente do camundongo (peso médio dos camundongos neste estudo - 20 g ou 0,02 kg, área de superfície corporal - 0.007 m^2) a ser administrada no homem normal (peso médio de homem normal - 60 kg, área de superfície corporal - 1,6 m^2) espera-se que seja 12,3 vezes menor do que o do camundongo. Se a dose a ser administrada no homem for

baseada na dose do AO que elicitou efeito de pico (20 mg/kg), o homem receberá uma dose hipotética de 1.6 mg/kg. O pré-tratamento com PTZ não bloqueou o efeito tipo ansiolítico do AO. O efeito tipo antidepressivo deste triterpeno foi atenuado pelo pré-tratamento com PCPA, AMPT, NAN-190, prazosina ou WAY100635. Os dados de ensaios *in vitro* e *ex vivo* da MAO mostraram que a AO não alterou a atividade desta enzima. A administração crônica de AO produziu um aumento no nível de fator BDNF hipocampal sem qualquer melhoria significativa sobre o efeito antidepressivo em relação ao tratamento agudo que não alterou o nível de BDNF.

Para dar continuidade à investigação da atividade biológica dos fitoconstituintes da FD, foram avaliadas as alterações comportamentais induzidas por MIE (um flavorizante natural). Os efeitos do MIE no teste geral da atividade farmacológica mostraram-se dependente da dose e da via de administração. Os relatos na literatura têm mostrado a atividade tipo anticonvulsivante do eugenol, metileugenol, isoeugenol, estragol e safrol (DALLMEIER; CARLINI, 1981). Em contraste, este derivado de fenilpropanóide (MIE) estudado não protegeu os animais contra a convulsão induzida por PTZ. Baseado no conhecimento do mecanismo de ação de PTZ, o efeito do MIE no SNC não envolveu o receptor $GABA_A$. No CCE e LCE, foi demonstrada a propriedade tipo ansiolítico do MIE neste estudo. Como a administração do MIE não protegeu os animais contra a convulsão induzida por PTZ, assume-se que a atividade biológica do MIE não pode ser atribuída ao mecanismo envolvendo o receptor $GABA_A$. O pré-tratamento dos animais com WAY100635 bloqueou o efeito tipo ansiolítico do MIE no LCE. No teste da natação forçada, o MIE reduziu o tempo de imobilidade. Este efeito demonstrou a propriedade tipo antidepressiva do MIE. O pré-tratamento dos

camundongos com *p*-clorofenilalanina atenuou o efeito tipo antidepressivo do MIE no TNF enquanto o pré-tratamento com α - metil - *p* - tirosina não alterou este efeito.

Os efeitos neurofarmacológicos da FD, AO ou MIE em camundongos foi consistente em alguns modelos utilizados. Um aumento na dose desta fração ou composto testado, geralmente, induz um efeito sedativo. Este efeito sedativo na dose elevada de FD talvez possa ser atribuído a um aumento da composição relativa do AO ou MIE. O fenômeno da resposta na forma de U (*U-shaped response pattern*) foi observado consistentemente após a administração da FD, AO ou MIE nos testes de natação forçada. Pela primeira vez, foi introduzido o "*model - dose induced – phenomenon*" (MDIP). A hipótese de MDIP poderia explicar não só a dose e a resposta na forma de U, mas também a perda de efeito na dose que induziu as alterações comportamentais em modelo diferente. Os resultados obtidos nestes experimentos - A) o pré-tratamento (i.p) com flumazenil antes do tratamento oral com a FD, - B) o pré-tratamento (ip) com PTZ (na dose não convulsiva e não ansiogênica) antes do tratamento oral com AO, e - C) a falha de MIE em proteger os animais contra a convulsão induzida por PTZ sugerem que o receptor $GABA_A$ não está envolvido no efeito tipo ansiolítico da FD. O efeito tipo antidepressivo da FD, AO e MIE, implica, consistentemente, a participação de monoaminas. Os nossos resultados mostraram evidências de efeito (s) tipo ansiolítico e/ou antidepressivo da FD, AO e MIE; e sugeriram mecanismos envolvendo as vias monoaminergicas.

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Anexos

Anexo 1. Parecer consubstanciado do Comitê de Ética em Pesquisa, Universidade Federal de Goiás (CEP/UFG)

SERVIÇO PÚBLICO FEDERAL

UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

UFG

PROTOCOLO 104/2008

Goiânia, 05 de dezembro de 2008

PARECER CONSUBSTANCIADO

I. IDENTIFICAÇÃO:

Título do projeto: "Fitoquímica a farmacologia de plantas medicinais do cerrado: Capacitação pessoal e busca do conhecimento visando a produção de fitofármacos"

Pesquisador Responsável: Elson Alves Costa

Pesquisador Participante: José Realino de Paula
Maria Tereza Freitas Bara

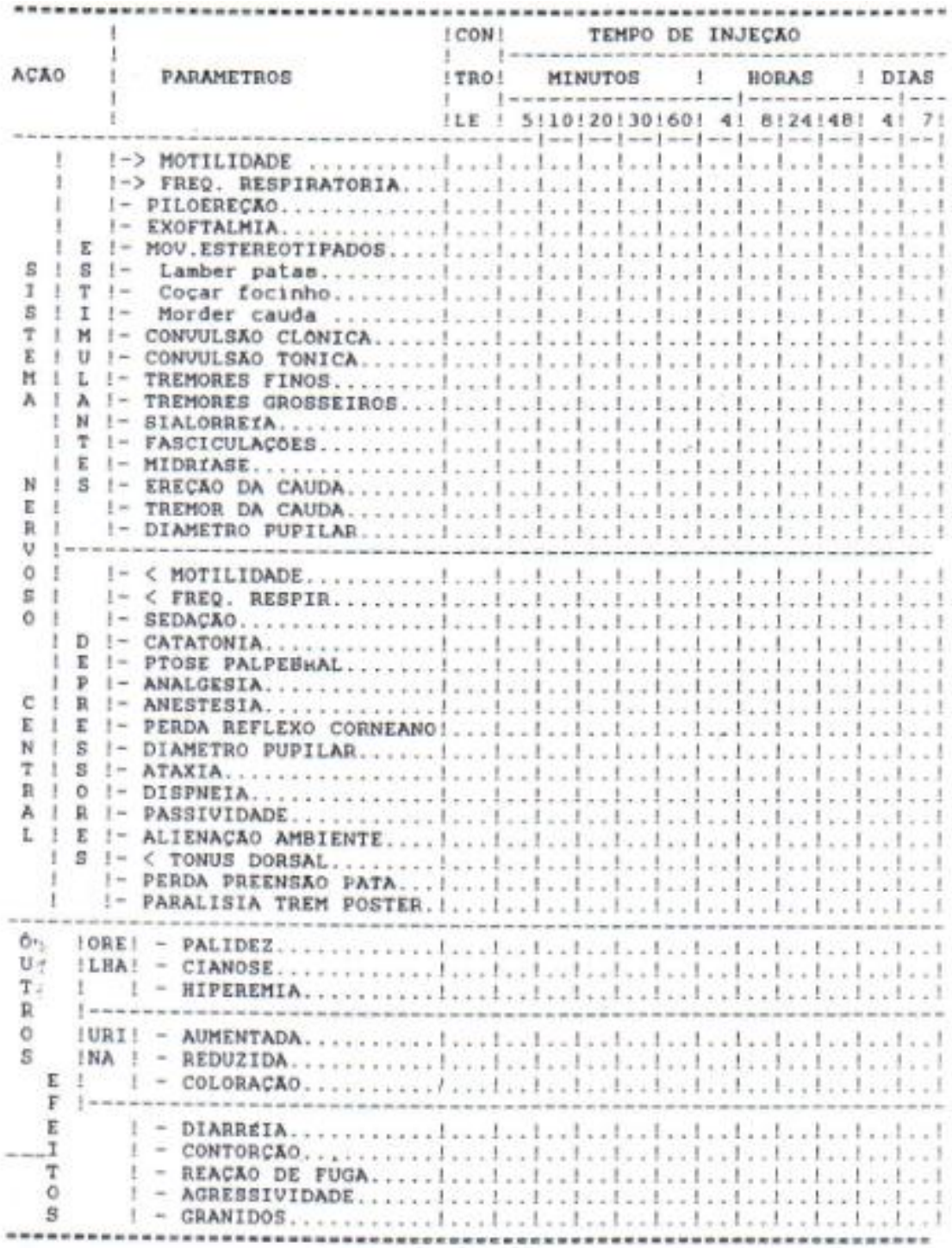
Local de realização: "DCiF/ICB"
Área Temática:

Informamos que o Comitê de Ética em Pesquisa da Universidade Federal de Goiás, após análise das adequações solicitadas, **Aprovou,** o projeto acima referido, e o mesmo foi considerado em acordo com os princípios éticos vigentes.

O pesquisador responsável deverá encaminhar ao CEP/UFG, relatórios da pesquisa, encerramento, conclusão (ões) e publicação (ões) de acordo com as recomendações da Resolução 196/96.

Profa Dra Rita Goreti Amaral
Coordenadora do CEP/UFG

Anexo 2. Ficha para o relatório do teste geral da atividade farmacológica



Anexo 3. Ficha para a pontuação das alterações comportamentais induzidas por pentilenetetrazol

	Parâmetros	**Pontuação**
1	Ausência de comportamento convulsivo	0 1 2 3 4 5 6 7 8
2	Mioclonia	
3	Vocalização	
4	*Straub*	
5	Akinesia	
6	Tremor e salto	
7	Paralisia dos membros posteriores	
8	Convulsões clônicas com perda do reflexo postural	
9	Rigidez/extensão tônica dos membros posteriores com a morte	
Outros parâmetros		
10	Latência ao primeiro mioclonia	Segundos
11	Duração da crise	Segundos
12	Sobrevivencia ou porcentagem dos animals protegidos	[(N - nd) /N] X 100

N - Número total do animal; nd - Número da morte relatada.

Anexo 4. Antidepressive - like property of dichloromethane fraction of *Pimenta pseudocaryophyllus* and relevance of monoamine metabolic enzymes

Abstract

Pimenta pseudocaryophyllus popularly referred to as craveiro is considered not only as a nerve tonic but also calming agent in different local preparations. Present study attempted to examine antidepressant like effect of dichloromethane fraction (DF), role of monoamine oxidase (MAO), tryptophan and tyrosine hydroxylase. Based on the research focus, tail suspension (TS), forced swimming (FS) and open field (OF) tests were conducted after oral administration of DF (125, 250 or 500 mg/Kg). Ex vivo assay of MAO was also conducted to evaluate inhibitory effect of DF (250 mg/Kg). Administration of DF elicits antidepressant - like behavioural response in both the TS and FS. However, DF 500 mg/Kg (highest dose) did not alter mice performance in these models. The data obtained in the open field showed a reduction in total crossing and rearing activity, these effects suggest motor incoordination and interference in TS and FS performance with this dose. Mice pretreatment with p-chlorophenylalanine methyl ester – PCPA (100 mg/kg, i.p - serotonin biosynthesis inhibitor) for four consecutive days or acute administration of α-methyl-p-tyrosine – AMPT (100 mg/kg, i.p. - catecholamine synthesis inhibitor) blocked anti-immobility effect of DF in the FS. In ex vivo assay of MAO, DF did not inhibit catabolic activity of MAO. Summarily, our findings support antidepressant-like activity of DF and suggests an effect that depends on monoamine biosynthesis.

Keywords: *Pimenta pseudocaryophyllus*; antidepressant–like effect; monoamine oxidase; tryptophan hydroxylase; tyrosine hydroxylase; dichloromethane fraction.

Introduction

Pimenta pseudocaryophyllus (Gomes) L.R. of Myrtaceae family is popularly known as craveiro-do-mato", "craveiro", "louro-cravo", "cataia", "chá-de-bugre", and "louro", "pau-cravo" [1, 2]. The genus *Pimenta* consists of approximately 15 known species of which only *Pimenta pseudocaryophyllus* occurs in brazillian flora. Popular applications (tranquilizer, nerve tonic, cold relief, diuretic, aphrodisiac, digestive and menstrual problems) of its leaf extract in different preparation have being reported especially in the county of Campos do Jordão, São Paulo-Brazil [1-6].

Previous neuropharmacological screening of essential oil, ethanolic leaf extract and active fractions of the *Pimenta pseudocaryophyllus* leaf demonstrated behavioural alterations in the open field, elevated plus maze, light dark box and barbiturate sleep induction tests without any form of motor incoordination [7-9]. Evaluation of the antidepressive like property of dichloromethane fraction (DF) of *Pimenta pseudocaryophyllus* is borne out of its anxiolytic property that has been associated with monoaminergic receptor [8].

The hypothesis of biogenic amine involvement in depression has produced several generations of antidepressant agents (monoamine oxidase inhibitors - MAOIs, Tricyclic antidepressants – TCAs, selective serotonin reuptake inhibitors - SSRIs, selective noradrenaline reuptake inhibitor - SNRI, atypical antidepressants among others). The fact that clinical responses to drug effects take weeks of sustained treatment [10] and occurrence of plethora of side effects make discovery of new compounds inevitable. Meanwhile, considering the fact that conventional antidepressant and natural product have demonstrated efficacy in the clinical treatment and

experimental model of anxiety [11-20], anxiolytic like property of dichloromethane fraction obtained from ethanolic leaf extract of *Pimenta pseudocaryophyllus* makes evaluation of its antidepressant effect imperative.

Thus, we hypothesize that the efficacy of aforementioned antidepressive drugs or natural products to treat anxiety or vice versa is a function of the active principles in these compounds and underlining mechanism of their neuropharmacological action. In this manner, the aim of this study was to investigate antidepressant-like property of DF on mice and involvement of metabolic enzymes (Tryptophan hydroxylase - TrOH, Tyrosine hydroxylase - TOH and Monoamine oxidase - MAO).

2. Material and methods

2.1. Preparation of DF and phytoconstituent analysis by HPLC

The leaf collection, identification, voucher specimen (herbarium) deposit, extraction, partitioning of ethanolic leaf extract to obtain DF as well as qualitative and quantitative analysis of this fraction by HPLC was achieved following the procedure described in our previous work [8].

2.2. Animals

Male albino Swiss mice (30 ± 5 g) were provided by Central Animal House of Federal University of Goiás (UFG). They were housed under controlled environmental conditions (22 ± 3 ^{o}C, 12 h light/dark cycle) and allowed free access to standard food and water. All experimental procedures were conducted with strict adherence to the regulations of ethical principles in animal research as adopted by the Brazilian society of

laboratory animal science. The experimental protocol was approved by research ethic council of the Federal University of Goiás (No. 104/08).

2.3. Drugs and administration

p-chlorophenylalanine methyl ester (PCPA – 100 mg/kg) and α-methyl-p-tyrosine (AMPT– 100 mg/kg) used to deplete monoamine (indolamine and catecholamine, respectively) were administered intraperitoneally to groups of mice; Imipramine (IMI - 30 mg/kg), Diazepam (DZP - 5 mg/kg, Cristália - Brazil), Clorgyline 15 mg/kg (an irreversible and selective inhibitor of monoamine oxidase A), Tranylcypromine 15 mg/kg (a non-selective and irreversible inhibitor of monoamine oxidase) used as standard drug were dissolved in 0.9% saline, dichloromethane fraction (DF – 125 to 500 mg/kg) was dissolved in vehicle (2% Polyoxyethylenesorbitan monooleate in 0.9% saline) while control animals received appropriate equivalent vehicle. Oleanolic acid (Sigma – EUA) was used as a standard for HPLC analysis of DF. All solutions were freshly prepared on test days and administered (10 mL/kg of mice body weight).

2.4. Pharmacological Procedure

2.4.1. Tail suspension test (TS)

According to the method described by Steru and his collaborators [21], duration of immobility following DF treatment was measured over 6 min of TS. In this model, mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm

from the tip of the tail. Mice were treated orally with DF (125, 250 or 500 mg/kg), IMI 30 mg/kg or vehicle prior to TS.

2.4.2. Forced swimming test (FS)

Assessment of antidepressant like effect of DF was conducted in FS. Pre-trial exposure (15 min, 24 h prior to the test) of mice to this apparatus was followed by a 6-min test period during which scoring of immobility time was realized. The FS was realized following the treatment procedure under item 2.4.1 and placement of mice in a cylinder (42 cm high, 18 cm in diameter) filled with water (24°C) up to 30 cm. Experimental subject was considered to be immobile when it ceased struggling and making minimum movements necessary to keep afloat [22]. In subsequent experiment mice were pretreated intraperitoneally with, 0.9% saline, PCPA 100 mg/kg for four consecutive days or AMPT 100 mg/kg 4 hours prior to the oral administration of Vehicle, DF (250 mg/kg), or IMI 30 mg/kg and were subjected to forced-swimming test to examine the effects of indolamine or catecholamine depletion respectively.

2.4.3. Open-field test (OF)

Mice were individually placed at the centre of the open field apparatus in a sound proof experimental room to measure rearing and locomotor activity during 5 min after oral administration of Vehicle, DF (500 mg/kg), or DZP (5 mg/kg). The floor of the OF is divided into 8 sector of equal area. Number of crossing and rearing were registered for further statistical analysis. The apparatus was cleaned with 10% alcohol after mouse exposure.

2.4.4. Ex vivo MAO assay by spectrophotometric method

Mice were treated acutely with DF (250 mg/kg), Clorgyline 15 mg/kg (a selective inhibitor of MAO-A) or Tranylcypromine 15 mg/kg (a non-selective and irreversible inhibitor of monoamine oxidase) and sacrificed by decapitation after 60 min. Brain tissues homogenates were prepared according to [53] and stored under -20°C in aliquots and used as the source of MAO within 48 h. Enzymatic activity was measured according to [54]. Protein concentration was estimated by using Bradford method [23].

2.5. Statistical Analysis

In order to compare level of significance between two groups, unpaired Student's t-test was used as described by Drummond and Tom [24, 25]. To compare more than two groups, we used ANOVA followed by Dunnet´s test to compare test with control group or student-Newman–Keuls to compare all pairs of means. All values of $P<0.05$ was considered to be significant.

