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Bárbara Nunes Krum

EFEITO DO ANTIPSICÓTICO HALOPERIDOL EM *Caenorhabditis elegans* E DO EXTRATO BRUTO DE *Piper methysticum* EM CAMUNDONGOS SOBRE PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Doutora em Farmacologia.**

Orientadora: Profª. Drª. Roselei Fachinetto

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Bárbara Nunes Krum

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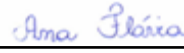

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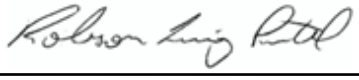

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2021

“Sem sonhos, a vida não tem brilho.
Sem metas, os sonhos não têm alicerces.
Sem prioridades, os sonhos não se tornam reais”.

Augusto Cury

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RESUMO

EFEITO DO ANTIPSICÓTICO HALOPERIDOL EM *Caenorhabditis elegans* E DO EXTRATO BRUTO DE *Piper methysticum* EM CAMUNDONGOS SOBRE PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS

AUTORA: Bárbara Nunes Krum
ORIENTADORA: Roselei Fachinetto

Transtornos psicóticos, como a esquizofrenia e as psicoses induzidas por substâncias de uso abusivo (por exemplo, anfetamina), são caracterizados pelo aparecimento de delírios, alucinações, entre outros e, seus sintomas estão associados à hiperativação da via dopaminérgica mesolímbica. Os antipsicóticos antagonizam os receptores de dopamina (DA) do tipo D_2 nesta via e por isso são utilizados para o tratamento dos sintomas psicóticos. Os fitoterápicos também vêm sendo utilizados como alternativas ao tratamento de sintomas decorrentes de patologias do sistema nervoso central (SNC). *Piper methysticum* (*P. methysticum*), popularmente chamado de Kava Kava, se enquadra neste contexto pois possui ação em receptores gabaérgicos, dopaminérgicos, sobre a enzima monoaminoxidase (MAO), entre outros. Pesquisas em modelos animais, como em *Caenorhabditis elegans* (*C. elegans*) e roedores, vêm sendo realizadas para melhor compreender os sintomas, tratamento farmacológico e seus mecanismos, bem como a utilização de possíveis adjuvantes à terapia farmacológica dos transtornos psicóticos. Desta forma, o primeiro objetivo deste estudo foi investigar os efeitos do antipsicótico haloperidol no sistema dopaminérgico em *C. elegans*. Os animais foram expostos ao haloperidol (80 ou 160 µM) durante 1-6 dias e, avaliações comportamentais, moleculares e morfológicas foram realizadas. O haloperidol aumentou a sobrevivência, diminui o comportamento locomotor e os níveis de DA nesses animais, mas não alterou a expressão dos genes *dop-1*, *dop-2* e *dop-3* nem a morfologia dos neurônios dopaminérgicos cefálicos. Após a retirada do fármaco, o comportamento locomotor retornou a níveis de atividade basal. Os efeitos do haloperidol em *C. elegans* parecem estar associados a modulação de receptores do tipo D_2 de DA. Em relação ao fitoterápico Kava Kava, o extrato bruto foi utilizado para investigar os seus efeitos em um modelo animal de psicose induzido por anfetamina sobre alterações comportamentais e na atividade da enzima MAO. Camundongos receberam por gavagem o extrato de Kava (40 mg/Kg) ou veículo (óleo de milho); 2 h após, anfetamina (1,25 mg/Kg) ou veículo (NaCl 0,9%) pela via intraperitoneal. 25 min depois, testes comportamentais mostraram que o extrato de Kava causou efeito ansiolítico no teste de labirinto em cruz elevado, aumentou a atividade locomotora no teste de campo aberto e diminui a atividade da MAO-A no córtex e da MAO-B no hipocampo dos animais. O extrato evitou os efeitos da anfetamina no comportamento estereotipado e o co-tratamento aumentou o número de entradas nos braços no teste de labirinto em Y e na atividade da MAO-B no estriado de camundongos. Entretanto, a hiperlocomoção induzida pela anfetamina não foi alterada pelo tratamento prévio com o extrato. A interação social não foi modificada. Os resultados mostram que o extrato de Kava preveniu o aparecimento do comportamento estereotipado induzido por anfetamina em camundongos, o qual poderia ser investigado como um possível adjuvante à terapia farmacológica para minimizar sintomas psicóticos em pacientes. Por fim, o terceiro objetivo deste estudo investigou o efeito do extrato de Kava (10-400 mg/kg, administrado por gavagem) sobre a atividade da MAO em diferentes estruturas do cérebro de camundongos e sobre alterações comportamentais após 21 dias de tratamento, bem como, em ensaios *in vitro* (10-100 µg/mL do extrato) utilizando homogeneizado de cérebro de camundongos. O extrato de Kava aumentou o percentual de entradas nos braços abertos do labirinto em cruz elevado e diminuiu a atividade da MAO-B no córtex (10 mg/Kg) e na região contendo a substância negra (10 e 100 mg/Kg) em ensaios *ex vivo*. *In vitro*, o extrato de Kava inibiu reversivelmente a atividade da MAO-B com IC_{50} de 14,62 µg/mL, aumentou os valores de K*m* (10, 30 µg/mL) e diminuiu a V*max* (100 µg/mL). Assim, o extrato apresentou diferentes efeitos sobre a MAO-B dependendo da estrutura cerebral avaliada, o qual pode ser promissor em patologias onde a MAO-B esteja envolvida. A totalidade de resultados apresentados nesta tese revelaram resultados úteis e condicionadas a psicose, informações as quais tornam-se relevantes para estudos futuros relacionados a este transtorno.

Palavras-chave: Anfetamina. Kava Kava. Monoaminoxidase. Nematoides. Psicoses. Sistema dopaminérgico.

ABSTRACT

EFFECTS OF THE ANTIPSYCHOTIC HALOPERIDOL IN *Caenorhabditis elegans* AND OF THE CRUDE EXTRACT OF *Piper methysticum* IN MICE ON BEHAVIORAL AND BIOCHEMICAL PARAMETERS

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ADVISOR: Roselei Fachinetto

Psychotic disorders, as schizophrenia and substance-induced psychoses (amphetamine, for example), are characterized by the appearance of delusions, hallucinations and other, and their symptoms are associated with hyperactivation of the mesolimbic dopaminergic pathway. Antipsychotics antagonize D2-type dopamine (DA) receptors in this pathway and due to it, they have been used for the treatment of psychotic symptoms. Herbal medicines have also been used as alternatives to the treatment of symptoms of central nervous system (CNS) disorders. *Piper methysticum* (*P. methysticum*), named Kava Kava, fits in this context because it has action on GABAergic and dopaminergic receptors, on the enzyme monoamine oxidase (MAO) and others. Research in animal models, such as in *Caenorhabditis elegans* (*C. elegans*) and rodents, has been carried out to better understand the symptoms, pharmacological treatment and its mechanisms, as well as the use of possible adjuvants to the pharmacological therapy of psychotic disorders. Thus, the first main of this study was to investigate the effects of the antipsychotic haloperidol on the dopaminergic system in *C. elegans*. The animals were exposed to haloperidol (80 or 160 µM) for 1-6 days and behavioral, molecular and morphological assays were performed. Haloperidol increased survival, decreased locomotor behavior and DA levels in these animals, but it did not alter neither *dop-1*, *dop-2* and *dop-3* genes expression, nor the morphology of cephalic dopaminergic neurons. Moreover, locomotion speed recovered to basal conditions upon haloperidol withdrawal. Haloperidol's effects on *C. elegans* seem to be related to modulation of the D2-type DA receptor. Regarding the herbal medicine Kava Kava, the crude extract was used to investigate its effects in an animal model of psychosis-like symptoms induced by amphetamine on behavioral changes and on MAO activity. Mice received Kava extract (40 mg/Kg) or vehicle (corn oil) by gavage; 2 h later, amphetamine (1.25 mg/kg) or vehicle (0.9% NaCl) by an intraperitoneal injection. 25 min later, behavioral tests showed that Kava extract exhibited anxiolytic effect in the elevated plus-maze test, increased the locomotor activity in the open-field test and decreased MAO-A activity in the cortex and MAO-B activity in the hippocampus of the animals. Kava extract prevented the effects of amphetamine on stereotyped behavior and, the co-treatment increased the number of entries into arms in Y maze test as well as MAO-B activity in striatum of mice. However, the hyperlocomotion induced by amphetamine was not altered by previous treatment with the extract. The social interaction was not modified for the treatment. The results showed that Kava extract avoided the increase of stereotyped behavior induced by amphetamine in mice, which could be investigated as a possible adjuvant in the pharmacological therapy to minimize psychotic symptoms in patients. Lastly, the third main of this study investigated the effect of Kava extract (10-400 mg/kg, by gavage) on MAO activity in different mouse brain structures and on behavioral changes after 21 days of treatment as well as, in *in vitro* assays (10-100 µg/ml of the extract) in mouse brain homogenate. Kava extract increased the percentage of entries into the open arms in the plus-maze test and decreased MAO-B activity in the cortex (10 mg/kg) and in the region containing the substantia nigra (10 and 100 mg/kg) in assays *ex vivo*. *In vitro*, Kava extract reversibly inhibited MAO-B activity with IC_{50} of 14.62 µg/mL, increased K*m* values (10, 30 µg/ml) and decreased V*max* (100 µg/ml). Thus, the extract showed different effects on MAO-B isoform depending on the brain structure evaluated, which could be promissory in pathologies where MAO-B is the pharmacological target. The totality of results presented in this thesis revealed useful results conditioned to psychosis, information which becomes relevant for future studies related to this disorder.

Keywords: Amphetamine. Kava Kava. Nematodes. Psychosis. Dopaminergic system.

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LISTA DE ABREVIATURAS

5-HT	Serotonina
5-HIAA	Ácido 5-hidroxi-indolacético
ADH	Aldeído desidrogenase
ANVISA	Agência Nacional de Vigilância Sanitária
CA1	Região CA1 do hipocampo
C. elegans	*Caenorhabditis elegans*
CID-10	Classificação Internacional de Doenças 10ª revisão
CPFDL	Córtex pré-frontal dorsolateral
CPFVM	Córtex pré-frontal ventromedial
CREB	Proteína de ligação ao elemento de resposta ao AMP cíclico
DA	Dopamina
D_2R	Receptor de dopamina subtipo 2
DOPAC	Ácido diidroxi-fenilacético
DSM	Manual Diagnóstico e Estatístico de Transtornos Mentais
EEG	Eletroencefalograma
FDA	*Food and Drug Administration*
GABA	Ácido γ-aminobutírico
GLU	Glutamato
H_2O_2	Peróxido de hidrogênio
IRM	Ressonância magnética
MAO	Monoaminoxidase
NA	Noradrenalina
NMDA	N-metil-D-aspartato
OMS	Organização Mundial da Saúde
P. methysticum	*Piper methysticum*
SNC	Sistema nervoso central
SUS	Sistema Único de Saúde
TEP	Tomografia de emissão de pósitrons
TVMA	Transportador vesicular de monoaminas
VTA	Área tegmental ventral

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APRESENTAÇÃO

No item **INTRODUÇÃO** está descrito uma exposição sucinta sobre as ideias essenciais, situando o assunto em um contexto global, trabalhadas nesta tese. No item **REFERENCIAL TEÓRICO** será apresentada uma breve revisão literária da temática que fundamenta esse trabalho, bem como destacando as pesquisas básicas que explicam ou compreendem o objeto do estudo.

Os **RESULTADOS** que fazem parte desta tese estão apresentados sob a forma de três artigos, os quais se encontram no item ARTIGOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências, encontram-se nos próprios ARTIGOS e representam a íntegra deste estudo. O artigo 1 está disposto na forma que foi publicado na edição da Revista Científica *Molecular Neurobiology*. O artigo 2 está disposto na forma que foi publicado na edição da Revista Científica *Journal of Ethnopharmacology*. O artigo 3 está disposto na forma que foi publicado na edição da Revista Científica *Journal of Traditional and Complementary Medicine.*

O item **DISCUSSÃO** apresenta uma interpretação de todos os dados encontrados e que fazem parte desta tese.

O item **CONCLUSÃO**, encontrada no final desta tese, apresenta os comentários gerais sobre este trabalho.

As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REFERENCIAL TEÓRICO e DISCUSSÃO** desta tese.

1 INTRODUÇÃO

O aparecimento de transtornos psicóticos, como a esquizofrenia, transtorno depressivo maior com características psicóticas, transtorno esquizoafetivo, entre outros, é uma condição de saúde mental que requer muita atenção. Na clínica, transtornos psicóticos são caracterizados pelo aparecimento de alucinações, delírios, distúrbios de pensamento ou da fala e comportamento desorganizado (LIEBERMAN; FIRST, 2018; MILLER; DRUSS, 2013). Os antipsicóticos (típicos e atípicos), são os fármacos de primeira escolha atualmente utilizados para o tratamento desses sintomas (LIEBERMAN; FIRST, 2018). Em relação a etiologia dos transtornos psicóticos, ela está fortemente associada com fatores genéticos bem como fatores ambientais, complicações obstétricas ou perinatais e devido ao abuso de drogas psicoestimulantes como a anfetamina (GULOKSUZ et al., 2015; HENQUET et al., 2005; LIEBERMAN; FIRST, 2018; SPAUWEN et al., 2004, 2006). A anfetamina atua aumentando os níveis sinápticos de dopamina (DA) no sistema nervoso central (SNC), acarretando também na liberação de outros neurotransmissores como serotonina (5-HT) e noradrenalina (NA), e assim, podendo provocar o aparecimento de sintomas psicóticos em seus usuários (CAPELA et al. 2009; GREEN et al. 2003; YAMAMOTO et al. 2010). Neste contexto, a anfetamina vem sendo utilizada em animais experimentais a fim de mimetizar um modelo de psicose nos mesmos (MABROUK et al., 2014). Em contrapartida ao uso de medicamentos alopáticos, existe o consumo de medicamentos fitoterápicos, os quais vem sendo utilizados como uma alternativa para o tratamento dos mais variados tipos de sintomas decorrentes de algumas patologias (NEWMAN; CRAGG, 2007). *Piper methysticum* (*P. methysticum*) é um exemplo de composto natural utilizado como fitoterápico. *P. methysticum*, é popularmente denominado de Kava Kava devido à presença das kavalactonas, principais constituintes ativos do seu extrato bruto (SMITH, 1983, 1984) com ampla ação sobre o SNC de mamíferos (CAIRNEY, MARUFF, CLOUGH, 2002). Dentre os mecanismos propostos para a ação do extrato de Kava, destaca-se a atuação em receptores do tipo A do ácido γ-aminobutírico ($GABA_A$), em receptores tipo 2 de DA e como um inibidor da enzima monoaminoxidase (MAO) (BAUM et al, 1998; UEBELHACK, FRANKE, SCHEWE, 1998). A enzima MAO é encontrada na membrana externa de mitocondrias e possui duas isoformas chamadas de MAO-A e MAO-B, as quais são responsáveis pela desaminação oxidativa de aminas biogênicas (COHEN; FAROOQUI; KESLER, 1997; LI et al., 2014; VINDIS et al., 2001; YOUDIM e BAKHLE, 2006). Compostos capazes de

inibir as isoformas da MAO são utilizados no tratamento de doenças do SNC tais como depressão e doença de Parkinson (BORTOLATO; CHEN; SHIH, 2008; LI et al., 2014; YOUDIM e BAKHLE, 2006). Pesquisas com modelos animais como o *Caenorhabditis elegans* (*C. elegans*) e roedores têm sido utilizados na pesquisa científica para melhor compreender mecanismos farmacológicos, fisiopatologia de doenças/transtornos e seus sintomas, entre outros.

Portanto, resumidamente, este trabalho consiste em avaliar os efeitos de dois compostos (haloperidol e *P. methysticum*) utilizando dois modelos animais (*C. elegans* e camundongos), ambos condicionados ao termo psicose. Primeiramente, foram avaliados os efeitos sobre o sistema dopaminérgico bem como as alterações comportamentais e de marcadores moleculares e morfológicos específicos causados pelo antipsicótico típico haloperidol em *C. elegans*. Por fim, também foram investigados os efeitos do extrato bruto de Kava em um modelo animal de psicose induzido por anfetamina, bem como o seu potencial efeito inibitório sobre a enzima MAO, ambos utilizando camundongos como modelo animal. Os resultados encontrados neste trabalho certamente contribuem para a obtenção de novos achados científicos.

2 REFERENCIAL TEÓRICO

2.1 PSICOSES

2.1.1 Epidemiologia

Psicose é um complexo de sintomas que pode incluir delírios, alucinações, distúrbios de pensamento ou da fala e comportamento desorganizado, cuja evolução e o aparecimento desses sintomas pode variar de acordo com o transtorno subjacente de cada paciente (LIEBERMAN; FIRST, 2018; MILLER; DRUSS, 2013). A esquizofrenia, o transtorno bipolar e a depressão com sintomas psicóticos são os transtornos mais comuns, que apresentam como característica a psicose, e, geralmente, tem início na adolescência ou no início da idade adulta, entre 15 e 25 anos de idade (VAN OS; KAPUR, 2009). Os sintomas de transtornos delirantes e psicoses associados a doenças neurodegenerativas surgem na meia-idade e durante a senescência, respectivamente. Já em psicoses secundárias decorrentes do uso abusivo de drogas ou medicamentos, bem como após o surgimento de alguma condição médica geral (febres, convulsões, entre outros), os sintomas podem ocorrer em qualquer faixa etária. Estima-se que 3% da população em geral apresente algum tipo de transtorno psicótico ao longo da vida, sendo 0,21% devido a alguma condição médica geral. Na infância, psicoses são incomuns, cerca de 0,2 a 0,4 a cada 10.000 crianças apresentam sintomas, os quais geralmente são em decorrência de psicoses secundárias (COURVOISIE; LABELLARTE; RIDDLE, 2001; REIMHERR; MCCLELLAN, 2004). Por fim, aproximadamente um caso de psicose pós-parto em cada 500 a 1000 nascimentos são relatados, que podem estar associados a históricos de transtornos bipolares, depressão pré-mórbida, entre outros (WOLFGANG; JURGEN, 2012).

2.1.2 Fisiopatologia

O termo “psicose”, palavra de origem grega para condição “anormal da mente”, tem sido utilizado de diferentes maneiras na clínica médica ao longo dos anos. O termo foi atribuído pela primeira vez em 1845 pelo médico Ernst von Feuchtersleben, o qual designou-o a pessoas cujo seu funcionamento mental encontrava-se prejudicado, ou seja, quando não conseguiam desenvolver

tarefas cotidianas. No início dos anos 80, o termo psicose passou a ser empregado para pessoas que apresentavam prejuízo no teste de realidade (interrupção da capacidade de distinguir entre a experiência interna da mente e a realidade externa do meio ambiente). Em 1994, alucinações, delírios, pensamentos desorganizados, frases sem sentido, associações de clang (palavras rimadas), ecolalia (repetição de palavras) e comportamento motor anormal (estereotipia, posturas bizarras e flexibilidade cerosa) foram designados como sintomas típicos de psicoses e, até hoje, servem como base para diagnósticos. Psiquiatras e profissionais da saúde utilizam os sistemas de Classificação Internacional de Doenças 10ª revisão (CID-10) e o Manual Estatístico e Diagnóstico de transtornos Mentais 5ª edição (DSM-V) para distinguir os diferentes tipos de psicose. Atualmente, transtornos psiquiátricos são denominados como síndromes clínicas em vez de doenças e levam em consideração a duração dos sintomas, perfil dos sintomas, relação entre sintomas psicóticos e episódios de humor perturbado e a causa (se os sintomas são devido ao uso de alguma substância ou devido a alguma doença que acometa o cérebro). Na clínica atual, "sintoma psicótico" refere-se a uma disfunção cognitiva ou perceptual (principalmente delírios e alucinações), enquanto que "transtornos psicóticos" denota a uma condição na qual os sintomas psicóticos atendem a critérios específicos de um determinado transtorno (LIEBERMAN; FIRST, 2018; WOLFGANG; JURGEN, 2015).

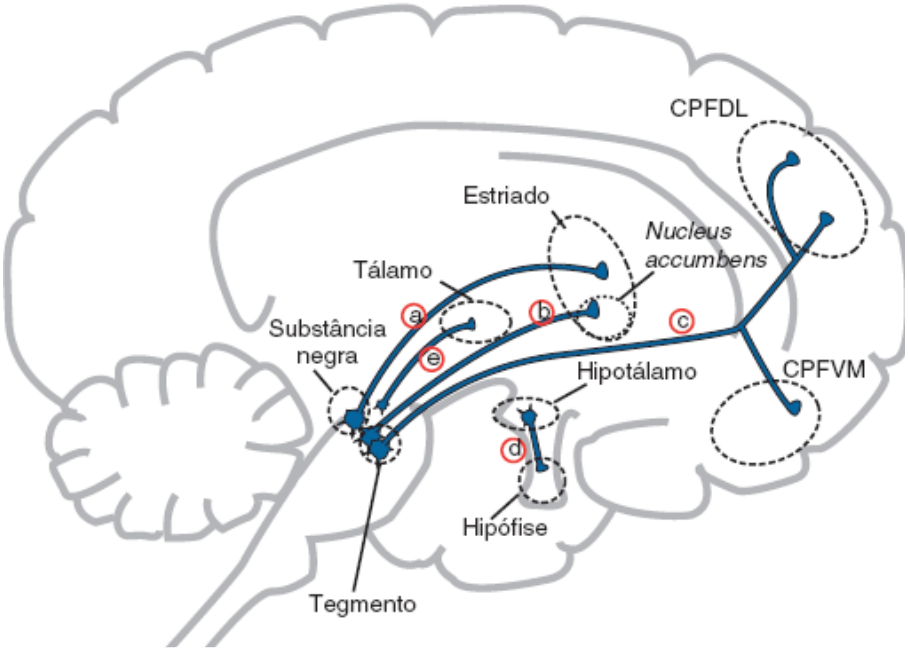
Psicoses podem ser classificadas de forma arbitrária em 3 grupos: 1- idiopáticas (esquizofrenia, transtorno esquizoafetivo, transtorno bipolar com características psicóticas, transtorno depressivo maior com características psicóticas, transtorno delirante, transtorno esquizofreniforme, transtorno psicótico breve e psicose pós-parto), 2- devido a condições médicas (condições neurológicas, metabólicas, endócrinas e outras) ou 3- tóxicas [psicose induzida por substâncias recreativas (álcool, cocaína, anfetaminas), psicose induzida por toxinas (monóxido de carbono, inseticidas organofosforados) e psicose iatrogênica (anestésicos, analgésicos)] (LIEBERMAN; FIRST, 2018). Psicoses secundárias são denominadas psicoses provenientes de psicoses tóxicas ou psicoses devido a condições médicas. A principal diferença da psicose secundária para a idiopática, inclui ausência de histórico familiar, rápido declínio da capacidade funcional dos níveis pré-mórbidos, início abrupto de sintomas sem causas esclarecidas, aparecimento de dores de cabeça, convulsões ou alucinações visuais, olfativas ou táteis. Essas classificações são baseadas em dados da literatura, as quais podem apresentar modificações com a descoberta de novos achados científicos (LIEBERMAN; FIRST, 2018).

O surgimento dos sintomas psicóticos nas suas mais variadas causas e manifestações fisiopatológicas ocorre devido a alterações na neurotransmissão glutamatérgica, GABAérgica e dopaminérgica no hipocampo, mesencéfalo, corpo do estriado e córtex pré-frontal. O excesso de níveis sinápticos de DA e glutamato (GLU) aumentam a estimulação pós-sináptica, cujos efeitos resultam no aparecimento de psicoses (MOGHADDAM; JAVITT, 2012; HOWES; KAPUR, 2009). As bases moleculares desses distúrbios incluem receptores hipofucionais de N-metil D-aspartato (NMDA) para o GLU e deficiência de interneurônios GABAérgicos inibitórios que alteram o equilíbrio excitatório-inibitório mediado pelo GLU e DA (LIEBERMAN; FIRST, 2018; LODGE; GRACE, 2011; NAKAZAWA, et al., 2012).

2.1.2.1 Hipóteses dos neurotransmissores

A hipótese dopaminérgica é a mais comumente utilizada para explicar a fisiopatologia da psicose (STANDAERT; GALANTER, 2013). Segundo esta hipótese, os sintomas psicóticos aparecem devido porá alteração nos níveis de DA que ocorre em lugares específicos do cérebro diretamente relacionados com a neurotransmissão dopaminérgica das vias mesolímbica e mesocortical (Figura 1 b, c) (STANDAERT; GALANTER, 2013; TODA; ABI-DARGHAM, 2007). A hipótese dopaminérgica também é apoiada pela eficácia dos antagonistas de receptores de DA (antipsicóticos) no tratamento de sintomas psicóticos, bem como de evidências de que o uso de drogas psicostimulantes (metanfetamina, cocaína, anfetamina) que aumentam a liberação de DA na via mesolímbica podem induzir sintomas psicóticos em seus usuários (ANGRIST; GERSHON, 1970; CERETTA et al., 2016; CREESE; BURT; SNYDER, 1996; YAMAMOTO et al., 2010).

Figura 1 - Vias dopaminérgicas



Fonte: (adaptado de STAHL, 2014). (a) via dopaminérgica nigroestriatal (b) via dopaminérgica mesolímbica (c) via mesocortical (d) via dopaminérgica tuberoinfundibular (e) via dopaminérgica talâmica. Córtex pré-frontal dorsolateral (CPFDL); Córtex pré-frontal ventromedial (CPFVM).

Como demostrado na figura 1, a via dopaminérgica mesolímbica (b) projeta-se da área tegmental ventral do mesencéfalo para o *nucleus accumbens*, área envolvida no aparecimento de sintomas como alucinações, delírios, fala desorganizada e comportamento bizarro, devido ao excesso de DA nessa via. A via dopaminérgica mesocortical (c) também se projeta da área tegmental ventral do mesencéfalo, entretanto envia axônios para áreas do córtex pré-frontal dorsolateral (CPFDL) e córtex pré-frontal ventromedial (CPFVM) e está envolvida ao aparecimento de sintomas como apatia, anedonia, tristeza, depressão, prejuízos na memória, aprendizado, atenção e linguagem originados a partir de uma diminuição de DA nessa via (ABI-DARGHAM, 2004; BRENNAN; HARRIS; WILLIAMS, 2013; MOORE; WEST; GRACE, 1999; STAHL, 2014; STANDAERT; GLANTER, 2013). Responsável pelo controle e função motora, a via dopaminérgica nigroestriatal (a) se projeta da substância negra para os núcleos da base ou estriado e está envolvida com o aparecimento dos efeitos colaterais (sintomas extrapiramidais) após terapia farmacológica (MARSDEN, 2006; STANDAERT; GLANTER, 2013).

A hipótese glutamatérgica também tem sido relacionada como uma das causas do aparecimento de sintomas psicóticos. Esta hipótese é condicionada a hiperatividade de neurônios glutamatérgicos (devido a déficits de interneurônios GABA e hipofunção de receptores NMDA no hipocampo), os quais poderiam desencadear um "desequilíbrio" na neurotransmissão mediada pela DA, acetilcolina e GABA, entre outros neurotransmissores que vêm sendo implicados na patofisiologia desse transtorno (DEUTSCH et al, 1995, 2003a,b, 2005; LIEBERMAN; FIRST, 2018; MASTROPAOLO et al., 2004).

