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AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DA *Valeriana officinalis* E DO DISSELENETO DE DIFENILA FRENTE À TOXICIDADE INDUZIDA POR ROTENONA EM *Drosophila melanogaster*

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Jéssie Haigert Sudati

Santa Maria, RS, Brasil

2012

AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DA *Valeriana officinalis* E DO DISSELENETO DE DIFENILA FRENTE À TOXICIDADE INDUZIDA POR ROTENONA EM *Drosophila melanogaster*

Jéssie Haigert Sudati

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elaborada por
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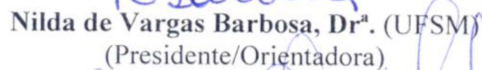

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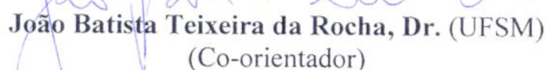

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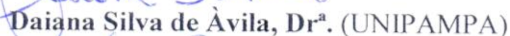

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"Eu não procuro saber as respostas,
procuro compreender as perguntas".

Confúcio

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DA *Valeriana officinalis* E DO DISSELENETO DE DIFENILA FRENTE À TOXICIDADE INDUZIDA POR ROTENONA EM *Drosophila melanogaster*

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Dentre as várias aplicações terapêuticas dos antioxidantes, destaca-se a ação neuroprotetora, uma vez que, o estresse oxidativo (EO) é reconhecido como um dos eventos envolvidos nos danos celulares que ocorrem na maioria das doenças neurológicas, incluindo a doença de Parkinson (DP). Consequentemente, a procura por antioxidantes naturais e/ou sintéticos que possam ser eficazes no tratamento de distúrbios neurológicos tem crescido muito ao longo dos últimos anos. Neste contexto, vários trabalhos têm evidenciado o potencial antioxidante do composto orgânico de selênio disseleneto de difenila (DPDS) *in vitro* e *in vivo*; mas, ainda, são escassos estudos acerca da atividade antioxidante da planta *Valeriana officinalis* (*V. officinalis)*. Da mesma forma, ainda não há pesquisas sobre os possíveis efeitos benéficos desses agentes em modelos de doenças neurológicas, como a DP, utilizando a *Drosophila melanogaster* (*D. melanogaster*), uma espécie que vem sendo usada com bastante confiabilidade na reprodução de modelos de disfunção dopaminérgica. Assim, o objetivo deste trabalho foi avaliar a atividade antioxidante *in vitro* da *V. officinalis*, bem como, os efeitos oriundos da suplementação com o extrato da raiz desta planta e com DPDS sobre alterações comportamentais e bioquímicas induzidas pela exposição ao pesticida rotenona em *D. melanogaster*. Como resultados, verificamos que o extrato etanólico de *V. officinalis* inibiu a geração de TBARS causada por diferentes agentes pró-oxidantes, em homogeneizado de tecido cerebral de rato *in vitro*; diminuiu a degradação da desoxirribose e a geração de espécies reativas de oxigênio (ERO), causada pelo ácido quinolínico (AQ); as moscas expostas à rotenona tiveram um desempenho significativamente inferior ao grupo controle nos testes comportamentais de escalada e campo-aberto (número de cruzamentos e tempo de imobilidade), bem como, uma maior incidência de mortalidade. O tratamento com *V. officinalis* foi eficaz em reduzir esses efeitos, exceto frente à diminuição do número de cruzamentos. A exposição à rotenona diminui a viabilidade celular e o conteúdo de tiol protéico das moscas, que foi normalizada aos níveis do controle pelo tratamento com *V. officinalis*. A rotenona aumentou a expressão de mRNA das enzimas Superóxido dismutase (SOD), Catalase (CAT) e Tirosina hidroxilase (TH) quando comparado ao grupo controle e a alteração observada na expressão da SOD e CAT foi restaurada pelo tratamento com *V. officinalis*; a suplementação com DPDS não foi eficaz em oferecer proteção contra as alterações locomotoras e bioquímicas induzidas por rotenona. Além disso, o DPDS induziu *per se* um aumento na produção de ERO e uma diminuição na taxa de sobrevivência das moscas. De forma geral, os dados obtidos mostram que a *V. officinalis* pode ser considerada um agente neuroprotetor promissor, uma vez que foi eficaz em reduzir os danos oxidativos causados por diferentes pró-oxidantes e os efeitos tóxicos causados pela exposição à rotenona. Assim, o uso do extrato desta planta pode ser benéfico na redução de complicações neurológicas associadas ao EO. Com relação ao uso do DPDS, mais estudos voltados para as concentrações utilizadas são necessários, tendo em vista que, na concentração testada neste modelo experimental, o mesmo não ofereceu proteção contra os efeitos danosos da rotenona, potencializou o efeito do pesticida sobre a taxa de mortalidade e exibiu efeitos tóxicos *per se.* De forma geral, esses resultados contribuem para o avanço das pesquisas voltadas para a toxicologia e farmacologia de produtos naturais e sintéticos e para a triagem de agentes que ofereçam neuroproteção e possam ser promissores para auxiliar na terapêutica de doenças neurodegenerativas, incluindo a DP.

Palavras-chave: Rotenona. *Valeriana officinalis*. Disseleneto de difenila. *Drosophila melanogaster.* Parkinson.

ABSTRACT

Thesis of Doctor's Degree
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

THERAPEUTIC POTENCIAL EVALUATION OF *Valeriana officinalis* AND DIPHENYL DISELENIDE ON ROTENONE INDUCED TOXICITY IN *Drosophila melanogaster*

AUTHOR: Jéssie Haigert Sudati
ADVISOR: Nilda de Vargas Barbosa
CO-ADVISOR: João Batista Teixeira da Rocha
DATE AND PLACE OF THE DEFENSE: Santa Maria, March, 2nd, 2012.

Among various antioxidant therapeutic applications, neuroprotective action is highlighted sinceoxidative stress is recognized as one of the events involved in cell damage which occur in most of neurological disorders, including Parkinson's disease (PD). Consequently, the search for natural and/or synthetic antioxidants which may be effective in the treatment of neurological disorders has grown over the past years. In this context, several studies have shown the antioxidant potential of selenium organic compound diphenyl diselenide (DPDS) *in vitro* and *in vivo*, but there are still few studies talking about antioxidant activity of *Valeriana officinalis* (*V. officinalis)*. Likewise, there is no research on the possible beneficial effects of these agents in models of neurological diseases such as PD using *Drosophila melanogaster* (*D. melanogaster*), an specie that has been used with great reliability in the reproduction of dopaminergic dysfunction models. Thus, the objective of this study was to evaluate the *in vitro* antioxidant activity of *V. officinalis*, as well as, the effect of supplementation of extract from roots of this plant and DPDS on behavioral and biochemical changes induced by pesticide rotenone exposure in *D. melanogaster.* As a result, we verify that: ethanolic extract from *V. officinalis* inhibited the generation of TBARS caused by various pro-oxidants agents in rat's brain homogenate *in vitro*, diminished deoxyribose degradation and generation of reactive oxygen species (ROS) caused by quinolinic acid (QA); flies exposed to rotenone were significantly lower than control group in behavioral tests of climbind and open-field (number of crossings and immobility time) and higher incidence of mortality. *V. officinalis* treatment was effective in reducing these effects, except against the decrease in number of crossings. Exposure to rotenone decreased in flies cell viability and non protein thiol content, but *V. officinalis* treatment normalized to the control levels. Rotenone increased mRNA expression on superoxide dismutase (SOD), catalase (CAT) and tyrosine hydroxilase (TH) enzymes, which were restored by treatment with *V. officinalis;* DPDS supplementation was not effective in offering protection against locomotor and biochemical alterations induced by rotenone. In addition, DPDS *per se* induced an increase in ROS production and decreased survival rate of flies. In general, data showed that *V. officinalis* may be a promising neuroprotective agent, since it was effective in reducing the oxidative damage caused by different neurotoxic agents and toxic effects caused by rotenone exposure. Thus, the use of this plant extract may be beneficial in reducing neurological disorders associated to the oxidative stress. In relation to the use of DPDS, further studies aimed at the concentrations used are necessary, given that the concentration tested in this work did not offer protection against rotenone damage effects, DPDS potentiated the effect of this pesticide on mortality and exhibited toxic effects *per se*. Overall, these results contribute to the advancement of research focused on the toxicology and pharmacology of natural and synthetic products and screening for agent that provide neuroprotection and may be promising to assist in the treatment of neurodegenerative diseases, including PD.

Keywords: Rotenone. *Valeriana officinalis*. Diphenyl diselenide. *Drosophila melanogaster.* Parkinson.

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LISTA DE ABREVIATURAS

3-ANP – Ácido 3 -Nitropropiônico
6-OHDA – 6-Hidroxidopamina
AQ – Ácido Quinolínico
ATP – Adenosina Trifosfato
BHT – Hidroxitolueno butilado
CAT – Catalase
CCD – Cromatografia em Camada Delgada
cDNA – DNA complementar
D. melanogaster – Drosophila melanogaster
DCFH –DA – Diacetato de Diclorofluoresceína
DP – Doença de Parkinson
DPDS – Disseleneto de difenila
DNA – Ácido Desoxiribonucléico
DNAse - Desoxiribonuclease
EDTA – Ácido Etilenodiaminotetracético
EO – Estresse Oxidativo
ERO – Espécies Reativas de Oxigênio
Fe^{2+} ou Fe (II) – Íon Ferroso
Fe^{3+} ou Fe (III) – Íon Férrico
GABA - Ácido γ-aminobutírico
GPx – Glutationa peroxidase
H_2O_2 – Peróxido de Hidrogênio
HPLC – High-Performance Liquide Chromatography
MDA – Malondialdeído
MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)
mRNA – Ácido Ribonucléico mensageiro
NADH – Nicotinamida Adenina Dinucleotídio
NMDA – N-Metil-D-Aspartato
NPS – Nitroprussiato de Sódio
$OH^{\cdot -}$ – Radical Hidroxila
PL – Peroxidação Lipídica
qPCR – Quantitative Polymerase chain reaction (reação de polimerização em cadeia)
RL – Radicais Livres
-SH – Grupo Tiol
SNC – Sistema Nervoso Central
SOD – Superóxido Dismutase
TBARS – Espécies Reativas ao Ácido Tiobarbitúrico
TH – Tirosina hidroxilase
V. officinalis – Valeriana officinalis

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APRESENTAÇÃO

No item **INTRODUÇÃO** consta uma revisão da literatura sobre os temas trabalhados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigo e manuscritos, os quais se encontram no item **ARTIGOS CIENTÍFICOS**. Neste constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens **DISCUSSÃO E CONCLUSÕES,** encontradas no final desta tese, apresentam descrições, interpretações e comentários gerais sobre os artigos científicos incluídos neste trabalho.

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

1 INTRODUÇÃO

A doença de Parkinson (DP) é uma desordem neurodegenerativa crônica e progressiva que afeta cerca de 2% da população mundial acima dos 65 anos (Lang and Lozano, 1998a, Hirth, 2010). Este distúrbio de elevada incidência é caracterizado por alterações motoras como tremor de repouso, rigidez muscular e bradicinesia, oriundos principalmente da perda seletiva e progressiva de neurônios dopaminérgicos, incluindo principalmente aqueles da região da parte compacta da substância negra (Greenamyre, 2001). Outra alteração celular característica na DP é a deposição de densos agregados protéicos denominados "corpos de *Lewy*", os quais são constituídos basicamente pelo acúmulo da proteína α-sinucleína (Spillantini et al., 1998). Estudos têm demonstrado que dentre as alterações bioquímicas envolvidas na neurodegeneração na DP, destacam-se a morte de neurônios dopaminérgicos, processo inflamatório, depleção de tióis, excitoxicidade glutamatérgica, disfunção mitocondrial e apoptose; e que tais fenômenos podem ser oriundos e/ou culminar com o estresse oxidativo (EO) (Siderowf and Stern, 2003). Embora os tratamentos atuais, os quais são baseados principalmente na administração de Levodopa e agonistas dopaminérgicos, melhore consideravelmente a qualidade de vida dos pacientes com DP, a etiologia e a cura desta doença continuam ainda desconhecidas. Sabe-se que, além da predisposição genética e fatores relacionados à idade, fatores ambientais também podem induzir o aparecimento e o desenvolvimento de sintomas de parkinsonismo. De fato, estudos epidemiológicos mostram que as intoxicações por neurotoxinas ambientais como o MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a rotenona e o paraquat, estão envolvidas com alguns casos de desenvolvimento da DP. Neste contexto, um modelo bastante utilizado e relativamente simples experimentalmente para a indução dos sintomas da DP é a intoxicação por rotenona (Cicchetti et al., 2009), um inibidor específico do complexo NADH desidrogenase mitocondrial (complexo I). Os distúrbios causados por este efeito inibitório em nível de metabolismo energético, função mitocondrial e danos oxidativos são eventos bem descritos nas alterações celulares observadas na patogênese da DP (Uversky, 2004).

Da mesma forma, a utilização da mosca da fruta *D. melanogaster* como modelo para estudo de mecanismos moleculares envolvidos em doenças neurológicas têm sido bem descrito na literatura (Bonini and Fortini, 2003, Nichols, 2006). Já foi demonstrado que a mosca possui

homologia entre cinco dos seis genes relacionados à DP em humanos (Whitworth et al., 2006). Apesar de a *D. melanogaster* apresentar diferença na anatomia do sistema nervoso central (SNC) e na distribuição de neurônios dopaminérgicos em relação aos vertebrados, muitas características em nível celular no desenvolvimento neuronal são conservadas entre vertebrados e invertebrados. Esta conservação faz com que a utilização de *D. melanogaster* como modelo experimental seja considerado um poderoso sistema *in vivo* para o estudo de disfunção neuronal. Especificamente com relação à DP, inúmeras pesquisas vêm sendo realizadas com *D. melanogaster* e exposição a pesticidas como a rotenona.

Nas últimas décadas, a procura por compostos naturais e/ou sintéticos com ação antioxidante tem aumentado notavelmente. Dentre as várias aplicações terapêuticas dos antioxidantes, ressalta-se a ação neuroprotetora, uma vez que o SNC exibe uma maior vulnerabilidade e susceptibilidade aos insultos oxidativos.

Com ênfase no uso de compostos naturais com ação neuroprotetora, sabe-se que os extratos da raiz da planta *Valeriana officinalis* (*V. officinalis),* nome vulgar Valeriana, são comumente usados na medicina popular para o tratamento da insônia, doença associada ao sono (Houghton, 1999, Cirelli, 2006). Estudos têm sugerido a participação de receptores gabaérgicos e purinérgicos para explicar a atividade farmacológica da *V. officinalis* (Muller et al., 2002, Schumacher et al., 2002, Ortiz et al., 2006). Contudo, os mecanismos moleculares envolvidos na ação da planta continuam sendo explorados. Neste sentido, já foi demonstrado que alguns componentes da *V. officinalis* exibem propriedades antioxidantes (Malva et al., 2004). No entanto, são escassos os trabalhos acerca dos mecanismos de ação, bem como dos efeitos da *V. officinalis* em modelos experimentais *in vivo.* Com relação à DP, dados da literatura relatam o efeito citoprotetor dessa planta em um modelo experimental de Parkinson *in vitro* induzido por rotenona (Oliveira DM et al., 2009). Entretanto, ainda não há trabalhos avaliando os efeitos da *V. officinalis* frente à DP em modelos *in vivo* e com *D. melanogaster*. Assim, a realização de pesquisas que investiguem se os possíveis efeitos antioxidantes de extratos da *V. officinalis* podem oferecer neuroproteção em modelos experimentais *in vivo* de desordens neurológicas, tal como a DP, podem contribuir efetivamente para a busca por tratamentos a base de terapias, as quais geralmente não induzem toxicidade.

Além dos antioxidantes naturais, compostos químicos de síntese relativamente simples com atividade antioxidante, também tem sido alvo de pesquisas associadas com a

procura de efetivos agentes terapêuticos. O DPDS é um composto orgânico de selênio (Se) que tem sido bastante investigado nas últimas décadas devido a sua propriedade antioxidante. Tal propriedade deve-se principalmente ao fato do mesmo mimetizar a ação de enzimas como as isoformas da glutationa peroxidase (GPx) (Alberto EE, 2009) e do sistema tiorredoxina redutase (TrxR) (Freitas AS, 2010), as quais têm papéis importantes na defesa celular contra danos oxidativos. Além da atividade antioxidante, propriedades anti-inflamatórias, anticarcinogênicas e antidiabetogênicas têm sido atribuídas a este composto em diferentes modelos experimentais de patologias humanas com roedores (Nogueira et al., 2004). No entanto, assim como outras formas de Se, o DPDS, quando em concentrações relativamente altas, inibe enzimas sulfidrílicas *in vivo* e *in vitro*; promove a depleção de grupos tióis e induz efeitos neurotóxicos, hepatotóxicos e genotóxicos (Barbosa et al., 2006, Barbosa et al., 2008, Rocha, 2010). Especificamente em modelos experimentais usando *D. melanogaster*, um recente estudo mostrou que o composto diminui a atividade e a transcrição gênica da enzima sulfidrílica δ-aminolevulinato desidratase em doses relativamente altas (Golombieski et al., 2008). No entanto, estudos vinculados aos possíveis efeitos farmacológicos do composto em modelos de patologias humanas usando *D. melanogaster* são raros. Da mesma forma, investigações do possível efeito neuroprotetor do composto frente a sintomas de parkinsonismo em modelos experimentais cruzando exposição à rotenona e *D. melanogaster* ainda não se encontram descritos na literatura.

1.1 Doenças neurodegenerativas

Embora o encéfalo humano compreenda somente cerca de 2% do peso corporal total, ele requer aproximadamente 20% do suprimento de oxigênio, tornando-se, assim, um órgão bastante susceptível aos danos oxidativos quando comparado aos demais (Halliwell, 1992). Além disso, apresenta um nível relativamente baixo de enzimas antioxidantes e é rico em ácidos graxos poliinsaturados e aminoácidos excitatórios, propiciando uma maior probabilidade para a ocorrência de PL (Reiter, 1995). Assim, muitas desordens neurodegenerativas, independente da região cerebral afetada, têm sua causa, desenvolvimento ou complicações associadas ao EO. Dentre estas, podemos citar as doenças de Alzheimer, Parkinson e Huntington (Simonian and

Coyle, 1996). A probabilidade de desenvolvimento de doenças neurodegenerativas, as quais ocorrem principalmente entre a 6ª e 8ª década de vida, aumentou dramaticamente em virtude do considerável aumento na expectativa de vida da população. Consequentemente, vários pesquisas estão focadas para a busca de compostos antioxidantes potenciais que sejam efetivos em reverter o EO e, assim, atuar na cura e/ou prevenção de uma série de doenças e suas complicações (Auluck and Bonini, 2002, Bastianetto et al., 2008). Dentre os compostos amplamente estudados atualmente, destacam-se os produtos naturais e os compostos químicos de síntese relativamente simples.

1.1.1 Doença de Parkinson

A DP é considerada a segunda desordem neurodegenerativa mais comum depois da doença de Alzheimer (Lang and Lozano, 1998b). Este distúrbio de elevada incidência que acomete cerca 1 a 2% da população acima dos 65 anos é caracterizado clinicamente por alterações motoras, tais como: tremor de repouso, instabilidade postural, bradicinesia e rigidez muscular (Przedborski, 2005). O tremor parkinsoniano é o sintoma inicial em cerca de 60-70% dos pacientes, sendo a característica mais evidente da doença, embora não seja necessariamente incapacitante (Poewe, 2006). Pacientes com DP também apresentam dificuldades na programação e execução de movimentos, e em estágio mais avançado exibem dificuldades em iniciar movimentos. Já, a rigidez muscular pode ser definida como um aumento da resistência da articulação durante um movimento passivo (Dauer and Przedborski, 2003). Além disso, sintomas como ansiedade, depressão, distúrbios do sono, fadiga, e declínio cognitivo podem ser apresentados por pessoas com DP (Dauer and Przedborski, 2003, Fahn et al., 2004). A prevalência de distúrbios do sono nos pacientes com DP é elevada e geralmente consiste em insônia noturna, fragmentação do sono e hipersonolência durante o dia, piorando a qualidade de vida do paciente (Korczyn, 2006).

Em termos de etiologia, a DP é caracterizada pela degeneração progressiva de neurônios dopaminérgicos que apresentam seus corpos celulares na parte compacta da substância negra (SNpc) e projetam seus axônios em direção ao estriado (Price et al., 1978) pela presença de

inclusões intracitoplasmáticas ricas em α-sinucleína, denominados corpos de *Lewy*, nos neurônios da substância negra e por alterações na enzima tirosina hidroxilase (TH) (Perez and Hastings, 2004). Dentre outros eventos moleculares envolvidos na degeneração neuronal e na patogênese da DP, destacam-se: processo inflamatório, depleção de tióis, excitoxicidade glutamatérgica, disfunção mitocondrial e apoptose; os quais são oriundos e/ou culminam com o EO.

De particular importância, o envolvimento da dopamina e das ERO são fatores centrais na morte de neurônios dopaminérgicos na DP, uma vez que a dopamina é uma molécula muito reativa e quando em níveis extracelulares elevados, pode se auto-oxidar e induzir a formação do ânion superóxido ($O_2 \cdot^-$), de H_2O_2 e de quinonas reativas (Lohr, 1991, Yu et al., 2005). Assim, uma das possíveis causas da neurodegeneração dopaminérgica pode ser proveniente desses insultos oxidativos. A perda destes neurônios, que normalmente são ricos em neuromelanina, resulta em despigmentação da substância negra (Dauer and Przedborski, 2003).

Microscopicamente, além da degeneração dopaminérgica e a consequente redução nos níveis de dopamina, pacientes com DP apresentam inclusões citoplasmática nos neurônios da substância negra denominadas de corpos de *Lewy*, em homenagem ao seu descobridor Fredrich Lewy. Os corpos de *Lewy* são estruturas esféricas organizadas em um núcleo denso de hialina e circundadas por um halo claro, medindo aproximadamente 15 µm de diâmetro, e compostas por numerosas proteínas, incluindo α-sinucleína, parkina, ubiquitina e neurofilamentos. Estas estruturas não são exclusivas da DP e podem ser encontradas em indivíduos com doença de Alzheimer, na demência com corpos de *Lewy* ou ainda como um achado acidental patológico em pessoas saudáveis de idade avançada (Schulz and Falkenburger, 2004). O papel dos corpos de *Lewy* na morte neuronal ainda é controverso, mas sabe-se que sua presença perturba o funcionamento normal dos neurônios, interrompendo a ação de importantes mensageiros químicos como, por exemplo, acetilcolina e dopamina (Perez and Hastings, 2004).

