UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA TOXICOLÓGICA

Bárbara Nunes Krum

Piper methysticum ALTERA A ATIVIDADE LOCOMOTORA E PARÂMETROS BIOQUÍMICOS EM *Drosophila melanogaster*

Santa Maria, RS, Brasil
2017

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

Orientadora: Profª. Drª. Roselei Fachinetto

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Aprovado em 08 de fevereiro de 2017:
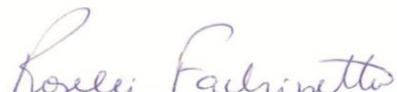
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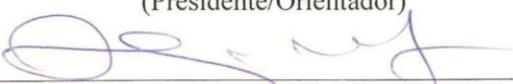
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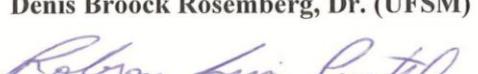
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Santa Maria, RS
2017

AGRADECIMENTOS

Primeiramente, agradeço a Deus pela proteção, bênçãos e por estar sempre guiando meus passos.

Agradeço meu pai Jairo, minha mãe Roselaine e meus irmãos Débora e Daniel pelo afeto, suporte e por estarem sempre presentes, apoiando todas as minhas decisões.

A professora Dra. Roselei Fachinetto, pelo exemplo profissional, pelos ensinamentos, dedicação e oportunidades proporcionadas a mim.

Aos amigos e colegas de laboratório, Ana Paula, Getúlio, Janaína, Jeane, Caroline, Larissa, Luis, Talita, Camila, Juliane, Alcindo, Elizete e Catiuscia pelos momentos alegres, descontraídos e divertidos. Obrigada por passarem a diante seus conhecimentos e ensinamentos além do auxilio na realização dos meus experimentos, nos momentos em que necessitei de ajuda sempre pude contar com o apoio de todos vocês, certamente foram de extrema importância para a minha formação. Lab 5209 sempre vai estar no meu coração!

As minhas amigas Vanessa, Andressa, Bianca e Camilla pelos inesquecíveis momentos de alegria, cumplicidade e apoio nas horas mais difíceis, sempre que precisei vocês estavam ao meu lado. Presentes que a faculdade me proporcionou, amo vocês!

Á Universidade Federal de Santa Maria, ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica e a CAPES pela bolsa concedida.

Enfim, meu muito obrigada a todos aqueles que de alguma forma me apoiaram e incentivaram para que eu chegasse até aqui.

"Como o pássaro que voa livre e segue o sol sem perder o rumo, o ser humano deve traçar sua vida num horizonte aberto, amando e aceitando desafios".

(Sérgio Mendes).

RESUMO

Piper methysticum ALTERA A ATIVIDADE LOCOMOTORA E PARÂMETROS BIOQUÍMICOS EM *Drosophila melanogaster*

AUTORA: BÁRBARA NUNES KRUM
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O *Piper methysticum* (*P. methysticum*)*,* conhecido popularmente como Kava kava, é utilizado para o tratamento da ansiedade devido a sua ação sobre o Sistema Nervoso Central (SNC). Entretanto seu mecanismo de ação ainda está sendo investigado. A fim de minimizar e substituir o uso de roedores em alguns modelos experimentais, a *Drosophila melanogaster* (*D. melanogaster*) é um exemplo de modelo alternativo usado nexte contexto, principalmente em estudos genéticos, doenças do SNC e para o screening de drogas com potencial farmacológico. Diante disso, o presente estudo investigou os efeitos de *P. methysticum* sobre as alterações comportamentais e bioquímicas em *D. melanogaster*. Para realização dos testes as *D. melanogaster* foram expostas a diferentes concentrações do extrato bruto de *P. methysticum* (0.001, 0.01, 0.1, 1 e 10 mg/mL). Na curva de sobrevivência, houve um aumento significativo no número de mortes das *D. melanogaster* na concentração de 0.001, 1 e 10 mg/mL. Para os teses de geotaxia negativa, parâmetros de estresse oxidativo e imunorreatividade da enzima tirosina hidroxilase (TH) foram realizados tratamentos de 5 ou 12 dias com as mesmas concentrações de *P. methysticum* utilizadas na curva de sobrevivência. No ensaio de geotaxia negativa, houve uma diminuição da atividade locomotora das *D. melanogaster* nas concentrações de 1 e 10 mg/mL durante 5 dias de tratamento e uma diminuição na concentração de 10 mg/mL durante 12 dias de tratamento com a planta. Após isso, as *D. melanogaster* foram tratadas com *P. methysticum* na concentração de 0.1 mg/mL durante 5 ou 12 dias e após foram colocadas em um novo meio de tratamento contendo 5 ou 500 μM de reserpina por 2 dias. Esta associação potencializou a redução na atividade locomotora das *D. melanogaster* nos dois tempos avaliados, indicando assim, um possível sinergismo entre os dois compostos utilizados. Nos parametros de estresse oxidativo, houve um aumento nos níveis de tiol proteico e de especies reativas de oxigênio e nitrogênio na concentração de 10 mg/mL somente para o tratamento de 12 dias com o extrato da planta. No ensaio de imunorreatividade da TH, houve um aumento nos níveis de TH na concentração de 0.001 mg/mL e uma diminuição destes níveis na concentração de 10 mg/mL após 5 dias de tratamento. Após 12 dias de tratamento houve uma diminuição nos níveis de TH nas concentrações de 1 e 10 mg/mL da planta. Os dados encontrados sugerem que a diminuição da sobrevivência das *D. melanogaster* assim como da sua atividade locomotora*,* poderiam estar associadas a diminuição da imunorreatividade da enzima TH e consequentemente um possivel envolvimento do sistema monoaminérgico. Entretanto mais estudos devem ser realizados a fim de verificar com maior clareza os efeitos de *P. methysticum* em *D. melanogaster.*

Palavras-chave: Kava kava. Modelo alternativo. Reserpina. Dopamina. Geotaxia negativa. Tirosina hidroxilase.

ABSTRACT

Piper methysticum ALTERS LOCOMOTOR ACTIVITY AND BIOCHEMICAL PARAMETERS IN *Drosophila melanogaster*

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Piper methysticum (*P. methysticum*), popularly known as Kava Kava, is used for the treatment of anxiety due to its action on the Central Nervous System (CNS). However, its action mechanism continues being investigated. In order to minimize and replace the use of rodents in some experimental models, *Drosophila melanogaster* (*D. melanogaster*) is an example of alternative model, used mainly in genetic studies, CNS diseases and the screening of drugs with pharmacological potential. Therefore, the present study investigated the effects of *P. methysticum* on behavioral and biochemical changes in *D. melanogaster*. To perform the tests *D. melanogaster* were exposed to different concentrations of the crude extract of *P. methysticum* (0.001, 0.01, 0.1, 1 e 10 mg/mL). In the survival curve, there was a significant increase in the number of deaths of *D. melanogaster* at the concentration of 0.001, 1 and 10 mg/mL. For negative geotaxis test, oxidative stress parameters and tyrosine hydroxylase (TH) immunoreactivity, treatments of 5 or 12 days were performed with the same concentrations of *P. methysticum* used in the survival curve. Negative geotaxis test, there was a decrease in the locomotor activity of *D. melanogaster* at concentrations of 1 and 10 mg/mL during 5 days of treatment and a decrease in the concentration of 10 mg/mL during 12 days of treatment with the plant. After this, *D. melanogaster* were treated with 0.1 mg/mL *P. methysticum* for 5 or 12 days and then placed in a new treatment medium containing 5 or 500 μM reserpine for 2 days. This association potentiated the reduction in the locomotor activity of *D. melanogaste* in the two evaluated times, indicating a possible synergism between the two compounds. In the oxidative stress parameters, there was an increase in the levels of protein thiol and reactive oxygen and nitrogen specimens at the concentration of 10 mg/mL only for the treatment of 12 days with the plant. TH immunoreactivity assay, there was an increase in TH concentrations at 0.001 mg/mL and a decrease of TH levels at the 10 mg/mL concentration after 5 days of treatment. After 12 days of treatment there was a decrease in TH levels at concentrations of 1 and 10 mg/ mL of the extract. The data suggest that the decrease of the survival of the *D. melanogaster* as well as the it locomotor activity could be associated with a decrease of the immunoreactivity of the TH enzyme and consequently a possible involvement of the monoaminergic system. However, further studies should be carried out in order to verify the effects of *P. methysticum* on *D. melanogaster*.

Keywords: Kava kava. Alternative model. Reserpine. Dopamine. Negative Geotaxia. Tyrosine hydroxylase.

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MANUSCRITO:

LISTA DE ABREVIATURAS

3-MT	3-metoxitiramina
aaNAT	arilalquilamina N-acetiltransferase
AMPc	3,5-adenosina-monofosfato-cíclico
ANVISA	Agência Nacional de Vigilância Sanitária
CB1	receptor canabinóide do tipo 1
COMT	catecol-O-metiltransferase
DA	dopamina
DOPAC	3,4-ácido dihidroxifenilacético
D. melanogaster	*Drosophila melanogaster*
GABA	ácido gama-aminobutírico
GAD	enzima glutamato descarboxilase
HVA	ácido homovanílico
MAO	enzima monoaminoxidase
NADA	N-acetil dopamina
NBAD	N-β- alanil dopamina
P. methysticum	*Piper methysticum*
PI3K	fosfatidilinositol-3-quinase
SNC	sistema nervoso cental
TH	tirosina hidroxilase
TVMA	transportador vesicular de monoaminas

SUMÁRIO

APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrita uma revisão sucinta sobre os temas abordados neste trabalho.