Podemos também mencionar a hipótese serotoninérgica, a qual está associada a hiperativação de receptores de serotonina $5HT_{2A}$ e/ou ao aumento na expressão dos receptores $5\text{-}HT_{2A}$. Ambos os mecanismos promovem um aumento na liberação de GLU, processo no qual pode ativar ainda mais a via dopaminérgica mesolímbica e levar ao aparecimento de sintomas psicóticos. (STAHL, 2016; STAHL. 2018).

Existem ainda evidências de que uma desregulação de neurotransmissores inibitórios GABAérgicos poderia contribuir para progressiva excitotoxicidade vista nos pacientes que apresentam sintomas psicóticos (JENTSCH; ROTH, 1999; NAKAZAWA et al., 2012). Estudos sugerem que essa desregulação também poderia provocar alterações na transmissão interneuronal gabaérgica com consequente exacerbação da função dopaminérgica (AHN et al., 2001; CARLSSON et al., 2001), hiperfunção de receptores glutamatérgicos (HOSAK; LIBIGER, 2002) e indução de sintomas psicóticos (HINTON; JOHNSTON, 2008).

2.1.3 Etiologia

A etiologia da psicose (desde experiências psicóticas transitórias até a expressão de psicose persistente) tem sido designada como uma etiologia múltipla envolvendo fatores genéticos (hereditariedade, polimorfismos genéticos), ambientais (doenças autoimunes, estresse ambiental), complicações obstétricas ou perinatais (infecções, prematuridade, hipóxia, fumo) e uso abusivo de drogas (anfetamina, cocaína). Além disso, existem evidências de que um aumento da exposição a alguns desses fatores pode contribuir para o surgimento de sintomas psicóticos aparentemente inexistentes (GULOKSUZ et al., 2015; HENQUET et al., 2005; SPAUWEN et al., 2004, 2006).

A hereditariedade é um fator preditivo para o aparecimento de transtornos psicóticos idiopáticos. Esses transtornos são caracterizados por concordância de aproximadamente 50% de

certos *loci* genéticos entre gêmeos idênticos e entre pais e filhos. Acredita-se que a incidência de transtornos psicóticos nesses indivíduos seja de 10 a 15 vezes mais alta do que a incidência na população em geral (LIEBERMAN; FIRST, 2018; POLDERMAN et al., 2015). Neste sentido, em associação aos fatores genéticos, doenças autoimunes e inflamatórias podem contribuir para o surgimento de psicoses, uma vez que, anticorpos podem estimular ou bloquear a função de neurotransmissores no cérebro, principalmente do GLU. Aproximadamente 30% de indivíduos com lúpus eritematoso sistêmico (doença autoimune) apresentam sintomas psicóticos, no qual seus anticorpos são dirigidos contra o DNA que reagem com a subunidade NR_2 do receptor NMDA e consequentemente alteram a atividade do mesmo (FAUST et al., 2010; LAUVSNES; OMDAL, 2012). A exposição durante o neurodesenvolvimento (por exemplo, infecções maternas, toxicidade de drogas, deficiências nutricionais, complicações no parto, trauma pós-natal) também está associada ao desenvolvimento de transtornos psicóticos subsequentes provavelmente pela interação com fatores genéticos (LIEBERMAN; FIRST, 2018). Por fim, o uso de drogas psicostimulantes, como a cocaína, anfetamina, cetamina, fenciclidina, possuem efeitos sobre o sistema nervoso que mimetizam sintomas psicóticos, caracterizado por níveis elevados ou desregulados de DA e/ou GLU em lugares específicos do cérebro (MOGHADDAM; JAVITT, 2012; HOWES; KAPUR, 2009; STANDAERT; GALANTER, 2013). Estudos também revelam que o aparecimento de episódios psicóticos após o uso de drogas possa se tornar crônica e desencadear o surgimento de uma psicose primária em indivíduos que são geneticamente predispostos a desenvolver esse transtorno (BERMAN et al., 2009; FLUYAU; MITRA; LORTHE, 2019; MCKETIN et al., 2016). Somado a isso, casos de abuso sexual e emocional, negligência e exposição a violência, bullying, entre outros, principalmente na infância e adolescência também têm sido implicados no desenvolvimento de psicoses (SCHREIER et al., 2009; TROTTA et al., 2013; VARESE et al., 2012).

2.1.3.1 Anfetamina

Sintetizada pela primeira vez em 1927 pelo químico GA Alles, a anfetamina (α-metilfenetilamina) é uma droga psicoestimulante com ação indireta sobre o SNC (GUTTMANN; SARGENT, 1937; HEAL et al., 2013). Sua ação psicoestimulante ocorre devido ao aumento dos níveis sinápticos de DA através de mecanismos indiretos, acarretando também na liberação de

outros neurotransmissores como 5-HT e NA, pois a anfetamina atua no transporte reverso dessas monoaminas através dos seus respectivos transportadores, bem como inibindo a recaptação de neurotransmissores no terminal axônico pré-sináptico (CAPELA et al. 2009; GREEN et al. 2003; YAMAMOTO et al. 2010). A anfetamina também pode atuar inibindo a atividade da enzima MAO evitando a degradação de aminas biogênicas. Além disso, como a anfetamina também tem afinidade pelo transportador vesicular de monoaminas (TVMA) (Teng et al., 1998), ela evita a translocação de monoaminas para as vesículas de armazenamento intraneuronal (BROWN; HANSON; FLECKNSTEIN, 2001; HEAL et al., 2013; YOUNG; GLENNON, 1986).

Assim, a anfetamina estimula todo o eixo cerebrospinal, o córtex, o tronco cerebral e o bulbo, aumentando o estado de alerta, diminuindo a fadiga e o apetite, causando insônia e em altas concentrações pode produzir comportamento estereotipado (CARVALHO et al., 2012; WHALEN; FINKEL; PANAVELIL, 2016). Neste contexto, ela vem sendo consumida de forma abusiva por humanos e, em consequência disso, acaba desenvolvendo rápida dependência física e psicológica nos seus usuários (GORDIAN; FORSTER, 2015). Drogas psicoestimulantes como a anfetamina também podem promover psicoses (devido ao aumento de DA na via dopaminérgica mesolímbica) em indivíduos normais ou agravar os sintomas de indivíduos que já apresentam algum transtorno psicótico (SAGUD et al., 2009; VOLLENWEIDER et al., 1998; WHALEN; FINKEL; PANAVELIL, 2016).

Diante de todos os efeitos que a anfetamina pode apresentar, frequentemente, ela vem sendo utilizada em animais experimentais a fim de mimetizar um modelo de psicose nos mesmos, possibilitando assim uma melhor compreensão dos seus mecanismos envolvidos bem como servindo como modelo para a busca de possíveis tratamentos farmacológicos (CERETTA et al., 2016; FEATHERSTONE; KAPUR; FLETCHER, 2007; KRUM et al., 2020).

2.1.4 Diagnóstico

O diagnóstico de transtornos psicóticos é baseado em critérios clínicos, portanto, são avaliados predominantemente a história (idade, alimentação, histórico familiar e social), o comportamento (humor, fala, afeto, processos do pensamento), relatórios subjetivos (frequência e características das alucinações) e resultados do exame do estado mental do paciente (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; SNYDERMAN; ROVNER, 2009). Testes de

neuroimagem, eletroencefalograma (EEG), avaliações genotípicas, toxicológicas e sorológicas são realizadas somente em pacientes que apresentam alguma doença neurodegenerativa preexistente associada ao aparecimento dos sintomas psicóticos, após o primeiro episódio de sintomas psicóticos ou devido ao uso abusivo de drogas, entre outras condições médicas. No entanto, esses testes não são específicos ou sensíveis suficientemente para diagnosticar casos isolados de transtornos psicóticos, embora apresentem diferenças nos resultados entre grupos de pacientes psicóticos de pessoas saudáveis (LIEBERMAN; FIRST, 2018).

O teste de neuroimagem através da tomografia por emissão de pósitrons (TEP) e a ressonância magnética (IRM) detecta algumas anormalidades em pacientes com transtornos psicóticos, como reduções no volume e espessura de regiões cerebrais (STEEN et al, 2006). Em pacientes esquizofrênicos, por exemplo, na TEP também é possível observar níveis aumentados de DA no estriado ventral e níveis diminuídos no córtex frontal, enquanto na IRM podemos detectar níveis aumentados de GLU nas regiões temporais pré-frontal e medial (KEGELES et al., 2010; KRAGULJAC et al., 2014; POELS et al., 2014). O teste neurofisiológico eletroencefalograma (EEG) é utilizado principalmente em pacientes cujos sintomas psicóticos aparecem pela primeira vez e não se sabe a causa primária para o acometimento desse sintoma, no qual, podem ser observados potenciais eliciados por eventos motores, sensoriais e cognitivos anormais nesses pacientes. Por fim, podem ser realizados testes sorológicos que inclui hemograma completo, perfil metabólico (função renal e hepática, níveis de eletrólitos e glicose), testes de função tireoidiana, hormônio da paratireoide, cálcio, vitamina B_{12}, folato e niacina, detecção do vírus da imunodeficiência humana e sífilis, presença de anticorpos e imunoglobulinas IgG contra a subunidade NR1 do receptor NMDA de GLU em amostra de sangue ou líquido cefalorraquidiano, bem como, testes de toxicologia da urina (presença de drogas lícitas e ilícitas) e, testes genotípicos (expressão de genes de interesse) (LEVENSON, 2005; LIEBERMAN; FIRST, 2018).

2.1.5 Tratamentos disponíveis

Atualmente os antipsicóticos são utilizados como fármacos de primeira escolha para o tratamento dos sintomas psicóticos. A eficácia clínica desses fármacos consiste principalmente no antagonismo de receptores de DA do tipo D_2 (ocupação de 60-75%) em regiões subcorticais do cérebro, modificando a neurotransmissão dopaminérgica (KAPUR; SEEMAN, 2001; MEYER,

2019). No entanto, sua eficácia farmacológica pode variar dependendo do transtorno subjacente de cada paciente (MIYAMOTO et al., 2012). Os antipsicóticos são classificados em típicos ou atípicos baseados em seus efeitos clínicos e mecanismo de ação (MEYER, 2019; STOCKMEIER et al., 1993).

Fármacos antipsicóticos típicos ou de 1ª geração, como a clorpromazina, haloperidol, flufenazina, perfenazina, loxapina, foram os primeiros fármacos eficazes para o tratamento da esquizofrenia após uma descoberta casual em 1950 (STAHL, 2014). Esses fármacos são eficazes na redução dos sintomas psicóticos (sintomas positivos) devido à ação antagonista em receptores dopaminérgicos do tipo D_2 na via dopaminérgica mesolímbica. Em contrapartida, eles estão diretamente relacionados ao aparecimento de efeitos motores extrapiramidais (acatisia, distonia, tremores e discinesia tardia - decorrente da alta taxa de ocupação de receptores de DA D_2 na via dopaminérgica nigroestriatal), hiperplolactinemia (devido à ação antagonista em receptores de DA D_2 na via dopaminérgica tuberoinfundibular) e embotamento cognitivo (devido à ação antagonista em receptores de DA D_2 na via dopaminérgica mesocortical) (KAPUR; SEEMAN, 2001; KINON; LIEBERMAN, 1996; MEYER, 2019).

Os fármacos antipsicóticos atípicos ou de 2ª geração, como a risperidona, clozapina, olanzapina, aripiprazol, lurasidona, possuem ação antagonista em receptores de serotonina 5-HT_{2A} e em receptores D_2 de DA. Estes fármacos, por exemplo, possuem eficácia sobre os sintomas positivos, negativos e cognitivos da esquizofrenia e reduzem o risco de efeitos motores extrapiramidais em comparação com os antipsicóticos típicos, efeitos estes associados aos diferentes perfis de ligação a receptores que estes fármacos apresentam. Entretanto, estão associados ao aumento de ganho de peso e distúrbios no metabolismo da glicose e de lipídeos podendo levar ao desenvolvimento de doenças cardiometabólicas (KINON; LIEBERMAN, 1996; LIEBERMAN; KANE; JOHNS, 1989; MEYER, 2019). A clozapina, interessantemente, tem sido indicada para o tratamento de sintomas psicóticos refratários (pacientes com resposta parcial ou nenhuma resposta a outros agentes antipsicóticos). Contudo, ela também está associada ao aparecimento de convulsões, miocardite, agranulocitose, apesar de produzir poucos efeitos motores extrapiramidais (MEYER, 2019; MIYAMOTO et al., 2012).

Por fim, os fármacos antipsicóticos possuem formas farmacêuticas orais de curta duração, que requerem administração diária, e injetáveis de curta e longa duração. As formulações injetáveis de ação prolongada facilitam a adesão do paciente ao tratamento e são administradas

periodicamente, por exemplo, a cada 4 semanas para o decanoato de haloperidol (via intramuscular) (KISHIMOTO et al., 2018; MEYER, 2019). Fármacos antipsicóticos associados com outros agentes (adjuvantes) como os inibidores da MAO-B, antioxidantes, hormônios, estabilizantes do humor, antidepressivos entre outros, também têm sido examinados na tentativa de amenizar os sintomas psicóticos em pacientes onde a monoterapia não é totalmente eficaz (CORRELL et al., 2017).

2.1.5.1 Haloperidol

O haloperidol, 4-[4-(4-clorofenil)-4-hidroxi-1-piperidil]-1-(4-fluorofenil)-1-butanona, é uma butirofenona pertencente a classe dos antipsicóticos típicos (CREESE; BURT; SNYDER, 1996). Este medicamento foi descoberto em 1958 pelo médico e farmacologista Paul Janssen e aprovado em 1967 pela agência regulatória americana *Food and Drug Administration* (FDA) para o tratamento de transtornos psiquiátricos (BYMASTER et al., 1996; ANDREASSEN; JORGENSEN, 2000; JASSEN, 1998). Atualmente, devido ao seu baixo custo, ampla disponibilidade, especificidade, potência e longa duração, o haloperidol tornou-se um dos medicamentos antipsicóticos mais vendidos no mundo para combater sintomas psicóticos decorrentes da esquizofrenia, transtorno bipolar, depressão psicótica, entre outros (PILLAI et al., 2008). No Brasil, o haloperidol encontra-se presente na lista de assistência farmacêutica do Sistema único de Saúde (SUS), regulamentado pela portaria GM/MS nº 1.555, de 30 de julho de 2013 para ser utilizado como agente antipsicótico (esquizofrenia aguda e crônica, confusão mental aguda), agente antiagitação psicomotora (mania, demência, alcoolismo) e antiemético (náuseas e vômitos, quando terapêuticas mais específicas não foram suficientemente eficazes) (ANVISA).

A eficácia farmacológica do haloperidol é atribuída ao antagonismo dos receptores D_2 na via dopaminérgica mesolímbica (DOLD et al., 2015; MEYER, 2019). Além disso, ele pode atuar com menor atividade nos receptores dopaminérgicos D_1, D_3, D_4, serotoninérgicos 5-HT_{2A} e adrenérgicos α_1 (KEILHOFF et al, 2010). No entanto, sua utilização acaba comprometida devido ao aparecimento de efeitos adversos, com os efeitos motores extrapiramidais (parkinsonismo, acatisia, acinesia, distonia aguda e discinesia tardia), prolongamento do intervalo QT, arritmias, hipotensão, hipotermia, retenção urinária, entre outros (TRAN et al., 1997).

Dada a complexidade do nosso sistema nervoso, é difícil estabelecer quais mecanismos e alterações neuronais estão associadas às mudanças comportamentais individuais causadas pelo haloperidol. Assim, modelos experimentais têm sido frequentemente utilizados na tentativa de elucidar com maior clareza os seus mecanismos, efeitos adversos e comportamentais (BENVEGNÚ et al., 2012; FACHINETTO., 2007; KRUM et al., 2020).

2.2 PRODUTOS NATURAIS

Produtos naturais a base de plantas contém uma variedade de compostos biologicamente ativos, os quais ao longo dos anos vêm sendo utilizados empiricamente pelo homem como remédio para tratar inúmeras doenças, bem como servindo de fonte para novos achados científicos e desenvolvimento de medicamentos fitoterápicos (CRAGG; NEWMAN, 2013; DIAS; URBAN; ROESSNER, 2012; HARVEY, 2008).

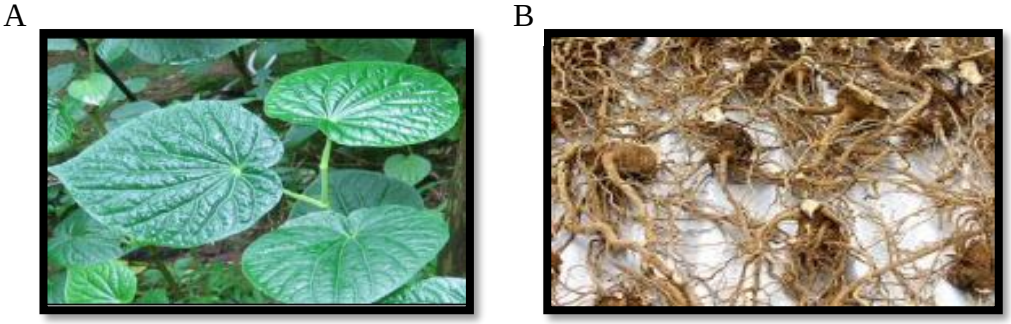
Segundo a Organização Mundial da Saúde (OMS), em 1985 aproximadamente 65% da população mundial fazia uso de medicamentos à base de plantas para cuidados primários a saúde (FARNSWORTH et al., 1985). Entre os anos de 1981 até 2010, aproximadamente 34% dos fármacos aprovados pela FDA, baseados em pequenas moléculas, eram produtos naturais ou derivados diretos dos mesmos (CARTER, 2011; CRAGG; NEWMAN, 2013; MISHARA; TIWARI, 2011). Neste contexto, pesquisas com produtos naturais foram e continuam sendo realizadas e, através destas, importantes fármacos foram descobertos, como a quelina, da *Ammi visnaga* (L) Lamk, planta que também levou ao desenvolvimento da cromolina (na forma de cromoglicato de sódio) um fármaco antialérgico; a galegina, da *Galega officinalis L* que serviu de modelo para a síntese de metformina e outros antidiabéticos do tipo biguanidina; a quinina, da *Cinchona officinalis* como antimalárico; a papaverina, da *Papaver somniferum* que serviu de base para o verapamil usado no tratamento da hipertensão, a morfina e a codeína, também da *Papaver somniferum* conhecidas como fonte de analgésicos, entre outros (BUSS; WAIGH, 1995; CRAGG; NEWMAN, 2012; FABRICANT; FARNSWORTH, 2001). No entanto, apesar da gama de plantas existentes, apenas 6% (± 300.000 espécies) já foram farmacologicamente exploradas (BALANDRIN; KINGHORN; FARNSWORTH, 1993; FABRICANT; FARNSWORTH, 2001; RASKIN et al., 2002).

Assim, investigar mecanismos farmacológicos de compostos ativos derivados de produtos naturais/plantas, bem como aprofundar os já conhecidos podem servir de base para a elaboração de fármacos eficazes ou adjuvantes para o tratamento de uma infinidade de doenças e, desta forma, melhorar a qualidade de vida dos pacientes que necessitam de terapia medicamentosa.

2.2.1 Piper methysticum

P. methysticum é um composto natural, encontrado na forma de arbusto, pertencente a família Piperacea (pimenta) e nativo das regiões etnogeográficas das Ilhas do Pacífico Melanésia, Micronésia e Polinésia (JUSTO; SILVA, 2008; SARRIS et al., 2012; SINGH; SINGH, 2002). Nesta região, *P. methysticum* é consumido sob a forma de bebida a partir da maceração do seu rizoma (Figura 2) combinado com leite de coco ou água (NORTON; RUZE, 1994). A bebida assim preparada é usada durante cerimônias religiosas e culturais para fins medicinais ou em eventos sociais como uma bebida inebriante capaz de promover relaxamento e bem-estar (BIBLIA et al., 2002; SARRIS; LAPORTE; SCHWEITZER, 2011).

Figura 2 - *Piper methysticum*



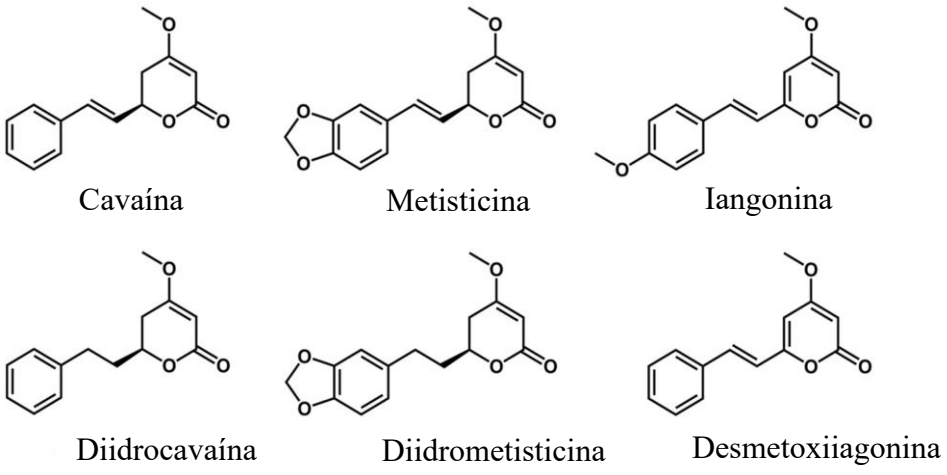
Fonte: (Adaptado de JUSTO; SILVA, 2008). A – Arbusto; B – Rizoma.

Por conseguinte, *P. methysticum* também se tornou popular em países ocidentais e europeus devido às suas propriedades sedativas, antiestresse e ansiolíticas (SINGH; SINGH, 2002). No Brasil seu extrato bruto vem sendo comercializado na forma de cápsulas desde o ano de 2001 quando foi liberado como medicamento fitoterápico pela Agência Nacional de Vigilância Sanitária (ANVISA) como uma alternativa ao uso de benzodiazepínicos (ARKOWITZ; LILIENFELD, 2013). Desde então sua prescrição vem aumentando, o qual chegou a estar presente na lista dos 10

medicamentos fitoterápicos mais vendidos em todo o país no início dos anos 2000 (BARBOSA; LENARDON; PARTATA, 2013).

Os principais compostos responsáveis pela ação farmacológica do *P. methysticum* são denominados de kavalactonas ou kavapironas. Esses compostos exercem aproximadamente 95% da atividade terapêutica do extrato e são os responsáveis pela denominação popular "Kava Kava" atribuído a ele (SMITH, 1983, 1984). A quantidade de kavalactonas pode variar conforme o local de origem, condições de crescimento e parte da planta (raiz, caule e folhas) que diminui progressivamente em direção às partes aéreas do arbusto (SINGH; SINGH, 2002). Aproximadamente 15 kavalactonas estão presentes no extrato bruto de Kava (SMITH, 1983, 1984), porém apenas 6 delas, a cavaína, metisticina, iangonina, diidrocavaína, diidrometisticina e desmetoxiiagonina (Figura 3) possuem grau de significância (GAUTZ et al, 2006;. JIANG et al, 2007; LI et al., 2011). Os demais constituintes presentes no extrato incluem: aminoácidos, minerais (alumínio, ferro, magnésio, potássio, cálcio e sódio, três chalconas (flavokavains A, B e C) e compostos fenólicos (SINGH; SINGH, 2002; XIAN-GUO et al., 1997).

Figura 3 - Estrutura química das kavalactonas



Fonte: (Adaptado de LI et al., 2011).

Com relação aos efeitos farmacológicos do extrato de Kava, destaca-se sua ação sobre os receptores $GABA_A$, mecanismo que está diretamente relacionado à diminuição dos sintomas de ansiedade, insônia ou estresse em pacientes que fazem uso do seu extrato (SARRIS et al., 2012;

SAVAGE et al., 2015). Numerosas proteínas incluindo canais de Na^+ e Ca^{2+} voltagem-dependentes, receptores μ e δ opioides, receptor tipo 2 de DA, receptores tipo 1 e 2 de histamina, receptor tipo 1 canabinóide e enzima MAO (isoforma B) também são alvos moleculares do extrato. Além disso, o extrato de Kava também parece atuar na modulação dos neurotransmissores DA e NE diminuindo a recaptação neuronal dos mesmos (BAUM et al, 1998; CAIRNEY, MARUFF, CLOUGH, 2002; GLEITZ et al., 1997; LIGRESTI et al., 2012; MUNTE et al., 1993; UEBELHACK, FRANKE, SCHEWE, 1998).

Em relação aos efeitos adversos causados por Kava podemos encontrar erupções cutâneas, queda de cabelo, amarelamento da pele, vermelhidão nos olhos, depressão/tendências suicidas, perda de apetite e hepatotoxicidade [associada com alterações nas enzimas do sistema citocromo P450 (CYP1A2, CYP2C19, CYP3A), presença das chalconas, dose utilizada, tempo de tratamento e o tipo de solvente usado para sua extração] (ANVISA; BEHL et al., 2011; OLSEN; GRILLO; SKONBERG, 2011; TESCHKE; GENTHNER; WOLFF, 2009; TESCHKE; SARRIS; LEBOT, 2011). No entanto, apesar dos relatos de suposta hepatotoxicidade e demais efeitos adversos causados pelo extrato, revisões sistemáticas e meta-análises realizadas nos últimos 15 anos encontraram um claro risco-benefício positivo para o extrato, tendo em vista a falta de evidências causais diretas (PITTLER; ERNST, 2003; SARRIS; LAPORTE; SCHWEITZER, 2011; WHITE, 2018).

Em ensaios clínicos, o extrato de Kava demonstrou superioridade em relação ao placebo em reduzir sintomas de ansiedade e, em geral, é bem tolerado, com efeitos adversos leves a moderados (SARRIS et al., 2009; SARRIS et al., 2013a; SARRIS et al., 2013b). A farmacocinética e a farmacodinâmica do seu extrato bruto ainda não estão totalmente esclarecidas (ANVISA, 2021). Como principal ação, o extrato fornece efeitos ansiolíticos agudos como os benzodiazepínicos. Com relação aos demais efeitos farmacológicos deste extrato, estes ainda são preliminares e pouco conhecidos, como os estudos mostrando as propriedades analgésicas, anticonvulsivantes, anticâncer, antipsicóticas e sobre a enzima MAO (ABU, N. et al., 2012; CAIRNEY; MARUFF; CLOUGH, 2002; JAMIESON; DUFFIELD, 1990). Portanto estudá-los e compreendê-los é de extrema relevância.

2.2.1.1 Enzima monoaminoxidase

A enzima MAO é encontrada na membrana externa de mitocondrias presentes em neurônios, células da glia e em tecidos periféricos de mamíferos (BACH et al., 1988; BLAZEVIC et al., 2017). Esta enzima é responsável pela desaminação oxidativa de aminas biogênicas como neurotransmissores e hormônios (COHEN et al., 1997; YOUDIM; BAKHLE, 2006). O processo de desaminação oxidativa envolve aminas primárias, secundárias e terciárias a seus aldeídos e suas aminas livres correspondentes, acompanhadas da geração de peróxido de hidrogênio (H_2O_2). O aldeído é rapidamente metabolizado pela enzima aldeído desidrogenase (ADH) em metabólitos acídicos (5-HIAA da 5-HT ou ácido diidroxi-fenilacético (DOPAC) da DA) que são comumente usados para mensurar a atividade da MAO *in vitro* e *ex vivo* (YOUDIM; BAKHLE, 2006).

