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Gerlânia de Oliveira Leite

# AVALIAÇÃO DA *Vanillosmopsis arborea* Baker E (-)-α-BISABOLOL FRENTE A PARÂMETROS OXIDATIVOS E TOXICOLÓGICOS

Santa Maria, RS.
2017

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

Orientadora: Profª. Drª Caroline Wagner
Co-orientadora: Profª. Drª Roselei Fachinetto

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**Aprovada em 18 de Agosto 2017:**

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Santa Maria, RS.
2017

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“Matar o sonho é matarmo-nos. É mutilar a nossa alma. O sonho é o que temos de realmente nosso, de impenetravelmente e expugnavelmente nosso.”

(Fernando Pessoa)

# RESUMO

## AVALIAÇÃO DA *Vanillosmopsis arborea* Baker E (-)-α-BISABOLOL FRENTE A PARÂMETROS OXIDATIVOS E TOXICOLÓGICOS

AUTORA: Gerlânia de Oliveira Leite
ORIENTADORA: Caroline Wagner

A *Vanillosmopsis arborea* Baker pertence à família *Asteraceae* e apresenta reconhecido valor econômico devido às suas propriedades anti-inflamatórias, provenientes do sesquiterpeno (-)-α-bisabolol (BISA), o qual está presente em concentrações elevadas no óleo essencial de sua madeira (OEVA). O BISA é utilizado em uma grande variedade de produtos dermatológicos. Alguns estudos sugerem que os efeitos farmacológicos deste sesquiterpeno, bem como do OEVA, podem estar relacionados a sua atividade antioxidante. A rotenona, um pesticida que atua como inibidor do complexo I da cadeia transportadora de elétrons mitocondrial, tem sido utilizada em modelos experimentais de parkinsonismo por causar aumento na produção de espécies reativas de oxigênio (EROs). O objetivo deste estudo foi avaliar a atividade antioxidante de *V. arborea*, bem como o potencial antioxidante, neuroprotetor e toxicológico do (-)-α-bisabolol em diferentes modelos experimentais. Primeiramente, foi avaliado o efeito *in vitro* do OEVA e extrato de *V. arborea* na produção de substâncias reativas ao ácido tiobarbitúrico (TBARS) induzido por Fe(II) e oxidação da diclorofluoresceína (DCFH) em homogeneizado de cérebro de ratos, o potencial antioxidante através do teste de sequestro do radical DPPH (2,2-difenil-1-picril-hidrazil) e a atividade quelante. Os extratos de *V. arborea* apresentam atividade antioxidante *in vitro* demonstrado através da diminuição da peroxidação lipídica induzida por Fe(II), e também pela capacidade de sequestrar o radical DPPH. Além disso, o OEVA e BISA reduziram a oxidação da DCFH induzida pelo $H_2O_2$. O efeito do BISA foi também avaliado em modelo de citotoxicidade e genotoxicidade em células monocucleares e hemácias de sangue periféricos (CMSP) humano. O BISA reduziu a viabilidade celular, causou dano nuclear e atividade hemolítica somente quando as células foram expostas à altas concentrações. Após, o efeito antioxidante e neuroprotetor do BISA foi avaliado *in vivo* utilizando *Drosophila melanogaster* como organismo modelo. As moscas da fruta foram expostas à rotenona e/ou BISA e foram observados parâmetros comportamentais, utilizando os testes de geotaxia negativa e mortalidade. Neste modelo, também foi determinada a immunorreatividade da tirosina hidroxilase (TH), a expressão dos genes superóxido dismutase (SOD), catalase e Keap 1 (relacionados à via de sinalização do estresse oxidativo), além do conteúdo de tióis e atividade do complexo I mitocondrial. O BISA não demonstrou toxicidade no modelo de *D. Melanogaster.* O BISA diminuiu a mortalidade e a perda da atividade locomotora em *D. Melanogaster* expostas a rotenona. A alteração na expressão de RNAm da SOD causada pela rotenona foi normalizada pelo tratamento com BISA (250 µM). O co-tratamento com BISA não modificou os outros parâmetros testados. Em conclusão a *V. arborea* apresenta atividade antioxidante. O BISA, principal constituinte do óleo essencial, demonstrou atividade antioxidante e neuroprotetora, além de não demonstrar toxicidade em modelo experimental utilizando *D. melanogaster*. A citotoxicidade e a genotoxicidade deste composto foi observada somente em altas concentrações e por um longo período de exposição em CMSP. Assim sendo, nossos resultados mostram que o BISA protege contra a toxicidade induzida por rotenona. Nossos estudos demonstraram que *V. arborea* e seu composto majoritário apresentam efeito antioxidante, neuroprotetor, com baixa toxicidade e sugere sua potencialidade como possível agente terapêutico.



**Palavras-chave:** *Vanillosmopsis arborea*. (-)-α-bisabolol. estresse oxidativo.

# ABSTRACT

## EVALUATION OF *Vanillosmopsis arborea* Baker AND (-) - α-BISABOLOL ON OXIDATIVE AND TOXICOLOGICAL PARAMETERS.

AUTHOR: Gerlânia de Oliveira Leite
ADVISOR: Caroline Wagner

*Vanillosmopsis arborea* Baker belongs to the Asteraceae family and has a recognized economic value due to its anti-inflammatory properties, derived from sesquiterpene (-)-α-bisabolol (BISA), which is present in high concentrations in the essential oil of its wood (OEVA). BISA is used in a wide variety of dermatological products. Some studies suggest that the pharmacological effects of this sesquiterpene, as well as the OEVA, may be related to its antioxidant activity. Rotenone, a pesticide which acts as mitochondrial inhibitor of the complex I from electron chain transporter, has been used in experimental models of parkinsonism by causing an increase in the production of reactive oxygen species (ROS). The aim of this study was to evaluate the antioxidant activity of *V. arborea*, as well as the antioxidant, neuroprotective and toxicological potential of (-)-α-bisabolol in different experimental models. Firstly, it was evaluated the *in vitro* effect of the OEVA and the extract of de *V. arborea* on the Fe (II)-induced thiobarbituric acid (TBARS) production and dichlorofluorescein (DCFH) oxidation in rat brain homogenates, the antioxidant potential through the DPPH radical scavenging test (2 , 2-diphenyl-1-picrylhydrazyl) and the chelating activity. The extracts of *V. arborea* presented antioxidant activity *in vitro* demonstrated through a decrease in lipid peroxidation induced by Fe(II), and by its ability in to scavenging DPPH radical. Moreover, the OEVA and BISA reduced the oxidation of DCFH induced by $H_2O_2$.The effect of BISA was also in a model of cytotoxicity and genotoxicity in peripheral blood mononuclear (PBMC) and red blood cells from humans. The BISA reduced the cellular viability, caused a nuclear damage and hemolytic activity only when the cells were exposed to high concentrations. After, the antioxidant and neuroprotective effect of BISA was evaluated *in vivo* using *Drosophila melanogaster* as organism model. The fruit flies were exposed to rotenone and/or BISA and it were observed behavioral parameters using negative geotaxis and mortality tests. In this model, it were also determined the expression of genes of superoxide dismutase (SOD), catalase and Keap 1 (related to the oxidative stress signaling pathway), also the thiol content and mitochondrial I complex activity. BISA showed no toxicity in the *D. melanogaster* model. BISA decreased the mortality and increased the locomotor activity in *D. melanogaster* exposed to rotenone. The alteration in the expression of SOD mRNA caused by rotenone was normalized by treatment with BISA (250 μM). BISA did not change the other parameters tested. In conclusion, *V. arborea* presents antioxidant activity. BISA, the main constituent of the essential oil, demonstrated antioxidant and neuroprotective activity, besides not showing toxicity in an experimental model using *D. melanogaster*. The cytotoxicity and genotoxicity of this compound was observed only at high concentrations and over a long period of exposure to PBMC. Therefore, our results show that BISA protects against the toxicity induced by rotenone. Our study reports the antioxidant, neuroprotective effects of *V. arborea* and its major compound, with low toxicity and suggests its potential as a possible therapeutic agent.


**Keywords**: *Vanillosmopsis arborea*. (-) - α-bisabolol. oxidative stress.

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## LISTA DE ABREVIATURAS

ATP – Adenosina Trifosfato
AChE – Acetilcolinesterase
BChE – Butirilcolinesterase
BISA – (-)-α-Bisabolol
BHA – Hidroxianisol de butila
BHE – Barreira hematoencefálica
BHT – Hidroxitolueno de butila
CAT – Catalase
CCD – Cromatografia em Camada Delgada
cDNA – DNA complementar
CMSP – Células mononucleadas de sangue periférico
DCFH –DA – Diacetato de Diclorofluoresceína
DP – Doença de Parkinson
DNA – Ácido Desoxiribonucléico
DNAse - Desoxiribonuclease
DPPH – 2,2-difenil-1-picril-hidrazil
ECVAM – Centro Europeu para a Validação de Métodos Alternativos
EDTA – Ácido Etilenodiaminotetracético
EO – Estresse Oxidativo
EROs – Espécies Reativas de Oxigênio
$Fe^{2+}$ ou Fe (II) – Íon Ferroso
$Fe^{3+}$ ou Fe (III) – Íon Férrico
GSH – Glutationa reduzida
GPx – Glutationa peroxidase
GST – Glutationa S-transferase
$H_2O_2$ – Peróxido de Hidrogênio
HPLC – *High-Performance Liquide Chromatography*
MDA – Malondialdeído
MPTP - *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*
MTT – Brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio)
NADH – Nicotinamida Adenina Dinucleotídio
NMDA – N-Metil-D-Aspartato
$O_2^{\bullet -}$ – Ânion superóxido
$^{\bullet}OH$ – Radical hidroxila
OEVA – Óleo essencial da *Vanillosmpsis arborea*
OMS – Organização Mundial de Saúde
PL – Peroxidação Lipídica
PCR – *Polymerase chain reaction* (reação em cadeia de polimerase)
RDC – Resolução da Diretoria Colegiada
RL – Radicais Livres
RNAm – Ácido Ribonucléico mensageiro
-SH – Grupo Tiol
SNC – Sistema Nervoso Central
SOD – Superóxido Dismutase
TBARS – Substâncias Reativas ao Ácido Tiobarbitúrico
TH – Tirosina hidroxilase

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# 1. INTRODUÇÃO

O uso de produtos naturais, principalmente derivados de plantas, é uma fonte tradicional de agentes terapêuticos para um amplo espectro de doenças. Com os avanços científicos, hoje já é possível extrair compostos bioativos purificados, permitindo a utilização específica do composto que apresenta a atividade desejada (CALIXTO, 2000; CALIXTO, 2005; CRAGG; GROTHAUS; NEWMAN, 2014). Considerando os produtos naturais, o uso medicinal das plantas pela população de diferentes partes do mundo tem encontrado respaldo nos estudos científicos, que comprovam a eficácia destas plantas em vários modelos experimentais (MACIEL et al., 2002; CALIXTO, 2005; VEIGA, 2005).

A elucidação dos componentes ativos presentes nas plantas, bem como seus mecanismos de ação, vem sendo um dos maiores desafios para a química farmacêutica, bioquímica e a farmacologia (GEBHARDT, 2000). As plantas contêm inúmeros constituintes e seus extratos e óleos essenciais, quando testados podem apresentar efeitos sinérgicos entre os diferentes princípios ativos devido a presença de compostos de classes ou estruturas diferentes contribuindo para a sua atividade.

Dentre os produtos naturais, destacamos os derivados da *Vanillosmopsis arborea* Baker, a qual é uma Asteraceae de reconhecido valor econômico que possui propriedades anti-inflamatórias, provenientes do sesquiterpeno (-)-α-bisabolol (BISA), presente em teores elevados no óleo essencial de sua madeira (OEVA), o qual apresenta cerca de 80,43% de (-)-α-bisabolol (SANTOS et al., 2010).

O BISA é um álcool sesquiterpeno encontrado no óleo de camomila e outras plantas, e tem sido utilizado em uma ampla variedade de produtos dermatológicos (GOMES-CARNEIRO et al., 2005). Alguns estudos (BRAGA et al., 2009; ROCHA et al., 2010; ROCHA et al., 2011a; LUQMNA et al., 2011) sugerem que os efeitos farmacológicos deste sesquiterpeno, bem como do OEVA, pode estar relacionado a sua atividade antioxidante.

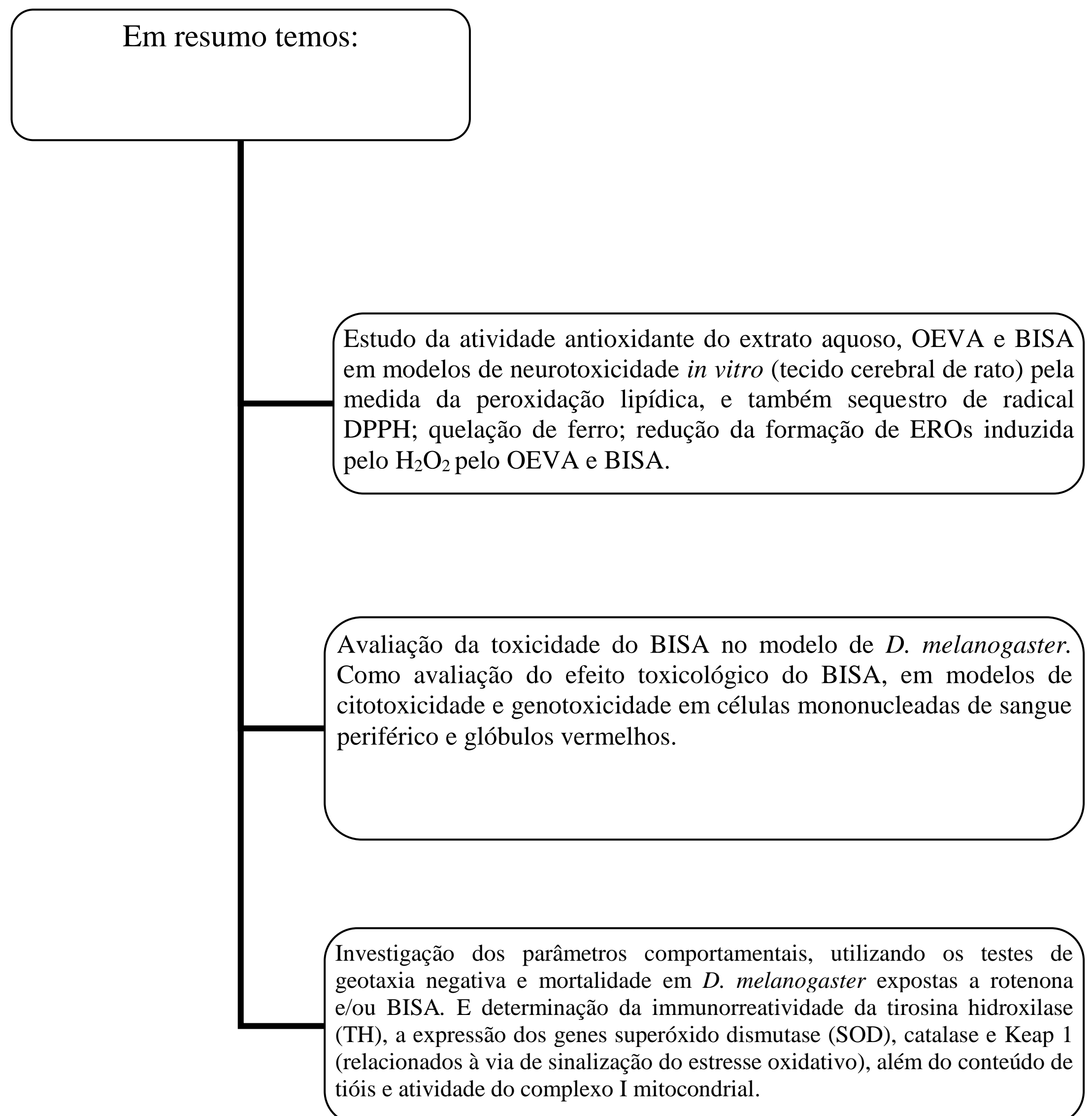
Evidências crescentes sugerem que muitas doenças degenerativas, como: disfunção cerebral, câncer, doenças cardíacas e declínio do sistema imunológico, poderiam ser o resultado do dano celular causados pelos radicais livres e que os antioxidantes podem desempenhar um papel importante na prevenção destas doenças (ARUOMA, 1998). Algumas atividades biológicas do óleo de *V. arborea* Baker já foram estudados e podem ser relacionadas à atividade antioxidante sendo que o (-)-α-bisabolol apresentou também uma atividade antioxidante bem descrita (BRAGA et al., 2009; ROCHA et al., 2010; 2011a; SAMPAIO et al., 2016).