3. Results

The chromatograms in figure 1a (sample - DF) and b (reference drug – oleanolic acid) showed relative composition of oleanolic acid (OA) to be 7.82 % in respect of the concentration of DF injected [Relative composition of OA = [(Cr x As x 100) ÷ (Cs x Ar) %] where Cr - concentration of reference drug, Ar - area under reference drug curve, Cs - concentration of sample, As - area under sample curve]. In the pharmacological tests, like imipramine (30 mg/kg), DF (125 or 250 mg/kg) significantly reduced immobility in the TS and FS as shown in figure 2 a and b respectively. As shown in figure 3 a and b, DF 500 mg/kg and diazepam (DZP 5 mg/kg) altered the number of crossings ($^{*}p<0.05$ and $^{**}p< 0.01$ respectively) and rearings ($^{*}p<0.05$ and $^{**}p< 0.01$ respectively) in the

open-field test (OF) significantly when compared with the vehicle treated group. Reduction in these parameters is an indication of motor incoordination by DF 500 mg/kg or DZP 5 mg/kg treatment. Effect of biosynthetic enzymes inhibition that result in indolamine (serotonin) depletion was shown in the FS with p-chlorophenylalanine methyl ester (PCPA100 mg/kg, i.p.) pretreatment for four consecutive days followed by acute oral administration of vehicle, DF (250 mg/kg) or imipramine (30 mg/kg) as described under item 2.4.2. Figure 4a showed a reduction in antidepressant - like effect of DF ($^{\#}p<0.001$) with PCPA pretreatment. Administration of this tryptophan hydroxylase (TrOH) inhibitor alone did not elicit significant behavioural alteration in the FS. Similar observation was made with the α-methyl-p-tyrosine (AMPT 100 mg/kg, i.p.) pretreatment, an inhibitor of tyrosine hydroxylase (TOH), the rate-limiting enzyme for catecholamine biosynthesis, administered 4 h before the FS. Figure 4 b shows blockade of DF anti-immobility effect by AMPT ($^{\#}p<0.05$). Effect of DF on brain MAO as investigated showed, unlike Clorgyline and Tranylcypromine that reduced MAO activities to 17.7±6.4 % ($p<0.001$) and 47±9.0 % ($p<0.01$) respectively, its ineffectiveness to inhibit MAO activity (103.7±8.8 %, $P> 0.05$).With this result, it is obvious that DF do not have inhibitory effect on any of the MAO isoform (A and B).

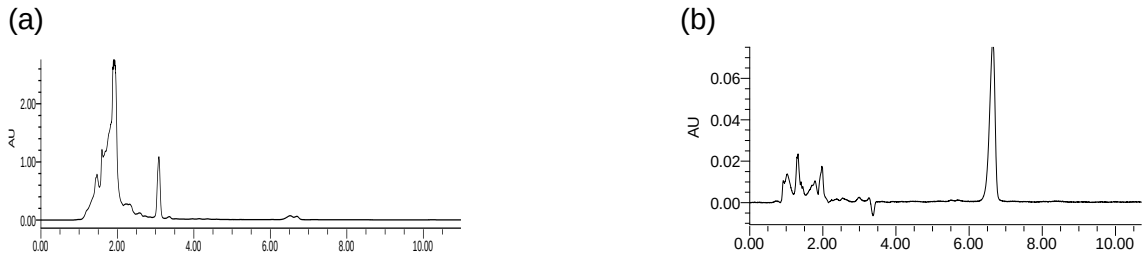


Figure 1. HPLC chromatogram showing (a) Oleanolic acid peak detected in dichloromethane fraction of *Pimenta pseudocaryophyllus*, (b) Reference drug (Oleanolic acid - Sigma).

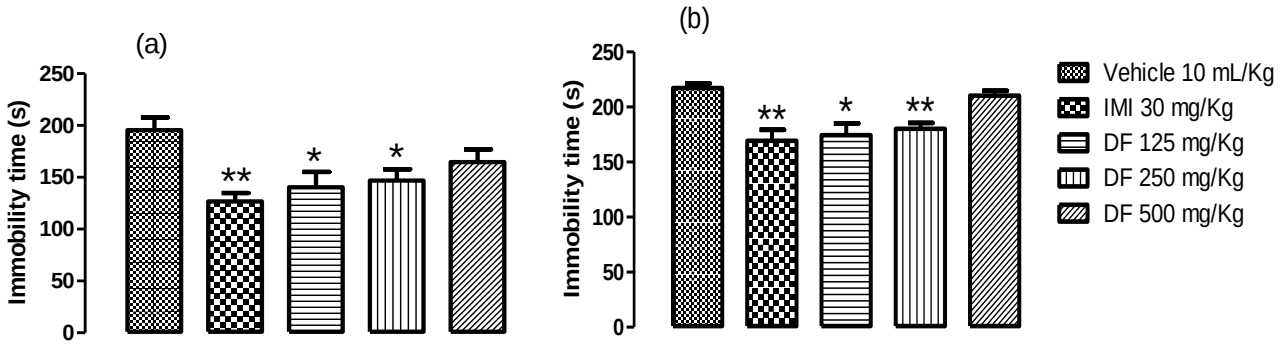


Figure 2. The effect of dichloromethane fraction (DF), imipramine (IMI) or vehicle administration on the immobility (a) in the TS; (b) FS. Data are presented as mean of immobility time in seconds ± S.E.M. (n=10). All differences from the control group are considered to be significant at $p < 0.05$, or $p < 0.01$ as denoted by * or ** respectively. Except for control group, lack of symbol * on the bar indicates $p > 0.05$.

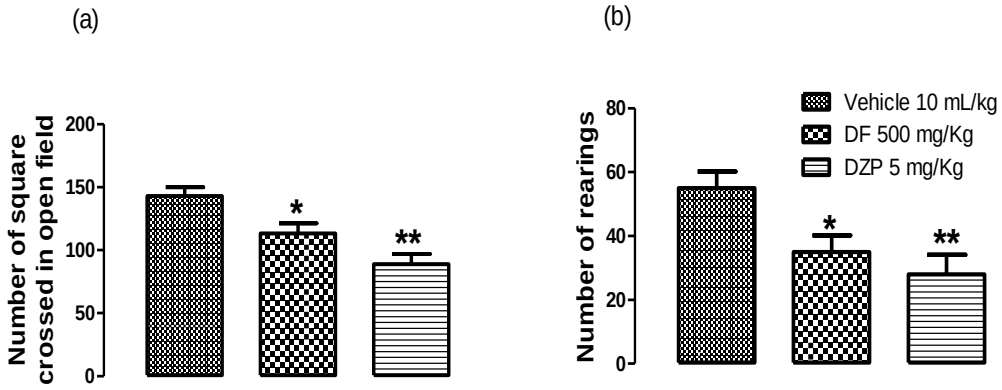


Figure 3. Effect of dichloromethane fraction (DF), diazepam (DZP) or vehicle oral treatments in the open-field test. Values are expressed as mean ± S.E.M (n=10±2).

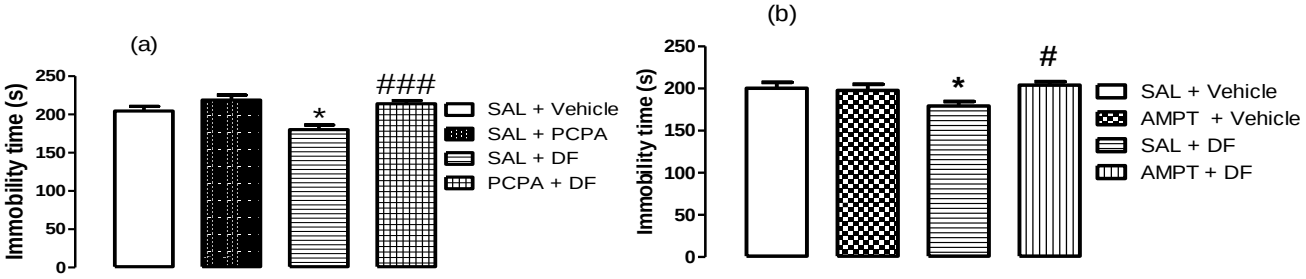


Figure 4. The effect of dichloromethane fraction 250 mg/kg (DF), imipramine (IMI) or vehicle administration on the immobility after pretreatment with (a) PCPA 100 mg/Kg; (b) AMPT 100 mg/Kg in FS. Data are presented as mean of immobility time in seconds ± S.E.M. (n=10±2). All differences from the control group are considered to be significant at $p < 0.05$, or $p < 0.01$ as denoted by * or ** respectively while # ($p < 0.05$) or ### (0.001) represent reversion of anti-immobility effect by PCPA or AMPT pretreatments. Except for control group, lack of symbol * or # on the bar indicates $p > 0.05$.

4. Discussion

Previous phytochemical analysis of the fractions obtained through liquid-liquid partitioning of the ethanol leaf extract of *P. pseudocaryophyllus* showed the presence of triterpenes, flavonoids, besides the (E)-methyl isoeugenol which constitute almost all of the essential oil (approximately 94%) [26]. In the dichloromethane fraction (DF), notable phytoconstituents identified with appropriate standards are oleanolic acid and methylisoeugenol as earlier reported [8]. Being one of the major phytoconstituents found and isolated, quantitative analysis of oleanolic acid showed a relative composition of approximately 8%. Meanwhile, estimation of DF doses administered in present study is based on its effective dose in previously published [8] and unpublished data.

The neuropharmacological activity of dichloromethane fraction - DF had been demonstrated and associated to the involvement of serotonergic pathway [8]. As earlier stated, investigation of antidepressant-like effects of DF is partly reinforced with the hypothesis that anxiolytic property of DF could be linked with its putative antidepressive action. In order to screen antidepressant effect of DF, tail suspension test (TS) was conducted. TS is a predictive and well established animal model of antidepressant activity [21] that permits investigation of anti-immobility property of a novel molecule. Significant reduction in immobility time in TS and consistent reduction of this parameter in the forced swimming tests can be associated with antidepressant like effect of this fraction. These results are similar to those obtained with imipramine treatment (norepinephrine/serotonin reuptake inhibitor) that is known to elicit an antidepressant response in FS [27]. FS remains one of the most effective and widely acceptable preclinical animal models [28, 22].

Acute treatment with the standard drug or DF seems not to satisfy the aspect of face validity in this test considering the notion that therapeutic actions of antidepressant drugs evolve gradually with chronic treatment [29 - 31]. However, preliminary study showed that anti-immobility activity of DF at the doses tested in this work was not significantly different as compared to the chronic treatment (data not shown). This may be of clinical benefit as cases of non-adherence and risk attached to chronic treatment can be drastically reduced. In contrary to the position of some authors that immobility is an adaptive coping mechanism to conserve energy [32, 33], we share the opinion that immobility reflects behavioural despair and a reliable means of demonstrating predictive validity [34, 35].

The insinuations that acquirement of anxiolytic properties by antidepressants following chronic administration [36] may not truly represent the cellular processes involved. In this study, we were able to show in contrast to this assertion, antidepressive effect of DF (a fraction that has shown anxiolytic like property) with an acute treatment. In essence, this result can be attributed to the presence of active principles that are capable of eliciting antidepressive like activity. Oleanolic acid among other constituents may have played a role in this activity as there are reports that demonstrated antidepressive effect of some triterpenes [37, 52] and linked oleanolic acid to CNS mediated antinociceptive effect [38].

However, the intriguing nature of DF 500 mg/Kg (highest dose) insignificance effect in antidepressive models (TS and FS) led us to its evaluation in the open field. In this animal model, parameters (locomotor activity and rearing - which some authors considered as vertical movement) that are susceptible to the effects of myorelaxant or

sedative agent were evaluated to augment information obtained on antidepressant models. Interestingly, reduction in these parameters as a result of DF 500 mg/Kg administration is an indication of motor incoordination. Moreover, CNS stimulatory effect is also one of the commonly found false positive effects of natural or synthetic product in these models. However, previous results [8, 9] did not show any form of motor alteration (psychostimulatory or sedative) after DF 250 mg/Kg oral treatment.

In an attempt to investigate possible mechanism of action involved, biosynthetic enzymes was hypothesized to influence the synaptic level of monoamine. Metabolic activities of citosolic enzymes like tryptophan hydroxylase (TrOH) and tyrosine hydroxylase (TOH) indirectly influence monoaminergic transmission. TrOH and TOH are rate limiting enzymes in serotonin and norepinephrine synthesis respectively. Evidences in the literature showed that inhibition of norepinephrine and serotonin synthetic enzymes blocked antidepressant effect of desipramine or fluoxetine respectively and elicit a rapid return of symptoms in depressed patients [39, 40]. Serotonin and noradrenaline depletion approach has been utilized [41] in animal model to elicit depressive like behaviour. Mice were depleted of serotonin with the parachlorophenylalanine - PCPA (tryptophan hydroxylase inhibitor) for 4 days [42] while α-methyl-p-tyrosine - AMPT was used to deplete catecholamine storage [40, 43]. Pretreatment with these biosynthetic enzymes inhibitors in this research abolished antidepressive like response to DF treatment. These results are in agreement with the results in the literature that showed increase in affective disorder symptoms due to inhibition of monoamine synthesis by PCPA and AMPT [44, 45].

Reduction in immobility time in this research is a reflection of an increase in swimming and struggling. According to Millan [46], dual norepinephrine and serotonin reuptake inhibitors may produce persistent effects on both noradrenergic and serotonergic neurotransmission for greater efficacy and a more rapid onset of action. Anti-immobility response which could be regarded as a measure of physiological alterations to acute DF treatment may be associated with the development of synergy among neural pathways.

Moreover, activity of drugs on MAO has been employed in the treatment of depression. In the treatment of this neural disease, monoamine oxidase inhibitors especially MAO A inhibitors (clorgyline, moclobemide) has proved to be more effective compare to MAO B inhibitor like selegiline [47, 48]. MAO A is acknowledged for its preferential catabolic activity on 5-HT and NE (substrate). Research has also demonstrated antidepressant action of vast number of medicinal plant extract among which is *Hypericum perforatum,* that inhibits monoamine oxidase (A and B) [49]. Unlike DF treated group, data obtained on MAO ex vivo assay showed significant reduction in enzymatic activity with the clorgyline and tranylcypromine treatment as compared to the vehicle treated group. Based on the experimental data and standard drugs used (clorgyline 15 mg/Kg- an irreversible and selective inhibitor of monoamine oxidase A [50] and tranylcypromine 15 mg/Kg - a non-selective and irreversible inhibitor of monoamine oxidase - MAO [51]), we can infer that DF is not an effective inhibitor of MAO.

In conclusion this work reveals antidepressive - like property of dichloromethane fraction and integrates new findings of possible mechanisms underlining antidepressant

action with a growing body of evidence on vital role of monoamine biosynthetic enzyme. Subsequent preclinical study will be focused on active principles that are responsible, toxicological study and dose extrapolation for possible clinical trial.

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Anexo 5. Plurality of anxiety and depression alteration mechanism by oleanolic acid

Abstract

Our study sought to evaluate the anxiolytic and antidepressant activities of OA as well as the neural mechanisms involved. Animal models like barbiturate sleep - induction, light dark box (LDB), elevated plus maze (EPM), forced swimming test (FST), tail suspension test (TST) and open field (OF) test were conducted. Male Albino Swiss mice were treated orally with vehicle 10 mL/kg, fluoxetine 20 mg/kg, imipramine 15 mg/kg, diazepam 1 mg/kg or OA 5–40 mg/kg. Pretreatment (i.p) of animals with pentylenetetrazole (PTZ) 20 mg/kg, 1-(2-methoxyphenyl)-4-[4- (2-phthalimido) butyl]piperazine hydrobromide (NAN-190) 0.5 mg/kg, p-chlorophenylalanine methyl ester (PCPA) 100 mg/kg or α-methyl-p-tyrosine (AMPT) 100 mg/kg, WAY100635 (WAY) 0.3 mg/kg, prazosin (PRAZ) 1 mg/kg, yohimbine (YOH) 2 mg/kg as well as monoamine oxidase (MAO) assay and hippocampal brain derived neurotrophic factor (BDNF) quantification were carried out. OA potentiatiated hypnotic effect of barbiturate and demonstrated anxiolytic effect in both the LDB and EPM. This effect was not reversed by PTZ. Acute and/or chronic oral treatment of mice with OA (5 - 20 mg/kg) elicited antidepressant effect in the FST and TST without interfering with the locomotor activity. Antidepressant effect of OA was attenuated by NAN-190, AMPT, PCPA, WAY and PRAZ. Although MAO activity remained unaltered by OA, chronic administration of OA augmented hippocampal BDNF level. These findings demonstrate multiple mechanisms of anxiolytic and antidepressant effect of OA.

Keywords: Oleanolic acid, Anxiolytic, Antidepressant, Monoamine, Metabolic enzymes, Neurotrophic factor

Introduction

Depression and anxiety are widely acclaimed as psychiatric disorders of global concern. These mood disorders or their comorbidity remains one of the most debilitating psychiatry diseases that can compromise human welfare (Yi et al., 2013). The global socioeconomic burdens and suffering from mood disorders are of tremendous impact and concern in the society (Nikola et al., 2012). In clinical practice, continuous search for new pharmacotherapies remains a correct strategy towards the discovery of drugs with better pharmacological profile (improved efficacy and faster action). Currently, the clinical outcomes of the application of many over - the - counter psychotropic drugs are characterized by cases of side effects, non-response to treatment and non-adherence to prolong treatment (Markou and Cryan, 2012). The global access to some natural products that have been considered to help control neural disorders (Ravindran et al., 2009) could provide therapeutic options. Considering the fact that mood disorders involve complex neural dysregulation, a clinically safe agent (Hunan, 1975; Singh et al., 1992) with multiple neural mechanism could offer a better treatment. This kind of agent may be preferable (clinically) to the combination of drugs.

Oleanolic acid – OA is a ubiquitous secondary metabolite and common ingredient in many fruits and herbs. This pentacyclic triterpene has been widely consumed for many centuries without health hazard (Newman and Cragg, 2007; Michael et al., 2007). Studies have shown anxiolytic and antidepressant - like effects of isolated triterpenoids like α amirin and β amirin (Rodrigues et al., Chen et al., 2005 Woode et al., 2001). However, being natural products, relative abundance of these triterpenoid varied from species to species. Although the previous studies on the organic extracts as well as the

folkloric use of leaf extract of *Pimenta pseudocaryophyllus* (Gomes) L. R. Landrum – Myrtaceae suggest the possibility of CNS modulation by OA (Fajemiroye et al., 2012 and 2013), the specific neuropharmacological activities of this compound remain uninvestigated. For the purpose of our study, the dose of OA was estimated from previous studies in our laboratory on the organic extract (Fajemiroye et al., 2012). The organic extract that demonstrated anxiolytic and antidepressiant - like activities (Fajemiroye et al., 2012 and 2013) in animal model composed of OA (relative composition - 8 %) among other phytoconstituents. This organic extract was administered orally at an effective dose of 250 mg/kg. Hence, we considered the dose of 20 mg/kg (8 % of 250 mg/kg) to be an equivalent of OA that participated in the neuropharmacological activities of the organic extract. In this study, we decided to work with inferior, intermediate and superior doses of OA.