A enzima MAO possui duas isoformas, as quais foram identificadas e denominadas como MAO-A e MAO-B (BACH et al.,1988; FIMBERG; RABEY, 2016). A diferenciação dessas isoformas foi primeiramente definida através da sensibilidade que cada isoforma tinha a um determindo substrato e a um inibidor, antes da sua caracterização molecular (BACH et al.,1988; FINBERG E RABEY, 2016). A isoforma MAO-A preferencialmente desamina aminas hidroxiladas como a NA e 5-HT e é inibida pelo inibidor seletivo irreversível clorgilina. Já a isoforma MAO-B desamina aminas não hidroxiladas tais como benzilamina e beta-feniletilamina e é seletivamente inibida pela rasagilina, pargilina e em baixas concentrações de selegilina (FIEDOROWICZ; SWARTZ, 2004, FIMBERG; RABEY, 2016, YOUDIM e BAKHLE, 2006). Aminas como adrenalina, DA, triptamina e tiramina são substratos de ambas as isoformas na maioria das espécies (YOUDIM e BAKHLE, 2006). Na sua conformação molecular, ambas as enzimas são diméricas em suas formas ligadas à membrana, apresentando uma similaridade de 70% de identidade sequencial. Segundo dados cristalográficos e bioquímicos, a principal diferença entre as isoformas da MAO está na estrutura de seus sítios ativos (BINDA; MATTEVI; EDMONDSON, 2011; EDMONDSON et al., 2009; FOWLER et al., 2007). A proporção das isoformas da enzima também pode variar de tecido para tecido. No tecido cerebral, a MAO-A é encontrada predominantemente em regiões com alta densidade de neurônios catecolaminérgicos como *locus coeruleus,* substância negra e regiões pariventriculares do hipotálamo. Já a MAO-B é preferencialmente expressa em neurônios serotoninérgicos (células do núcleo dorsal da *rafe*) e em astrócitos (SHIH; CHEN; RIDD, 1999; WESTLUND et al., 1985).

A enzima MAO parece exercer um papel importante na fisiopatologia de alguns distúrbios neurológicos e psiquiátricos devido à sua função no metabolismo das catecolaminas. Agentes inibidores da MAO impedem o metabolismo de neurotransmissores e assim aumentam a disponibilidade dos mesmos (FISAR, 2016). Desta forma, esses agentes são utilizados como uma ferramenta para o tratamento de doenças do SNC afim de amenizar os sintomas como os da depressão, transtornos de ansiedade, transtorno do déficit de atenção e hiperatividade, e nas doenças de Parkinson e Alzheimer (BORTOLATO; CHEN; SHIH, 2008; YOUDIM e BAKHLE, 2006). No entanto, ao longo do tratamento, os fármacos inibidores da MAO usados na clínica podem demonstrar eficácia reduzida e efeitos adversos, gerando assim a necessidade de busca por novos compostos. Sabe-se hoje, que alguns compostos provenientes de produtos naturais apresentam mecanismos de inibição para ambas as isoformas da MAO, como por exempo, as kavalactonas e chalconas presentes no extrato bruto de Kava. A enzima MAO é um dos alvos farmacológicos de Kava e, neste trabalho, sua interação com a enzima será amplamente abordado (COETZEE et al., 2016; GUO et al., 2016; LIANG et al., 2016; SCOLIO et al., 2014; PRINSLOO et al., 2019; YOUDIM e BAKHLE, 2006; ZARMOUH et al., 2016).

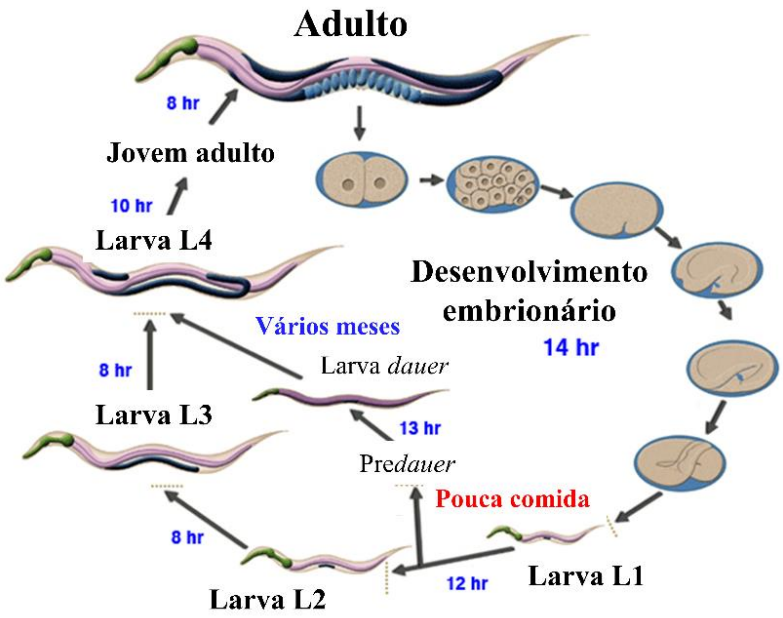
2.3 MODELOS ANIMAIS

A utilização de modelos animais experimentais tem contribuído significativamente para a compreensão e o avanço da medicina durante milênios. Diversos tipos de animais experimentais como *C. elegans*, *Drosophila melanogaster*, *Danio rerio*, camundongos, ratos, entre outros, têm sido usados em estudos pré-clínicos (ELLENBROEK; YOUN, 2016; FONTANA et al., 2018; NIGON; FÉLIX, 2017; YAMAGUCHI; YASHIDA, 2018). Ao longo dos anos, técnicas cirúrgicas, desenvolvimento de vacinas, antibióticos, alterações genéticas, e mais uma variedade de achados científicos foram observados utilizando animais experimentais (HADDAD, 2004; KING, 2012; SABIN, 1965). A pesquisa animal contribui para a melhora da saúde e qualidade de vida da população em geral. Em relação ao SNC, modelos animais experimentais nos permitem observar e compreender alterações neuroquímicas e estruturais do SNC diretamente relacionados com determinada doença/ transtorno, desenvolver novas estratégias terapêuticas mais eficazes e compreender as já existentes (WADENBERG; HICKS, 1999).

2.3.1 *Caenorhabditis elegans*

C. elegans é um nematoide microscópico de vida livre (1-2 mm de comprimento) que se alimenta de micróbios, principalmente bactérias, e possui uma vida útil curta (2-3 semanas) com alta taxa reprodutiva (~300 ovos/por hermafrodita) (ALTUN; HALL, 2009). Diferentes tipos de cepas desses animais são cultivados em placas de *petri* com ágar ou em culturas líquidas, ambas contendo bactéria como alimento (LEWIS; FLEMING, 1995). *C. elegans* é encontrado na forma de hermafrodita (XX - reprodução através de autofecundação, com progênie geneticamente idêntica) ou como macho (XO - surge do acasalamento entre um hermafrodita e um macho ou raramente por não disjunção espontânea na linha germinativa hermafrodita) (ZARKOWER, 2006). O seu ciclo de reprodução completo compreende entre 2,5 e 3 dias (22˚C) e tem início a partir da ovoposição de um hermafrodita (desenvolvimento embrionário), para a eclosão dos seus ovos e desenvolvimento de quatro estágios larvais (L1, L2, L3 e L4) até a idade adulta (jovem adulto e adultos) (Figura 4) (ALTUN; HALL, 2009).

Figura 4 – Ciclo de vida de *C. elegans* a 22˚C



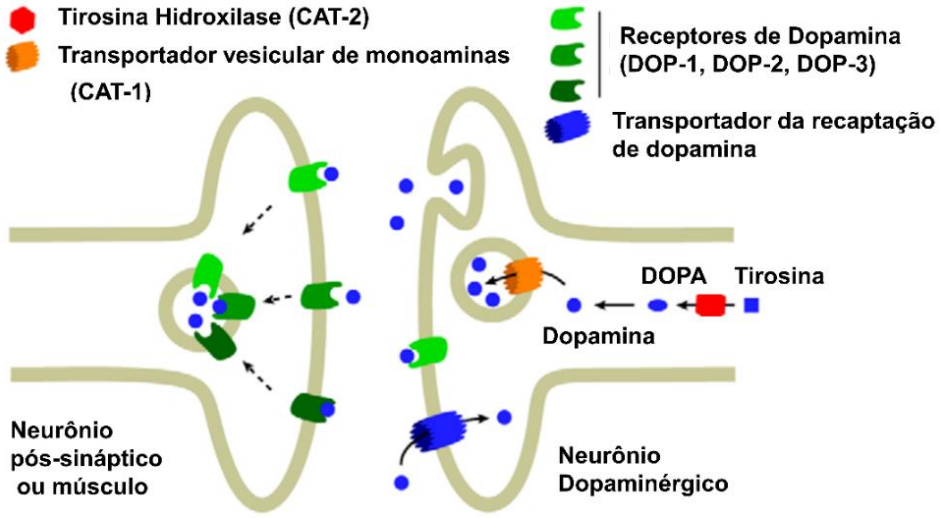
Fonte: (Adaptado de © Wormatlas, ALTUN; HALL, 2009).

O fim de cada estágio larval é marcado pela síntese de uma nova cutícula (composta por colágeno e secretada pelo epitélio subjacente) que envolve o verme na parte externa e reveste a faringe e o reto, juntamente com a cessão do bombeamento faríngeo e um breve letargo do animal (ALTUN; HALL, 2009). Interessantemente, essas cepas também podem sobreviver a condições ambientais adversas, pois mudam para um estágio larval chamado *dauer*, podendo aumentar sua expectativa sua de vida de quatro a oito vezes (CASSADA; RUSSELL, 1975).

C. elegans possui uma anatomia simples, com aproximadamente 1000 células somáticas. Além disso, ele é geneticamente tratável, o que possibilita a realização da fusão de genes como, por exemplo, da proteína fluorescente verde – GFP – que associada ao seu revestimento corporal transparente permitem a visualização da morfologia celular e padrões de expressão de proteínas *in vivo* (ENGLEMAN; KATNER; NEAL-BELIVEAU, 2016). Eles também expressam genes homólogos referentes a algumas funções biológicas básicas e vias bioquímicas (apoptose, resposta ao estresse e vias de sinalização celular) a de organismos superiores (BLAXETER, 2011; CULETTO; SATTELLE, 2000; MALIN; SHAHAM, 2015; RODRIGUEZ et al., 2013). Além disso, eles possuem uma série de comportamentos como locomoção, defecação, postura de ovos, comportamento social, aprendizado, memória e respondem a estímulos como cheiro, sabor, tato e temperatura (RANKIN, 2002; DE BONO, 2003).

O sistema nervoso de *C. elegans* é um sistema bem caracterizado incluindo a presença de receptores, transportadores vesiculares, canais iônicos e outros componentes sinápticos funcionalmente semelhante ao dos mamíferos (BARGMANN, 1998; WHITE et al., 1976). Um bom exemplo para isso, são os 8 neurônios dopaminérgicos dentre os 302 neurônios presentes em um hermafrodita, os quais expressam proteínas da síntese de DA e a maioria das proteínas envolvidas em seu transporte vesicular, liberação, recaptação e sinalização (pré-sináptica e pós-sináptica) análogas aos de vertebrados (BONO; VILLU MARICQ, 2005; SAWIN; RAGANATHAN; HORVITZ, 2000). Além disso, *C. elegans* possuem receptores dopaminérgicos acoplados a proteína G, os quais são classificados em *dop-1* (proteína G estimulatória) receptor semelhante ao receptor D_1 de vertebrados e *dop-2* e *dop-3* (proteína G inibitória) semelhante ao receptor D_2 de vertebrados. Além disso, os receptores do tipo D_1 e D_2 são expressos principalmente nos neurônios motores do cordão ventral (Figura 5) (BENEDETTO et al., 2010).

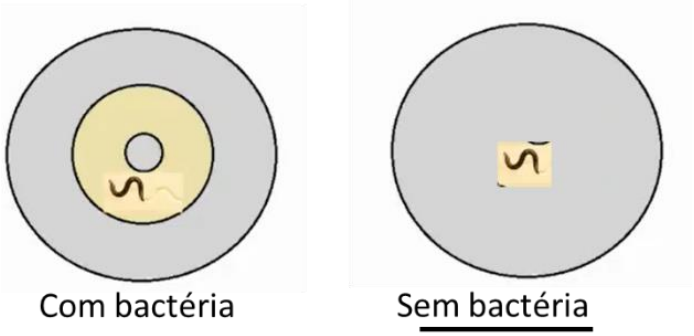
Figura 5 – Proteínas envolvidas na sinapse dopaminérgica em *C. elegans*



Fonte: (Adaptado de BENEDETTO et al., 2010).

Em *C. elegans*, a DA participa do controle de funções fisiológicas como a saciedade alimentar, postura e produção de ovos, locomoção, a qual pode ser avaliada pelo teste de resposta de desaceleração basal, por exemplo, (Figura 6), entre outros (HILLS; BROCKIE; MARICQ, 2004; MERSHA et al., 2013; BONO; VILLU MARICQ, 2005; SAWIN; RAGANATHAN; HORVITZ, 2000).

Figura 6 – Comportamento de resposta de desaceleração basal



Fonte: (adaptado de SAWIN et al., 2000). Resposta de desaceleração basal. Para realização do teste, *C. elegans* são lavados três vezes com tampão apropriado para remover bactérias residuais e então colocados no centro de placas de *petri* na ausência ou presença de um anel de bactérias. Após habituação de cinco minutos, o número de curvas corporais de cada verme é contabilizado durante 20 s para determinar sua taxa de imobilidade. Os resultados são expressos como a diferença (Δ) entre o número de curvas corporais na ausência (a) e na presença (p) de bactérias em placas frescas (Δ= a-p).

Sabe-se também que um desbalanço na sinalização de DA tem sido associada a distúrbios neurológicos e mudanças comportamentais (SOLANTO, 2002; WISE, 2004). Outros neurotransmissores como o GABA, acetilcolina, 5-HT e GLU também apresentam interações com o sistema dopaminérgico em *C. elegans* (BROWNLEE; FAIRWEATHER, 1999).

Desta forma, o modelo genético de *C. elegans* tem sido frequentemente utilizado em pesquisas a fim de explorar a ação mecanicista de drogas, novos alvos terapêuticos e comportamento animal. Além disso, a manutenção em laboratório de cepas de *C. elegans* é fácil, barata e passível de análises de alto rendimento. Inúmeras características que o torna um ótimo modelo animal experimental.

2.3.2 Roedores

Roedores como ratos e camundongos têm sido um dos modelos animais mais utilizados na pesquisa científica nos últimos anos. O uso de roedores nos permite compreender processos celulares, perturbações genéticas, bem como testar terapias farmacológicas. No geral, esses animais têm um importante papel na compreensão da etiologia, fisiopatologia e farmacologia de doenças neuropsiquiátricas (ELLENBROEK; YOUN, 2016; PARKER, PETERSON, 2018). Neste contexto, roedores são frequentemente utilizados em estudos pré-clínicos, pois nos permitem compreender mecanismos de ação de novos fármacos ou dos já utilizados na clínica e observar alterações comportamentais geradas pelos mesmos (WADENBERG; HICKS, 1999). Além disso, a utilização de camundongos tem sido frequentemente usada como um modelo animal de diversas doenças. Por exemplo, esses animais são frequentemente utilizados como modelo animal de psicose, uma vez que modificações ambientais ou administração de fármacos como a anfetamina geram alterações comportamentais nesses animais que mimetizam os sintomas apresentados por pacientes com transtornos psicóticos (ARGUELO; GOGOS, 2006; CARCELES-CORDON et al., 2020; HARTMANN, et al., 2019; JONES; WATSON; FONE, 2011; LEE et al., 2020).

Desta forma, o uso de roedores é importantíssimo para melhorar a compreensão das alterações neuroquímicas e estruturais do SNC e a ação mecanicista e comportamental gerado por determinado fármaco.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar os efeitos do antipsicótico haloperidol em *C. elegans* e do extrato bruto de Kava em camundongos sobre o comportamento e a atividade da enzima MAO.

3.2 OBJETIVOS ESPECÍFICOS

- Investigar as interações do antipsicótico haloperidol sobre o sistema dopaminérgico em *C. elegans* através de avaliação comportamental e de marcadores moleculares e morfológicos específicos.

- Avaliar os efeitos do extrato bruto de Kava em um modelo de psicose induzido por anfetamina em camundongos.

- Analisar o potencial efeito inibitório do extrato bruto de Kava sobre a atividade da enzima MAO em ensaios *in vitro* e *ex vivo* em camundongos.

4 RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. O **artigo 1** está disposto na forma que foi publicado na edição da Revista Científica *Molecular Neurobiology*. O **artigo 2** está disposto na forma que foi publicado na edição da Revista Científica *Journal of Ethnopharmacology*. O **artigo 3** está disposto na forma que foi publicado na edição da Revista Científica *Journal of Traditional and Complementary Medicine*.

4.1 ARTIGO 1

Artigo

Haloperidol Interactions with the *dop-3* Receptor in *Caenorhabditis elegans*

Bárbara Nunes Krum; Airton C. Martins Jr; Libânia Queirós; Beatriz Ferrer; Ginger L. Milne; Félix Alexandre Antunes Soares; Roselei Fachinetto; Michael Aschner.

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Haloperidol Interactions with the *dop-3* Receptor in *Caenorhabditis elegans*

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Abstract

Haloperidol is a typical antipsychotic drug commonly used to treat a broad range of psychiatric disorders related to dysregulations in the neurotransmitter dopamine (DA). DA modulates important physiologic functions and perturbations in *Caenorhabditis elegans* (*C. elegans*) and, its signaling have been associated with alterations in behavioral, molecular, and morphologic properties in *C. elegans*. Here, we evaluated the possible involvement of dopaminergic receptors in the onset of these alterations followed by haloperidol exposure. Haloperidol increased lifespan and decreased locomotor behavior (basal slowing response, BSR, and locomotion speed via forward speed) of the worms. Moreover, locomotion speed recovered to basal conditions upon haloperidol withdrawal. Haloperidol also decreased DA levels, but it did not alter neither *dop-1*, *dop-2*, and *dop-3* gene expression, nor CEP dopaminergic neurons' morphology. These effects are likely due to haloperidol's antagonism of the D2-type DA receptor, *dop-3*. Furthermore, this antagonism appears to affect mechanistic pathways involved in the modulation and signaling of neurotransmitters such as octopamine, acetylcholine, and GABA, which may underlie at least in part haloperidol's effects. These pathways are conserved in vertebrates and have been implicated in a range of disorders. Our novel findings demonstrate that the *dop-3* receptor plays an important role in the effects of haloperidol.

Keywords Basal slowing response · Dopamine signaling · Lifespan · Locomotor behavior · Neurotransmitter · Worm

Introduction

The powerful genetic model *Caenorhabditis elegans* (*C. elegans*) is frequently used in research to explore mechanistic action of drugs, new drug targets, and animal behavior. *C. elegans* is a microscopic worm (1–2 mm in length), with short and productive life cycle (2–3 weeks). It has transparent body lining, which can be readily visualized through green fluorescence protein (GFP) to study genes of interest. It is genetically tractable; thus, it is possible to study different transgenic strains including those that express human proteins, and use them as a tool to study disorders of the nervous system [1]. The *C. elegans* nervous system has 302 neurons, 8 of which are dopaminergic. These neurons are classified into 3 types, 2 ADEs (anterior deirids), 4 CEPs (cephalic sensilla), and 2 PDEs (posterior deirids) [2]. They contain the requisite genes for dopamine (DA) synthesis, and most of the proteins involved in its packing, release, reuptake, and signaling, analogous to vertebrates [3].

The synaptic transmission of DA modulates many important physiologic functions in different species. Altered DA signaling (diminished or excessive) has been directly

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associated with brain disorders and behavioral changes in both invertebrates and vertebrates that can lead to onset of diseases such as Parkinson's disease, atypical movements, and diseases associated with attention-deficit hyperactivity disorder, impulse control, and reward, including schizophrenia and addiction [4–8]. DA regulates neuronal functions and participates in a wide array of behaviors such as locomotion and food sensation in *C. elegans* [3,9,10]. The locomotion, both forward and backward, is regulated by motoneurons in ventral nerve cord and the head ganglia of *C. elegans* [11]. When worms are on bacterial food, head neurons (CEPs and ADEs) [11, 12] and midbody neurons (PDEs) [13] release DA, causing a switch in behavior, mostly to forward locomotion with frequent reversals and slower movement [14, 15].

In *C. elegans*, DA is transduced by G protein-coupled receptors (GPCRs). These receptors are classified in *dop-1* ($G\alpha_q$, stimulatory G protein; $G\alpha_{olf}$, G protein involved in olfaction) as a D1-like receptor, *dop-2* ($G\alpha_{i/o}$, inhibitory G protein), and *dop-3* ($G\alpha_{i/o}$, inhibitory G protein) as a D2-like receptor, while *dop-4* ($G\alpha_s$, G protein), as an invertebrate-specific receptor [16–18]. *Dop-1* receptor has been involved in tap habituation and locomotion behavior [19, 20], *dop-2* auto-receptor in associative learning [21, 22] and anterior touch habituation, *dop-3* receptor in negative regulation of locomotion [20, 23], and *dop-4* receptor in general enhancement of avoidance responses [24]. Furthermore, D1-like and D2-like receptors are expressed mainly on ventral cord motor neurons [25]. Studies have shown that *C. elegans* locomotion is modulated via both types of dopaminergic receptors, which have their function in apposition to each other [20]. D1-like and D2-like receptors are found in analogous pathways in mammalian brain, oppositely modulating acetylcholine and γ-aminobutyric acid (GABA) release as well [14, 26]. There are several studies addressing issues pertinent to the dopaminergic system in *C. elegans* [27–33]. However, studies on the effects of antipsychotics on the dopaminergic system are scant and not exclusive to haloperidol, a typical antipsychotic [34–40].

Haloperidol is one of the oldest antipsychotic drug used in the treatment of psychiatric illnesses as schizophrenia, bipolar disorder, and psychotic depression [41], which continues to be largely used around the world. It acts mainly through D2-type dopamine receptor antagonism and at a lower activity on D1, D3, D4, serotoninergic receptors (5-HT_2A), and adrenergic receptors (α1) [42]. Pharmacological efficacy of haloperidol occurs mainly by blockade of D_2 receptors on the mesolimbic pathway. However, it can also cause QT interval prolongation, arrhythmias, and movement disorders such as parkinsonism, akathisia, akinesia, acute dystonia, and tardive dyskinesia, which are associated to the supra-regulation of D_2 receptors in the nigrostriatal pathway due to antagonism effects of haloperidol in these receptors [43]. Thereby, in order to explain the neurological alterations leading to the production of adverse effects poorly understood caused by antipsychotics as haloperidol, the use of animal models has been shown to be effective in clarifying the mechanisms associated with these alterations [44–49].

Thus, the main goal of this study was to investigate the effect of haloperidol on the dopaminergic system, with *C. elegans* as animal model, addressing specific behavioral, molecular, and morphological parameters. Here, we show that haloperidol antagonized the D2-like receptor *dop-3*. It not only increased worm lifespan but also decreased basal slowing response (BSR) and locomotion speed, concomitant with decreased DA levels. However, it did not alter neither *dop-1*, *dop-2*, and *dop-3* gene expression nor CEP dopaminergic neurons, morphology. Our novel findings reveal the involvement of haloperidol in the modulation of DA receptor *dop-3* and on DA signaling investigation in *C. elegans*.

Materials and Methods

Reagents

Haloperidol (CAS Number: 52-86-8) was purchased from Sigma (St. Louis, MO, USA). TaqMan primers used for real-time quantitative reverse transcription PCR (qRT-PCR) analysis were obtained from Life Technologies (Carlsbad, CA, USA). Agar, peptone, and agarose used in culture and test media preparation were purchased from BD (Franklin Lakes, NJ, USA). Trizol and SuperSignal West Pico chemiluminescent substrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

C. elegans Maintenance and Synchronization

C. elegans strains used in this study were as follows: wild-type (WT) N2 (+); BY200 *Pdat-1::gfp* (vtIs1), *dat-1::*GFP*(vtls1) V* and LX636, *dop-1(vs101)*; LX702, *dop-2(vs105)*; LX703, *dop-3(vs106)*; CB1112, *cat-2(e1112) II* loss-of-function mutants. The strains were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA), except BY200 *Pdat-1::gfp* (vtIs1) that was kindly provided by the Blakely laboratory, Vanderbilt University Medical Center. *C. elegans* was grown and maintained at 20 °C in 8P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 25 mM potassium phosphate buffer (pH 6), 0.013 mM cholesterol (in 95% ethanol), 1 mM $MgSO_4$, 1.25 mL nystatin/L) seeded with NA22 *Escherichia coli* (food source). Throughout the experiments, worms were maintained on nematode growth medium plates (NGM 51.3 mM NaCl, 17.0 g bactoagar/L, 2.5 g bactopeptone/L, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM potassium phosphate buffer (pH 6.0), 0.013 mM cholesterol

(in 95% ethanol), 1.25 mL nystatin/L, 5 mg streptomycin sulfate/L) seeded with OP50 *Escherichia coli* [50].

Synchronization of worms was performed recurring to hypochlorite solution (5 mL sodium hypochlorite, 1.25 mL 10 M sodium hydroxide, and 18.75 mL distilled water). Eggs were isolated with a 30% sucrose gradient, washed with sterile water, and resuspended in M9 buffer (42 mM Na_2HPO4, 22 mM KH_2PO4, 8.5 mM NaCl, and 1 mM $MgSO_4$). After 13 h, worms in the first larval stage (L1) were transferred to OP50-seeded NGM plates until they reached the four larval stage (L4). L4 worms were used for haloperidol exposure, as described below.

Haloperidol Exposure

The stock solution of haloperidol (20 mM) was prepared in dimethyl sulfoxide (DMSO) and dissolved in distilled water. Diluted acetic acid p.a. (1:10,000) was also added to the final solution to maximize solubility on the plates. The final concentration of haloperidol into the plate was calculated based on the total volume of each plate, which was included 6.4 mL NGM/agar plus 200 μl of drug-solution poured over the surface of 60 mm agar plates previously seeded with bacteria OP50. All the plates were allowed to dry and equilibrate overnight before use. L4-stage worms were placed on control plates (0.01% DMSO plus acetic acid p.a. (1:10,000) - haloperidol vehicle) or haloperidol treated plates with final concentrations of 80 μM and 160 μM [38, 39]. For lifespan assays, worms were transferred to fresh plates every day during egg laying. After it, worms were transferred every 4 days until their death. In the experiments performed during 1–6 days, worms were transferred to fresh plates every day until the end of the experiment. The concentrations used are in accordance with previous studies [38, 39].

Lifespan

For the lifespan analysis, wild-type worms at L4 larval stage were exposed to vehicle or haloperidol (80 μM and 160 μM) in OP50-seeded NGM plates from day 0 to their death. Worms were observed and counted daily to score the total number of dead and alive worms per plate. During the first week, worms were transferred to fresh plates every day to avoid mixing of different generations. When reproduction rates declined, worms were moved every 4 days. Worms were scored as dead when they did not respond to a mechanical stimulus, and censored when they displayed extruded internal organs, crawled off the plate, or died because of hatching progeny inside the uterus. The experiment was performed in four independent preparations (20 worms each).

Basal Slowing Response

BSR or food-sensing response is a behavioral assay that evaluates the functionality of the worms' dopaminergic system [2]. Briefly, L4 larvae WT N2, *dop-2*, and *dop-3* strains were exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 day in OP50-seeded NGM plates. After exposure, BSR test was carried out. For this, worms were washed three times with S-basal buffer (100 mM NaCl, 5 mg/L cholesterol, 50 mM KPO_4, pH 6.0) to remove residual bacteria, and placed on the center of a 60-mm NGM plates in the absence or presence of an OP50 bacteria ring. After habituation (5 min), the number of body bends of each worm was counted during 20 s to determine its immobility rate. According to Sawin et al. [2], worms with normal DA content move slower in the presence of bacteria than in the absence of bacteria. The results were expressed as the difference (Δ) between the number of body bends in the absence (a) and presence (p) of bacteria on fresh plates (Δ = a-p). The *cat-2*, a strain with reduced levels of DA, was used as positive control. The experiment was performed in four independent preparations.