As seções **MATERIAIS E MÉTODOS**, **RESULTADOS**, **DISCUSSÃO DOS RESULTADOS** e **REFERÊNCIAS BIBLIOGRÁICAS**, encontram-se nesta dissertação e estão apresentados sob a forma de manuscrito científico o qual representa a íntegra deste estudo.

O item **CONCLUSÕES** encontrado no final desta dissertação, apresenta comentários gerais sobre o manuscrito contido neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** desta dissertação.

INTRODUÇÃO

1.1 Piper methysticum

Durante muitos anos, estudos tem dado atenção à utilização de produtos naturais como fontes de antioxidantes e também como uma alternativa para o tratamento dos mais variados tipos de doenças (NEWMAN e CRAGG, 2007). O *Piper methysticum (P. methysticum)* é um exemplo de especíe de planta usado nexte contexto, o qual é utilizado principalmente para o tratamento da ansiedade e insônia (GARRETT et al., 2003; REX; MORGENSTERN; FINK, 2002).

P. methysticum é uma planta perene, com forma de arbusto, pertencente a família Piperacea (pimenta), nativo das ilhas do pacífico e comumente encontrado na Melanésia, Micronésia e Polinésia (JUSTO e SILVA, 2008; SARRIS et al., 2012; SINGH e SINGH, 2002). Na medicina popular, *P. methysticum* é utilizado na forma de bebida, preparada a partir de suas folhas e rizoma (Figura 1) para fins recreativos em todo o Pacífico, na América do Norte e Europa (BALICK e LEE, 2002; BRUNTON, 1998; RYCHETNIK e MADRONIO, 2011; SINGH, 1992) devido a sua ação em promover relaxamento e sensação de bem-estar (BEHL et al., 2011).

Figura 1- Folhas e rizoma de *P. methysticum*

Fonte: (JUSTO e SILVA, 2008).

No Brasil, o *P. methysticum* vem sendo comercializado desde 2001, quando foi liberado como medicamento fitoterápico pela Agência Nacional de Vigilância Sanitária (ANVISA), o qual é utilizado na medicina humana como alternativa ao uso de

benzodiazepínicos com a finalidade de aliviar sintomas de ansiedade, insônia e estresse (ARKOWITZ e LILIENFELD, 2013). Desde então, sua prescrição para pacientes com esses sintomas vem aumentando com o passar dos anos, estando presente na lista dos 10 medicamentos fitoterápicos mais vendidos em todo o país (BARBOSA; LENARDON; PARTATA, 2013). Dados da literatura também demonstram sua ação como anti-inflamatório, (WU et al., 2002), analgésico (AMARAL et al., 2005), anticonvulsivante (GLEITZ et al., 1996), espasmolítico (MEYER, 1965) e em receptores canabinóides (a kavalactona yagonina possui a afinidade com os receptores do tipo CB1) (LIGRESTI, 2012).

Esta planta possui o nome popular de Kava Kava, devido aos seus principais constituintes, as kavalactonas, as quais são responsáveis por 95% da sua atividade terapêutica (SMITH, 1983, 1984). Seu extrato possui em torno de 15 kavalactonas (SMITH, 1983, 1984), no entanto apenas 6 delas possuem grau de significância. Dentre elas encontram-se a cavaína, metisticina, iangonina, diidrocavaína, diidrometisticina e desmetoxiiagonina (Figura 2), (GAUTZ et al, 2006;. JIANG et al, 2007; LI et al., 2011).

Figura 2 - Estrutura química das kavalactonas
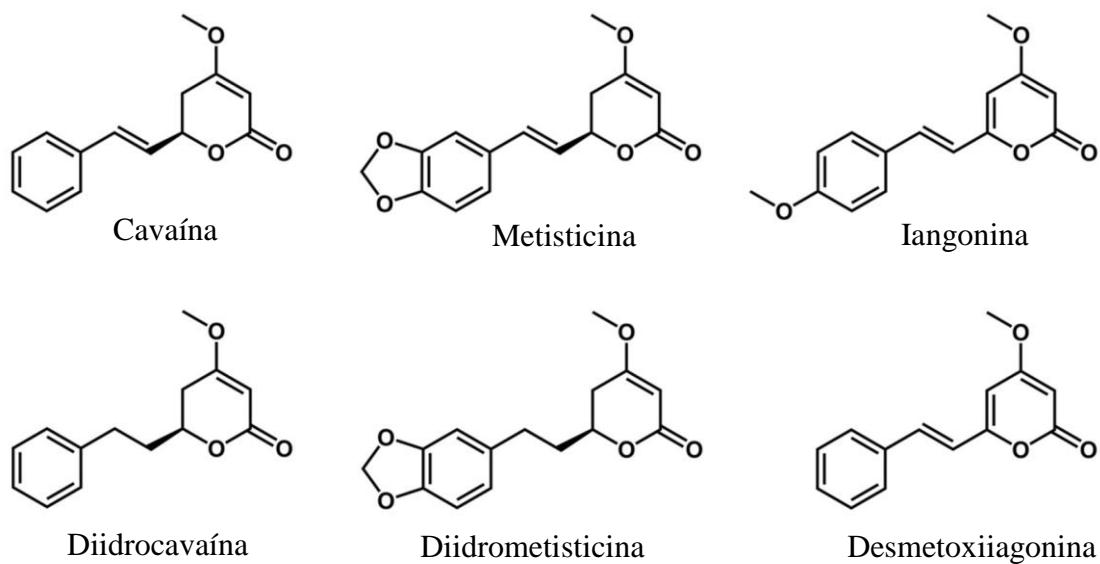
Fonte: (LI et al., 2011).

As kavalactonas, principais contituintes ativos presentes no extrato bruto de *P. methysticum*, possuem ação sobre o sistema nervoso central (SNC), principalmente na neurotransmissão gabaégica e dopaminérgica, e com menor intensidade na neurotransmissão

serotoninérgica e glutamatérgica (REX; MORGENSTERN; FINK, 2002; SHOMAN et al., 2015).

Além da sua utilização como ansiolítico e tranquilizante devido a sua atuação em receptores gabaérgicos (REX et al., 2002; SHOMAN et al., 2015), *P. methysticum* também possui ação sobre o sistema dopaminérgico (DINH, 2001). Estudos demonstram que doses elevadas do extrato do rizoma de *P. methysticum* são capazes de aumentar os níveis de dopamina (DA) no núcleo accumbens de ratos, assim como doses baixas do extrato bruto são capazes de diminuí-los (BAUM et al., 1998). Entretanto, existem poucos estudos na literatura sobre o envolvimento de *P. methysticum* e o sistema dopaminérgico.

Apesar de ser uma planta muito utilizada clinicamente e em estudos com animais experimentais, quando consumida cronicamente, *P. methysticum* é capaz de causar efeitos indesejáveis como erupção cutânea, queda de cabelo, amarelamento da pele, vermelhidão nos olhos, perda de apetite e principalmente elevada hepatotoxicidade (ANVISA; BEHL et al., 2011; SARRIS et al., 2010; TESCHKE; SCHWARZENBOECK; ESCHKE, 2008; TESCHKE; SARRIS; LEBOT, 2011). A hepatotoxicidade de *P. methysticum* está associada com alterações nas enzimas do sistema citocromo P450, uso concomitante ao abuso de álcool, deficiência genética de alguma enzima hepática e a alguns componentes tóxicos da planta, os quais podem causar alterações da glicoproteína-P e esgotamento da glutationa hepática (NERURKAR; DRAGULL; TANG, 2004; WU et al., 2002).

A farmacocinética e a farmacodinâmica do extrato bruto de *P. methysticumm* ainda não estão totalmente esclarecidas. Atualmente, há um ensaio clínico de fase III em andamento com *P. methysticum*, o qual tem como objetivo estabelecer sua eficácia e a segurança em pacientes com diagnóstico de transtorno de ansiedade generalizada, podendo assim ser utilizado com maior segurança (SAVAGE et al., 2015).

1.2 MODELOS ALTERNATIVOS

Atualmente, modelos alternativos tem sido utilizados com maior frequência na pesquisa científica. Esses modelos foram desenvolvidos considerando o princípio dos 3 Rs criado por Russel e Burch, ou seja qualquer modelo que possa ser usado a fim de substituir, reduzir ou refinar o uso de alguns animais na pesquisa biomédica, ensaios ou ensino. A partir de então, houve um crescimento no desenvolvimento de métodos alternativos ao uso de animais de experimentação (RENAMA).

Existem diversos modelos alternativos utilizados para complementar o uso de mamíferos. Animais vertebrados como peixes, anfíbios, répteis e pássaros são alguns exemplos (MCGREW; MCGREW, 1985), assim como o uso de bactérias e leveduras, geralmente utilizados para estudos de genética e metabolismo bioquímico (NAWAZ et al., 1992). Dentre os invertebrados, os insetos são os representantes majoritários, sendo a *Drosophila melanogaster (D. melanogaster)* a espécie mais utilizada, principalmente para estudos genéticos e sobre o SNC (HODGKIN e HUXLEY, 1952; NICHOLS, 2006).

Estudos com *D. melanogaster* tiveram início desde o ano de 1908 com Thomas Hunt Morgan, que descobriu uma mosca com olhos brancos entre diversas moscas com olhos vermelhos, desenvolvendo a teoria da hereditariedade mendeliana e o conceito de que são os cromossomos que transportam seu material genético. Em 1970 Seymour Benzer verificou a capacidade das *D. melanogaster* em exercer comportamentos complexos como (NICHOLS, 2006), entre outras inúmeras descobertas com o passar dos anos. Pesquisas recentes referentes às *D. melanogaster* demonstram sua utilização em estudos sobre o SNC, em modelos de doenças neurodegenerativas (NICHOLS, 2006; BRAND e LIVESEY, 2011; PINTO-TEIXEIRA KONSTANTINIDES; DESPLAN, 2016) e também como uma alternativa para a descoberta de novos farmacos (PANDEY e NICHOLS, 2011).