No estudo da atividade biológica é importante a seleção de bioensaios para a detecção do efeito específico. Os sistemas de ensaio devem ser simples, sensíveis e reprodutíveis. Os bioensaios podem envolver organismos inferiores (microorganismos e microcrustáceos, entre outros), ensaios bioquímicos visando alvos moleculares (enzimas e receptores) e cultura de células animais ou humanas. Contudo, o teste adequado dependerá da doença alvo (MACIEL et al., 2002; CALIXTO, 2005). As pesquisas científicas estudam os organismos elucidando os numerosos processos biológicos. Isto decorre do fato de que muitos aspectos da biologia são similares na maioria dos organismos (FERREIRA et al., 2005; YANG et al., 2006; NICHOLS, 2006). O rato é o principal organismo modelo para estudar as funções gênicas. O grande número de linhagens e dados disponíveis sobre este organismo o torna um excelente modelo para estudos de doenças humanas (FERREIRA et al., 2005). A *Drosophila melanogaster* é um organismo-modelo que tem sido amplamente utilizada em vários modelos para estudos de doenças humanas (YANG et al., 2006). Embora os seres humanos e moscas são apenas remotamente relacionados considerando a escala evolutiva, quase 75% dos genes relacionados as doenças em humanos são análogos na mosca, tornando-se um sistema modelo para estudos preliminares acerca dos mecanismos envolvidos nas doenças humanas. Além disso, estudos *in vitro* com BISA mostram muitas vantagens, especialmente no que se refere à compreensão dos mecanismos biológicos e dos efeitos tóxicos e citotóxico. (CAVALIERI et al., 2004; DARRA et al., 2008; CAVALIERI et al., 2009; CHEN et al., 2010; CAVALIERI et al., 2011; ANTER et al., 2011; RIGO et al., 2016).

Dentre os modelos de neurotoxicidade estudados em *D. melanogaster*, a exposição à rotenona é bem reconhecida por sua ação neurotóxica (RAVIKUMAR et al., 2009; RAVIKUMAR et al., 2010; LIU et al., 2005; SUDATI et al., 2013; SUBRAMANIAN et al., 2014). A Rotenona é um composto extraído de plantas leguminosas, utilizada como inseticida botânico e controle populacional de peixes. A rotenona é altamente lipofílica, o que a torna capaz de atravessar a barreira hematoencefalica (BHE) e todas as outras membranas celulares e também pode se acumular em organelas subcelulares como as mitocôndrias (BOVÉ; PERIER, 2012), sendo potente inibidor do complexo I mitocondrial. A inibição do complexo I leva a um aumento da produção das espécies reativas de oxigênio (EROs), potencializando seus efeitos deletérios na célula (UVERSKY, 2004).

Assim sendo, a realização de estudos que investiguem os possíveis efeitos antioxidantes, neuroprotetores e bem como o potencial toxicológico da *V. arborea* Baker e (-)-α-bisabolol podem proporcionar proteção em modelos experimentais *in vivo* de doenças neurológicas. Além disso, esses estudos podem contribuir efetivamente para a busca por

tratamentos à base de terapias naturais para patologias relacionadas ao estresse oxidativo e neurotoxicidade. Para melhor compreender o nosso trabalho se divide da seguinte maneira:



## 1.1. OBJETIVOS

### 1.1.1. Objetivo Geral

- ✓ Avaliar o potencial antioxidante, neuroprotetor, bem como o possível efeito tóxico do extrato aquoso e óleo essencial da *V. arborea,* seu principal constituinte, (-)-α-bisabolol (BISA) em diferentes modelos experimentais.

### 1.1.2. Objetivos Específicos

- ✓ Verificar a atividade antioxidante (*in vitro*) e neuroprotetora do extrato aquoso ou óleo essencial da *V. arborea* e do BISA através de diferentes parâmetros de estresse oxidativo em homogeneizado de cérebro de rato;
- ✓ Investigar os efeitos antioxidantes e tóxicos do BISA em *D. melanogaster;*
- ✓ Avaliar o efeito neuroprotetor *in vivo* do BISA sobre a sobrevivência de *D. melanogaster* expostas à rotenona;
- ✓ Avaliar o efeito toxicológico do BISA através de ensaios de citotoxicidade e genotoxicidade em CMSP, bem como, genotoxicidade e mutagênico em hemácias.

# 2. REFERENCIAL TEÓRICO

## 2.1. PLANTAS MEDICINAIS

O conhecimento sobre plantas medicinais simboliza muitas vezes o único recurso terapêutico de várias comunidades e grupos étnicos. A utilização de plantas no tratamento e na cura de doenças é tão antigo quanto a espécie humana. Atualmente nas regiões mais pobres do país e até mesmo nas grandes cidades brasileiras, plantas medicinais são comercializadas em feiras livres, mercados populares e encontradas em quintais residenciais (MACIEL et al., 2002).

Geralmente, o conhecimento popular é desenvolvido por grupamentos culturais que ainda convivem intimamente com a natureza, observando-a de perto no seu dia-a-dia, e explorando suas potencialidades, mantendo vivo e crescente esse patrimônio pela experimentação sistemática e constante (ELISABETSKY, 1997).

O Brasil é reconhecido por sua biodiversidade. Essa riqueza biológica torna-se ainda mais fundamental porque está aliada a uma sociodiversidade que envolve vários povos e comunidades, com visões, saberes e práticas culturais próprias. No ponto de vista do uso terapêutico das plantas, esses saberes e práticas estão intrinsecamente relacionados aos territórios e seus recursos naturais, como parte integrante da reprodução sociocultural e econômica desses povos e comunidades. Neste sentido, é imprescindível promover o resgate, o reconhecimento e a valorização das práticas tradicionais e populares de uso de plantas medicinais e remédios caseiros, como elementos para a promoção da saúde, conforme preconiza a Organização Mundial da Saúde, 2006.

No Brasil, o uso das plantas medicinais foi disseminado principalmente pela cultura indígena. É um país rico em diversidade cujo território possui cinco principais biomas sendo designados como Floresta Amazônica, Cerrado, Mata Atlântica, Pantanal e Caatinga. Consequentemente, o Brasil é uma rica fonte de produtos terapêuticos. Entretanto, este potencial para a descoberta de plantas como fonte de novos fármacos é pouco explorado ou regulamentado, contrastando com o que ocorre em países como Alemanha, Estados Unidos e Canadá (CALIXTO, 2000; RATES, 2001; VEIGA-JUNIOR; MELLO, 2008).

Dados da Organização Mundial de Saúde (OMS) mostram que cerca de 80% da população mundial fez uso de algum tipo de erva na busca de alívio de alguma sintomatologia ou enfermidade desagradável. Uma porcentagem desse total, pelo menos 30% deu-se por indicação médica. Esta prática tradicional, ainda existente entre os povos de todo o mundo, e tem, inclusive, recebido incentivo da própria OMS (MACIEL et al., 2002).

São consideradas plantas medicinais aquelas que têm efeito definido sobre doenças ou sintomas. Segundo Di Stasi (1996), representam uma fonte inesgotável de medicamentos usualmente utilizados, assim como uma rica fonte de novas substâncias com atividade biológica potencial, exercendo algum tipo de ação farmacológica. Elas podem ser classificadas com sua ordem de importância, iniciando-se pelas plantas empregadas diretamente na terapêutica, seguidas daquelas que constituem matéria-prima para manipulação e, por último, as empregadas na indústria para obtenção de princípios ativos ou como precursores em semi-síntese. As plantas medicinais têm sido utilizadas tradicionalmente para o tratamento de várias doenças. Seu emprego é extenso e abrange desde o combate ao câncer até a microrganismos patogênicos (CALIXTO, 2000). As plantas, além de seu uso na medicina popular com finalidades terapêuticas, têm contribuído, ao longo dos anos, para a obtenção de vários fármacos, e atualmente utilizados na clínica, como a emetina, a vincristina, a colchicina e a rutina. A cada ocasião são relacionadas na literatura novas moléculas, algumas de relevante ação farmacológica como a forscolina, o taxol e a artemisinina (CECHINEL, 1998).

A triagem de uma planta para estudo farmacológico é um passo muito importante. A escolha pode ser feita de várias maneiras através do uso tradicional, dos componentes químicos, da seleção randomizada ou da combinação de mais de um critério. A estratégia mais comum é a utilização das fontes naturais na medicina popular, que é conhecida como etnofarmacologia (RATES, 2001; CARLINI, 2003; 2006; ALBUQUERQUE; HANAZAKI, 2006).

## 2.2. ÓLEOS ESSENCIAIS E ANTIOXIDANTES

De acordo com Vertuani (2004), antioxidantes são substâncias que retardam a velocidade da oxidação, através de um ou mais mecanismos, tais como inibição de radicais livres e complexação de metais. Eles podem ser sintéticos ou naturais e, para serem utilizados em alimentos, devem ser seguros para a saúde. Alguns dos antioxidantes sintéticos mais importantes são hidroxianisol de butila (BHA) e o hidroxitolueno de butila (BHT), já entre os naturais destacam-se ácido ascórbico, vitamina E e β-caroteno (RICE-EVANS et al., 1996).

De acordo com Laguerre et al. (2007), um importante desafio para a pesquisa industrial nos últimos anos é a busca por antioxidantes naturais para produtos alimentícios, cosméticos e farmacêuticos. Esses antioxidantes são obtidos, sobretudo, de produtos de origem vegetal: compostos fenólicos, ácido ascórbico e carotenóides. Nesses produtos, há um grande interesse pelo estudo da oxidação lipídica, em virtude da deterioração que este tipo de

dano oxidativo pode causar (rancificação, perda de aromas e formação de *off-flavors*, rejeição do consumidor).

Os efeitos desejados de compostos antioxidantes são a inativação de radicais livres, a complexação com íons metálicos e/ou a redução de hidroperóxidos a compostos estáveis que não produzam radicais livres ou formem compostos de decomposição tóxicos (LAGUERRE et al. 2007). Muitos compostos de ocorrência natural são conhecidos por exibirem largo espectro de atividade biológica, destacando-se as propriedades antioxidantes. Os óleos essenciais são produtos naturais constituídos de diversas moléculas de interesse que apresentam efeitos antioxidantes. Sua importância comercial e em pesquisas científicas está associada à sua viabilidade de usos e aplicações como compostos naturais biologicamente ativos (MIGUEL, 2010).

Óleos essenciais são definidos como uma mistura complexa de diversas substâncias voláteis e lipofílicas, extraídas de diferentes partes de plantas. Dentre os compostos orgânicos constituintes encontram-se hidrocarbonetos, álcoois, aldeídos, cetonas, fenóis, ésteres, ácidos orgânicos entre outros, os quais apresentam como principal característica a volatilidade, o que os difere dos lipídeos (SIMÕES; SPITZER, 1999). Seus constituintes variam desde hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, éteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, até compostos com enxofre. Na mistura, tais compostos apresentam-se em diferentes concentrações; normalmente, um deles é o composto majoritário, existindo outros em menores teores e alguns em baixíssimas quantidades (SIMÕES, 2007).

O interesse na utilização de muitos óleos essenciais está associado às diversas potencialidades biológicas como atividade antibacteriana, antifúngica e antioxidante. Além disso, aspectos sensoriais como aroma e odor são interessantes para a utilização desses produtos como aromatizantes na indústria farmacêutica e de alimentos (TEISSEDRE; WATERHOUSE, 2000). Da mesma maneira, a atividade antioxidante de óleos essenciais e seus constituintes pode ter papel importante na prevenção de doenças associadas ao estresse oxidativo e danos celulares causados por radicais livres (MIGUEL, 2010). A ação antioxidante dos óleos essenciais tem adquirido uma grande relevância, uma vez que essas substâncias têm potencial antioxidante e são capazes de retardar os efeitos deletérios das espécies reativas ao organismo, tais como câncer, doenças cardiovasculares, envelhecimento da pele dentre outras. Muitos óleos essenciais já possuem ação antioxidante relatada como citronela (*Cymbopogon nardus*), canela (*Cinnamomum zeylanicum*) e gengibre (*Zingiber officinale*) (ANDRADE et al., 2012).

A eficiência antioxidante de óleos essenciais e seus constituintes variam de acordo com a composição e pureza dos compostos, sendo que a composição depende de fatores ambientais, de processamento e conservação. As características como volatilidade, odor e os aspectos referentes à estabilidade dos compostos e interações com outros compostos, quando aplicados em diferentes sistemas, são fatores relevantes para o estudo (BURT, 2004).