Locomotion Speed

WT N2 and *dop-1*, *dop-2*, and *dop-3* (loss-of-function mutant strains for motor neurons) were used to evaluate several parameters of speed (moving average speed, forward, and backward speed). Thus, L4 larvae were exposed to vehicle or haloperidol (80 μM and 160 μM) for 1–6 days (days 1, 2, 4, and 6) in NGM plates. The speed parameters were analyzed separately for each strain by advanced software using WormLab 3.1 software (MBF Bioscience, Williston, VT, USA). Briefly, plates were placed under the microscope with a mono digital camera (DFK 72AUC02) attached and videos (60 s with 1600 × 1200 pixel resolution) were taken at 5 frames/s. Moving average speed refers to the speed of a particular worm, averaged across a number of frames (known as the size of the moving window), with reduced effects of noise, and a latency in speed measurement. Forward speed (the worm moves forward—head direction, if the sign is positive) and backward speed (the worm moves backward—tail direction, if the sign is negative) were the locomotion parameters analyzed. In the same video captured for speed analysis, size parameters such as length (from head to tail along the central axis), width (cross-section averaged over entire length), area (region within worm's contour), and wavelength (total length between the positive and the negative stationary points multiplied by 2) were also analyzed by WormLab software for the control and treated worms (Table S1, illustrating size parameters of WT N2 and *dop-1*, *dop-2*, *dop-3*). Worms were daily transferred to fresh plates containing vehicle or haloperidol soon after WormLab analyses. The experiment was performed

in four independent preparations (plates with 10–15 worms each).

Moving average speed was also evaluated in WT N2 worms (L4 stage) in presence (day 1) or absence (day 2) of haloperidol in OP50-seeded NGM plates. It was performed in order to evaluate whether after haloperidol withdrawal the moving average speed would continue to reflect haloperidol's effects. Firstly, worms were exposed to vehicle or haloperidol (80 μM and 160 μM). After 24 h of exposure, the plates were placed under a microscope and moving average speed was measured. Then, the plates were washed with M9 buffer and worms were added immediately to new plates without haloperidol (withdrawal, all plates), according to their respective groups. Worms remained in the fresh plates without haloperidol for 1 more day (24 h). At the end of the day 2, moving average speed was measured again. The experiment was carried out in four independent worm preparations (plates with 10–15 worms each).

Dopamine Content Analysis

To measure DA levels, 200,000 worms per treatment in L4 stage were exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 and 4 days in OP50-seeded NGM plates. Briefly, worms were washed off the plates with M9, transferred for tubes, and collected from the pellets, after centrifugation at 2500 rpm for 2 min. Then, the samples were frozen in liquid nitrogen and stored at − 80 °C. C*at-2* mutant strain was used as positive control. DA content was determined by a highly sensitive and specific liquid chromatography/mass spectrometry (LC/MS) methodology following derivatization of analytes with benzoyl chloride (BZC) [51]. The analysis was carried out in the Vanderbilt University Neurochemistry Core Laboratory using LC/MS, as previously described [52, 53], and it comprised three independent worm preparations.

RNA Isolation and Real-Time Polymerase Chain Reaction

Worms in L4 stage were exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 and 4 days in OP50-seeded NGM plates. To extract RNA, about 200 worms per condition were washed with M9 buffer and lysed with Trizol (1 mL) by mechanical homogenization (politron) three times. The samples were centrifuged (14,000 rpm, 10 min, 4 °C), the supernatant was removed, and chloroform (200 μL) was added to each tube. After further centrifugation, the top phase was collected and transferred to RNA-free polypropylene tubes followed by precipitation using isopropanol alcohol at − 20 °C for 1 h. Glycogen (2 μL) was added before isopropanol to allow RNA pellet. The precipitate was washed with 75% ethanol (300 μL) and then resuspended in RNase-free water. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and an equal RNA amount was converted to cDNA synthesis using the HighCapacity cDNA Reverse Transcription kit (Thermo Scientific). Real-time PCR was performed using thermocycler CFX96 real-time system (Bio-Rad). cDNAs from the above were used as templates and pre-designed TaqMan probes (Life Technologies) were used to detect *C. elegans dop-1*, *dop-2*, and *dop-3* (Ce02494345_m1; Ce02479624_m1; Ce02496463_m1, respectively) gene expression, while *ama-1* (Ce02462735_g1) was used as a housekeeping gene for normalization. Change in expression was determined using the $2^{-\Delta\Delta Ct}$ method [54]. All the real-time PCR were performed in a final volume of 20 μL1X of the TaqMan Universal PCR Master mix (Applied Biosystems). For both genes and endogenous control, the experiment was performed in five independent worm preparations.

Neurodegeneration Assay

BY200 worms were exposed to vehicle or haloperidol (80 μM and 160 μM) from 1 to 6 days (days 1, 2, 4, and 6) in OP50-seeded NGM plates. Fifteen worms were mounted on 4% agarose pads and anesthetized with 5 μL of 3 mM levamisole hydrochloride adapted from Sulston [55], with some modifications. Neuronal defects in the CEP dopaminergic neurons were evaluated with an epifluorescence microscope (Nikon Eclipse 801, Nikon Corporation, Tokyo, Japan) equipped with a Lambda LS Xenon lamp (Sutter Instrument Company) and Nikon Plan Fluor 20 × dry and Nikon Plan Apo 60 × 1.3 oil objectives. Each worm was scored for absence (0) or presence (1) of morphological changes. Shrunken and/or lost soma, puncta formation along dendritic processes, and loss of dendrites were used as represent degeneration parameters (morphological changes) [56]. The results were expressed as percentage of degenerated worms [57]. Each experiment was performed in four independent preparations.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Data were analyzed by one-way or two-way analysis of variance (ANOVA), followed by a post hoc Tukey's test, when appropriate. Survival curve was analyzed by the log-rank (Mantel-Cox) test. Results were considered statistically significant when $p < 0.05$.

Results

Haloperidol Extends Worm Lifespan

The lifespan of WT N2 worms exposed to vehicle or haloperidol (80 μM and 160 μM) was analyzed from day 0 to their

death. The results showed a significant effect in the lifespan of worms exposed to both haloperidol concentrations tested (Fig. 1). Control WT N2 worms had a mean lifespan of 14.26 ± 0.49 days, whereas for haloperidol groups, the mean lifespan was extended to 16.81 ± 0.81 days at 80 μM (18%) and to 17.46 ± 0.64 days at 160 μM (22%).

Haloperidol Alters BSR

BSR behavior depends on the functionality of the dopaminergic neurons, and it is measured by the change in body bends *per* 20 s. The WT N2, *dop-2*, and *dop-3* strains were exposed to haloperidol in this behavioral test (Fig. 2). Two-way ANOVA revealed a significant effect in the difference between the number of body bends in the absence and presence of bacteria in WT N2 worms (at highest concentration) and in *dop-2* strain (both concentrations), when compared with their respective control ($F(2, 27) = 10.28$, $p < 0.05$). There was no difference among the groups for the *dop-3* strain. A significant difference among the strains used ($F(2, 27) = 11.61$, $p < 0.05$), as well as an interaction between concentration and strains ($F(4, 27) = 6.98$, $p < 0.05$) were found. *Cat-2* loss-of-function mutant strain was used as positive control and showed also an impairment in the behavioral test ($F(3, 12) = 29.12$, $p < 0.05$).

Haloperidol Decreases Speed Parameters and This Effect Did Not Remain After Haloperidol Withdrawal in WT N2 Worms

WT N2 and *dop-1*, *dop-2*, and *dop-3* loss-of-function mutant strains were exposed to haloperidol for 1, 2, 4, and 6 days in NGM plates. Speed parameters were calculated separately for each strain by using WormLab software. Two-way ANOVA (with haloperidol concentration and exposure duration as variables) showed a significant effect of haloperidol on moving average speed ($F(2, 36) = 8.913$, $p < 0.05$) and forward speed ($F(2, 36) = 6.84$, $p < 0.05$) in WT N2 worms (Fig. 3a and b, respectively), with no change in backward speed (Fig. 3c). The time of exposure had a significant effect on the moving average speed ($F(3, 36) = 11.25$, $p < 0.05$), forward speed ($F(3, 36) = 14.49$, $p < 0.05$), and backward speed ($F(3, 36) = 15.35$, $p < 0.05$) with a significant interaction between haloperidol concentration and exposure duration ($F(6, 36) = 4.509$, $p < 0.05$). For *dop-1* strain, a significant effect of the time of exposure on the moving average speed ($F(3, 36) = 18.83$, $p < 0.05$), forward speed ($F(3, 36) = 21.70$, $p < 0.05$), and backward speed ($F(3, 36) = 3.665$, $p < 0.05$) was found (Fig. 3d, e, and f, respectively). Analogous to findings in the *dop-1* strain, haloperidol's time of exposure caused a significant effect on moving average speed ($F(3, 36) = 11.54$, $p < 0.05$), forward speed ($F(3, 36) = 11.87$, $p < 0.05$), and backward speed ($F(3, 36) = 8.897$, $p < 0.05$) in the *dop-2* strain (Fig. 3g, h, and i, respectively). In the *dop-3* strain, haloperidol (160 μM) had a significant effect on the moving speed average ($F(2, 36) = 4.947$, $p < 0.05$) (Fig. 3j) with the time of exposure causing changes in the moving average speed ($F(3, 36) = 8.35$, $p < 0.05$), forward speed ($F(3, 36) = 18.43$, $p < 0.05$), and backward speed ($F(3, 36) = 5.762$, $p < 0.05$) (Fig. 3j, k, l, respectively).

Regarding the size parameters, there were no significant changes in WT N2 and *dop-1*, *dop-2*, and *dop-3* strains exposed to haloperidol for 1–6 days (see Table S1).

Lastly, for 2 days, moving average speed was measured in the presence (day 1) and absence (day 2) of haloperidol. Two-

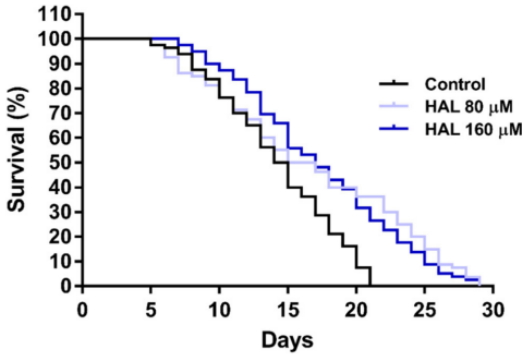

Fig. 1 Lifespan of L4-synchronized WT N2 worms exposed to vehicle or haloperidol (80 μM and 160 μM) in NGM plates. Worm survival was scored each day until death. Survival rate was determined by Mantel-Cox log-rank test. HAL, haloperidol

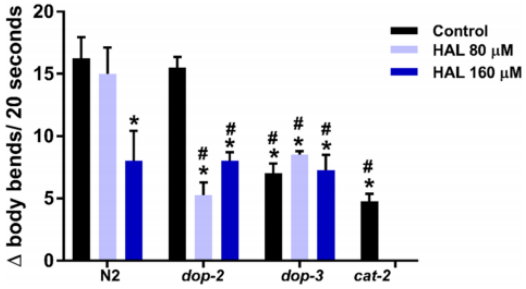

Fig. 2 Basal slowing response (BSR) was performed in WT N2 and *dop-2*, *dop-3*, and loss-of-function mutant strains exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 day. L4-synchronized worms were treated in NGM plates, and after 1 day, the plates were washed off with S-basal and the difference between the number of body bends in 20s intervals was counted for each worm. *Cat-2* loss-of-function mutants were used as positive control. Data were expressed as mean ± standard error of mean (SEM; $n = 4$). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. $^{*}p < 0.05$ compared with WT N2 control group. $^{\#}p < 0.05$ compared with *dop-2* control group. HAL, haloperidol

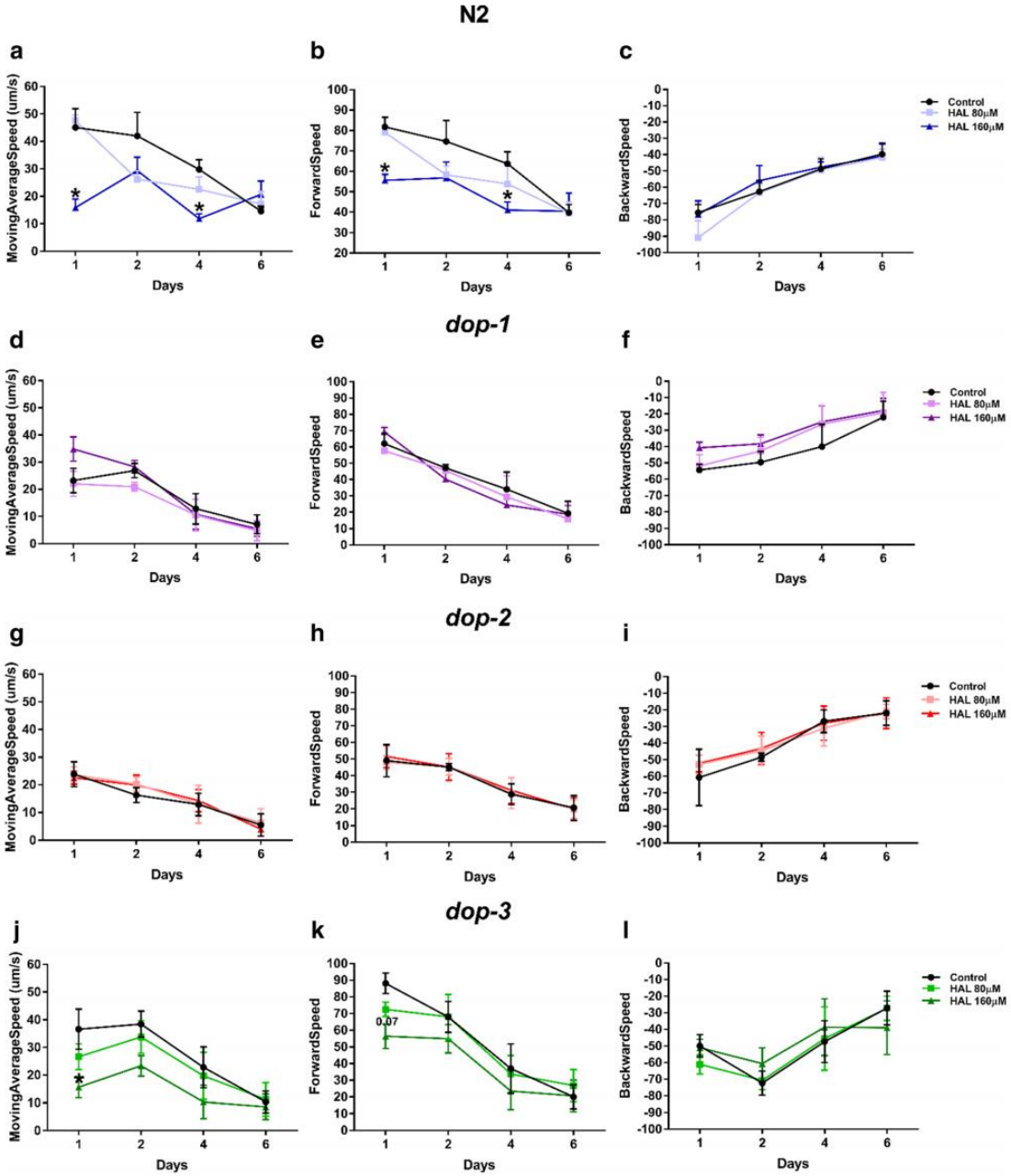

Fig. 3 Speed parameters of WT N2 and *dop-1*, *dop-2*, *dop-3*, loss-of-function mutant strains exposed to vehicle or haloperidol (80 μM and 160 μM/1–6 days) in NGM plates. **a**, **d**, **g**, **j** Moving average speed. **b**, **e**, **h**, **k** Forward speed. **c**, **f**, **i**, **l** Backward speed. Data were expressed as mean ± standard error of mean (SEM; $n = 4/10–15$ worms per n). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. *$p < 0.05$ compared with control group for the same day. HAL, haloperidol

way ANOVA showed a significant effect of haloperidol concentration on moving average speed ($F\ (2, 18) = 9.191$, $p < 0.05$). However, haloperidol withdrawal abolished this effect (Fig. 4).

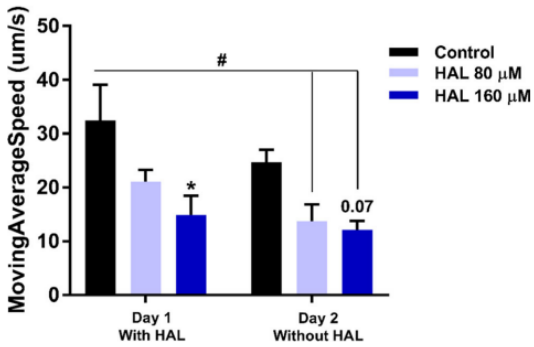

Fig. 4 Moving average speed of WT N2 worms exposed to vehicle or haloperidol (80 μM and 160 μM) was measured at the end of the day 1 (24 h of haloperidol exposure) and at the end of the day 2 (24 h after the haloperidol withdrawal). Data were expressed as mean ± standard error of mean (SEM; $n = 4/10–15$ worms per n). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. $^{*}p < 0.05$ compared with control group for the same day. $^{\#}p < 0.05$ compared with control group on day 1. HAL, haloperidol

Haloperidol Decreases Dopamine Levels

One-way ANOVA showed significant effects on DA levels in WT N2 worms exposed to haloperidol for 1 day (80 and 160 μM) ($F(3, 8) = 15.26$, $p < 0.05$) (Fig. 5a). However, after 4 days of exposure, these changes were no longer observed for both haloperidol concentrations tested (Fig. 5b). The levels of DA in *Cat-2*, used as positive control, decreased when compared with control on both days.

Haloperidol Does Not Change Dopaminergic Receptors' Gene Expression and Dopaminergic Neurons' Morphology

One-way ANOVA showed no changes in *dop-1*, *dop-2*, and *dop-3* gene expression after 1 and 4 days of exposure to haloperidol (80 and 160 μM) (Fig. 6). In addition, in both haloperidol concentration used and exposure duration, it did not show significant effects on CEP dopaminergic neurons' morphology in BY200 worms (Fig. 7).

Discussion

Haloperidol is the typical antipsychotic most commonly used in Europe, Asia, and the United States of America (USA) for the treatment of psychosis [58, 59]. However, it causes a range of adverse effects [43]. The nematode *C. elegans* has been used as a model animal organism to study neurotransmitter signaling and animal behavior, given its high experimental versatility and ease of handling. Moreover, *C. elegans* exhibits high conservation with the human genome, making it an optimal model for the discovery of novel mechanisms of action and the identification of novel drug targets [39, 60, 61]. The overall goal of this study was to evaluate the main pharmacological effects of haloperidol on the dopaminergic system, with the nematode *C. elegans* as a model system. Our novel findings established that haloperidol increased the lifespan in WT N2 worms and decreased the difference between the number of body bends (BSR) in the absence and presence of food in WT N2 and *dop-2* strains. Haloperidol also decreased the moving average speed in WT N2 and *dop-3* strains. Furthermore, the speed of locomotion returned to basal levels upon haloperidol withdrawal. Haloperidol reduced DA levels at both concentrations tested after 1 day of exposure but did not cause changes neither in *dop-1*, *dop-2*, and *dop-3* gene expression nor in CEP dopaminergic neurons on none of the evaluated days. To our knowledge, this is the first time that *dop-3* receptors are shown to mediate the pharmacological effects of haloperidol.

A limited number of studies have previously assessed the effects of antipsychotics such as the haloperidol in *C. elegans*, and even fewer have addressed its specific effects on the dopaminergic system [28, 37, 40, 62, 63]. Accordingly, first, we evaluated the effect of haloperidol on worms' lifespan in the presence of food. It is believed that food limits lifespan through nutrition and by providing sensory perception [64]. Worms have sensory endings exposed to the external milieu, which can sense food by mechanosensation (sensation that activates DA release from their 8 dopaminergic neurons). In the presence of food, DA signaling is activated by tactile perception, suppressing octopamine signaling and consequently decreasing worm lifespan. In the absence of food, DA signaling ceases and octopamine signaling is activated via the serotonin receptor *ser-3* [65]. Lastly, DA signaling seems to suppress octopamine signaling [65]. In *C. elegans*, DA signaling in the presence of food occurs via *dop-2* and *dop-3* receptors in SIA neurons (cholinergic neuron), which inhibit the activation of cyclic AMP response element binding protein (CREB). On the other hand, in the absence of food, it occurs in *dop-3* receptors via RIC neurons (octopaminergic neuron), which increase octopamine release and, via *ser-3* in SIA neurons, promoting CREB activation [21, 65]. In the present study, haloperidol, a D2 antagonist, extended worm lifespan at both tested concentrations. Accordingly, haloperidol seems to act on DA signaling in *C. elegans* via *dop-2* and *dop-3* receptors in SIA neurons. The antagonism of D2-like receptor by haloperidol could be inducing CREB activation after the recognition of food, and in turn, it would be increasing their lifespan, as illustrated in Fig. 8a (adapted from published data; [21, 65, 66]).

According to Allen et al. [26], DA signaling also participates on "slowing" and locomotion behavior in *C. elegans*. In the present study, we performed two tests, BSR and locomotion speed, to evaluate locomotor behavior of the animals. BSR or food-sensing response is a behavior that requires

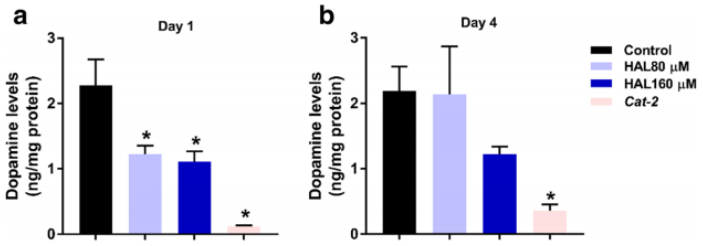

Fig. 5 DA levels in WT N2 worms exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 day (**a**) and 4 days (**b**) in NGM plates. *Cat-2* loss-of-function mutants were used as positive control. Data were expressed as mean ± standard error of mean (SEM; $n = 3$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. $*p < 0.05$ compared with control group. HAL, haloperidol

acetylcholine and GABA signaling, but it is modulated by endogenous DA (via CEPs, ADEs, and PDE neurons), as illustrated in Fig. 8b. Thus, the test mainly evaluates the functionality of the D2-type dopaminergic neurons [26, 67]. When worms have normal DA content, they move slower in the presence than in the absence of bacteria [2]. For the test, well-fed WT N2 and D2-like mutant strains (*dop-2* and *dop-3*) were used. WT N2 worms in the control group slowed their locomotion rate in presence of food. When WT N2 worms were exposed to haloperidol, the highest concentration caused an increase in locomotion rate in presence of bacteria (and a decrease in the difference of body bends of the worms in the absence versus presence of food). In the *dop-2* strain, the same occurs at both haloperidol concentrations, and for *dop-3*, there was no difference among the groups. These results suggest the involvement of the *dop-3* receptors in haloperidol's effect in *C. elegans*.

Speed parameters were also assessed in *C. elegans*, which is characterized by coordinated dorsal-ventral muscle contractions in a sinusoidal fashion [68]. It is another locomotor behavior that requires an intact dopaminergic system. Here, along with WT N2 and mutants lacking the D2-like receptor (*dop-2* and *dop-3*), we also exposed mutants lacking the D1-like receptor, and we analyzed moving average speed, forward speed, and backward speed of worms exposed to haloperidol. D1 and D2 are the DA receptors involved on locomotor

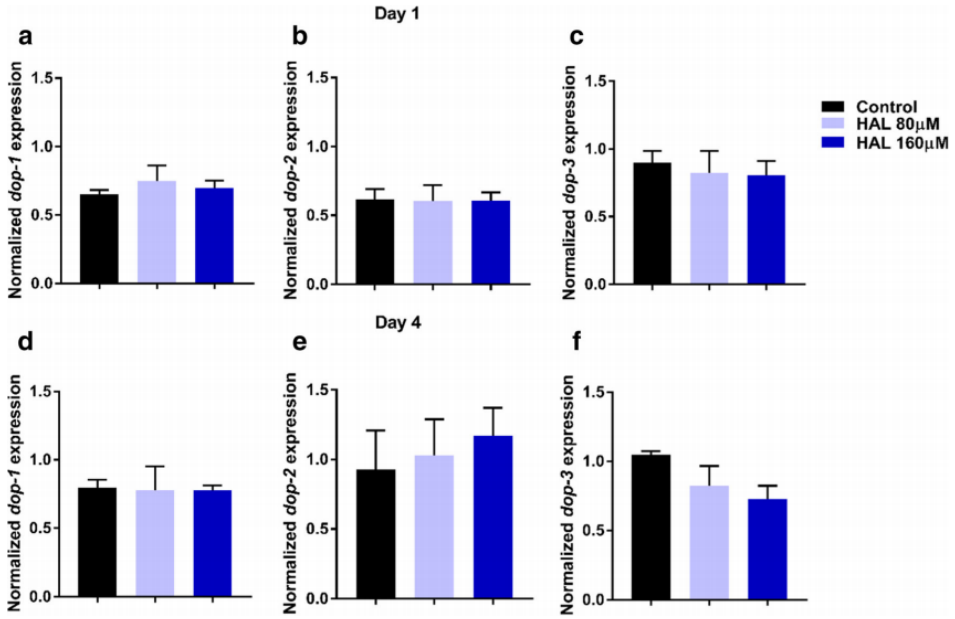

Fig. 6 mRNA expression of *dop-1*, *dop-2*, and *dop-3* genes in WT N2 worms exposed to vehicle or haloperidol (80 μM and 160 μM) for 1 day (**a**) *dop-1*, (**b**) *dop-2*, and (**c**) *dop-3* strains and 4 days (**d**) *dop-1*, (**e**) *dop-2*, and (**f**) *dop-3* strains. *Ama-1* was used as a housekeeping gene to normalize the data. Data were expressed as mean ± standard error of mean (SEM; $n = 5$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. $*p < 0.05$. HAL, haloperidol

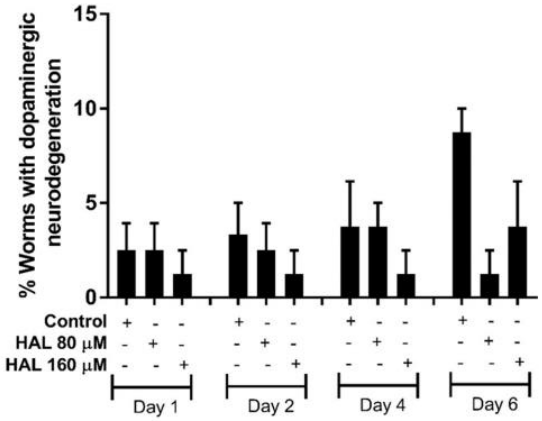

Fig. 7 Dopaminergic neurodegeneration in BY200 worms. Data show the percentage of worms with degenerating on cephalic (CEP) dopaminergic neurons in the head of L4-synchronized worms exposed to vehicle or haloperidol (80 µM and 160 µM) during 1–6 days in NGM plates. Data were expressed as mean ± standard error of mean (SEM; $n = 4/15$ worms per n). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. $^{*}p < 0.05$. HAL, haloperidol

behavior [14, 26]. To measure and analyze locomotor behavior, we used an efficient video-tracking system (WormLab software). We observed a decrease in moving average speed in WT N2 worms (on day 1 and 4) and in *dop-3* mutants (only on day 1) generated at the highest concentration of haloperidol. This decrease in moving average speed seems to be directly related to a decrease in forward speed, since there were no changes in backward speed. In *dop-1* and *dop-2* mutants, there were no differences among the groups. These results reinforce the notion that the effects of haloperidol are related to *dop-3* receptors. We posit that DA modulates (via CEPs, ADEs, and PDE neurons) *C. elegans* movement via D1- and D2-like receptors, and these receptors suppress acetylcholine and GABA release by acting in the cholinergic and GABAergic motoneurons themselves (Fig. 8b) [26, 29]. Thus, the physiological role of the *dop-3* receptor in the motor neurons is to inhibit acetylcholine release into the neuromuscular junction and attenuating muscle contraction. The motoneurons also release GABA, which leads to the relaxing of the opposing muscle. Both neurotransmitters, GABA and acetylcholine, prevent the decreasing in locomotion. Lastly, the action of DA in *Dop-1* receptor leads to opposite effects, increasing the locomotion in the worms [26, 29]. In this study, locomotor behavior test predicts the involvement *dop-3* receptor in the antagonistic effects of haloperidol, which might be causing an abnormal (increase) acetylcholine and GABA release into the neuromuscular junction and, consequently, decreased locomotor activity in *C. elegans*.