Mediante a todas as características mencionadas acima, a *D. melanogaster* se enquadra neste contexto e atualmente exerce um papel de extrema importância para a pesquisa científica, tornando-se um bom organismo modelo alternativo a ser empregado.

1.3 *Drosophila melanogaster*

Pertencentes ao filo *Arthropoda,* as *D. melanogaster*, conhecidas como mosca da fruta, são animais invertebrados, com genética amplamente conhecida, tempo de geração rápida e de fácil manejo (CELNIKER e RUBIN, 2003).

Sua genética e biologia molecular nos fornecem informações sobre muitos aspectos de processos do desenvolvimento dos mamíferos, assim como uma significante semelhança quando comparadas com humanos, a qual pode chegar a aproximadamente 65%(BERNARDS e HARIHARAN, 2001; NICHOLS, 2006). As *D. melanogaster*, possuem 4 cromossomos, sendo 3 deles responsáveis por carregarem a maior parte dos seus genes (NICHOLS, 2006). Além disso, as *D. melanogaster* também possuem alguns comportamentos complexos, podendo serem combinados a estudos de cunho genético, de eletrofisiologia e farmacológico a

fim de ajudar a esclarecer algumas funções e circuitos do SNC (GREESPAN e DIERICK, 2004; GREENSPAN e VAN SWINDEREN, 2004; NICHOLS 2006).

Outra vantagem entre as *D. melanogaster*, além do seu fácil manuseio é que elas possuem uma alta taxa de reprodução (NICHOLS, 2006). Uma única *D. melanogaster* é capaz de colocar centenas de ovos dentro de poucos dias. O seu ciclo reprodutivo ocorre de 10-12 dias a uma temperatura de 25 °C. Cerca de 24 horas após a oviposição, ocorre a eclosão dos ovos e a formação das larvas de primeiro estágio chamadas de L1. Passado mais um dia, ocorre à formação de larvas de segundo estágio (L2) e sucessivamente, após mais 24 horas a formação de larvas de terceiro estágio (L3). Passando-se mais 72 horas após a formação de larvas L3, as mesmas rastejam para fora do meio de cultura e tornam-se pupas, nas quais permanecem na forma imóvel. Durante os próximos 4-7 dias, ocorrem diversas transformações nas *D. melanogaster*, até a eclosão das pupas e a sua formação propriamente dita (figura 3A) (NECKAMEYER e ARGUE, 2012; NICHOLS, 2006). Além disso, seu tempo de geração e seu custo relativo quando comparados com outros modelos animais como *Zebrafish* e camundongos, podem ser de 900 a 10.000 vezes respectivamente, mais baratos (Figura 3B) (NECKAMEYER e ARGUE, 2012).

Figura 3- Ciclo reprodutivo (A), tempo de geração e custo relativo (B) de *D. melanogaster*
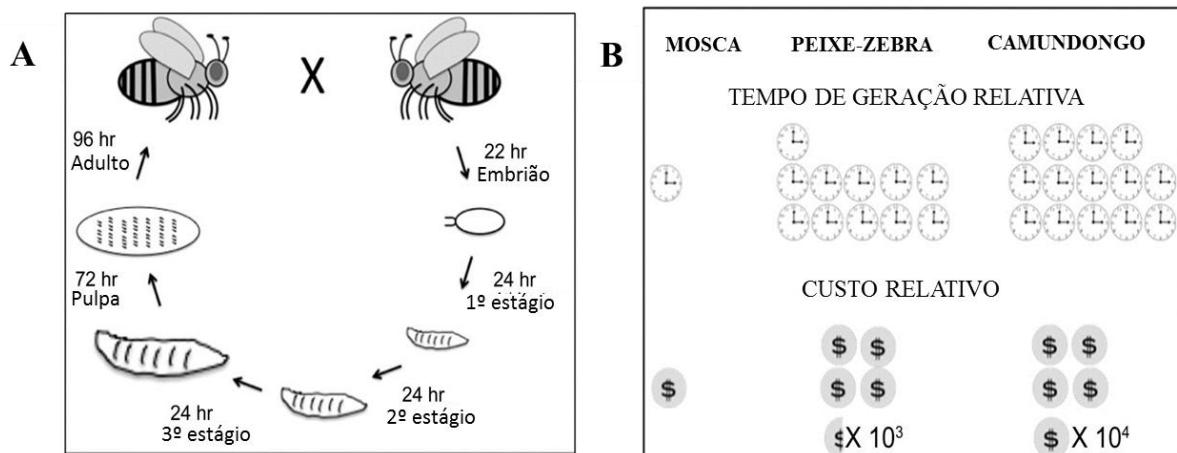
Fonte: (Adaptado de NECKAMEYER e ARGUE, 2012).

Devido a essas características, *D. melanogaster* permitem que pesquisadores consigam realizar análises de diferentes processos fisiológicos, toxicidades de compostos, processos neurofarmacológicos encontrados em doenças humanas, entre outras inúmeras possibilidades de pesquisa (NICHOLS, 2006). Devido a isso é que elas consistem em um dos modelos mais utilizados atualmente para a avaliação preditiva do potencial terapêutico/toxicológico de

compostos químicos e fitoquímicos, assim como para descoberta dos alvos moleculares desses compostos (GIRISH e MURALIDHARA, 2012).

1.3.1 *D. melanogaster* e o Sistema Nervoso Central

O SNC das *D. melanogaster* é dividido em lobos ópticos, cérebro central e cordão ventral (PINTO-TEIXEIRA KONSTANTINIDES; DESPLAN, 2016) com caracterização e genética bem esclarecida (BRAND e LIVESEY, 2011). A neurogênese das *D. melanogaster* tem início na sua fase embriogênica, onde ocorre a diferenciação celular. Estas células adquirem um destino neuronal, as quais são chamadas de neuroblastos ou ocorre a formação de células epidérmicas, responsáveis pela formação do exoesqueleto das *D. melanogaster* (PINTO-TEIXEIRA KONSTANTINIDES; DESPLAN, 2016). Os neuroblastos da fase larval são as principais células responsáveis pela formação do seu SNC, sendo aproximadamente 90% dos seus neurônios produzidos nesta fase (FROLDI e CHENG, 2016; TRUMAN e BATE, 1988; TRUMAN; TAYLOR; AWAD, 1993). Com o passar da fase larval para a fase adulta, há uma divisão dos neuroblastos, dando origem a neurônios e células gliais. As *D. melanogaster* possuem aproximadamente 100.000 nêuronios, os quais são capazes de formar circuitos funcionais responsáveis por processos fundamentais como por exemplo a locomoção, visão, aprendizagem, memória, alimentação, agressão, olfato entre outros (MAO e DAVIS, 2009; NICHOLS, 2006; STRAUSFELD, 1976).

Assim como em humanos, as *D. melanogaster* possuem neurotransmissores capazes de produzir respostas através de receptores acoplados a proteína G, assim como por canais ionotrópicos. Serotonina, dopamina (DA), glutamato, ácido gama-aminobutírico (GABA), acetilcolina, histamina, adenosina e neuroquininas são alguns dos neurotransmissores presentes no seu SNC. Entretanto, as mesmas não possuem resposta adrenérgica, a qual é substituida pela monoamina octopamina (NICHOLS, 2006). Dentre os neurotransmissores acima citados, cada um possui um importante papel na vida das *D. melanogaster,* principalmente quando relacionadas a sua atividade comportamental. Como por exemplo a serotonina que está diretamente relacionada com a sua alimentação, agressão, cortejo, sono, aprendizagem e memória (ALEKSEYENKO et al., 2010; DIERICK e GREENSPAN, 2007; NECKAMEYER, 2010; SITARAMAN et al., 2008), a acetilcolina com a aprendizagem, memória e ciclo circadiano (GU e O'DOWD, 2006; HAMASAKA et al., 2007), a DA com atividade locomotora, excitação e ciclo circadiano (FOLTENYI et al., 2007; HIRSH et al., 2010) e assim por diante.

A DA além de ser um dos principais neurotransmissores presentes nas *D. melanogaster,* também é um bom exemplo de conservação genética entre as espécies. A DA encontrada em humanos é semelhante a DA presente nas *D. melanogaster* (YAMAMOTO e SETO, 2014). As *D. melanogaster* possuem genes que estão diretamente envolvidos na síntese, transporte, secreção, metabolismo e sinalização da DA (YAMAMOTO e SETO, 2014; YAMAMOTO e VERNIER, 2011).

Tanto os metabólitos de DA quanto a própria DA desempenham um papel muito importante na formação do exoesqueleto pigmentado das *D. melanogaster,* encontrando-se diretamente envolvida na sua regulação da ecdise larval, pupal e metamorfose das *D. melanogaster* (BAI et al., 2011; GANGER et al., 2000; MAYER et al., 2012; PARK et al., 2004; SRIVASTAVA, 2005; WRIGHT, 1987). Sua síntese ocorre a partir da conversão de tirosina em L-dopa através da enzima tirosina hidroxilase (TH), a qual é codificada pelo gene pálido presente nas *D. melanogaster* na presença do cofator tetrahidrobiopterina (JURGRNS et al.,1984). Após isto, ocorre a conversão de L-Dopa a DA, mediada pela enzima dopa descarboxilase (CLARCK et al., 1978). As duas enzimas responsáveis pela formação da DA são de extrema importância para o seu desenvolvimento normal (REGNA et al., 2016). Estudos relatam que mutações dessas enzimas pode levar a letalidade embionária das *D. melanogaster*, ou causar déficis comportamentais nas mesmas quando a depleção dessas enzimas ocorrer somente no SNC (BUDNIK e WHITE, 1987; RIEMENSPERGER et al., 2011). A TH é um marcador específico da síntese de DA, a redução dos seus níveis pode causar déficits cognitivos nos animais (SANTOS et al., 2013).