Pela utilização crescente na indústria de alimentos, cosméticos e farmacêutica, o cultivo de espécies aromáticas e a extração de óleos essenciais constituem importantes atividades econômicas. Embora a utilização maior ocorra nas áreas de alimentos (condimentos e aromatizantes de alimentos e bebidas) e cosméticos (perfumes e produtos de higiene), também em farmácias, drogas vegetais ricas em óleos voláteis são empregadas *in natura* para a preparação de infusões e/ou sob a forma de preparações galênicas simples. (SIMÕES, 2007). Farmacologicamente, os óleos essenciais são conhecidos por seu poder antisséptico, bactericida, virucida e fungicida, antimicrobianos, analgésicos, sedativos, anti-inflamatórios, antiespasmódicos e anestésico local, funcionando também como antihelmínticos e antiprotozoários (BASSOLÉ e JULIANI, 2012; YORK et al., 2012).

A atividade dos óleos essenciais está associada à característica lipofílica das moléculas que os constituem. Estas moléculas atravessam a membrana celular e rompem a estrutura da camada de fosfolipídios que constituem as membranas, podendo levar a um extravasamento de moléculas e à lise celular (YORK et al., 2012). A habilidade de difusão através de membranas dá a estas moléculas vantagens em agir sobre componentes celulares, estabelecendo uma opção valiosa na procura por componentes bioativos (ALVIANO et al., 2012).

## 2.3. *Vanillosmopsis arborea* Baker

A chapada do Araripe destaca-se no Nordeste brasileiro pela sua geomorfologia e geologia e estende-se dos limites de Pernambuco ao Ceará. A biodiversidade da chapada com suas riquezas naturais atrai uma intensa atividade antrópica que resulta em degradação e risco de extinção. Entre as espécies vegetais empregadas com a finalidade curativa pelas comunidades residentes na área de cerrado da Chapada do Araripe, evidencia-se o Candeeiro, como é mencionado popularmente. Essa planta pertence ao Gênero *Vanillosmopsis,* família Asteraceae. Conforme a Flora brasiliensis: *Vanillosmopsis brasiliensis; Vanillosmopsis capitata; Vanillosmopsis erythropappa; Vanillosmopsis pohlii; Vanillosmopsis polycephala; Vanillosmopsis discolor e Vanillosmopsis arborea.* (LIMA, 2006). O gênero *Vanillosmopsis* é

representado por sete espécies nativas do Brasil, algumas delas de valor econômico devido ao teor de óleo, que é muito similar ao óleo de camomila (MATOS et al., 1988).

A espécie *Vanillosmopsis arborea* Baker (Figura 1A) é uma arvoreta, com até 4m de altura, uma Asteraceae que possui propriedades anti-inflamatórias, provenientes do sesquiterpeno (-)-α-bisabolol, presente em concentrações elevadas no óleo essencial do tronco de sua madeira (Figura 1B). Trata-se de uma madeira de boa qualidade, muito resistente às intempéries e com alto teor de óleo essencial, atributo que promove sua queima provocando chama intensa, justificando o nome popular "Candeeiro" (CAVALCANTI; NUNES, 2002). Na cultura popular são atribuídas ao Candeeiro propriedades repelentes contra mosquitos (FURTADO et al., 2005).

O óleo do candeeiro é rico em (-)-α-bisabolol, presente em produtos dermatológicos, e além de apresentar atividades antimicrobiana, antifúngica e anti-inflamatória, possui também baixa toxicidade (MATOS et al, 1998). O (-)-α-bisabolol apresenta-se como 80,43% de sua composição. O restante da composição, propanoato de etila (5,87%), etanoato de propila (9,00%), o-metil-eugenol (2,39%), óxido-bisabolol (2,31%), totalizando 100% na identificação do óleo essencial (SANTOS, 2009).

Estudos já demonstraram atividades biológicas com o óleo de *V. arborea* Baker, demonstrando efeito gastroprotetor do óleo essencial (LEITE et al., 2009); atividade antinociceptiva visceral e anti-inflamatória tópica através dos modelos clássicos de edema de pata, edema de orelha, contorções induzido por formalina e/ou ácido acético; modelo alternativo de dor aguda visceral que emprega a instilação intracolônico de capsaicina, óleo de mostarda ou formalina ou ciclofosfamida, que produz comportamentos relacionados à dor visceral, e no modelo de dor corneal (INOCÊNCIO LEITE et al., 2014; LEITE et al., 2011a; SANTOS et al., 2009; 2015; MENEZES et al., 1990); atividades antimicrobianas através das técnicas de difusão de disco e contato gasoso, antioxidante em modelo de sequestro de radical DPPH e, ansiolítica, sedativa, depressora do sistema nervoso central em modelos comportamentais (campo aberto e tempo de sono induzido por pentobabital) (CRAVEIRO, 1989; SANTOS et al., 2009); propriedades repelentes e larvicidas (FURTADO et al., 2005; COSTA et al., 2010); atividade antimalárica (MOTA, 2009); atividade leishmanicida (COLARES et al., 2013). Sendo que grande parte destes trabalhos foi realizado pelo grupo de pesquisa da Universidade Regional do Cariri, inclusive pela própria autora.

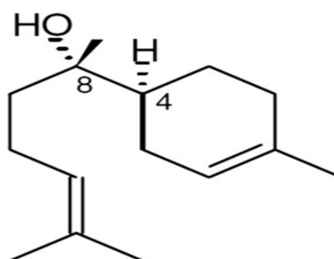
Pesquisas mostraram que a potencialidade do óleo essencial do caule de *V. arborea* (OEVA) pode estar relacionado ao alto teor de (-)-α-bisabolol, uma vez que os estudos químicos mostraram que o OEVA contém 80,43% de (-)-α-bisabolol (SANTOS et al., 2010).

A análise química do óleo essencial do caule revelou a presença de estragol, p-elemeno, metil-eugenol, p-cubebeno, p-himachaleno, p-maalieno, 6-guaieno, p-bisaboleno, elemicino, α-cadinol e α-bisabolol – (principal constituinte) (MATOS et al., 1988).

Figura 01 - *Vanillosmopsis arborea* Baker (A) e estrutura química do (-)-α-bisabolol (B)

**A**

**B**




Fonte: Laboratório de Pesquisa de Produtos Naturais - URCA

## 2.4. (-)-α-BISABOLOL

O (-)-α-bisabolol é um álcool sesquiterpênico monocíclico insaturado que possui fórmula molecular $C_{15}H_{26}O$, peso molecular 222,371 g/mol e sinonímia de levomenol. É encontrado no óleo de camomila e outras plantas (GOMES-CARNEIRO et al., 2005). O alto teor de (-)-α-bisabolol no óleo essencial de *V. arborea* torna um possível sucessor ao óleo de *Matricaria chamomilla* (CAVALCANTI et al., 2002). Seu uso principal é em produtos dermatológicos, pois além de apresentar atividades antimicrobiana, antifúngica e anti-inflamatória, possui também baixa toxicidade (LIMA et al., 2006).

O (-)-α-Bisabolol foi isolado pela primeira vez em 1951 por Isaac e colaboradores a partir de flores de camomila (*Matricaria chamomilla*, Asteraceae). O (-)-α-bisabolol tem sido amplamente utilizado como ingrediente em formulações cosméticas, tais como cremes pós-barba, loções de corpo-e-mão, desodorantes, batons, cuidados com o sol e depois do sol,

produtos de cuidados com o bebê e cremes para esporte (KAMATOU;VILJOEN, 2010; GOMES-CARNEIRO et al., 2005).

Destacam-se entre as atividade biológica do (-)-α-bisabolol as atividades anti-inflamatória, anti-irritante, antimicrobiana e propriedades não-alergênicas (KAMATOU;VILJOEN, 2010). Os estudos mostram que o (-)-α-bisabolol apresenta atividade mutagênica/antimutagênica, antitumoral (CAVALIERI et al., 2004; GOMES-CARNEIRO et al., 2005; GANZERA et al., 2006; DARRA et al., 2008; CAVALIERI et al., 2009; CHEN et al., 2010; MAGNELLI et al., 2010; SILVA et al., 2010; COSTARELLI et al., 2010; CAVALIERI et al., 2011; ANTER et al., 2011; SEKI et al., 2011; BONIFÁCIO et al., 2012; RIGO et al., 2016); é bloqueador nervoso periférico via canais de sódio (ALVES et al., 2010) e bloqueador de cálcio (SIQUEIRA et al., 2012); cicatrizante (VILLEGAS et al., 2001), clareador da pele (KADIR et al., 1991; LEE et al., 2010); antiulcerogênico e antioxidante (TORRADO et al., 1995; BRAGA et al., 2009; BEZERRA et al., 2009; ROCHA et al., 2010; ROCHA et al., 2011a; LUQMNA et al., 2011), antinociceptivo e anti-inflamatório (ROCHA et al., 2011b; LEITE et al., 2011b; KIM et al., 2011; LEITE et al., 2012; MAURYA et al., 2014; MELO et al., 2015; 2017; BARRETO et al., 2016; TEXEIRA et al., 2017); Efeito ansiolítico (TABARI, et al., 2017); Efeito nefroprotetor (SAMPAIO et al., 2016) e leishmanicida (MORALES-YUSTE et al., 2010; ROTTINI et al., 2015).

A atividade gastroprotetora está associada ao efeito antioxidante do (-)-α-bisabolol levando a um aumento da biodisponibilidade dos grupos sulfidrílicos gástricos, redução da formação de substâncias reativas ao ácido tiobarbitúrico (MDA), aumento da atividade da SOD e redução do influxo de células inflamatórias (neutrófilos) na mucosa gástrica. Esses achados mostram que (-)-α-bisabolol é capaz de diminuir o estresse oxidativo e inflamatório, como também evidenciam o efeito protetor de (-)-α-bisabolol em marcadores de estresse oxidativo em eritrócitos (ROCHA et al., 2010; TORRADO et al., 1995; BRAGA et al., 2009; ROCHA et al., 2011a; LUQMNA et al., 2011).

(-)-α-Bisabolol apresenta um efeito citotóxico dependente da dose e tempo de exposição sobre linhagens celulares malignas de glioma de rato e de humano, induzindo estes a apoptose, sendo esta se relacionada à concentração intracelular (CAVALIERI et al., 2004; 2009). E as análises de concentrações intracelulares de (-)-α-bisabolol nas células de glioma humano evidenciaram que este é distribuído rapidamente em frações na membrana, núcleo e citosol; sugerindo que essa distribuição rápida ocorre, pois uma vez absorvido em *rafts lipídicos* (domínios de membrana dinâmicos e enriquecidos com esfingolipídeos e esterol,

que possuem importante papel no transporte de proteínas intracelular e fusão de membrana), este é transportado para o interior da célula (CAVALIERI et al., 2004; 2009; 2011).

## 2.5. OS FITOTERÁPICOS E PARÂMETROS FARMACOLÓGICOS

O uso de plantas para o tratamento de enfermidades vem sendo relatado há séculos, já sendo uma tradição em muitas culturas (BHATTARAM et al, 2002). Em muitos países, os fitoterápicos não são classificados como as demais drogas industriais, as quais precisam rigorosamente passar por uma bateria de ensaios garantindo sua qualidade antes de serem lançadas ao mercado. Eles geralmente são vendidos como suplemento dietético, privando o paciente de informações sobre sua farmacocinética e farmacodinâmica, além de muitas vezes, serem auto administrados pelo próprio paciente, sem o conhecimento de um médico (CALIXTO, 2000; CARLINI, 1983).

O uso tradicional das plantas medicinais baseado em conhecimentos populares, juntamente com a crença de que por ser natural não causa efeitos adversos, fez com que raras plantas medicinais fossem avaliadas com ensaios pré-clínicos e clínicos, a fim de garantir sua eficácia (TUROLLA; NASCIMENTO, 2006). O uso racional de fitoterápicos deve ser apoiado por investigações laboratoriais apropriadas e testes clínicos (CRAVOTTO et al, 2010).

No Brasil, a legislação que rege os medicamentos fitoterápicos vem sofrendo alterações. A Agência Nacional de Vigilância Sanitária elaborou normas para a regulamentação desses medicamentos, desde a Portaria n. 6 de 1995, a qual estabelece prazos para a apresentação de dados comprovando a eficácia e segurança dos fitoterápicos comercializados pelas indústrias farmacêuticas, passando pela Resolução da Diretoria Colegiada (RDC) n. 17 de 2000, e a Resolução RDC n. 48 de 16 de março de 2004, em vigor, que dispõe do registro desses medicamentos (TUROLLA; NASCIMENTO, 2006). A comprovação da eficácia e segurança dos produtos naturais abrange também testes para a detecção de efeitos citotóxicos, genotóxicos e mutagênicos, os quais são o enfoque desta pesquisa.

As *D. melanogaster* têm sido utilizadas para ensaios de toxicidade, como um método alternativo para o uso de animais, e tornou-se um excelente modelo alternativo para vários ensaios (RAND 2010; TIWARI et al. 2011). A mosca-da-fruta, *D. melanogaster*, vem sendo amplamente utilizada em estudos bioquímicos e toxicológicos e por não apresentar imposições éticas no uso de animais em pesquisa, tem sido o modelo de escolha para estudos de triagem de compostos. Neste sentido, a busca por modelos economicamente acessíveis e de

fácil manipulação que demonstrem resultados positivos em relação à toxicidade a *D. melanogaster* é um bom representante que pode ser submetido aos diferentes protocolos de exposição a partir de sua dieta, como já previamente demonstrado em estudos que se baseiam na investigação dos efeitos tóxicos (ARAÚJO PINHO et al., 2014; CUNHA et al., 2015).

Muitos estudos têm sugerido o estresse oxidativo como mecanismo bioquímico central na toxicidade de diversos agentes ambientais (FRANCO et al., 2010; LIU et al., 2003). Alguns contaminantes podem aumentar a produção de EROs, e modificar a capacidade antioxidante celular. Este desequilíbrio entre a produção de agentes antioxidantes e as defesas pode danificar fosfolipídios na membrana plasmática, DNA e proteínas. (BERRA, MENCK; 2006; HALLIWELL, 1994; ARAÚJO PINHO et al., 2014).

Sob a expectativa da investigação de compostos naturais e de novas moléculas com propriedades antioxidantes, muitas pesquisas são direcionadas na busca por alternativas à substituição de antioxidantes sintéticos utilizados, ou na tentativa de fazer associações entre eles objetivando a diminuição em suas aplicações industriais (FEJES et al., 1998; NÚÑES et al., 2001; SAYADI, 2005). Entre os antioxidantes naturais mais utilizados, podem ser citados tocoferóis, compostos fenólicos, extratos de plantas e Óleos Essenciais (RAMALHO; JORGE, 2006; WANG et al., 2008).