Embora a maioria dos casos de DP seja esporádica, a identificação de algumas formas de DP familiar, tanto autossômica dominante como recessiva despertou o interesse para o envolvimento genético na patogênese da DP (Whitworth et al., 2006). Há, pelo menos, seis genes descritos como causa de parkinsonismo familiar: *SNC/* α-sinucleína – PARK1, *UCH-L*1 – PARK5 e Dardarin/ *LRRK2– PARK8*, os quais apresentam padrão de herança autossômico dominante; e *parkin – PARK2*, *PINK1 – PARK6* e *DJ1-PARK7*, de herança autossômica recessiva (Gwinn-Hardy, 2002). Mutações no *lócus* PARK1 levam à identificação de agregados de α-

sinucleína na DP, enquanto mutações no *lócus* PARK2 causam deficiência na proteína parkina e degeneração nigroestriatal predominante sem corpos de *Lewy* (Polymeropoulos et al., 1997), mutações no lócus PARK7 são indicadores de alterações oxidativas (Meulener et al., 2005). Em geral, modelos animais *in vivo* e culturas celulares *in vitro* vinculam as mutações desses genes a prejuízos na estrutura e função mitocondrial e na resposta ao EO, reforçando o envolvimento desses dois fatores na patogênese da DP. Essas proteínas parecem interagir com proteínas mitocondriais ou, até mesmo, estarem presentes na mitocôndria, sugerindo uma ação direta das mesmas na função mitocondrial (Yang et al., 2006).

De acordo com alguns autores, fatores não-genéticos, como a exposição a toxinas ambientais, podem estar envolvidos com a gênese da DP e, até mesmo, anteceder a hereditariedade na etiologia DP (Collins and Neafsey, 2002). No entanto, apesar dos avanços da Medicina e da investigação científica nesta área, a etiologia da DP continua ainda por ser esclarecida.

1.1.1.1 Modelos experimentais no estudo da DP

Em termos de mecanismos, os modelos experimentais baseados na exposição à neurotoxinas ambientais têm encontrado importantes evidências que suportam o envolvimento da disfunção mitocondrial e da excitotoxicidade em doenças neurodegenerativas (Mattson and Magnus, 2006). Particularmente com relação à DP, a exposição acidental ao MPTP, um inibidor do complexo I da cadeia respiratória mitocondrial, constituiu uma das primeiras evidências entre disfunção mitocondrial e o desenvolvimento de sintomas de Parkinson esporádico (Beal, 2007). Desde então, o uso de inibidores do complexo I, os quais induzem características típicas da DP, vem sendo muito útil a nível experimental para a compreensão dos mecanismos de morte celular que ocorrem ao longo do desenvolvimento desta doença. A inibição do complexo I mitocondrial pode culminar com um aumento da produção de ERO, um decréscimo na produção de ATP, alterações na homeostase do cálcio, excitotoxicidade e abertura do poro de transição mitocondrial (Norenberg and Rao, 2007). Dentre as toxinas mais utilizadas experimentalmente, destacam-se, além do MPTP, a rotenona e o paraquat (Dauer and Przedborski, 2003). Dentre as aplicações

destas neurotoxinas em modelos animais, podemos destacar o uso da *D. melanogaster* como um modelo bastante confiável nas pesquisas que envolvem o estudo da DP (Whitworth et al., 2006, Jeibmann and Paulus, 2009).

1.1.1.2 *Drosophila melanogaster*

A utilização da mosca da fruta *D. melanogaster* no estudo de doenças neurodegenerativas humanas oferece várias vantagens, entre elas os inúmeros genes em comum com os humanos, conservando aspectos relacionados às vias metabólicas e sinalizadoras a nível celular, bem como certos aspectos comportamentais (ritmos circadianos, aprendizagem e memória) (Benton, 2008; Hirth, 2010). Nos últimos anos, tem sido um organismo modelo na área da genética para a descoberta de fármacos, pois as similaridades entre o mecanismo de ação de drogas, os parâmetros comportamentais e as respostas genéticas fazem da *D. melanogaster* um sistema atrativo para o estudo dos processos neurofarmacológicos relevantes para as doenças humanas (Nichols, 2006). Embora possua uma distribuição de neurônios dopaminérgicos no SNC de forma diferenciada a dos vertebrados, a *D. melanogaster* demonstra conservação de muitos processos celulares e moleculares de desenvolvimento e função neuronal. Como prova disso, o sequenciamento de seu genoma revelou a homologia entre cinco dos seis genes relacionados à DP com humanos (Whitworth et al., 2006). Assim, o sistema dopaminérgico presente na *D. melanogaster* (Figura 1) está associado ao controle locomotor como ocorre em humanos. Consequentemente, é aceitável concluir que danos nos neurônios dopaminérgicos de *D. melanogaster* levam a déficits locomotores comparáveis aos que ocorrem na DP (Lima and Miesenbock, 2005). Além disso, fármacos empregados no tratamento dos sintomas de DP em humanos também se mostram eficazes em modelos usando *D. melanogaster* (Pendleton et al., 2002).

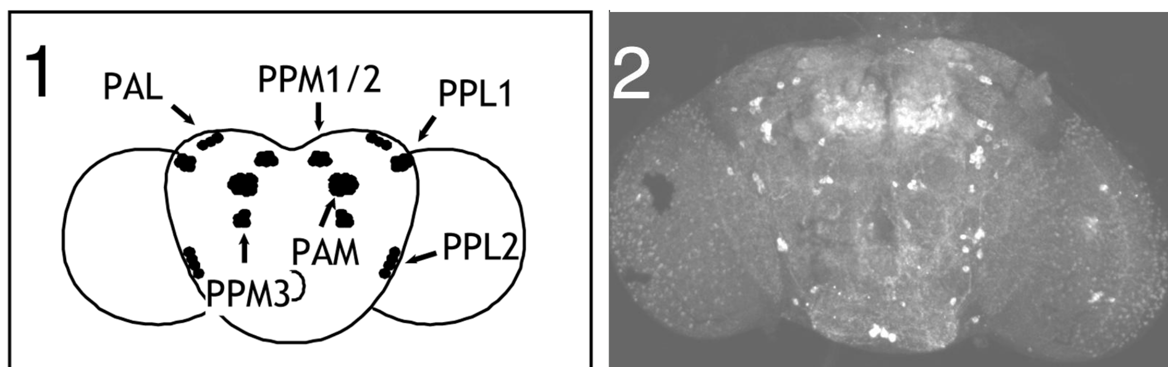

Figura 1 – Neurônios dopaminérgicos presentes no cérebro de *D. melanogaster* adulta. (1) Representação esquemática dos *clusters** dopaminérgicos. (2) Neurônios dopaminérgicos determinados através de marcador para TH em microscopia confocal. Fonte: Adaptação de parte do artigo publicado na revista "The Journal of Neuroscience (2004) 24(48):10993–10998 (Coulom and Birman, 2004)". **Abreviações: PPL1 e PPL2, junção posterior lateral 1 e 2; PPM1/2, junção posterior medial 1 e 2; PPM3, posterior medial 3; PAL, anterior lateral ; e PAM, anterior medial.*

1.1.1.3 Estresse oxidativo

As espécies reativas de oxigênio (ERO) são produzidas normalmente durante o metabolismo celular aeróbico. As ERO incluem o peróxido de hidrogênio (H_2O_2), bem como radicais livres, tais como ânion superóxido ($O_2^{\bullet -}$), radical hidroxila ($^{\bullet}OH$) entre outros. Estas moléculas são normalmente neutralizadas pelos sistemas antioxidantes presentes nos organismos. As defesas antioxidantes podem ser tanto enzimáticas (catalase, superóxido dismutase, GPX, etc) quanto não-enzimáticas (glutationa, tocoferóis (vitamina E), ácido ascórbico (vitamina C)). Assim, o EO pode resultar tanto de um aumento na produção de ERO quanto da redução da capacidade antioxidante celular total, ou seja, a ocorrência de um dano oxidativo depende de um desequilíbrio entre a produção de ERO e a atividade e os níveis de defesas antioxidantes (Finkel and Holbrook, 2000). De acordo com Halliwell e Gutteridge, o termo antioxidante significa: "substância que, quando presente em baixas concentrações comparado a um substrato oxidável, significativamente previne a oxidação deste substrato". Assim, o EO é uma condição celular ou fisiológica caracterizada por elevadas concentrações de ERO, as quais podem causar danos às estruturas moleculares de componentes celulares, tais como, lipídios, proteínas, carboidratos e

DNA com consequente alteração funcional e prejuízo das funções vitais em diversos tecidos ou órgãos (Halliwell, 2007).

Sabe-se que os pró-oxidantes, responsáveis pela indução de EO, auxiliam na investigação da atividade antioxidante de possíveis agentes farmacológicos que possam vir a atuar como terapêuticos no tratamento de doenças associadas ao EO. Um importante método para tal avaliação é a medida da peroxidação lipídica (PL), a qual consiste no ataque das ERO aos lipídios insaturados das membranas biológicas. Esta interação pode causar modificações estruturais nos lipídios de membrana, as quais alteram a permeabilidade e favorecem processos que culminam com a lise celular (Laguerre et al., 2007). Neste método as substâncias antioxidantes são testadas frente a diversos pró-oxidantes os quais induzem a PL através de diferentes mecanismos (Puntel et al., 2005, Wagner et al., 2006, Puntel et al., 2007, Pereira et al., 2009). Dentre os pró-oxidantes neurotóxicos, podemos destacar alguns, tais como: *(i)* Fe(II) (Laguerre et al., 2007), *(ii)* nitroprussiato de sódio (NPS) (Bates et al., 1991, Bolanos and Almeida, 1999); *(iii)* ácido quinolínico (AQ) (Foster et al., 1983), (*iv*) ácido 3-nitropropiônico (3-ANP) (Tunez et al., 2004). Esses agentes, conhecidos por estimularem a geração de RL, através de mecanismos diversos, tornam-se importantes para estudar de forma mais detalhada o mecanismo de ação de promissores compostos com ação antioxidante.

1.1.1.4 Rotenona

A Rotenona (Figura 2) é o agente mais tóxico da classe dos rotenóides (compostos citotóxicos naturais extraídos de plantas tropicais, tal como a *Derris elliptica*) e é amplamente utilizada como pesticida (Tanner et al., 2011). A exposição a esse rotenóide causa parkinsonismo em ratos e pode predispor humanos à DP (Cannon and Greenamyre, 2010). Por ser uma molécula altamente lipofílica, acessa rapidamente organelas celulares, como as mitocôndrias, onde atua como um inibidor específico, de alta afinidade, da NADH desidrogenase mitocondrial (complexo I) (Cicchetti et al., 2009). Consequentemente, a rotenona tem sido considerada em diversas pesquisas como um efetivo agente na reprodução dos sintomas e alterações celulares causadas pela DP em modelos experimentais *in vivo* e *in vitro* (Cannon and Greenamyre, 2010). Evidências *in vitro* mostram que a rotenona induz apoptose, acúmulo e agregação de α-sinucleína

e ubiquitina e também EO (Sherer et al., 2002). Especialmente em *D. melanogaster*, a exposição a doses subletais de rotenona causa perda seletiva de neurônios dopaminérgicos e disfunção locomotora dose-dependente (Coulom and Birman, 2004).

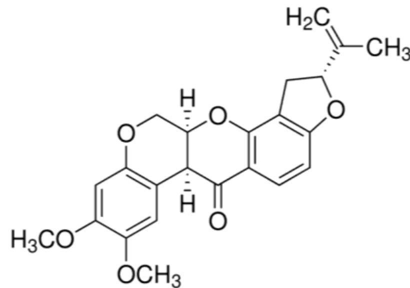

Figura 2 - Estrutura química da rotenona.

1.2 Terapias Antioxidantes Naturais e Sintéticas

Dentre as várias aplicações terapêuticas dos antioxidantes, ressalta-se a ação neuroprotetora, uma vez que, o SNC exibe uma maior susceptibilidade aos insultos oxidativos quando comparado aos demais órgãos e sistemas. Consequentemente, o uso e a procura por compostos naturais e/ou sintéticos, que possuam ação antioxidante e sejam promissores no tratamento contra as diversas doenças que acometem o SNC, vem crescendo continuamente nas últimas décadas.

1.2.1 *Valeriana officinalis*

V. officinalis (nome popular, Valeriana) é uma planta medicinal amplamente reconhecida devido às suas propriedades sedativas e calmantes (Houghton, 1999), mais popularmente nos Estados Unidos e na Europa. Os extratos da raiz da planta *V. officinalis* são comumente usados na medicina popular para o tratamento da insônia, doença associada ao sono, que pode culminar com o aparecimento de distúrbios centrais como ansiedade, variação de humor e disfunções

psiquiátricas e que está, pelo menos em parte, associada com uma diminuição das defesas antioxidantes e, consequentemente, com o desenvolvimento de EO em nível de SNC (D'Almeida et al., 1998, Cirelli, 2006). Estudos têm evidenciado que o tratamento crônico com *V. officinalis* produz efeitos suaves na indução do sono sem causar danos oxidativos ao SNC (Hallam et al., 2003, Fachinetto et al., 2007). Diferentes mecanismos têm sido propostos para explicar a atividade farmacológica da *V. officinalis;* entre eles incluem-se a interação com os receptores dos neurotransmissores ácido γ-aminobutírico (GABA), adenosina, entre outros (Mueller CE et al., 2002, Schumacher BSS et al., 2002, Ortiz et al., 2006). No entanto, o preciso mecanismo de ação envolvido no efeito neuroprotetor da planta ainda não se encontra completamente elucidado. Alguns componentes da *V. officinalis* demonstraram ser efetivos agentes contra a PL *in vitro* devido às suas propriedades antioxidantes (Malva et al., 2004). Porém, estas evidências da atividade antioxidante da planta foram obtidas somente contra a indução de PL provocada por Fe(II)/ascorbato. Assim, estudos adicionais acerca dos possíveis mecanismos envolvidos na neuroproteção oriunda do uso da planta, bem como, trabalhos que investiguem o seu potencial terapêutico em modelos experimentais *in vivo* de desordens neurológicas se tornam importantes.

Figura 3 – *Valeriana officinalis*.

1.2.2 Disseleneto de Difenila como um Antioxidante Sintético

O elemento Se foi descoberto em 1818 pelo químico sueco J. J. Berzelius, e encontra-se no grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}). Em 1842 ocorreu o primeiro relato de toxicidade do Se e em 1943 um relato sobre sua ação carcinogênica. Esse elemento foi considerado tóxico até Schwarz e Foltz (1957) identificar sua presença como um micronutriente em bactérias, mamíferos e pássaros. O papel bioquímico de Se ficou bem estabelecido após o descobrimento de que ele faz parte da enzima antioxidante GPx (Rotruck et al., 1973). Desde a descoberta das funções do Se para o sistema biológico enzimático, tornou-se necessário esclarecer a importância desse elemento para as selenoenzimas. Deste modo, muitos pesquisadores têm investigado uma gama de compostos orgânicos de Se que possuem ação antioxidante principalmente através do mecanismo mimético a certas enzimas, tais como as isoformas da GPx (Alberto EE, 2009) e do sistema TrxR (Freitas AS, 2010), as quais têm papéis importantes na defesa celular contra danos oxidativos.

A síntese de compostos químicos relativamente simples com atividade antioxidante, também tem sido alvo de pesquisas associadas com a procura de efetivos agentes terapêuticos. Entre eles, podemos citar o DPDS (Figura 3) que é um composto orgânico sintético de Se bastante investigado nas últimas décadas. Além da atividade antioxidante; propriedades anti-inflamatória, anticarcinogênica e antidiabetogênica têm sido referenciadas ao DPDS em diferentes modelos experimentais de patologias humanas com roedores (Nogueira et al., 2004). Neste contexto, cabe ressaltar o sucesso obtido com o Ebselen (1,2-fenil-1,2-benzisoselenazol-3(2H)-one), um composto orgânico de Se de baixa toxicidade e mimético à enzima GPx, na sua aplicação clínica no tratamento de isquemia cerebral (Yamaguchi et al., 1998, Ogawa et al., 1999) e patologias relacionadas (Saito et al., 1998), indicando a potente ação neuroprotetora desse composto. Dessa forma, os disselenetos são promissores antioxidantes, uma vez que possuem algumas características químicas e bioquímicas análogas ao Ebselen, entre elas a atividade mimética à GPx, reatividade com grupos tióis (-SH) e formação de selenosulfeto e selenol (-SeH), bem como dissulfetos (Barbosa et al., 1998, Brandao et al., 2006). Além disso, o DPDS possui atividade mimética à GPx cerca de duas vezes maior que o Ebselen (Spector et al.,

1989) e também apresenta menor toxicidade em roedores (Nogueira et al., 2004). Ainda, o DPDS possui atividade anti-inflamatória, anti-nociceptiva e neuroprotetora em modelos *in vitro* de isquemia e toxicidade induzida por glutamato (Nogueira et al., 2004). No entanto, assim como outras formas de Se, o DPDS quando em concentrações relativamente altas, inibe enzimas sulfidrílicas *in vivo e in vitro* e promove a depleção de grupos tióis (Barbosa et al., 2006). Especificamente em modelos de *D. melanogaster*, um recente estudo mostrou que o composto, em doses relativamente altas, diminui a atividade e a transcrição gênica da enzima sulfidrílica δ-aminolevulinato desidratase (Golombieski et al., 2008). No entanto, estudos vinculados aos possíveis efeitos farmacológicos do composto em modelos de patologias humanas usando *D. melanogaster* são raros. Da mesma forma, investigações sobre o possível efeito neuroprotetor do composto frente a sintomas de parkinsonismo em modelos experimentais cruzando exposição à rotenona e *D. melanogaster* ainda não se encontram descritos na literatura.

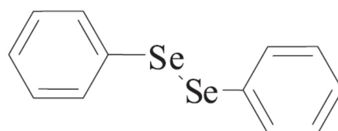

Figura 4 – Estrutura química do DPDS.

1.3 Justificativa

A grande maioria das doenças crônicas e degenerativas em humanos, incluindo a DP, tem durante sua origem e/ou progressão o envolvimento de eventos oxidativos, os quais são, em parte, responsáveis pelas principais injúrias moleculares que ocorrem a nível celular. Com ênfase em tais parâmetros; faz-se de vital importância a realização de estudos que investiguem a ação antioxidante de novos compostos naturais ou sintéticos que possam exibir propriedades neuroprotetoras em modelos *in vivo* de doenças neurodegenerativas. Da mesma forma, a procura e o uso de modelos experimentais relativamente simples, de baixo custo financeiro e de efetividade comprovada em termos de exposição e reprodutibilidade também são de similar

importância. Neste sentido, este trabalho visa buscar, através de terapias antioxidantes (naturais e sintéticas), formas de tratamentos que possam atenuar; previnir e/ou retardar os efeitos deletérios causados pela DP; através da investigação dos possíveis efeitos benéficos oriundos do uso de extratos da planta *V. officinalis* e do composto DPDS sobre alguns sintomas de parkinsonismo e alterações bioquímicas induzidas pela exposição à rotenona em *D. melonogaster*, uma espécie que vem sendo usada ultimamente com bastante confiabilidade na reprodução de modelos de disfunção neurológicas dopaminérgicas. Além disso, a execução deste estudo poderá contribuir de forma significativa na "triagem" de compostos (naturais e/ou sintéticos) que sejam de fácil obtenção, não causem toxicidade e que possam ser promissores em termos de farmacologia central.

1.4 Objetivos

1.4.1 Objetivos Gerais

- O objetivo geral deste estudo consiste em avaliar a atividade antioxidante *in vitro* da tintura de raiz *V. officinalis*, bem como avaliar os possíveis efeitos terapêuticos desta planta e do organocalcogênio sintético, DPDS, sobre alterações associadas à transcrição gênica, parâmetros bioquímicos e comportamentais em *D. melanogaster* expostas ao pesticida rotenona.

1.4.2 Objetivos Específicos

Análises *in vitro*

- Identificar os compostos químicos presentes na raiz da *V. officinalis*;

- Avaliar *in vitro* a atividade antioxidante da tintura de raiz da *V. officinalis* através de diferentes parâmetros de EO em tecido cerebral de ratos;

Análises *in vivo*

- Avaliar o efeito do tratamento com extrato de raiz da *V. officinalis* e DPDS sobre a taxa de sobrevivência de *D. melanogaster* expostas à rotenona;
- Através de diferentes testes comportamentais, investigar os possíveis efeitos neuroprotetores oriundos da ingestão de extrato aquoso de raiz de *V. officinalis* e do DPDS sobre alterações motoras em *D. melanogaster* expostas à rotenona;

Análises *ex vivo*

- Analisar o efeito do tratamento com extrato da raiz da *V. officinalis* e do DPDS sobre parâmetros bioquímicos relacionados ao EO em *D. melanogaster* expostas a rotenona;
- Avaliar a viabilidade celular em *D. melanogaster* expostas a rotenona e submetidas ao tratamento com *V. officinalis* e DPDS;
- Analisar o efeito dos tratamentos na expressão de alguns genes de interesse na DP presentes em *D. melanogaster.*

2 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico (1) e manuscritos (2). Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos próprios artigos e manuscritos. O **artigo** está disposto na forma que foi publicado na revista Neurochemical Research. Os **manuscritos** estão dispostos na forma em que normalmente se submete para publicação.

2.1 Artigo 1: "Atividade antioxidante *in vitro* da *Valeriana officinalis* frente à diferentes agentes neurotóxicos".

ARTIGO 1

Neurochemical Research (2009) 34:1372–1379
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In vitro Antioxidant Activity of *Valeriana officinalis* Against Different Neurotoxic Agents

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ORIGINAL PAPER

In vitro Antioxidant Activity of *Valeriana officinalis* Against Different Neurotoxic Agents

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Abstract *Valeriana officinalis* L. (Valerian) is widely used as a traditional medicine to improve the quality of sleep. Although *V. officinalis* have been well documented as promising pharmacological agent; the exact mechanisms by which this plant act is still unknown. Limited literature data have indicated that *V. officinalis* extracts can exhibit antioxidant properties against iron in hippocampal neurons in vitro. However, there is no data available about the possible antioxidant effect of *V. officinalis* against other pro-oxidants in brain. In the present study, the protective effect of *V. officinalis* on lipid peroxidation (LPO) induced by different pro-oxidant agents with neuropathological importance was examined. Ethanolic extract of valerian (0–60 μg/ml) was tested against quinolinic acid (QA); 3-nitropropionic acid; sodium nitroprusside; iron sulfate ($FeSO_4$) and Fe^{2+}/EDTA induced LPO in rat brain homogenates. The effect of *V. officinalis* in deoxyribose degradation and reactive oxygen species (ROS) production was also investigated. In brain homogenates, *V. officinalis* inhibited thiobarbituric acid reactive substances induced by all pro-oxidants tested in a concentration dependent manner. Similarly, *V. officinalis* caused a significant decrease on the LPO in cerebral cortex and in deoxyribose degradation. QA-induced ROS production in cortical slices was also significantly reduced by *V. officinalis*. Our results suggest that *V. officinalis* extract was effective in modulating LPO induced by different pro-oxidant agents. These data may imply that *V. officinalis* extract, functioning as antioxidant agent, can be beneficial for reducing insomnia complications linked to oxidative stress.