The neural mechanism of anxiolytic and antidepressant drugs have been associated to their action on receptors, metabolic processes, modulation of cellular, neurotrophic factors processes among others (Kennett, 1992; Machado et al., 2013). The brain-derived neurotrophic factor (BDNF) has been reported to be an important target of antidepressant drugs. An increase in mRNA encoding BDNF and hippocampal BDNF protein levels (Castrén et al, 2007) was attributed to the antidepressant effect. The reduction in hippocampal BDNF levels in learned helplessness rats (Itoh et al., 2004) and the restoration of this neurotrophic factor with imipramine treatment also support the role of this neurotophic factor in mood disorder and mechanism of antidepressant drugs.

Considering the high concentration of oleanolic acid in the organic extract that was studied, we assume that this compound is responsible for the effect of the acute oral dose of this extract. We hypothesized that acute administration of equivalent dose of OA should possess anxiolytic and antidepressant - like effects. The therapeutic potential and susceptibility of OA to chemical modification on its C-3 hydroxy, the C-12–C-13 double bond and the C-28 carboxylic acid to produce series of new synthetic oleanane triterpenoids (Sporn et al., 1997, 2002, 1998, 2011) make the investigation of neuropharmacological activity of OA essential for new drug discovery. Hence, our study focuses on animal model of anxiety and depression among other bioassays. Additionally, pharmacological tools were employed to delineate the neural mechanism of this compound.

Methods

Experimental subjects

Male Swiss mice (20 ± 3 g; 5 weeks old) were provided by central animal house, Federal University of Goiás. Animals were kept in a mini-intra-laboratory facility cage (10 animals per cage of size 320 × 180 × 160 cm) during 7 day-acclimatization period under controlled environmental conditions (23 ± 1°C, 12 hr light-dark cycle). Mice were provided with free access to standard chow and water. In this study, minimum number of mice (naïve) that permits adequate statistical analysis and interpretation of results were used. Behavioural sessions were conducted between 1200 hours and 1800 hours in compliance with the approved experimental protocol (number 104/08) as certified by Ethical Committee of Federal University of Goiás and international laws of the care and use of laboratory animals. All experimental procedure minimizes noise and animal

suffering. All the studies involving animals were reported as recommended in Kilkenny et al. (2010).

Drugs and Treatment

OA - Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) was purchased from Sigma-Aldrich (St-Quentin-Fallavier, France), DZP – diazepam (Cristália, Itapira, SP, Brazil), PTZ – pentylenetetrazole and fluoxetine (Sigma-Aldrich, St. Louis, MO, USA), IMI imipramine (Cristália, Itapira, SP, Brazil). PCPA - p-chlorophenylalanine, NAN-190 - 1-(2-methoxyphenyl)-4-[4-(2-phthalimido) butyl]piperazine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA), WAY100635 (WAY) - N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclohexanecarboxamide - (Sigma-Aldrich, St. Louis, MO, USA), TRAN - Tranylcypromine (Synth, Diadema, SP, Brazil), PRAZ - Prazosin (Cristália, Itapira, SP, Brazil), YOH - Yohimbine (Sigma-Aldrich, St. Louis, MO, USA), Tween 80 - 2% (Polyoxyethylenesorbitan monooleate, Sigma-Aldrich, St. Louis, MO, USA). Drugs were dissolved in a vehicle [a mixture of 0.9% NaCl and 5% (v/v) Tween-80 (polyoxyethylene sorbitan monooleate) and administered orally (p.o.) or intraperitoneally (i.p) in a volume of 0.1 ml per 10 g of mice body weight. In barbiturate sleep induction, mice were treated orally with vehicle 10 mL/kg, OA (5 - 40 mg/kg) or DZP (1 mg/kg) 1h prior to sodium pentobarbital (40 mg/kg, i.p.) injection. The light dark box, elevated plus maze, Open field, forced swimming and tail suspension tests were performed 1 h following oral administration of OA (5, 10, 20 or 40 mg/kg), IMI (15 mg/kg), or vehicle. Drug pretreatments (PTZ 20 mg/kg, NAN-190 0.5 mg/kg, WAY 0.3 mg/kg, prazosin 1 mg/kg, yohimbine 2 mg/kg) were carried out intraperitoneally 30 min prior to oral administration of OA or vehicle. To selectively deplete 5-HT, animals were

pretreated with the tryptophan hydroxylase inhibitor PCPA (100 mg/kg, i.p) once a day for four consecutive days before behavioral testing. To deplete newly synthesized pools of NE and DA, mice were treated with a single dose of tyrosine hydroxylase inhibitor, AMPT (100 mg/kg, i.p) 4 h prior to behavioural testing. To quantify hippocampal BDNF, mice were decapitated on the 14th day of the oral administration of vehicle 10 mL/kg, OA 20 mg/kg or fluoxetine 20 mg/kg. The whole hippocampus were carefully dissected out from each hemisphere and stored at −80°C for enzyme-linked immunosorbent assay (ELISA). All control animals received vehicle on the same regimen as the treated groups.

Behavioural tests

Barbiturate sleep induction

The Barbiturate sleep induction was carried out essentially as previously described (Carlini and Burgos, 1979; Fajemiroye et al., 2012). Mice were treated orally with vehicle 10 mL/kg, OA (5 - 40 mg/kg) or diazepam (1 mg/kg) prior to sodium pentobarbital (40 mg/kg, i.p.) injection (1hr interval). Time taken for the loss of righting reflex (sleep latency) and voluntary recovery of the righting reflex (sleep duration) were recorded and analysed to detect CNS depression or stimulation.

Evaluation of Anxiolytic - like property

Light dark box test (LDB)

The LDB apparatus used in this study consisted of a dark compartment (20×30 cm) and an illuminated compartment (40×30 cm). The two compartments were demarcated by a partition with an opening (4×4 cm) through which the animal could transit between the two compartments. Mice were treated orally with vehicle (10 mL/kg), OA (10, 20 or

40 mg/kg) or DZP (1 mg/kg). Experimental animals were placed at the centre of the light area facing the opening of the dark area after 1 hr of oral treatment. The apparatus was cleaned thoroughly with 10 % alcool between trials. The number of transitions between the two compartments and the time spent in the light area were recorded over a 5 min period (Crawley and Goodwin, 1980).

Elevated plus maze test (EPM)

The elevated plus-maze is a widely used behavioural model to measure anxiolytic-like effect of a compound (Pellow et al., 1985). The EPM apparatus (Insight Scientific Equipment, SP, Brazil) consisted of two open arms (30 x 5 x 0.5 cm) and two closed arms (30 x 5 x 15 cm) connected by a common central platform (5 x 5 cm). The maze was located above the ground at a height of 60 cm. Mice were treated orally with vehicle (10 mL/kg), OA (10, 20 or 40 mg/kg) or DZP (1 mg/kg). Sixty minutes after the oral treatment, mice were placed individually at the centre of the plus maze with their head facing the direction of the enclosed arms and videotaped for 5 min. The apparatus was cleaned thoroughly with 10 % ethanol between trials.Time spent and number of entries with all four paws inside the open arms were recorded for statistical analysis.

Forced swimming test (FST) and Tail suspension test (TST

Mice were submitted to a modified forced swimming test described by Porsolt et al. (1977). Animals were subjected to acute (single oral dose) with vehicle, imipramine 15 mg/kg or OA (5, 10, 20 or 40 mg/kg) and chronic (daily oral dose for 14 days). This was followed by placing the mice individually in a cylindrical water container (42 cm in height, 18 cm in diameter) filled with water up to 30 cm at 24 ± 2°C. Initially, the mouse attempts to escape but eventually adopts an immobility posture, characterized by the

lack of active movement except that which is necessary to keep the mice afloat. After mice exposure, the container was cleaned with 10% ethanol solution to prevent biasness that could emanate from sensory stimulation by odour from faeces and urine. In a separate experiment with naïve mice, a modified version of the tail suspension test validated by Steru et al. (1985) was conducted. Mice were randomly allocated to treatment conditions described in FST and suspended (using an adhesive tape placed 2 cm from the tip of the tail) at about 50 cm above the foor. The TST share similar basic principle with FST in that mice develop an immobile posture when placed in an inescapable stressful situation after initial escape-oriented movements. Acute administration of an antidepressant drug prior to the exposure of experimental subject to TST prolong active escape-directed behaviours (Cryan et al., 2005). A 6-min test session was videotaped and the immobility time was later scored and analyzed. The immobility time was scored during the last 4 minutes of the FST and TST.

Open field exploratory activity

Animals were treated orally with OA (20 or 40 mg/kg), DZP (1 or 5 mg/kg) or vehicle 10 mL/kg as described in previous work (Fajemiroye et al., 2012) and placed in a circular open field [πr^2 (base area) = 62.80 cm^2 with a 50 cm high wooden wall]. The base area was divided into 8 equal sectors. A 5 - min test session was videotaped in a sound proof experimental room. Parameters like crossing and rearing activity were later scored.

Mechanism of antianxiety - like effect

After 30 min of PTZ 20 mg/kg, i.p (a subconvulsive, non-anxiogenic dose and competitive antagonist of $GABA_A$ receptor) or saline solution 10 mL/kg, i.p.

pretreatments, mice were treated orally with vehicle 10 mL/kg or OA 20 mg/kg and exposed to LDB following 1 h interval.

Mechanism of antidepressant – like effect

Mice were pretreated intraperitoneally with PCPA 100 mg/kg (serotonin depletor) or saline solution for four consecutive days prior to the oral administration of OA 20 mg/kg or vehicle prior to FST. In a separate experiments, animals were pre-treated (i.p) with a single dose of AMPT 100 mg/kg (catecholamine depletor) 4 h prior to the oral administration of OA 20 mg/kg or vehicle; NAN-190 0.5 mg/kg (non-selective 5-HT1A receptor antagonists) pretreatment 30 minutes prior to the oral administration of OA 20 mg/kg or vehicle; additional groups were pretreated with saline solution 4 h or 30 minutes prior to the oral administration of OA 20 mg/kg or vehicle. Animals were later subjected to FST to examine the effects of monoamine in the antidepressant - like property of OA. The regimen of PCPA in this study is known to deplete about 60 % of endogenous storage of serotonin content without altering the noradrenaline or dopamine levels (Kwon et al., 2010; Redrobe et al., 1998a and Redrobe et al., 1998b). The protocols used in the depletion of dopamine and noradrenaline in this study were similar to those used in numerous other studies some of which had the residual content of cathecolamine assayed. Mayorga et al. (2001) demonstrated that AMPT (tyrosine hydroxylase inhibitor) reduces 57% of dopamine and 53% of noradrenaline levels in mice without affecting the levels of serotonin. Hence, even though we did not measure residual dopamine and noradrenaline levels in the present study, there are strong precedents to assume that the agent worked as expected. Moreover, behavioural

response to anti-immobility effect of OA in the PCPA and AMPT-pretreated group supports the presumption that there was a depletion of the monoamine.

Effects of* WAY100635*, prazosin and yohimbine pretreatments on antidepressant - like property of OA

The effects of WAY100635 - WAY (a selective antagonist of 5-HT1A), prazosin - PRAZ (α1- adrenoceptor antagonist) or yohimbine - YOH (α2- adrenoceptor antagonist) pretreatments on antidepressant - like property of OA were investigated. Mice were pretreated intraperitoneally with WAY 0.3 mg/kg, PRAZ 1 mg/kg, YOH 2 mg/kg or saline solution 30 minutes prior to the oral administration of OA 20 mg/kg or vehicle. Animals were later subjected to FST to examine the effects of drug pretreatments on the antidepressant - like property of OA.

Monoamine oxidase - MAO assay by spectrophotometric method

For *ex vivo* assay, groups of mice were subjected to acute oral treatment of OA 20 mg/kg and tranylcypromine - TRAN 15 mg/kg (a non-selective and irreversible inhibitor of MAO). The animals were sacrificed by decapitation after 1 h. Naïve untreated mice were used for *in vitro* assay. A whole brain tissues homogenates were prepared 1:20 (w/v) in ice-cold potassium phosphate buffer, with a mechanical homogenizer (Turrax). Homogenates were centrifuged at 1200 x g and 4 ^{o}C for 7 min. This procedure was repeated with the supernatant at 12500 x g and 4 ^{o}C for 15 min. The resulting pellet was suspended in 1.5 mL homogenization buffer and recentrifuged at 12500 x g and 4 ^{o}C for 15 min; the resulting pellets were resuspended in 1.0 mL homogenization buffer, stored under -20°C in aliquots and used as the source of MAO within 48 h. Total protein concentration was estimated using the method of Bradford (Bradford, 1976). The

measurement of *in vitro* and *ex vivo* activity of MAO were conducted using a modified spectrophotometric method (Holt et al. 1997; Stafford et al., 2007 and Fajemiroye et al., 2013).

Hippocampal protein extraction and BDNF quantification

Mice received single oral dose (acute administration) or daily oral dose (14 consecutive days – chronic administration) of OA 20 mg/kg, fluoxetine 20 mg/kg or vehicle (10 mL/kg) and sacrificed to collect their left and right hippocampi. Hippocampal tissues were homogenized in lysis buffer (NaCl 1mM; EDTA 4 mM, Tris-HCl 100 mM; albumin 2%, Triton-X 100 2%, thrimerosal 0.01%, pH 7.0, glycerol 10%, protease inhibitor cocktail - GE) in ratio 1:40 w/v. After centrifugation (16800 x g, 4°C, 35 min), the supernatant was stored at -80°C. Hippocampal BDNF was measured by ELISA kit (BDNF Emax® ImmunoAssay System kit, Promega, Madison, WI, USA) according to the manufacturer's instructions. Normalization of total protein level of sample were measured by using bovine serum albumin as a standard (Bradford, 1976). The coefficients of variation (CV) obtained for intra-assay (3.7 %) and the inter-assay (6.8%) showed a good precision.

Statistical analyses

Experimental data were expressed as group mean ± S.E.M. In keeping with the experimental hypotheses, a one-way ANOVA was used to detect the effect of drug treatment (an independent variable) on animal behaviour (a dependent variable) and followed by pairwise comparisons (Dunnett´s test as post hoc) of individual treatment groups to vehicle treated group. Two-way analysis of variance (ANOVA) as detailed by Neter et al. (1990) was used to detect the effect of treatment period/drugs (independent

variables) or pretreatment/drug factors (independent variables) on the immobility time (a dependent variable). Pairwise followup comparisons of individual treatment groups were carried out using Bonferroni test as post hoc test. Significant difference was set at p value less than 0.05 (Drummond and Tom, 2011).

Results

Barbiturate Sleep Potentiation

In the present study OA treatment did not elicit alteration in sleep latency (the time it takes to lose righting reflex) [$F (5, 51) = 1.31$, $p > 0.05$, one-way ANOVA, Fig. 1A]. However, statistical analysis of sleep duration showed a significant effect of OA [$F (5, 51) = 8.92$, $p < 0.001$, one-way ANOVA, Fig. 1B]. Like DZP 1 mg/kg ($p < 0.001$), a post-hoc test (Dunnett´s test) revealed a significant increase in sleep duration (potentiation of the hypnotic effect of sodium pentobarbital) by OA 20 mg/kg ($p < 0.05$) and 40 mg/kg ($p < 0.001$).

Behavioural responses in the LDB and EPM

The results illustrated in Fig. 2 A [$F (4, 41) = 7.85$, $p < 0.001$, one-way ANOVA] and Fig. 2B [$F (4, 41) = 15.97$, $p < 0.001$, one-way ANOVA] demonstrated significant alterations in the number of transitions and time spent in the light area of LDB, respectively. The time spent in the light area of LDB was increased by OA at 10 and 20 mg/kg ($p < 0.05$). OA at 40 mg/kg reduced the number of transition significantly ($p < 0.05$]. OA elicited significant alteration in the time spent and number of entries into the open arms of EPM [$F (4, 41) = 9.63$, $p < 0.001$ and $F (4, 41) = 4.65$, $p < 0.01$, one-way ANOVA; Fig. 2C and Fig. 2D, respectively]. Post-hoc test (Dunnett´s test) revealed that OA increased the time spent in the open arms of EPM at 20 and 40 mg/kg ($p < 0.01$). Pairwise

comparisons with Dunnett´s test showed that the number of transitions (Fig. 2A) and percentage of open arms entries (Fig. 2D) remained unaltered by OA up to 20 mg/kg ($p > 0.05$).

Effects of OA treatment in the forced swimming and tail suspension tests

The significant effects of OA administrations on immobility time in the FST are shown in Fig. 3A [$F (5, 54) = 5.15$, $p < 0.001$, one-way ANOVA]. Dunnett´s test showed significant reduction in immobility time by OA at 5 mg/kg ($p < 0.05$), 10 and 20 mg/kg ($p < 0.01$). Fig. 3 B demonstrated the effect of treatment period (chronic or acute administration of drugs; independent variable) and drug (independent variable) on immobility time (dependent variable) in the forced swimming test. Statistical analysis did not show interaction between the independent variables [$F (2, 54) = 0.09$, $p > 0.05$, two-way ANOVA]. However, treatment period and drugs showed significant effect [$F (1, 54) = 4.316$, $p < 0.05$ and $F (2, 54) = 29.50$, $p < 0.001$, respectively; two-way ANOVA]. Bonferroni post hoc test showed that the anti-immobility response was independent of the doses of drug (OA 10 or 20 mg/kg dose) administered and treatment period [$F (1, 36) = 1.59$, $p > 0.05$]. In the tail suspension test, one way ANOVA showed significant effect of OA on immobility time as shown in Fig. 3C [$F (5, 54) = 4.20$, $p < 0.01$]. Dunnett´s test showed significant reduction in immobility time by OA at 5, 10 mg/kg ($p < 0.05$), and 20 mg/kg ($p < 0.01$).

Spontaneous motor activity in mice

Fig. 4A showed the effects of OA administration on the number of sector traversed and number of rearings activities [$F (4, 45) = 7.97$, $p < 0.001$ and $F (4, 45) = 3.75$, $p < 0.05$, respectively, one-way ANOVA]. Unlike OA 40 mg/kg and DZP 5 mg/kg ($p < 0.05$),

Dunnett´s test showed that OA 20 mg/kg and DZP 1 mg/kg did not alter the number of sector traversed ($p > 0.05$). At 20 and 40 mg/kg, OA elicited a reduction in the number of rearings ($p < 0.05$; Fig 4 B).