The moving average speed assessment performed in absence and presence of haloperidol for 2 days was also analyzed. On day 1, worms exposed to haloperidol (at the highest

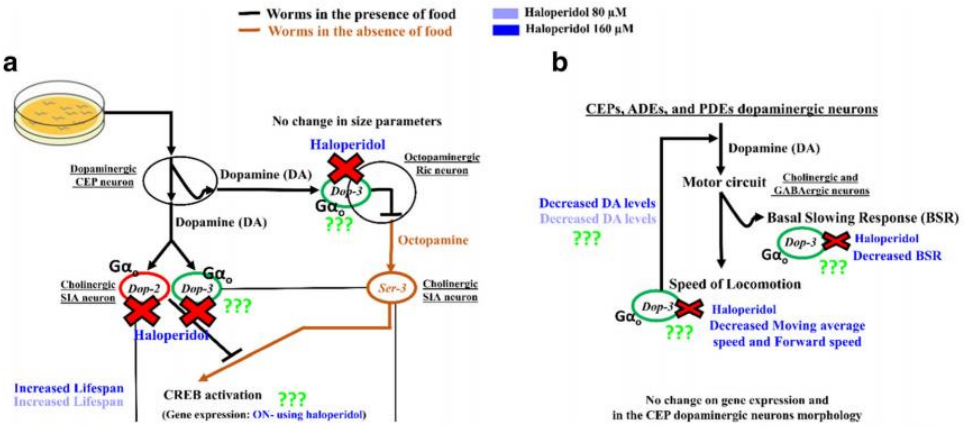

Fig. 8 Illustration of the experimental results performed in this study after haloperidol exposure. Lifespan, BSR, speed of locomotion, DA levels, mRNA expression, and neurodegeneration were performed using WT N2 and *dop-1*, *dop-2*, and *dop-3* mutant strains exposed to 80 µM and 160 µM of haloperidol. **a** CREB activation by haloperidol. In the presence of food, DA is released via CEP dopaminergic neurons that activates D2-like receptors in the cholinergic (SIA) and octopaminergic (RIC) neurons, suppressing octopamine signaling and inhibiting CREB activation. However, the antagonism of D2-like receptor by haloperidol might induce CREB activation, contributing to the increase of worm's lifespan, without affecting their size parameters. **b** Locomotor response by haloperidol. In well-fed animals, DA is released by ADEs, PDEs, and CEPs dopaminergic neurons that regulate *C. elegans* movement via DA receptors. These receptors modulate acetylcholine and GABA signaling in motor circuit. Physiologically, *dop-3* DA receptor prevent the decrease in locomotor behavior. The possible antagonism of *dop-3* receptor by haloperidol might decrease locomotor behavior in BSR and speed of locomotion in *C. elegans*. Consequently, the decrease in DA levels might be due to increased cholinergic and GABAergic signaling by antagonism of haloperidol. However, it did not show changes neither *dop-1*, *dop-2*, and *dop-3* gene expression nor CEP dopaminergic neurons

concentration) showed a decrease in moving average speed, when compared with the control group. However, on day 2, the worms that received only the vehicle in all groups showed no changes in locomotor behavior, reinforcing that the binding of haloperidol to molecular targets of pharmacological action is reversible in *C. elegans*.

DA levels was also measured in worms exposed to haloperidol on day 1 and day 4. In both haloperidol concentrations, WT N2 worms and *cat-2* loss-of-function mutant strain had reduced DA levels when compared with the control group on day 1. On day 4, there was a decrease in DA levels only in *cat-2* loss-of-function mutants. The decrease in DA levels after exposure to haloperidol is possibly due to an increase in cholinergic and GABAergic signaling secondary to the antagonistic effects of DA on *dop-3* receptor, since DA and acetylcholine/GABA act in opposition to each other [26, 29]. Interestingly, the decrease in DA levels in worms exposed to 80 μM haloperidol did not change any of the behavioral parameters. It may be because 80 μM haloperidol could be not sufficient to change these parameters. On the other hand, both concentrations of haloperidol increased the worms' lifespan (mentioned above), but these concentrations did not change their size parameters (see Table S1). These data suggest that DA negatively regulates body size in *C. elegans* via the *dop-3* receptors as well as through suppression of octopamine signaling via *ser-3* and *ser-6*, as demonstrated in previously reported by Nagashima et al. [69]. Therefore, we corroborate that the antagonism of *dop-3* receptor by haloperidol likely regulates body size in worms.

In addition, we evaluated the mRNA expression of *dop-1*, *dop-2*, and *dop-3* genes in WT N2 worms exposed to haloperidol for 1 or 4 days. However, on both days, there were no significant differences among the groups in any of the genes analyzed. In rodents, chronic haloperidol treatment led to increased D2 mRNA expression [70–72], while shorter treatments failed to show differences in D2 mRNA levels. In the present study, the results indicate that haloperidol exposure up to 4 days did not to alter dopaminergic receptors mRNA expression in *C. elegans*.

Lastly, we failed to observe haloperidol-induced morphological neurodegeneration in CEP neurons in BY200 worms. This seems to be related to the decrease in DA levels, since oxidation and degradation by enzyme-catalyzed oxidation of DA and the ensuing cellular damage would also be decreased [73]. In a corroborating study, McCormick et al. [40] have also shown that D2-like antagonism might be a mechanism involved in the potential neuroprotective effect of antipsychotic drugs by preventing tau aggregation and the ensuing neurotoxicity in *C. elegans*.

In conclusion, haloperidol increased *C. elegans* lifespan, decreased BSR and speed parameters, altered forward speed, and decreased DA levels, yet it did not alter neither *dop-1*, *dop-2*, and *dop-3* gene expression, nor CEP dopaminergic neurons morphology. Combined, these effects indicate the involvement of D2-like DA receptor *dop-3* in mediating haloperidol's effects in *C. elegans*. Our study capitalizes on the advantages inherent to the *C. elegans* model, and combines comprehensive behavioral, molecular, and morphological analyses germane to the dopaminergic system. Moreover, our findings serve as a basis for testing novel drugs and further research as to clarify other mechanisms inherent to haloperidol's efficacy, and its therapeutic potential for a broad range of psychiatric disorders.

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Authors' Contributions Krum BN: conceptualization, investigation, writing- original draft preparation, conducted all the experiments with the support of Martins AC, Queirós L, Ferrer B, and Milne GL. Soares FAA: conceptualization, writing - review and editing, visualization. Fachinetto R: supervision, writing - review & editing. Aschner M: conceptualization, supervision, resources, writing - review & editing.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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4.2 ARTIGO 2

Artigo

Kava decreases the stereotyped behavior induced by amphetamine in mice

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Kava decreases the stereotyped behavior induced by amphetamine in mice

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ABSTRACT

Ethnopharmacological relevance: Kava extract (*Piper methysticum*) is a phytotherapic mainly used for the treatment of anxiety. Although the reported effects of Kava drinking improving psychotic symptoms of patients when it was introduced to relieve anxiety in aboriginal communities, its effects on models of psychosis-like symptoms are not investigated.

Aim of the study: To investigate the effects of Kava extract on behavioral changes induced by amphetamine (AMPH) and its possible relation with alterations in monoamine oxidase (MAO) activity.

Materials and methods: Mice received vehicle or Kava extract by gavage and, 2 h after vehicle or AMPH intraperitoneally. Twenty-five minutes after AMPH administration, behavioral (elevated plus maze, open field, stereotyped behavior, social interaction and Y maze) and biochemical tests (MAO-A and MAO-B activity in cortex, hippocampus and striatum) were sequentially evaluated.

Results: Kava extract exhibited anxiolytic effects in plus maze test, increased the locomotor activity of mice in open field test and decreased MAO-A (in cortex) and MAO-B (in hippocampus) activity of mice. Kava extract prevented the effects of AMPH on stereotyped behavior and, the association between Kava/AMPH increased the number of entries into arms in Y maze test as well as MAO-B activity in striatum. However, Kava extract did not prevent hyperlocomotion induced by AMPH in open field test. The social interaction was not modified by Kava extract and/or AMPH.

Conclusion: The results showed that Kava extract decreased the stereotyped behavior induced by AMPH at the same dose that promotes anxiolytic effects, which could be useful to minimize the psychotic symptoms in patients.

1. Introduction

Schizophrenia is a chronic psychiatric illness that affects approximately 1% of the population around the world (Millier et al., 2014; Mueser and McGurk, 2004; Saha et al., 2005). It is characterized by positive (hallucinations, delusions), negative (apathy, social withdrawal) and cognitive (cognitive impairment) symptoms (Brennan et al., 2013; Insel, 2010; Mueser and McGurk, 2004; Nagai et al., 2011). Although the etiology of schizophrenia remains not completely elucidated, it is considered a multifactorial neurodevelopmental disorder

Abbreviations: AMPH, amphetamine; ANOVA, analysis of variance; CEUA, Ethic Committee on Animal Use; TCA, trichloroacetic acid; CNS, central nervous system; CONCEA, National Council of Control of Animal Experimentation; DA, dopamine; GABA, γ-gamma-aminobutyric acid; HPLC, High-performance liquid chromatography; H_2O_2, hydrogen peroxide; 4-HQ, 4- hydroxyquinoline; 5-HT, serotonin; MAO, monoamine oxidase; NaOH, sodium hydroxide; NE, noradrenaline; SEM, standard error of mean.

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associated with genetic and environmental factors (Broome et al., 2005; Van Os et al., 2010). Dopamine (DA), glutamate, serotonin (5-HT) and γ-aminobutyric acid (GABA) are some of the neurotransmitters involved in schizophrenia (Brennan et al., 2013). The alterations in these neurotransmitter circuits have been associated with hyperactivation of the mesolimbic dopaminergic pathway and a decrease in the mesocortical dopaminergic pathway with consequent appearance of schizophrenia symptoms (Abi-Dargham et al., 2000; Kucinski et al., 2011; Janowsky and Risch, 1979; Lieberman et al., 1990).

Amphetamine (AMPH) is a psychostimulant drug that acts by increasing the synaptic levels of DA, 5-HT and noradrenaline (NE) in the central nervous system (CNS) (Tonge, 1974; Vogel et al., 1985). In rodents, AMPH is a pharmacological agent used as a tool to mimic psychosis-like symptoms, allowing a better understanding of its mechanisms as well as finding agents with therapeutic potential for schizophrenia (Ceretta et al., 2018; Featherstone et al., 2007; Jones et al., 2011). AMPH may act either by increasing the processes of release or decreasing the re-uptake and metabolism of monoamines (DA, 5-HT and NE) (Faraone, 2018; Heal et al., 2013). The monoamine oxidase (MAO) enzyme catalyzes the oxidative deamination of the monoamines in their corresponding aldehydes with formation of hydrogen peroxide (H_2O_2) and ammonia (Cohen et al., 2002; Vindis et al., 2001). Alterations in the levels and the consequent metabolism of monoamines are related to the appearance of various neurological diseases including schizophrenia (Meltzer and Stahl, 1976).

Piper methysticum is a perennial shrub from Piperaceae (pepper) family, also called Kava due to the presence of kavalactones, which are the main constituents of its extract (Rex et al., 2002; Sarris et al., 2012). The crude extract of Kava has been used as ceremonial and social drink in the Pacific islands and, in the phytotherapy, as an effective short-term treatment of anxiety (Sarris et al., 2011; Singh and Singh, 2002). Furthermore, it has other medicinal uses which include actions anti-stress and sedative (Singh and Singh, 2002). The main mechanism associated to the anxiolytic effects of Kava on CNS of mammals is through the $GABA_A$ modulation (Chua et al., 2016; Sarris et al., 2011). However, other targets to Kava extract were also demonstrated as binding DA type-2 receptor, blockage of voltage-gated sodium and calcium ion channels, reduction of the neuronal reuptake of DA and NE, as well as an MAO-B inhibitor (Cairney et al., 2002; Dinh et al., 2001a; Laporte et al., 2011; Ligresti et al., 2012; Uebelhack et al., 1998). Of particular importance to the present study, evidences in the literature showed the possible antipsychotic effects of Kava extract improving the psychotic symptoms of patients from aboriginal communities in north Australia from Oceania when the Kava drinking was introduced to relieve anxiety (Cawte, 1986). Corroborating, Kava reduced psychotic symptoms in patients (Cairney et al., 2002; Cawte, 1986) and caused motor alterations as dyskinesia which are clinical signs of central DA antagonism (Cairney et al., 2002; Schelosky et al., 1995). Experimental data demonstrated that Kava extract could alters the DA levels in the *nucleus accumbens* of rats (Sällström Baum et al., 1998b) and bind to DA type-2 receptor (Dinh et al., 2001a) suggesting the action of Kava components on dopaminergic system. Despite of case-related reporting the effects of Kava on psychotic symptoms in patients, its effects were not investigated in a model of psychosis-like symptoms induced by AMPH in rodents. Based on the above-mentioned evidence, the research for new therapeutic agents as well as possible pharmacological adjuvants to use in the treatment of schizophrenia is relevant. Thus, the present study aimed to investigate the effects of the crude extract of Kava on behavioral changes induced by AMPH in mice and whether these effects are associated with alterations in MAO activity.

2. Materials and methods

2.1. Drugs

The crude extract of Kava rhizome (*P. methysticum*) was obtained from Huakang Biotechnology Development (China-manufacturer's lot HK20160415) with approximately 30% of kavalactones (according to the supplier's report). High-performance liquid chromatography (HPLC) was used to characterize the compounds of the extract. All reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) or other with high quality and purity.

2.2. Quantification of phenolics and flavonoids compounds by HPLC-DAD

The quantification of phenolic and flavonoid compounds was carried out as previously described (Peroza et al., 2013).

2.3. Animals

Thirty-two male Albino Swiss mice (2 months of age, 25–35 g) from the Central Animal House-holding of Federal University of Santa Maria were used in this study. The animals were housed in polycarbonate cages (four or five per cage) with free access to water and food, in temperature-controlled room (22 ± 2 °C) and on 12-h light/dark cycle with light on at 7:00 a.m. All experiments were performed in accordance to the guidelines of the National Council of Control of Animal Experimentation (CONCEA) and the experimental procedures were approved by the Ethic Committee on Animal Use of Federal University of Santa Maria - Brazil, under the protocol number CEUA 1637290415.

2.4. Experimental design

Mice were randomly assigned to one of four groups (eight mice/group): (I) Control, (II) Kava extract, (III) AMPH, or (IV) Kava extract + AMPH. The animals were acclimated for 1 h to the behavioral room before starting the drugs administration. The animals of groups I and III received pretreatment with vehicle of Kava extract (corn oil) and the animals of groups II and IV received pretreatment with Kava extract (40 mg/kg) orally by gavage. Two hours after, the animals in groups I and II received vehicle of AMPH (NaCl 0.9%) and the animals in groups III and IV received 1.25 mg/kg of AMPH by an intraperitoneal injection (i.p.) (Behl et al., 2011; Ceretta et al., 2016; Figueira et al., 2015). To mimic what happens clinically, the dose administered of Kava extract to the animals was calculated by allometric conversion (Reagan-Shaw et al., 2007) and it corresponds similarly to the dose commonly used by humans (±200 mg/60 Kg). The behavioral evaluation began 25 min after the administration of AMPH or its vehicle as previously described Figueira at al (Figueira et al., 2015) with minor modifications. The animal received the drugs administration and passed by all behavioral tests during approximately 40 min in the following sequence: Elevated plus maze, Open field test, Stereotypy, Social interaction test and Y maze test. Then, the total time that the animal was maintained in the experiment considering the acclimation, drug administration, behavioral analysis until euthanasia was approximately 4.5 h. During the experimental assays, all possible efforts were made to avoid animal stress as appropriate supply of water, food, temperature, humidity and light, environmental enrichment, low noise levels and others. Immediately after behavioral analysis, the animals were conducted to another experimental room and euthanized by cervical dislocation; their brains were rapidly dissected in cortex, hippocampus and striatum. The brain structures were frozen in powdered dry ice and, then stored at −80 °C to perform the MAO activity. The experimental design is depicted in Fig. 2.

2.5. Behavioral analysis

For the behavioral observations, the experimenters were blind regarding to the treatment conditions. The inter-rater reliability between 2 observers is greater than 90%. The calculated α value was significant for $p < 0.05$.

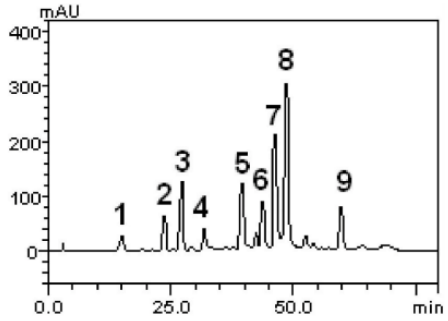

Fig. 1. High performance liquid chromatography profile of Kava extract. Panel A: Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rosmarinic acid (peak 4), rutin (peak 5), isoquercitrin (peak 6), quercitrin (peak 7), quercetin (peak 8) and kaempferol (peak 9).

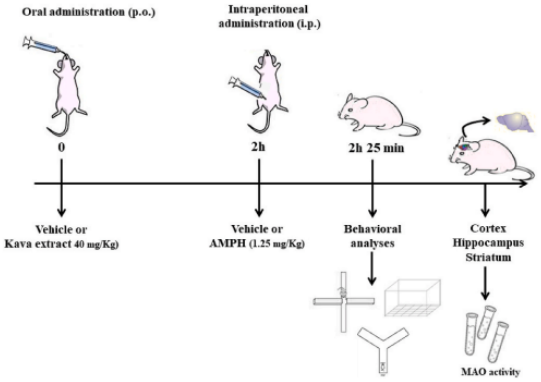

Fig. 2. Experimental design of the acute treatment with Kava extract and/or AMPH. Firstly, mice received 40 mg/kg of Kava extract or vehicle by gavage and, after 2 h 1.25 mg/kg of AMPH or vehicle intraperitoneally. Twenty-five minutes after last administration of AMPH or vehicle, behavioral analyses were performed. Brain dissections were carried out after euthanasia of animals to perform MAO activity. AMPH: amphetamine.

2.5.1. Elevated plus maze

To evaluate the anxiety-like state caused by treatments, mice were exposed to an elevated plus maze (Anchan et al., 2014; Treit et al., 1993). The apparatus consisted of four arms, two open and two closed arms (30 cm L × 5 cm W). For closed arms, walls (17 cm H) were positioned 38.5 cm from the floor. The animals were placed in the center of the apparatus to quantify the time spent into open or closed arms and the number of head dips during a 5 min session. The time spent on open arm and the entries into the open arms were calculated and expressed in percentage, as follows: time spent or number of entries into the open arm/total time or total number of the entries into closed and open arm X 100, respectively (Fachinetto et al., 2007).

2.5.2. Open field test

Open field test was performed to verify possible changes in spontaneous locomotor and exploratory activity in treated mice (Anchan et al., 2014; Archer, 1973). The animals were placed individually in the center of an open field arena (44 cm L × 44 cm W × 44 cm H) divided into 16 equal areas. The number of lines crossed (locomotor activity), frequency of rearing (exploratory activity) and number of times the animal crosses the central square were measured during 5 min without habituation period.

2.5.3. Stereotypy

To evaluated stereotyped behavior in treated mice, the animals were placed in glass cages (20 cm L x 20 cm W x 19 cm H) and during 5 min the following parameters were taken to account stereotypy score: sniffing, grooming, nail biting, circling (Figueira et al., 2015; Machado et al., 2006). Scores from 0 to 4 were attributed for these parameters, as follow: 0) absence or 1) presence of one type of abnormal movements; 2) presence of two types of abnormal movements; 3) presence of three types of abnormal movements; 4) presence of four types of abnormal movements.

2.5.4. Social interaction test

Social behavior of mice treated with Kava extract and/or AMPH was performed in an open field arena (44 cm L × 44 cm W × 44 cm H) with a floor divided into 16 equal areas during 15 min. Pairs of unfamiliar mice receiving the same treatment were placed on opposite sides into the arena and the time of social interaction active (sniffing and following) or passive (when animals lie next to each other within a distance of 5 cm from skin to skin) and number of contacts of the animals treated were quantified as previously described by Calzavara et al. (2011) with some modifications.

2.5.5. Y maze test

Spatial working memory of mice treated with Kava extract and/or AMPH was evaluated by Y maze test. The apparatus consisted of three equal arms (A, B and C arms) (30 cm L x 6 cm W x 2 cm H) positioned 31 cm from the floor. The animals were individually placed at the end of the A arm and then the sequence in which the animal goes through the arms was quantified during 8 min. The series of arm entries (considered complete when the hind paws of the mice had been completely placed in the arm) was recorded visually. Correct alternation was defined as successive entries into the three different arms, on overlapping triplet sets for example ABC, BCA, CAB. The percentage of alternation was calculated as total of alternations/(total arm entries − 2) x 100 (Ceretta et al., 2016; Dall'Igna et al., 2007; Monte et al., 2013).

2.6. Tissue preparation and MAO activity

To carry out MAO activity *ex vivo* the brain structures were homogenized in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, pH 7.4) and, the homogenate was used to determine the protein quantity as previously described Lowry (1951). The reaction mixture containing brain homogenates (0.25 mg of protein), 250 nM pargyline (selective MAO-B inhibitor) or 250 nM clorgyline (selective MAO-A inhibitor) was pre-incubated at 37 °C during 20 min. The reaction was started by the addition of 60 μM kynuramine in the reaction mixture and incubated for more 30 min at 37 °C. The reaction was stopped with 10% trichloroacetic acid (TCA). The samples were centrifuged at 500*g* for 8 min and 1 mL of the supernatant was mixed with 1 mL of NaOH (1 N) (Busanello et al., 2017; de Freitas et al., 2018; Soto-Otero et al., 2001). The product of the reaction was measured in fluorimeter at 315 nm for excitation and 380 nm for emission by measuring the kynuramine oxidation to 4-hydroxyquinoline (4-HQ) (Morinan and Garratt, 1985). Results were expressed in nmol of 4-HQ per milligram of protein per minute.

2.7. Statistical analysis

Data were expressed as mean + standard error of the mean (SEM) and analyzed by two-way analysis of variance (ANOVA) (with AMPH and Kava as factors) followed by Tukey's *post hoc* test when appropriate. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. HPLC analysis

HPLC fingerprinting of the Kava extract showed an elution diagram when the peaks were grouped into three regions based on the UV absorption profile. These regions showed typical patterns of UV absorption, supporting the presence of gallic acid (14.35min; peak 1), chlorogenic acid (23.98 min; peak 2), caffeic acid (27.15 min; peak 3), rosmarinic acid (32.54 min; peak 4), rutin (39.08min; peak 5), isoquercitrin (44.26; peak 6), quercitrin (46.35min; peak 7), quercetin (49.13min; peak 8) and kaempferol (60.02min; peak 9), (Fig. 1 and Table 1).

3.2. Effects of Kava extract and/or AMPH on elevated plus maze test in mice

In plus maze test, Kava treatment induced a change in behavior since two-way ANOVA revealed a significant main effect in the percentage of time spent into the open arms [(1,28) = 33.82 and $p < 0.05$; Fig. 3A], number of entries into the open arms [(1,28) = 19.40 and $p < 0.05$; Fig. 3B] and in the number of head dips [(1,28) = 21.97 and $p < 0.05$; Fig. 3C]. The treatment with AMPH altered the behavior regardless Kava pre-treatment in the percentage of time spent into the open arms [(1,28) = 9.11 and $p < 0.05$; Fig. 3A] and in the number of head dipping [(1,28) = 5.71 and $p < 0.05$; Fig. 3B] without significant effects in the number of entries in the open arms [(1,28) = 3.50 and $p = 0.071$; Fig. 3B].

3.3. Effects of Kava extract and/or AMPH on open field test in mice

In open field test, two-way ANOVA revealed a significant main effect of Kava in the number of crossings [(1,28) = 11.43 and $p < 0.05$; Fig. 4A], rearings [F (1, 28) = 15.80; $p < 0.05$; Fig. 4B] and in the number of entries into the center of arena [F (1, 28) = 20.23; $p < 0.05$; Fig. 4C]. Two-way ANOVA also revealed a significant main effect of AMPH in the number of crossings in the open field arena [(1,28) = 6.22 and $p < 0.05$; Fig. 4A].

3.4. Effects of Kava extract and/or AMPH on stereotypy in mice

Two-way ANOVA revealed a significant main effect of Kava [F (1, 28) = 14.40; $p < 0.05$; Fig. 5], AMPH [F (1, 28) = 10.00; $p < 0.05$; Fig. 5] and the interaction [F (1, 28) = 10.00; $p < 0.05$; Fig. 5]. *Post hoc* analysis demonstrated that AMPH increased the stereotypy in mice compared with control group and the pre-treatment with Kava avoided the AMPH effects. Kava *per se* did not altered the stereotyped behavior in mice (Fig. 5).

Table 1
Phenolics and flavonoids composition of Kava extract.

Compounds	Kava extract		LOD	LOQ
	mg/g	Percent	μg/mL	μg/mL
Gallic acid	23.75 ± 0.03 a	2.37	0.017	0.056
Chlorogenic acid	50.93 ± 0.01 b	5.09	0.038	0.125
Caffeic acid	85.68 ± 0.02 c	8.56	0.031	0.102
Rosmarinic acid	21.16 ± 0.07 a	2.11	0.014	0.047
Rutin	87.49 ± 0.06 c	8.74	0.023	0.075
Isoquercitrin[a]	63.17 ± 0.04 d	6.31	-	-
Quercitrin[a]	112.45 ± 0.05 e	11.24	–	–
Quercetin	152.68 ± 0.01 f	15.26	0.007	0.023
Kaempferol	61.73 ± 0.03 d	6.17	0.041	0.135

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$.

[a] Quantified as quercetin.