Keywords *Valeriana officinalis* · Ethanolic extract · Pro-oxidant agents · TBARS · Deoxyribose degradation · Oxidative stress

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Introduction

Insomnia is considered the most frequent sleep illness and affects nearly all populations throughout the world, particularly the elderly [1]. Numerous surveys conducted in countries around the world report that ~30–40% of adults have problems on initiating or maintaining sleep [2–4]. Insomnia symptoms are particularly relevant, given the prevalence of mood and anxiety disorders among individuals with chronic insomnia and the risk that insomnia poses for the subsequent development of psychiatric disorders [5, 6]. The precise cellular and molecular mechanism(s) which underlie(s) the etiology and progression of insomnia are still not fully understood. However, oxidative stress has been implicated as a major cause of cellular injures in a vast variety of clinical abnormalities particularly in the central nervous system [7–9]. In fact, it has been reported that sleep deprivation impairs antioxidant defense, leading to oxidative damage by causing imbalance between oxidants and antioxidant defenses [10, 11].

Extracts from the roots of *Valeriana officinalis* (*V. officinalis* L., Valerianaceae) have long been used in

alternative medicine for the treatment of insomnia and are the most well recognized herbal sedatives worldwide, mainly in United States and Europe [12, 13]. Most of the evidence from randomized clinical trials, with repeated administration, suggests that valerian extract produces mild sleep-inducing effects, without causing alteration of the sleep architecture or significant residual effects [14–16].

Many potential mechanisms for the pharmacological action of *V. officinalis* have been proposed based on their agonistic effects via GABA, adenosine, barbiturate and benzodiazepine receptors [17–19]. Indeed, the pharmacological properties of some components of *V. officinalis* are believed to be associated with their antioxidant activities. Recent literature data have supported a cytoprotective effect for *V. officinalis* extracts in a in vitro model of Parkinson's disease [20]. Furthermore, alcoholic extracts of valerian root have been considered promising pharmacological agent against lipid peroxidation (LPO), however this antioxidant activity was analyzed only against iron/ascorbate-induced LPO [21].

Thus, considering that insomnia can be associated with an increase in oxidative stress and that *V. officinalis* is widely used as sleep-promoting agent; the present study was planned to investigate the protective effects of ethanolic extract of *V. officinalis* (as well as its possible action mechanism) against LPO induced by different pro-oxidant agents. As the antioxidant activity of different class of compounds can vary greatly depending on the pro-oxidant used, we determined the effect of *V. officinalis* extract against neurotoxic agents such as quinolic acid and nitropropionic acid. Subsequently, it was analyzed whether the protective action of *V. officinalis* could involve Fenton reaction and/or reactive oxygen species production.

Experimental Procedure

Chemicals

Tris–HCl, QA, thiobarbituric acid, malonaldehyde bis-(dimethyl acetal; MDA) gallic acid and valeric acid (minimum 99%) were obtained from Sigma (St Louis, MO, USA). Sodium nitroprusside was obtained from Merck (Darmstadt, Germany). Ferrous sulphate, ethylenediamintetracetic (EDTA), chloridric acid and acetic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil).

Plant Material

A standard tincture of *V. officinalis* (10 g of valerian roots per 100 ml of ethanol) was obtained from Bio extracts (São Paulo, Brazil) and tested at concentrations of 0–60 μg/ml.

Pro-oxidant Agents

The 3-nitropropionic acid (3-NPA), quinolinic acid (QA), sodium nitroprusside (SNP), iron sulfate ($FeSO_4$), EDTA and H_2O_2 were used as pro-oxidant agents in in vitro assays.

Animals

Male wistar rats (±3 months old), weighing between 270 and 320 g, from our own breeding colony (Animal Householding, UFSM, Brazil) were kept in cages with free access to foods and water in a room with controlled temperature (22°C ± 3) and in 12-h light/dark cycle with lights on at 7:00 a.m. The animals were maintained and used in accordance to the guidelines of the Brazilian association for laboratory animal science (COBEA).

Preparation of Brain Homogenates

On the day of the experiments the rats were sacrificed by decapitation and the encephalic tissue (whole brain) was rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000*g* to yield a pellet that was discarded and a low-speed supernatant (S1) that was used in the experiments.

Preparation of Cortical Slices

Rats were decapitated and the left cerebral hemisphere was used for preparation of cortical slices. Cortexes were dissected and coronal slices (0.4 mm thickness) were obtained from the parietal area using a McIlwain tissue chopper.

Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) production was determined as described by Ohkawa et al. [22] and Puntel et al. [23]. Aliquots of the homogenate (200 μl) from tissues were incubated at 37°C in a water bath in the presence of different concentrations of ethanolic extract of *V. officinalis* (0–60 μg/ml) and with the respective pro-oxidant agents. Color reaction was developed by adding 200 μl 8.1% SDS (sodium dodecyl sulphate) to the reaction mixture containing S1 from rat brain. This was subsequently followed by the addition of 500 μl of acetic acid/HCl (pH 3.4) mixture and 500 μl 0.6% thiobarbituric acid (TBA). This mixture was incubated at 100°C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde (MDA).

Deoxyribose Degradation

Deoxyribose degradation was determined by method of Halliwell et al. [24]. Deoxyribose is degraded by hydroxyl radicals with the release of thiobarbituric acid (TBA) reactive substances. Deoxyribose (3 mM) was incubated at 37°C for 30 min with 50 mM potassium phosphate (pH 7.5) plus iron sulfate ($FeSo_4$) (0.1 mM) and/or H_2O_2 (1 mM) to induce deoxyribose degradation and *V. officinalis* at a concentration of 0–12 µg/ml. After incubation, 0.4 ml of TBA 0.8% and 0.8 ml of TCA 2.8% were added, and the tubes were heated for 20 min at 100°C and spectrophotometric measured at 532 nm.

FOX Assay

Lipid peroxidation (LPO) was determined in cortex of rat's brain according to Monserrat et al. [25]. The method is based on the oxidation of Fe^{2+} by lipid hydroperoxides (FOX reactive substances) at acid pH in the presence of the Fe^{3+} complexing dye, xylenol orange (Sigma). Samples were homogenized (1:20 w/v) in 100% cold (4°C) methanol. The homogenate was then centrifuged at 1,000*g*, for 10 min at 4°C. The supernatant was collected and used for LPO determination (580 nm). Cumene hydroperoxide (CHP; Sigma) was employed as standard.

ROS Measurement

Slices from cortex were incubated during 2 h at 37°C in a buffer containing artificial cerebrospinal fluid (ACSF; in mM: NaCl, 120; KCl, 0.5; $NaHCO_3$, 35; $CaCl_2$, 1.5; $MgCl_2$, 1.3; NaH_2PO_4, 1.25; glucose, 10) bubbled with 95% O_2 + 5% CO_2 and *V. officinalis* (10, 20, 40 µg/ml) on the presence or absence of QA (1 mM), in a final volume of 2 ml. At the end of incubation, slices were homogenized and an aliquot of 1 ml was collected in order to read the ROS production. About 10 µM of 2′,7′-dichlorofluorescein diacetate (DCHF-DA) was added to supernatants and samples were read after 1 h. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) as described by Pérez-Severiano et al. [26].

Phytochemical Analysis of *Valeriana officinalis* Extracts

Silica gel 60 F254-precoated TLC plates (Merck, Germany) were used. Dichloromethane (DCM) extracts were dissolved in methanol and 10–30 µl of this solution was used for chromatography. As a reference solution 10 mg of vanillin and 10 µl of anisaldehyde were dissolved in 10 ml of methanol. Alternatively, the reference solution was composed of reference valepotriates (1 mg/ml). The plate was developed twice over a 10 cm path using hexane-methyl ethyl ketone 8:2 as the mobile phase. The spots were visualized under UV light (254 nm) after spraying with a solution of 0.1% 2,4-dinitrophenylhydrazine (DNPH) in hydrochloric acid (25%)–glacial acetic acid 1:1, subsequently heated at 110°C for 10 min. It was confirmed the presence of valtrate (Rf 0.7), dihidrovaltrate (Rf 0.65) and acetoxivaltrate (Rf 0.55) in commercial extract used in this study (data not shown).

Quantification of Valeric Acid and Identification of Gallic Acid by HPLC Analysis

High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto-Sampler (SIL-20A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD SPD-M20A and Software LC solution 1.22 SP1. Reverse phase chromatographic analyzes were carried out in isocratic conditions using C-18 column (4.6 mm × 250 mm) packed with 5 µm diameter particles, the mobile phase was methanol: water (80:20 v/v), 0.5% H_3PO_4; pH 2. The mobile phase was filtered through a 0.45 µm membrane filter and then degassed by an ultrasonic bath prior to use. Stock solutions of valeric acid standard reference were prepared in the HPLC mobile phase at a concentration range of 3.12–50.0 mg/ml. All solutions and samples were first filtered through a 0.45 µm membrane filter (Millipore). Quantification was carried out by the integration of the peaks using external standard method at 220 nm. The flow rate was 1.5 ml/min and the injection volume was 10 µl. The chromatographic peaks were confirmed by comparing their retention time and DAD-UV spectra with those of the reference standards and by spiking the isolated compounds in the plant sample. The presence of gallic acid in the plant was confirmed by the HPLC (290 nm; injection volume = 5 µl; flow rate = 1 ml/min; column = C18; mobile fase = methanol; H_2O and 0.4% acetic acid) in comparison with a standard reference of gallic acid (data not shown). All chromatographic operations were carried out at ambient temperature and in triplicate.

Statistical Analysis

Values were expressed as mean ± SEM. Statistical analysis were performed by one-way ANOVA, followed by Duncan's multiple range tests when appropriated. When data did not present homogeneity of variance, they were log transformed. The results were considered statistically significant for $P < 0.05$.

Results

HPLC Analyzes

According to obtained cromatograms, it was found a peak with a retention time (r.t.) at 2.57 min corresponding to valeric acid ($y = 668.6x + 3{,}361$, $r = 0.9960$; Fig. 1a). Valeric acid concentration was 6.11 mg/ml in the analyzed sample (10 mg/ml). Additionally, a peak (r.t. = 2.74 min) can be attributed to the presence of gallic acid in the tincture of *V. officinalis* used in this work (data not shown).

TBARS Production

3-NPA × V. officinalis

3-NPA caused a significant increase on TBARS production in brain homogenates (S1) when compared to the control ($P < 0.05$) and its pro-oxidant effect was abolished by *V. officinalis* in a concentration dependent-manner (Fig. 2).

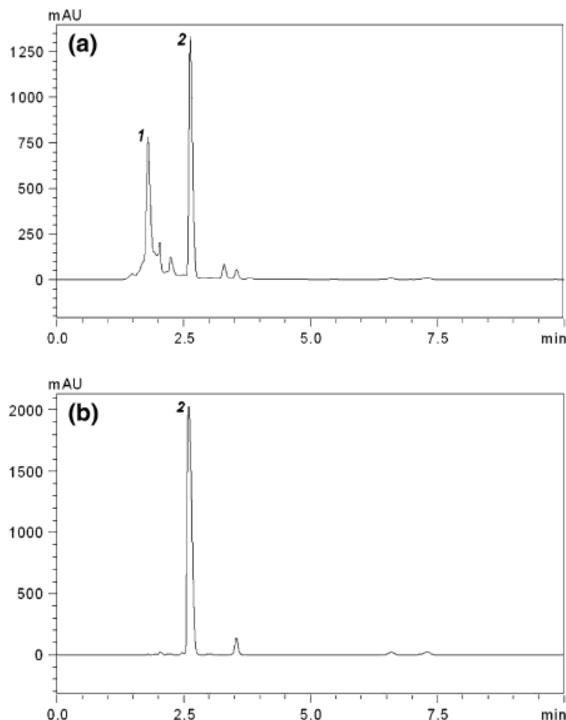


Fig. 1 **a** High performance liquid chromatography of *V. officinalis* tincture. *1* Represents an unknown peak; *2* corresponds to valeric acid peak. **b** Represents a high performance liquid chromatography of valeric acid (peak *2*) used as standard reference. Chromatographic conditions are described in the experimental section

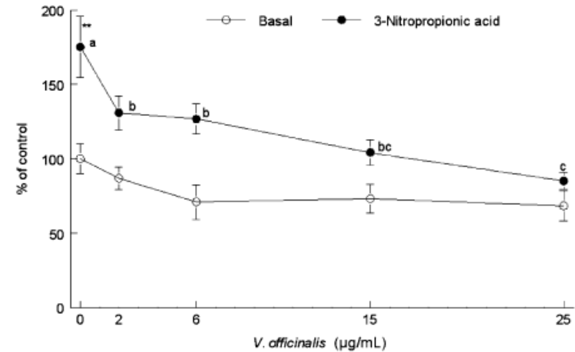


Fig. 2 Effects of *V. officinalis* on 3-NPA (2 mM)-induced TBARS production in brain homogenates. Values are expressed as mean ± SEM of 3–4 independent experiments performed in duplicate or triplicate. *a*, *b*, *c* Represent differences in relation to 3-NPA-induced TBARS production when compared to those induced by 3-NPA and treated with *V. officinalis*. **Significant difference between 3-NPA-induced TBARS and basal values

QA × V. officinalis

QA produced a significant increase on TBARS formation in brain homogenates that was blocked by *V. officinalis* ($P < 0.05$); however there was no significant differences among the extract concentrations (Fig. 3). Under basal conditions, *V. officinalis* also caused a significant reduction on TBARS production in relation to control ($P < 0.05$).

SNP × V. officinalis

SNP induced a significant increase on TBARS formation in brain homogenates ($P < 0.05$) that was reduced in a concentration dependent-manner to basal levels by *V. officinalis* extract ($P < 0.05$; Fig. 4).

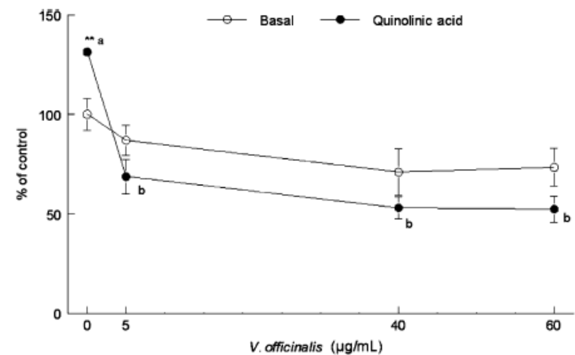


Fig. 3 Effects of *V. officinalis* on QA (2 mM)-induced TBARS production in brain homogenates. Values are expressed as mean ± SEM of 3–4 independent experiments performed in duplicate or triplicate. *a*, *b* Represent differences in relation to QA-induced TBARS levels when compared to those induced by QA and treated with *V. officinalis*. **Significant difference between QA-induced TBARS and basal values

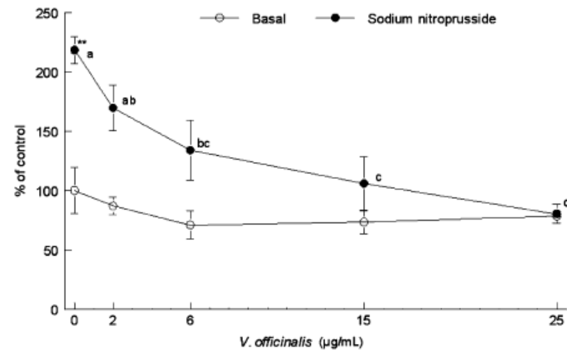


Fig. 4 Effects of *V. officinalis* on SNP (5 μM)-induced TBARS production in brain homogenates. Values are expressed as mean ± SEM from 3 to 4 independent experiments performed in duplicate or triplicate. *a*, *b*, *c* Represent differences in relation to SNP-induced TBARS values when compared to those induced by SNP and treated with *V. officinalis*. **Significant difference between SNP-induced TBARS and basal values

Fe^{2+} × *V. officinalis*

Statistical analyzes revealed that Fe^{2+} induced a significant stimulation in brain TBARS levels ($P < 0.05$), which were partially reduced by *V. officinalis* extract in a concentration-dependent manner ($P < 0.05$; Fig. 5).

Fe^{2+}/EDTA × *V. officinalis*

Fe^{2+}/EDTA caused a significant increase in TBARS levels ($P < 0.05$; Fig. 6) that were reduced by *V. officinalis* ($P < 0.05$). The presence of BHT did not modify TBARS production during color development (Fig. 6b) when compared to assay without BHT (Fig. 6a). The lack of effect of BHT can be attributed to the presence of SDS,

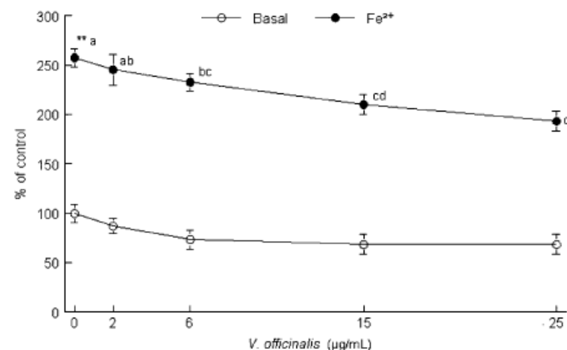
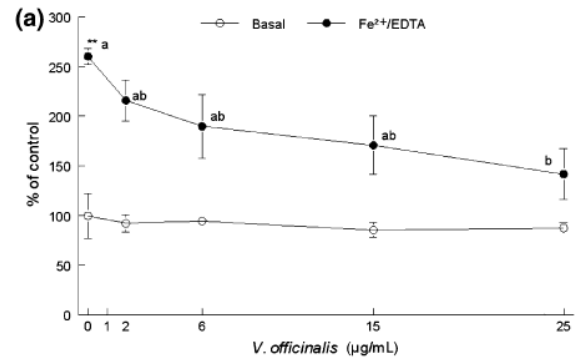


Fig. 5 Effects of *V. officinalis* on Fe^{2+} (10 μM)-induced TBARS production in brain homogenates. Values are expressed as mean ± SEM from 3 to 4 independent experiments performed in duplicate or triplicate. *a*, *b*, *c*, *d* Represent differences between Fe^{2+}-induced TBARS when compared to those induced by Fe^{2+} and treated with *V. officinalis*. **Significant difference between Fe^{2+}-induced TBARS and basal values



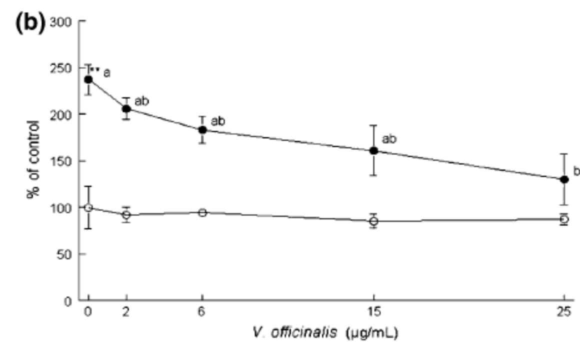


Fig. 6 Effects of *V. officinalis* on Fe^{2+}/EDTA (100 μM)-induced TBARS production without BHT (**a**) and with BHT (**b**) in brain homogenates. Values are expressed as mean ± SEM from 3 to 4 independent experiments performed in duplicate or triplicate. *a*, *b*, *c* Represent differences in relation Fe^{2+}/EDTA-induced TBARs when compared to those induced by Fe^{2+}/EDTA and treated with *V. officinalis*. **Significant difference between Fe^{2+}/EDTA-induced TBARS and basal values

which reduces considerably the production of TBARS during color development. In a previous study of our laboratory, TBARS production was about 25–35% higher in tubes that were boiled in the absence of SDS (SDS added after color development) [23].

Deoxyribose Degradation × *V. officinalis*

Deoxyribose degradation was stimulated 2.5 times by H_2O_2 and 3.5 times by Fe^{2+} plus H_2O_2. *V. officinalis* caused a significant decrease in deoxyribose degradation induced by H_2O_2 (about 20%, $P < 0.05$) and by Fe^{2+} plus H_2O_2 (about 35%, $P < 0.05$; Fig. 7).

Fox Assay

Fe^{2+}/EDTA produced an increase on cortical lipid oxidation when compared with basal condition ($P < 0.05$) and *V. officinalis* diminished the Fe^{2+}/EDTA pro-oxidant effect ($P < 0.05$; Fig. 8).

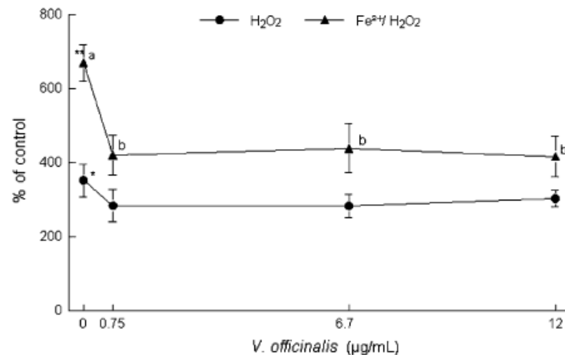


Fig. 7 Effects of *V. officinalis* on H_2O_2 (1 mM) and/or Fe^{2+}/H_2O_2-induced deoxyribose degradation. The deoxyribose was incubated for 20 min with H_2O_2 or Fe^{2+}/H_2O_2 in the presence or absence of *V. officinalis*. Values are expressed as mean ± SEM from 3 to 4 independent experiments performed in duplicate or triplicate. *, **Represent differences in relation Fe^{2+}plus H_2O_2, H_2O_2-induced deoxyribose degradation when compared to basal values. *a*, *b* Represent differences in relation Fe^{2+}/H_2O_2-induced deoxyribose degradation when compared to those induced by Fe^{2+}/H_2O_2 and treated with *V. officinalis*

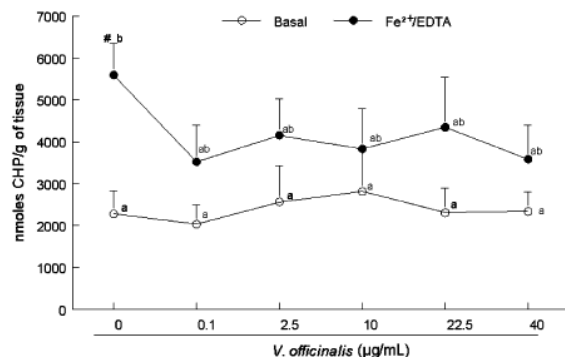


Fig. 8 Effect of *V. officinalis* on Fe^{2+}/EDTA (100 µM) induced LPO. Values are expressed as mean ± SEM of 3–4 independent experiments performed in quadruplicate. *a*, *b* Represent differences among all groups. #Significant difference in relation to Fe^{2+}/EDTA-induced LPO when compared to the basal values (without Fe^{2+}/EDTA and *V. officinalis*)

ROS Production

The incubation of brain cortical slices with QA (1 mM) caused an increase in ROS production when compared to basal conditions ($P < 0.05$) and *V. officinalis* blocked the pro-oxidant effect of QA ($P < 0.05$; Fig. 9).