Mechanism of antianxiety- like property

Fig. 5 A and B demonstrated the effect of pretreatment (SAL or PTZ - independent variable) and treatment (Vehicle, OA 20 mg/kg or DZP 1 mg/kg – independent variable) on time spent in the light compartment and number of transition (dependent variables) of the LDB. As shown in Fig. 5B, statistical analysis revealed interaction between the independent variables [$F (2, 54) = 19.92$, $p < 0.001$, two-way ANOVA] on time spent in the light compartment of the LDB. Pairwise comparisons with Bonferroni post hoc test showed an increase in the time spent in the light compartment of the groups SAL + OA ($p < 0.05$) and SAL + DZP ($p < 0.05$) as compared to the one obtained in the group that received SAL + Vehicle. Unlike SAL + DZP vs PTZ + DZP ($p < 0.05$), the main effect of OA on time spent in the light compartment remained unaltered with PTZ pretreatment (i.e SAL + OA vs PTZ + OA, $p > 0.05$). In Fig. 5A, the data obtained on the number of transition revealed interaction between the independent variables [$F (2, 54) = 6.07$, $p < 0.01$; two-way ANOVA]. Pairwise comparisons with Bonferroni post hoc test did not show changes in the number of transition in the light compartment in the group treated with OA (i.e SAL + OA vs SAL + Vehicle, $p > 0.05$). The reference drug, diazepam, produced an increase in the number of transition (i.e SAL + DZP vs SAL + Vehicle, $p < 0.05$). The effect of DZP on the number of transition in the light compartment was attenuated with PTZ pretreatment (i.e SAL + DZP vs PTZ + DZP, $p < 0.05$).

Mechanism of antidepressant - like property

The effects of NAN – 190 (0.5 mg/kg), PCPA 100 mg/kg or AMPT 100 mg/kg pretreatments (three separate experiments – EXP 1, 2 and 3, respectively) on the behavioral response to OA in the FST are shown in Fig. 6. Separate analysis of EXP 1 showed the effect of pretreatment (SAL or NAN-190 - independent variable) and treatment (vehicle or OA 20 mg/kg – independent variable) on the immobility time (dependent variable) in the FST. The data obtained on immobility time revealed interaction between the independent variables [$F (1, 36) = 6.02$, $p < 0.05$, two-way ANOVA]. Pairwise comparisons with Bonferroni post hoc test showed a decrease in immobility time by OA treatment (i.e SAL + OA vs SAL + Vehicle, $p < 0.01$). The anti-immobility effects of OA was partially blocked by NAN-190 pretreatment [i.e SAL + OA vs NAN-190 + OA, ($p < 0.05$) and SAL + Vehicle versus NAN-190 + OA ($p < 0.05$)]. EXP 2 showed the effect of pretreatment (SAL or PCPA) and treatment (Vehicle or OA 20 mg/kg) on the immobility time in FST. The data obtained on immobility time revealed an interaction between the independent variables [$F (1, 36) = 8.79$, $p < 0.01$; two-way ANOVA]. Pairwise comparisons with Bonferroni post hoc test showed a decreease in immobility time in the group with OA treatment (i.e SAL + OA vs SAL + Vehicle, $p < 0.05$). The main effect of OA was attenuated by PCPA pretreatment (i.e SAL + Vehicle vs PCPA + OA, $p > 0.05$). EXP 3 showed the effect of pretreatment (SAL or AMPT) and treatment (Vehicle or OA 20 mg/kg) on the immobility time in FST. The data obtained on immobility time revealed interaction between the independent variables [$F (1, 36) = 5.72$, $p < 0.05$; two-way ANOVA]. Pairwise comparisons with Bonferroni post hoc test showed a decrease in immobility time with OA treatment (i.e the SAL + OA vs SAL +

Vehicle, $p < 0.01$). AMPT pretreatment elicited partial attenuation of the main effect of OA [i.e SAL + OA vs AMPT + OA ($p < 0.05$) and SAL + Vehicle vs AMPT + OA ($p < 0.05$)]. In all the 3 experiments, NAN, PCPA or AMPT pretreatments in combination with vehicle treatment did not alter animal behaviour at the dose tested [i.e NAN + Vehicle, PCPA+ Vehicle or AMPT+ Vehicle versus SAL+ Vehicle ($p > 0.05$)]. Fig 6 was used to show all the data in order to facilitate comprehension.

Effects of WAY, prazosin and yohimbine pretreatments on antidepressant – like property of OA

Fig. 7 showed the effect of pretreatment (SAL, PRAZ, WAY or YOH- independent variable) and treatment (vehicle or OA 20 mg/kg – independent variable) on the immobility time (dependent variable) in the FST. The data obtained on immobility time revealed an interaction between the independent variables [$F (3, 72) = 3.51$, $p < 0.05$; two-way ANOVA]. Pairwise comparisons with Bonferroni post hoc test showed that OA administration decreased the immobility time (i.e SAL + OA vs SAL + Vehicle, $p < 0.01$). Pretreatment with yohimbine did not attenuate anti - immobility effect of OA [i.e SAL + OA vs YOH + OA ($p > 0.05$)]. Bonferroni post test revealed a blockade of the main effect of OA with WAY pretreatment [SAL + Vehicle vs WAY + OA ($p > 0.05$)] and PRAZ pretreatment [SAL + Vehicle versus PRAZ + OA ($p > 0.05$)]. Pretreatments with WAY, PRAZ or YOH prior to vehicle administration did not alter animal behaviour at the dose tested [i.e WAY + Vehicle, PRAZ + Vehicle or YOH + Vehicle versus SAL+ Vehicle ($p> 0.05$)].

Hippocampal BDNF levels

Fig. 8 demonstrated the effect of treatment period (acute or chronic administration - independent variable) and drugs (Vehicle, OA 20 mg/kg or Fluoxetine 20 mg/kg – independent variable) on the level of hippocampal BDNF (dependent variable). A two-way ANOVA revealed an interaction between the independent variables [$F(2, 30) = 20.18$, $p < 0.001$]. Bonferroni post test showed that an increase in hippocampal BDNF level by OA or fluoxetine treatment depend on treatment period (level of significance for OA - $p < 0.001$ or fluoxetine - $p < 0.05$, Fig. 8).

Effects of OA on the activities of catabolic enzymes

The *in vitro* assay of MAO activity showed that OA did not alter catabolic activity of MAO (Fig. 9 A). A sharp drop in the activity of this enzyme at the highest concentration 1 mM of OA (Fig. 9 A) could be a mere precipitation of protein. Fig. 9 B showed the bar graph of *ex vivo* assay of MAO. A one-way ANOVA showed alteration in the MAO activity [$F(2, 12) = 25.03$, $p < 0.001$]. Pairwise comparisons with Dunnett´s test revealed significant reduction in the MAO activity by tranylcypromine ($p < 0.001$). In contrast, MAO activity remained unaltered by OA (Dunnett´s test as post hoc test, $p > 0.05$).

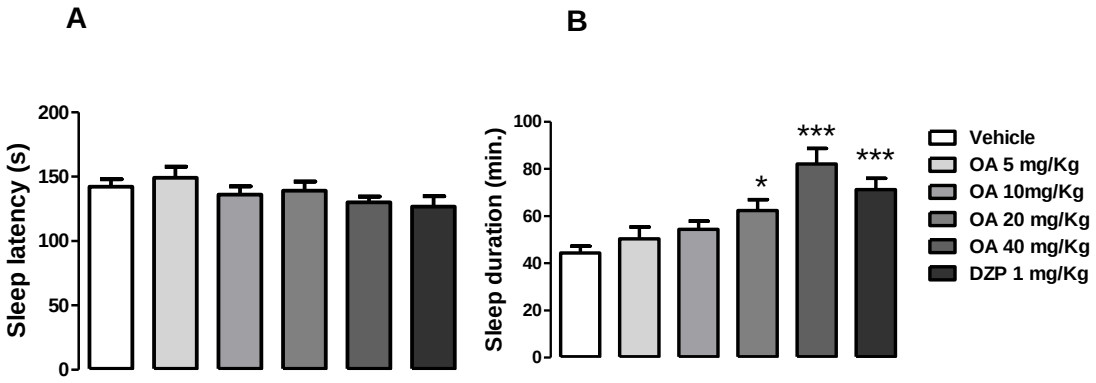

Figure 1. Effect of oral administration of oleanolic acid – OA 5, 10, 20 or 40 mg/kg, diazepam – DZP 1 mg/kg or vehicle 10 mL/kg on latency (A) and duration (B) of sodium pentobarbital induced hypnosis. Results are expressed as mean ± SEM; n = 8-10 in each group. *$p < 0.05$ and and ***$p < 0.001$ vs vehicle treated group (one way ANOVA followed by Dunnett's test).

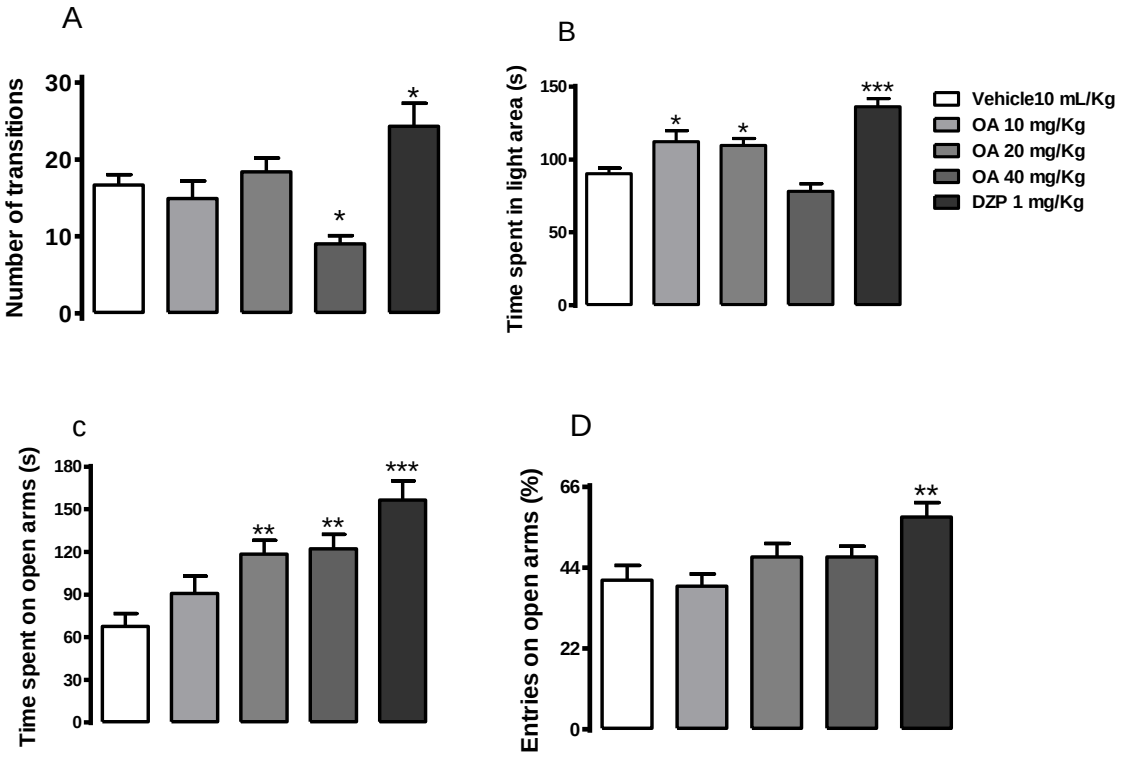

Figure 2. Effect of oral administration of oleanolic acid – OA 10, 20 or 40 mg/kg, diazepam – DZP 1 mg/kg or vehicle 10 mL/kg on the number of transition in the light dark box - LDB (A), time spent in the light area of LDB (B) and time spent on the open arms of the elevated plus maze – EPM (C) and percentage of entries into the open arms (D). Results are expressed as mean ± SEM; n =8-10 in each group; *p < 0.05, **p < 0.01and ***p < 0.001 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

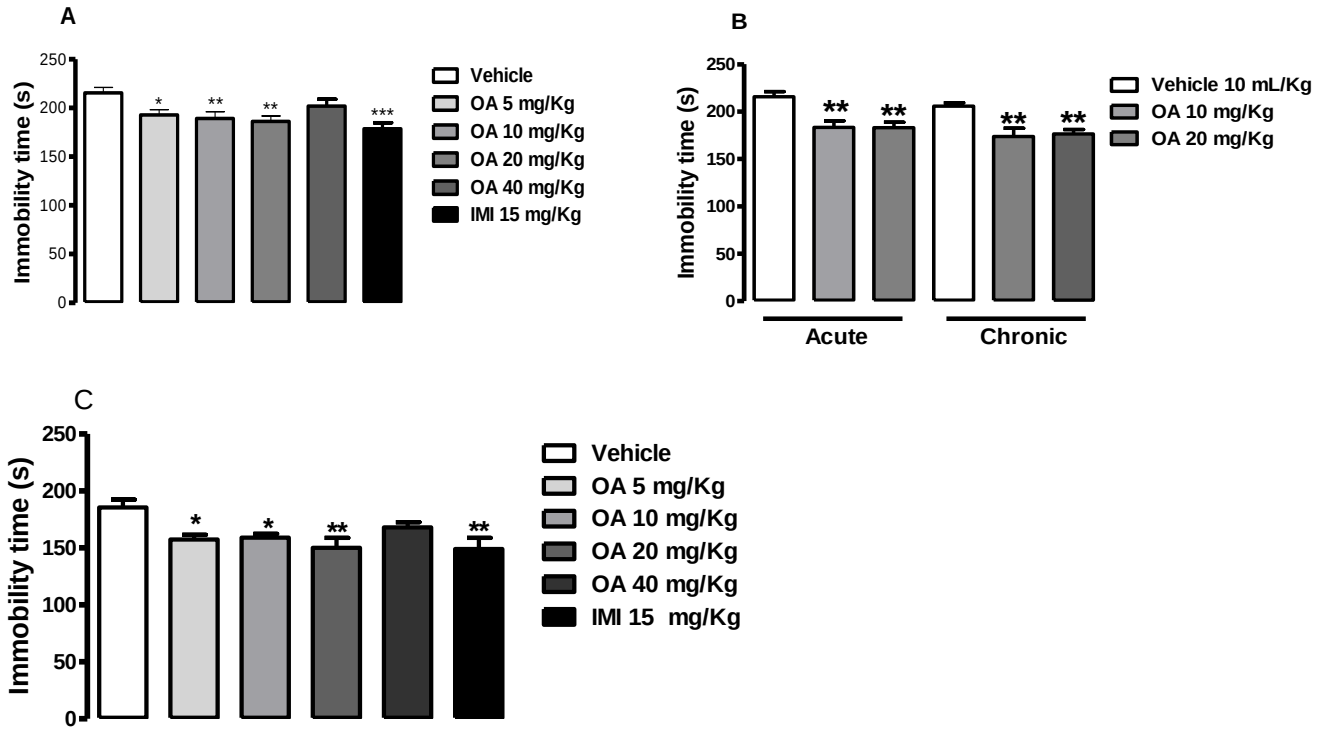

Figure 3. (A) The effect of acute oral administration of oleanolic acid – OA 5, 10, 20 or 40 mg/kg, imipramine – IMI 15 mg/kg or vehicle 10 mL/kg on the immobility in the forced swimming test – FST (one way ANOVA followed by Dunnett´s test). (B) Bar graph showing the effect of acute and chronic oral administration of OA 10 and 20 mg/kg on the immobility in the FST (two way ANOVA followed by Bonferroni test). (C) The effect of oral administration of oleanolic acid – OA 5, 10, 20 or 40 mg/kg, imipramine – IMI 15 mg/kg or vehicle 10 mL/kg on the immobility in the tail suspension test (one way ANOVA followed by Dunnett´s test). All data are expressed as mean ± S.E.M of 8-10 mice. *$p < 0.05$, **$p<0.01$ or ***$p<0.001$ vs vehicle treated group.

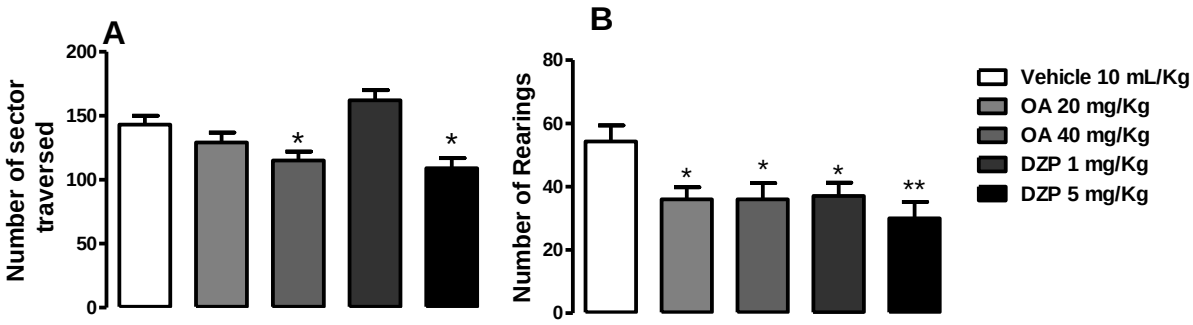

Figure 4. Effect of oral administrations of oleanolic acid – OA 20 or 40 mg/kg, diazepam – DZP 1 and 5 mg/kg or vehicle 10 mL/kg on the number of sector traversed (A) and the number of rearings (B) by mice in the open-field. Each column represents the mean ± SEM of 10 animals. *p < 0.05, **p < 0.01 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

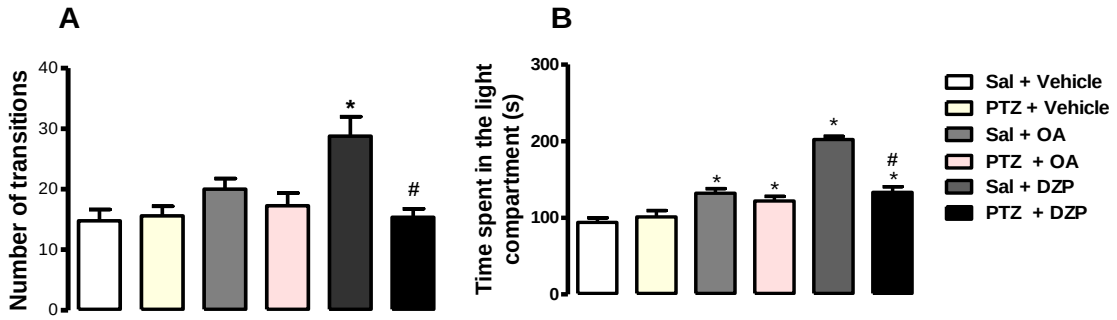

Figure 5. Behavioral responses [(A) number of transitions and (B) time spent in the light compartment] to OA 20 mg/kg, vehicle 10 mL/kg or DZP 1 mg/kg (administered orally 60 min before testing) after pretreatment with PTZ 20 mg/kg or SAL 10 mL/kg (administered intraperitoneally 90 min before testing). Data are expressed as mean ± SEM, n= 10 (two way ANOVA followed by Bonferroni test). * p < 0.05 versus vehicle treated group; # p < 0.05 SAL + DZP vs PTZ + DZP. SAL – saline solution, PTZ – pentylenetetrazole, OA – oleanolic acid, vehicle, DZP – diazepam.