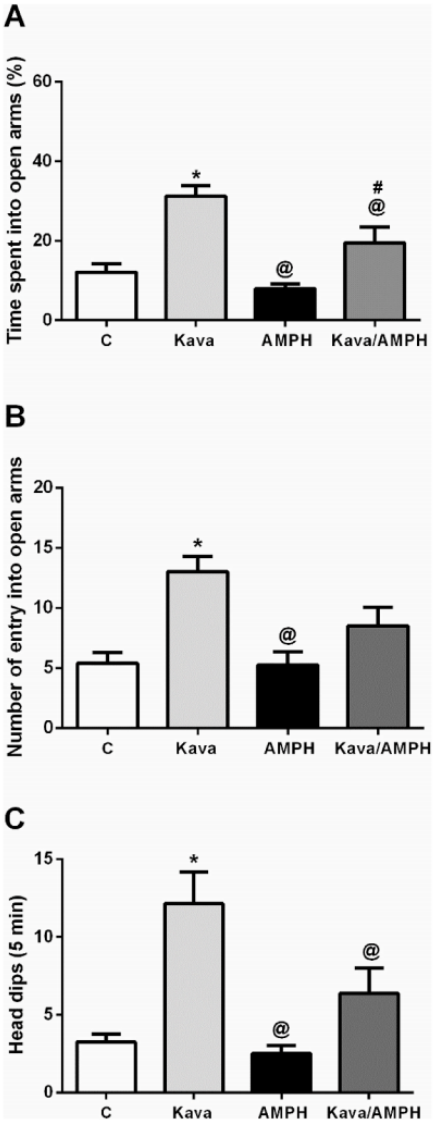

Fig. 3. Plus maze test. (A) Percentage of the time spent on the open arms, (B) number of entries into the open arms and (C) number of head dips in mice treated with Kava extract and/or AMPH during 5 min. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 8). $^{*}p < 0.05$, when compared with control group. $^{@}p < 0.05$, when compared with Kava group. $^{\#}p < 0.05$, when compared with AMPH group. C: control, AMPH: amphetamine.

3.5. Effects of Kava extract and/or AMPH on social interaction test in mice

Regarding social interaction test, statistical analysis did not found alterations neither in number of contacts nor in time of social interaction in mice treated when compared with control group (Fig. 6).

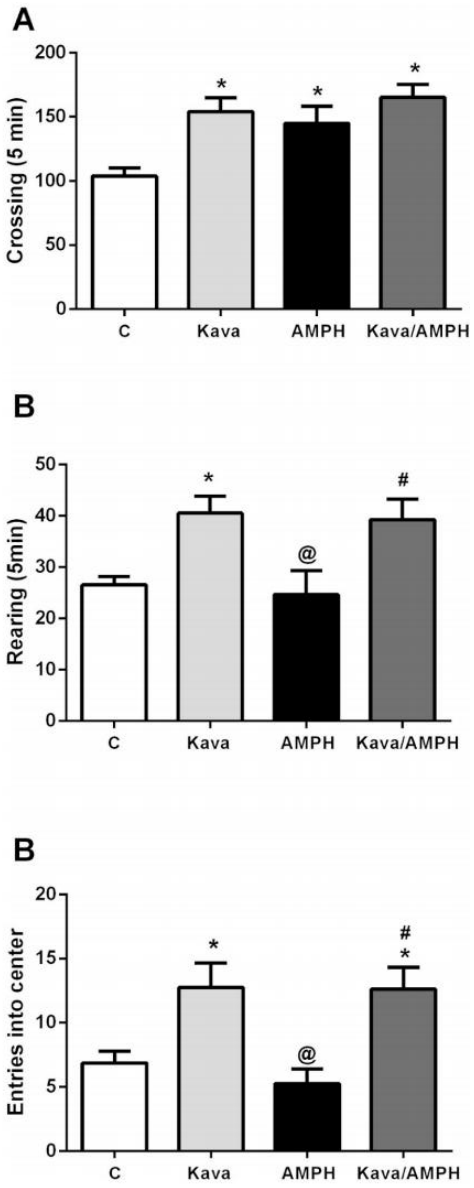

Fig. 4. Open field test. Number of (A) crossing and (B) rearing in mice treated with Kava extract and/or AMPH during 5 min. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 8). $^{*}p < 0.05$, when compared with control group. $^{@}p < 0.05$, when compared with Kava group. $^{\#}p < 0.05$, when compared with AMPH group. C: control, AMPH: amphetamine.

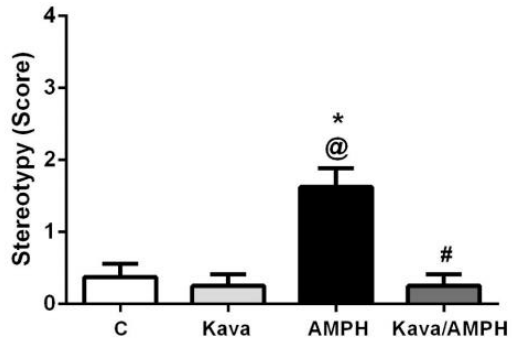

Fig. 5. Stereotypy. Score from 0 to 4 were attributed for stereotyped behaviors (sniffing, grooming, nail biting and circling) in mice treated with Kava extract and/or AMPH during 5 min. Scores: 0) absence or 1) presence and one type of abnormal movements; 2) presence and two types of abnormal movements; 3) presence and three types of abnormal movements; 4) presence and four types of abnormal movements. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 8). $^{*}p < 0.05$, when compared with control group. $^{@}p < 0.05$, when compared with Kava group. $^{\#}p < 0.05$, when compared with AMPH group. C: control, AMPH: amphetamine.

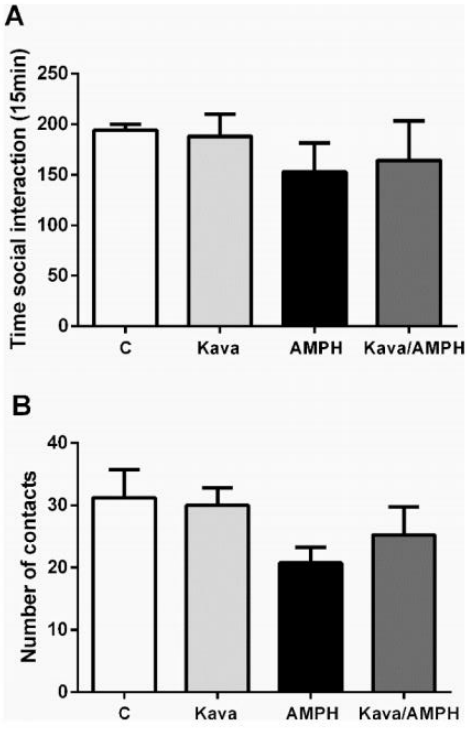

Fig. 6. Social interaction test. In open field arena the (A) time social interaction and (B) number of contacts in mice (animals tested in pairs) treated with Kava extract and/or AMPH during 15 min. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 4). AMPH: amphetamine.

3.6. Effects of Kava extract and/or AMPH on Y maze test in mice

Spatial working memory of mice treated with Kava extract and/or AMPH was evaluated in the Y maze test (Fig. 7). There were no significant effects of Kava extract and AMPH treatments in the percentage of correct alternations (Fig. 7A). A significant main effect of Kava was found in the number of entries into the arms [$F(1, 28) = 5.99$; $p < 0.05$;

Fig. 7B]. AMPH did not present significant effects on the number of entries into the arms [F (1,28) = 3.24 p = 0.08; Fig. 7B].

3.7. Effects of AMPH in mice pretreated with Kava extract on MAO activity in the cortex, hippocampus and striatum

MAO-A and MAO-B activity were evaluated in cortex, hippocampus and striatum of mice. A significant main effect of Kava was detected on MAO-A activity in cerebral cortex [F (1, 28) = 6.31; $p < 0.05$; Fig. 8A] as well as in MAO-B activity in hippocampus [F (1, 28) = 5.18; $p < 0.05$; Fig. 8E]. In striatum, a main effect of AMPH [F (1, 28) = 11.51; $p < 0.05$; Fig. 8E] was observed to MAO-B activity. No changes were observed in MAO-A activity in hippocampus and striatum even as for MAO-B activity in cortex (Fig. 8B, C, D).

4. Discussion

The present study aimed to investigate whether the crude extract of Kava could protect against behavioral alterations induced by AMPH in mice and, whether changes in MAO activity could be involved in its effects. The present results showed that AMPH produced an increase in behavioral responses as locomotor activity and stereotyped behavior without altering social interaction and spatial working memory. The pre-treatment with Kava extract avoided the increase of stereotyped behavior but did not prevent against hyperlocomotion induced by AMPH in mice. Moreover, the pre-treatment with Kava extract was associated with an increase in the number of entries into arms in Y maze test in those animals that received AMPH. Kava extract increased the frequency and the percentage of time spent on the open arm of the elevated plus maze as well as on the number of head dipping. It also increased locomotor and exploratory activity with an increase in the number of entries into the center of the open field. Kava extract decreased the activity of MAO-A in cortex and MAO-B in hippocampus, while AMPH increased the MAO-B activity in the striatum of animals pre-treated with Kava extract.

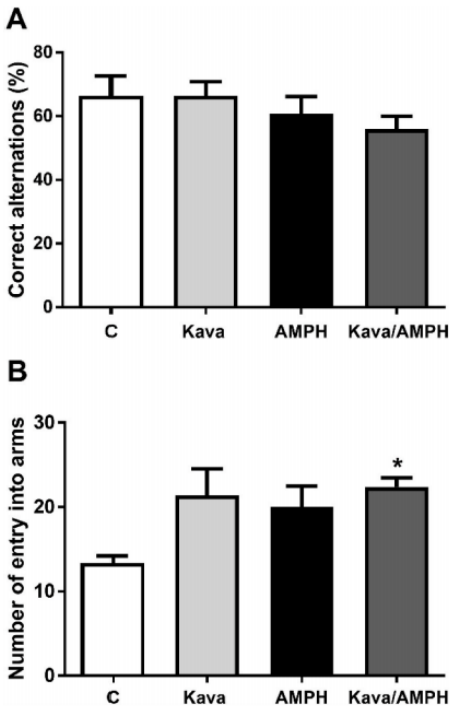

Fig. 7. Y maze test. Analyses of spatial working memory by (A) percentage of correct alternations and (B) number of entry into arms in mice treated with Kava extract and/or AMPH during 8 min. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 8). *$p < 0.05$, when compared with control group. C: control, AMPH: amphetamine.

Schizophrenic patients frequently present additional conditions as anxiety and major depression associated with psychotic symptoms (Sim et al., 2006). Approximately 65% of patients with schizophrenia exhibit anxiety symptoms (Sim et al., 2006). In this context, Kava extract could also play an important role in alleviating these symptoms due its action on GABA receptors (Laporte et al., 2011; Rex et al., 2002; Sarris et al., 2012; Singh and Singh, 2002) since GABAergic interneurons of ventral tegmental area are involved in the control of dopaminergic neurons from mesolimbic pathway (Yang and Tsai, 2017). The possible anti-psychotic effect of Kava was suggested in aboriginal communities in north Australia from Oceania when the Kava drinking was introduced to relieve anxiety and improved the psychotic symptoms of patients (Cawte, 1986). Furthermore, case-related reported the reduction of psychotic symptoms in patients (Cairney et al., 2002; Cawte, 1986) and appearance of motor alterations as dyskinesia in patients taking Kava extract suggesting its pharmacological effects as dopaminergic antagonist (Cairney et al., 2002; Schelosky et al., 1995). Despite of the mentioned clinical evidences and a study showing the Kava extract can bind in the type 2 dopaminergic receptors (Dinh et al., 2001a), its effects were not investigated in a model of psychosis-like symptoms induced by AMPH in rodents.

The present study was the first to investigate the effects of Kava extract on behavioral alterations induced by AMPH in mice as well as its possible effects on MAO activity. In this context, firstly we performed plus maze test to confirm the effectiveness of Kava extract, since this test evaluates anxiolytic-like behaviors in the animals (Anchan et al., 2014; Treit et al., 1993). As results, Kava extract induced a change in behavior increasing the percentage of time spent into the open arms, number of entries into the open arms and in the number of head dips, confirming that Kava extract at the dose used was reaching the CNS leading to an anxiolytic-like behavior. AMPH altered the behavior regardless Kava pre-treatment in the percentage and frequency of time spent into the open arms and in the number of head dipping. After, we carried out specific behaviors that could be modified in animal models of psychosis-like symptoms (Pogorelov et al., 2017).

In open field test, Kava extract increased the number of crossings, rearings and the number of entries into the center of open field. Confirming the anxiolytic effect of Kava (Kraeuter et al., 2019), the animals that received Kava extract presented a high number of entries into the center of open field. AMPH also increased the number of crossings in the open field arena and this effect was not modified by pre-treatment with Kava extract. The increase of locomotor activity caused by Kava extract *per se* suggests that it could be interacting with the same pathway of AMPH, which is demonstrated by the associated group Kava/AMPH that remained unchanged even with the combination of the two compounds. When the stereotyped behavior was evaluated, the administration of AMPH increased the stereotyped behavior of the mice and the pre-treatment with Kava extract prevented the appearance of these symptoms. These results suggest a decrease in dopaminergic activity. Both, hyperlocomotion and stereotyped behaviors (represented by unusual behaviors such as sniffing, grooming, nail biting, circling and immobility) in the animals, are associated to an hyperdopaminergic state in the mesolimbic pathway (mainly in the *nucleus accumbens*) (Ceretta et al., 2016; Ellenbroek and Cools, 2006; Featherstone et al., 2007; Figueira et al., 2015; Robinson and Becker, 1986; Saito et al., 2014; Wolgin, 2012). Taken together, it is hypothesized that either direct and/or indirect dopaminergic mechanisms are contributing to these behavioral effects. This hypothesis is based in the studies of

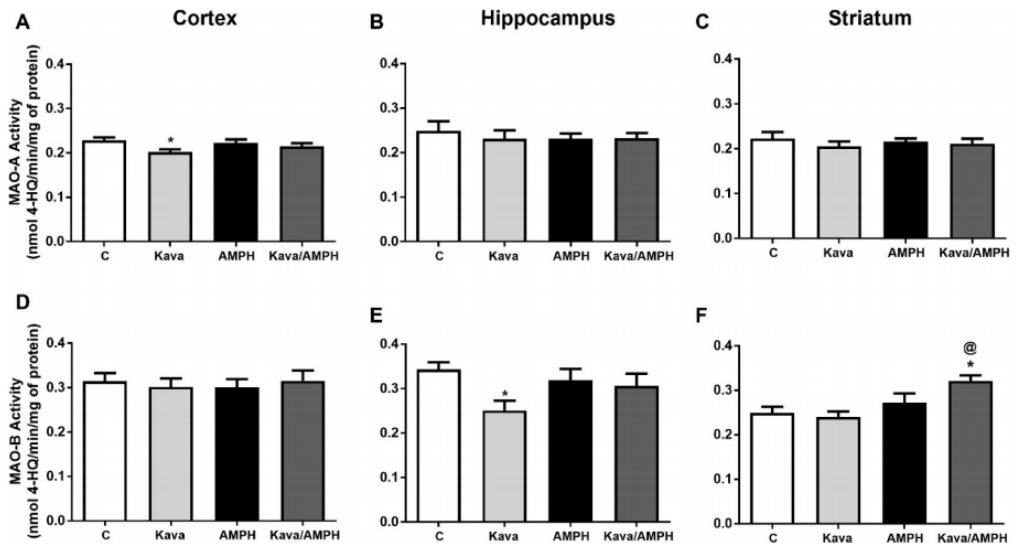

Fig. 8. MAO-A (A–C) and MAO-B activity (D–F) in cortex (A, D), hippocampus (B, E) and striatum (C, F) of mice treated with Kava extract and/or AMPH. Kynuramine was used as substrate and clorgyline (MAO-A) and pargyline (MAO-B) as a selective inhibitor. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean + standard error of mean (n = 8). $^{*}p < 0.05$, when compared with control group. $^{@}p < 0.05$, when compared with Kava group. C: control, AMPH: amphetamine.

Sällström Baum et al., which demonstrated that Kava extract can cause an increase or decrease in DA levels in *nucleus* accumbens of rats (Sällström Baum et al., 1998) as well as the inhibitory potential on MAO-B (Prinsloo et al., 2019) and the binding with the type 2 dopaminergic receptors (Dinh et al., 2001b) *in vitro*. All these effects vary depending on the dose and the parts of plant used to prepare Kava extract (Dinh et al., 2001a; Sällström Baum et al., 1998). Regarding the differential effects of Kava in hyperlocomotion and stereotyped behavior, similar effects were observed when tested diphenyl diselenide (Figueira et al., 2015) and gabapentin (Ceretta et al., 2016) in AMPH model. Diphenyl diselenide avoided the effects of AMPH on number of crossings and potentiated AMPH effects on stereotyped behavior (Figueira et al., 2015) while gabapentin prevented the AMPH-induced stereotyped behavior without effects on the number of crossings (Ceretta et al., 2016). In fact, data from literature suggest that occurs a behavioral sensitization to AMPH in a differential manner leading to different responses observed for both stereotypy and hyperlocomotion despite of both behavior to be related to same brain regions (Robinson and Becker, 1986).

As previously mentioned, MAO is an enzyme that catalyzes the oxidative deamination of monoamines. It is present in two isoforms (MAO-A and MAO-B) in the most mammalian tissues (Shih et al., 1999; Tipton et al., 2012). MAO-A has high affinity for the hydroxylated amines as serotonin, norepinephrine, and by the inhibitor clorgyline. Whereas MAO-B has high affinity for the non-hydroxylated amines as beta-phenylethylamine, benzylamine and by the inhibitors selegiline, pargyline, rasagiline and low concentrations of selegiline. The amines DA, tyramine, epinephrine and tryptamine have affinity for both isoforms (Finberg and Rabey, 2016; Youdim et al., 2006; Youdim and Bakhle, 2006). Regarding degradation of DA, it can vary in relation to the specie and the tissue under consideration. In the rodent striatum, DA is metabolized preferentially by MAO-A isoform under basal conditions and, by both (MAO-A and MAO-B) in high concentrations (Fornai et al., 1999; Youdim and Bakhle, 2006).

In vitro studies demonstrate that Kava extract inhibits preferentially MAO-B (Prinsloo et al., 2019; Uebelhack et al., 1998); however, the effects of Kava extract on MAO activity *in vivo* are scarce. As AMPH also inhibits the activity of both isoforms of MAO (Faraone, 2018; Miller et al., 1980; Robinson, 1985) we investigated the possible effects on both isoforms since a combined effect of Kava extract and AMPH could be involved in the behavioral changes found in this study. However, besides an effect of Kava extract on MAO-A activity in cortex and MAO-B activity in hippocampus and striatum, no statistical correlation was found between the activity of MAO and locomotion and stereotyped behavior (data not shown). Since the *in vitro* binding of Kava extract (Uebelhack et al., 1998) and AMPH (Miller et al., 1980) are reversible, the binding with the enzyme is highly dynamic which could explain the lack of effects on MAO activity in most of evaluated tissues.

Additionally, it was performed the evaluation of social interaction and Y maze (spatial working memory). As expected, AMPH does not produce alterations in both tests, which is in agreement with other studies (Featherstone et al., 2007; Jones et al., 2011; Kameda et al., 2013; Kane et al., 2011; Lieberman et al., 1987). In Y maze test, there was no significant effects of Kava extract/AMPH on the percentage of correct alternations. However, a significant effect of Kava extract increasing the number of entries into the arms was observed which could be related to the increased locomotor activity present in the animals in the open field test.

5. Conclusion

Taken together, the present study demonstrated that the Kava extract prevented the appearance of stereotyped behavior induced by AMPH in mice, suggesting a potential therapeutic in psychotic symptoms. Furthermore, Kava extract decreases the stereotyped behavior at the same dose which could help to alleviate anxiety symptoms found in patients.

Co-authors contribution

De Freitas, C. M.; Ceretta, A. P. C.; Barbosa, C. P.; Reis, E. de M.: Behavioral and biochemical analysis. Scussel, R.; Corneo, E. S.; Machado-de-Avila, R. A.: Biochemical analysis. Boligon, A: carried out HPLC analysis. Krum, B.: Fachinetto, R.: investigation and

writing–original draft preparation.

Declaration of competing interest

The authors declare that there are no conflicts of interest associated with this study.

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4.3 ARTIGO 3

Artigo

Ex vivo and *in vitro* inhibitory potential of Kava extract on monoamine oxidase B activity in mice

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Ex vivo and *in vitro* inhibitory potential of Kava extract on monoamine oxidase B activity in mice

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ABSTRACT

Background and aim: This study investigated the effect of Kava extract (*Piper methysticum*), a medicinal plant that has been worldly used by its anxiolytic effects, on monoamine oxidase (MAO) activity of mice brain after 21 days of treatment as well as anxiolytic and locomotor behavior. Furthermore, the *in vitro* inhibitory profile of Kava extract on MAO-B activity of mouse brain was evaluated.
Experimental procedure: Mice were treated with Kava extract (10, 40, 100 and 400 mg/kg) for 21 days by gavage. After behavioral analysis (plus maze test and open field), MAO activity in different mouse brain structures (cortex, hippocampus, region containing the substantia nigra and striatum) were performed. MAO-B inhibitory profile was characterized *in vitro*.
Results: The treatment with Kava extract (40 mg/kg) increased the percentage of entries of mice into the open arms. *Ex vivo* analysis showed an inhibition on MAO-B activity caused by Kava extract in cortex (10 mg/kg) and in the region containing the substantia nigra (10 and 100 mg/kg). *In vitro*, Kava extract also reversibly inhibited MAO-B activity with IC_{50} = 14.62 μg/mL and, increased *Km* values at the concentrations of 10 and 30 μg/mL and decreased *Vmax* value at 100 μg/mL.
Conclusion: Kava extract showed different effects on MAO-B isoform depending on the brain structure evaluated. Therefore, the use of Kava extract could be promissory in pathologies where MAO-B is the pharmacological target.
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1. Introduction

The perennial shrub named Kava (*Piper methysticum* - G. Forst) is native from the South Pacific islands countries and, the herb extracted of its roots or rhizome has been used in modern phytotherapy as a recreational kava-drinking due to its relaxing properties and as a natural alternative to replace anxiolytic and sleeping drugs.[1,2] In addition to its anxiolytic/hypnotic effects, studies with Kava extract already showed analgesic and anticancer properties *in vitro* and *in vivo*, as well as improved of the psychotic symptoms.[3–5] The presence of different components in Kava extract have been reported. Among them fifteen kavalactones may be found in the crude extract, being six kavalactones the most abundant - kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin. In lower quantities, Kava chalcones (flavokavains A, B and C - a class of open chain flavonoids), amino acids, minerals and phenolic compounds are also present among the chemicals already isolated from the extract.[6–8] A variety of biological mechanisms of these secondary metabolites from Kava extract have been demonstrated, incluing positive modulation of gamma-aminobutyric acid type A ($GABA_A$) receptors, affinity by DA type-2 and opioid receptors, reduction in

Abbreviations: 4-HQ, 4-hydroxyquinoline; 5-HT, serotonin; AMPH, amphetamine; CEUA, Ethic Committee on Animal Use; CNS, central nervous system; CONCEA, National Council of Control of Animal Experimentation; DA, dopamine; GABA, gamma-aminobutyric acid; HPLC, High performance liquid chromatography; MAO, Monoamine oxidase; NE, norepinephrine.

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reuptake of neuronal norepinephrine (NE) and dopamine (DA), inhibition of voltage-gated Na^{+} and Ca^{2+} ion channels, and inhibitory effects on monoamine oxidase -B (MAO-B) *in vitro*.[4,9,10]

MAO is a flavoenzyme found in the outer mitochondrial membrane of cells present in the central nervous system (CNS) and peripheral tissues of mammals.[11,12] It catalyzes the oxidative deamination of biogenic and dietary amines to their corresponding aldehydes with subsequent formation of hydrogen peroxide and ammonia.[13–15] MAO has two isoforms, MAO-A and MAO-B, which are differentiated by their specificities, substrates and inhibitors.[16,17] MAO-A isoform preferentially metabolizes hydroxylated amines as NE and serotonin (5-HT) and, it is selectively inhibited by clorgyline.[17,18] Whereas MAO-B metabolizes non-hydroxylated amines as benzylamine and beta-phenylethylamine and, it is selectively inhibited by rasagiline, pargyline and low concentrations of selegiline.[17,18] Amines as epinephrine, DA, tryptamine and tyramine are substrates for both MAO isoforms in the majority of the species.[19] In this sense, MAO inhibitors have a long-standing use as anti-parkinsonian and anti-depressant drugs that improve quality of life for patients.[20] Recently, Krum et al. demonstrated that Kava extract avoided the increase of stereotyped behavior in a model of psychosis-like symptoms induced by amphetamine (AMPH) in mice.[8] Furthermore, the acute administration of Kava extract inhibited MAO isoforms in cortex and hippocampus of mice.[8] Given the changes in MAO activity observed, we have now analyzed its effects on this enzyme after 21 days of treatment with Kava extract in mice.

Thus, the main goal of the present study was to evaluate the effect of 21 days of treatment with Kava extract on MAO-A and MAO-B isoforms in different mouse brain structures. Also, the behavioral changes with the doses used were evaluated as well as the *in vitro* inhibitory profile of Kava extract on MAO-B activity in mouse brain.

2. Material and methods

2.1. Plant material and chemicals

The crude extract of Kava rhizome (*Piper methysticum* – with approximately 30% of kavalactones) was obtained from Huakang Biotechnology Development (China - manufacturer's lot HK20160415). The composition of the extract was previously described by Krum et al.[8] All reagents were purchased from Sigma (Sigma-Aldrich, São Paulo, SP, Brazil) or other with high quality and purity.

2.2. Animals

Male Albino Swiss mice weighing between 25 and 35 g (2 months of age) from breeding colony of experimental house of Federal University of Santa Maria were used in this study. The animals were housed in polycarbonate cages (44 cm × 30 cm/4–5 per cage) with free access to water and food, in a temperature-controlled room (22 ± 2 °C) and under a 12-h light/dark cycle (lights on at 7:00 a.m.). All the animal experimentation was conducted in accordance with the guidelines of the National Council of Control of Animal Experimentation (CONCEA) and, the experimental procedures were approved by the Ethic Committee on Animal Use of Federal University of Santa Maria, Brazil under the protocol number CEUA 1637290415.

2.3. In vivo experimental design

Mice were randomly assigned into five groups (8 animals per group): (I) control, (II) Kava extract 10 mg/kg, (III) Kava extract 40 mg/kg, (IV) Kava extract 100 mg/kg and (V) Kava extract 400 mg/kg. Kava extract 40 mg/kg corresponds to the dose commonly used by humans (±200 mg/60 kg), which was calculated by allometric conversion.[21] Based on that, a pharmacological curve was carried out. Animals received Kava extract or its vehicle (corn oil) orally by gavage in a volume of 5 mL/kg once a day for 21 days.[22] Behavioral assessment was performed on day 22 of the experimental period (24 h after the last administration of Kava extract or vehicle) and all possible efforts were taken to avoid animal stress (environmental enrichment, low noise levels, appropriate supply of water, food, humidity, light, temperature, and others). After, the mice were euthanized by cervical dislocation in another experimental room and, rapidly, their brains were dissected in cortex, hippocampus, region containing the substantia nigra and striatum, frozen in powdered dry ice and stored at −80 °C for *ex vivo* analysis (MAO-A and MAO-B activity).