Discussion

Insomnia is a condition that affects a large percentage of population around the world causing impairment in normal

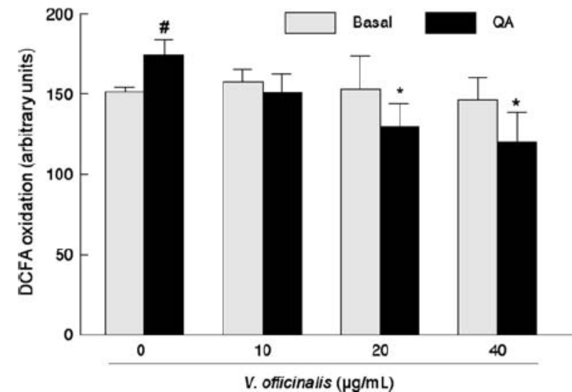


Fig. 9 Effect of QA (1 mM)-induced ROS production in rat brain cortical slices. Data of ROS levels are presented as fluorescence intensity emission. Data are expressed as mean ± SEM of three different experiments. #Significant differences between QA-induced ROS production and basal values. *Significant differences between QA-induced ROS values when compared to those induced by QA and treated with *V. officinalis*

life style [1]. The neuropathophysiologic basis of insomnia remains unclear, but several lines of evidence suggest that this disorder is associated with changes in the homeostasis of neurotransmitters and can be associated with an increase in oxidative stress [9–11]. In this way, an over production of reactive oxygen species can promotes a deregulation in physiological cellular performance and increase the susceptibility to disease.

In folkloric medicine the pharmacological agent *V. officinalis* is widely suggested as a possible alternative to benzodiazepine treatment against insomnia [27, 28]. Besides, a recent study from our laboratory demonstrated that *V. officinalis* seems not produce any oxidative damage to CNS after chronic in vivo administration [29]. Of potential pharmacological significance, the results presented here have indicated that tincture of *V. officinalis* prevents oxidative damage in brain preparations induced by different pro-oxidants QA, SNP, NPA, Fe(II) and Fe(II)/EDTA complex. The data obtained with iron are somewhat similar to that published recently by Malva et al. [21], where they have shown that *V. officinalis* extract protected cultured hippocampal neurons from iron/ascorbate-induced LPO and neurotoxicity in a model of Parkinson diseases [20]. Brain is extremely sensitive to iron overload and intracerebral injection of Fe(II) causes neurotoxic effects [30]. Here we have expanded the findings of Malva et al. [21] to other neurotoxic pro-oxidants. The efficacy and the antioxidant potency of some extracts varied depending on the pro-oxidant used [31–33], tincture of *V. officinalis* showed to be in accordance with it. In fact, we have observed that it was more effective against NPA, QA and SNP than Fe(II) or Fe(II)/EDTA complex, which may

indicate that *V. officinalis* extract can have a more wide-ranging antioxidant activity in the central nervous system. In line with this, *V. officinalis* also blocked the ROS production in brain cortical slices induced by QA.

Many studies have tested the effects of medicinal plants in in vivo models of different pathologies and preclinical tests frequently confirm the folk use of the plant extracts [34]. However, for the majority of plants, including *V. officinalis*, the exact mechanisms by which the extracts act are unknown. There are many studies showing that *V. officinalis* modulates anxiety and insomnia [35] possibly by interacting with different neurotransmitter systems [17–19, 35]. However, the occurrence of oxidative stress in insomnia and anxiety [1] can indicate that *V. officinalis* could exert its pharmacological effects, at least in part, via modulation of oxidative stress. In this vein, here we have demonstrated that *V. officinalis* is a potent antioxidant against neurotoxins that act via distinct mechanisms [30, 36–42]. We have also observed that *V. officinalis* modulates the pro-oxidant effects of QA in brain homogenates and in cortical slices where the cells are more preserved. In fact, literature has indicated that *V. officinalis* can inhibit MK-801 binding to brain membranes [19]; suggesting an interaction between *V. officinalis* and NMDA receptors [40, 43–46].

In summary, our findings suggest that *V. officinalis* extract was an effective modulator of LPO induced by different neurotoxic pro-oxidant agents. Indeed, these data may imply that *V. officinalis* extract, by functioning as antioxidant agent, may be beneficial for reducing insomnia complications linked to oxidative stress.

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References

1. World Health Organization (1992) The ICD-10 classification of mental and behavioral disorders. World Health Organization, Geneva
2. Ancoli-Israel S, Roth T (1999) Characteristics of insomnia in the United States: results of the 1991 National Sleep Foundation Survey. I Sleep 22:S347–S353
3. Sateia MJ, Doghramji K, Hauri PJ, Morin CM (2000) Evaluation of chronic insomnia. An American academy of sleep medicine review. Sleep 23:243–308
4. Ohayon M (2002) Epidemiology of insomnia: what we know and what we still need to learn. Sleep Med Rev 6:97–111. doi:10.1053/smrv.2002.0186
5. Markesbery WR (1997) Oxidative stress hypothesis in Alzheimer’s disease. Free Radic Biol Med 23:134–147. doi:10.1016/S0891-5849(96)00629-6
6. Riemann D, Voderholzer U (2003) Primary insomnia: a risk factor to develop depression? J Affect Disord 76:255–259. doi:10.1016/S0165-0327(02)00072-1
7. Halliwell B, Gutteridge JHC, Cross CE (1992) Free radicals, antioxidants and human disease: where we are now? J Lab Clin Med 119:598–620
8. Halliwell B, Gutteridge JMC (eds) (1999) Free radicals in biology and medicine, 3rd edn. Oxford University Press, Oxford, p 936
9. Olanow CW (1993) A radical hypothesis for neurodegeneration. Trends Neurosci 16:439–444. doi:10.1016/0166-2236(93)90070-3
10. Cirelli C (2006) Cellular consequences of sleep deprivation in the brain. Sleep Med Rev 10:307–321. doi:10.1016/j.smrv.2006.04.001
11. D’Almeida V, Lobo LL, Hipolide DC, De Oliveira AC, Nobrega JN, Tufik S (1998) Sleep deprivation induces brain region-specific decreases in glutathione levels. NeuroReport 9(12):2853–2856
12. Houghton PJ (1999) The scientific basis for the reputed activity of valerian. J Pharmacol 51:505–512. doi:10.1211/0022357991772772
13. Richman A, Witkowski JP (1998) 4th Annual herbal products sales survey. Whole Foods 21:19–26
14. Gyllenhaal C, Merritt SL, Peterson SD, Block KI, Gochenour T (2000) Efficacy and safety of herbal stimulants and sedatives in sleep disorders. Sleep Med Rev 4:229–251. doi:10.1053/smrv.1999.0093
15. Meoli AL, Rosen C, Kristo D, Kohrman M, Gooneratne N, Aguillard RN, Fayle R, Troell R, Touwnsend D, Claman D, Hoban T, Mahowald M (2005) Clinical practice review committee of the American academy of sleep medicine. Oral nonprescription treatment for insomnia: an evaluation of products with limited evidence. J Clin Sleep Med 2:173–187
16. Hallam KT, Olver JS, McGrath C, Norman TR (2003) Comparative cognitive and psychomotor effects of single doses of *Valeriana officinalis* and triazolam in healthy volunteers. Hum Psychopharmacol 18:619–625. doi:10.1002/hup.542
17. Mueller CE, Brattstrom A, Abourashed EA, Koetter U (2002) Interactions of valerian extracts and a fixed valerian-hop extract combination with adenosine receptors. Life Sci 71:1939–1949. doi:10.1016/S0024-3205(02)01964-1
18. Schumacher BSS, Hoelzl J, Khudeir N, Hess S, Mueller CE (2002) Lignans isolated from valerian: identification and characterization of a new olive oil derivative with partial agonistic activity at A1 adenosine receptors. J Nat Prod 65:1479–1485. doi:10.1021/np010464q
19. Ortiz JG, Rassi N, Maldonado PM, González-Cabrera S, Ramos I (2006) Commercial valerian interactions with [^{3}H]-Flunitrazepam and [^{3}H]MK-801 binding to rat synaptic membranes. Phytother Res 20(9):794–798. doi:10.1002/ptr.1960
20. Oliveira DM, Barreto G, De Andrade DEV, Saraceno E, Aon-Bertolino L, Capani F, El Bacha RS, Giraldez LD (2009) Cytoprotective effect of *Valeriana officinalis* extract on an in vitro experimental model of Parkinson disease. Neurochem Res 34:215–220. doi:10.1007/s11064-008-9749-y
21. Malva JO, Santos S, Macedo T (2004) Neuroprotective properties of *Valeriana officinalis* extracts. Neurotox Res 6(2):131–140
22. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. doi:10.1016/0003-2697(79)90738-3
23. Puntel RL, Nogueira CW, Rocha JBT (2005) *N*-Methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity in vitro. Neurochem Res 30:417–424. doi:10.1007/s11064-005-2617-0
24. Halliwell B, Gutteridge JMC, Aruoma OI (1987) The deoxyribose method: a simple “Test-tube” assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 165:215–219. doi:10.1016/0003-2697(87)90222-3
25. Monserrat JM, Geracitano LA, Pinho GLL, Vinagre TM, Faleiros M, Alciati JC, Bianchini A (2003) Determination of lipid

peroxides in invertebrates tissues using the Fe(III). Xylenol orange complex formation. Arch Environ Contam Toxicol 45:177–183. doi:10.1007/s00244-003-0073-x
26. Pérez-Severiano P, Rodríguez-Pérez M, Pedraza-Chaverrí J, Maldonado PD, Medina Campos ON, Ortíz-Plata A, Sánchez-García A, Villeda-Hernández J, Galván-Arzate S, Aguilera P, Santamaría A (2004) S-allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats. Neurochem Int 45:1175–1183. doi:10.1016/j.neuint.2004.06.008
27. Kupfer DJ, Reynolds CFIII (1997) Current concepts: management of insomnia. N Engl J Med 336:341–346. doi:10.1056/NEJM199701303360506
28. Holz J, Godau P (1989) Receptor binding studies with *Valeriana officinalis* on the benzodiazepine receptor. Planta Med 55:642. doi:10.1055/s-2006-96221
29. Fachinetto R, Villarinho JG, Wagner C, Pereira RP, Ávila DS, Burger ME, Calixto JB, Rocha JBT, Ferreira J (2007) *Valeriana officinalis* does not alter the orofacial dyskinesia induced by haloperidol in rats: role of dopamine transporter. Prog Neuro-psychopharmacol Biol Psychiatry 31:1478–1486. doi:10.1016/j.pnpbp.2007.06.028
30. Bostanci MO, Bağirici F (2008) Neuroprotective effect of aminoguanidine on iron-induced neurotoxicity. Brain Res Bull 76:57–62. doi:10.1016/j.brainresbull.2007.11.011
31. Oboh G, Puntel RL, Rocha JBT (2007) Hot pepper (Capsicum annuum, Tepin and Capsicum chinese, Habanero) prevents Fe2+-induced lipid peroxidation in brain—in vitro. Food Chem 102:178–185. doi:10.1016/j.foodchem.2006.05.048
32. Pereira RP, Fachinetto R, Prestes AS, Puntel RL, Silva GNS, Heinzmann BM, Boschetti TK, Athayde ML, Bürger ME, Morel AF, Morsh VM, Rocha JBT (2009) Antioxidant effects of different extracts from *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citrates*. Neurochem Res (in press). doi:10.1007/s11064-008-9861-z
33. Wagner C, Fachinetto R, Dalla Corte CL, Brito VB, Severo D, Dias GOC, Morel AF, Nogueira CW, Rocha JBTR (2006) Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. Brain Res 1107:192–198. doi:10.1016/j.brainres.2006.05.084
34. Rates SMK (2001) Plants as source of drugs. Toxicon 39:603–613. doi:10.1016/S0041-0101(00)00154-9
35. Abourashed EA, Koetter U, Brattstrom A (2004) In vitro binding experiments with a valerian, hops, and their fixed combination extract (Ze91019) to selected central nervous system receptors. Phytomedicine 11:633–638. doi:10.1016/j.phymed.2004.03.005
36. Brouillet E, Hantraye P, Ferrante RJ, Dolan R, Leroy-Willig A, Kowall NW, Beal MF (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. Proc Natl Acad Sci USA 92:7105–7109. doi:10.1073/pnas.92.15.7105
37. Tũnez I, Montilla P, Muñoz MC, Feijoo M, Salcedo M (2004) Protective effect of melatonin on 3-nitropropionic acid-induced oxidative stress in synaptosomes in an animal model of Huntington's disease. J Pineal Res 37:252–256. doi:10.1111/j.1600-079X.2004.00163.x
38. Bates JN, Baker MT, Guerra R, Harrison DG (1990) Nitric oxide generation from nitroprusside by vascular tissue. Biochem Pharmacol 42:157–165. doi:10.1016/0006-2952(91)90406-U
39. Chen J, Chang B, Williams M, Murad F (1991) Sodium nitroprusside degenerates cultured rat striatal neurons. NeuroReport 2:121–123. doi:10.1097/00001756-199103000-00002
40. Loiacono RE, Beart PM (1992) Hippocampal-lesions induced by microinjection of the nitric-oxide donor nitroprusside. Eur J Pharmacol 216:331–333. doi:10.1016/0014-2999(92)90381-D
41. Bellé NAV, Dalmolin GD, Fonini G, Rubin MA, Rocha JBT (2004) Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. Brain Res 1008:245–251. doi:10.1016/j.brainres.2004.02.036
42. Santamaría A, Ríos C (1993) MK-801, an *N*-Methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. Neurosci Lett 159:51–54. doi:10.1016/0304-3940(93)90796-N
43. Goda K, Kishimoto R, Shimizu S, Hamane Y, Ueda M (1996) Quinolinic acid and active oxygens. Possible contribution of active oxygens during cell death in the brain. Adv Exp Med Biol 398:247–254
44. Graf E, Mahoney JR, Bryant RG, Eaton JW (1984) Iron catalyzed hydroxyl radical formation: stringent requirement for free iron coordination site. J Biol Chem 259:3620–3624
45. Gogvadze V, Walter PB, Ames BN (2003) The role of Fe^{2+}-induced lipid peroxidation in the initiation of the mitochondrial permeability transition. Arch Biochem Biophys 414:255–260
46. Zago MP, Verstraeten SV, Oteiza PI (2000) Zinc in the prevention of Fe^{2+} initiated lipid and protein oxidation. Biol Res 33(2):143–150

2.2 Manuscrito 1: “*Valeriana officinalis* atenua a toxicidade induzida por rotenone em *Drosophila melanogaster”.*

MANUSCRITO 1

Valeriana officinalis Attenuates the Rotenone-Induced Toxicity in *Drosophila melanogaster*

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ABSTRACT

The fruit fly *Drosophila melanogaster* has been widely used in neurotoxin-based models of neurodegenerative diseases. In this study, we investigated the neuroprotective effects of *Valeriana officinalis* (*V. officinalis)*, a plant commonly used as herbal medicine, against motor deficits, oxidative stress, mortality and toxicity induced by rotenone in *D. melanogaster*. Adult wild-type flies were concomitantly exposed to rotenone (500 μM) and *V. officinalis* aqueous extract (10 mg/mL) in the food during 7 days. The rotenone-fed flies had a worse performance in the negative geotaxis assay (i.e. climbing capability) and open-field test (i.e. mobility time) and a higher incidence of mortality when compared to control groups. These effects were abolished by *V. officinalis* treatment. However, the decreased number of crossings observed in the flies exposed to rotenone was not modified by *V. officinalis.* Rotenone caused a marked reduction on the protein-thiol content in the homogenates of flies as well as a decrease in the cellular viability in flies with and without homogenate procedure. *V. officinalis* treatment offered protection against these effects induced by rotenone. In relation to gene expression, we observed that the flies exposed to rotenone had a significant increase in the mRNA expression of antioxidant enzymes superoxide dismutase (SOD) and Catalase (CAT) and also in the tyrosine hydroxylase gene (TH) when compared to the control groups. In these parameters, only the antioxidant genes were normalized by *V. officinalis* treatment. Our results suggest that *V. officinalis* extract is effective in reducing the toxicity induced by rotenone in *D. melonogaster* as well as confirm the utility of this model to investigate potential therapeutic strategies against neurodegeneration.

Keywords: *Drosophila melonogaster,* Rotenone, *V. officinalis,* Parkinson's disease

1 Introduction

Parkinson´s disease (PD) is the most common neurodegenerative movement disorder, affecting 1 to 2% of the population above the age of 60 years. The pathological hallmarks of PD include selective loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies in surviving dopamine neurons (Spillantini et al., 1998, Cannon and Greenamyre, 2010). Lewy bodies are cytoplasmic inclusions composed mainly of alpha-synuclein, which are believed to disrupt the brain's normal functioning in PD. Although it is well known that environmental exposures and individual genetic susceptibility may determine the onset of PD symptoms; the precise cellular and molecular mechanism(s) responsible for the neurodegeneration processes remain still unknown. However, free radical overproduction and abnormalities in mitochondria function have emerged as critical mediators of the neuronal damage in PD (Sherer et al., 2002, Rama Rao et al., 2007, Valko et al., 2007). Knowing that oxidative stress plays a central role in PD disease; numerous studies have been conducted to investigate the potential neuroprotective action of antioxidants in neurotoxin-based models of PD. Though several synthetic antioxidants are available, a growing trend has been targeting toward the use of medicinal plants as antioxidants since herbal compounds are generaly free of adverse effects.

The root extracts of plant *Valeriana officinalis* (*V. officinalis* L., Valerianaceae) have been used for centuries in popular medicine for the treatment of sleep disorders, anxiety and epilepsy (Hadley and Petry, 2003). Despite the mechanisms, most of the studies show that *V. officinalis* modulate anxiety and insomnia possibly by interacting with different neurotransmitter systems (Malva et al., 2004, Ortiz et al., 2006, Sudati et al., 2009). However, with emphasis in neurodegenerative disorders, there are not published data on the possible protective effect of *V. officinalis*. Though there are many models of neurodegenerative disease it is difficult to mimic certain features of the disease properly *in vivo*. Regarding to PD, it is well recognized that the rotenone model of PD accurately reproduces many aspects of the pathology in rats. However, this model provides some limitations that are mainly related to the variability of percentage of animals that development nigrostriatal lesion and the level of lesion (Cannon and Greenamyre, 2009). Consequently, there is a need for improvement of current models and development of new models. In this way, it has been amply accepted the use of the fruit fly *Drosophila melanogaster* (*D. melanogaster*) as a model to study molecular mechanisms involved in neurological diseases,

including PD (Hirth, 2010). Notably, the fly has homology among five of the six genes related to PD in humans (Whitworth et al., 2006). In view of the probable advantages of the rotenone model on flies in terms of reproducibility and experimental design, we aimed to investigate the possible neuroprotective effects of *V. officinalis* on rotenone-induced toxicity in *D. melanogaster*. In this work, it was specially evaluated the potential of *V. officinalis* powder root in attenuating lethality, toxicity, movement disorders and some parameters of oxidative stress induced by rotenone exposure in the flies.

2 Materials and Methods

2.1 Drugs

Chemicals, including Rotenone, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2’,7’-dichlorofluorescein diacetate (DCHF-DA) were obtained from Sigma Chemical Company (St Louis, MO, USA). All other reagents were commercial products of the highest purity grade available. The standard powder of *V. officinalis* roots was obtained from Bio extracts (Ribeirão Preto-SP, Brazil) and tested as aqueous extract at concentration of 10 mg/mL.

2.2 Plant Material

Powder from roots of *Valeriana officinalis* was obtained from Bio extracts (São Paulo , Brazil), aquous extract was obtained after 15 min of infusion using water.

2.2 *Drosophila* Stock

D. melanogaster wild-type was obtained from the National species Stock Center, Bowling Green, OH, USA. The flies were reared on agar medium (1% w/v brewer's yeast; 2% w/v sucrose; 1% w/v powdered milk; 1% w/v agar; 0.08% v/w Nipagin) at constant temperature and humidity (23°C±1°C; 60% relative humidity, respectively). The flies were reared in 2.5cm x 6.5cm vials containing 5 mL of medium at constant temperature, humidity (and 60% and under 12h dark/light cycle. All experiments were performed with the same strain.

2.3 Rotenone exposure and *V. officinalis* treatment

Flies (both gender, 1-2 days old) were divided into five groups: (1) control; (2) ethanol; (3) rotenone; (4) *V. officinalis* and (5) rotenone plus *V. officinalis*. Rotenone (dissolved in ethanol 98%) and *V. officinalis* (aqueous extract) were added into the food`s flies at final concentration of 500 μM and 10 mg/mL, respectively. Two controls were used (with and without ethanol). The

total food medium contained a volume of 1% of ethanol, *V. officinalis* aqueous extract, rotenone or rotenone plus *V. officinalis*. The flies were exposed to its respective treatments during 7 days (Moreira et al., 2012) and the vials containing flies were maintained in an incubator at 23°C±1°C before being used for different assays.

The concentration of rotenone used in this protocol was based in a survival curve (0.1, 0.5 and 1 mM), and corresponds to the concentration that caused approximately 50% death of the flies after 7 days of exposure (data not shown). The choice of *V. officinallis* aqueous extract concentration was based on previous observations, which show that *V. officinallis* aqueous extract in the range of (5- 20 mg/mL) did not cause overt signals of toxicity in flies (data not shown).

2.4 Valeric Acid and Gallic Acid quantification

High performance liquid chromatography (HPLC) was performed according to the methodology previously described (Sudati et al., 2009). The aqueous extract (10 mg/mL) from *V. officinalis* roots was analyzed. Stock solutions of valeric acid, gallic acid and quercetin were used as standard reference. The chromatographic peaks were confirmed by comparing their retention time and UV spectra (200 to 400 nm) and with those of the reference standards. All chromatographic operations were carried out at ambient temperature and in triplicate.

2.5 *In vivo* assays

2.5.1 Survival rate

The survival rate was evaluated by counting daily of the number of living flies until the end of the experimental period (7 days). Around 150 flies per group were included in the survival data.

2.5.2 Negative Geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously (Feany and Bender, 2000). The flies (both gender) were sorted under ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 5 flies each). After 10 minutes of recovery from cold exposure, flies were gently tapped to the bottom of the column. The flies that reached the top of the column (6 cm) and the flies that remained at the bottom were counted separately during 6 seconds. The scores represent the mean of the numbers of flies at the top (n_{top}) as percentage of the total number of flies (n_{tot}). Around 15 flies were included for each treatment performed and the total number of flies contained in the negative geotaxis data (45 per group) represents the sum of three independent experiments.