Após ser sintetizada no citoplasma, a DA é conduzida através da membrana pelos transportadores vesiculares de monoaminas (TVMA) (GREER et al., 2005). A reserpina é um exemplo de fármaco capaz de bloquear a ação desse transportador e é usualmente utilizada em estudos de doenças neurodegenerativas e no envolvimento do sistema dopaminérgico das *D. melanogaster* (BAINTON et al., 2000; PENDLETON et al., 2005; CANHG et al., 2006). Esse neurotransmissor é capaz de se ligar a quatro tipos de receptores acopladas a proteína G em *D. melanogaster*, sendo dois deles do tipo D1 (DopR e DopR2) atuando na ativação da 3´5´-adenosina-monofosfato-cíclico (AMPc), um receptor do tipo D2 (D2R) inibindo AMPc e um receptor não-canônico (DopEcR), o qual é similar aos receptore ß-adrenérgicos dos mamíferos, que ativam vias de AMPc e fosfatidilinositol-3-quinase (PI3K) (FENG et al., 1996; HAN et al., 1996; HEAR et al., 2002; SRIVASTAVA et al., 2005), conforme figura 4.

Figura 4- Diagrama esquemático da dinâmica e sinalização da DA em (A) cutícula e (B) cérebro de *D. melanogaster* e (C) cérebro de mamíferos
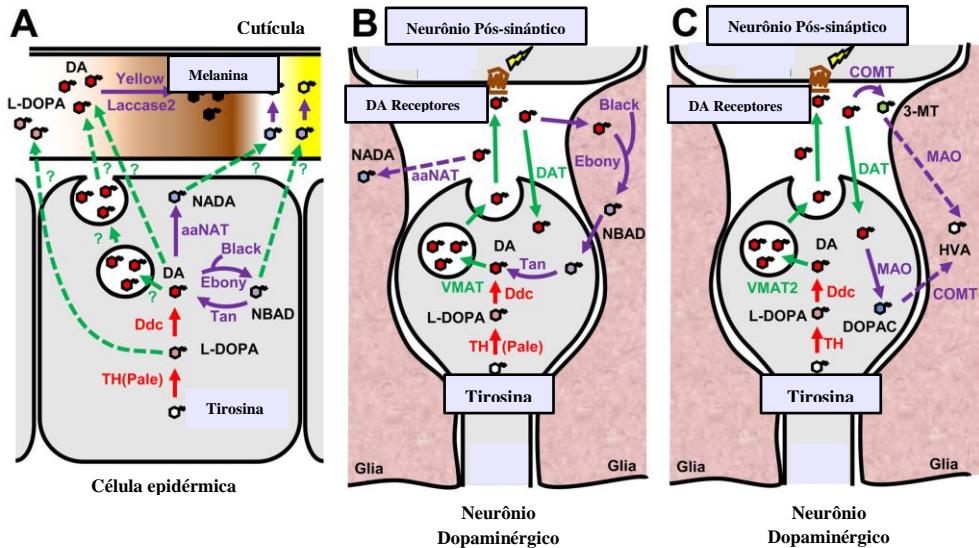
Fonte: (Adaptado de YAMAMOTO e SETO, 2014).

A ação de fármacos (agonistas ou antogosnistas de receptores de DA) ou disturbios fisiológicos que levem a níveis elevados ou diminuidos da DA são capazes de afetar a atividade basal e locomotora das *D. melanogaster* (YAMAMOTO e SETO, 2014; YELLMAN et al., 1997).

1.4 RESERPINA

A reserpina é um alcaloide isolado das raízes da planta <u>*Rauwolfia serpentina*</u> e utilizada na clínica como anti-hipertensivo, entretanto atualmente seu uso é obsoleto (AL-BLOUSHI et al., 2009). Em mamíferos, a reserpina possui como principal mecanismo de ação a inibição do TVMA, interferindo diretamente no estoque de aminas biogênicas das vesículas sinápticas (DOYLE et al., 1955; METZGER et al., 2002) assim como, na capacidade de armazenamento de monoaminas como a norepinefrina, serotonina e DA (GOODMAN e GILMAN, 2010). A reserpina também está diretamente relacionada com a diminuição da imunorreatividade da enzima TH e no aumento da proteína alfa-sinucleína (DE FREITAS et al., 2016; LEE et al., 2015; SANTOS et al., 2013). Devido a esses relatos, atualmente ela vem sendo utilizada como indutor para modular a doença de Parkinson experimentalmente (ABÍLIO et al., 2004; BILSKA e DUBIEL, 2007; NAIDU et al., 2004; RECKZIEGEL et al., 2016).

Quando administrada em roedores, alterações como movimentos de mascar no vazio (MMVs), protrusões de língua, tempo de tremor facial, catalepsia (ABÍLIO et al., 2004; BURGER et al., 2004; BUSANELLO et al., 2011; FARIA et al., 2005; NEISEWANDER; CASTAÑEDA; DAVIS, 1994; PEREIRA et al., 2011), hipolocomoção e rigidez muscular podem ser observadas (DOYLE et al., 1955; FERNANDES et al., 2012). Acredita-se que esses efeitos transcorrem através de interferências no metabolismo da DA e na acumulação de produtos neurotóxicos provenientes do seu metabolismo oxidativo em estruturas cerebrais que participam do controle dos movimentos, como o estriado (ALUF et al., 2011; FERNANDES et. al., 2012).

Em *D. melanogaster,* os sintomas causados pela reserpina são em partes semelhantes aos encontrados em roedores (LEE et al., 2015). A inibição de TVMA, diminuição dos níveis da enzima TH, aumento da proteína alfa-sinucleína e alterações na atividade locomotora, são alguns dos sintomas presentes em modelos animais de vertebrados e invertebrados (LEE et al., 2015).

Diante dos relatos citados acima, neste trabalho, foi de extrema importância realizar estudos preliminares sobre os efeitos do extrato bruto de *P. methysticum* utilizando como organismo modelo as *D. melonogaster*, através de análise comportamental e parâmetros bioquímicos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste trabalho consistiu em investigar os efeitos do extrato bruto do rizoma de *Piper methysticum* sobre alterações comportamentais e bioquímicas em *Drosophila melanogaster*.

2.2 OBJETIVOS ESPECÍFICOS

Em *D. melanogaster*:

- Mensurar os efeitos do extrato de *P. methysticum* sobre a sua sobrevivência.

- Avaliar o efeito do extrato de *P. methyticum* ou sua associação com reserpina sobre a atividade locomotora.

- Verificar possíveis alterações do extrato de *P. methysticum* sobre parâmetros de estresse oxidativo.

- Determinar possíveis alterações causadas pelo extrato de *P. methysticum* sobre a imunoreatividade da enzima TH.

3 RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito, o qual se encontra aqui organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio manuscrito. O **manuscrito** está disposto na forma em que será submetido para publicação na revista **Journal of Etnopharmacology.**

3.1 MANUSCRITO

Piper methysticum ALTERA A SOBREVIVÊNCIA E COMPORTAMENTO DE ESCALADA EM *Drosophila melanogaster:* ASSOCIAÇÃO COM ESTRESSE OXIDATIVO E TIROSINA HIDROXILASE

MANUSCRITO

Piper methysticum ALTERS SURVIVAL AND CLIMBING BEHAVIOR IN *Drosophila melanogaster*: ASSOCIATION WITH OXIDATIVE STRESS AND TIROSINE HIDROXILASE

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Piper methysticum ALTERS SURVIVAL AND CLIMBING BEHAVIOR IN *Drosophila melanogaster*: ASSOCIATION WITH OXIDATIVE STRESS AND TIROSINE HIDROXILASE

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ABSTRACT

Piper methysticum (*P. methysticum*) is a plant commonly used to treat anxiety with action in some neurotransmitters including dopaminergic system in mammals. The aim this study was to investigate the effects of the extract of *P. methysticum* on survival rate and locomotor activity an *Drosophila melanogaster* (*D. melanogaster*) and if it is related to changes in oxidative stress and/or tirosine hydroxilase immunorreactivity. We also investigated if reserpine, a monoamine depleting agent could alter the locomotor response promoted by *P. methysticum*.. In the survival curve there was an increase in mortality of the flies at the concentrations of 0.001, 1 and 10 mg/mL. According to this, all others tests were conducted in two times, 5 or 12-days of treatment. *P. methysticum* extract decreased the locomotion of the flies at 1 and 10 mg/mL concentration in 5-days and at 10 mg/mL concentration in 12-days of treatment. On oxidative stress parameters there was only an increase in DCFH-DA and proteic thiol levels at 10 mg/mL concentration in 12-days of treatment. In the immunoreactivity of the tyrosine hydroxylase (TH) there was an increase at 0.001 mg/mL concentration and a decreased at 10 mg/mL concentration after 5-days of treatment, while for 12-days of treatment there was a decrease at 1 and 10 mg/mL concentration. When the flies were treated with 0.1 mg/mL of the extract associated with 5 or 500 μM of reserpine, there was a synergic effect between the two compouds on locomotor activity of the flies. In conclusion, *P. methysticum* extract seems to be altering oxidative stress parameters the monoaminergic system which could be associated to locomotor and survival of the *D. melanogaster*. .