Outra forma de análise de toxicidade é o uso de células sanguíneas humanas. O sangue humano possui vários tipos celulares com propriedades estruturais e metabólicas bem particulares, sendo um tecido importante na avaliação de estudos farmacológicos e toxicológicos, parâmetro para avaliação de riscos (SANTOS, 2009).

Dentre elas, as CMSP são de fácil obtenção e cultivo, e por isso são amplamente utilizadas para análises citogenéticas. Como esse tipo celular é abundante na circulação sanguínea, estas ficam expostas a agentes mutagênicos e podem servir de marcadores de danos recentes, sendo desta forma utilizadas a muitos anos para avaliar efeito cito e genotóxicos (MALUF e RIEGEL, 2011). Um dos ensaios mais utilizados para avaliação da viabilidade ou toxicidade celular, é o teste de MTT (brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio). Este ensaio ocorre a partir da determinação da função mitocondrial, medindo a atividade enzimática da enzima succinato desidrogenase (MOSSANN, 1983). Para verificar se ocorreu dano a nível nuclear nestas células, um teste bastante utilizado é o ensaio cometa, o qual baseia-se na detecção microscópica dos fragmentos de DNA de célula (MOLLER, 2005). O teste da hemólise, é muito utilizado por diversos autores para avaliar efeitos tóxicos de diferentes plantas, que pode ocorrer através da solubilização da membrana plasmática do eritrócito (APARÍCIO et al., 2005), ou promover à hemólise através da

oxidação da hemoglobina, dando origem a meta-hemoglobina (BUKOWSKA; KOWALKA, 2004).

## 2.6. ESTRESSE OXIDATIVO E DEFESAS ANTIOXIDANTES

Atualmente, existe um grande interesse no estudo de moléculas e substâncias com ação antioxidante, devido, principalmente, às descobertas sobre o efeito do excesso dos radicais livres no organismo. A oxidação é parte fundamental da vida aeróbica e do metabolismo e, assim, os radicais livres são produzidos naturalmente ou por alguma disfunção biológica. As espécies reativas de oxigênio (EROs) incluem o peróxido de hidrogênio ($H_2O_2$), bem como o ânion superóxido (O2•-), radical hidroxila (•OH), entre outros (Quadro 01). Estas moléculas são normalmente neutralizadas pelos sistemas antioxidantes enzimáticos e não-enzimáticos presentes nos organismos (FINKEL; HOLBROOK, 2000).

A produção de espécies reativas de oxigênio (EROs), de nitrogênio (ERN), entre outras espécies reativas, é parte integrante do metabolismo humano e é observada em diversas condições fisiológicas. As EROs têm importante função biológica, como na fagocitose, fenômeno em que essas espécies são produzidas para eliminar o agente agressor. Por outro lado, quando sua produção é exacerbada, o organismo dispõe de um eficiente sistema antioxidante que consegue controlar e restabelecer o equilíbrio. O estresse oxidativo resulta do desequilíbrio entre o sistema pró e antioxidante com predomínio dos oxidantes, com dano consequente (FINKEL; HOLBROOK, 2000; VALKO et al., 2007).

| **Quadro 1 : Redução do oxigênio a espécies reativas** | |
|---|---|
| $O_2 + e^- \rightarrow O_2^{-\cdot}$ | Radical superóxido |
| $O_2^{-\cdot} + H_2O \rightarrow HO_2^{-\cdot} + OH$ | Radical hidroperoxil |
| $HO_2^{-\cdot} + e^- + H \rightarrow H_2O_2$ | Peróxido de hidrogênio |
| $H_2O_2 + e^- \rightarrow .OH + OH^-$ | Radical hidroxila |

O estresse oxidativo (EO) pode resultar tanto de um aumento na produção de EROs quanto da redução da capacidade antioxidante celular total, ou seja, a ocorrência de um dano oxidativo depende de um desequilíbrio entre a produção de EROs e a atividade e os níveis de defesas antioxidantes (FINKEL; HOLBROOK, 2000). Quando o estresse oxidativo ocorre, as células tentam neutralizar os efeitos oxidantes e restaurar o equilíbrio redox pela ativação ou silenciamento dos genes que codificam enzimas de defesa, fatores de transcrição e proteínas estruturais (BIRBEN, 2012).

Estudos ao longo dos últimos anos demonstraram que as EROs participam ativamente de uma gama diversificada de processos biológicos, incluindo crescimento anormal da célula, a indução e manutenção do estado fisiológico, a morte celular programada e a senescência celular (FINKEL, 2003). A produção de EROs, é parte integrante do metabolismo humano e é observada em diversas condições fisiológicas. As EROs têm importante função biológica, como na fagocitose, fenômeno em que essas espécies são produzidas para eliminar o agente agressor. Por outro lado, quando sua produção é exacerbada, o organismo dispõe de um eficiente sistema antioxidante que consegue controlar e restabelecer o equilíbrio. Os mecanismos de defesa antioxidante podem ser tanto enzimático (catalase, superóxido dismutase, GPX, etc) quanto não-enzimático (glutationa, vitamina E, ácido ascórbico, etc). No sistema enzimático, as SOD, enzimas abundantes em células aeróbicas, agem sobre o ânion superóxido ($O2^{\cdot-}$), dismutando-o a $H_2O_2$ (LAGUERRE et al., 2007). A GPX inativa os peróxidos lipídicos e de hidrogênio, sendo na mitocôndria de mamíferos a principal defesa contra o $H_2O_2$. A CAT é uma enzima, presente nos peroxissomas e também atua inativando o $H_2O_2$. O acúmulo dessa espécie reativa em ($H_2O_2$) possibilita, por meio das reações de *Fenton* e *Haber-Weiss,* a geração do radical hidroxila (OH•), contra o qual não existe defesa enzimática (YOSHIHARA, et al, 2010).

As EROs quando produzidas em excesso podem ocasionar uma grande variedade de reações deletérias no organismo podendo assim lesionar diferentes estruturas celulares como exercer efeitos citotóxicos sobre os fosfolipídios de membrana resultando em peroxidação lipídica, oxidação protéica e alterações na atividade das enzimas antioxidantes (ALLEN, 1998; FINKEL; HOLBROOK, 2000; HALLIWELL; GUTTERIDGE, 2007; BIRBEN, 2012).

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

De acordo com seu mecanismo de ação os antioxidantes podem ser classificados como eliminadores de radicais livres (reagem com radicais livres interrompendo a propagação da cascata de reações); como varredores de oxigênio (desativam o oxigênio singlete que pode iniciar uma nova cadeia de propagação de radicais livres), ou como quelantes de íons metálicos capazes de catalisar a peroxidação lipídica (SÁNCHEZ-MORENO et al., 1999).

Devido à importância do EO na patogênese de inúmeras doenças e a consequente demanda por novas substâncias com propriedades antioxidantes, vários métodos são utilizados para avaliar a presença e o dano associado ao EO. Um exemplo destes ensaios para

detectar a peroxidação lipídica é o método TBARS (espécies reativas ao ácido tiobarbitúrico) que consiste numa medida do produto de peroxidação lipídica tecidual (OHKAWA et al., 1979). As espécies reativas podem ser determinadas pela oxidação de 2',7'-diclorofluoresceína diacetato (DCFDA) como um índice geral de produção de EROs (PEREZ-SEVERIANO, et al., 2004). A molécula de DPPH é caracterizada como um radical livre estável sintético sendo sua inibição usada como um indicador da atividade antioxidante *in vitro* (HATANO et al., 1988).

Outra forma de estudo é através de modelos experimentais alternativos, usada por exemplo como um modelo de dano ao sistema neurológico (RUBIN 2000; BIER 2005). Esses animais possuem características que o tornam um excelente modelo para estudar a função de genes relacionados a diversas doenças, incluindo os envolvidos em doenças neurodegenerativas (NILCHOLS, 2006; PANDEY e NILCHOLS, 2011). A *D. melanogaster* possui sensibilidade a substâncias tóxicas como rotenona (SUDATI et al., 2013; RAVIKUMAR et al., 2009; 2010) e é utilizada por exemplo como modelo para estudo de poluentes para avaliar o potencial terapêutico de plantas e compostos fitoquímicos (FRANCO et al., 2010; ARAÚJO PINHO et al., 2014).

## 2.7. ROTENONA

Os pesticidas são definidos como qualquer substância ou mistura de substâncias que destina-se a prevenir, destruir, repelir ou mitigar pragas (USEPA, 2010). Pesticidas consistem em várias classes e subclasses de compostos que apresentam uma vasta gama de estruturas quimicamente diversas. No entanto, os pesticidas nem sempre são seletivos para suas espécies-alvo pretendidas, e efeitos adversos sobre a saúde pode ocorrer em espécies não alvo, incluindo os seres humanos. A toxicidade dos pesticidas foi claramente demonstrada por alterar uma variedade de funções fisiológicas. Além disso, as evidências sugerem que exposição a pesticidas aumenta o risco de câncer e doenças neurodegenerativa, e contribui para vários efeitos adversos associados à reprodução e toxicidade (FRANCO et al., 2010; LIU et al., 2003).

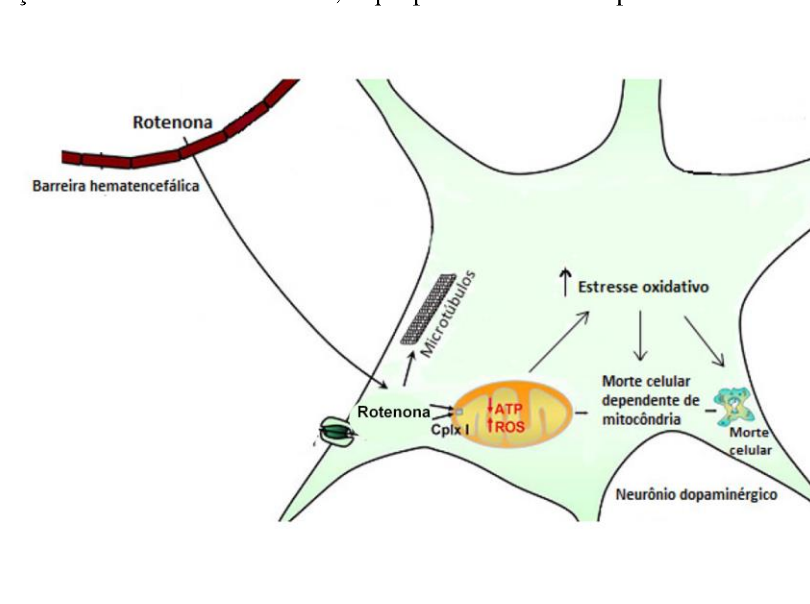
A rotenona é um complexo de cetona que ocorre naturalmente sendo extraído a partir das raízes de espécies *Lonchocarpus* (KOTAKE, 2003; UVERSKY, 2004). Ela é facilmente degradada pela exposição à luz solar, água e solo, o que torna vantajoso o uso como um pesticida (UVERSKY, 2004; BOVE et al., 2005). Quando ingerida por seres humanos, tem absorção lenta e incompleta no estômago e intestinos e, após absorvida, é degradada pelo

efeito de primeira passagem no fígado (BOVE et al., 2005). Desta forma, é necessário ingerir uma grande quantidade deste composto para ocorrer dano hepático (BOVE et al., 2005).

A rotenona é um inibidor clássico do complexo I da cadeia respiratória, prejudicando a fosforilação oxidativa (BOVÉ; PERIER, 2012). Isto provoca a redução na síntese de ATP e a formação de EROs que danificam o próprio complexo I e outras macromoléculas celulares (SANDERS; GREENAMYRE, 2013). Paralela à sua ação na respiração mitocondrial, a rotenona também inibe a formação de microtúbulos a partir da tubulina. A inibição do proteassomo tem sido relatada após exposição crônica à rotenona *in vitro* e *in vivo* (BOVÉ; PERIER, 2012).

A rotenona quando administrada em animais experimentais, atravessa com facilidade a barreira hematoencefálica e ultrapassa as membranas celulares dos neurônios, estabelecendo-se no encéfalo e dentro das mitocôndrias dos neurônios, causando danos neurológicos. Pesquisas experimentais com o pesticida indicam que vários fatores estão envolvidos na sua neurotoxicidade: inibição do complexo I da cadeia respiratória mitocondrial e diminuição na síntese de ATP intracelular, aumento da atividade microglial, estresse oxidativo, indução da morte por apoptose, inibição da atividade do proteassoma e interação direta com a proteína alfa-sinucleína (DAUER; PRZEDBORSKI, 2003; BETABERT et al., 2003; UVERSKY, 2004; CICCHETTI et al., 2009). (Figura –2)

FIGURA 02 - Mecanismo de ação da rotenona. A rotenona é um composto lipofílico que pode atravessar a BHE e outras membranas biológicas. Acumula-se nas mitocôndrias, onde inibe o complexo I (Cplx I) da cadeia respiratória mitocondrial. A inibição do complexo I conduz à diminuição nos níveis de ATP, aumento na produção de EROs e à ativação da via de morte celular dependente de mitocôndria. A rotenona também pode induzir a despolimerização de microtúbulos celulares, o que pode contribuir no processo de morte celular.




Fonte: Adaptado de BASSANI, 2013.

Betarbet e colaboradores (2000) demonstraram que a administração sistêmica de rotenona em ratos resulta em degeneração progressiva da substância negra e do estriado. Eles também demonstraram a presença de α-sinucleína nos neurônios dopaminérgicos. Estudos demonstraram que ocorre expressão de α-sinucleína nos corpos celulares e axônios de neurônios do sistema nervoso entérico e que ela aumenta progressivamente mesmo após finalizar as administrações em roedores. Outras alterações gastrointestinais como diminuição da motilidade e da absorção também são encontradas em animais tratados sistemicamente com rotenona (DROLET et al., 2009 e GREENE et al., 2009).

Em *Drosophila,* a rotenona leva a degradação de neurônios dopaminérgicos (NASSEL e ELEKES, 1992, COULOM e BIRMAN 2004). 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 e tipos celulares são conservadas entre vertebrados e invertebrados tornando a *D. melanogaster* como modelo experimental interessante para o estudo de disfunção neuronal.