2.5.3 Open-field

Open-field task was performed according to the method described by Hirth (2010), with some modifications. Each wild-type fly was kept in an arena divided by squares (1 cm x 1 cm) measuring 9 cm of diameter, which can be covered by petri dish. The fly's activity and movement was recorded with a video camera and the distance travelled was recorded by the resulting trajectory during a given time-window (60 sec), which was calculated throughout the number of squares crossed by each single fly analyzed. Video-assisted movement tracking records locomotor behavior allows the quantification of immobility (time spends without locomotion) and distance travelled (number of square crossed). Around 15 flies were included for each treatment performed and the total number of flies contained in the open-field data (45 per group) represents the sum of three independent experiments.

2.6 *Ex vivo* assays

2.6.1 Homogenate preparation

Around 20 flies per group were immobilized by chilling on ice and then decanted into a chilled mortar. Whole body of flies was manually homogenized in ice-cold Tris/HCl buffer (pH 7.4, 0.1 M), 1:10 (flies/volume (μL)). The homogenate was filtered through sieve with nylon

mesh (pore size/10 mm), centrifuged at 3000 g for 3 min at 4 °C, and the supernatant was used for biochemical assays (each n= 20 flies/200 µL).

2.6.2 RNA Isolation and Analysis of mRNA Expression

Approximately 2 µg of total RNA from 20 flies per group was extracted using Trizol® reagent (Invitrogen®) accordingly to the manufacturer´s suggested protocol. The primers sequences for genes of Catalase, Superoxido dismutase and Tyrosine hydroxilase were designed. Gene specific primer sequences were based on published sequences in GenBank Overview (http://www.ncbi.nlm.nih.gov/genbank/) designed with Primer3 program version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and custom made by Invitrogen®. After quantification, total RNA was treated with DNase I (Invitrogen®). The cDNA was synthesed with M-MLV reverse transcriptase enzyme and random primer (Invitrogen) accordingly to the manufacturer´s suggested protocol. Quantitative real-time polymerase chain reaction were performed in 20 µL PCR mixture containing 1 µl RT product (cDNAs) as template, 1x PCR Buffer, 25 µM dNTPs, 0.2 µM of each primer, 1.5-2.5 mM $MgCl_2$, 0.1x SYBR Green I (Molecular Probes®) and 1U Taq DNA Polymerase (Invitrogen®). The thermal cycle was carried in a Thermocycler StepOne Plus (Applied Biosystems) and the protocol used was the following: activation of the Taq DNA polymerase 95°C for 5 min followed of 40 cycles of 15 s at 95°C, 15 s at 60°C and 25 s at 72°C. SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems), and the CT value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each well, analyzed in quadruplicate, a ΔC T value was obtained by subtracting the Actin and GPDH C T value from the C T value of the interest gene. The ΔC T mean value obtained from the control group of each gene was used to calculate the ΔΔCt of the respective gene ($2^{-\Delta\Delta Ct}$). All experiments were calculated in relation to both gene controls: Tubulin and GPDH. Three independent experiments were performed.

2.6.3 Cell viability evaluation

Cell viability was evaluated by dehydrogenase activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This analysis was performed in whole body of flies in two differente manners (with and without homogenate procedure),

according to the method described by (Babot et al., 2005). The ratio values were standardized per protein content and expressed as percentage in relation to the control.

2.6.4 Thiol determination

Total and non-protein thiol content were estimated based on a spectrophotometric method using Ellman`s reagent, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) (Ellman, 1959). For non-protein thiol, the homogenate was precipitated with TCA 10% followed by centrifugation at 3000 g for 3 min at 4 °C. The supernatant samples were measured espectrophotometrically at 412 nm. A standard curve was constructed for each measurement using GSH. Thiol levels were expressed as % in relation to the control.

2.6.5 Isolation of mitochondria

After time exposed to the treatment (described previously), mitochondria from wild type *D. melanogaster* was isolated according to Miwa and Brand, 2005, with minnor modifications. Briefly, about 50 flies were immobilized by chilling on ice, and gently pressed with a pestle in a chilled mortar containing a little isolation medium (250 mM sucrose, 5 mM Tris – HCl, 2 mM EGTA, 1% (w/v) bovine serum albumin, adjusted to pH 7.4 at 4°C) then passed through absorbent muslin and collected into a tube (1.5 mL) and immediately centrifuged at 150xg for 3 min at 4°C. The supernatant was passed through one layer of muslin and recentrifuged at 9000xg for 10 min. The supernatant was discarded and the pellet was carefully resuspended with a little more isolation medium to give about 30 mg protein/ml. The detailed characteristics of mitochondria prepared in this way were reported (Miwa et al., 2003). This study used only freshly prepared mitochondria, and experiment was started as soon as possible after the isolation procedure.

2.6.5.1 Measurement of mitochondrial H_2O_2 production

H_2O_2 released from mitochondria was detected using the Ampliflu red fluorescent dye, which reacts with H_2O_2 in the presence of horseradish peroxidase, producing highly fluorescent

resourcing. Horseradish peroxidase (0.2 U/ml) and Amplifu red reagent (1 μM) were added to the assay medium and then mitochondria (≈0.1 mg/ml) were applied. H_2O_2 formation was initiated by addition of pyruvate (5 mM) and fluorescence was detected at 30 °C in a Shimadzu spectrophotometer. The excitation and emission fluorescent wavelengths were 550 and 585 nm, slit (3 and 5), respectively. The positive control signal was produced by addition of known amounts of H_2O_2 at the end of each experiment.

2.6.6 Protein determination

Protein concentrations in the whole body homogenates were determined by the method of (Bradford, 1976), using bovine serum albumin as the standard.

2.7 Statistical analyses

Data of behavior testing were analyzed by nonparametric methods, using Kruskal-Wallis (kw) followed by Dunn's Multiple Comparisons Test when appropriate. Survival data was analyzed statistically by the X^2 method and Fisher's exact test. All other parameters were analyzed by Two-way ANOVA followed by Duncan Multiple Range Test when appropriate. Differences between groups were considered significant when $p<0.05$. Data of non-parametric analysis are represented as medians and ranges (interquartile interval) and the data of parametric analysis as means and S.E.M.

3 Results

3.1 HPLC Analyzes

HPLC fingerprinting of the aqueous extract of *V. officinalis* (10 mg/ml) showed an elution diagram when the peaks were grouped in regions based on the UV absorption profile. The regions of chromatogram revealed the presence of valeric acid (2.29 mg/ml, peak 2), gallic acid (0.47 mg/ml, peak 3) and quercetin (0.11 mg/ml, peak 4) (Figure 1).

3.2 Effect of *V. officinalis* on Survival rate of flies exposed to Rotenone

Data of figure 2 show that rotenone exposure to flies was associated with a gradual percentage of death until the end of experimental period when compared to the control group. In contrast, rotenone exposure practically did not cause death in the flies maintained with *V. officinalis* treatment. *V. officinalis* alone did not alter the survival rate of the flies when compared to the control groups (Figure 2).

3.3 Effect of *V. officinalis* on Negative Geotaxis assay in the flies exposed to rotenone

Dunn's Multiple Comparisons Test revealed that the climbing behavior performed in the flies exposed to rotenone was significantly lower (kw=10.77; p=0.029) than control group; indicating that the flies rotenone-exposed presented difficulty to move to the top of the tube (Figure 3). This effect of rotenone was abolished by *V. officinalis* treatment. Kruskal-Wallis test also showed that there was not statistical difference in this parameter between *V. officinalis* and control groups (kw=10.77; $p > 0.05$).

3.4 Effect of *V. officinalis* on Open-field test in the flies exposed to rotenone

In open-field evaluation, Kruskal-Wallis test followed by Dunn's Multiple Comparisons Test revealed that the flies exposed to rotenone had a significant decrease in the number of crossings (kw=50.66; p=0.0001) when compared to the control groups and that this effect did not

modified by concomitant treatment with *V. officinalis* (kw=50.66; $p>0.05$) (Figure 4A). No significant difference was observed between *V. officinalis* and control groups in this parameter (kw=50.66; $p>0.05$).

Dunn's Multiple Comparisons Test revealed that the immobility parameter, evaluated by time spent without moving, was significantly increased in the flies exposed to rotenone (kw=17.90; $p=0.0013$) when compared to control groups (Figure 4B). This effect of rotenone was blunted by *V. officinalis* treatment. Kruskal-Wallis test also indicated that there was no significant difference between *V. officinalis* and control groups in this evaluation (kw=17.90; $p>0.05$).

3.5 mRNA Expression of SOD, CAT and TH Genes

Two-way ANOVA followed by *Post Hoc* comparisons showed that flies exposed to rotenone had a significant increase in the mRNA expression of SOD and CAT when compared to the values found in the control groups (Figure 5A and 5B). These effects induced by rotenone exposure on antioxidant genes were normalized by *V. officinalis* treatment. Likewise to rotenone, the mRNA expression of CAT was increased in the flies treated with *V. officinalis* alone (Figure 5B). Rotenone exposure also caused an increase in mRNA expression of TH, which did not modify by concomitant treatment with *V. officinalis* (Figure 5C).

3.6 Effect of *V. officinalis* on MTT reduction in homogenates and body region of flies exposed to rotenone

Two-way ANOVA followed by *Post Hoc* comparisons showed that rotenone exposure caused a significant decrease on MTT reduction in the fly homogenates and that this effect was blunted by *V. officinalis* treatment (Fig. 6). To check if the homogenate procedure could influence on this results, since the mechanic process may release some enzymes that can cause reduction of MTT, we performed the same assay without homogenate procedure. Likewise to homogenate samples, the cell viability evaluated by MTT reduction was reduced in the flies exposed to rotenone and this effect was abolished by *V. officinalis* treatment (data not shown).

3.7 Effect of *V. officinalis* on thiol content in homogenate of flies exposed to rotenone

Two-way ANOVA followed by *Post Hoc* comparisons showed that rotenone exposure diminished significantly the protein-thiol content of the flies when compared to the control groups (Figure 7). *V. officinalis* treatment was effective in restoring the thiol content in the flies exposed to rotenone. No significant difference was observed among the groups on the non-protein thiols levels (data not shown).

3.8 Effect of *V. officinalis* on H_2O_2 mitochondrial production in mitochondria of flies exposed to rotenone

Two-way ANOVA followed by *Post Hoc* comparisons showed that H_2O_2 mitochondrial formation was increased in the flies exposed to rotenone ($p<0.05$) compared to control groups. This effect rotenone-induced was abolished by *V. officinalis* treatment (Figure 8).

4 Discussion

In recent years, *D. melanogaster* has been widely used as a model in screening of phytotherapics for the treatment of neurodegenerative diseases, including PD (Hosamani and Muralidhara, 2009, Jeibmann and Paulus, 2009). With this in mind, the present study was delineated to evaluate the possible neuroprotective efficacy of *V. officinalis* against the toxicity caused by rotenone exposure in *D. melanogaster*. We have found that the aqueous extract from roots of *V. officinalis* was effective in offering protection against motor impairments, mortality, toxicity and some oxidative stress parameters that were altered by rotenone.

In our experimental protocol, exposure of *D. melanogaster* to rotenone reproduced key aspects of PD as for example locomotor deficits. In fact, the flies exposed to rotenone exhibited a worse performance in a negative geotaxis assay (climbing) and in the open-field test (crossing numbers and mobility time). Interestingly, most of these movement impairments were ameliorated by *V. officinalis* treatment. Indeed, *V. officinalis* enhanced the survival rate of the flies exposed to rotenone. These benefic effects of *V. officinalis* observed here are in accordance with the data of a recent study where this plant was effective in preventing oral movement deficits induced by reserpine in rats, which are associated with neuropatologies as orofacial dyskinesia and tardive dyskinesia (Pereira et al., 2011).

A growing body of evidence suggests that pesticides may play a role in Sporadic PD in humans. Although, little be known about the precise mechanism action of these agents in *D. melanogaster*; the involvement of mitochondrial dysfunction, specially complex I deficiency, is well supported by epidemiological studies demonstrating that the exposure to neurotoxins such as rotenone and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) leads to the PD like pathology and symptoms (Coulom and Birman, 2004, Goldman et al., 2011). Accordingly, numerous studies in *in vivo* rodent models have shown that these pesticides can provoke dopaminergic neurodegeneration, decrease of striatal dopamine levels and occurrence of Lewy bodies and that these events are usually accomplished by disturbances in GSH homeostasis (Wang et al., 2009, Garrido et al., 2011). Indeed, alterations in gene expression like tyrosine hydroxylase, alpha-synuclein, PARKIN, PINK1 or DJ-1, which are involved in the sporadic forms of PD, has been associated with the toxicity of these compounds (Rajput and Rajput, 2007, Wang et al., 2009). Thus, it is widely accepted that the effects induced by rotenone encompass

oxidative stress through mitochondrial dysfunction, increasing reactive oxygen species (ROS) production, which triggers cellular events involved in the cell death including DNA and proteins of neural cells damage (Cicchetti et al., 2009).

Despite mechanisms in *D. melanogaster*, here we verify that rotenone promoted an increase in the mRNA expression of antioxidant enzymes SOD and CAT and in the mRNA expression of TH. These results differ from some experimental protocols, where these parameters are commonly decreased after pesticide exposure. However, they may represent a compensatory response of organisms to oxidative insults, since rotenone exposure was associated with mitochondrial H_2O_2 production enhanced. Indeed, rotenone reduced significantly the cell viability of the flies and the protein-thiol content in the fly homogenates. However, no change was observed on the non-protein thiols levels in the flies. Taken together, these data suggest a possible implication of oxidative stress in the rotenone-based model with *D. melanogaster.*

It is well established that *V. officinalis* is a medicinal plant effective in improving anxiety and sleep disorders. However; few investigations have evaluated the effects of *V. officinalis* in experimental neurodegeneration. In fact, we found only one study pointing a cytoprotective role for *V. officinalis* extracts in an *in vitro* model of PD (Oliveira DM et al., 2009). Thus, our data show, for the first time, *in vivo V. officinalis* effectiveness in reducing the deleterious effects of rotenone in both behavior and oxidative stress parameters. It is reasonable to assume that the neuroprotection offered by *V. officinalis* in behavior and biochemical parameters may be associated to antioxidant properties of plant due its phenolic and flavonoid constituents. Corroborating this idea, a recent study performed to evaluate the antioxidant effects of the plant *in vitro* demonstrated that *V. officinalis* tincture inhibited thiobarbituric acid reactive substances and deoxyribose degradation induced by different pro-oxidant in brain rats and that this effect was in a concentration concentration-dependent manner (Sudati et al., 2009).

In conclusion, our findings suggest that *V. officinalis* extract is effective in reducing the toxicity induced by rotenone in *D. melanogaster* as well as confirm the utility of this model to investigate potential therapeutic strategies that may be effective *in vivo* mammalian models of neurodegeneration. Moreover, these data implied that the use of *V. officinalis* extract as herbal medicine may be beneficial for attenuating PD complications.

Acknowledgments

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REFERENCES

Babot Z, Cristofol R, Sunol C (2005) Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on GABAA receptors and niflumic acid-sensitive chloride channels. Eur J Neurosci 21:103-112.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Cannon JR, Greenamyre JT (2009) NeuN is not a reliable marker of dopamine neurons in rat substantia nigra. Neurosci Lett 464:14-17.

Cannon JR, Greenamyre JT (2010) Neurotoxic in vivo models of Parkinson's disease recent advances. Prog Brain Res 184:17-33.

Cicchetti F, Drouin-Ouellet J, Gross RE (2009) Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci 30:475-483.

Coulom H, Birman S (2004) Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. J Neurosci 24:10993-10998.

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-77.

Feany MB, Bender WW (2000) A Drosophila model of Parkinson's disease. Nature 404:394-398.

Garrido M, Tereshchenko Y, Zhevtsova Z, Taschenberger G, Bahr M, Kugler S (2011) Glutathione depletion and overproduction both initiate degeneration of nigral dopaminergic neurons. Acta Neuropathol 121:475-485.

Goldman SM, Quinlan PJ, Ross GW, Marras C, Meng C, Bhudhikanok GS, Comyns K, Korell M, Chade AR, Kasten M, Priestley B, Chou KL, Fernandez HH, Cambi F, Langston JW, Tanner CM (2011) Solvent exposures and parkinson disease risk in twins. Ann Neurol.

Hadley S, Petry JJ (2003) Valerian. Am Fam Physician 67:1755-1758.

Hirth F (2010) *Drosophila melanogaster* in the study of human neurodegeneration. CNS Neurol Disord Drug Targets 9:504-523.

Hosamani R, Muralidhara (2009) Neuroprotective efficacy of Bacopa monnieri against rotenone induced oxidative stress and neurotoxicity in *Drosophila melanogaster*. Neurotoxicology 30:977-985.

Jeibmann A, Paulus W (2009) *Drosophila melanogaster* as a model organism of brain diseases. Int J Mol Sci 10:407-440.

Malva JO, Santos S, Macedo T (2004) Neuroprotective properties of Valeriana officinalis extracts. Neurotox Res 6:131-140.

Miwa S, Brand MD (2005) The topology of superoxide production by complex III and glycerol 3-phosphate dehydrogenase in Drosophila mitochondria. Biochim Biophys Acta 1709:214-219.

Miwa S, St-Pierre J, Partridge L, Brand MD (2003) Superoxide and hydrogen peroxide production by Drosophila mitochondria. Free Radic Biol Med 35:938-948.

Oliveira DM, Barreto G, De Andrade DEV, Saraceno E, Aon-Bertolino L, Capani F, El Bacha RS, LD G (2009) Cytoprotective effect of Valeriana officinalis extract on an in vitro experimental model of Parkinson disease. Neurochemical Research 34.

Ortiz JG, Rassi N, Maldonado PM, Gonzalez-Cabrera S, Ramos I (2006) Commercial valerian interactions with [3H]Flunitrazepam and [3H]MK-801 binding to rat synaptic membranes. Phytother Res 20:794-798.

Pereira RP, Fachinetto R, de Souza Prestes A, Wagner C, Sudati JH, Boligon AA, Athayde ML, Morsch VM, Rocha JB (2011) Valeriana officinalis ameliorates vacuous chewing movements induced by reserpine in rats. J Neural Transm 118:1547-1557.

Rajput A, Rajput AH (2007) Old age and Parkinson's disease. Handb Clin Neurol 84:427-444.

Rama Rao KV, Reddy PV, Hazell AS, Norenberg MD (2007) Manganese induces cell swelling in cultured astrocytes. Neurotoxicology 28:807-812.

Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT (2002) An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J Neurosci 22:7006-7015.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A 95:6469-6473.

Sudati JH, Fachinetto R, Pereira RP, Boligon AA, Athayde ML, Soares FA, de Vargas Barbosa NB, Rocha JB (2009) In vitro antioxidant activity of Valeriana officinalis against different neurotoxic agents. Neurochem Res 34:1372-1379.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44-84.

Wang CS, Burke JR, Steffens DC, Hulette CM, Breitner JC, Plassman BL (2009) Twin pairs discordant for neuropathologically confirmed Lewy body dementia. J Neurol Neurosurg Psychiatry 80:562-565.

Whitworth AJ, Wes PD, Pallanck LJ (2006) Drosophila models pioneer a new approach to drug discovery for Parkinson's disease. Drug Discov Today 11:119-126.

LEGENDS FOR FIGURES

Figure 1- High performance liquid chromatography of *V. officinalis* aqueous extract. Peak 1 represents an unknown peak; peak 2, 3 and 4 correspond to valeric acid, gallic acid and quercetin, respectively. Chromatographic conditions are described in the experimental section.

Figure 2 - Effect of *V. officinalis* on survival rate of flies exposed to rotenone. Data were collected every 24 h for each group during 7 days. The numbers of surviving flies are represented as % of control. The total number of flies (150 per group) represents the sum of three independent experiments. *represents significant difference in relation to the control group (Fischer's exact probability test, $p < 0.05$).

Figure 3- Effect of *V. officinalis* on geotaxic response (climbing) of flies exposed to rotenone during 7 days. The total number of flies (≈ 50 per group) represents the sum of three independent experiments. Values are expressed as median and range (interquartile interval). *represents significant difference in relation to the control group (Kruskal-Wallis test followed Dunn' Multiple Comparisons test, $p < 0.05$).

Figure 4- Effect of *V. officinalis* on locomotor and exploratory activities evaluated by open-field of the flies exposed to rotenone. Two parameters were analyzed: (A) exploratory activity evaluated by counting of the crossing numbers and (B) immobility time evaluated by counting of the time spent without moving. The total number of flies (≈ 50 per group) represents the sum of three independent experiments. Values are expressed as median and range (interquartile interval). *represents significant difference in relation to the control group (Kruskal-Wallis test followed Dunn' Multiple Comparisons test, $p < 0.05$).

Figure 5- qPCR relative gene expression for three different primers: superoxide dismutase (SOD, graph A), catalase (CAT, graph B) and Tirosine hydroxilase (TH, graph C). Results are expressed as mean±S.E.M (n=3). *represents significant difference in relation to control group ($P < 0.05$, Two-way ANOVA followed Duncan multiple range Test).

Figure 6 - Effect of *V. officinalis* on cell viability in homogenate of flies exposed to rotenone. *represents significant difference in relation to control group (n=6). Values are expressed as mean±S.E.M ($P < 0.05$, Two-way ANOVA followed by Duncan multiple range Test).

Figure 7- Effect of *V. officinalis* on protein-thiol content in homogenate of flies exposed to rotenone. *represents significant difference in relation to control group (n= 9). Values are expressed as mean±S.E.M ($P < 0.05$, Two-way ANOVA followed by Duncan multiple range Test).

Figure 8- Hydrogen peroxide (H_2O_2) production of isolated mitochondria from *D. melanogaster*. (a) probe, (b) control, (c) Val, (d) Rot and (e) Rot+Val. *represents significant difference in relation to control group (n=3). Values are expressed as mean±S.E.M ($P < 0.05$, Two-way ANOVA followed by Duncan multiple range Test).