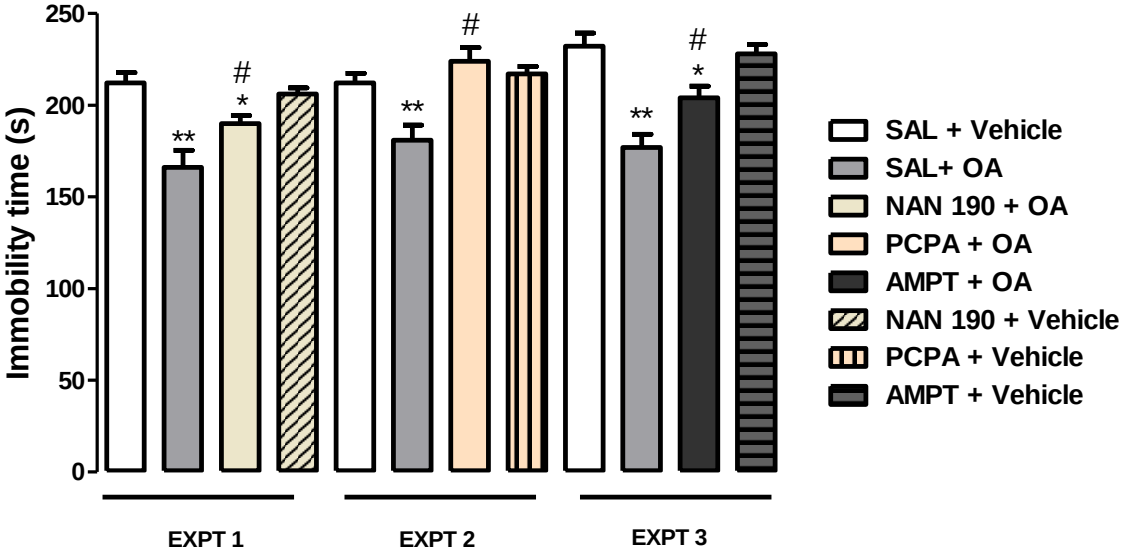

Figure 6. Behavioral response [Immobility time] to OA 20 mg/kg or vehicle 10 mL/kg (p.o) after pretreatment with SAL 10 mL/kg or NAN-190 0.5 mg/kg (i.p, Experiment – Expt 1); SAL 10 mL/kg or PCPA 100 mg/kg (i.p, Experiment – Expt 2); SAL 10 mL/kg or AMPT 100 mg/kg (i.p, Experiment – Expt 3) . Data are expressed as mean ± SEM, n= 10 (two way ANOVA followed by Bonferroni test). * $p < 0.05$, **$p < 0.01$ vs vehicle treated group; # $p < 0.05$ SAL + OA vs NAN-190 + OA, PCPA + OA or AMPT + OA (two way ANOVA followed by Bonferroni test).

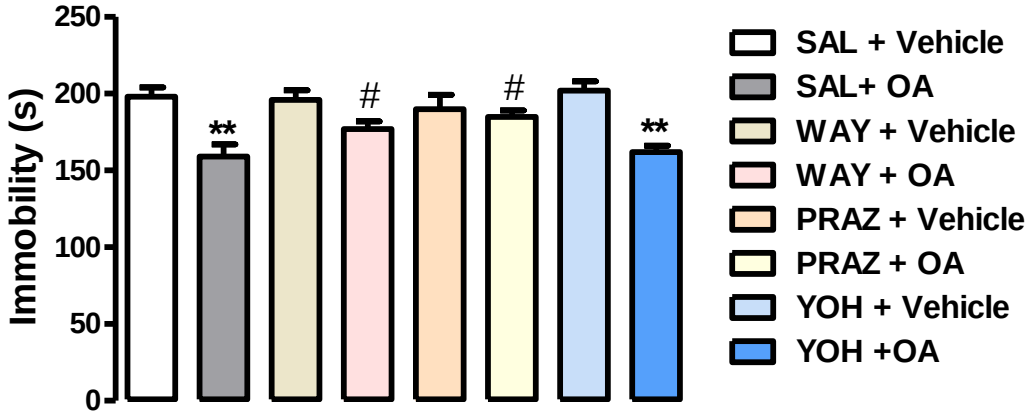

Figure 7. Behavioral response [Immobility time] to OA 20 mg/kg or vehicle 10 mL/kg (p.o) after pretreatment with SAL 10 mL/kg, WAY100635 (WAY) 0.3 mg/kg, prazosin (PRAZ) 1 mg/kg or yohimbine (YOH) 2 mg/kg (i.p). Data are expressed as mean ± SEM, n= 10 (two way ANOVA followed by Bonferroni test). **$p < 0.01$ vs vehicle treated group; # $p < 0.05$ SAL + OA versus WAY + OA, PRAZ + OA or YOH + OA (two way ANOVA followed by Bonferroni test).

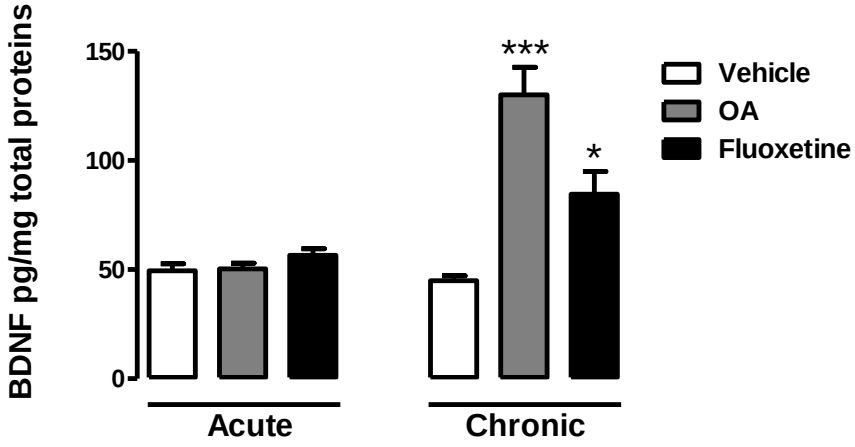

Figure 8. Bar graph showing the effect of oral administration (acute and chronic) of oleanolic acid – OA 20 mg/kg, fluoxetine 20 mg/kg, or Vehicle 10 mL/kg on the level of hippocampal BDNF. Data are expressed as mean of pg/mg of protein ± S.E.M, n = 6. Value of *p<0.05 or ***p < 0.001 vs vehicle treated group (two way ANOVA followed by Bonferroni test).

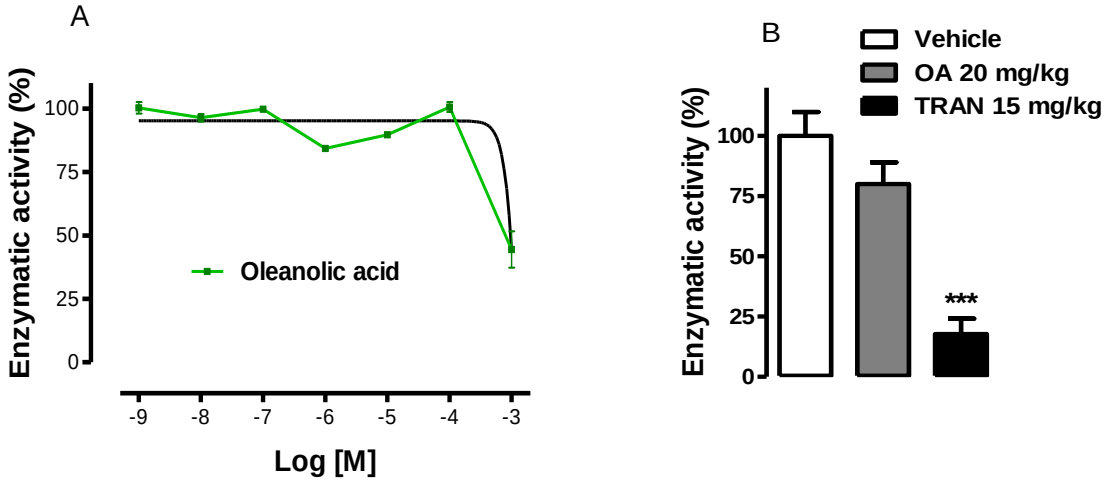

Figure 9. (A) *In vitro* measurement of MAO activity, (B) *ex vivo* measurement of MAO catabolic. Data are expressed as mean enzymatic activity ± SEM (%), n= 5 (one way ANOVA followed by Dunnett´s test). TRAN – tranylcypromine

Discussion

The dose range in our study was estimated from the relative composition of OA in the organic extract that was studied in our laboratory. The dose of organic extract was extrapolated from popular application of the leaf extract as a calming agent (Fajemiroye et al., 2012). In the current study, statistical analysis of time it takes the animal to lose their righting reflex (sleep latency) was not altered by OA administraton. The duration of sodium pentobarbital induced hypnosis was potentiated by OA in a dose dependent manner. According to Fujimori (1965), an agent that prolongs hypnotic effect of barbiturate is considered as a CNS depressant. This result is relevant to present investigations since a CNS stimulant could enhance animal performance in the FST test to produce a false positive effect.

Oral administration of OA (10, 20, or 40 mg/kg) prior to to LDB testing did not alter the number of transition in this apparatus. In contrast, OA 10 or 20 mg/kg elicited an increase in time spent at the light area of LDB. According to Young and Johnson (1991), the measurement of time spent in the light zone is the most consistent and useful parameter for assessing antianxiety - like activity in the LDB. OA at 40 mg/kg did not alter the time spent at the light area of LDB. Though it is intriguing to substantiate the contributing factor to the loss of anxiolytic - like effect of OA at 40 mg/kg, a reduction in the number of transition and the degree of CNS depression (as suggested by the potentiation of hypnotic effect – $p < 0.001$) suggest sedative effect at 40 mg/kg.

Furthermore, mice were exposed to EPM in order to investigate the antianxiety - like effect of OA . In this model, the normal tendency of the animals to stay in the closed arms can be enhanced by compounds that promote open arms inherent aversion.

Aversive response of the mice in this paradigm can be associated with the anxiety provoking or aggravating stimuli such as novelty, height, open space among other factors. However, an anxiolytic agent reduces the aversive behaviour to open arms (Hogg, 1996). In the present study, mice treated with OA increased the time spent in the open arm of EPM without altering the number of open arm entries. The effects of OA seem to have reached a plateau at 20 mg/kg in both the LDB and EPM. Our data showed that OA 10 and 20 mg/kg did not alter the number of transition in the LDB and open arm entry in EPM (spatial parameters in these models). Considering this spatial behaviour, we hypothesized that the anxiolytic like property of OA was predominated by temporal parameters.

The slight inconsistency of the anxiolytic like effect of OA in relation to the organic extract (Fajemiroye et al., 2012) could be attributed to the synergistic effect of the mixture of OA and other phytoconstituents. The process of isolation could perhaps leads to a change in the chemical form of OA (unconjugated or conjugated form). OA can occurs as a free acid or aglycone precursor in which it is linked to sugar chains (Jacob and Alain 2012; Liu, 1995, Szakiel et al., 2003 and 2005). The observed differences in the biological activity of OA could also be associated with distinctive underlying neural mechanism of OA and organic extract.

Moreover, in order to investigate antidepressant - like property of OA, mice were subjected to FST. Oral administration of OA produced a reduction in immobility time 5 - 20 mg/kg. In an attempt to satisfy face validity of this model and verify if a prolonged treatment could enhance anti - immobility response, we conducted chronic oral administration of OA before the exposure of animals to FST. However, a single oral

dose of OA elicited anti – immobility effect with the same level of significance as compared to chronic administration. In this model, the effect of OA at 40 mg/kg was not significant.

In this study, the antidepressant - like effect of the OA (5 - 40 mg/kg) was also evaluated in the TST. The data in this model were consistent with that of FST as the peak effect of OA was observed at 20 mg/kg. At 40 mg/kg, OA showed tendency of a reduction in immobility time even though it was not significant. Although The FST and TST are widely used as animal models for screening antidepressant activity of drugs (Cryan et al., 2005), their sensitivity to the pharmacological effects of drugs varies. According to Cryan et al. (2005), both the FST and TST are similar in the constructs that they assess even though the biological substrates that underlie the observed behaviour may be different. These models often offer converging data on a potential antidepressant (Porsolt, 2000; Renard et al., 2003).

In the open field paradigm, OA 40 mg/kg produced a significant reduction in the number of sector traversed (crossing) and number of rearing activities. Though OA reduced the number of rearing activities at 20 mg/kg, the number of sector traversed remained unaltered at this dose. Diazepam at 1 mg/kg showed slight increase in the number of sector traversed and reduced the number of rearing activities. At 5 mg/kg, diazepam reduced both of these parameters in the open field. These results suggest that OA at 40 mg/kg interfered with locomotor activity of the animal.

The open field data and other animal models in this study demonstrate the critical role of dosage to biological response. On the basis of repeated experiments, there emerges clear and consistent evidence indicating that OA at 40 mg/kg could not

produce antidepressant - like effect. The insignificance effect of OA at 40 mg/kg in both the FST and TST could be attributed to myorelaxant or sedative effect as clearly observed in the open field. Profound potentiation of barbiturate sleep and reduction in the number of transitions in the LDB reinforce the suggestion of an interference with locomotion activity of animal. In contrast, antidepressant - like property of OA at lower doses is devoid of stimulatory effect by using the data on parameters like sleep duration, number of entry in the LDB and number of open arm entry in the EPM, number of sector traversed and number of rearing in the OF.

Meanwhile, unravelling the U-shaped dose - response in the FST and TST still poses some challenges. The phenomenon of U-shaped pattern of response could be a model - dose - induced phenomenon (MDIP) since, unlike FST and TST, OA produced a dose dependent response in the barbiturate sleep test and EPM model. The hypothesis of MDIP could explain not only the U-shaped dose - response but also the loss of effect at the highest dose of OA (40 mg/kg) in the FST and TST. The sedative effect at 40 mg/kg could have potentiated the hypnotic effect of sodium pentobarbital and enhanced dose dependent effect in the barbiturate sleep - induction model. In contrast, an anti - immobility property in FST and TST is sensitive to an agent or drug dose that interferes with locomotor activities. In order to explain the appearance of u-shaped dose-response curve in these models of depression, a biphasic effect of OA could also be hypothesized (i.e a dose dependent effect up till optimal dose and a loss of anti - immobility property at supra optimal dose). The hypothesis of MDIP and biphasic effect still need to be study extensively with OA or any other drugs that share similar pharmacological profile.

The peak effect of OA at 20 mg/kg in the present study is in agreement with the optimal effect of this compound at this dose as reported by Yi et al (2013). We also agree that an optimal dose in mice may not necessarily translate to an optimal response in the clinic. According to the Food and Drug Administration (Center for Drug Evaluation and Research, 2012), the extrapolation of animal dose to human dose is correctly performed by: dose administered to animal x animal Km/human Km. The value of Km is derived through the division of body weight by body surface area - BSA (m^2). The comparison of the division of average weight of the mice in our study (20 g or 0.02 kg) by the equivalent value of BSA - 0.007 m^2 (Center for Drug Evaluation and Research, 2012) and the division of the average normal human weight 60 kg by the equivalent value of BSA 1.6 m^2 shows that Km value of human is 12.3 times higher. Hence, the dose administered in human is expected to be 12.3 times lesser than the one in mice.

The mechanism of anxiolytic drugs are commonly linked with gabaergic system (especially $GABA_A$ receptors). These receptors have been associated with the binding sites of DZP (Squires et al., 1979). According to Gielen et al. (2012), agonists of benzodiazepine binding site could potentiate the effect of $GABA_A$ by increasing the apparent affinity of $GABA_A$ receptor for GABA. In this study, we conducted a preliminary screening of subconvulsive doses of PTZ to determine a non-anxiogenic dose that is capable of attenuating anxiolytic like effect of DZP. The pretreatment of PTZ 20 mg/kg (a subconvulsive and non-anxiogenic dose) blocked the anxiolytic like effect of DZP in the LDB. However, the anxiolytic like effect of OA was not blocked by PTZ pretreatment. Although the binding site on $GABA_A$ and precise definition of its pharmacological role still remain contentious, PTZ is considered to be a competitive antagonist of $GABA_A$

receptor (Huang et al., 2001). Our results suggest that $GABA_A$ receptor was not involved in the anxiolytic like effect of OA.

The present study also evaluated the involvement of receptors, metabolic processes and neurotrophic factors in the antidepressant - like effect of OA. In order to ensure that the pretreatment of drugs did not interfere with the locomotion activity of the animal, we carried out a preliminary test in the open field after the treatment of animals with PCPA, AMPT, NAN-190, WAY100635, prazosin and yohimbine (data not shown). These pharmacological tools did not alter the number of sector traversed (crossing) and number of rearing activities at the dose tested. In this study, pretreatments with PCPA, AMPT or NAN-190 abolished the anti-immobility response to OA treatment.

Since studies have shown that NAN-190 could also block α1 and α2-adrenoceptors besides 5-HT1A, we employed prazosin (α1 – adrenoceptor antagonist), yohimbine (α2 – adrenoceptor antagonist) and WAY100635 (a selective antagonist at the 5-HT1A receptors) pretreatments to investigate the participation of these receptors in the antidepressant - like effect of OA. Unlike yohimbine, prazosin pretreatment attenuated the anti - immobility effect of OA in the FST. Hence, these results suggest the contribution of α-1 adrenergic receptors to antidepressant - like effects of OA. Further experiments also examined the participation of 5-HT1A receptors in the antidepressant - like effects of this compound. The administration of WAY100635 blocked the anti - immobility effect of OA in the FST, thereby suggesting the participation of this 5-HT1A receptor subtype in the antidepressant - like effect of OA.