Key-words: Kava kava. Locomotor activity. Wester blot. Dopamine. Oxidative stress.

1 Introduction

Piper methysticum (*P.methysticum*) is a plant native from South Pacific Islands (Sarris et al., 2012). It is popularly known as Kava Kava due to its major constituents, the kavalactones, responsible for 95% of the total therapeutic activity of the plant (Gautz et al, 2006). The main kavalactones are kawain, dihydrokawain, yangonin, desmethoxyyagonin, methysticin, and dihydromethysticin (Jiang et al., 2007; Li et al., 2011).

Therapeutically, *P. methysticum* has been used primarily to treat anxiety disorders (Pittler and Ernst, 2000; Sarris et al., 2009) since its mechanisms were related to dopaminergic, gabaergic, serotonergic and glutamatergic neurotransmission (Malsch and Kieser, 2001; Singh and Singh, 2002). It was demonstrated that high doses of kavain and desmethoxyyangonin increased dopamine (DA) levels in the *nucleus accumbens* of rats, while low doses of kavain caused a decrease in DA levels (Baum et al., 1998). Reserpine is another example of compound which also acts in the monoaminergic system. It depletes monoamine levels including DA by blocking the vesicular monoamine transporter, changes the immunocontent of the enzyme tyrosine hydroxylase (TH) in mammals (De Freitas et al., 2016; Metzger et al. 2002). All these effects are associated to a loss in locomotor activity being reserpine used as a model of Parkinsonism (Busanello et al., 2011). The impairment in locomotor activity has been reported also in *D. melanogaster* (Lee et al., 2015) and *Caenorhabditis elegans* (Reckziegel et al. 2016; Saharia et al., 2012) including with changes in proteins as DA transporter in *Caenorhabditis elegans* (Reckziegel et al. 2016).

In order to test the mechanism of action of drugs and/or its toxicity, the use of alternative models have increased significantly. Thus, invertebrate animals like *Drosophila melanogaster* (*D. melanogaster*), also known as fruit fly, are often used due to its rapid generation time, genetic widely known and ease of handling (Nguyen et al., 2014; Nichols, 2006; Pandey and Nichols, 2011), for the screening of drugs with pharmacological potential (Girish and Muralidhara, 2012; Rao, 2016; Venkareddy and Muralidhara, 2015). The main models that have been used in flies are related to neurodegenerative and genetic disorders, which could be carried out due to its high genetic homology with mammals (Coulom and Birman, 2004; Inamdar et al., 2012), as some basic biological, physiological, and neurological properties (Barron et al., 2010; Liu et al., 2012). As in mammals, in flies the DA has an important role in the locomotor activity, stereotyped behaviors, sleep, arousal and oxidative stress. (Andretic et al., 2005; Krstić et al., 2009; Lebestky et al., 2009; Sammelhack and Wang, 2009; Hanna et al., 2015). The DA is self-oxidizing catecholamine and are directly

related with the oxidative stress because it known to produce more free radicals than other types of neurotransmitters (Ueda et al., 2000; Hald and Lotharius, 2005; Hanna et al., 2015). The TH found in flies is the enzyme responsible for converts tyrosine to DA during catecholamine synthesis (Neckameyer and White, 1993). For these characteristics the flies are currently used for predictive evaluation of the therapeutic potential/toxicity, behavioral and enzymatic activities of phytochemicals (Girish and Muralidhara, 2012).

Considering the data showing that *P. methysticum* alters the levels of some neurotransmitters which are important to survival and locomotion of *D. melanogaster* we tested if *P. methysticum* could alter these parameters in *D. melanogaster* and if it is related to changes in oxidative stress and/or tirosine hydroxilase immunorreactivity. We also investigated if reserpine, a monoamine depleting agent could alter the locomotor response promoted by *P. methysticum*.

2 Materials and methods

2.1 Plant material and Chemicals

The rhizome crude extract of *P.methysticum* was purchased from HUAKANG BIOTECHNOLOGY DEVELOPMENT (China). The extract containing 30% of kavalactones (according to the supplier's report). Reserpine was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and was dissolved in ethanol 1%. All other reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) or others with high quality and purity.

1.2 D. melanogaster culture

D. melanogaster wild-type (Harwich strain) used in this study were obtained from the National Species Stock Center (Bowling Green, OH, USA). The flies were maintained and reared in standard medium of corn meal containing 8.9% sugar v/w, 0.66% salt v/w, 8.9% wheat germ v/w, 1.33% milk powder v/w, 44.54% of coarse cornmeal v/w, 35.63% medium cornmeal v/w and 0.08% nipagin v/w with controlled temperature and humidity (23 ± 2 ° C, 60% relative humidity, respectively) and dark / light cycle of 12 hours, with the start of the light period 07:00.

2.3 Experimental protocol

To perform the treatment with *P. methysticum*, 50 flies with 1-5 days old were placed in each glass bottle (10cmx3cm) containing agar medium (1% agar v/w, 1% milk powder v/w, 2% sucrose v/w and 0.08% nipagin® v/w) with temperature and humidity controlled (23 ± 2 º C, 60% relative humidity, respectively) and dark / light cycle of 12 hours. The flies were separated in the following groups: (1) Control, (2) *P. methysticum* 0.001 mg/mL, (3) *P. methysticum* 0.01 mg/mL (4) *P. methysticum* 0.1 mg/mL (5) *P. methysticum* 1 mg/mL and (6) *P. methysticum* 10 mg/mL. *P. methysticum* extract was diluted in water and the control group contained only agar medium. The concentrations were chosen from a preliminary curve with differents concetrations of the extract of *P. methysticum.*

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2.4 *In vivo* assays

2.4.1 Lethality Response

In the survival curve, the death of the flies was quantified every day during five days. After this period the flies were counted every other day until the death of all of them. After the count of death , data were analyzed and plotted as percentage of alive flies during treatment. Two days were chosen to perform the behavioral and biochemical tests, according with figure 1A.

Time 1 – day 5 (last day before the start of death of the flies).

Time 2 – day 12 (beginning of significant deaths of the flies).

Survival rate was carried out in triplicate, with 50 flies in each treatment.

2.4.2 Negative geotaxis

Negative geotaxis was determined according to the method previously described by Pendleton et al. (2002) with some modifications. This test was performed to evaluate the locomotor activity in climbing of the flies. Flies were transferred to falcon tubes with diameters of 120 x 17 mm containing a horizontal line around these tubes with 6 cm tall from bottom of the container. After a period of 30 min of acclimation due to the brief anesthesia with ice, the falcon tube was gently beaten in order that the flies stayed on the bottom of the flask. The number of flies that have crossed the mark of 6 cm after 4 seconds was measured.

For each test, it was performed three to five independent experiments with 10 flies in each group. All experiments were performed and recorded in an environment with controlled temperature (23 ±2 ºC).

Firstly, the negative geotaxis test was performed with different concentrations *of P. methysticum* for 5 or 12 days of treatment based on the survival curve. After this experiment, we also investigate if the association of *P. methysticum* with reserpine, a drug well established for reducing monoamine levels and locomotor activity of the flies, could cause a synergistic effect with *P. methysticum* on locomotor activity. For this, it was chosen the concentration of 100 μg/mL of *P. methysticum*, which did not cause changes in the locomotor activity of the flies. This concentration was associated with 5 or 500 μM of reserpine (concentration without and with effect on the locomotion of the flies, respectively). Thus, the treatment was performed for 5 or 12 days with the extract of *P. methysticum* and, after the flies were treated for 2 days with reserpine, totalizing 7 or 14-days of treatment, as depicted in Figure 1B.The extract of *P. methysticum* or reserpine were diluted in water or ethanol 1%, respectively. The control group contained agar medium or agar medium with ethanol 1%, respectively. The concentrations of reserpine used were based on unpublished data of our group.

2.5 Ex vivo assays

2.5.1 Homogenate preparation

After the treatments, 50 flies from each concentration of *P. methysticum* were immobilized on ice and manually homogenized in 1 mL of Tris/HCl buffer (pH 7.4, 0.1 M) (flies/volume (mL)). The homogenates were centrifuged at 4000 rpm for 10 min, and the supernatant was used for biochemical assays. All tests were performed in duplicate.

2.5.2 Assessment of 2, 7-dichlorodihydrofluorescein diacetate oxidation (DCFH-DA)

DCFH-DA assay was performed to determine the level of reactive oxygen and nitrogen species (RONS) in samples (100 μg protein), according to the methodology described by Pérez-Severiano et al. (2004) with modifications. Supernatants were incubated with DCFH-DA and the fluorescence of the samples was determined at 488 nm for excitation and 520 nm for emission in spectrofluorimeter. The results were defined as the difference

between the fluorescence at 30 and 15 min of the reaction and expressed as DCFH-DA oxidation/mg of protein.

2.5.3 Thiol levels determination

Thiol levels were determined as previously described by Sudati et al. (2013) with minor modification. For the evaluation of total thiol levels one aliquot of supernatant containing 100 μg of protein was used. For the evaluation of non-protein-thiol levels the supernatant (100 μg of protein) was pretreated with 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 min. After addition of 5,5'- dithiobis (2-nitrobenzoic acid) (DTNB) in the samples, the chromogen formed was measured spectrophotometrically (λ = 412 nm). The results were expressed in μmol/mg of protein.