Figure 1

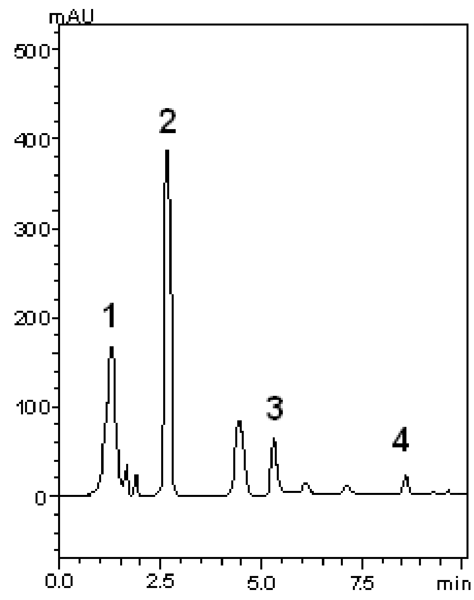


Figure 2

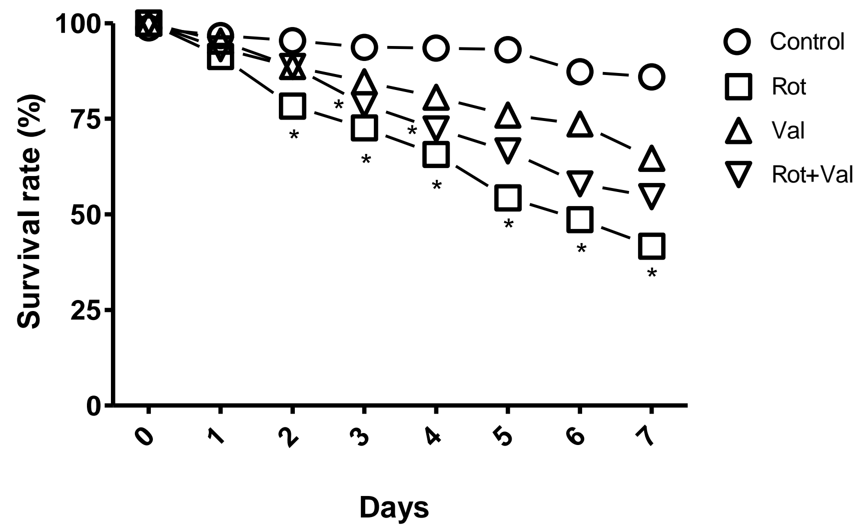


Figure 3

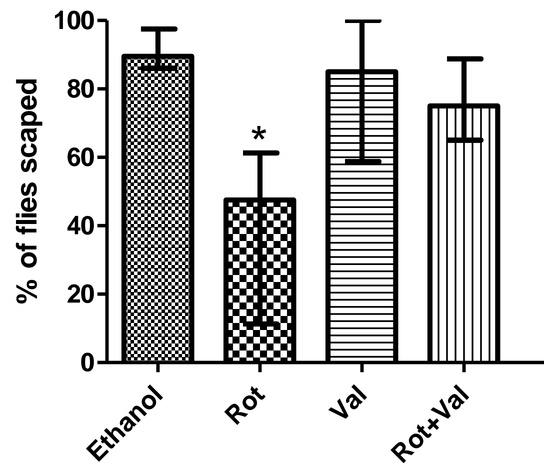


Figure 4

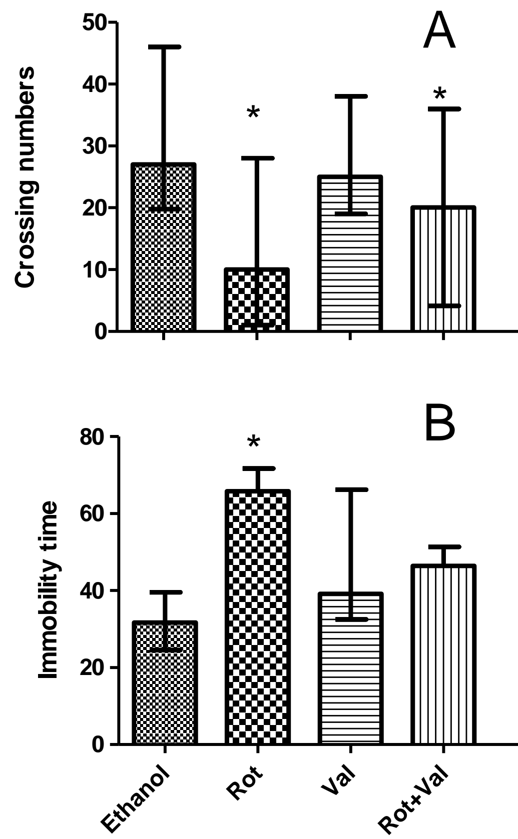


Table 1 – Sequences of qPCR primers.

Primer	Sequence
Sod LEFT	5´GGAGTCGGTGATGTTGACCT 3´
Sod RIGHT	5´GTTCGGTGACAACACCAATG 3´
Catalase LEFT	5´ACCAGGGCATCAAGAATCTG 3´
Catalase RIGHT	5´AACTTCTTGGCCTGCTCGTA 3´
Thiore LEFT	5´TACAAGCCCACGGAGTTCTT 3´
Thiore RIGHT	5´GTCAGGCCAGACTTCAAAGC 3´
GPDH LEFT	5´ ATGGAGATGATTCGCTTCGT 3´
GPDH RIG HT	5´ GCTCCTCAATGGTTTTTCCA 3´
Tubulin LEFT	5´ ACCAATGCAAGAAAGCCTTG 3´
Tubulin RIGHT	5´ ATCCCCAACAACGTGAAGAC 3´

Figure 5

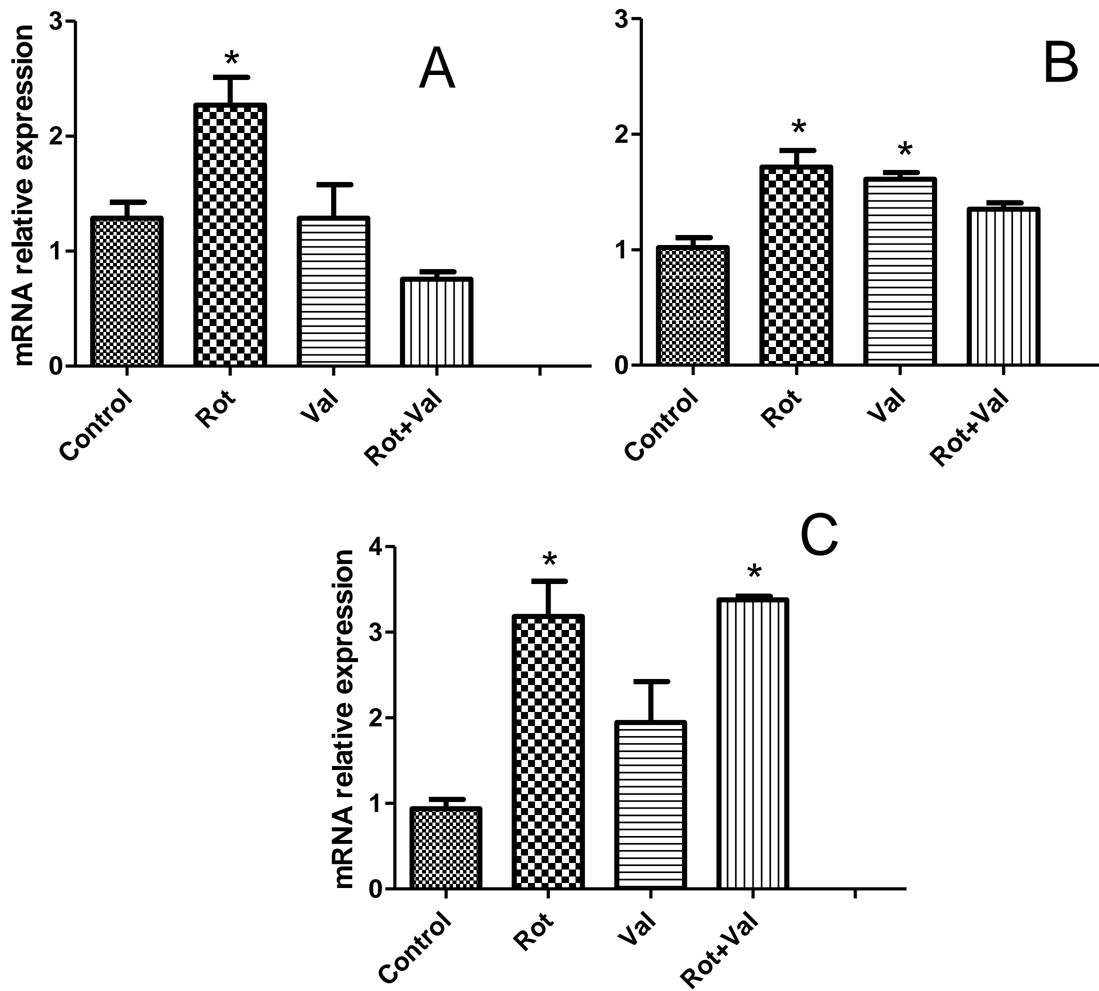


Figure 6

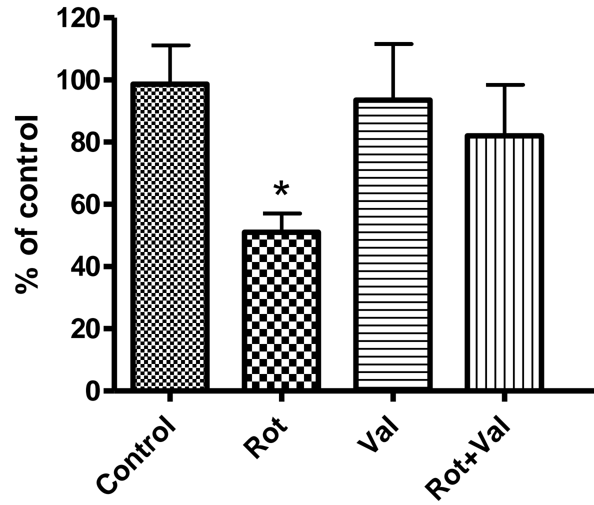


Figure 7

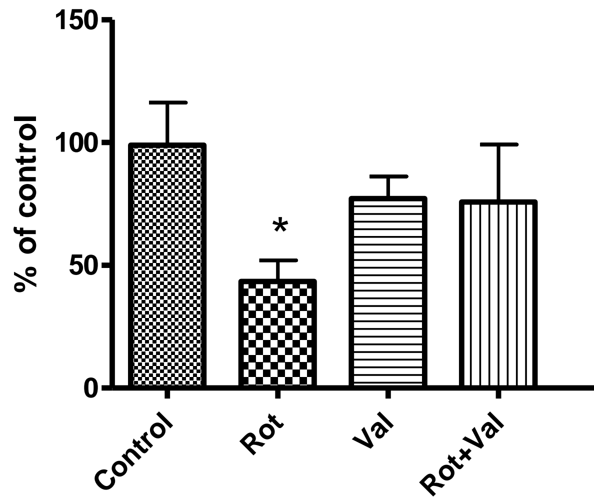
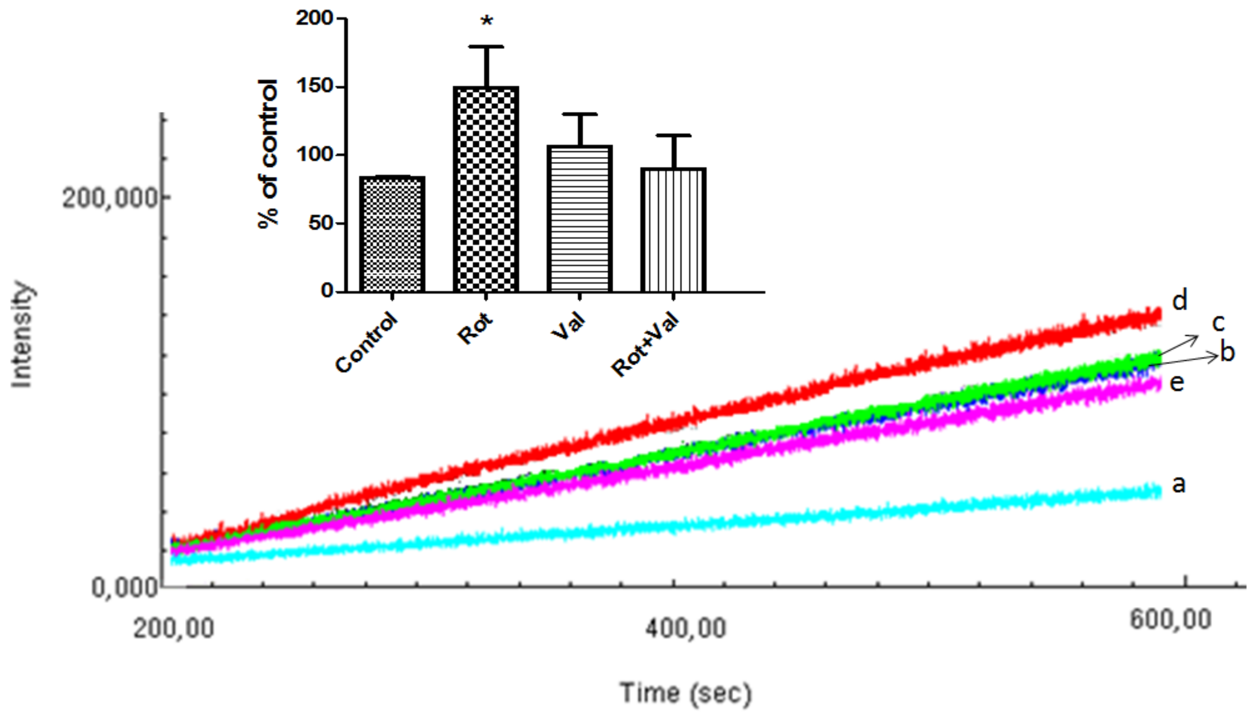


Figure 8



2.3 Manuscrito 2: “Efeito da dieta de disseleneto de difenila em *Drosophila melanogaster* expostas à rotenona: parâmetros comportamentais e bioquímicos”.

MANUSCRITO 2

(Em preparação, dados preliminares)

Effects of dietary diphenyl diselenide in *Drosophila melanogaster* exposed to Rotenone: behavior and biochemical parameters

Jéssie H. Sudati, Francielli A. Vieira, Sandra S.Pavin,
João Batista T. da Rocha, Nilda V. Barbosa

1 INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by a variety of motor and non-motor symptoms with no proven successful therapy (Greenamyre and Hastings, 2004). The cause of this disorder remains unknown, but many studies have related the chronic neurodegeneration with marked formation of ubiquitin- and α-synuclein-positive cytoplasmic inclusions known as Lewy bodies and selective loss of dopaminergic neurons in the substantia nigra (Dawson and Dawson, 2003).

Epidemiological studies have been indicated that exposure to agricultural pesticides represents a significant risk factor for Sporadic PD development (Langston, 2002). In this sense, it has been demonstrated that rotenone, a broad spectrum pesticide, induces Parkinson-like symptoms *in vitro* and *in vivo* (Betarbet et al., 2000, Coulom and Birman, 2004). This lipophilic compound freely crosses cell membranes and accesses the mitochondria, where triggers cellular damage via complex I inhibition (Betarbet et al., 2000, Coulom and Birman, 2004).

The fruitfly *Drosophila melanogaster* has been recognized as a powerful organism for modeling human neurodegenerative diseases (Bilen and Bonini, 2005). At least ~75% of human disease genes have *Drosophila* homologues (Reiter et al., 2001). Indeed to analyze of gene functions *in vivo*, *Drosophila* has other manifold advantages as rapid generation cycle (10–14 days) with a short life span (50–60 days); suitability for genetic analysis, abundant genetic information, complex behavior processes including learning and memory and little labor and cost-effective to maintain fly stocks (Hirth, 2010). In this way, *Drosophila* models of PD have already been established and have successfully provided valuable insights into the elucidation of pathomechanisms and development of new therapies for this disease. Although levodopa and dopamine agonists are the basis for symptomatic PD's treatment, many studies have been suggested natural and synthetic antioxidant therapies as alternatives to treat this disorder (Auluck and Bonini, 2002, de Oliveria et al., 2009, Liu et al., 2010).

A variety of seleno organic compounds have been considered potent antioxidant and neuroprotective agents (Commandeur et al., 2001, Klotz and Sies, 2003). In accordance, results of clinical trials showed the benefit of the synthetic selenium-containing heterocycle ebselen (PZ51; 2-phenyl-1,2-benzisoselenazol-3(2H)-one) in the treatment of patients with acute ischemic stroke or aneurysma subarachnoid hemorrhage (Yamaguchi et al., 1998, Ogawa et al.,

1999). Consequently, diselenides are good candidates to become antioxidant agents because they have some chemical and biochemical characteristics in common with ebselen, i.e., they exert glutathione peroxidase-like activity and can react with –SH groups, forming selenosulfide or selenol (−SeH) as well as disulfides (Barbosa et al., 1998, Brandao et al., 2006). The simplest diaryl diselenide, diphenyl diselenide (DPDS), is a synthetic organoseleno compound with potential role associated to its therapeutic or toxic action (Rocha, 2010). Interestingly, DPDS has been shown to be more active as glutathione peroxidase and thioredoxin reductase mimic than ebselen (Spector et al., 1989, Nogueira et al., 2004, Sausen de Freitas et al., 2010). Indeed, DPDS exhibits anti-inflammatory, anti-nociceptive and specially neuroprotective effects in several *in vivo* experimental models when used at low doses (Nogueira et al., 2004). On the order hand, at high concentrations, DPDS can be toxic to rodents, yeast, bacteria and fruitfly (Maciel et al., 2000; Moreira Rosa et al., 2005; Brito et al., 2006; Golombieski et al., 2008). Actually, acute or prolonged exposure to relatively high doses of DPDS causes marked inhibition of hepatic and renal δ-ALA-D from mice, rats as well as inhibits *Drosophila* δ-ALA-D gene transcription and enzyme activity (Golombieski et al., 2008).

Based on the evidence addressed previously, this study was delineated to investigate the effects of dietary DPDS in *Drosophila* intoxicated with rotenone; in order to establish whether in the concentration tested the compound could be considered an therapeutic or a toxic supplement.

2 MATERIALS AND METHODS

2.1 Drugs

Rotenone, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCHF-DA) and DPDS were obtained from Sigma Chemical Company (St Louis, MO, USA). All other reagents were commercial products of the highest purity grade available.

2.2 *Drosophila* Stock

Drosophila melanogaster wild-type (1-2 days old) was obtained from the National species Stock Center, Bowling Green, OH, USA. The cornmeal food for flies was made from cornmeal, sugar, yeast, and agar according to Klein et al, 1999. The flies were reared in 2.5cm x 6.5cm vials containing 5 mL of cornmeal medium and flies were maintained in an incubator at constant temperature, humidity (23°C±1°C and 60% relative humidity) and under 12h dark/light cycle before being used for different assays.

2.3 Rotenone exposure and DPDS treatment

Adult flies (female and males) were divided into five groups: (1) control; (2) ethanol; (3) rotenone; (4) DPDS and (5) rotenone plus DPDS. Rotenone and DPDS were dissolved separately in ethanol (98%) and after were added into the food`s flies at final concentration of 0.5 and 1 mM, respectively. Two controls were used (group with and without ethanol). The total food medium contained a total volume of 1% of ethanol. The flies were exposed to respective treatments during 7 days. The concentration of rotenone corresponds to the concentration that caused approximately 50% death of the flies up to 7 days of exposure (data not shown).

2.4 Survival rate

The survival was evaluated by counting daily of the number of living flies until the end of the experimental period (7 days). Around 100 flies per group were included in the survival data.

2.5 Negative Geotaxis

Locomotor function of flies was performed with a negative geotaxis assay as described previously by (Feany and Bender, 2000). Flies (female/male) were sorted under ice anesthesia

and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 5 flies each). After 10 minutes of recovery from cold exposure, flies were gently tapped to the bottom of the column. The flies that reached the top of the column (6 cm) and the flies that remained at the bottom were counted separately during 6 seconds. The scores represent the mean of the numbers of flies at the top (n_{top}) and at the bottom (n_{bot}) as percentage of the total number of flies (n_{tot}). Around 15 flies were included for each treatment performed and the total number of flies contained in the negative geotaxis data (45 per group) represents the sum of three independent experiments.

2.6 Homogenate preparation

Twenty-four hours after the end of treatments, around 20 flies per group were immobilized by chilling on ice and then decanted into a chilled mortar. Whole body of flies was manually homogenized in ice-cold Tris/HCl buffer (pH 7.4, 0.1 M), 1:10 (flies/volume (μL). The homogenate was filtered through sieve with nylon mesh (pore size/1 mm), centrifuged at 3000 g for 3 min at 4 °C, and the supernatant was used for *in vitro* biochemical assays, where each n= 20 flies/200 μL.

2.7 Cell viability evaluation

Cell viability was evaluated by dehydrogenase activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This analysis was performed in intact body of flies according to the method described by (Babot et al., 2005). The ratio values were standardized per protein content and expressed as percentage in relation to the control.

2.8 Thiol determination

Total and non-protein thiol content were estimated based on a spectrophotometric method using Ellman`s reagent, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) (Ellman, 1959). For non-protein thiol, the fly homogenate was precipitated with TCA 10% followed by centrifugation at 3000 g for 3 min at 4°C. The supernatant samples were measured espectrophotometrically at 412 nm. A standard curve was constructed for each measurement using GSH. Thiol levels were expressed as % in relation to the control.

2.9 Reactive Oxygen species (ROS) determination

The generation of ROS was determined fluorimetrically in fly homogenate using the membrane permeable fluorescent dye 2’,7’-dichlorofluorescein diacetate (DCHF-DA) (Hempel et al., 1999). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured at 488 nm for excitation and 525 nm for emission, with slit widths of 1.5 nm. The values are expressed as percentage in relation to the control.

2.10 Protein determination

Protein concentrations in the whole body homogenates were determined by the method of (Bradford, 1976), using bovine serum albumin as the standard.

2.11 Statistical analyses

Survival data was analyzed statistically by the X2 method and Fisher’s exact test. All other parameters were analyzed by Two-way ANOVA followed by Duncan Multiple Range Test when appropriate. Differences between groups were considered significant when $p<0.05$. Data of statistical analysis are represented as means and S.E.M.

3 RESULTS

3.1 Survival rate

Rotenone caused a progressive decrease on the survival rate of the flies when compared to control groups (Figure 1). From day 4, the percentages of death were more pronounced in the flies exposed to rotenone and concomitantly supplemented with DPDS ($p<0.05$). Indeed, dietary DPDS caused per se a significant reduction on the survival rate of the flies after 7 days of supplementation.

3.2 Negative Geotaxis assay

Two-way ANOVA revealed a main effect of rotetone [F(1,8=111, $p<0.05$] on negative geotaxis test (Figure 2). Post hoc comparisons showed that the climbing response of the flies exposed to rotenone was significantly decreased when compared to control groups ($p<0.05$). This climbing behavior rotenone-induced was not modified by dietary DPDS ($p<0.05$). No statistical difference was observed between control and DPDS groups.