Feany e Bender (2000) desenvolveram um modelo genético utilizando moscas para simular o observado em humano com Parkinson. Estes autores provaram que o aumento na expressão da forma normal de α-sinucleína em neurônios era capaz de reproduzir três aspectos chaves: início tardio, depleção seletiva de neurônios dopaminérgicos e produção de agregados citoplasmáticos contendo α-sinucleína, semelhantes aos corpos de Lewy observados em humanos com DP. As moscas transgênicas também apresentaram déficits motores progressivos (perda precoce da capacidade de escalar).

Estudos como este mostram que a *D. melanogaster* representa um excelente modelo para estudar as doenças neurodegenerativas (LESSING e BONINI 2009; LU e VOGEL, 2009), e avaliar o potencial terapêutico dos fitoquímicos.

### 2.8. *Drosophila melanogaster*

O inseto *D. melanogaster* é popularmente conhecido como mosca da fruta sendo pertencente à Ordem Díptera e a Família *Drosophilidae* (NICHOLS, 2006)**.** Uma das principais preocupações dos pesquisadores é a redução no número de animais de laboratório para pesquisas devido a questões éticas. O uso da mosca da fruta, *D. melanogaster*, é recomendado pelo Centro Europeu para a Validação de Métodos Alternativos (ECVAM) para a promoção dos 3R (redução, refinamento e substituição) do uso de animais de laboratório em estudos de toxicidade (BENFORD et al., 2000). As moscas possuem sistemas que controlam absorção de nutrientes, armazenamento e metabolismo e estes sistemas foram relatados ser

análogos aos dos seres humanos (BAKER et al., 2007). As moscas possuem alta sensibilidade a substâncias tóxicas e são úteis para estudos de toxicidade, bem como avaliar a ação biológica de agentes farmacológicos (ADEDARA et al., 2005).

Embora os seres humanos e moscas sejam evolutivamente distantes, quase 75% dos genes relacionados às doenças em humanos são similares à mosca, tornando-se um sistema modelo razoável para estudo de doenças no ser humano (NICHOLS, 2006).

Assim, nosso trabalho tem como objetivo avaliar o potencial farmacológico da *V. arborea,* e principal constituinte*:* (-)-α-bisabolol em diferentes modelos animais relacionados à atividade antioxidante e neuroproteção, bem como seu possível efeito tóxico.

# 3. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de DOIS ARTIGOS E UM MANUSCRITO: O Artigo: **In vitro antioxidant activity investigation of *Vanillomopsis arborea* Baker aqueous extracts, essential oil and isolated compound: (-)-α -bisabolol** que está disposto na forma que foi publicado na revista **Pharmacologia** e o artigo: **Protective effect of (-)-α-Bisabolol on rotenone-induced toxicity in *Drosophila melanogaster*** que está disposto na forma que foi publicado na revista **Canadian Journal of Physiology and Pharmacology**. O Manuscrito: **Evaluation of toxicity of (-)-α-bisabolol in different experimental models** que está disposto na forma que foi submetido na revista **Toxicology Research**. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos próprios manuscritos.

## 3.1. ARTIGO 1

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## Research Article

# *In vitro* Antioxidant Activity Investigation of *Vanillomopsis arborea* Baker Aqueous Extracts, Essential Oil and Isolated Compound: (-)-α-bisabolol

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## Abstract

**Background and Objective:** *Vanillosmopsis arborea* Baker (candeeiro) is a native plant from the Northeast of Brazil. Recently, this plant attracted interest of researchers due to its pharmacological properties, however, there is no underling mechanism established for its properties. So, the aim of this study was to investigate the antioxidant potential of this plant, since oxidative stress is in the core of the development of the diseases that *V. arborea* shows to be efficient in counteract. **Materials and Methods:** For this purpose, used aqueous extracts from bark, trunk and leaves for the plant, as well as the essential oil from the truck, in a set of oxidative stress models. **Results:** The main results obtained here demonstrate that aqueous extract from leaves are able to reduce Fe(II)-induced lipid peroxidation. However, when tested for iron chelation, none of the extracts shows any effect. For this reason, performed free radical scavenging test, by the quenching of 1′1′-diphenil-2-picrylhydrazyl (DPPH). It was observed that both aqueous extract from leaves and essential oil from the trunk were capable to scavenge free radical, indicating a direct effect of the plant on free radicals. Due to high (-)-α-bisabolol content in the essential oil and in the aqueous extract from leaves, hypothesized that this compound could be a central character in the antioxidant activity of the plant. So, performed a 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation test with the essential oil and the (-)-α-bisabolol, which confirm the suggestion that (-)-α-bisabolol could be a major responsible for the antioxidant activity of *V. arborea*. **Conclusion:** Thus, *V. arborea* Baker could be considered an effective agent in the prevention of various diseases associated with oxidative stress and (-)-α-bisabolol is suggested to have prominent role in the plant properties.

Key words: Oxidative stress, *V. arborea* Baker, (-)-α-bisabolol, antioxidant, candeeiro essential oil

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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## INTRODUCTION

*Vanillosmopsis arborea* Baker (Asteraceae), popularly known as "candeeiro", is a small tree native to Brazil, which grows in the Araripe National Forest, in the state of Ceará. It has received considerable interest in the past years possibly due to its economic values. Of particular importance, it has high oil content and its wood has a strong odor of chamomile and burns easily with a strong flame[1]. In folk medicine, *Vanillosmopsis arborea* is used as a repellent[2].

In the past decade, more attention have been paid on the pharmacological activities of *V. arborea* essential oil (EOVA). Studies have demonstrated that EOVA exhibit anti-inflammatory[3,4], antinociceptive[4,5,6], gastroprotective[7], larvicidal[8], antibiotic[9], antimalarial[10] and antileishmanial activities[11]. To knowledge, there is however, no studies on the antioxidant activities of the EOVA that may at least it part, justify such activities.

The major chemical constituents found in different parts of *Vanillosmopsis arborea* include phenolic compounds (apigenin, quercetin, luteolin and their glucosides)[1,12], while (-)-α-bisabolol (BISA) (Fig. 1) has been reported as the pharmacologically active principle of its essential oil[4,12,13]. The BISA is a monocyclic sesquiterpene alcohol found in chamomile (*Matricaria recutita*) and other plants and it has been widely used in dermatological and cosmetic formulations[14]. The BISA was shown to exhibit apoptosis-inducing action in malignant tumor cells[15] and to inhibit the activities of major human drug-metabolizing enzymes[16]. It possesses antimutagenic[14] and antipeptic[17] properties and has the potential to modulate the activity of antibiotics[9]. Although, BISA has been reported to exhibit gastroprotective effect via diverse mechanisms of action including reduction of lipid peroxidation and superoxide dismutase activity, there is limited information on the antioxidant activity of BISA against hydrogen peroxide, a source of hydroxyl radicals[18].

Free radicals are constantly produced in living organisms and detoxified by antioxidants. However, they can cause oxidative damage to important cellular compartments when present in excess. Indeed, consumption of foods antioxidants have been reported to have health-promotion and disease-prevention effects[19], since they can delay the development human diseases, specially the Reactive Oxygen Species (ROS)-mediated ones[20,21]. In addition, natural and synthetic antioxidant compounds have been reported to afford protection in a variety of *in vitro* and *in vivo* models of toxicity[22-28], highlighting the importance of evaluating the antioxidant activity of plant extracts and/or chemicals for potential therapeutic approach.

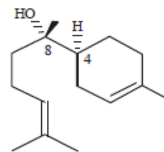


Fig. 1: Chemical structure of (-)-α-bisabolol

Given the interesting biological activities of *V. arborea* and that of the active principle of its essential oil, (-)-α-bisabolol (BISA) and considering the scarcity of information in regard to their antioxidant activity, the present study aimed to investigate the antioxidant activity of aqueous extract from different parts of *V. arborea* (leaves, trunk and bark) in chemical and biological models. In addition, the effect of EOVA and its active component were tested against $H_2O_2$-induced ROS generation in stomach mucus as well as the characterization of the chemical constituents of those aqueous extracts.

## MATERIALS AND METHODS

**Chemicals:** (-)-α-bisabolol, tris-HCl, thiobarbituric acid (TBA), 1,1-diphenil-2-picrylhydrazyl (DPPH), gallic acid and malonaldehyde bis-dimethyl acetal (MDA) were obtained from Sigma (St. Louis, MO, USA). Iron sulfate ($Fe_2SO_4$), ascorbic acid, rutin, caffeic acid, gallic acid, chlorogenic acid, chloridric acid and acetic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil). The $CH_3CN$ and MeOH (HPLC grade) were from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). All laboratory chemicals used in this study were of analytical grade.

**Plant material and extracts preparation:** *Vanillomopsis arborea* was collected in Crato City, state of Ceará, Brazil. The plant material was identified by Dr. Arlene Pessoa and a voucher specimen was deposited under the number 18639 at the Herbarium "Dardano de Andrade Lima" of the Universidade Regional do Cariri (URCA). The aqueous extracts of leaves, bark and trunk were obtained by infusion in hot water at 100°C and they were prepared prior to use.

**Essential oil analysis:** Oil analysis was performed using a Shimadzu GC-17 A/MS QP5050A (GC/MS system): DB-5HT capillary column (30 m×0.251 mm, 0.1 μm film thickness); helium carrier gas at 1.7 mL min$^{-1}$, injector temperature 270°C, detector temperature 290°C, column temperature 60°C (2 min) to 180°C (1 min) and at 4°C min$^{-1}$. Then 180-260°C; at 10°C min$^{-1}$ (10 min). Scanning speed was

0.5 scan/sec from 40-450 m/z. Split ratio (1:30). Injected volume: About 1 μL of 5 mg $mL^{-1}$ solution ethyl acetate. Solvent cut time = 3 min. The mass spectrometer was operated using 70 eV of ionization energy. Identification of individual components was based on their mass spectral fragmentation based on two computer library MS searches (Wiley 229), retention indices and comparison with published data. The EOVA was extracted from chopped plant trunk by steam distillation and analyzed at the Natural Products Research Laboratory of the Regional University of Cariri (URCA). Freshly chopped trunk was placed in a glass flask connected at one end to a glass vessel with water and at the other end to a water-cooled condenser. When the water was boiled, steam percolated through the barks and was collected in the condenser. After condensation, the essential oil was separated from the aqueous phase with its solutes.

**Animals:** Male Wistar rats (3.0-3.5 months of age and weighing (270-320 g)) had free access to food and water and were maintained in a room with controlled temperature (22±3°C) and on a 12 h light/dark cycle. The animals were maintained and used in accordance to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

**Tissue preparation:** Rats were killed by decapitation and the encephalic tissue-brain was rapidly dissected and placed on ice. Tissue was immediately homogenized in cold 10 mM tris-HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000×g to yield a pellet that was discarded and a low-speed supernatant (S1) was used for the lipid peroxidation assay[29].

**Lipid peroxidation assay:** Lipid peroxidation was determined by measuring the production of TBARS according to the method of Ohkawa[30] with slights modifications[29]. The homogenate (100 μL) was pre-incubated with or without 50 μL of freshly prepared pro-oxidant (iron sulfate, 10 μM) agent and different concentrations of the plant extracts together with an appropriate volume 10 mM tris–HCl, pH 7.4 to give a total volume of 300 μL at 37°C. After 1 h of incubation at 100°C, the color reaction was developed by adding subsequently, 200 μL of 8.1% Sodium Dodecyl Sulphate (SDS), 500 μL of 1.33 M acetic acid buffer (pH 3.4) and 500 μL of 0.6% TBA (thiobarbithuric acid). After cooling the tubes, the absorbance was read at 532 nm in a spectrophotometer. The results were expressed as nmol of MDA/g tissue.

**Iron chelation assay:** The ability of the aqueous extracts from the leaves, trunk and bark of *V. arborea* to chelate Fe (II) was determined using a modified method of Puntel *et al.*[31]. Briefly, 20 μL of freshly prepared 150 μM $FeSO_4$ were added to a reaction mixture containing 168 μL of 0.1 M tris–HCl (pH 7.4), 218 μL saline and different concentrations of aqueous extract of the plant. The reaction mixture was incubated for 5 min, before the addition of 13 μL of 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in the spectrophotometer.

**DPPH radical scavenging activity:** The potential of essential oil from the trunk of *V. arborea* and aqueous extracts from the leaves, the trunk and the bark of *V. arborea* to scavenge the DPPH radical was evaluated according to Hatano[32]. The absorbance was measured at 517 nm using spectrophotometer and the percent inhibition was calculated in relation to the control. Ascorbic acid known as standard antioxidant was used as reference.

**Determination of ROS formation in stomach:** The levels of ROS in stomach mucus were measured by the oxidation of 2',7'-dichlorofluorescein (DCFH) as described by Wang and Joseph[33]. The mucus obtained from rat was homogenized in cold 5 mM tris-HCl, pH 7.4 (1/20, w/v) and then was pre-incubated with different concentrations of essential oil of *V. arborea*, (-)-α-bisabolol or vitamin E, as a positive control. Experiments were carried out in a standard reaction medium containing $H_2O_2$ (20 μL/30%), 20 μL of mucus, tris-HCl (5 mM), pH 7.4, DCFH-DA (5 μM). The fluorescence emission of DFC resulting from DCFH oxidation was monitored in three Independent experiment for 600 sec (10 min) at 480 and 525 nm, excitation and emission wavelengths, respectively, using spectrofluorimeter (Fluorescence spectrophotometer, Hitachi F-2000). The results were expressed as percent of controls.

**Quantification of primary constituents:** Chromatographic analyses were carried out in isocratic conditions using RP-$C_{18}$ column (4.6×250 mm) packed with 5 μm diameter particles. The mobile phase consisted of methanol-acetonitrile-water (45:10:45, v/v/v) containing 1.0% acetic acid. The flow rate was 0.8 mL $min^{-1}$, injection volume 50 μL and the wavelength 257 nm. The mobile phase was filtered through a membrane filter 0.45 μm and then degassed by an ultrasonic sound before use. The solutions of standards (rutin, caffeic

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acid, chlorogenic acid, gallic acid and (-)-α-bisabolol) were prepared in the same mobile phase of HPLC. The concentration range for the standard curves used was 0.0125-0.200 mg $mL^{-1}$. The chromatographic peaks were confirmed by comparing its retention time with those of reference standards and quantification was performed by peak integration using the external standard method. The calibration curve for caffeic acid was: Y = 12153x-21513 (r = 0.9983), the curve of gallic acid was: Y = 109130x-526314 (r = 0.9998) and the curve of rutin was: Y = 102171x-16949 (r = 1). All chromatographic operations were performed at room temperature and in triplicate.