2.5.4 Catalase activity

Catalase activity was determined according to the method previously described by Aebi (1984). It was monitored the disappearance of hydrogen peroxide (H_2O_2) in the presence of the supernatant containing 100 μg protein with phosphate buffer pH 7.0 at 25 ° C. The reading was carried out in λ= 240 nm. The enzymatic activity was expressed in μmol H_2O_2/mg protein/min.

2.5.5 Western Blot

Flies were homogenized with lysis buffer (4 % sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, 0.5 mM Na_2VO_4, 2 μg/mL aprotinin, 0.1 mM benzamidine, 0.1 mM PMSF). Samples were boiled for 6 min and centrifuged at 8000 rpm at 4 °C for 10 min. The supernatant was used to determine protein concentration using the Lowry method. Then, the samples (90 μg of protein) were mixed with 10% glycerol and 8% 2-mercaptoethanol and resolved by 10% SDS-PAGE. The samples were transferred into nitrocellulose membrane (Millipore, USA). Proteins on the membrane were stained with a Ponceau solution (0.5% Ponceau plus 5% glacial acetic acid in water), as a loading control (Romero-Calvo et al. 2010). After staining, the membranes were dried and scanned for quantification. Right after, membranes were then processed, blocked with 1% bovine serum albumin, and incubated overnight with an anti-TH (1:7500; Millipore). After, the membranes were incubated with

alkaline phosphatase-coupled secondary antibody (1:10000; Millipore). The reaction was determined by a colorimetric assay using nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate (Trevisan et al. 2013). The membranes were dried, scanned, and quantified. Finally, all values were normalized using Ponceau quantification (Romero-Calvo et al. 2010). The results of tyrosine hydroxylase were expressed as relative optical density as described by De Freitas et al. (2015).

2.5.7 Protein determination

The amount of protein in the samples was determined by following the methodology previously described by Lowry et al. (1951) using bovine serum albumin as standard.

2.6 Statistical Analyses

Data are presented as mean ± SEM and were statistically analyzed by one-way or two-way analysis of variance (ANOVA), followed by Tukey's or Dunnett 's test. The lifespan of the flies was determined by comparing the survival curves with a log-rank (Mantel–Cox) test. The results were considered significant when $p < 0.05$.

3 Results

3.1. Effect of P. methysticum extract on survival rate of the flies

Flies were exposed to different concentrations of the extract *P. methysticum* (Fig. 2A) and the time until that all the flies were dead was observed. The data were analyzed and quantified using a chart area under the curve (Fig. 2B). The extract of *P. methysticum* decreased the survival of the flies at concentrations of 0.001 mg/mL, 1 and 10 mg/mL when compared with control group ($F_{(5,12)} = 48.98$, $p < 0.05$; Fig. 1B). It was observed a hormetic effect considering the tested concentrations with a concentration-dependent at highest concentrations.

3.2 Effects of P. methysticum extract on negative geotaxis behavior

There was a decrease in the climbing behavior of the flies at the concentrations of 1 and 10 mg/mL after 5-days of treatment (fig. 3A) (F (5,24) = 12.28, p<0.05) as well as at a concentration of 10 mg/mL after 12-days treatment (Fig. 3B) (F (5,18) = 11.52, p<0.05) with extract of *P. methysticum*, when compared with control group.

3.3 Effects of P. methysticum on oxidative stress parameters

There was no significant difference in the DCFH-DA levels among the flies treated during 5-days with different concentration of *P. methysticum* extract, while after 12-days of treatment there was a significant increase in DCFH-DA levels at the concentration of 10 mg/mL of *P. methysticum* when compared with control group (F (5,18) = 5.79, p<0.05; Table 1). The results were similar when we analyzed the protein thiol levels. Flies treated for 5-days did not present significant difference, while after 12-days of treatment where there was a significant increase in the protein thiol levels at the concentration of 10 mg/mL of *P. methysticum* when compared with control group (F (5,18) = 4.04, p<0.05; Table 1). The non-protein thiol levels and catalase activity were also analyzed for 5-days and 12-days of treatment. However, there was no significant difference (Table 1).

3.4 Effects of P. methysticum extract in TH immunoreactivity

We also investigated the effects of *P. methysticum* on TH (limiting enzyme of dopamine production) immunoreactivity. The treatment during 5-days with *P. methysticum* caused an increase in the expression of TH at concentration 0.001 mg/mL and a decrease at 10 mg/mL (F (5,12) = 10.93 p<0.05), when compared with control group (Fig. 4A). 12-days of treatment with *P. methysticum* caused a decrease at the concentration 1 and 10mg/mL, when compared with control group (F (5,12) = 8.21, p<0.05) (Fig. 4B).

3.2 Effects of P. methysticum extract and/or reserpine on negative geotaxis behavior

Post-hoc analysis demonstrated that the treatment with 0.1 mg/mL *P. methysicum* + 5 µM of reserpine, caused a decrease in the locomotor activity of the flies after 7-days of treatment (fig. 5A) (F (3,8) = 4.596, p<0.05) when compared with control group. However, the the extract of *P. methysticum* at a concentration of 0.1 mg/mL or 5 µM of reserpine alone did not change the climbing in flies. The same profile of response was observed when the flies were treated with *P. methysticum* extract during 12 days plus 2 days with 5 µM of reserpine (fig. 5B) (F (3,8) = 13.59, p<0,05).

Statistical analysis demonstrated that the treatment with 500 µM of reserpine or *P. methysticum* + 500 µM reserpine, caused a decrease in the locomotor activity of the flies (fig. 5C) (F (3,16) = 176.16 p<0.05) when compared with control and extract group. The extract of *P. methysticum* at a concentration of 0.1 mg/mL did not change the climbing behavior in flies. The same profile of response was observed when the flies were treated with *P. methysticum* extract during 12 days plus 2 days with 500 µM of reserpine (fig. 5d) (F (3,8) = 236.9, p<0.05;). Furthermore, there was a significant interaction between *P. methysticum* and reserpine treatment (F(1,8) = 12.186, p<0.05; fig. 1D) without effects of *P. methysticum* alone.

4 Discussion

The present study demonstrated for the first time the effect of the crude extract of *P. methysticum* in a *D. melanogaster* model. *P. methysticum* extract changed the lifespan, motor deficits and TH immunoreactivity, as well as increased some parameters of oxidative stress. Furthermore, there was a synergistic effect on climbing behavior of flies with the association between 0.1 mg/mL *P. methysticum* extract with 500 µM of reserpine.

The first aim of this study was to evaluate if the treatment with *P. methysticum* extract could alter the survival rate of flies. The period required to the occurrence of death of all flies that were put in analyze was of approximately 60 days. It was observed an increase in the mortality of flies at the lowest concentration and at the two highest concentrations of *P. methysticum* extract suggesting a hormetic response. According to studies reported in the literature, the death of the flies may be mainly to the lack or decrease of dopamine present them, as well as associated with other factors (De Lucca et al., 2003; Hanna et al., 2015).

Considering a previous study showing that *P. methysticum* and its components alters dopamine levels in a different manner when tested in low or high doses in rodents our data suggest that some modifications in monoaminergic system can be occurring (Baum et al., 1998). Furthermore, we observed that after 12 days of treatment with the extract the mortality was prominent mainly in the two highest concentrations tested. Thus, we choose a time of 5-days, where the number of flies dead was little, to compare with the time of 12-days, because it was observed the a significant mortality found in this period. Our purpose with both times was investigate the mechanisms of the extract comparing them.

Literature data have pointed alterations in the locomotor activity of the flies could be associated with dysfunctions on the monoaminergic system, where dopamine is the main neurotransmitter responsible for this function of the flies (Mustard et al., 2010; Nichols, 2006; Hanna et al., 2015). Thus, we evaluated the locomotor activity through of climbing behavior of the flies in the presence of different concentrations of *P. methysticum* extract in both times, 5- and 12-days of treatment. Negative geotaxis test showed a decrease in the climbing behavior of the flies in the two highest concentrations of the extract after 5-days of treatment while that after 12-days there was a decrease only at a highest concentration of *P. methysticum* extract. Adult flies have in each hemisphere of its brain six dopaminergic neuronal sets present in the central nervous system (PPM3, PPL2, PPL1, PAM, and PAL, PPM ½) (Coulom and Birman, 2004). They also have VMAT and dDAT (vesicular monoamine transporter and dopamine transporter, respectively), which are responsible for the maintenance of the homeostasis of the dopamine and therefore act on its locomotion and cognition (Giros et al., 1996). Accordingly, studies carried out by Hanna et al. (2015), showed that mutant flies with high levels of dopamine exhibited climbing ability better than wild-type controls, as well as flies with impaired dopamine synthesis had less climbing ability than control flies. Then, alterations in monoamine homeostasis could be involved in the responses found on negative geotaxis of flies under treatment with *P. methysticum.*

It is known that alterations in the metabolism of monoamines can lead to oxidative events with consequent production of reactive oxygen species (Ueda et al., 2000; Hanna et al., 2015). From this, we checked if oxidative stress parameters could be altered in the period where the death and locomotor alteration were occuring. Then, oxidative stress parameters were evaluated after 5-days or 12-days of treatment with *P. methysticum* extract, where there was an increase in DCFH-DA production and proteic thiol levels only at the highest concentration during 12-days of treatment with *P. methysticum* extract. These results suggest that the increase of DCFH-DA production could be contributing to deficit locomotor and

consequent death of the flies, while the increase in proteic thiol levels could be an attempt to compensate the oxidative changes caused by the extract, however it does not seem to be enough.