3.3 Cell viability

Two-way ANOVA revealed a main effect of DPDS on cell viability of the flies [F(1,24=13.6, $p<0.05$]. Post Hoc comparisons showed that the flies exposed to rotenone had a significant reduction on cell viability when compared to the control groups ($p<0.05$; Figure 3). Dietary DPDS was not effective in offering protection against this effect induced by rotenone. No significant difference was observed between DPDS and control groups.

3.4 Thiol content

Two-way ANOVA revealed a significant main effect of rotenone [F(1,8=10.25, $p<0.05$] and DPDS [F(1,8=30.75, $p<0.05$] on total protein thiol levels of the flies (Figure 4A). *Post Hoc* comparisons showed that the rotenone exposure caused a significant reduction on total thiol protein of the flies when compared to control groups ($p<0.05$), which was restored by dietary DPDS. Indeed, the DPDS caused per se an increase in the total thiol protein levels of the flies. There was no difference among the groups on non-protein thiol content parameter (Figure 4B).

3.5 ROS levels

Two-way ANOVA revealed a significant main effect of rotenone [F(1,8=8.58, p<0.019] and DPDS [F(1,8=16, p<0.039] on ROS production. Post Hoc comparisons indicated that ROS levels were significantly increased in the flies exposed to rotenone and that dietary DPDS was not effective in ameliorating this effect rotenone-induced (p<0.05). Similarly, the flies supplemented with DPDS also had the ROS levels increased when compared to the values found in the control groups (p<0.05).

REFERENCES

Auluck PK, Bonini NM (2002) Pharmacological prevention of Parkinson disease in *Drosophila*. Nat Med 8:1185-1186.

Babot Z, Cristofol R, Sunol C (2005) Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on $GABA_A$ receptors and niflumic acid-sensitive chloride channels. Eur J Neurosci 21:103-112.

Barbosa NB, Rocha JB, Zeni G, Emanuelli T, Beque MC, Braga AL (1998) Effect of organic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. Toxicol Appl Pharmacol 149:243-253.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3:1301-1306.

Bilen J, Bonini NM (2005) *Drosophila* as a model for human neurodegenerative disease. Annu Rev Genet 39:153-171.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Brandao R, Santos FW, Farina M, Zeni G, Bohrer D, Rocha JB, Nogueira CW (2006) Antioxidants and metallothionein levels in mercury-treated mice. Cell Biol Toxicol 22:429-438.

Brito VB, Folmer V, Puntel GO, Fachinetto R, Soares JC, Zeni G, Nogueira CW, Rocha JB (2006) Diphenyl diselenide and 2,3-dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. Brain Res Bull 68:414-418.

Commandeur JN, Rooseboom M, Vermeulen NP (2001) Chemistry and biological activity of novel selenium-containing compounds. Adv Exp Med Biol 500:105-112.

Coulom H, Birman S (2004) Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. J Neurosci 24:10993-10998.

Dawson TM, Dawson VL (2003) Molecular pathways of neurodegeneration in Parkinson's disease. Science 302:819-822.

de Oliveria DM, Barreto G, De Andrade DV, Saraceno E, Aon-Bertolino L, Capani F, Dos Santos El Bacha R, Giraldez LD (2009) Cytoprotective effect of *Valeriana officinalis* extract on an in vitro experimental model of Parkinson disease. Neurochem Res 34:215-220.

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-77.

Feany MB, Bender WW (2000) A *Drosophila* model of Parkinson's disease. Nature 404:394-398.

Golombieski RM, Graichen DA, Pivetta LA, Nogueira CW, Loreto EL, Rocha JB (2008) Diphenyl diselenide [(PhSe)2] inhibits *Drosophila melanogaster* delta-aminolevulinate dehydratase (delta-ALA-D) gene transcription and enzyme activity. Comp Biochem Physiol C Toxicol Pharmacol 147:198-204.

Greenamyre JT, Hastings TG (2004) Biomedicine. Parkinson's-divergent causes, convergent mechanisms. Science 304:1120-1122.

Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM (1999) Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med 27:146-159.

Hirth F (2010) *Drosophila melanogaster* in the study of human neurodegeneration. CNS Neurol Disord Drug Targets 9:504-523.

Klotz LO, Sies H (2003) Defenses against peroxynitrite: selenocompounds and flavonoids. Toxicol Lett 140-141:125-132.

Langston JW (2002) Parkinson's disease: current and future challenges. Neurotoxicology 23:443-450.

Liu L, Xu H, Jiang H, Wang J, Song N, Xie J (2010) Ghrelin prevents 1-methyl-4-phenylpyridinium ion-induced cytotoxicity through antioxidation and NF-kappaB modulation in MES23.5 cells. Exp Neurol 222:25-29.

Moreira Rosa R, de Oliveira RB, Saffi J, Braga AL, Roesler R, Dal-Pizzol F, Fonseca Moreira JC, Brendel M, Pegas Henriques JA (2005) Pro-oxidant action of diphenyl diselenide in the yeast Saccharomyces cerevisiae exposed to ROS-generating conditions. Life Sci 77:2398-2411.

Nogueira CW, Zeni G, Rocha JB (2004) Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem Rev 104:6255-6285.

Ogawa A, Yoshimoto T, Kikuchi H, Sano K, Saito I, Yamaguchi T, Yasuhara H (1999) Ebselen in acute middle cerebral artery occlusion: a placebo-controlled, double-blind clinical trial. Cerebrovasc Dis 9:112-118.

Reiter LT, Potocki L, Chien S, Gribskov M, Bier E (2001) A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. Genome Res 11:1114-1125.

Nogueira CW, Rocha JBT (2010) Diphenyl Diselenide a Janus-Faced Molecule. Journal of Brazilian Chemical Society 21:2055-2071.

Sausen de Freitas A, de Souza Prestes A, Wagner C, Haigert Sudati J, Alves D, Oliveira Porciuncula L, Kade IJ, Teixeira Rocha JB (2010) Reduction of diphenyl diselenide and analogs by mammalian thioredoxin reductase is independent of their gluthathione peroxidase-like activity: a possible novel pathway for their antioxidant activity. Molecules 15:7699-7714.

Spector A, Wilson SR, Zucker PA, Huang RR, Raghavan PR (1989) The synthesis of glutathione peroxidase analogs. Lens Eye Toxic Res 6:773-780.

Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H (1998) Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. Stroke 29:12-17.

LEGENDS FOR FIGURES

Figure 1 - Effect of DPDS on survival rate of flies exposed to rotenone. Data were collected every 24 h for each group during 7 days. The numbers of surviving flies are represented as % of control. The total number of flies (100 per group) represents the sum of three independent experiments. *represent significant difference in relation to the control group (Fischer's exact probability test, $p < 0.05$).

Figure 2 - Effect of DPDS on climbing response of flies exposed to rotenone during 7 days. The total number of flies (45 per group) represents the sum of three independent experiments. *represent significant difference in relation to the control group (Two-way ANOVA followed Duncan Multiple Range Test, $p < 0.05$). Values are expressed as mean±S.E.M

Figure 3 - Effect of DPDS on cell viability in intact body of flies exposed to rotenone. *represents significant difference in relation to control groups (n=8-10). Values are expressed as mean±S.E.M (Two-way ANOVA followed by Duncan Multiple Range Test, $P < 0.05$).

Figure 4 - Effect of *DPDS* on protein (A) and non protein-thiol (B) content in homogenate of flies exposed to rotenone. *represents significant difference from control groups (n=4). Data are expressed as mean±S.E.M ($P < 0.05$, Two-way ANOVA followed by Duncan multiple range Test).

Figure 5 - Effect of DPDS on ROS production in homogenate of flies exposed to rotenone. *represents significant difference from control groups (n=4). Data are expressed as mean±S.E.M (Two-way ANOVA followed by Duncan multiple range Test, $P < 0.05$).

Figure 1

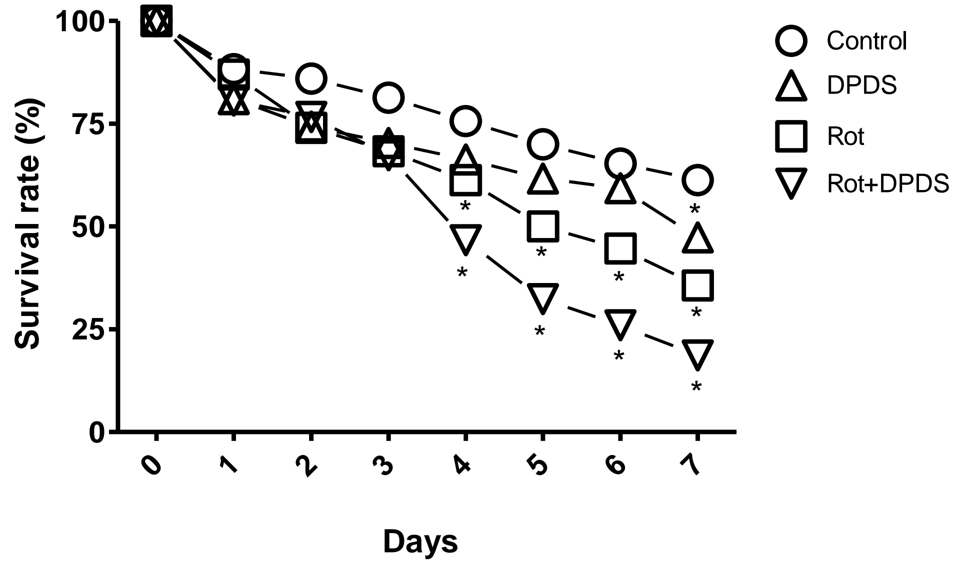


Figure 2

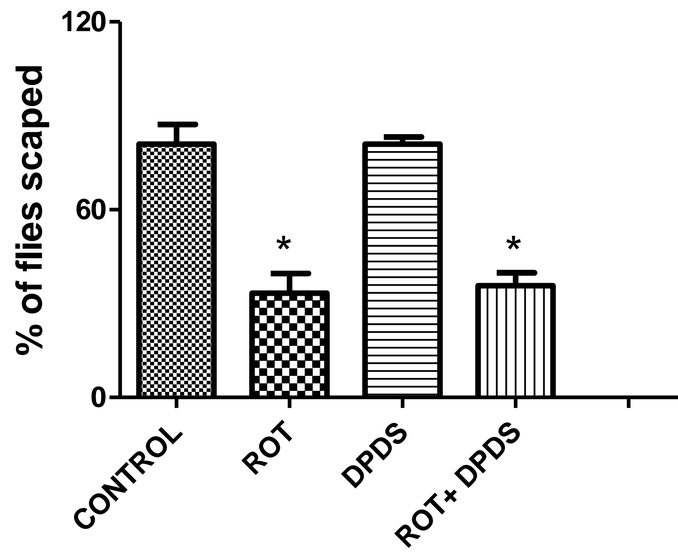


Figure 3

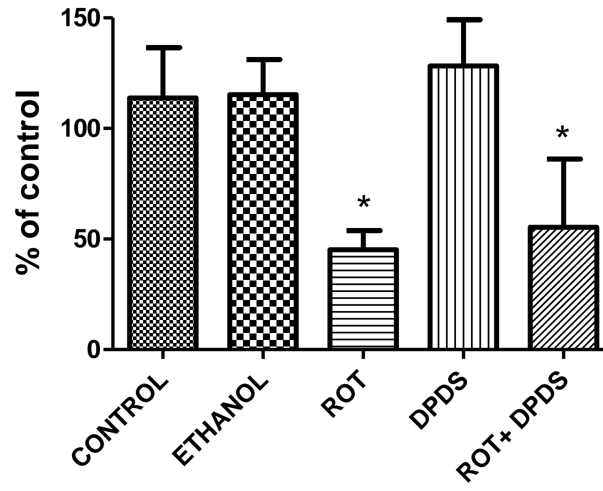


Figure 4

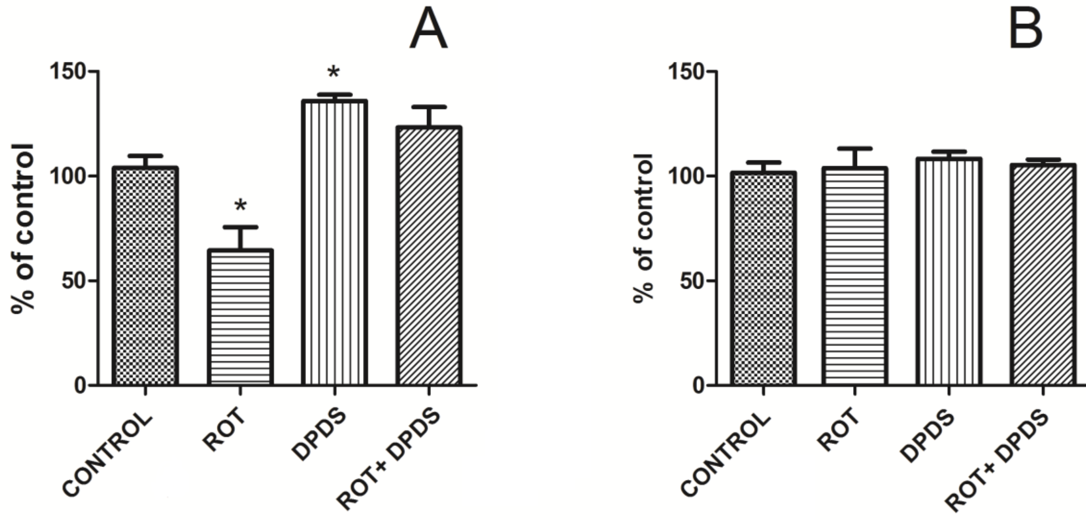
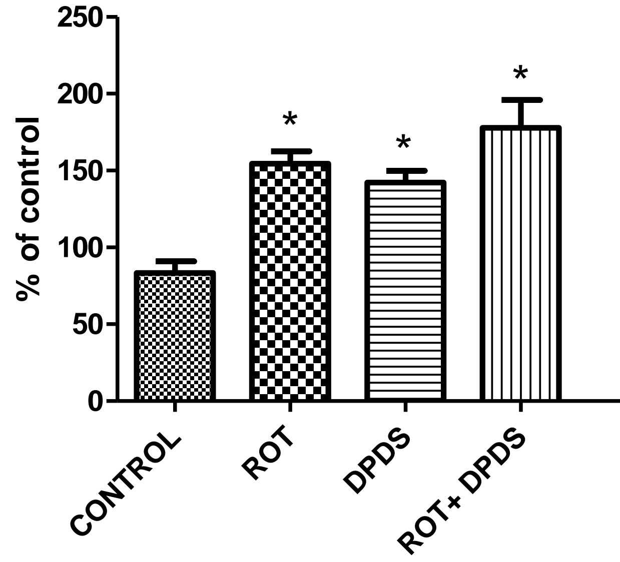


Figure 5



3 DISCUSSÃO

Nos últimos anos, a mosca da fruta *D. melanogaster* vem sendo amplamente utilizada modelos experimentais na busca por novos agentes terapêuticos, principalmente no auxílio da prevenção e/ou cura de doenças neurológicas, incluindo a DP (Hosamani and Muralidhara, 2009, Jeibmann and Paulus, 2009). Nesse contexto, este trabalho teve como objetivo avaliar o potencial terapêutico da planta *V. officinalis* e do organocalcogênio DPDS frente aos efeitos neurotóxicos do pesticida rotenona em *D. melanogaster.* Como o DPDS já possui atividade antioxidante bem estabelecida *in vitro* e *in vivo* (Posser et al., 2006, Posser et al., 2008, Hassan et al., 2009, Brandao et al., 2010); e poucos estudos foram realizados a respeito da eficácia da *V. officinalis* frente à geração ERO (Malva et al., 2004), a primeira etapa do presente estudo foi delineada para avaliar a composição química, bem como, o potencial antioxidante da *V.officinalis in vitro.* Considerando a alta suscetibilidade do cérebro ao dano causado por RL, a relação existente entre doenças neurodegenerativas e EO e o papel da *V. officinalis* em nível central, o tecido cerebral foi escolhido para avaliar os efeitos do extrato etanólico da *V. officinalis* frente aos insultos oxidativos induzidos por diferentes agentes pró-oxidantes através do ensaio de TBARS. Nesta avaliação foram usados agentes cuja ação neurotóxica está ligada a mecanismos diversos, como por exemplo: *(i)* Fe(II) que se oxida a íon férrico (Fe(III)) e, na presença de H_2O_2, estimula a reação de Fenton, formando o radical hidroxila ($OH\cdot^-$) (Laguerre et al., 2007); *(ii)* NPS, cuja toxicidade está associada à liberação de cianeto e/ou óxido nítrico, o qual pode gerar ânion peroxinitrito ($ONOO^-$) (Bates et al., 1991, Bolanos and Almeida, 1999); *(iii)* AQ, um agonista seletivo de receptores glutamato do subtipo NMDA, que causa um aumento na concentração de cálcio citosólico, na depleção de ATP e GABA (Foster et al., 1983) e leva à formação de radical superóxido e EO (Rios and Santamaria, 1991, Belle et al., 2004) e (*iv*) 3-ANP, que possui ação tóxica devido à inibição irreversível da succinato desidrogenase e consequente disfunção mitocondrial (Tunez et al., 2004). Os resultados obtidos nesses parâmetros mostraram que o extrato de *V. officinalis* foi eficaz em inibir a PL causada por todos os agentes pró-oxidantes testados. No entanto, o efeito antioxidante exibido pelo extrato variou com relação ao tipo de pró-oxidante utilizado, ou seja, a ação antioxidante foi mais efetiva contra os danos induzidos pelo 3-ANP, AQ e NPS quando comparado ao Fe(II) e ao complexo Fe(II)/EDTA. Ainda neste trabalho,

foi verificado que a *V. officinalis* também exibiu efeito protetor contra a degradação da desoxirribose (reação de Fenton) e a geração de ERO induzida por AQ em fatias de córtex cerebral de ratos. De forma geral, os dados encontrados na primeira parte deste estudo indicaram a efetividade da *V. officinalis* em oferecer proteção contra a PL cerebral *in vitro* e serviram como base para a escolha do uso desta planta como agente antioxidante/neuroprotetor em estudos *in vivo* de doenças neurológicas relacionadas com o EO, como por exemplo, a DP. Com relação à modelo experimental *in vivo* de DP e terapia natural utilizando a *V. officinalis*, não há dados na literatura. No entanto, um recente estudo demonstrou o efeito citoprotetor da *V. officinalis* em um modelo de Parkinson *in vitro*, onde foi especialmente avaliado a viabilidade celular de neuroblastomas frente ao dano causado pela rotenona (Oliveira e *cols*, 2008). Assim, com ênfase na ação da *V. officinalis* sobre as complicações causadas pela DP, nossas pesquisas foram direcionadas para um protocolo experimental *in vivo* envolvendo o pesticida rotenona e a mosca da fruta *D. melanogaster*, os quais vêm sendo amplamente utilizados na reprodução de sintomas associados à DP (Jeibmann and Paulus, 2009). Neste trabalho foi observado que os danos motores, os danos oxidativos e a taxa de mortalidade induzidos pela exposição das moscas ao pesticida foram significativamente reduzidos pelo tratamento concomitante com *V. officinalis.* Na avaliação da função locomotora, a perfomance das moscas expostas à rotenona foi significativamente alterada, tanto no teste de escalada quanto no campo-aberto (cruzamentos e imobilidade), quando comparada ao grupo controle. A maioria destas alterações locomotoras observadas nas moscas expostas a rotenona foram normalizadas pelo tratamento com *V. officinalis.* O tratamento ainda foi eficaz em reduzir a taxa mortalidade, tornando as moscas mais resistentes à exposição ao pesticida. De acordo com os nossos resultados, recentemente foi demonstrado a efetividade da *V. officinalis* em previnir os danos motores causados pela reserpina em um modelo de discnesia orofacial em ratos (Pereira et al., 2011).

Vários estudos têm relatado que a exposição a neurotoxinas ambientais, via disfunção mitocondrial, pode ser responsável pelo aparecimento de casos esporádicos de DP em humanos, podendo causar morte de neurônios dopaminérgicos, formação de corpos de *Lewy* (Coulom and Birman, 2004, Goldman et al., 2011) e até mesmo alterações em importantes genes ligados à DP (Rajput and Rajput, 2007, Wang et al., 2009). Em termos de mecanismos, neste estudo foi verificado que a exposição à rotenona causou aumento na expressão de mRNA das enzimas antioxidantes SOD e CAT e na enzima TH, os quais podem ter sido decorrentes de uma resposta

compensatória aos insultos oxidativos induzidos pelo pesticida como: o aumento da produção mitocondrial de H_2O_2 e a diminuição do conteúdo de tiol protéico e da viabilidade celular. É importante ressaltar, que essas alterações não foram observadas nas moscas concomitantemente tratadas com *V. officinalis*. Esses dados sugerem que a ação tóxica da rotenona em *D. melanogaster,* está possivelmente associada com a geração de ERO e com os eventos moleculares provocados pela ação dos radicais livres nas células, bem como, comprovam a efetividade do uso *in vivo* da *V. officinalis* como um agente antioxidante/neuroprotetor neste modelo de indução de sintomas de DP. Conforme verificado pela análise da composição do extrato, é provável que os efeitos exibidos pela *V. officinalis* sejam oriundos dos seus constituintes fenólicos, os quais são reconhecidos como agentes antioxidantes. Em conjunto, os dados dos trabalhos realizados com *V. officinalis*, nos permitem sugerir que a ingestão de *V. officinalis* pode ser considerada benéfica para proteger o SNC de insultos oxidantes, bem como, um promissor agente terapêutico para o tratamento de doenças neurodegenerativas relacionadas ao EO em humanos, tal como a DP.