**Statistical analysis:** The results are expressed as Mean±SEM (standard error of mean). Statistical analysis was performed using a one or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison or Bonferroni posttests multiple comparison test when appropriated. Data from TBARS, DPPH and Iron chelation assays were analyzed by one-way ANOVA, while, that of DCFH oxidation was analyzed by two-way ANOVA. The results were considered statistically significant for $p<0.05$.

## RESULTS

**Quantification of primary constituents:** The quantification of the constituents of the extracts by HPLC showed that in the aqueous extract of the bark the major constituents are gallic acid (7.25%) and BISA (2.08%); in the aqueous extract of the leaves are gallic acid (11.03%), chlorogenic acid (5.23%) and BISA (4.12%) and in the trunk aqueous extract of the trunk are gallic acid (6.56%) and BISA (2.34%) (Fig. 2a-c).

**Lipid Peroxidation (LP):** The iron-induced lipid peroxidation was reduced by aqueous extract from leaves of *V. arborea* as shown in Fig. 2. Statistical analyzes revealed that $Fe^{2+}$ induced a significant stimulation in brain LP levels ($p<0.05$), which were reduced by aqueous extract of *V. arborea* Baker leaves in a concentration-dependent manner $p<0.05$ (Fig. 3a). In contrast, the aqueous extracts from trunk and bark are not able in prevent lipid peroxidation in brain homogenates in tested conditions (Fig. 3b and c).

**Iron chelation assay:** The extracts tested did not showed any properties on the chelation of iron (II) chelation as showed in the Fig. 4.

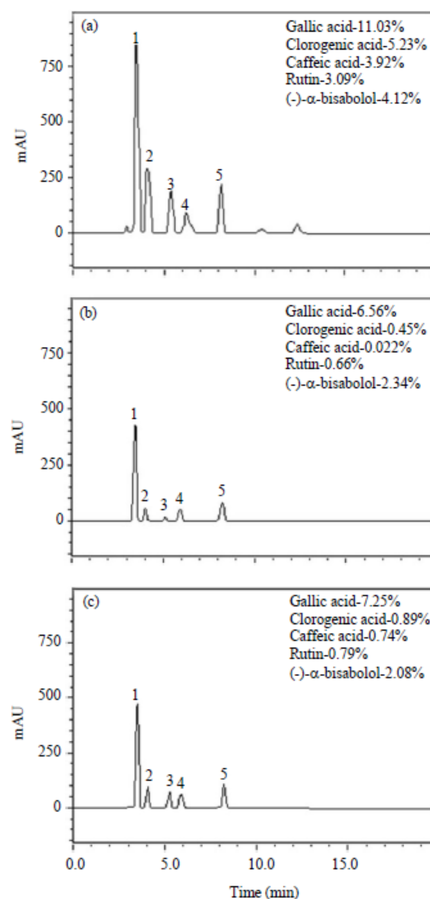


Fig. 2(a-c): Major components from aqueous extracts from *V. arborea* determined by HPLC analysis, (a) Aqueous leaves, (b) Aqueous trunk and (c) Aqueous bark

**DPPH radical scavenging:** The leaves aqueous extract and EOVA inhibited DPPH radical, with the maximal observable effect of 40 and 60% radical scavenging for essential oil trunk and aqueous leaves extract (Fig. 5a, b). In contrast, aqueous extract from trunk did not reduce DDPH radical (Fig. 5c). Bark aqueous extract moderately reduced DPPH radical and its maximal effects was about 40% inhibition (Fig. 5d).

**Determination of ROS formation in stomach:** Data show ROS formation in the mucus was markedly increased in the presence of $H_2O_2$, as expected. The EOVA or (-)-α-bisabolol

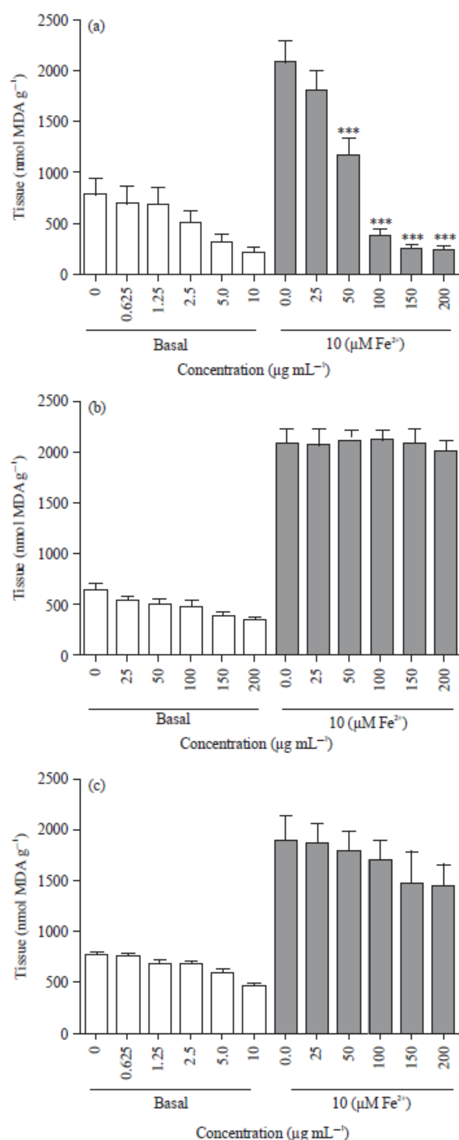


Fig. 3(a-c): Effects of different aqueous extracts from (a) Leaves, (b) Trunk and (c) Barks of *V. arborea* Baker on $Fe^{2+}$ (10 µM)-induced LP production in brain homogenates. Values are expressed bas Mean±SEM from 3-4 independent experiments performed in duplicate. ***$p<0.001$ vs. $Fe^{2+}$-induced TBARS

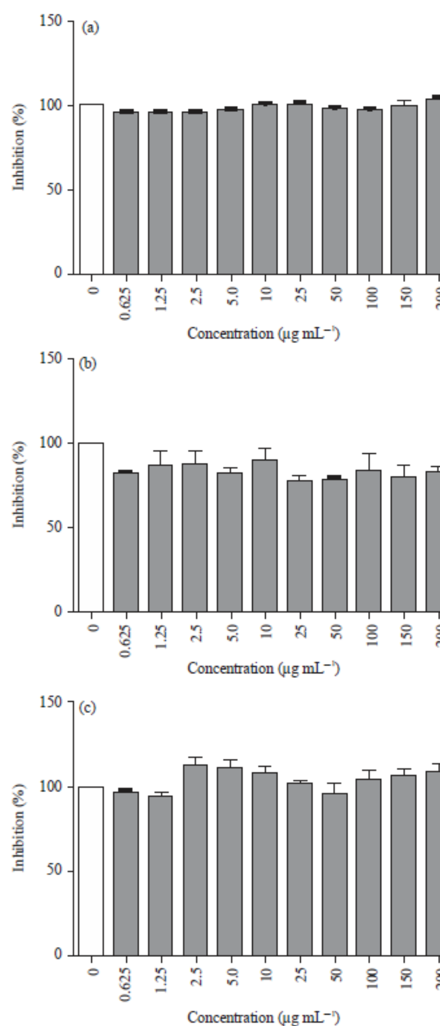


Fig. 4(a-c): Effects of different aqueous extracts from (a) Leaves, (b) Trunk and (c) Barks of *V. arborea* Baker on iron chelation. Values are expressed bas Mean±SEM from 3-4 independent experiments performed triplicate

added in the presence of $H_2O_2$ caused a reduction in ROS production in a concentration-dependent manner (Fig. 6).

## DISCUSSION

In this study, tested the effect of the plant *V. arborea* against well-known pro-oxidant agents, to investigate the effects of this plant and search for new potential antioxidants from natural sources. The results suggested that *V. arborea* leave extract and EOVA presented a markedly antioxidant

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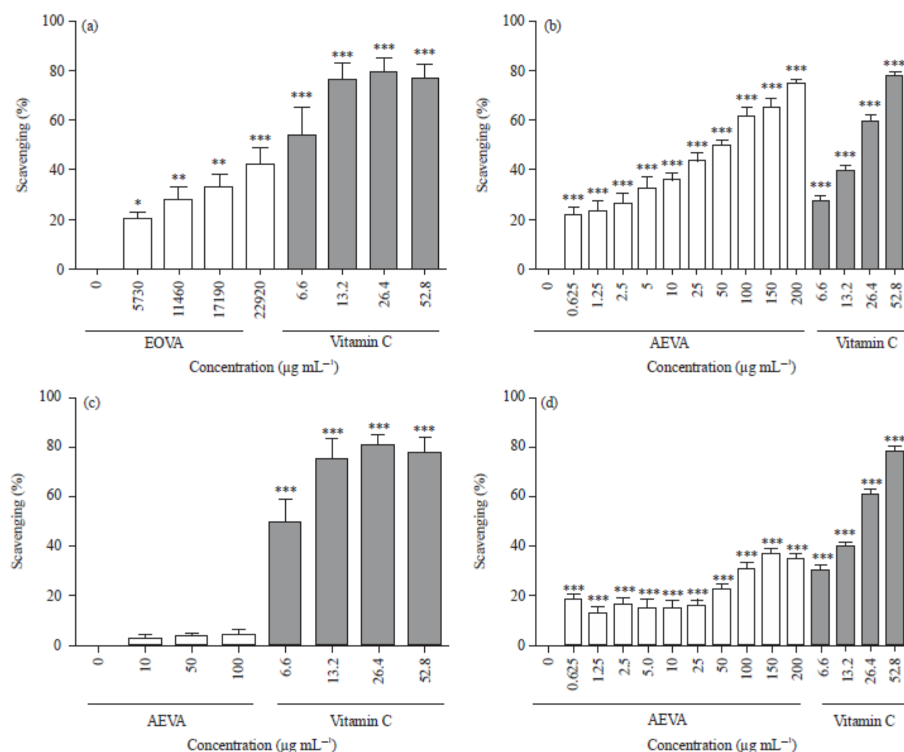


Fig. 5(a-d): DPPH scavenging activity of (a) Essential oil of trunk of *V. arborea* Baker (EOVA) and effects of different aqueous extracts from (b) Leaves, (c) Trunk and (d) Barks of *V. arborea* Baker on DPPH test. The results are expressed as percentage of inhibition and ascorbic acid was used as a positive control. Values are expressed as Mean±SEM from 3-4 independent experiments performed in triplicate. ***p<0.001 vs. control, **p<0.01 vs. control, *p<0.05 vs control

capacity by scavenging free radicals. In addition EOVA and their major compound (-)-α-bisabolol reduced ROS formation in stomach mucous exposed to $H_2O_2$.

The brain is particularly susceptible to free radical damage due its high consumption of oxygen and its relative low concentration of antioxidants enzymes and free radicals scavengers. Considering this, used cerebral tissue for the TBARS assay. The iron-induced lipid peroxidation was reduced by aqueous extract from leaves of *V. arborea*. In contrast, the aqueous extracts from trunk and bark are not able in prevent lipid peroxidation in brain homogenates in tested conditions. These results are probably due to the minor content of caffeic acid, rutin, chlorogenic acid and (-)-α-bisabolol in these plant structures compared, which the extract from the leaves (Fig. 2). Take into account that free iron in the cytosol and in the mitochondria can cause considerable oxidative damage by increasing ROS production[34] via stimulation of fenton reaction[35], attribute the *V. arborea* effect in decrease/prevent lipid peroxidation to a possible free radical scavenging capacity of the plant contents, once this data do not show any iron chelation properties of all plant extracts, as shown in Fig. 4.

The 1,1-diphenil-2-picrylhydrazyl (DPPH) radical has been widely used to test the free radical scavenging ability of various natural products and has been accepted as a model compound for free radicals[32,36]. This hypothesis gathered strength when tested the plant extracts in a radical scavenging model, with the DPPH radical scavenging test. Data demonstrated that that leaves aqueous extract presented higher scavenging activity than bark and trunk. Here, introduced to our study essential oil of the plant (EOVA); this extract from the truck by practical laboratorial reasons, as well as because of the increased oil yield. Additionally, the inhibition of lipid peroxidation by *V. arborea* extracts showed

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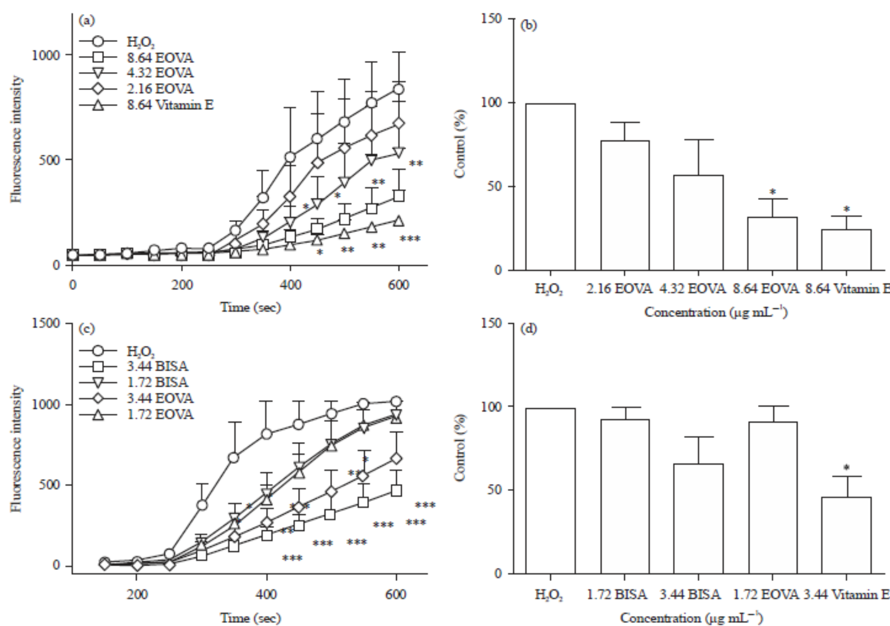


Fig. 6(a-d): Effects of essential oil of trunk of *V. arborea* Baker (EOVA) and (-)-α-Bisabolol in mucous ROS generation during 600 sec. The mucous were exposed to (20 μL/30%) $H_2O_2$ in the presence or absence of different concentration of (a-b) EOVA or (a-d) (-)-α-bisabolol. Data are expressed as Mean±SEM and are calculated as percent control for five independent assays (graphic showing fluorescence intensity versus time and the graph shows the second fluorescence intensity difference)

a relation with its phenol content (Fig. 2), suggesting once more that the effects are related with these compounds, which is also confirmed in the literature[37]. Especially important was the effect of EOVA from the trunks in the DPPH radical scavenging test.