In order to investigate our hypothesis that *P. methysticum* extract could be altering monoaminergic system, we quantified TH enzyme since alterations in monoamines, particularly dopamine, have been associated with locomotor activity and death of the flies (Nichols, 2006; 2011; Hanna et al., 2015). For this, the immunoreactivity of TH enzyme, a limiting enzyme for the synthesis of dopamine in the flies, was performed. After 5-days of treatment with *P. methysticum* extract, there was an increase TH immunoreactivity at the concentration lowest and a decrease at the highest concentration. Moreover, after 12-days of treatment, there was a decrease at the two highest concentrations. We believe that the increase of TH immunoreactivity in the lowest concentration could be due to a possible compensatory mechanism of the flies treated with *P. methysticum* extract. Considering our results, *P. methysticum* could be influencing a reduction in TH immunoreactivity mainly at the highest concentration tested in both times of treatment evaluated suggesting that it is occuring neurotoxicity to dopaminergic neurons of the flies.

In order to corroborate with our hypothesis that *P. methysicum* extract is capable of causing alterations on monoaminergic system, we used a pharmacological agent, reserpine, to evaluate if *P. methysticum* extract could alter the effects of it. The reserpine is a well established drug to inhibiting the vesicular monoamine transporter interfering in the biogenic amines stock in the synaptic vesicles (Doyle et al., 1955; Metzger et al., 2002) and in the decrease of the immunoreactivity levels of the TH enzyme and locomotor activity of mice (De Freitas et al., 2016) and invertebrates as *D. melanogaster* (Lee et al., 2015). According with the results found, the association between *P. methytisticum* extract (0.1 mg/mL) and 5 μM of reserpine, caused a decrease on locomotor activity of the flies after 7 or 14-days treatment while the same concentration of the extract or reserpine alone did not promote any alteration in climbing behavior. Regarding to the association between *P. methytisticum* extract (0.1 mg/mL) and 500 μM of reserpine, there was also a decrease in locomotor activity after 7 or 14-days of treatment with a significant interaction after 14 days of treatment. These results suggest a summation of effects between both compounds, with a synergic effect after 12-days of treatment with *P. methytisticum* extract (0.1 mg/mL) plus 2-days of treatment with 500 μM reserpine. Therefore, our results indicate a possible action of *P. methysticum* extract on the monoaminergic system, since reserpine has direct action on this system (Doyle et al., 1955; Metzger et al., 2002; De Freitas et al., 2016).

In conclusion, *P. methysticum* extract seems to be acting on monoaminergic neurotransmission in *D. melanogaster*, since the the highest concentration of the extract used was able to cause alterations in lifespan, climbing behavior, ROS production, proteic thiol and TH immunoreactivity. Furthermore, *P. methysticum* extract showed a synergic effect with a pharmacological monoamine depletor suggesting its effects on monoaminergic agents. All these effects were influenced by time of treatment with *P. methysticum*. However, further studies are necessary to understand if there are other mechanisms associated with the effects of *P. methysticum* extract in *D. melanogaster* as well as their signaling.

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FIGURE CAPTIONS

Fig.1 Experimental design on Negative geotaxis. Different concentrations of *P. methysticum* treatment (A) and 0.1 mg/mL de *P. methysticum* associated with 5 or 500 µM of reserpine (B). Square represents the days where geotaxis negative assay was performed and circle represents the days where biochemical assays were carried out.

Fig. 2 Survival rate. Flies 1-5 days old were exposed the different concentrations of *P. methysticum* (0.001, 0.01, 0.1, 1, 10 mg/mL) and their lifespan were observed until the occurrence of death of all of them. Three independent experiments with 50 flies in each group was used for achievement this experiment. Survival rate (A) was determined by survival curves Mantel–Cox log-rank test and area under the curve (B) was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values are expressed as mean ± SEM of three independent experiments. *$p< 0.05$ when compared with control group, #$p< 0.05$ when compared with 1 mg/mL group and %$p< 0.05$ when compared with all the groups.

Fig. 3 Effect of *P. methysticum* (0.001, 0.01, 0.1, 1, 10 mg/mL) on negative geotaxis (climbing 6 cm in 4s) with flies after 5-days (A) and 12-days (B) of treatment. Values are expressed as mean ± SEM (n=10 flies for replicate, 4-5 replicates were used for 5 and 12-days treatment). Significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *$p < 0.05$, when compared with control group.

Table 1 Oxidative stress parameters in flies treated with *P. methysticum* (0.001, 0.01, 0.1, 1 and 10 mg/mL) during 5-days or 12-days . Values are expressed as mean ± SEM (n=50 flies for replicate, 3-4 replicates were used for 5-days and 12-days treatment). Significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *$p< 0.05$, when compared with control group.

Fig. 4 Effect of *P. methysticum* (0.001,0.01, 0.1, 1 and 10 mg/mL) on TH immunoreactivity after 5-days (A) or 12-days (B). Values are expressed as mean ± SEM (n=100 flies for replicate, 3 replicates were used for 5 and 12-days of treatment). Significance was determined

by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. *$p < 0.05$ compared with control group.

Fig. 5 Effect of 0.1 mg/mL of *P. methysticum* or its association with reserpine on negative geotaxis (climbing 6 cm in 4s). Flies after 7-days (A) or 14-days (B) of treatment with *P. methysticum* and/or 5µM de reserpine and 7-days (C) or 14-days (D) of treatment with *P. methysticum* and/or 500 µM de reserpine. Values are expressed as mean ± SEM (n=10 flies for replicate, 3-5 replicates were used for treatment). Significance was determined by two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *$p<0.05$, when compared with control group, [#]$p<0.05$, when compared with reserpine group, [%]$p<0.05$, when compared with *P. methysticum* group.

Fig. 1
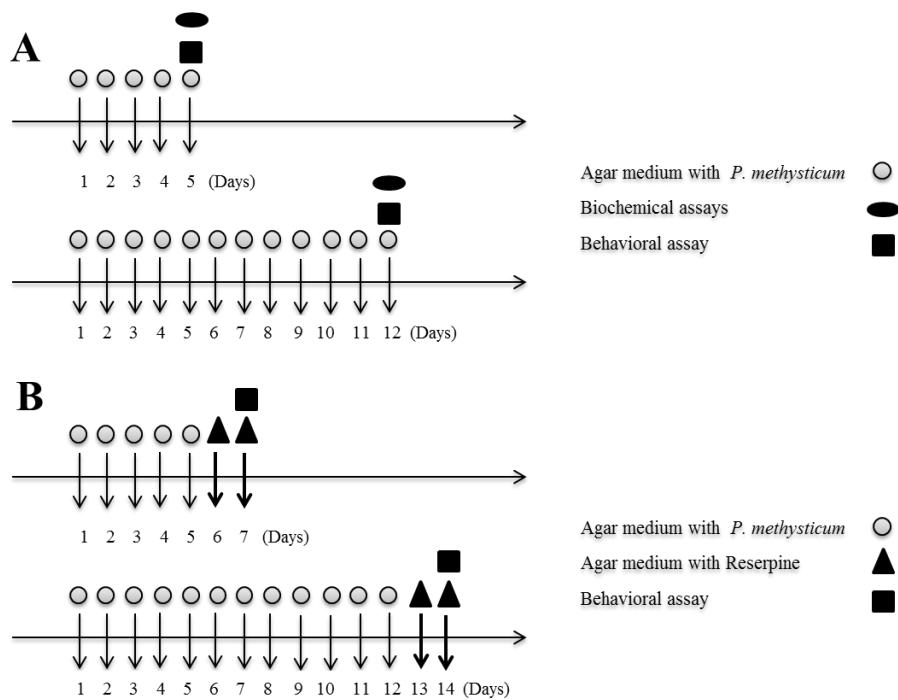
**Fig. 2**
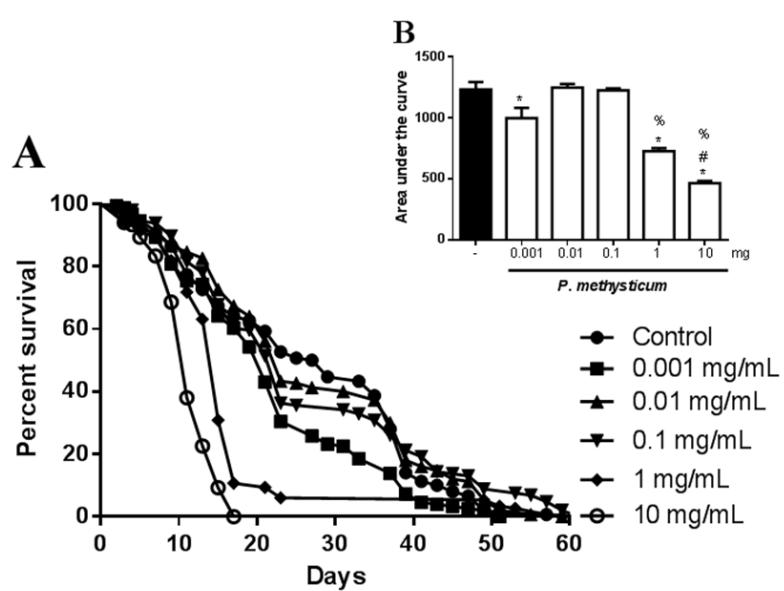
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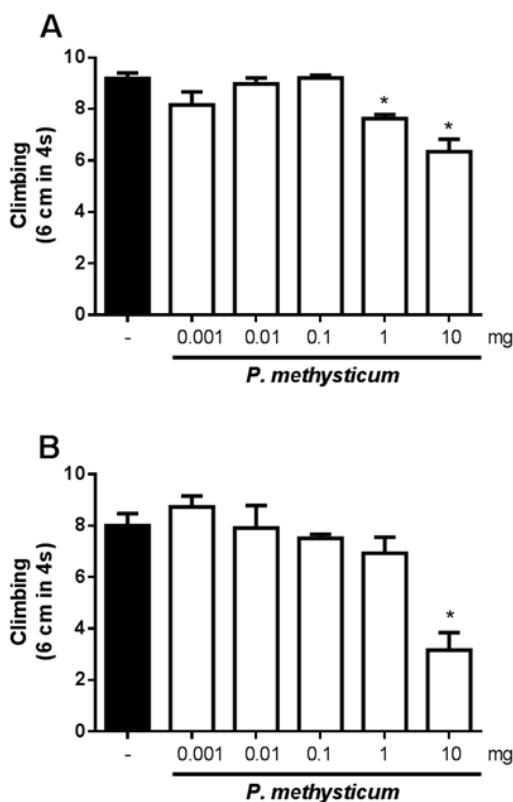
Table 1