The function of 5-HT1A receptors have been associated with the overlapping abnormalities in anxiety and depression, and explain the comorbidity of these

psychiatric disorders (Nutt and Stein, 2006). The data in behavioural models suggest the involvement of monoamine and complex interaction with biological system. The blockade of cathecolamine transport system may provide a more conclusive behavioural alteration as previous work showed unaltered level of 4-hydroxy-3-methoxyphenylglycol and 3,4-dihydroxyphenylacetic acid metabolites in the brain hippocampus and cortex after administration of OA (Yi et al., 2013). The inconsistency in the results from behaviour and HPLC assay by Yi and colaborators (2013) further support the plurality of the effects of OA.

The data of *in vitro* and *ex vivo* MAO assays showed that the activity of MAO remained unaltered by OA. The seemingly reduction in MAO activity *in vitro* at 1 mM could be considered to be unspecific or a precipitation of protein since this compound did not demonstrate effective inhibition at low concentrations. Ineffectiveness of OA on MAO activity could explain unaltered level of 5-hydroxyindoleacetic acid (5-HIAA) as reported by Yi et al (2013). However, an increase in 5-HT level (assessed HPLC analysis) in frontal cortex and hippocampus could be attributed to platelets induced release of serotonin (Lee et al., 2007).

Despite the acceptance of monoamine hypothesis of depression in an attempt to provide a pathophysiologic explanation of the actions of antidepressants, there are still some vital issues such as why the antidepressant effect of drugs are always delayed? Why antidepressants are also effective in anxiety disorders? Alternatively, why all drugs that enhance monoamine transmission are sometimes not effective in depression? In an attempt to elucidate the mechanism of antidepressant drugs, the neurotrophins have been associated with the pathophysiology of depression and the mechanisms of

antidepressant drugs (Duman, 2004; Martinowich et al., 2007). In the present study, since the acute administration of OA induced antidepressant – like effect without upregulation of hippocampal BDNF, we hypothesized that the effect of OA was independent of hippocampal BDNF level. This hypothesis is further supported by the chronic administration of OA that elicited an increase in hippocampal BDNF without any significant improvement on the antidepressant like effect as compared to acute treatment that did not alter the level of BDNF.

Currently the therapeutic application of OA or its derivatives is still very limited. The pharmacological potential of OA may introduce new class of antidepressant drugs in clinical practices. The challenges to the treatments of anxiety and depression are associated with the fact that the available drugs are still far from producing optimal effects in several patients. In addition, several cases of side effects and non-adherence to chronic administration of the conventional antidepressant have remained largely unresolved by medical practitioners. A widely prescribed drug like fluoxetine (antidepressant drug and serotonin selective reuptake inhibitor) induces several undesirable effects and possesses pharmacotherapy limitations. The diazepam (agonist of benzodiazepines site and a potent anxiolytic drug) produces side effects like sedation, amnesic effect, tolerance and withdrawal symptoms (Garner et al., 2009). Despite the desire of producing a greater efficacy through drug combination, many patients still respond poorly. The complexity of the pathophysiology of this diseases and plurality of underlying mechanism of anxiety and depression make OA not only a potential therapy but also as a pharmacological and chemical tool.

Our findings showed evidences of anxiolytic and antidepressant - like properties of OA and suggested monoamine mechanism. As our data are not sufficient to exclude the possibility of side effects that could emanate from multiple interaction of this biomolecule, its susceptibility to chemical modification offers limitless opportunity towards the synthesis of anxiolytic and antidepressant drugs with desirable pharmacological profile.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Anexo 6. Anxiolytic and antidepressant like effects of natural food flavour (E) - methyl isoeugenol

Abstract

(E) - methyl isoeugenol (MIE) is a natural food flavour that constitutes 93.7 % of its essential oil *Pimenta pseudocaryophyllus* leaf. The leaf extracts of this species are used as a calming agent. As a ubiquitous food additive, application of MIE in treating mood disorders seems to be globally attractive. Hence, we sought to evaluate general pharmacological activities, anticonvulsant, anxiolytic and antidepressant like effects and possible mechanisms of MIE actions. Administration of MIE was carried out prior to the exposure of male Swiss mice to general behavioural tests, barbiturate sleep, PTZ - induced convulsion, light dark box - LDB, elevated plus maze - EPM, wire hanging, open field - OF and forced swimming test - FST. Involvement of monoamine system was studied through mice pretreatment with WAY100635 (antagonist of 5-HT1A), α-methyl-p-tyrosine (AMPT; depletor of catecholamine) or p - chlorophenylalanine (PCPA; serotonin depletor storage). There was no record of neurotoxic effect or animal's death in the course of general pharmacological tests. MIE at 250 and 500 mg/kg potentiated hypnotic effect of sodium pentobarbital. However, MIE did not protect against PTZ - induced convulsion. Except for MIE at 500 mg/kg, parameters evaluated in the LDB, EPM and OF demonstrated anxiolytic like property of MIE. This effect was blocked by WAY100635 pretreatment. MIE at 500 mg/kg elicited a reduction in locomotor activity of the mice in the OF. Anti - immobility effect of MIE 250 mg/kg in the FST suggested its antidepressive like property. Unlike AMPT, pretreatment with PCPA reversed antidepressant like effect of MIE. Our findings demonstrated anxiolytic and

antidepressant like properties of (E)-methyl isoeugenol and suggested the participation of serotonergic pathways.

Keywords: food flavour, (E) - methyl isoeugenol, serotonergic pathways, anxiety, depression

1. Introduction

Mood disorders belong to the most common psychiatric diseases with lifetime prevalence of up to 20% worldwide.[1] Considering the low remission rates with current treatments (about 30%) and high rate of non-response to the currently available first-line medication, the development of new therapeutic agents becomes a necessity.[2,3] The cases of non-adherence to prolong treatment of these diseases[4] could be overcome through the consumption of a functional food. This food could provide basic nourishment and health benefit.[5,6] Since time immemorial, Plants of medicine and food. Plant resources in traditional societies, especially wild greens, serve dual purposes as food and medicine.[7] Studies on the potential health benefit aspects of traditional foods show that such plants have specific pharmacological effects.[7] The gathering or cultivation, preparation, and consumption of these species are rooted in the emic perceptions of the natural environments coupled with available resources, local cuisine and medical practices, taste appreciation, and cultural heritage.[8-15] The links between food and medicine among different cultures were evident in the superb work of Etkin and Ross[16] on the medicinal plant uses among the Hausa ethnic group in Nigeria, where out of 235 noncultivated medicinal plants, 63 taxa were also used as food. Studies have demonstrated how the overlap of food and medicine are related to the ingestion of phytochemicals and explain diverse cultural food behaviours and health outcomes[9,17-20.]

Over several decades, essential oils (also known as volatile oils) from plants have been used in the form of aromatherapy to balance the mind, body and spirit as well as to prevent or cure diseases.[21] Popular use of aromatic plants for healing cut

across many cultures, including ancient China, India, and Egypt.Essential oils and their isolated compounds have been reported to possess psychotropic effects.[22] The essential oil mixture of the Chinese herbal prescription SuHeXiang Wan (SHXW) and the *Aconus gramineus* rhizome protect against epilepsy.[23,24] Barocelli and collaborators[25] demonstrated an analgesic activity of the essential oil of Lavender. Reinaldo has documented anticonvulsant activity of essential Oils and their Constituents in his work.[30] This documentation showed that common essential oil constituents such as eugenol, methyleugenol, isoeugenol[26] possess anticonvulsant property in experimental models. α-Asarone, a phenylpropanoid, also presented effective anticonvulsant activity.[27] Sell and Carlini demonstrated anesthetic action of methyleugenol and other eugenol derivatives found in the volatile oil fraction of *Myristica pagans* in mice.[28]

Paula and collaborators reported the presence of a phenylpropanoid derivative (E) - methyl isoeugenol (MIE) and its predominance (93.9%) in the essential oils of *Pimenta pseudocaryophyllus*.[29] The characteristic fragrances of this species have been attributed to the presence of MIE.[31] Previous ethnopharmacological and neuropharmacological studies have reported nerve tonic and calming properties as well as *anxiolytic* and antidepressant like activities of an organic extract and essential oils of *P. pseudocaryophyllus*.[32-35] Hence, in the present study we sought to evaluate the effect of MIE on the CNS (depressive or stimulatory) and investigate anticonvulsive, antianxiety and antidepressive like properties of MIE. The neural mechanisms of MIE were studied by using appropriate pharmacological tools.

2. Material and Methods

2.1 Drugs and Treatment

(E) - methyl isoeugenol (MIE; Sigma-Aldrich, St. Louis, MO, USA), diazepam (DZP; Cristália, Itapira, SP, Brazil), buspirone (BUS; Cristália, Itapira, SP, Brazil), pentylenetetrazole (PTZ; Sigma-Aldrich, St. Louis, MO, USA), imipramine (IMI; Cristália, Itapira, SP, Brazil), p-chlorophenylalanine (PCPA; Sigma-Aldrich, St. Louis, MO, USA), α-methyl-p-tyrosine (AMPT; Sigma-Aldrich, St. Louis, MO, USA), Polyoxyethylenesorbitan monooleate (Tween 80; Sigma-Aldrich, St. Louis, MO, USA), N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclohexanecarbox-amide (WAY100635 or WAY; Sigma-Aldrich, St. Louis, MO, USA), were used in the present study. Drugs were prepared freshly and dissolved in a vehicle [a mixture of 0.9% NaCl and 2% (v/v) Tween-80 (polyoxyethylene sorbitan monooleate)]. Mice received 0.1 mL per 10 g b.wt. (10 mL/kg) orally. All control animals received vehicle on the same regimen as the treated groups.

2.2 Animals

Experimental animals were male Swiss mice (27 - 35 g) provided by central animal house, Federal University of Goiás. Animals were kept for acclimatization under 23 ± 2°C (12 hr light-dark cycles) with access to standard diet and water *ad libitum*. Experiments were carefully conducted by a trained researcher to minimize animal´s pain or distress in compliance with the experimental protocol (number 104/08) as approved by the Ethical Committee of the Federal University of Goiás and in agreement with the relevant national and international laws.[36]

2.3 Pharmacological approaches

2.3.0 General pharmacological test

This test was conducted by using a modified method that was adopted by Malone.[37] This preliminary test permits us to observe general behavioural change, estimate effective doses in our subsequent tests and report any sign of MIE - induced toxicity. Animals were treated through subcutaneous - s.c, intraperitoneal - i.p, or oral - p.o route with MIE (4, 20, 100 or 500 mg/kg) or vehicle and observed periodically for 7 days.

2.3.1 Sodium pentobarbital sleep induction

Mice (n = 10) were treated orally with vehicle 10 mL/kg, MIE (125, 250 or 500 mg/kg) or diazepam (1 mg/kg) 1 hour prior to the intraperitoneal administration of sodium pentobarbital (50 mg/kg). Sleep latency and duration (time to the loss of righting reflex and voluntary recovery of the righting reflex, respectively) were recorded as parameters to assess the depression or stimulation of CNS*.*

2.3.2 Pentylenetetrazol-induced seizure test

The anticonvulsant activity of MIE was evaluated by using the model of pentylenetetrazol-induced seizure. Mice were randomly divided into five groups (n = 10) and subjected to oral administration of vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 3 mg/kg). After 1 hr of drug administrations, pentylenetetrazol (PTZ 70 mg/kg i.p.) was administered to each animal. Behavioural changes in the animals were videotaped for 30 minutes and analyzed later. Parameters like latency or threshold to the first myoclonic, duration of the seizure were recorded. The survival (%) is calculated by using the formula; [(N - nd)/N] X 100 where N indicate total number of animal; nd, the number of death recorded. The severity of the seizure was taking as a

measure of collective changes in mice behaviour (myoclonic jerks, vocalization, straub, akinesia, tremor, leap, paralysis, clonic seizure, rigidity and tonic extension of the hind limbs with death). A trained researcher scored each of this behavioural parameter.

2.3.3 Light dark box test (LDB)

Mice were treated orally with vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 1 mg/kg). The animals were placed at the centre of the light area facing the opening of the dark area after 1 hr of oral treatment. The number of transitions between the two compartments and the time spent in the light area were recorded for 5 min.[38]

2.3.4 Elevated plus maze test (EPM)

Groups of mice (n=10) were treated orally with vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 1 mg/kg). The animals were later placed individually at the centre of the plus maze (after 1hr of oral administration) and observed for 5 min.[39] The time spent and the numbers of entries into the open arms were recorded for statistical analysis.

2.3.5 Wire Hanging Test

The wire hanging test is an in vivo preclinical model to evaluate pharmacological effect of drugs on motor function (motor impairment or coordination) of experimental animal. Mice were randomly divided into five groups (n = 10) and subjected to the oral administration of vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 3 or 5 mg/kg). The test begins with the animal hanging from an elevated wire by their forepaws at a height of ~20 cm above the floor to prevent the animal from climbing down. The animal is placed at the centre of the wire; the time that elapsed until the

animal fell was recorded three times and the cutoff time was set at 60 s. The latency to the falls was recorded and analyzed.

2.3.6 Open field exploratory activity

After oral administration of MIE (125, 250 or 500 mg/kg), diazepam (DZP 1 mg/kg) or vehicle, mice were exposed to a circular open field (a 50 cm high wooden wall with the division of the base area of 62.80 cm^2 into 8 equal sectors). The apparatus was clean up with 10 % alcohol at the end of each experiment. Parameters like total crossing, immobility time, number of grooming, rearing activity, crossing at the centre and time spent at the centre were scored in the course of 5 min and later analyzed statistically.

2.3.7 Forced Swimming Test

The detail of the FST in the present study has been described in our previous study.[35] All animals were subjected to swimming for 6 min, and the duration of immobility was recorded during the final 4-min interval of the test. The immobility period was considered to be the time spent by the mouse floating in the water and making only those movements necessary to keep its head afloat. The test sessions were recorded by a video camera while the parameter (immobility time) was later scored and analyzed.

2.3.8 Mechanism of anxiolytic like effect of MIE

After 30 minutes of N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclo-hexane-carboxamide - WAY100635 0.3 mL/kg, i.p or NaCl 0.9% - SAL, i.p pretreatments, mice were treated orally with vehicle 10 mL/kg or MIE 250 mg/kg prior to their exposure to EPM.

2.3.9 Mechanism of antidepressive like effect of MIE

In experiment 1, mice were pretreated intraperitoneally with NaCl 0.9% - SAL or AMPT 100 mg/kg and treated orally with vehicle or MIE (250 mg/kg) after 4 hours interval. In experiment 2, mice were pretreated intraperitoneally with NaCl 0.9% - SAL or PCPA 100 mg/kg for four consecutive days prior to vehicle or MIE treatments. Following 1 hour of oral treatment in both experiments 1& 2, animal were subjected to forced-swimming test.

2.4 Statistics analysis

Parametric data are expressed as means ± S.E.M following appropriate statistical analysis (Unpaired Student's *t*-test, one way ANOVA followed by the Dunnett´s test as post hoc test or two way ANOVA followed by the Bonferroni as post hoc test. Analysis of convulsions severity scores was realized by using Kruskal-Wallis test followed by Dunn's multiple comparison tests. Quantitative data are expressed as the median and interquartile range (Q1–Q3). Significance difference between or among groups were set at $p < 0.05$.[40]

3. Results

3.1.0 General pharmacological test of MIE effect on mice

In the general pharmacological test, the effects elicited (abdominal contortion, environmental alienation, ataxia, sedation, analgesia, loss of paw grip, an increase and a reduction in exploratory activity by MIE 100 or 500 mg/kg were time and route of administration (s.c, i.p or p.o, table 1) dependent. At MIE 500 mg/kg, we observed sedation, analgesia and loss of paw grip via s.c. In addition, at this dose, sedation was observed via i.p or p.o route (table1). However, all these behavioural manifestations

disappeared after 4 hours. These pharmacological effects of MIE did not lead to animal death in the course of 7 day - observation irrespective of the administration route and dose (table1).

3.1.1 Effect of MIE on sleep induced by sodium pentobarbital

The administration of diazepam 1 mg/kg or MIE 500 mg/kg elicited a decrease in the sleep latency ($F (4, 42) = 7.5$, $p < 0.001$, one way ANOVA, fig. 1A). Also fig. 1B demonstrated a dose dependent increase in sleep duration after oral treatment of MIE [$F (4, 42) = 9.6$, $p < 0.001$, one way ANOVA]. The Dunnett´s post-hoc test revealed a significant increase in sleep duration by MIE 250 mg/kg ($p < 0.05$) and MIE 500 mg/kg ($p < 0.001$).

3.1.2 Pentylenetetrazol-induced seizure test

One way analysis of variance showed significant increase in the latency to the myoclonic convulsion [$F (4, 45) = 11.04$, $p < 0.001$, fig. 2A]. Post-hoc test (Dunnett´s test) did not show significant alteration in the latency to the myoclonic convulsion by MIE treatment 125, 250 or 500 mg/kg ($p > 0.05$), unlike diazepam - DZP 3 mg/kg ($p < 0.001$) as compared to the vehicle treated group (fig 2A). One way analysis of variance showed a change in seizure duration [$F (4, 45) = 13.93$, $p < 0.001$ fig. 2B]. Except for DZP 3 mg/kg ($p < 0.001$), seizure duration was not altered significantly by MIE treatments ($p > 0.05$, Dunnett´s post hoc test, fig. 2B). The severity of convulsion [fig. 2C, represented by median (25^{th} percentile – 75^{th} percentile)] induced by PTZ was not influenced significantly by MIE administration [MIE at 125 mg/kg, 15.5 (11-17); 250 mg/kg, 18 (14 – 25.5); 500 mg/kg, 20 (15.5 – 23.0); however, DZP 3 mg/kg, 6.5 (3.2 – 9.7) shows a significant decrease in severity as compared to vehicle treated group 15.5

(10.7 – 21.2). Also, % of animal protected by the administration of MIE (125, 250 or 500 mg/kg) dwindled (60, 40 and 30 %, respectively) or DZP 3 mg/kg – 100 % as compared to the vehicle treated group 60 % (fig. 2D). The scoring of seizure severity and protection against its occurrence is displayed on table 2.