2.4. Behavioral assays

2.4.1. Elevated plus maze

Elevated plus maze test was conducted to evaluate the possible anxiolytic-like effect caused by Kava extract in mice.[23] The apparatus consisted of four arms, two open and two closed (30 cm L × 5 cm W and for closed arms, walls with 17 cm H) elevated 38.5 cm from the floor. The animals were placed in the center of the apparatus and the time spent and the number of entries into open or closed arms and, the number of head dips during 5 min were evaluated. The time spent on open arm and the number of entries into the open arms were calculated and demonstrated in percentage, as follows: time spent or number of entries into the open arm/total time or total number of the entries into closed and open arm X 100, respectively.[24]

2.4.2. Open field test

To verify possible changes in spontaneous locomotor and exploratory activity caused by Kava extract, mice were placed individually in the center of an open field arena (44 cm L × 44 cm W × 44 cm H) divided into 16 squares (4 rows of 4). The number of lines crossed (locomotor activity) and frequency of rearing (stand-up responses on two paws - exploratory activity) were measured during 5 min with no habituation period.[23,25,26]

2.4.3. Ex vivo MAO activity

To measure MAO activity *ex vivo*, mouse brain structures such as cortex, hippocampus, region containing the substantia nigra and striatum were homogenized in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, pH 7.4) and, the homogenate was used to determine the protein quantity.[27] The reaction mixture containing brain structures homogenates (0.25 mg of protein) plus 250 nM pargyline (selective MAO-B inhibitor) or 250 nM clorgyline (selective MAO-A inhibitor) was pre-incubated at 37 °C for 20 min. After, kynuramine (60 μM) was added to start the reaction and incubated for additional 30 min at 37 °C. The reaction was stopped with 10% trichloroacetic acid (TCA). The samples were centrifuged at 500×*g* for 8 min. 1 mL of the supernatant was mixed with 1 mL of NaOH (1 N) and used to estimate the MAO-A and MAO-B activity. The product of reaction 4-hydroxyquinoline (4-HQ) was determined in fluorimeter at 315 nm for excitation and 380 nm for emission.[28] Results were expressed in nmol of 4-HQ per milligram of protein per minute.[8,29,30]

2.5. In vitro experimental protocol

For *in vitro* assays, the potential inhibitory effect of Kava extract on MAO activity (MAO-A and MAO-B) and the reversibility of MAO

activity inhibition were tested in total mouse brains. The extract was diluted in ethanol 1% (vehicle) and tested at different concentrations (10, 30, 100 μg/mL). A control without ethanol was used to verify if the ethanol *per se* could cause alterations in MAO activity.

2.5.1. In vitro MAO activity

Kava extract was tested for its *in vitro* inhibitory potential on MAO-A and MAO-B activity. Briefly, brains were homogenized in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, and 3.6 mM KCl, pH 7.4) and the homogenate was used for protein determination.[27] The activities of MAO-A and MAO-B isoforms were measured using 0.25 mg of protein in the presence of 250 nM clorgyline (selective MAO-A inhibitor) or 250 nM pargyline (selective MAO-B inhibitor) into the reaction mix along with different concentrations of Kava extract (10, 30, 100 μg/mL) or its vehicle. The mixture was pre-incubated at 37 °C for 20 min and then, 60 μM kynuramine (non-selective MAO substrate) was added to start the enzymatic reaction and incubated for additional 30 min at 37 °C. To stop the reaction, 10% TCA was added to the mixture. The samples were centrifuged at 500×*g* for 8 min. 1 mL of the supernatant was mixed with 1 mL of NaOH (1 N) and used to estimate the MAO-A and MAO-B activity. The product of reaction 4-HQ was determined in fluorimeter (315 nm for excitation and 380 nm for emission).[28] Results were expressed in nmol of 4-HQ per milligram of protein per minute.[30–32] The reversibility of MAO-B activity was carried out using the dialysis method.[33,34] Then, mixtures of outer buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, 1 mM dithiothreitol) and mice brain homogenates (0.25 mg of protein) in the absence or presence of Kava extract (100 μg/mL) were dialyzed at 25 °C for 24 h. For each 1 mL of mixture dialyzed, 40 mL of outer buffer was used. The outer buffer was replaced to a fresh buffer at 1, 2, 4 and 6 h after the start of dialysis. Nondialyzed mixtures were maintained under the same assay conditions. Dialyzed and non-dialyzed mixtures were simultaneously assayed for MAO-B activity as described above 24 h after the start of dialysis, to evaluate the reversibility of the inhibition produced by Kava extract. For kinetic experiments, different concentrations of kynuramine (1–100 μM) were used, and the MAO-B activity was determined in the absence or presence of different concentrations of Kava extract (10–100 μg/mL) to calculate K_m and V_{max} values.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test when appropriate. All values are expressed as mean + standard error of the mean (SEM). IC_{50} value was calculated by nonlinear regression using sigmoidal dose–response with a variable slope equation, K*m* (μM) and V*max* (nmol/min/mg protein) values by nonlinear regression using the Michaelis–Menten equation. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Kava extract causes anxiolytic-like effect without altering locomotor and exploratory behavior in mice

In plus maze test, one-way ANOVA reveled a significant effect of Kava extract in the percentage of the number of entries into the open arms [$F(4, 35) = 3.82$; $p < 0.05$; Fig. 1B] *Post hoc* analysis showed Kava extract (40 mg/kg) increased the percentage of number of entries into the open arms compared to control group. Kava extract at 100 and 400 mg/kg decreased the percentage of the number of entries into the open arms when compared to Kava extract 40 mg/kg. However, no significant effect was detected in the percentage of time spent into the open arms and in the number of head dips (Fig. 1A and C, respectively) in plus maze test.

In open field test, Kava extract did not cause significant effects neither in the number of crossing nor in the number of rearing (Fig. 1D and E).

3.2. Kava extract decreases MAO-B activity in cortex and substantia nigra of mice

MAO-A and MAO-B activities were evaluated in cortex, hippocampus, region containing the substantia nigra and striatum of treated mice. For MAO-A activity, no significant difference among the groups was found in the brain structures analyzed (Fig. 2A, B, C, D). On the other hand, one-way ANOVA showed a significant effect of Kava on MAO-B activity in cortex [$F(4, 20) = 3.80$; $p < 0.05$; Fig. 3A] and in the region containing the substantia nigra [$F(4, 20) = 4.08$; $p < 0.05$; Fig. 3C]. Kava extract decreased the MAO-B activity in cortex at dose of 10 mg/kg, and in region containing the substantia nigra at doses of 10 and 100 mg/kg compared with their respective control group. No changes were observed in MAO-B activity in hippocampus and striatum (Fig. 3B and D).

3.3. Kava extract inhibits MAO-B activity with a reversible profile in vitro

One-way ANOVA showed a significant effect of Kava extract on MAO-A [$F(5, 12) = 5.16$; $p < 0.05$; Fig. 4A] and MAO-B activity [$F(5, 12) = 26.46$; $p < 0.05$; Fig. 4B]. *Post hoc* analysis demonstrated that Kava extract 100 μg/mL decreased MAO-A activity only when compared to the concentrations at 3 and 10 μg/mL of the extract. To MAO-B, *post hoc* analysis showed Kava extract decreased MAO-B activity at concentrations of 10, 30 and 100 μg/mL compared to control and vehicle groups with an $iC_{50} = 14.62$ μg/mL. Furthermore, Kava extract decreased MAO-B in a concentration-dependent manner since *post hoc* analysis showed the concentrations of 30 and 100 μg/mL were different from 3 μg/mL.

The reversibility of the MAO-B inhibition induced by Kava extract was measured by using the dialysis method. The inhibition caused by Kava extract at 100 μg/mL on MAO-B activity was completely reversible after dialysis [$F(1,12) = 13.67$; $p < 0.05$; Fig. 4C]. Lastly, in the kinetic experiments, Kava extract caused an increase in the K*m* values at 10 and 30 μg/mL (23.05 ± 3.61 and 40.01 ± 7.83 μM, respectively) and decrease V*max* values at 100 μg/mL (0.259 ± 0.022 nmol/min/mg of protein) compared to K*m* and V*max* values in the absence of Kava extract (12.63 ± 1.54 and 0.419 ± 0.014 nmol/min/mg of protein, respectively) (Fig. 4D and Table 1).

4. Discussion

Pharmacological actions in CNS have been demonstrated to Kava extract and, our previous study demonstrated that Kava extract decreases stereotyped behavior and promotes alterations in MAO activity in mice[8]; however, these effects on MAO activity have not been fully explored yet. Kava extract at 40 mg/kg caused an increase in the percentage of entries into the open arm in plus maze test without altering locomotor activity of the mice in the open field test. The treatment with Kava extract for 21 days reduced the activity of MAO-B in cortex at a dose of 10 mg/kg and in the region containing the substantia nigra at the doses of 10 and 100 mg/kg without altering the activity of MAO-A in any brain structure of mice. *In vitro*, Kava extract reversibly inhibited the MAO-B activity with an $IC_{50} = 14.62$ μg/mL, increasing the K*m* values at 10 and 30 μg/mL and decreasing the V*max* values at 100 μg/mL.

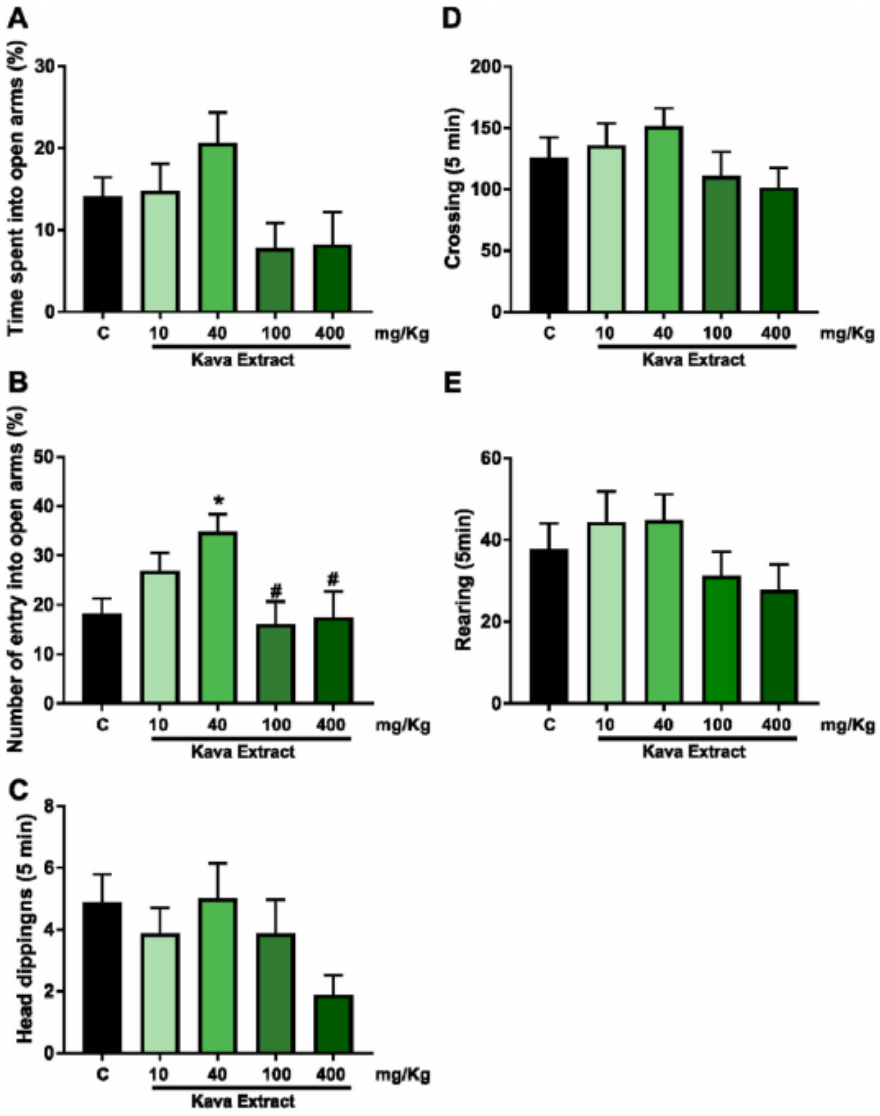

Fig. 1. Behavioral assays. (A) the percentage of the time spent on the open arms, (B) the percentage of the number of entries into the open arms and (C) the number of head dips in plus maze test, along with the number of (D) crossing and (E) rearing in the open field test were evaluated in mice, both for 5 min. The animals were treated with vehicle or Kava extract (10, 40, 100, 400 mg/kg) for 21 days. Data are expressed as mean + standard error of mean (n = 8). *$p < 0.05$, compared to control group. #$p < 0.05$, compared to kava extract (40 mg/kg) group.

The use of Kava extract in herbal medicine started in social ceremonies through Kava-drinking for centuries (approximately 3000 years ago).[35] The extract preparations (from parts of Kava plant) were made and used in local medicine for a range of illnesses such as sleeping difficulties, anxiety, convulsion, pain, fever, weight reduction, psychotic symptoms and others.[4,36,37] In the 1990s, Kava organic extract, now found in encapsulated and tincture forms, showed interest worldwide and it started to be used an over-the-counter preparations clinically proven treatment for anxiety, depression, insomnia and stress.[1,7,38] It is known that secondary metabolites of Kava extract such as kavalactones and chalcones have differents bioactivities. Kavain, for example, is implicated in a sudden relaxing effect while dihydrokavain and dihydromethysticin can cause nausea.[39] On the other hand, flavokavain A has been associated with anti-inflamatory and anticarcinogenic properties while flavokavain B with anti-inflamatory, anticarcinogenic, antinociceptive properties and hepatotoxicity.[39–41] In this context, *in vitro* studies demonstrated the six major kavalactones from Kava extract show MAO inhibition properties, which yangonin (with IC_{50} values of 1.29 μM/MAO-A and 0.085 μM/MAO-B) was the

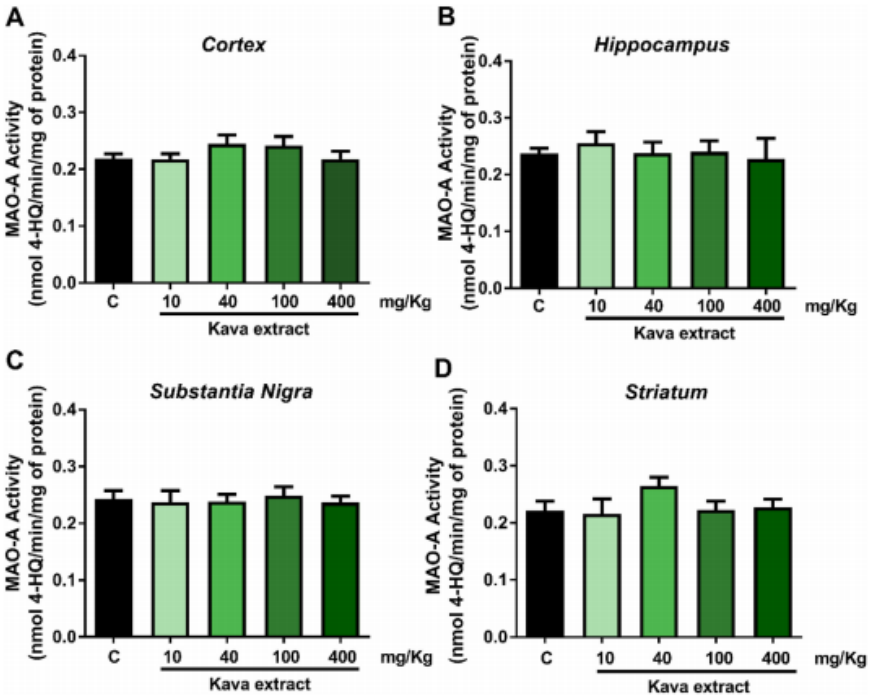

Fig. 2. MAO-A activity in (A) cortex, (B) hippocampus, (C) region containing the substantia nigra and (D) striatum in treated mice with vehicle or Kava extract (10, 40, 100, 400 mg/kg) for 21 days. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean + standard error of mean (n = 5).

most potent MAO inhibitor, followed by kavain (with IC_{50} values of 19 μM/MAO-A and 5.34 μM/MAO-B) and desmethoxyyangonin (with IC_{50} values of 4.44 μM/MAO-A and 2.51 μM/MAO-B).[42,43] Furthermore, other plant-derived metabolites may also show inhibitory activities on both isoforms of MAO, such as flavonoids, xanthones and alkaloids. The inhibitory activity on MAO enzyme of these secondary metabolites can be associated on their general structure activity relationship principles. The chalcones present in the Kava extract, have selectivity on MAO isoforms due substituents present in the B ring and double bond present in the 2 and 3 position of C ring, or electron donating hydrophilic hydroxyl group in the para position of B ring favoring the MAO-A inhibition, or absence of double bond between 2 and 3 positions of the C ring maintaining the non-planar nature of flavonoids and others [43].

Literature data also demonstrated that the dose used influences in the response produced by Kava on CNS of experimental animals.[44] In acute treatments, lower doses may produce mild stimulant effects while higher doses may cause sleepiness, ataxia and muscle relaxation.[44] Notwithstanding the Kava actions on GABAergic system are extensively studied and related to its main pharmacological use,[9,45] modulation on dopaminergic system[44,46] with consequent pharmacological effects were reported in humans[4,5,37] and experimental animals.[8] Recently, it was demonstrated that Kava reduced the stereotyped behavior induced by amphetamine in mice and had effects on MAO activity after a single administration of the extract[8] reinforcing the evidence of its action on other neurotransmitter systems beyond GABAergic system. However, of our knowledge, the effects of Kava extract on MAO activity *ex vivo* have not been fully investigated despite of *in vitro* evidence of its inhibitory effect on MAO-B.[10,42]

In the present study, mice were treated with Kava extract during 21 consecutive days to evaluate the effects of long-term treatment since there are few studies investigating the effects of Kava extract in chronic models. Firstly, we investigated the effects of Kava on behavioral effects in elevated plus maze test to evaluate if the doses used are promoting the anxiolytic effect which is reported in the literature.[1] Kava extract increased the percentage of entries in the open arms in elevated plus maze test at a dose of 40 mg/kg. Previously, Krum et al. demonstrated the single dose of 40 mg/kg also increased the number of entries of mice in the open field test[8] demonstrating this effect did not present tolerance with the repeated administrations at least up to 21 days of treatment. Interestingly, this dose corresponds to the equivalent dose used by humans calculated by allometric conversion (±200 mg/60 kg/day) for the relief of anxiety symptoms. Furthermore, the effects of Kava extract observed in plus maze test were not caused by an increase in locomotor activity since significant differences were not found in the open field test.

As it was previously demonstrated that Kava extract preferentially inhibited the activity of MAO-B *in vitro*[10,42] and, a single administration of Kava extract altered the MAO activity in brain regions of mice,[8] the main objective of the present study was to evaluate MAO-A and MAO-B activities in brain regions of mice treated with Kava extract for 21 days. Kava extract reduced the activity of MAO-B in the cortex at a dose of 10 mg/kg and in the region containing the substantia nigra at the doses of 10 and 100 mg/kg without altering the activity of MAO-A in any brain structure of mice. Interestingly, Kava extract seems to have different effects on MAO-B depending on the brain structure and time of treatment. Furthermore, the dose of Kava extract that

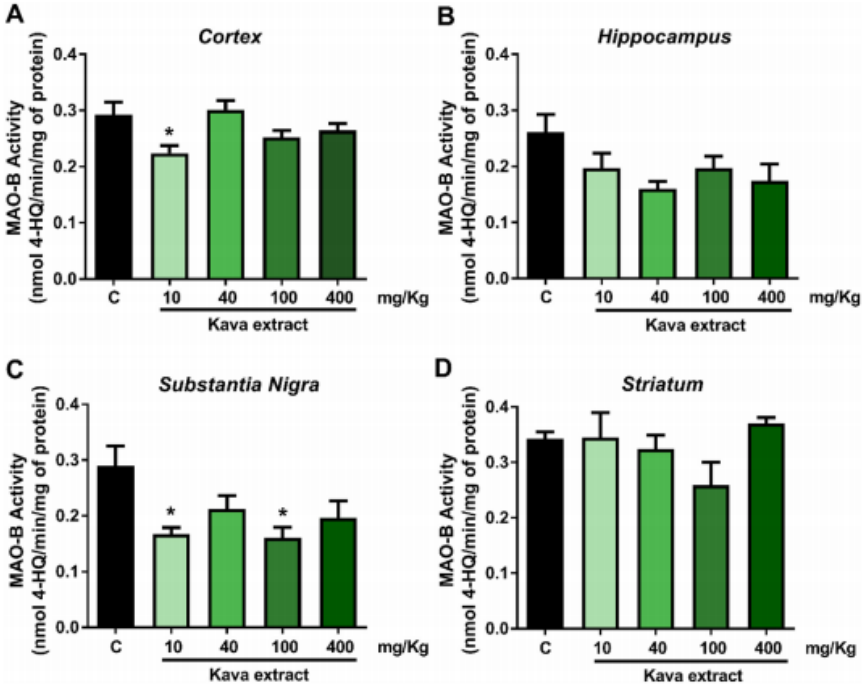

Fig. 3. MAO-B activity in (A) cortex, (B) hippocampus, (C) region containing the substantia nigra and (D) striatum in treated mice with vehicle or Kava extract (10, 40, 100, 400 mg/kg) for 21 days. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean + standard error of mean (n = 5). *$p < 0.05$, compared to control group.

showed anxiolytic affects was not the same dose that caused MAO-B inhibition. Krum et al.[8] demonstrated a decrease in MAO-A activity in cortex and MAO-B activity in hippocampus after a single dose of Kava extract in mice. Another important finding of the present study is the selectivity of Kava extract by MAO-B after long-term treatment once MAO-A inhibition is associated to higher probability of side effects.[19,47,48] Of our knowledge, this is the first study showing the inhibitory action of Kava extract *ex vivo* in a longer treatment in mice.

Considering it was found a reduction in the activity of MAO even after *ex vivo* analysis in the present and previous[8] studies from our group, an *in vitro* study was performed using the Kava extract to evaluate its effects on MAO activity from mice brain homogenates. Kava extract inhibited the activity of MAO-B with an iC_{50} of 14.62 μg/mL without altering the activity of MAO-A. The reversibility of the Kava extract binding to MAO-B was also confirmed since the percentage of activity was above of 80% after 24 h of dialysis[33] at the highest concentration tested (100 μg/mL). Moreover, Kava extract increased the K*m* values (10 and 30 μg/mL) and decreased the V*max* values (100 μg/mL) of MAO-B. Therefore, Kava extract was shown to be reversible and preferential MAO-B inhibitor. Our findings are in agreement with previous studies conducted by Uebelhack et al.[10] and Prinsloo et al.[42] Taken together, we suggest that the components of Kava extract can possess different bioavailability in the different regions of the brain when administered *in vivo*. Furthermore, even the binding of Kava extract with the enzyme is highly dynamic with reversible profile, a part of it continues bound during the *ex vivo* analysis maintaining the enzyme inhibited.

Monoamines are biological active compounds that act as neurotransmitters regulating several body functions such as behavioral, cognitive, motor and endocrine processes.[49] Biogenic amines are enzymatically metabolized by MAO which has participation in the regulation of their levels in mammals.[48,50,51] Currently, MAO inhibitors isocarboxazid, phenelzine, tranylcypromine, safinamide, rasagiline and selegiline are the medications approved by *Food and drug administration* (FDA) for treatment of depression, Parkinson's disease, and Alzheimer's disease.[52–55] Moreover, recent research with plant-derived compounds based on chalcones with pharmacophores from FDA approved drugs may become therapeutic possibility as MAO inhibitors.[54,56] In this sense, Kava chalcones (flavokavain A, B, C) could be contributing to the effects promoted by kavalactones, an MAO inhibitor in *in vitro* studies already known. However, there are still no studies about this possible effect.[42]

Previous studies have demonstrated the Kava extract can modulates monoamines as DA, NE, 5-HT in rodents[44] and zebrafish.[57] Furthermore, recently Krum et al.[8] demonstrated Kava extract decreased stereotyped behavior induced by psychostimulant amphetamine and MAO activity in mice suggesting a potential monoamine-modulating *in vivo*. Considering the reports of different pharmacological actions of Kava, its use should be cautious. In the same way that Kava extract could be used as a pharmacological agent (mainly as adjuvant – MAO-B inhibitor) for the treatment of pathologies as anxiety, Parkinson's disease, Huntington's disease, Alzheimer's disease, psychosis[4,8,18,19,37,50,58–60] it could be also used as abuse substance, since Kava extract has psychoactive effects by acting on the mesolimbic reward system.[57,61]

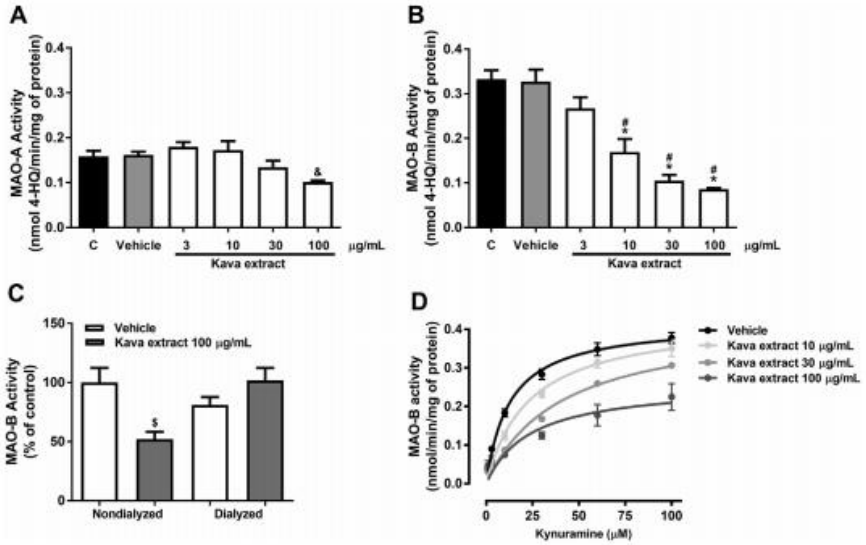

Fig. 4. Inhibitory potential *in vitro* of Kava extract (10, 30, 100 µg/mL) on (A) MAO-A and (B) MAO-B activities in mouse brain homogenates. Values are expressed as mean + standard error of mean (n = 3–4). MAO activity was analyzed by one-way followed by Tukey's post hoc test. $^{\&}p < 0.05$, compared to Kava extract 3 and 10 µg/mL $^{*}p < 0.05$, compared to control and ethanol group. $^{\#}p < 0.05$, compared to Kava extract 3 µg/mL. (C) reversibility of MAO-B inhibition caused by Kava extract (100 µg/mL) after 24 h of dialysis in mouse brain homogenates. Values are expressed as mean + standard error of mean (n = 3–4). Reversibility was analyzed by two-way ANOVA followed by Tukey's post hoc test. $^{\$}p < 0.05$, compared to nondialyzed control. (D) Substrate concentrations curve for *in vitro* MAO-B activity in the absence or presence of Kava extract (10, 30, 100 µg/mL). Km (µM) and Vmax values were calculated by nonlinear regression using the Michaelis–Menten equation (n = 6).

Table 1
Km and Vmax values in the absence or presence of Kava extract for MAO-B activity from mouse brain homogenates.

	Kava extract (µg/mL)	Km (µM)	Vmax (nmol/min/mg of protein)
MAO-B	0	12.63 ± 1.54	0.419 ± 0.014
	10	23.05 ± 3.61*	0.429 ± 0.022
	30	40.01 ± 7.83*	0.426 ± 0.034
	100	23.84 ± 6.12	0.259 ± 0.022*

Data were analyzed by test-t. *p < 0.05 when compared with control group.