Since, the EOVA showed an increased amount of (-)-α-bisabolol, we hypothesized whether this compound could be a major responsible for the antioxidant effects of plant, once it occurs in relatively high concentrations in the aqueous extract from leaves.

In order to test if (-)-α-bisabolol is a main responsible for the antioxidant effects observed used a model of oxidative stress in stomach mucous of rats. This choice was due to the major role of reactive oxygen species in the development of stomach pathogeneses, especially in gastric mucosal lesion associated with water immersion stress, anti-inflammatory drugs and ethanol-induced ulcers[38]. Under these conditions, there is an imbalance between formation and degradation of these species. The enzymatic antioxidant defenses and non-enzymatic cannot restrain the ROS increase, thus, may exert deleterious actions on the gastric mucosal epithelium.

Gastrointestinal tract cells have an antioxidant defense system capable of preventing the cytotoxicity of ROS through mechanisms that involve the action of enzymes and compounds with potential to scavenge free radicals. In the list of enzymes involved in this action are superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and catalase (Cat). The mucosa is also protected in a non-enzymatic mechanisms such as reduced glutathione (GSH), alpha-tocopherol (vitamin E), vitamin C, carotenoids, methionine and taurine, which can bind or reduce the oxygen radicals and prevent their harmful actions[39].

Choose here the DCFHDA oxidation test to measure the amount of ROS generated. Briefly, DCFH-DA is cleaved intracellular by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound

DCF. Data show ROS formation in the mucus was markedly increased in the presence of $H_2O_2$ as expected. The EOVA or (-)-α-bisabolol added in the presence of $H_2O_2$ caused a reduction in ROS production in a concentration-dependent manner (Fig. 6). Vitamin E, used here as a positive control, presented a markedly protection against ROS production. Taken together, the data suggest a major role of the (-)-α-bisabolol for the therapeutic properties of the *V. arborea*, acting as an antioxidant molecule and may be the responsible for the pharmacological properties previously observed to the plant here studied. This idea is reinforced by the previously showed gastroprotective effect of the (-)-α-bisabolol against ethanol- and indomethacin-induced ulcer in a model in mice, which effect as related to its antioxidant activity[40]. In addition, a previous study from the same research group make a relation between the gastroprotective effects of (-)-α-bisabolol and the reduction in lipid peroxidation in the gastric mucosa[41] and the gastroprotective activity presented by EOVA[7] can be related to antioxidant capacity, preventing the cytotoxicity of ROS.

## CONCLUSION

In conclusion, work demonstrates that *V. arborea,* aqueous extracts and essential oil, present significant antioxidant activity, acting through scavenging radical directly. In addition, demonstrated here that (-)-α-bisabolol is, at least in part, responsible for the therapeutic properties of *V. arborea*. Consequently, this plant could be used as a potential antioxidant agent for the prevention of diseases associated with oxidative damage.

## ACKNOWLEDGMENTS

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3.2. MANUSCRITO 1

**Evaluation of toxicity of (-)-α-bisabolol in different experimental models**

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**ABSTRACT**

(-)-α-Bisabolol (BISA), a sesquiterpene, is a naturally occurring volatile constituent found in the essential oil of several plants from the Asteraceae family, widely used as a herbal remedy due of its anti-inflammatory, antimicrobial, fungicidal, anti-spasmodic and anti-allergic properties. The aim of this work was to evaluate the effect of BISA on survival rate and ROS production in *Drosophila melanogaster* and the parameters of cytotoxicity and genotoxicity in peripheral human blood mononuclear and red blood cells (PBMCs). The BISA flies effect was evaluated using a survival rate of flies, ROS production by 2’7’-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation and antioxidant activity modulation by analyzing the catalase activity. Additionally, we analyzed cell viability (MTT assay), nuclear damage (comet test) in peripheral blood mononuclear cells (PBMCs) and the possible hemolytic activity of BISA to the red blood cells obtained from peripheral blood. The BISA demonstrated no toxicity in the *D. melanogaster* model. BISA caused cytotoxicity and genotoxicity when PBMC and red blood cells were exposed to higher concentrations, decreasing cellular viability, causing damage at nuclear level and presenting hemolytic activity only when the cells were exposed to high concentrations of BISA and for a long period. Thus, EOVA and BISA presented a satisfactory effect in animal models tested here, but presented a toxic effect in PBMCs and red blood cells, emphasizing that more studies are necessary to characterize the pharmacological activity and toxicity of BISA.

**Keywords:** (-)-α-Bisabolol; toxicity; oxidative damage; cell death.

## 1. Introduction

(-)-α-Bisabolol (BISA), is a monocyclic sesquiterpene alcohol which was first isolated in 1951 by Isaac and collaborators from the blossoms of chamomile (*Matricaria chamomilla*; Asteraceae) (Figure 1) [1]. BISA is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents [2]. Concerning its pharmacologic effects, previous studies showed that BISA demonstrated apoptosis-induced action in malignant tumor cells [3, 4]; inhibition of the activities of major human drug-metabolizing enzymes [5] and antimutagenic [6] and antipeptic properties [7], inhibition of peripheral nerve conduction [8], blockade of $Ca^{2+}$ [9] and $K^{+}$ channels [10], gastroprotective [10-12], anti-nociceptive and ant-inflammatory activities [13-15], leishimanicidal [16], antioxidant [11,17-18], wound healing [19] properties, inhibitory actions on α7-nicotinic acetylcholine receptors [20] and nephroprotective effects [21].

Increasing evidences have suggested that many degenerative diseases such as brain dysfunction, cancer, heart disease and immune system decline could be the result of cellulardamage caused by free radicals and that anti-oxidants may play an important role in disease prevention [22]. However, the most of the knowledge of biological effects of (-)-α-Bisabolol are due to its antioxidant activity [23]. (-)-α-Bisabolol becomes of paramount importance when studying these injuries, which possess an oxidative component.

The aim of this study was to screening the effect of (-)-α-Bisabolol in different experimental models. We test toxicity effect of (-)-α-Bisabolol in Drosophila model and genotoxic and cytotoxic activities of (-)-α-Bisabolol in culture in peripheral blood mononuclear and red cells.

## 2. Materials and methods

### Materiais

(-)-α-Bisabolol, Dimetilsulfoxide (DMSO), (4-5-dimetil) -2-5diphenyl tetrazolium (MTT), Histopaque – 1077®, 2',7'-dichlorofluorescein (DCFH) were obtained from Sigma – Aldrich (São Paulo, Brazil). Polysorbate 80 (Tween® 80) was provided by Delaware (Porto Alegre, Brazil). Dulbecco's modified Eagle's médium (DMEM), Fungizona, Penicillin / Streptomycin, trypsin solution 0.25% EDTA and fetal bovine serum (FBS) were obtained

from Gibco (Carlsbad, CA). Thiobarbituric acid (TBA), Tris–HCl was purchased from Merck® (Alemanhã). Butylated hydroxy-toluene (BHT) was purchased from Alpha Chemistry® (Porto Alegre, Brazil) and trichloroacetic acid (TCA) was obtained from Neon® (São Paulo, Brazil). The blue color of trypan and hydrogen peroxide were supplied by Nuclear® (São Paulo, Brazil). Culture plates were obtained from TPP® (USA) and culture medium RPMI from Vitrocell® (São Paulo, Brazil). All other products were obtained from standard commercial suppliers.

***Drosophila* stock**

*D. melanogaster* (Harwich strain) used in the present investigation were obtained from the National Species Stock Center (Bowling Green, OH, USA). The flies were reared in vials containing 3 mL agar medium (2%, w/v sucrose; 1%, w/v brewer's yeast; 1%, w/v powdered milk; 1%, w/v agar; 0.08%, v/w nipagin) at constant temperature and humidity (23 ± 1 ºC and 60% relative humidity, respectively) with a 12-hour light and dark cycle. All experiments were performed using the same strain.

**BISA exposure and *D. melanogaster* assays**

Flies (1–2 days old, male and female) were divided into four groups: (1) control; (2, 3, and 4) BISA (5, 25, and 250 µM, respectively); BISA was added into the fly food. The flies were exposed to the treated food for 7 days [24] and the vials maintained in an incubator at 23 ± 1 ºC until being processed for various assays.

**Survival Rate**

The vials were scored daily for mortality. The survival rate was based on the live flies counting each day until they die, and the survivors were transferred to freshly prepared food. The number of flies in the final calculation represents the sum of three independent experiments (20 flies/treatment/repetition).

**Dichlorofluorescein Oxidation**

For oxidation, 20 treated flies per group were homogenized in 200 µL Tris-HCL 10 mM, pH 7.4, and centrifuged at 4000 g for 10 min at 4ºC. The resulted supernatant was used for

2’,7’-dichlorofluorescein diacetate (DCFDA) oxidation [25]. The fluorescence emission of DCFH resulting from DCFH-DA oxidation was monitored at regular intervals at an excitation wavelength of 488 nm and an emission wavelength of 522 nm. The rate of DCF formation was calculated as of the DCF formation (AFU/mg protein) in relation to the control group.

**Catalase**

For enzyme activity, 20 treated flies per group were homogenized in 200 μL Tris-HCL 10 mM, pH 7.4, and centrifuged at 4000 g for 10 min at 4℃. The resulted supernatant was used for determination of catalase. Catalase activity was measured following the method of Aebi [26]. In 1 ml reaction mixture containing 0.3 M $H_2O_2$ (3%), 50 mM sodium phosphate buffer, pH 7.0. The reaction was initiated by adding an aliquot. The decrease in the absorbance of $H_2O_2$ was monitored for 150s at 240 nm. The enzyme activity was performed at room temperature (25±1℃) using a Thermo Scientific Evolution 60s UV–Vis spectrophotometer.

**Collection of human blood samples**

Peripheral blood samples were obtained from the Clinical Analysis Laboratory of the Franciscan University Center, under the approval of the Institutional Ethics Committee of the institution (CAAE: 31211214.4.0000.5306) without the identification data. The collection was performed through a venous catheter using tubes with Vacutainer®-type heparin, which were used to separate red cells and PBMC.

**Treatments carried out in PBMC**

To test the cytotoxic and genotoxic effects of the compound on cell viability and DNA damage, an experimental protocol similar to that described by Wilms et al. [27], in PBMC. The incubation medium containing the cells was used as a negative control was used without treatment and the incubation medium adding 100 mM of hydrogen peroxide was used as a positive control of cell damage.

**Separation of mononuclear cells**

The PBMC separations occurred by density gradient (Histopaque®-1077) by centrifugation, and the concentration of $2x10^5$ cells was obtained by counting in a Neubauer chamber with 0.4% Trypan blue.

**Cell viability (MTT)**

Cell viability was determined by the method described by Mosmann [28]. The cytotoxic activity in PBMC was evaluated by the colorimetric method, whose principle is based on the reduction of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) in colored product Dark purple, MTT-formazan, by the mitochondrial enzymes of viable cells. The decreasing in the absorbance of the tests relative to the negative control indicates cell death. The experiment was performed in triplicate on a 96-well ELISA. The plate was incubated at 37 ° C with 5% $CO_2$ for 72 hours, and the samples were analyzed in a spectrophotometer at 570 nm. The experiment was performed in triplicate and the results were expressed as a percentage of the control.

**Comet assay**

The comet test was performed according to Singh et al. [29], modified by García et al. [30]. On a glass slide previously covered with a 1.5% agarose layer, PBMC were incubated for 24 and 72 hours in a $CO_2$ oven and suspended in low melting agarose (Low Melting). The material was immersed in lysis solution (89 mL of lysis solution to 10mL of dimethyl sulfoxide and 1mL of Triton X-100), for the removal of membranes and cytoplasm from the cells. Next, the slides was incubated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA in distilled water) and electrophoresed for about 30 minutes at 25 V and 300 mA. Subsequently, the process of neutralization, fixation and staining was performed so that the genetic material was analyzed.

The analysis of each slide was done under an optical microscope and the cells was classified according with image format in four damage classes proposed by García et al. [30] and illustrated by Fronza et al. [31], ranging from 0 (none damage) to 4 (maximum damage), including also the classification of cellular apoptosis.

**Hemolysis**

For the assessment of hemolysis, total peripheral blood was collected with heparin anticoagulant. The Red blood cells were then centrifuged for 15 minutes at 1000 rpm and washed three times with PBS 1X. After washes, 400 µL of red cells were with drawn and transferred to another Falcon tube adding 1 ml of PBS 1X. Subsequently, 80 µL of the treatments (BISA) were added and then incubated for one hour at 37° C in a 5% $CO_2$ green house. The material was centrifuged for 5 minutes at 1000 rpm, the supernatant removed and transferred to a 96-well culture plate (ELISA). The reading was performed in an ELISA reader, at the wavelength of 405 nm. The negative control was done with the cells together with PBS, and the positive control cells were added with PBS 1X plus the red blood cell lysing solution (Ammonium chloride ($NH_4Cl$) plus potassium tricarbomate ($KHCO_3$); EDTA in milli-q water).

**Statistical analysis**

Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post hoc test or using a two-way ANOVA followed by Bonferroni post tests for survival curve. Differences between groups were considered to be significant when $p<0.05$.

## 3. Results

**Effect of BISA on survival rate of flies**

Drosophila treated with BISA (5, 25, and 250 µM) did not show any alterations in flies survival in relation to control up to 7 days of treatment (Figure 2).

**Effect of BISA on DCFDA Oxidation in flies**

The oxidation of the fluorescent dye DCFDA was used as a general index of ROS formation (Figure 3). Flies exposed to BISA condition did not presented a significant increase in DCFDA oxidation.

**Effect of BISA on catalase in homogenate of flies**

The activity of the enzyme catalase determined in control and flies exposed to BISA for 7 consecutive days are presented in Figure 4. There were no effect on catalase activity in flies exposed to BISA when compared with the control.

**Effect of BISA cell death in PBMC**

MTT assay demonstrated that when PBMC were incubated with BISA for up 24 hours, the viability percentage significantly decreased at the higher concentration (300 μg/mL) of BISA tested, as compared to the negative control ($p < 0.001$). In addition, cells exposed to BISA for 72 hours had a decrease on the viability significantly in relation to the negative control at the concentrations of 100 and 300 μg/mL ($p < 0.001$) (Figure 5B), demonstrating higher cellular damage when exposed to the BISA for a longer period of time (chronic damage). The BISA (1-30 μg/mL) increased cell viability when compared to the positive control. Hydrogen peroxide decreased cell viability in relation to the negative control ($p < 0.001$) at both incubation times.