3.1.3 MIE effects on mice behaviour in the light dark box - LDB

The treatment with MIE (in different doses) increased the number of transition with $F(4, 35) = 6.67$, $p < 0.001$, fig. 3A and time spent in the light area of the light-dark box with $F(4, 35) = 6.19$, $p < 0.001$, fig. 3B (one way ANOVA). The Dunnett post-hoc test showed a significant increase in transition by MIE at 125 mg/kg ($p < 0.05$) and 250 mg/kg ($p < 0.01$). The reference drug diazepam 1 mg/kg increased ($p < 0.01$) both of these parameters (fig. 3A & B).

3.1.4 Behavioural alterations elicited by MIE in the elevated plus maze - EPM

In the elevated plus maze, MIE administration at the dose of 500 mg/kg reduced total arm entries ($p < 0.05$) with $F(4, 45)$ value of 3.86 (one way ANOVA fig. 4A). The number of open arms entries was altered significantly [$F(4, 45) = 3.48$, $p < 0.05$, one way ANOVA, fig. 4B] by MIE 250 mg/kg and diazepam 1 mg/kg treatment ($p < 0.05$); Also, the time spent on the open arms was increased [$F(4, 45) = 4.99$, $p < 0.001$, one way ANOVA, figure 5 C] by MIE 125 mg/kg ($p < 0.05$), 250 mg/kg ($p < 0.01$) and diazepam 1 mg/kg ($p < 0.001$).

3.1.5 Effect of MIE on mice performance in the wire hanging test

MIE administration did not elicit significant changes in the values of latency of fall as represented by median (25^{th} percentile – 75^{th} percentile) on figure 5 [MIE 125 mg/kg, 25.0 (11.5 – 35.5); 250 mg/kg, 18 (10 – 54.7); 500 mg/kg, 7.5 (6.7 – 17.0)] or DZP 3

mg/kg 18.0 (14 – 53.4). However, DZP 5 mg/kg [8.0 (6.7 – 11.2)] reduced this parameter significantly as compared to the vehicle treated group 37.5 (9.0 – 52.5).

3.1.6 Effect of MIE on mice behaviour in the open field

The parameters evaluated in the open field were altered significantly by MIE or diazepam treatments; total crossing in the open field [$F(4, 45) = 8.07$, $p < 0.001$, fig 6A], freezing time [$F(4, 45) = 5.14$, $p < 0.01$, fig. 6 B], grooming activity [$F(4, 45) = 3.17$, $p < 0.05$, fig. 6 C], number of rearing [$F(4, 45) = 4.37$, $p < 0.05$, fig. 6 D], time spent at the centre of open field [$F(4, 45) = 4.18$, $p < 0.01$, fig. 6 E], and crossing at the centre of open field [$F(4, 45) = 4.81$, $p < 0.01$, fig. 6F] by using one way ANOVA. MIE 500 mg/kg reduced total crossing ($p < 0.05$) and number of rearing ($p < 0.01$) while the freezing time was increased ($p < 0.01$); Both MIE 250 mg/kg and diazepam 1 mg/kg reduced the number of grooming ($p < 0.05$ and $p < 0.01$, respectively). MIE 125, 250 mg/kg and diazepam 1 mg/kg increased the number of crossing at the centre of the open field ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively). The time spent at the centre of the open field was increased by MIE 250 mg/kg and diazepam 1 mg/kg ($p < 0.05$ and $p < 0.01$, respectively).

3.1.7 MIE effect on mice performance in the Forced Swimming Test

MIE or IMI administration elicited significant alteration in the immobility time in the FST [$F(4, 45) = 5.27$, $p < 0.01$, fig. 7]. Dunnett post hoc test showed significant reduction in immobility time by MIE 250 mg/kg ($p < 0.05$) and IMI 30 mg/kg ($p < 0.01$).

3.1.8 Mechanism of anxiolytic like property

The effect of pretreatment (SAL and WAY100635 - independent variables) and treatment (Vehicle, MIE 250 mg/kg and BUS 10 mg/kg – independent variable) on time

spent in the open arms (dependent variables, fig. 8A) and the percentage of open arms entries (dependent variables, fig 8B) of the EPM were demonstrated. The data obtained on time spent in the open arms of the EPM revealed interaction between the independent variables [$F(2, 54) = 6.39$, $p < 0.01$, two-way ANOVA]. In fig 8A, Bonferroni post hoc test showed an increase in the time spent in the light compartment by MIE [i.e SAL + MIE vs SAL + Vehicle, $p < 0.05$] and buspirone – BUS treatments [i.e SAL + BUS vs SAL + Vehicle, $p < 0.05$]. However, the effect of both MIE and BUS on this parameter was blocked completely by WAY100635 pretreatment [i.e SAL + MIE vs WAY100635 + MIE and SAL + BUS vs WAY100635 + BUS, $p < 0.05$]. The data obtained on the percentage of open arms entries revealed interaction between the independent variables [$F(2, 54) = 25.44$, $p < 0.01$, two-way ANOVA]. In fig 8B, Bonferroni post hoc test indicated an increase in the percentage of open arms entries in the groups SAL + MIE ($p < 0.05$) and SAL + BUS ($p < 0.05$) as compared to the group that received SAL + Vehicle (control group). The effect of BUS and MIE on the percentage of open arms entries were attenuated by WAY100635 pretreatment [i.e SAL + BUS vs WAY + BUS and SAL + MIE vs WAY + MIE, $p < 0.05$, respectively].

3.1.9 Mechanism of antidepressive like property

Figure 9A showed the effect of pretreatment (SAL or AMPT - independent variable) and treatment (Vehicle or MIE 250 mg/kg – independent variable) on the immobility time (dependent variables) in the forced swimming test - FST. The data obtained did not demonstrate interaction between the independent variables [$F(1, 36) = 6.02$, $p > 0.05$, using a two-way ANOVA] on the immobility time. Bonferroni post hoc test showed a decrease in immobility time in the groups SAL + MIE as compared to control group (i.e

SAL + Vehicle, $p < 0.05$). AMPT did not reverse the anti-immobility effect of MIE (i.e SAL + MIE versus AMPT + MIE showed a p value > 0.05). Figure 9 B showed the effect of pretreatment (SAL or PCPA) and treatment (vehicle or MIE 250 mg/kg) on the immobility time in FST. The data obtained on immobility time did not show interaction between the independent variables [$F (1, 36) = 2.14$, two-way ANOVA, $p > 0.05$]. Bonferroni post hoc test showed a decrease in immobility time in the group SAL + MIE ($p < 0.05$) but not in SAL + PCPA ($p > 0.05$) as compared to the control group (i.e SAL + Vehicle). PCPA pretreatment blocked the anti-immobility effect of MIE (i.e SAL + MIE versus PCPA + MIE, $p < 0.05$).

Table 1. General pharmacological tests

Observation time after acute administration	Dose (mg/kg)	Administration routes/Observations		
		s.c	**i.p**	**p.o**
15min	**4, 20 or 100**	N	N	N
	500	Reduced exploration	N	N
30 min	**4 or 20**	N	N	N
	100	Ataxia, contortion	Environmental alienation	Increased exploration
	500	Sedation, analgesia, loss of paw grip,	Sedation	Reduced exploration
1hr	**4 or 20**			N
	100	Effects after 30 min of administrations persists		Increased exploration
	500			Sedation
4 hr – 7 days	Total recovery from the effects of MIE administration without sign of toxicity in the course of the 7 day observation			

N - No observable behavioural alteration as compared to vehicle treated group

Table 2. Parameters for the scoring of PTZ induced behavioural alterations

	Parameter	Score
1	Absence of convulsive behaviour	0
2	Myoclonic jerks	1
3	Vocalization	2
4	Straub	3
5	Akinesia	4
6	Tremor and leap	5
7	Paralysis of hindlimbs	6
8	Clonic seizures with loss of righting reflex	7
9	Rigidity and tonic extension of the hind limbs with death	8
Other parameters		
10	Latency to first myoclonic jerk	seconds
11	Duration of crisis	seconds
12	Survival or percentage of animals protected	[(N - nd)/N] X 100

N - total number of animal; nd - number of death recorded.

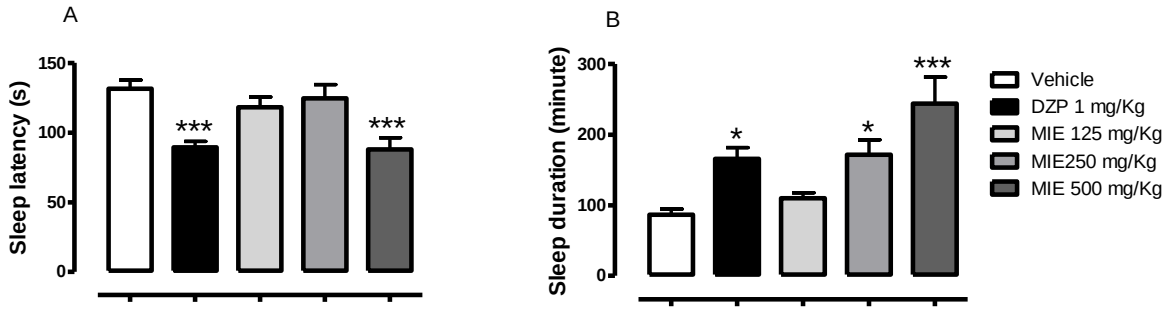

Figure 1. Effect of vehicle, diazepam – DZP 1 mg/kg or (E) - methyl isoeugenol (MIE) 125, 250 or 500 mg/kg on latency (A) and duration (B) of sodium pentobarbital (50 mg/kg) induced hypnosis. Results are expressed as mean ± SEM; n = 8-10 in each group. * and *** indicate $p < 0.05$ and $p < 0.001$ respectively as compared with vehicle treated group (One way ANOVA followed by Dunnett´s post hoc test).

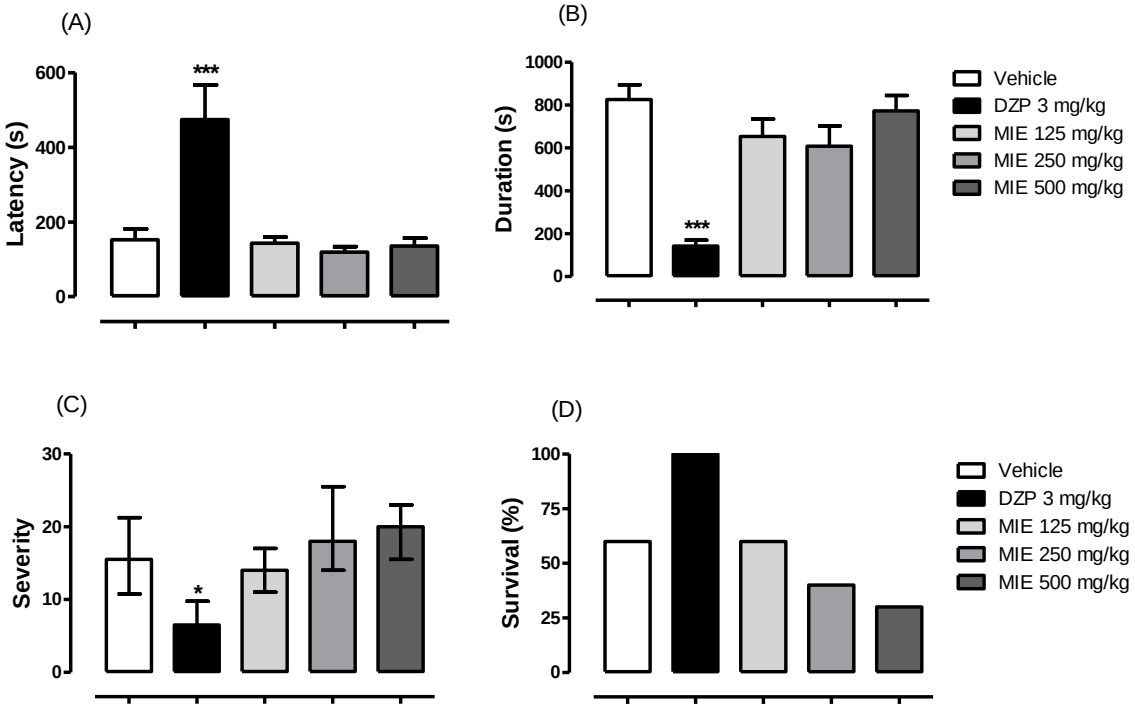

Figure 2. Data on the latency to the first myoclonic convulsion (A), and the duration of convulsion (B) were analyzed by one-way ANOVA followed by Dunnett as post hoc test. Data are represented as mean ± SEM, n=10; Non parametric data on the severity (C) were analyzed using Kruskal-Wallis test followed by Dunns as post hoc test (data are represented as median (25^{th} percentile – 75^{th} percentile), n=10. Bar graph (D) showed % of the animals that were protected against pentylenetetrazol (PTZ). * and *** indicate $p < 0.05$ and $p < 0.001$ respectively as compared with vehicle treated group

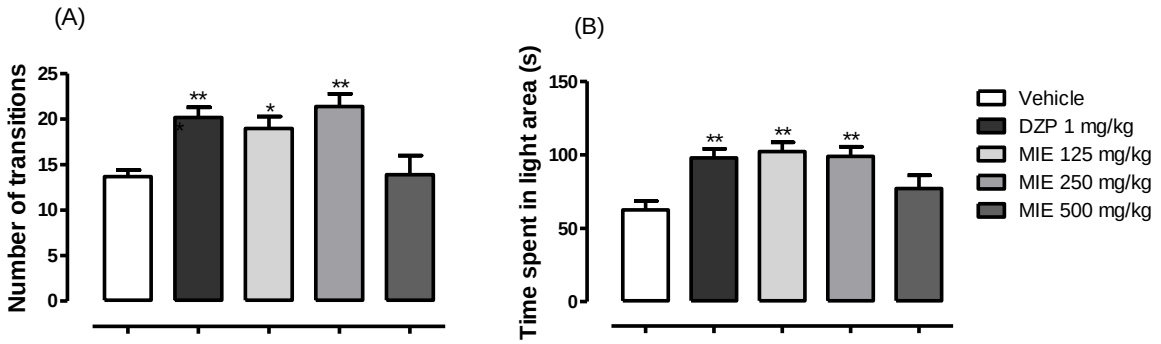

Figure 3. Effect of oral administration of vehicle, (E) - methyl isoeugenol (MIE) or diazepam (DZP) on the number of transition (A), and time spent in the light area (B) of the light dark box. Results are expressed as mean ± SEM; n = 8; *p < 0.05 and **p < 0.01versus vehicle treated group using one way ANOVA followed by Dunnett´s post hoc tests.

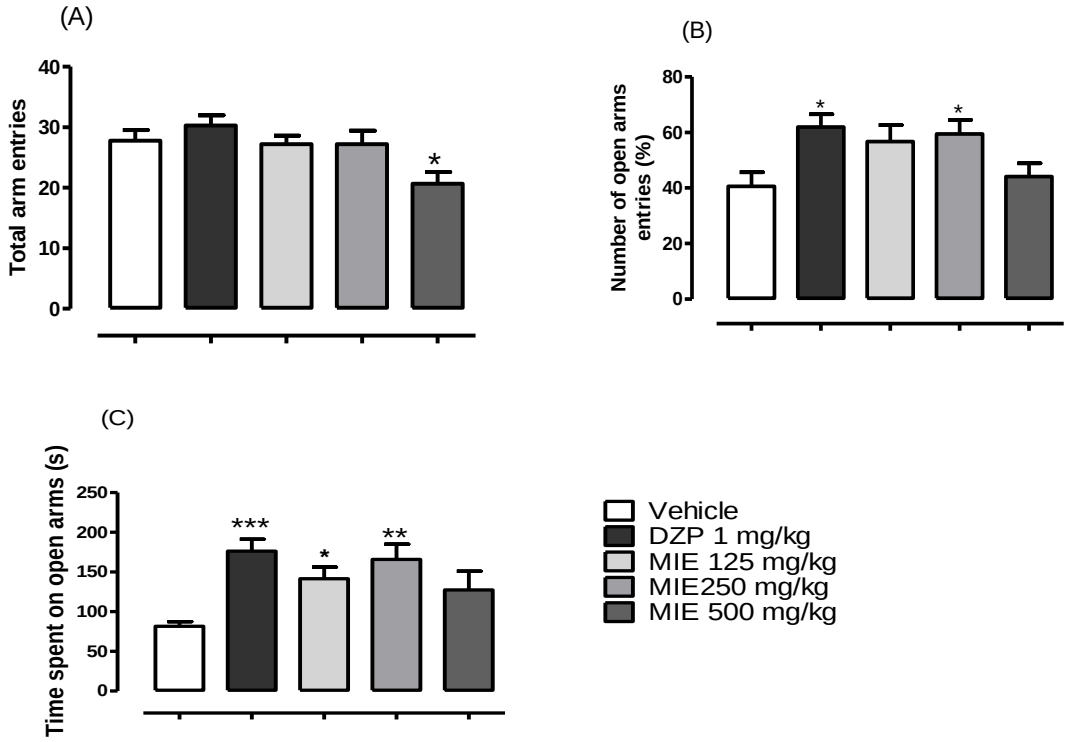

Figure 4. Effect of the oral administration of vehicle, diazepam (DZP), or (E) - methyl isoeugenol (MIE) on the mice behaviour in the elevated plus maze. Parameters like total arm entries (A), number of open arms entries (B), and time spent on the open arms (C) were evaluated. Results are expressed as mean ± SEM; n = 10; *p < 0.05, **p < 0.01 and ***p < 0.001 versus vehicle using one way ANOVA followed by Dunnett´s post hoc tests.

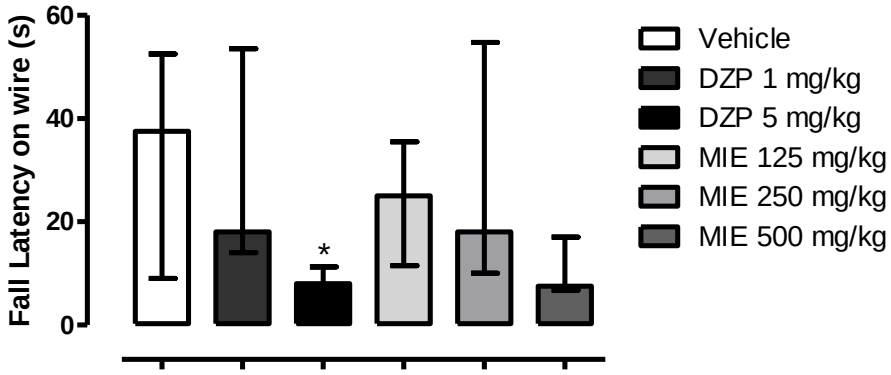

Figure 5. Effects of the oral treatment with vehicle, (E) - methyl isoeugenol (MIE) or diazepam (DZP) on motor activity of mice exposed to wire hanging test. Data are analyzed by Kruskal-Wallis test followed by Dunns as post hoc test and expressed as median (25^{th} percentile – 75^{th} percentile), n=10.