A busca por compostos com propriedades farmacológicas promissoras vai além dos antioxidantes naturais. Neste contexto, os compostos de Se vêm recebendo uma especial atenção, principalmente devido ao fato do elemento Se ser requerido nutricionalmente para diversas funções fisiológicas e apresentar potente atividade antioxidante por fazer parte do sítio ativo de classes de enzimas como a GPx e do sistema TrxR (Rotruck et al., 1973). No entanto, os estudos farmacológicos envolvendo compostos de selênio sempre vêm acompanhados de investigações toxicológicas, uma vez que a janela terapêutica entre a concentração que induz toxicidade e a que induz efeitos benéficos é muito pequena. Mesmo assim, um número relativamente grande de pesquisas relacionadas tem demonstrado a eficácia de compostos sintéticos de Se principalmente como antioxidantes, anticarcinogênicos e neuroprotetores (Commandeur et al., 2001, Klotz and Sies, 2003). Com importância clínica comprovada, destacam-se os estudos evidenciando o papel do organoselênio Ebselen como neuroprotetor (Yamaguchi et al., 1998, Ogawa et al., 1999). Desde então, os disselenetos, em geral, vêm sendo testados como promissores antioxidantes e neuroprotetores por possuírem relações químicas e bioquímicas em comum com este composto. Com ênfase na ação mimética à enzima GPx e ao substrato da TrxR, já foi verificado que DPDS exibe atividade superior ao Ebselen (Spector et al., 1989). Além disso, em modelos experimentais utilizando roedores este composto foi menos tóxico que o Ebselen e apresentou,

além da atividade antioxidante e neuroprotetora, uma série de outros efeitos farmacológicos *in vivo* em doses relativamente baixas (Nogueira et al., 2004; Nogueira & Rocha, 2010). Por outro lado, trabalhos avaliando o papel do composto em modelos experimentais em espécies como *D. melanogaster*, bem como, em doenças neurodegenerativas ainda são raros na literatura. Assim, outro foco deste estudo foi avaliar os efeitos do DPDS, como suplemento alimentar, sobre alguns distúrbios motores e bioquímicos em *D. melanogaster* expostas a rotenona. De forma geral, os resultados obtidos até o momento demonstraram que a suplementação com DPDS, na concentração de 1 mM, não ofereceu proteção contra os efeitos induzidos pela exposição a rotenona. Além disso, as moscas expostas a rotenona e suplementadas com DPDS tiveram uma taxa de mortalidade maior e a ingestão do composto causou *per se* um aumento nos níveis de ERO. Este efeito do composto pode indicar que a concentação testada foi relativamente alta e causou toxicidade em *D. melanogaster*. Está bem descrito na literatura que, em doses elevadas, o DPDS, bem como, outras formas de Se, exibe efeitos pró-oxidantes *in vitro* e *in vivo* em roedores (Nogueira e Rocha, 2010). Com relação a *D. melanogaster,* já foi observado que o DPDS inibe a atividade e a transcrição gênica da enzima sulfidrílica δ-aminolevulinato desidratase em *D. melanogaster* (Golombieski et al., 2008). No entanto, via suplementação na dieta, poucos estudos avaliaram os efeitos do DPDS; e diferente dos resultados obtidos com *D. melanogaster*, foi evidenciado que a ingestão crônica de DPDS não causou toxicidade em ratos (1-10 ppm) e em coelhos (0.3-30 ppm) (de Bem et al., 2007, Barbosa et al., 2008, Dias et al., 2012). Assim, mais estudos são necessários para estabelecer os efeitos farmacológicos e toxicológicos do DPDS em *D. melanogaster*.

Em resumo, os resultados obtidos nos trabalhos realizados mostram a potente ação antioxidante da *V. officinalis* em modelos de neurotoxicidade *in vitro* (tecido cerebral de rato) e *in vivo* (*D. melanogaster),* bem como, sugerem que o uso desta planta deve ser mais investigado, uma vez que, este pode ser benéfico para tratar as complicações da DP. Com relação, ao uso do DPDS para o tratamento da DP em *D. melanogaster*, é necessário a realização de mais experimentos *in vivo* e *ex vivo*, a fim de estabelecer o efeito dose/resposta deste composto para esta espécie.

4 CONCLUSÕES

Baseando-se nos resultados apresentados nesta tese, pode-se concluir:

Análises *in vitro*

- O extrato etanólico da *V. officinalis* apresentou quantidades representativas de ácido valérico, ácido gálico e quercitina.
- O extrato etanólico de *V. officinalis* inibiu a geração de TBARS causada por diferentes agentes pró-oxidantes, em homogeneizado de tecido cerebral de rato *in vitro*; diminuiu a degradação da desoxirribose e a formação ERO induzida pelo AQ**.**

Análises *in vivo*

- O extrato aquoso de *V. officinalis* foi eficaz em diminuir a mortalidade em *D. melanogaster* expostas à rotenona; enquanto a suplementação com DPDS, além de não oferecer proteção, causou *per se* um aumento na taxa de mortalidade;
- O tratamento com *V. officinalis* reduziu os efeitos tóxicos da rotenona associados aos distúrbios locomotores referentes ao teste de escalada e ao tempo de mobilidade; enquanto a suplementação com DPDS não modificou a alteração locomotora (teste e escalada) induzida por rotenona.

Análises *ex vivo*

- O tratamento com *V. officinalis* protegeu as moscas contra as alterações causadas pela rotenona nos níveis de tiol-protéico, na viabilidade celular e nos níveis de ERO; enquanto o DPDS não ofereceu proteção nos parâmetros bioquímicos analisados;
- O tratamento com *V. officinalis* foi eficaz em restaurar, aos níveis do controle, as alterações induzidas por rotenona na expressão das enzimas antioxidantes SOD e CAT.

.

5 PERSPECTIVAS

A partir dos resultados apresentados nesta tese, poderíamos realizar estudos com os seguintes objetivos:

- Quantificar os níveis de dopamina e seus metabólitos em *D. melanogaster* submetidas à intoxicação por rotenona e tratadas com os respectivos antioxidantes;
- Realizar imunohistoquímica de proteínas associadas com EO em amostras de *D. melanogaster* expostas à rotenona e tratadas com extrato aquoso da raiz de *V. officinalis* ou DPDS;
- Avaliar parâmetros de função mitocondrial em isolados mitocondriais de *D. melanogaster* expostas à rotenona e tratamentos;
- Avaliar os efeitos de pré e pós-tratamento com extrato aquoso da raiz de *V.officinalis* ou DPDS sobre parâmetros comportamentais e bioquímicos em *D. melanogaster* expostas à rotenona;
- Comparar os efeitos obtidos com DPDS neste modelo experimental com o uso de concentrações mais baixas do composto, a fim de fazer um comparativo com relação a dose/resposta nesta espécie.

DEMAIS TRABALHADOS REALIZADOS DURANTE O PERÍODO DO DOUTORADO:

1. Sudati JH, Rosseti IB, Alberto EE, Soares LC, Braga AL, Costa MS, Rocha JBT. Antifungal Evaluation of Different Diselenoamino Acid Compounds on *Candida albicans* Growth. (MANUSCRITO).

2. Sudati JH, Saraiva RA, Alberto EE, Wagner C, Soares LC, Nogara PA, Bueno DC, Freitas AS, Braga AL, Rocha JBT. New Diselenoamino Acid Derivatives: Evaluation of GPx Mimic Properties and TrxR Substrate Activity. (MANUSCRITO).

3. Pereira RP, Fachinetto R, Prestes AS, Wagner C, Sudati JH, Boligon AA, Athayde ML, Morsch VM, Rocha JBT. *Valeriana officinalis* ameliorates vacuous chewing movements induced by reserpine in rats. *Journal of Neural Transmission,* v. 118, p. 1547-1557, 2011.

4. Wagner C, Sudati JH, Nogueira CW, Rocha JBT. *In vivo* and *in vitro* inhibition of mice thioredoxin reductase by methylmercury. *BioMetals (Oxford), v. 23, p. 1171-1177, 2010.*

REFERÊNCIAS

Alberto EE SL, Sudati JH, Borges ACA, Rocha JBT, Braga AL (2009) Efficient Synthesis of Modular Amino Acid Derivatives Containing Selenium with Pronounced GPx-Like Activity. Eur J Org Chem 4211–4214

Auluck PK, Bonini NM (2002) Pharmacological prevention of Parkinson disease in Drosophila. Nat Med 8:1185-1186.

Barbosa NB, Oliveira C, Araldi D, Folmer V, Rocha JB, Nogueira CW (2008) Acute diphenyl diselenide treatment reduces hyperglycemia but does not change delta-aminolevulinate dehydratase activity in alloxan-induced diabetes in rats. Biol Pharm Bull 31:2200-2204.

Barbosa NB, Rocha JB, Wondracek DC, Perottoni J, Zeni G, Nogueira CW (2006) Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. Chem Biol Interact 163:230-238.

Barbosa NB, Rocha JB, Zeni G, Emanuelli T, Beque MC, Braga AL (1998) Effect of organic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. Toxicol Appl Pharmacol 149:243-253.

Bastianetto S, Krantic S, Quirion R (2008) Polyphenols as potential inhibitors of amyloid aggregation and toxicity: possible significance to Alzheimer's disease. Mini Rev Med Chem 8:429-435.

Bates JN, Baker MT, Guerra R, Jr., Harrison DG (1991) Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. Biochem Pharmacol 42 Suppl:S157-165.

Beal MF (2007) Mitochondria and neurodegeneration. Novartis Found Symp 287:183-192; discussion 192-186.

Belle NA, Dalmolin GD, Fonini G, Rubin MA, Rocha JB (2004) Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. Brain Res 1008:245-251.

Bolanos JP, Almeida A (1999) Roles of nitric oxide in brain hypoxia-ischemia. Biochim Biophys Acta 1411:415-436.

Bonini NM, Fortini ME (2003) Human neurodegenerative disease modeling using Drosophila. Annu Rev Neurosci 26:627-656.

Brandao R, de Oliveira R, Nogueira CW (2010) Association between diphenyl diselenide and cadmium chloride attenuates the toxicity of both in tissues of mice in vitro. Toxicol In Vitro 24:1736-1742.

Brandao R, Santos FW, Farina M, Zeni G, Bohrer D, Rocha JB, Nogueira CW (2006) Antioxidants and metallothionein levels in mercury-treated mice. Cell Biol Toxicol 22:429-438.

Cannon JR, Greenamyre JT (2010) Neurotoxic in vivo models of Parkinson's disease recent advances. Prog Brain Res 184:17-33.

Cicchetti F, Drouin-Ouellet J, Gross RE (2009) Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci 30:475-483.

Cirelli C (2006) Cellular consequences of sleep deprivation in the brain. Sleep Med Rev 10:307-321.

Collins MA, Neafsey EJ (2002) Potential neurotoxic "agents provocateurs" in Parkinson's disease. Neurotoxicol Teratol 24:571-577.

Coulom H, Birman S (2004) Chronic exposure to rotenone models sporadic Parkinson's disease in Drosophila melanogaster. J Neurosci 24:10993-10998.

D'Almeida V, Lobo LL, Hipolide DC, de Oliveira AC, Nobrega JN, Tufik S (1998) Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport 9:2853-2856.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.

de Bem AF, de Lima Portella R, Farina M, Perottoni J, Paixao MW, Nogueira CW, Teixeira Rocha JB (2007) Low toxicity of diphenyl diselenide in rabbits: a long-term study. Basic Clin Pharmacol Toxicol 101:47-55.

Dias GR, Vieira FA, Dobrachinski F, Bridi JC, Balk RD, Soares FA, Nogueira CW, Barbosa NB (2012) Diphenyl diselenide diet intake improves spatial learning and memory deficits in hypothyroid female rats. Int J Dev Neurosci.

Fachinetto R, Villarinho JG, Wagner C, Pereira RP, Avila DS, Burger ME, Calixto JB, Rocha JB, Ferreira J (2007) Valeriana officinalis does not alter the orofacial dyskinesia induced by haloperidol in rats: role of dopamine transporter. Prog Neuropsychopharmacol Biol Psychiatry 31:1478-1486.

Fahn S, Oakes D, Shoulson I, Kieburtz K, Rudolph A, Lang A, Olanow CW, Tanner C, Marek K (2004) Levodopa and the progression of Parkinson's disease. N Engl J Med 351:2498-2508.

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239-247.

Foster AC, Collins JF, Schwarcz R (1983) On the excitotoxic properties of quinolinic acid, 2,3-piperidine dicarboxylic acids and structurally related compounds. Neuropharmacology 22:1331-1342.

Freitas AS PA, Wagner C, Sudati JH, Alves D, Porciuncula LO, Kade IJ, Rocha JBT (2010) Reduction of Diphenyl Diselenide and Analogs by Mammalian Thioredoxin Reductase Is Independent of Their Gluthathione Peroxidase-Like Activity: A Possible Novel Pathway for Their Antioxidant Activity. Molecules 15:7699-7714.

Goldman SM, Quinlan PJ, Ross GW, Marras C, Meng C, Bhudhikanok GS, Comyns K, Korell M, Chade AR, Kasten M, Priestley B, Chou KL, Fernandez HH, Cambi F, Langston JW, Tanner CM (2011) Solvent exposures and parkinson disease risk in twins. Ann Neurol.

Golombieski RM, Graichen DA, Pivetta LA, Nogueira CW, Loreto EL, Rocha JB (2008) Diphenyl diselenide [(PhSe)2] inhibits Drosophila melanogaster delta-aminolevulinate dehydratase (delta-ALA-D) gene transcription and enzyme activity. Comp Biochem Physiol C Toxicol Pharmacol 147:198-204.

Greenamyre JT (2001) Glutamatergic influences on the basal ganglia. Clin Neuropharmacol 24:65-70.

Gwinn-Hardy K (2002) Genetics of parkinsonism. Mov Disord 17:645-656.

Hallam KT, Olver JS, McGrath C, Norman TR (2003) Comparative cognitive and psychomotor effects of single doses of Valeriana officianalis and triazolam in healthy volunteers. Hum Psychopharmacol 18:619-625.

Halliwell B (1992) Reactive oxygen species and the central nervous system. J Neurochem 59:1609-1623.

Halliwell B (2007) Biochemistry of oxidative stress. Biochem Soc Trans 35:1147-1150.

Hassan W, Ibrahim M, Nogueira CW, Braga AL, Mohammadzai IU, Taube PS, Rocha JB (2009) Enhancement of iron-catalyzed lipid peroxidation by acidosis in brain homogenate: comparative effect of diphenyl diselenide and ebselen. Brain Res 1258:71-77.

Hirth F (2010) Drosophila melanogaster in the study of human neurodegeneration. CNS Neurol Disord Drug Targets 9:504-523.

Hosamani R, Muralidhara (2009) Neuroprotective efficacy of Bacopa monnieri against rotenone induced oxidative stress and neurotoxicity in Drosophila melanogaster. Neurotoxicology 30:977-985.

Houghton PJ (1999) The scientific basis for the reputed activity of Valerian. J Pharm Pharmacol 51:505-512.

Jeibmann A, Paulus W (2009) Drosophila melanogaster as a model organism of brain diseases. Int J Mol Sci 10:407-440.

Korczyn AD (2006) Management of sleep problems in Parkinson's disease. J Neurol Sci 248:163-166.

Laguerre M, Lecomte J, Villeneuve P (2007) Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods, new trends and challenges. Prog Lipid Res 46:244-282.

Lang AE, Lozano AM (1998a) Parkinson's disease. First of two parts. N Engl J Med 339:1044-1053.

Lang AE, Lozano AM (1998b) Parkinson's disease. Second of two parts. N Engl J Med 339:1130-1143.

Lima SQ, Miesenbock G (2005) Remote control of behavior through genetically targeted photostimulation of neurons. Cell 121:141-152.

Lohr JB (1991) Oxygen radicals and neuropsychiatric illness. Some speculations. Arch Gen Psychiatry 48:1097-1106.

Malva JO, Santos S, Macedo T (2004) Neuroprotective properties of Valeriana officinalis extracts. Neurotox Res 6:131-140.

Mattson MP, Magnus T (2006) Ageing and neuronal vulnerability. Nat Rev Neurosci 7:278-294.

Meulener M, Whitworth AJ, Armstrong-Gold CE, Rizzu P, Heutink P, Wes PD, Pallanck LJ, Bonini NM (2005) Drosophila DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinson's disease. Curr Biol 15:1572-1577.

Moreira CG, Barbiero JK, Ariza D, Dombrowski PA, Sabioni P, Bortolanza M, Cunha CD, Vital MA, Lima MM (2012) Behavioral, neurochemical and histological alterations promoted by bilateral intranigral rotenone administration: a new approach for an old neurotoxin. Neurotox Res 21:291-301.

Mueller CE, Brattstrom A, Abourashed EA, U K (2002) Interactions of valerian extracts and a fixed valerian-hop extract combination with adenosine receptors. Life Science 71:1939–1949.

Muller CE, Schumacher B, Brattstrom A, Abourashed EA, Koetter U (2002) Interactions of valerian extracts and a fixed valerian-hop extract combination with adenosine receptors. Life Sci 71:1939-1949.

Nichols CD (2006) Drosophila melanogaster neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. Pharmacol Ther 112:677-700.

Nogueira CW, Rocha JBT (2010) Diphenyl Diselenide a Janus-Faced Molecule. Journal of Brazilian Chemical Society 21:2055-2071.

Nogueira CW, Zeni G, Rocha JB (2004) Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem Rev 104:6255-6285.

Norenberg MD, Rao KV (2007) The mitochondrial permeability transition in neurologic disease. Neurochem Int 50:983-997.

Ogawa A, Yoshimoto T, Kikuchi H, Sano K, Saito I, Yamaguchi T, Yasuhara H (1999) Ebselen in acute middle cerebral artery occlusion: a placebo-controlled, double-blind clinical trial. Cerebrovasc Dis 9:112-118.

Oliveira DM, Barreto G, De Andrade DEV, Saraceno E, Aon-Bertolino L, Capani F, El Bacha RS, LD G (2009) Cytoprotective effect of Valeriana officinalis extract on an in vitro experimental model of Parkinson disease. Neurochemical Research 34.

Ortiz JG, Rassi N, Maldonado PM, Gonzalez-Cabrera S, Ramos I (2006) Commercial valerian interactions with [3H]Flunitrazepam and [3H]MK-801 binding to rat synaptic membranes. Phytother Res 20:794-798.

Pendleton RG, Parvez F, Sayed M, Hillman R (2002) Effects of pharmacological agents upon a transgenic model of Parkinson's disease in Drosophila melanogaster. J Pharmacol Exp Ther 300:91-96.

Pereira RP, Fachinetto R, de Souza Prestes A, Puntel RL, Santos da Silva GN, Heinzmann BM, Boschetti TK, Athayde ML, Burger ME, Morel AF, Morsch VM, Rocha JB (2009) Antioxidant effects of different extracts from Melissa officinalis, Matricaria recutita and Cymbopogon citratus. Neurochem Res 34:973-983.

Pereira RP, Fachinetto R, de Souza Prestes A, Wagner C, Sudati JH, Boligon AA, Athayde ML, Morsch VM, Rocha JB (2011) Valeriana officinalis ameliorates vacuous chewing movements induced by reserpine in rats. J Neural Transm 118:1547-1557.

Perez RG, Hastings TG (2004) Could a loss of alpha-synuclein function put dopaminergic neurons at risk? J Neurochem 89:1318-1324.

Poewe W (2006) The natural history of Parkinson's disease. J Neurol 253 Suppl 7:VII2-6.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045-2047.

Posser T, Franco JL, dos Santos DA, Rigon AP, Farina M, Dafre AL, Teixeira Rocha JB, Leal RB (2008) Diphenyl diselenide confers neuroprotection against hydrogen peroxide toxicity in hippocampal slices. Brain Res 1199:138-147.

Posser T, Moretto MB, Dafre AL, Farina M, da Rocha JB, Nogueira CW, Zeni G, Ferreira Jdos S, Leal RB, Franco JL (2006) Antioxidant effect of diphenyl diselenide against sodium

nitroprusside (SNP) induced lipid peroxidation in human platelets and erythrocyte membranes: an in vitro evaluation. Chem Biol Interact 164:126-135.

Price KS, Farley IJ, Hornykiewicz O (1978) Neurochemistry of Parkinson's disease: relation between striatal and limbic dopamine. Adv Biochem Psychopharmacol 19:293-300.

Przedborski S (2005) Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism Relat Disord 11 Suppl 1:S3-7.

Puntel RL, Nogueira CW, Rocha JB (2005) N-methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity in vitro. Neurochem Res 30:417-424.

Puntel RL, Roos DH, Grotto D, Garcia SC, Nogueira CW, Rocha JB (2007) Antioxidant properties of Krebs cycle intermediates against malonate pro-oxidant activity in vitro: a comparative study using the colorimetric method and HPLC analysis to determine malondialdehyde in rat brain homogenates. Life Sci 81:51-62.

Rajput A, Rajput AH (2007) Old age and Parkinson's disease. Handb Clin Neurol 84:427-444.

Reiter RJ (1995) Oxidative processes and antioxidative defense mechanisms in the aging brain. FASEB J 9:526-533.

Rios C, Santamaria A (1991) Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem Res 16:1139-1143.

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179:588-590.

Saito I, Asano T, Sano K, Takakura K, Abe H, Yoshimoto T, Kikuchi H, Ohta T, Ishibashi S (1998) Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage. Neurosurgery 42:269-277; discussion 277-268.

Schulz JB, Falkenburger BH (2004) Neuronal pathology in Parkinson's disease. Cell Tissue Res 318:135-147.

Schumacher B, Scholle S, Holzl J, Khudeir N, Hess S, Muller CE (2002) Lignans isolated from valerian: identification and characterization of a new olivil derivative with partial agonistic activity at A(1) adenosine receptors. J Nat Prod 65:1479-1485.

Schumacher BSS, Hoelzl J, Khudeir N, Hess S, CE M (2002) Lignans isolated from valerian: identification and characterization of a new olive oil derivative with partial agonistic activity at A1 adenosine receptors. Journal of Natural Products 65:1479–1485.

Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT (2002) An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J Neurosci 22:7006-7015.

Siderowf A, Stern M (2003) Update on Parkinson disease. Ann Intern Med 138:651-658.

Simonian NA, Coyle JT (1996) Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36:83-106.

Spector A, Wilson SR, Zucker PA, Huang RR, Raghavan PR (1989) The synthesis of glutathione peroxidase analogs. Lens Eye Toxic Res 6:773-780.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A 95:6469-6473.

Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR, Comyns K, Richards MB, Meng C, Priestley B, Fernandez HH, Cambi F, Umbach DM, Blair A, Sandler DP, Langston JW (2011) Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect 119:866-872.

Tunez I, Montilla P, Del Carmen Munoz M, Feijoo M, Salcedo M (2004) Protective effect of melatonin on 3-nitropropionic acid-induced oxidative stress in synaptosomes in an animal model of Huntington's disease. J Pineal Res 37:252-256.

Uversky VN (2004) Neurotoxicant-induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration. Cell Tissue Res 318:225-241.

Wagner C, Fachinetto R, Dalla Corte CL, Brito VB, Severo D, de Oliveira Costa Dias G, Morel AF, Nogueira CW, Rocha JB (2006) Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. Brain Res 1107:192-198.

Wang CS, Burke JR, Steffens DC, Hulette CM, Breitner JC, Plassman BL (2009) Twin pairs discordant for neuropathologically confirmed Lewy body dementia. J Neurol Neurosurg Psychiatry 80:562-565.

Whitworth AJ, Wes PD, Pallanck LJ (2006) Drosophila models pioneer a new approach to drug discovery for Parkinson's disease. Drug Discov Today 11:119-126.

Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H (1998) Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. Stroke 29:12-17.

Yang Y, Gehrke S, Imai Y, Huang Z, Ouyang Y, Wang JW, Yang L, Beal MF, Vogel H, Lu B (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci U S A 103:10793-10798.

Yu S, Ueda K, Chan P (2005) Alpha-synuclein and dopamine metabolism. Mol Neurobiol 31:243-254.