In conclusion, the present study demonstrated the potential action of Kava extract as an MAO-B inhibitor *ex vivo*, which showed different potencies depending on the analyzed brain structure. It was confirmed for *in vitro* assays, where Kava extract reveled also to be a reversible and preferential MAO-B inhibitor. Therefore, Kava seems to act on monoaminergic system in mice. Its effects could be promissory as adjuvant therapeutic approaches in pathologies involving alterations in MAO-B activity.

Author's contribution

Conception and design of the work: BNK and RF; conducted experiments: BNK, CMF, AB, LFS; data analysis and interpretation: BNK and RF; statistical analysis: BNK; writing–original draft preparation: BNK. The authors declare that all data were generated in-house and that no paper mill was used.

Declaration of competing interest

All authors declare there are no potential financial, personal, or otherwise conflicts of interest.

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5 DISCUSSÃO

Inúmeras pesquisas vêm sendo realizadas, ano após ano, para melhor compreender os mecanismos envolvidos na etiologia/fisiopatologia das psicoses. Para isso, modelos animais, novos compostos e fármacos já utilizados na clínica são amplamente testados. Assim, para esse estudo, foram utilizados *C. elegans* e camundongos como modelos animais experimentais e os compostos haloperidol, anfetamina e o extrato de Kava (*P. methysticum*), divididos em três trabalhos que se correlacionam entre si na busca pelo entendimento de mecanismos de fármacos já utilizados como antipsicóticos (haloperidol) e novas propostas farmacológicas (extrato de Kava) considerando a problemática principal que é o tratamento das psicoses.

Assim, o primeiro trabalho deste estudo investigou os efeitos do haloperidol sobre componentes do sistema dopaminérgico em *C. elegans*. O haloperidol tornou-se um dos medicamentos antipsicóticos mais vendidos mundialmente para combater sintomas psicóticos em indivíduos que sofrem de algum tipo de psicose (PILLAI et al., 2008). No entanto, apesar dos seus inúmeros benefícios, o tratamento com haloperidol pode apresentar efeitos adversos bastante significativos nos pacientes que o utilizam, como o aparecimento de alterações motoras. Além disso, apesar dos estudos encontrados na literatura, ainda existem lacunas em relação aos mecanismos pelo qual esse fármaco atua, tanto em relação aos seus efeitos farmacológicos como para seus efeitos adversos. Modelos alternativos tem sido uma ferramenta importante para se estabelecer o mecanismo farmacológico de diversas substâncias de forma preliminar ao estudo em roedores. Desta forma, o modelo alternativo de *C. elegans* nos permite avaliar desde a expressão de genes de interesse, funções biológicas básicas, vias bioquímicas até o comportamento animal dada sua alta versatilidade experimental (BLAXETER, 2011; CULETTO; SATTELLE, 2000; ENGLEMAN; KATNER; NEAL-BELIVEAU, 2016; MALIN; SHAHAM, 2015; RODRIGUEZ et al., 2013). Os achados experimentais em relação aos mecanismos do haloperidol no modelo animal de *C. elegans* certamente servirão como base para estudos futuros relacionados a investigação de mecanismos de sinalização celular e de possíveis associações com outras terapias farmacológicas. Os resultados deste trabalho (artigo 1) demonstraram que o haloperidol aumentou a vida útil de cepas do tipo selvagem N2 e diminuiu a diferença entre o número de curvas corporais em cepas N2 e em cepas *knockout* para a subtipo *dop-2* do receptor de DA (um subtipo do receptor de DA tipo 2). O haloperidol também diminuiu a velocidade média móvel das cepas N2 e cepas *knockout* para a subtipo *dop-3* do receptor de DA (um subtipo do receptor de DA tipo 2). Em cepas

N2, a velocidade de locomoção voltou aos níveis basais após a retirada do haloperidol. O tratamento com haloperidol também reduziu os níveis de DA, mas não alterou a expressão gênica para os subtipos de receptores de DA *dop-1* (um subtipo do receptor de DA tipo 1), *dop-2, dop-3* bem como a morfologia dos neurônios dopaminérgicos cefálicos em cepas N2. Com base nos dados encontrados, o haloperidol parece modular os receptores de DA do tipo 2 em *C. elegans,* com uma possível ativação em *dop-2* e um antagonismo em receptores *dop-3* de DA. O presente estudo demonstrou pela primeira vez que os receptores de DA medeiam efeitos farmacológicos do haloperidol em *C. elegans.*

Sabe-se que a presença de alimento é um fator limitante na expectativa de vida de *C. elegans*. Esses animais possuem terminações sensoriais (mecanorreceptores) que ativam a liberação de DA via receptores *dop-2* e *dop-3* em neurônios da SIA (neurônio colinérgico), e consequentemente, suprimem a liberação de octopamina e inativam a proteína de ligação ao elemento de resposta ao AMP cíclico (CREB) na presença de alimento, assim, delimitando sua expectativa de vida (SMITH et al., 2008; SUO; CULOTTI; VAN TOL, 2009a, 2009b). O aumento da expectativa de vida das cepas N2 foi atribuído a modulação do haloperidol em receptores de DA tipo 2 (*dop-2* e *dop-3*) nos neurônios da SIA e consequente ativação de CREB após o reconhecimento do alimento.

A sinalização de DA também participa do comportamento de desaceleração basal e locomoção em *C. elegans* (ALLEN et al., 2011). A DA modula o movimento desses animais através da interação com seus receptores dopaminérgicos que suprimem a liberação de ACh e GABA, agindo nos próprios neurônios motores colinérgicos e GABAérgicos (ALLEN et al., 2011; REFAI; BLAKELY, 2019). Fisiologicamente, o papel do receptor *dop-3* em neurônios motores é inibir a liberação de ACh na junção neuromuscular, diminuindo a contração muscular e a liberação de GABA, levando ao relaxamento do musculo oposto. Ambos, ACh e GABA, impedem a diminuição da locomoção (ALLEN et al., 2011; OMURA et a., 2012). Neste trabalho foi observado, após exposição ao haloperidol, uma diminuição na resposta de detecção ao alimento (comportamento de desaceleração) em cepas *dop-3* e uma diminuição na velocidade média móvel (atividade de locomoção) em cepas N2 e *dop-3*. Esses resultados reforçam o envolvimento do haloperidol em receptores de DA do tipo 2. A avaliação da velocidade média móvel em *C. elegans* também foi realizada na presença (dia 1) e ausência (dia 2) de haloperidol, respectivamente. No dia 1, os animais expostos a concentração mais alta de haloperidol apresentaram diminuição na

atividade locomotora. Enquanto, no dia 2, os mesmos animais foram transferidos para placas contendo somente bactéria/ veículo e nenhuma alteração sobre a locomoção dos animais foi observada. Esses achados indicam que a ligação do haloperidol em seus alvos moleculares é reversível em *C. elegans*.

Após, os níveis de DA foram mensurados em cepas N2. Os animais que foram expostos ao haloperidol apresentaram níveis reduzidos de DA quando comparados com os animais expostos somente ao veículo. A diminuição dos níveis de DA após exposição ao haloperidol é possivelmente decorrente de um aumento na sinalização colinérgica e GABAérgica secundária aos efeitos na modulação dos receptores de DA tipo 2, uma vez que a DA e ACh/GABA atuam em oposição (ALLEN et al., 2011; OMURA et a., 2012). Por fim, a expressão gênica dos receptores *dop-1, dop-2, dop-3* e a morfologia de neurônios dopaminérgicos cefálicos (possível neurodegeneração) foram analisados após exposição ao haloperidol. Entretanto, não houve diferenças significativas entre os grupos em nenhum dos genes testados, nem na morfologia dos neurônios dopaminérgicos cefálicos. Em roedores, foram observadas alterações na expressão gênica de receptores D_2 em tratamentos crônicos com o haloperidol, enquanto para os tratamentos mais curtos não foram observadas diferenças significativas. Neste sentido, o tempo de exposição utilizado no presente estudo (4 dias) pareceu não ser suficiente para causar alterações nos genes avaliados. A morfologia dos neurônios cefálicos dopaminérgicos não foi alterada após exposição ao haloperidol, não demonstrando degeneração nesses neurônios. Esse resultado pode estar relacionado a diminuição dos níveis de DA, uma vez que a oxidação e a degradação de DA também estariam diminuídos (MOR; DANIELS; ISCHIROPOULOS, 2019).

Em conclusão, combinados, os efeitos encontrados neste trabalho revelam o envolvimento do receptor de DA tipo 2 na mediação dos efeitos do haloperidol em *C. elegans*. Esse estudo demonstram as vantagens inerentes ao modelo de *C. elegans* e combina análises comportamentais, moleculares e morfológicas abrangentes relacionadas com o sistema dopaminérgico. Além disso, esses resultados servem como base para testes com novos compostos bem como para pesquisas adicionais a fim de esclarecer outros mecanismos inerentes a eficácia do haloperidol e seu potencial terapêutico em transtornos psicóticos.

Para o segundo (artigo 2) e o terceiro (artigo 3) trabalho deste estudo, foram avaliados os efeitos do extrato bruto de Kava em um modelo animal de psicose induzido por anfetamina e seu potencial efeito inibitório sobre a atividade da enzima MAO, utilizando camundongos como

modelo animal. O extrato bruto de Kava, preparado a partir do seu rizoma, é de venda livre isento de prescrição médica no Brasil e consumido mundialmente para tratar sintomas de ansiedade e distúrbios nervosos, como estresse e insônia (DENTALI, 1997; LEBOT; MERLIN; LINDSTROM, 1992; SINGH, 2005). Em relação aos mecanismos farmacológicos, sabe-se que além da ação do extrato de Kava sobre os receptores $GABA_A$ (ansiolítico), alguns estudos sugerem o seu envolvimento na modulação do sistema dopaminérgico e em um potencial efeito inibitório sobre a enzima MAO (BAUM et al, 1998; SARRIS et al., 2012; UEBELHACK, FRANKE, SCHEWE, 1998). Neste contexto, transtornos psicóticos, são transtornos heterogêneos que conferem problemas na vida social e incapacidade ocupacional a indivíduos acometidos. O aparecimento de sintomas psicóticos nesses indivíduos tem sido fortemente atribuído a alterações na neurotransmissão de DA na via dopaminérgica mesolímbica (ANDREASEN, 1994; KUCINSKI et al 2011).

Deste modo, o segundo trabalho deste estudo teve como objetivo investigar se o extrato de Kava poderia evitar as alterações comportamentais induzidas por anfetamina em camundongos e se alterações na MAO poderiam estar envolvidas em seus efeitos. Os animais foram pré-tratados com extrato de Kava (40 mg/Kg, única administração, por gavagem) e posteriormente com anfetamina (1,25 mg/Kg, única administração, via intraperitoneal) (CERETTA et al., 2016; FIGUEIRA et al., 2015). Os animais tratados somente com o extrato de Kava, primeiramente, demonstraram um comportamento do tipo ansiolítico no teste do labirinto em cruz elevada, confirmando a eficácia do extrato sobre o SNC desses animais. Em seguida, foram realizados testes comportamentais descritos na literatura por mimetizarem alguns sintomas encontrados em pacientes acometidos por algum transtorno psicótico, como a esquizofrenia, a qual é caracterizada pelo aparecimento de sintomas positivos, negativos e cognitivos (POGORELOV et al., 2017).

Para avaliar a resposta ao tratamento sobre os sintomas similares aos sintomas positivos, foram realizados os testes de campo aberto e estereotipia. No teste de campo aberto, o extrato de Kava, anfetamina bem como o co-tratamento com esses compostos causaram um aumento na atividade locomotora dos animais. O extrato de Kava e o co-tratamento também levaram a um aumento no comportamento exploratório. Enquanto no teste de estereotipia, a administração de anfetamina causou um aumento no comportamento estereotipado dos animais e o pré-tratamento com o extrato de Kava impediu o aparecimento desses sintomas. Os resultados encontrados corroboram com outros estudos, os quais mostraram que a administração aguda de anfetamina leva

à hiperlocomoção e ao desenvolvimento de comportamentos incomuns (cheirar, roer unhas, aparecimento de movimentos circulares e imobilidade nos animais), os quais estão associados a um estado hiperdopaminérgico na via mesolímbica (principalmente no *nucleus accumbens*) (CERETTA et al., 2016; SAITO et al., 2014; WOLGIN, 2012). O aumento da atividade locomotora causado pelo extrato sugere que ele poderia estar interagindo na mesma via da anfetamina, pois quando realizado o co-tratamento não houve mudança de resposta em relação a cada tratamento isoladamente.

Em relação a enzima MAO, o extrato de Kava alterou a atividade da MAO-A (córtex) e da MAO-B (hipocampo e estriado) dos animais tratados. Estudos demostraram que *in vitro* o extrato de Kava assim como a anfetamina possuem efeitos inibitórios sobre esta enzima (FARAONE, 2018; MILLER; SHORE; CLARCKE, 1988; PRINSLOO et al., 2019). Entretanto, nenhuma correlação estatística foi observada entre as alterações comportamentais e os efeitos do extrato sobre a enzima MAO.

Os sintomas negativos e cognitivos [representados pelos testes de interação social e labirinto em Y (memória espacial de trabalho), respectivamente], são sintomas que estão diretamente relacionados à diminuição dos níveis de DA no córtex pré-frontal (ABI-DARGHAM, 2004; MARSDEN, 2006). Como esperado, a anfetamina não causou alterações comportamentais nos animais nos dois testes avaliados. Estes resultados estão de acordo com outros estudos, os quais reportam o uso da sensibilização à anfetamina como um modelo animal para avaliar somente os sintomas positivos de transtornos psicóticos (FEATHERSTONE; KAPUR; FLETCHER, 2007; JONES et al., 2011). No teste do labirinto em Y, os animais do grupo co-tratado apresentaram um aumento no número de entradas nos braços do labirinto. Sugere-se que esse efeito possa estar relacionado ao aumento da atividade locomotora observada nos animais no teste do campo aberto.

Assim, este protocolo experimental demonstrou pela primeira vez que o extrato de Kava evitou o aparecimento do comportamento estereotipado induzido pela anfetamina em camundongos, sugerindo um potencial efeito terapêutico do extrato sobre os sintomas psicóticos, além de aliviar sintomas de ansiedade que frequentemente estão associados nos mesmos pacientes (aproximadamente 65%) (SIM et al., 2006).

Baseado nos dados encontrados no trabalho anterior, o terceiro trabalho desta tese avaliou o efeito do extrato de Kava sobre a enzima MAO por meio de análises *ex vivo* em associação a ensaios comportamentais em camundongos e a atividade MAO/cinética *in vitro* usando

preparações de cérebro de camundongo. Para os ensaios *in vivo*, os animais foram tratados com diferentes doses do extrato de Kava durante 21 dias. Como no estudo anterior, a mesma dose do extrato (40 mg/Kg) causou um aumento no número de entradas nos braços abertos no teste do labirinto em cruz elevada. No entanto, não houve alterações nos parâmetros de locomoção e exploratórios nos mesmos animais em nenhuma das doses testadas. Após análise dos testes comportamentais, foi realizada a atividade das isoformas da MAO-A e MAO-B utilizando diferentes regiões cerebrais (córtex, hipocampo, região contendo a substância negra e estriado) desses animais. Como resultados, o extrato de Kava inibiu a isoforma MAO-B no córtex e na região contendo a substância negra, sem alterar a atividade da isoforma MAO-A. O extrato de Kava parece ter diferentes efeitos sobre a isoforma MAO-B, dependendo da estrutura cerebral avaliada e do tempo de tratamento. Isso pode ocorrer devido à concentração em que os componentes do extrato atingem as diferentes estruturas cerebrais.

Para confirmar e aprofundar os resultados encontrados nos experimentos *ex vivo*, foi investigado os efeitos do extrato de Kava sobre a atividade da MAO *in vitro*. Como resultado, o extrato também inibiu a isoforma MAO-B bem como, aumentou os valores de K*m* e diminuiu o valor de V*max*, apresentando uma ligação do tipo reversível (após 24h de diálise). A avaliação da atividade da enzima MAO é um mecanismo importante, pois algumas monoaminas (DA, 5-HT, NA) são metabolizadas por essa enzima e consequentemente, estão envolvidas na regulação de diversas funções corporais (DI GIOVANNI et al., 2016; YOUDIM; FRIDKIN; ZHENG, 2004). Estudos anteriores demostraram o potencial efeito central mediado por Kava e seu envolvimento na modulação do sistema monoaminérgico (BAUM et al., 1998; WANG et al., 2020). Como mencionado acima, as interações do extrato de Kava parece ser complexa, pois possui diferentes respostas dependendo da dose usada, tempo de tratamento, bem como da região do cérebro examinada. Portanto, esse extrato deve ser utilizado para fins terapêuticos com cautela. Da mesma forma que ele poderia ser usado como um possível adjuvante no tratamento de patologias como na doença de Parkinson, Alzheimer, ansiedade, psicoses entre outras, o extrato pode apresentar efeitos psicoativos, sendo usado como substância de abuso, atuando sobre o sistema de recompensa na via mesolímbica (KIM et al., 2016; KRUM et al., 2020; SINGH, 1992; WANG et al., 2020; YOUDIM; BAKHLE, 2006; VOLGIN et al., 2020; YOUDIM; EDMONDSON; TIPTON, 2006).

Os resultados encontrados neste estudo mostraram a potencial ação do extrato de Kava como um inibidor da MAO-B *ex vivo*, que apresentou diferentes potências dependendo da estrutura

cerebral analisada. Também foi confirmado em ensaios *in vitro* que o extrato é um inibidor reversível e preferrencial da MAO-B. O extrato de Kava parece atuar no sistema monoaminérgico em camundongos, portanto, seus efeitos podem ser promissores como terapia adjuvante em patologias onde a MAO-B esteja envolvida. No entanto, a dose do extrato a ser utilizada deve ser sempre analisada com cautela.

6 CONCLUSÃO

6.1 CONCLUSÕES ESPECÍFICAS

De acordo com os resultados apresentados nesta tese podemos concluir que:

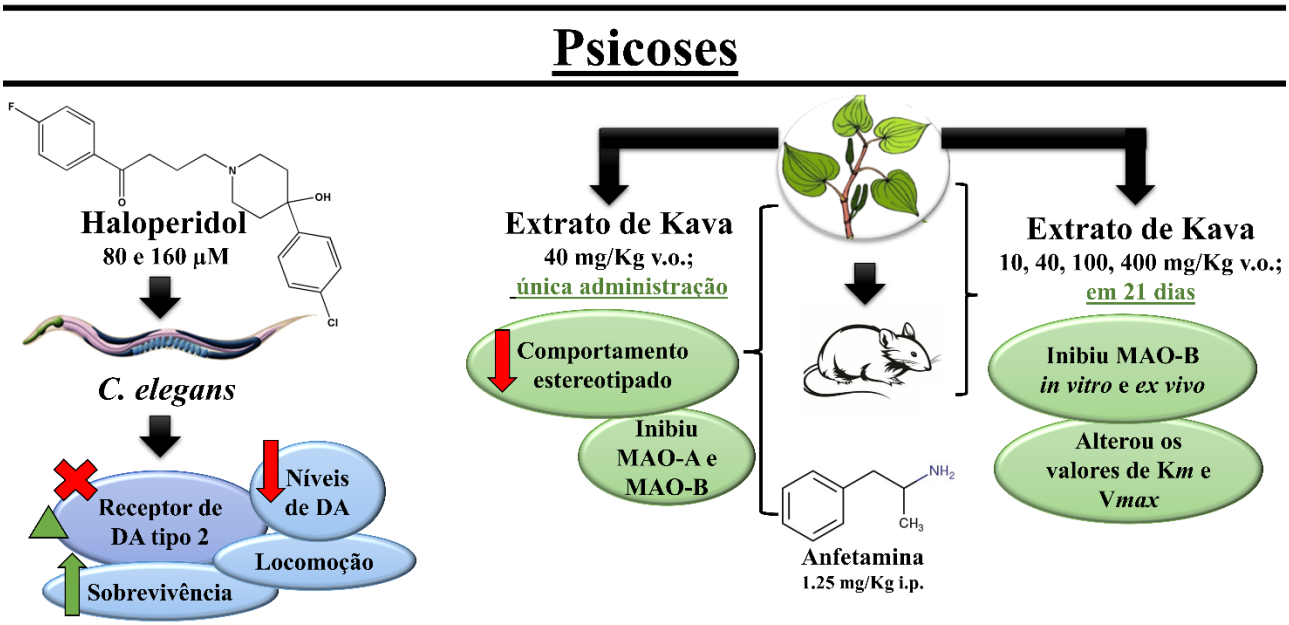
- A exposição do antipsicótico haloperidol promoveu modulação em receptores de DA do tipo 2, em *C. elegans*, mecanismo pelo qual gerou um aumento na expectativa de vida, uma diminuição na diferença entre o número de curvas corporais, na velocidade de locomoção e nos níveis de DA desses animais.

- A administração aguda do extrato bruto de Kava em um modelo de psicose impediu o aparecimento do comportamento estereotipado induzido por anfetamina em camundongos, sugerindo um potencial efeito terapêutico sobre sintomas psicóticos e inibiu a atividade da isoforma MAO-A no córtex e da MAO-B no hipocampo e estriado dos animais tratados.

- O extrato bruto de Kava inibiu a atividade da enzima MAO-B *in vitro* e no córtex e na substância negra de camundongos previamente tratados com o extrato, sugerindo ser uma terapia promissora em patologias onde a MAO-B esteja envolvida.

6.2 CONCLUSÃO FINAL

Figura 6 – Conclusão final da tese



Primeiramente investigou-se os efeitos do antipsicótico haloperidol sobre sistema dopaminérgico em *C. elegans* através de avaliações comportamentais, moleculares e morfológicas. O haloperidol aumentou a sobrevivência, alterou o comportamento locomotor e os níveis de DA nesses animais. Os efeitos do haloperidol em *C. elegans* parece estar relacionado a modulação de receptores do tipo D_2 de DA. Para os experimentos realizados com o extrato bruto de Kava, primeiro avaliou-se seus efeitos em um modelo de psicose induzido por anfetamina em camundongos e a atividade da enzima MAO em um ensaio *ex vivo*. O extrato impediu o aparecimento do comportamento estereotipado nos animais, sugerindo um potencial efeito terapêutico sobre sintomas psicóticos e inibiu a atividade da isoforma MAO-A no córtex e da MAO-B no hipocampo e o co-tratamento aumentou a atividade da isoforma MAO-B no estriado dos animais tratados. Por fim, investigou-se os efeitos do extrato bruto de Kava após 21 dias de tratamento em camundongos e a atividade das isoformas da MAO em ensaios *ex vivo* e *in vitro*. O extrato inibiu atividade da enzima MAO-B *in vitro* no córtex e na substância negra dos animais previamente tratados com o extrato, sugerindo ser uma terapia promissora em patologias onde a MAO-B esteja envolvida.

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ANEXO A – CERTIFICADO DE APROVAÇÃO DE PROJETO NA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação Farmacológica/ Toxicológica de Piper methysticum in vitro e in vivo", protocolada sob o CEUA nº 1637290415, sob a responsabilidade de **Roselei Fachinetto** *e equipe; Alcindo Busanello; Bárbara Nunes Krum; Caroline Pilecco Barbosa; Catiuscia Molz de Freitas; Getúlio Nicola Bressan; Larissa Finger Schaffer* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 19/05/2015.

We certify that the proposal "Pharmacologic/toxicologic evaluation of Piper methysticum in vitro e in vivo", utilizing 121 Heterogenics mice (121 males), protocol number CEUA 1637290415, under the responsibility of **Roselei Fachinetto** *and team; Alcindo Busanello; Bárbara Nunes Krum; Caroline Pilecco Barbosa; Catiuscia Molz de Freitas; Getúlio Nicola Bressan; Larissa Finger Schaffer* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 05/19/2015.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2015 a 08/2018 Área: Fisiologia E Farmacologia

Origem:	Não aplicável biotério				
Espécie:	Camundongos heterogênicos	sexo: Machos	idade:	2 a 2 meses	N: 121
Linhagem:	Swiss		Peso:	25 a 30 g	

Resumo: O Piper methysticum (P. methysticum) é uma planta conhecida popularmente como Kava kava, a qual é consumida na forma de chá e também está presente em formulações fitoterápicas. É utilizado principalmente para o tratamento de ansiedade, agitação e insônia, mas em doses elevadas alguns autores sugerem que ela cause hepatotoxicidade, gerando assim controvérsias em relação ao seu uso. Diante disso, uma avaliação farmacológica/ toxicológica de P. methysticum in vitro e in vivo será de extrema importância para verificar os efeitos causados pela planta. Os estudos serão realizados através de testes in vitro como a atividade da monoamina oxidase (MAO), a afinidade com receptores do tipo GABAA e a expressão de proteínas como a Erk1/2, p38 e Nrf-2, alterações na cascata Akt/GSK-3 e a expressão da enzima tirosina hidroxilase e do transportador de dopamina (DAT), através do ensaio de imunoreatividade. Também serão realizados testes in vivo através de uma curva dose resposta nas doses de 0, 10, 40, 100 e 400 mg/Kg que serão administradas por gavagem durante 21 dias de tratamento, com o objetivo de escolher uma dose ideal para utilização em outros experimentos e excluir a possibilidade da mesma exercer algum efeito tóxico aos animais. A melhor dose será escolhida por meios dos testes comportamentais (campo aberto e labirinto em cruz elevado) e através de testes bioquímicos com o soro dos animais e também com o cérebro, fígado e rim dos mesmos para avaliação de parâmetros de estresse oxidativo (EO). Por fim, após a escolha da dose, os efeitos da planta serão também analisados em um modelo de esquizofrenia induzido por anfetamina com uma dose 1,25 mg/Kg (administração intraperitoneal) em camundongos, onde serão realizados testes comportamentais (Campo aberto, Estereotipias, Labirinto em cruz elevado, Labirinto em Y e Preferência condicionada), a expressão de proteínas (Erk1/2, p38, Nrf-2, Akt/GSK-3, da enzima tirosina hidroxilase e DAT) e a atividade da MAO. Por meio desses experimentos, acredita-se que poderão ser esclarecidos alguns dos mecanismos de ação de Kava kava afim de que essa planta possa, futuramente, também ser utilizada em estudos de outras doenças neurodegenerativas.

Local do experimento: Laboratório do Departamento de Fisiologia e Farmacologia (Sala 5209)

Santa Maria, 30 de agosto de 2016

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax:
Horário de atendimento: das 8:30 ás 12h e 14h ás 17hs : e-mail: ceua.ufsm@gmail.com
CEUA N 1637290415

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO B – COMPROVANTE DE CADASTRO AO CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO (SisGen)

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A00421C

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A00421C**
Usuário:	**UFSM**
CPF/CNPJ:	**95.591.764/0001-05**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	

☑ **Pesquisa Científica** ☐ **Bioprospecção** ☐ **Desenvolvimento Tecnológico**

Espécie

Piper methysticum

Título da Atividade:	**AVALIAÇÃO FARMACOLÓGICA/TOXICOLÓGICA DE PIPER METHYSTICUM EM CAMUNDONGOS E EM DROSOPHILAS MELANOGASTER**

Equipe

Roselei Fachinetto	**UFSM**
Bárbara Nunes Krum	**UFSM**
Catiuscia Molz de Freitas	**UFSM**
Aline Augusti Boligon	**UFSM**
Alcindo Busanello	**UFSM**
Ana Paula Ceretta	**UFSM**
Elizete de Moraes Reis	**UFSM**