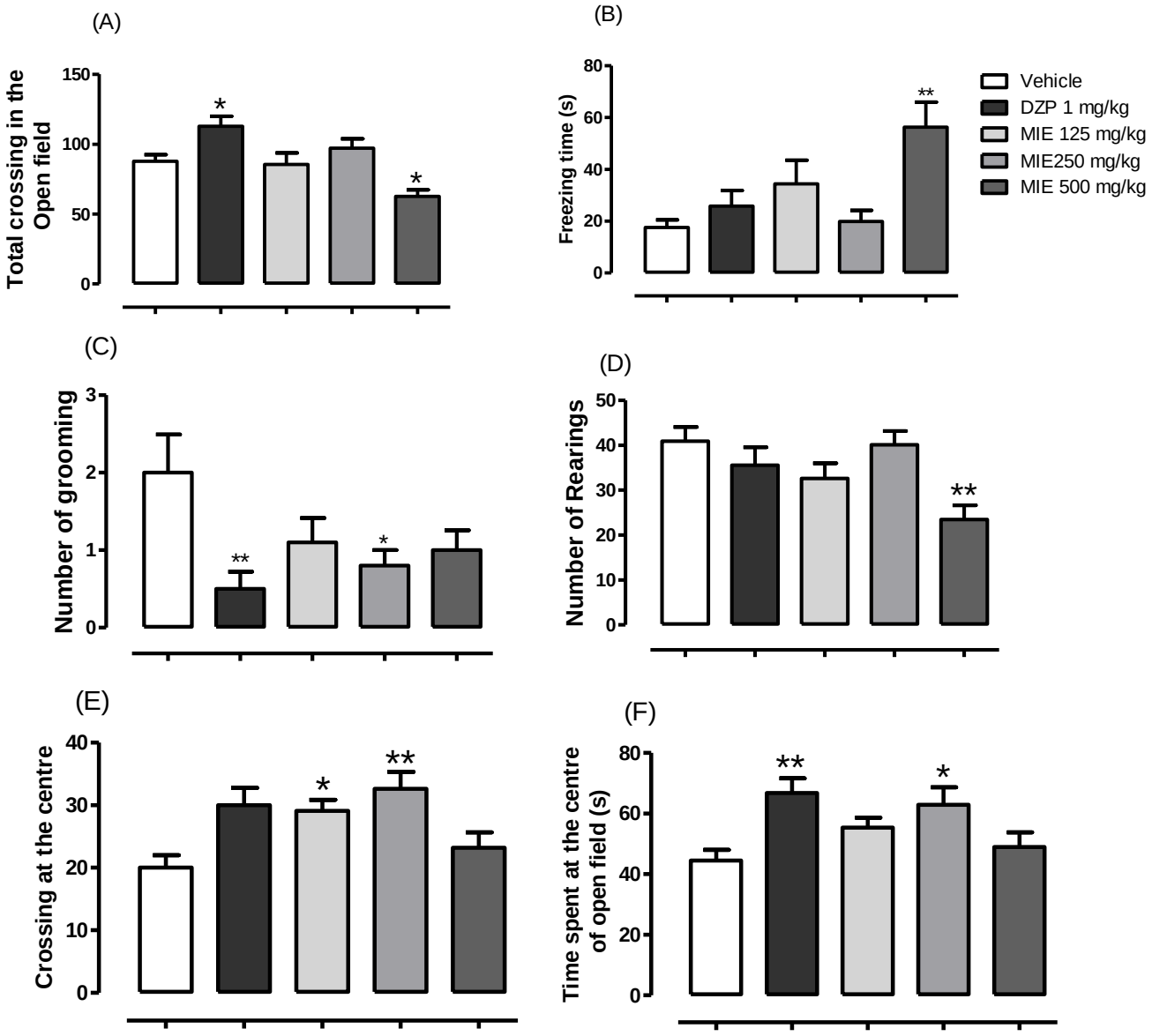

Figure 6. Effects of oral treatments of vehicle, diazepam (DZP) or (E) - methyl isoeugenol (MIE) on the total crossing (A), freezing time (B), number of grooming (C), number of rearing (D), crossing at the centre (E) and time spent at the centre (F) of the open-field. Each column represents mean ± SEM of 10 mice. *$p < 0.05$, **$p < 0.01$ as compared to the vehicle treated group (one-way ANOVA followed by Dunnett´ s post hoc test).

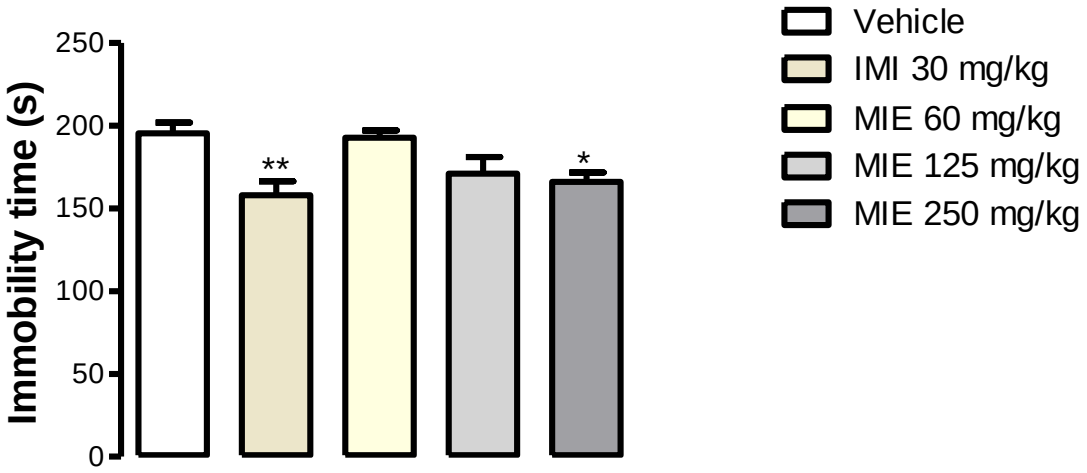

Figure 7. The effect of acute oral administration of vehicle, imipramine (IMI) or (E) - methyl isoeugenol (MIE) on the immobility time in the forced swimming test. Data are analyzed using one way ANOVA followed by Dunnett´s test as post hoc test (A). Each column represents the mean ± SEM of 10 mice. *p < 0.05, **p < 0.01 as compared to the vehicle treated group (one-way ANOVA followed by Dunnett´ s post hoc test).

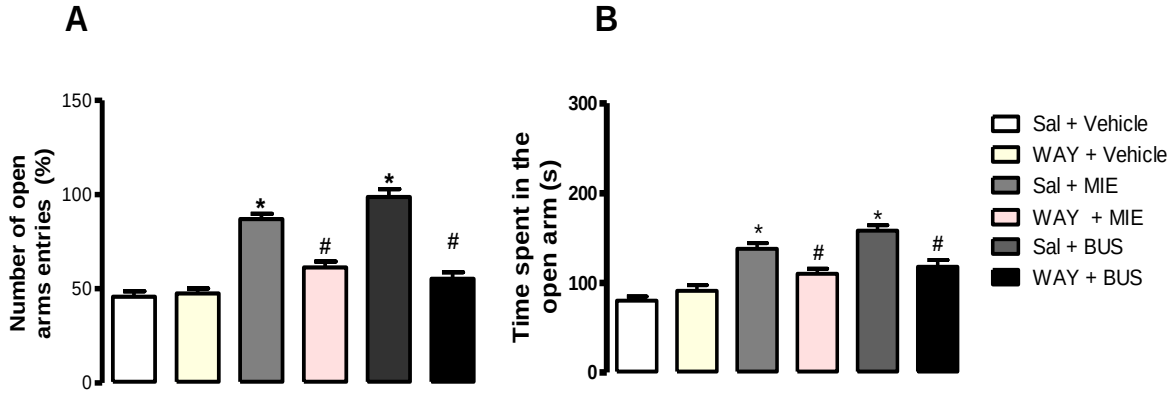

Figure 8. Effects of NaCl 0.9% (SAL) or WAY100635 0.3 mL/kg (WAY) pretreatment on the number of open arms entries (A) and time spent in the open arms (B) of EPM prior to oral treatments with vehicle, (E) - methyl isoeugenol (MIE) 250 mg/kg or buspirone (BUS) 10 mg/kg. Data were analyzed using two way ANOVA followed by Bonferroni post hoc test and expressed as mean ± SEM, n = 10. *p < 0.05 versus vehicle treated group while $^{\#}$p < 0.05 indicate significant reversal of MIE or BUS effect by WAY pretreatment.

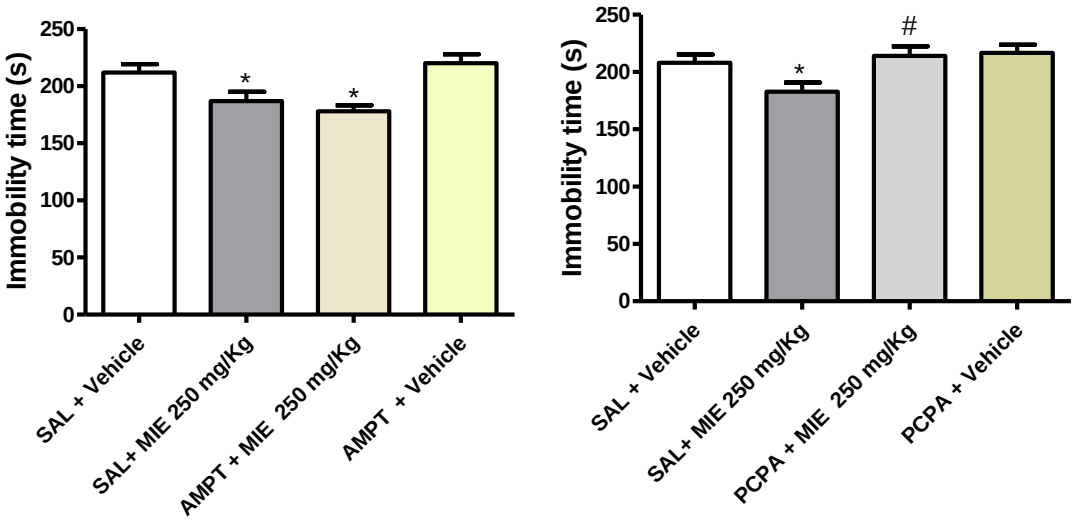

Figure 9. Effects of pretreatment with (A) NaCl 0.9% (SAL) or α-methyl-p-tyrosine 100 mg/kg (AMPT), (B) NaCl 0.9% (SAL) or p-chlorophenylalanine 100 mg/kg (PCPA) prior to oral administration of (E) - methyl isoeugenol – MIE 250 mg/kg or vehicle on the immobility time in forced swimming test. Data are presented as mean ± SEM (n= 10). *$p < 0.05$ versus vehicle treated group while $^{\#}p < 0.05$ indicate significant reversal of anti-immobility effect of MIE.

4. Discussion

The growing interest in natural product could be traced to their perceived safety.[41] Up to date, aromatic plants are largely explored as functional ingredients in the pharmaceutical, cosmetic, food and feed industries.[42] Despite the presence of (E)-methyl isoeugenol's (MIE) in the essential oil and crude extract that has been acclaimed to possess calming effect and anxiolytic like property,[32-35] there has been no pharmacological data on the biological activities of MIE to the best of our knowledge. Being a naturally occurring food flavour, therapeutic application of MIE for the treatment of neural disorders seems to be more acceptable to the use of available pharmacotherapies. Since food safety issues is crucial to human health,[43] the present study also revealed behavioural alterations that are elicited by MIE at different doses.

The report on the behavioural alteration in the general pharmacological tests seems to be dependent on dose and the route of administration. The oral treatment of MIE 100 mg/kg increased exploratory activity while at 500 mg/kg, MIE elicited sedative effect and a decrease in exploratory activity. The effects of intraperitoneal and subcutaneous administration of MIE were characterized by sedation. The oral route of administration and the dose ranges of MIE in our subsequent experiments were chosen based on the popular application of the leaf extract and previous work on the essential oil of *Pimenta pseudocaryophyllus*.[31]

The sodium pentobarbital sleep induction test showed an increase (in a dose dependent manner) in the sleep duration by oral administration of MIE. Potentiation of the hypnotic effect of barbiturate sleep is an indication of central nervous system depressive activity.[34] This data reinforces the assumption of MIE involvement in the

activity of the organic leaf extract.[35] Being a CNS depressive compound like diazepam, we hypothesized anti-seizure property of MIE. Our hypothesis is further supported by the antiseizure property of aromatic compounds like methyleugenol, eugenol and 1-nitro-2-phenylethane[30,44] that share similar chemical structure with MIE. We assumed that the presence of phenylpropanoid structure could be associated with their anti-convulsant property. Hence, we conducted pentylenetetrazole (PTZ) induced convulsion test; a predictive animal model that is widely used in the search for new antiepileptic drugs.[45,46] However, contrary to our expectation, oral administration of MIE did not protect against the PTZ induced convulsion. It is intriguing to observe that at the highest dose there was a decline in the percentage of animal protected, an increase in the severity and duration of convulsion. Since the dose of diazepam tested in this study did not induce sedative or myorelaxant effect, we hypothesized that the sedative tendency of MIE at the highest dose could be responsible for the potentiation of PTZ effects. Based on the mechanism of action of PTZ, the effect of MIE on CNS perhaps did not involve $GABA_A$ receptor.

The study of antianxiety like property of unknown compound could be achieved through environmental manipulation that elicits aversive behaviour and the resultant conflict with the innate desire of the animal to explore. The light dark box is an established animal model for the detection of compound with potential anxiolytic like property.[38] Parameters like number of transition between the light and dark compartments and time spent in the light area of the box are used to make antianxiety inference.[47] In this study, except for the highest dose, MIE induced an increase in the number of transition and time spent in the light area of LDB. The elevated plus maze

(EPM) was further used to detect possible anxiolytic and anxiogenic like effects.[48] Mice treated with MIE (125 or 250 mg/kg) increases time spent on the open arms. MIE at 500 mg/kg reduced total arm entry in the EPM. Based on these data there are indication that MIE interferes with motor coordination of the animals at the dose of 500 mg/kg.

In the wire hanging test, the effect of oral administration of MIE on motor activity of mice was evaluated. In this test, the data obtained on the latency of falls, after MIE administration, did not demonstrate significant alteration. These results further suggest that the anxiolytic-like effect of MIE at lower doses (125 and 250 mg/kg) did not elicit myorelaxant or sedative effect. However, the effect of MIE at 500 mg/kg on motor activity seems inconclusive based on the EPM data. In order to further unravel possible sedative effect of MIE at 500 mg/kg or stimulatory at 125 and 250 mg/kg, animals were exposed to the open field test. This animal model could be used to ascertain if the increase in number of transition in the LDB and an increase in the open arm entries are mere stimulatory response. This model is of importance to our subsequent study of MIE in the forced swimming model. An agent with stimulatory effect could reduce immobility time in the forced swimming test while sedative agent or dose could interfere with the animal performance in this model. Our data showed a reduction in rearing activity, total crossing and an increase in freezing time at MIE 500 mg/kg. These results suggest sedative or myorelaxant effect at this dose. On the other hand, MIE 125 or 250 mg/kg increased crossing and time spent at the centre significantly (effects that suggest a reduction in aversiveness and anxiety in this animal model) without significant increase in total crossing (sum of the crossing at the centre and periphery of open field). The observations in the open field further confirm anxiolytic like property of MIE at lower

doses (125 or 250 mg/kg) and sedative property at the highest dose of 500 mg/kg. These results seem to be consistent with the data under general pharmacological tests.

In the present study, a U-shaped dose - response and dose dependent response were reported with MIE administrations. The pattern of behavioural responses to the doses administered could be dependent on animal model or associated with physiological alterations. MIE at 500 mg/kg potentiated sleep induced and elicited a sedative like effect in the open field and EPM but could not alter behavioural response in the LDB. In contrast, at 250 mg/kg, the anxiolytic like effect of MIE on animal were consistent in the LDB, EPM and open field tests. On the basis of animal model, MIE effects were dose dependent in sodium pentobarbital sleep induction test while a U-shaped dose – response was reported in the LDB. On the whole, we hypothesized that different doses of drug (MIE) administered could produce physiological changes which can be observed through an alteration (specific or non-specific) in animal behaviour. However, the observation and quantification of behavioural responses are susceptible to the influence of external factors/ sensitive of animal models.

Since MIE administration did not protect the animals against PTZ induced convulsion, we assume non-involvement of $GABA_A$ receptor in its mechanism of actions. Hence, we decided to investigate the participation of serotonergic system by using pharmacological antagonist of 5-HT1A receptor. Evaluation of this particular receptor becomes interesting based on its involvement in the anxiolytic like property of the organic extract of *Pimenta pseudocaryophyllus*.[34] The neural mechanism of clinically prescribed anxiolytic drug like buspirone has been associated with 5-HT1A receptor.[49] The WAY100635, a selective antagonist of 5-HT1A, pretreatment blocked anxiolytic like

effect of MIE in the EPM. Interestingly, 5-HT1A receptor had been implicated in the antidepressant property of azapirones.[50,51] Previous study has demonstrated antidepressant like property of in our laboratory on the organic extract of *Pimenta pseudocaryophyllus*.[35] Hence, we proposed to study the effect of MIE administration on animal behavior in the forced swimming test (FST).

In the FST, the lowest dose of 60 mg/kg was introduced to substitute 500 mg/kg dose whose effect was characterized by sedation in the previous experiments. MIE at 250 mg/kg reduced the immobility time, thereby demonstrating antidepressive-like activity. In order to elucidate the involvement of monoamine in the effect of MIE, monoamine depletion approach were employed. The anti-immobility effect of MIE remained unaltered by α-methyl-p-tyrosine (depletor of catecholamine storage) pretreatment.[52,53] In contrast, pretreatment of mice with parachlorophenylalanine (serotonin depletor), attenuated anti-immobility effect of MIE.

Conclusion:

Our findings demonstrated anxiolytic and antidepressive like properties of (E)-methyl isoeugenol and suggested the participation of serotonergic pathways.

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