		Control	**1μg/mL**	**10μg/mL**	**100μg/mL**	**1mg/mL**	**10mg/mL**
DCFH-DA	5 days	414.0 ± 61.28	559.7 ± 33.94	507.8 ± 59.17	423.1 ± 27.12	516.9 ± 39.21	543.4 ± 71.09
	12 days	146.1 ± 13.13	130.4 ± 13.95	141.8 ± 21.04	146.1 ± 17.25	171.8 ± 19.02	$239.7 \pm 13.98^*$
Proteic thiol	5 days	10.01 ± 0.35	10.60 ± 0.73	10.38 ± 0.24	10.44 ± 0.93	9.185 ± 0.12	9.86 ± 0.38
	12 days	23.00 ± 0.81	29.50 ± 0.97	28.28 ± 1.87	24.15 ± 2.56	25.31 ± 1.94	$32.04 \pm 1.55^*$
Non-proteic thiol	5 days	0.97 ± 0.09	0.92 ± 0.05	0.91 ± 0.04	1.11 ± 0.11	0.99 ± 0.05	0.97 ± 0.04
	12 days	1.51 ± 0.20	1.77 ± 0.25	1.41 ± 0.02	1.55 ± 0.05	1.52 ± 0.17	2.06 ± 0.41
Catalase	5 days	42.30 ± 5.01	48.00 ± 6.66	42.00 ± 1.19	50.91 ± 1.39	43.47 ± 2.41	50.49 ± 3.40
	12 days	46.41 ± 2.38	85.29 ± 8.25	62.71 ± 16.8	77.19 ± 4.21	67.54 ± 3.46	67.89 ± 5.04

Fig.4
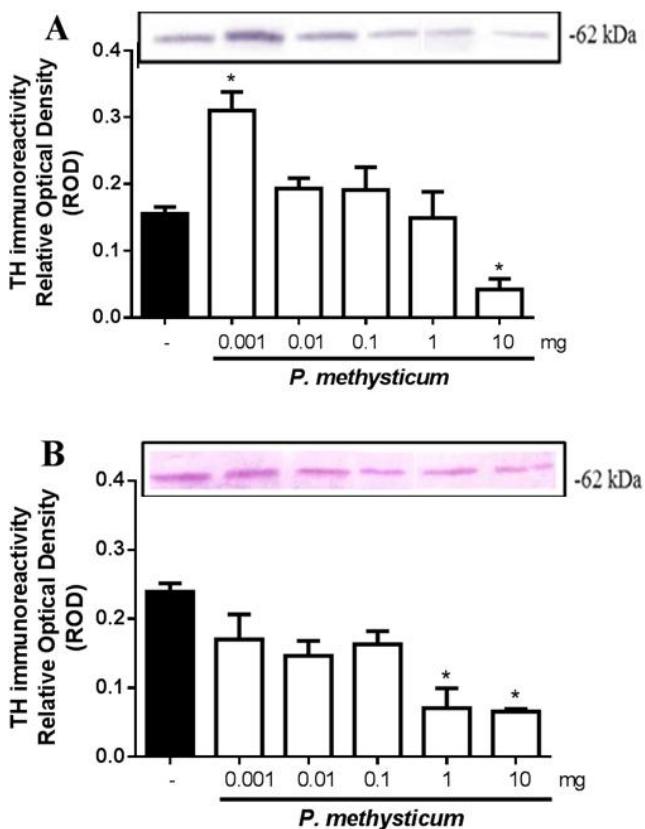
Fig. 5
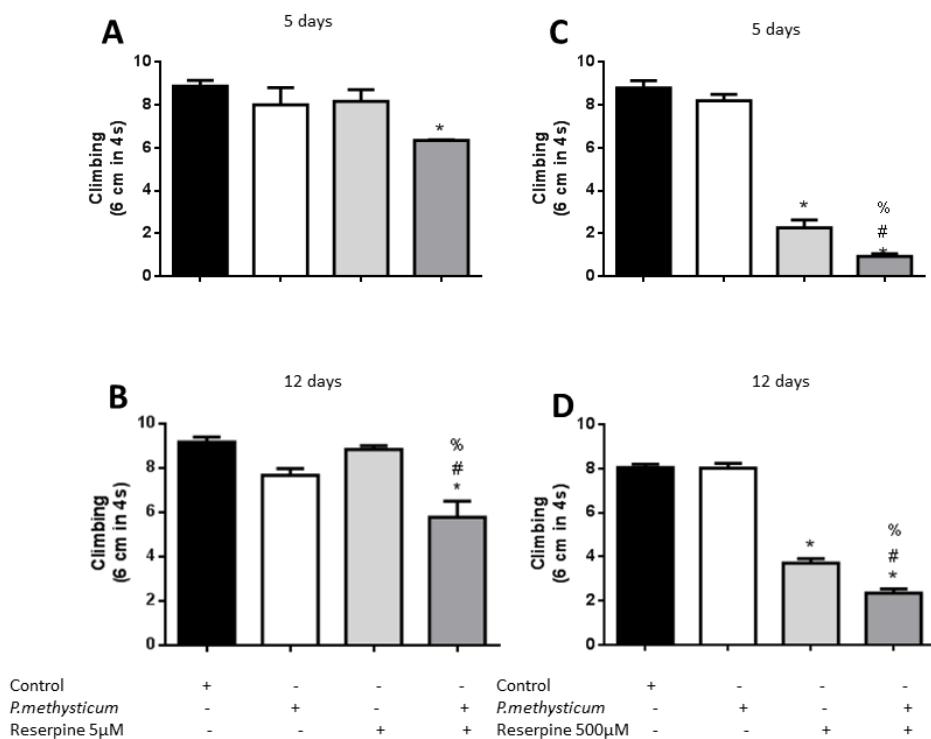
4 CONCLUSÕES ESPECÍFICAS

De acordo com os resultados apresentados nesta dissertação, podemos concluir que em *D. melanogaster:*

- O tratamento com *P. methysticum* nas concentrações de 1µg/mL e 1 e 10 mg/mL causou uma diminuição da sua sobrevivência;

- *P. methysticum* e em associação com reserpina diminuiu a atividade locomotora em ambos os tratamentos realizados. Aos efeitos encontrados, deve-se a uma possível disfunção referente ao seu sistema monoaminérgico;

- *P. methysticum* acarretou um aumento nos níveis de tiol proteico e de espécies reativas de oxigênio e nitrogênio somente na concentração de 10 mg/mL durante 12 dias de tratamento, demonstrando que as alterações motoras observadas não estão diretamente relacionadas às alterações oxidativas;

- Na presença de diferentes concentrações, *P. methysticum* não causou alterações na atividade da enzima acetilcolinesterase durante 5 e 12 dias de tratamento, indicando que a planta parece não estar alterando a sua metabolização de acetilcolina. Já para o ensaio de imunoreatividade da enzima TH*, P. methysticum* aumentou o imunoconteúdo desta na concentração de 1 µg/mL e diminuiu na concentração de 10mg/mL após 5 dias de tratamento. Já após 12 dias de tratamento houve uma diminuição no imunoconteúdo da TH na concentração de 1 e 10 mg/mL. Estes dados indicam que a alteração da atividade locomotora pode estar diretamente envolvida a alterações na TH.

5 CONCLUSÕES FINAIS

Os resultados encontrados neste estudo evidenciam o uso de *D. melanogaster* como um bom modelo para investigar mecanismos de ação e utilidades terapêuticas de diversos fitoterápicos como o *P. methysticum.* O extrato bruto desta planta*,* em determinadas concentrações causou alterações na sobrevivência, atividade locomotora, imunoreatividade da enzima TH e pequenas alterações de parâmetros oxidativos em *D. melanogaster*. Dessa forma, *P. methysticum* pode estar reduzindo a atividade dos neurônios dopaminérgicos conduzindo as alterações motoras encontradas. Todavia, mais estudos devem ser realizados para elucidar com maior clareza os mecanismos relacionados com as alterações encontradas neste trabalho.

6 PERSPECTIVAS

Efeitos de *P. methysticum* em um modelo animal de camundongos:

- Investigar sua ação em diferentes vias de sinalização, através de ensaios *in vitro,* como a atividade da enzima monoaminoxidase (MAO), binding ao receptor GABA, e expressão de proteínas como da enzima glutamato descarboxilase (GAD), Erk1/2, p38, Nrf-2 e Akt/GSK-3*;*

- Através de uma curva dose-resposta, estabelecer uma dose com efeito farmacológico com nenhum ou baixos efeitos tóxicos através de parâmetros comportamentais e bioquímicos;

- Avaliar o seu envolvimento em um modelo animal de parkinsonismo induzidos por reserpina;

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