**Effect of BISA DNA damage in PBMC**

Analyzes of nuclear damage by comet test demonstrated that when PBMC were incubated at BISA exposure for 24 hours, only the higher concentration tested demonstrated significant nuclear damage. However, when the cells are exposed for a longer period of time (72 hours) we can observe a significant damage in all concentration tested (Table 01).

**Effect of BISA in hemolysis test in total peripheral blood**

In the hemolysis evaluation, the highest concentration of BISA (300μg/ml) presented hemolytic activity significantly different of negative control ($p < 0.001$; Figure 6).

**4. Discussion**

In the current study, the results showed the BISA does not demonstrated toxicity in the *D. melanogaster* model. BISA caused cytotoxicity and genotoxicity when PBMC and red blood cells are exposed only to its higher concentrations, reducing cellular viability, causing damage at the nuclear level and presenting hemolytic activity.

*D. melanogaster* has been used for toxicity assays, as an alternative method to the use of vertebrate animals, and it has become an excellent alternative model for several assays [32-34]. The results showed none toxicity of this compound and demonstrates a potential in pharmacological application. Here, we have demonstrated that in a short period of exposure, concentrations of BISA are not able to induce mortality in Drosophila. In parallel to the induced mortality, flies exposed to BISA did not showed signs of oxidative stress, including ROS formation as well as changes in important antioxidant response systems.

Moreover, for better understanding of possible pro-oxidant effects of BISA action against oxidative damage, we assessed the activities of antioxidant enzyme catalase and the levels of ROS, in the flies. The protective role of catalase in the cells is related to the elimination of deleterious $H_2O_2$ [35-36]. In the present study, however, no significant difference was observed among the groups on the catalase levels. The production of reactive species was not altered for BISA.

As demonstrated in Figure 6, the higher concentrations of BISA resulted in a reduction on cellular viability, compared to the negative control. The cells exposed to the BISA for a time of 72 hours, demonstrated cellular damage. In previous studies, it was observed cytotoxic effect of BISA on several human cancer cell lines different from those mentioned [3, 37, 38, 39], and came to the conclusion that BISA-induced apoptosis in HepG2 cells in a dose- and time-dependent manner [40] and its uptake is mediated by lipid rafts on the plasma membrane [3]. The effectiveness of BISA as an agent against tumor cells is grounded on its capability to act on different layers of cell regulation to elicit different concurrent death signals, thereby neutralizing a variety of aberrant survival mechanisms leading to treatment resistance in neoplastic cell [41].

Our analyzes of nuclear damage through the comet assay demonstrated that when PBMC were incubated at BISA exposure for 24 hours, only the concentration 300 µg/ml demonstrated nuclear damage. However, when the cells are exposed for a period of 72 hours we observe a significant damage from the concentration of 10 µg/ml.

Similar to our study, Cavalieri et al. [37] demonstrated that BISA has a potent cytotoxic effect when correlating its dose and exposure time on malignant rat and human glioma cell lines, rapidly inducing them to apoptosis through the mitochondrial pathway

with no toxic effect on cells. In addition, these authors demonstrated that BISA is able to induce apoptotic cell death preferentially in tumor cells and suggest that its toxicity is strictly related to its intracellular concentration [38]. Intracellular concentrations of BISA in human glioma cells have shown that it is rapidly distributed in fractions in the membrane, nucleus and cytosol. The authors suggest that this rapid distribution occurs once it is absorbed into lipid rafts (dynamic membrane domains and enriched with sphingolipids and sterol, which play an important role in the transport of intracellular proteins and membrane fusion), this is transported into the cell quickly. Thus, according to previous work, BISA enters cells via lipid rafts and directly involves mitochondrial permeability transition pore opening, which is responsible for the reduced glutamate/malate supported oxygen consumption and leads to disruption of the mitochondrial membrane potential and programmed cell death [42], causing nuclear damage.

The hemolysis test has been used by several authors to evaluate the toxic effects of different plants. Our results showed that the concentration of the BISA (300µg/ml) was the most toxic, from this, it is emphasized that more concentrated doses of the compound can produce a hemolytic activity in total peripheral blood. We suggest that this toxicity can occur in two ways. One is the occurrence of solubilization of the plasma membrane of the erythrocyte [43]. The other mechanism comprises that reduced xenobiotic compounds, such as phenolic compounds, promote hemolysis through the oxidation of hemoglobin, giving rise to meta-hemoglobin [44]. Hemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. This hemolysis was related to concentration of BISA used.

## 5. Conclusion

The BISA demonstrated none toxicity and ROS generation in the *D. melanogaster* model. The compound caused toxicity for cells at longer period and higher concentrations only. Our data emphasizes this substance has important biological properties together with relative low toxicity in *in vivo* and *in vitro* models.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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**Figure Legends**

**Figure 1** – (-)-α-Bisabolol chemical structure.

**Figure 2 –** Effect of (-)-α-Bisabolol (5, 25, 250 μM) on survival rate of flies. Data were collected every 24 h for each group. The numbers of surviving flies are represented as % of control (mean ± SEM). The total number of flies (75 per group) represents the sum of three independent experiments, analyzed by two-way ANOVA followed by Bonferroni post tests.

**Figure 3 –** Effect of (-)-α-Bisabolol (5, 25, 250 μM) on *D. melanogaster* ROS formation. 2’,7’-dichlorofluorescein diacetate (DCFH) oxidation was used as a general index of reactive oxygen species (ROS) production. Results are expressed as mean ± SEM of the arbitrary fluorescence units emitted followed by DCFDA oxidation in flies’samples, analyzed by one-way ANOVA, followed by Dunnett's post hoc test.

**Figure 4 –** Effect of (-)-α-Bisabolol (5, 25, 250 μM) on Catalase (CAT) activity in homogenate of flies. Results are expressed as mean ± SEM of the in flies’samples, analyzed by one-way ANOVA, followed by Dunnett's post hoc test.

**Figure 5** – Effect of (-)-α-Bisabolol on the MTT assay in PBMC after 24 hours of treatment (A) and after 72 hours of treatment (B). Data were expressed as a percentage of the negative control (C-). C +: positive control ($H_2O_2$-100 mM). Cell viability percentage values are expressed as mean ± SD, analyzed by one-way ANOVA, followed by Dunnett's

post hoc test. ** ($p < 0.01$), *** ($p < 0.001$) represents statistical difference when compared to the negative control.

**Figure 6 –** Effect of (-)-α-Bisabolol on the hemolysis test in total peripheral blood. Data were expressed as percentage of positive control ($H_2O_2$-100 mM). C-: negative control. The hemolysis percentage values are expressed as mean ± SD, analyzed by one-way ANOVA, followed by Dunnett's post hoc test. *** ($p < 0.001$) represents statistical difference when compared with the negative control.

**TABLE 1** – Comet assay damage analyzed by microscopy. Comparisons between positive control, negative control and concentrations of (-)-α-Bisabolol.

| TREATMENTS After 24 Hours | Damage 0 | Damage 1 | Damage 2 | Damage 3 | Damage 4 | Total nuclei analyzed (100) DI |
|---|---|---|---|---|---|---|
| **C-** | 98 | 2 | | | | 0.02 |
| **C+** | 50 | 10 | 10 | 10 | 20 | 0.50 |
| **1** | 98 | 2 | | | | 0.02 |
| **3** | 95 | 5 | | | | 0.05 |
| **10** | 96 | 4 | | | | 0.04 |
| **30** | 94 | 6 | | | | 0.06 |
| **100** | 92 | 8 | | | | 0.08 |
| **300** | 88 | 03 | 05 | 02 | 02 | 0.12 |
| **TREATMENTS After 72 Hours** | **Damage 0** | **Damage 1** | **Damage 2** | **Damage 3** | **Damage 4** | **Total nuclei analyzed (100) DI** |
| **C-** | 96 | 2 | 2 | | | 0.04 |
| **C+** | 30 | 15 | 12 | 23 | 20 | 0.70 |
| **1** | 93 | 02 | 01 | 03 | 1 | 0.07 |
| **3** | 90 | 03 | 02 | 02 | 3 | 0.10 |
| **10** | 75 | | 5 | 10 | 7 | 0.22 |
| **30** | 60 | | 10 | 10 | 10 | 0.30 |
| **100** | 50 | | 17 | 20 | 13 | 0.50 |
| **300** | 30 | | 30 | 20 | 20 | 0.70 |

Damage Index (DI): Σ (1,2,3,4 classification of comet damage) / 100 cells

**Figure 1** – (-)-α-Bisabolol chemical structure.

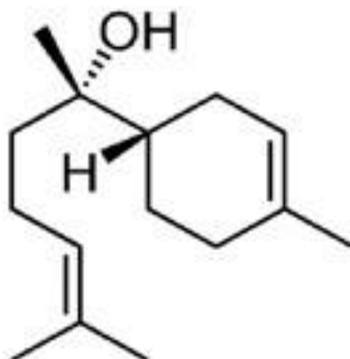



**Figure 2** – Effect of (-)-α-Bisabolol (5, 25, 250 μM) on survival rate of flies. Data were collected every 24 h for each group. The numbers of surviving flies are represented as % of control (mean ± SEM). The total number of flies (75 per group) represents the sum of three independent experiments, analyzed by two-way ANOVA followed by Bonferroni post tests.

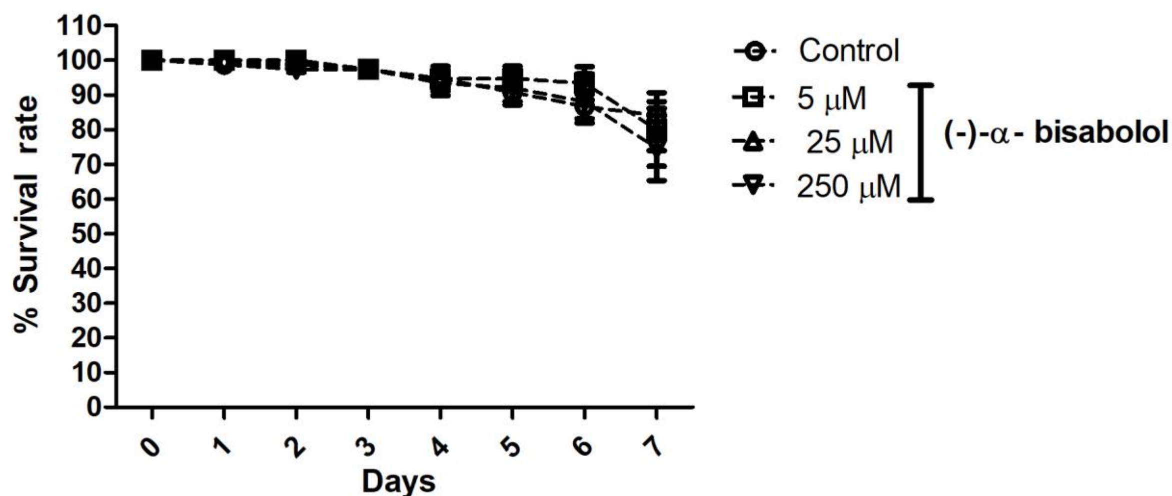


**Figure 3** – Effect of (-)-α-Bisabolol (5, 25, 250 μM) on *D. melanogaster* ROS formation. 2',7'-dichlorofluorescein diacetate (DCFH) oxidation was used as a general index of reactive oxygen species (ROS) production. Results are expressed as mean ± SEM of the arbitrary fluorescence units emitted followed by DCFDA oxidation in flies'samples, analyzed by one-way ANOVA, followed by Dunnett's post hoc test.

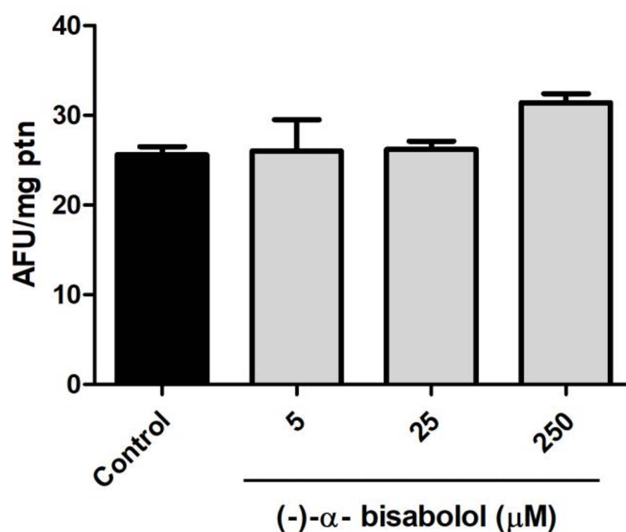



**Figure 4** – Effect of (-)-α-Bisabolol (5, 25, 250 μM) on Catalase (CAT) activity in homogenate of flies. Results are expressed as mean ± SEM of the in flies'samples, analyzed by one-way ANOVA, followed by Dunnett's post hoc test.

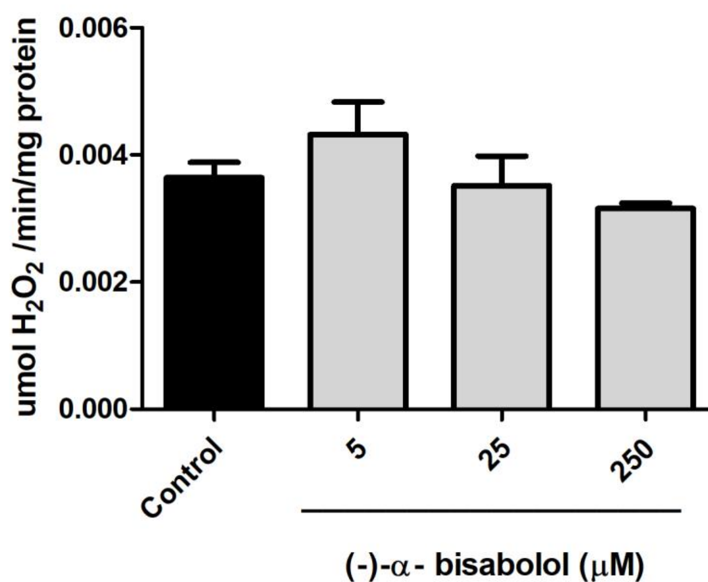


**Figure 5** – Effect of (-)-α-Bisabolol on the MTT assay in PBMC after 24 hours of treatment (A) and after 72 hours of treatment (B). Data were expressed as a percentage of the negative control (C-). C +: positive control ($H_2O_2$-100 mM). Cell viability percentage values are expressed as mean ± SD, analyzed by one-way ANOVA, followed by Dunnett's post hoc test. ** (p <0.01), *** (p <0.001) represents statistical difference when compared to the negative control.

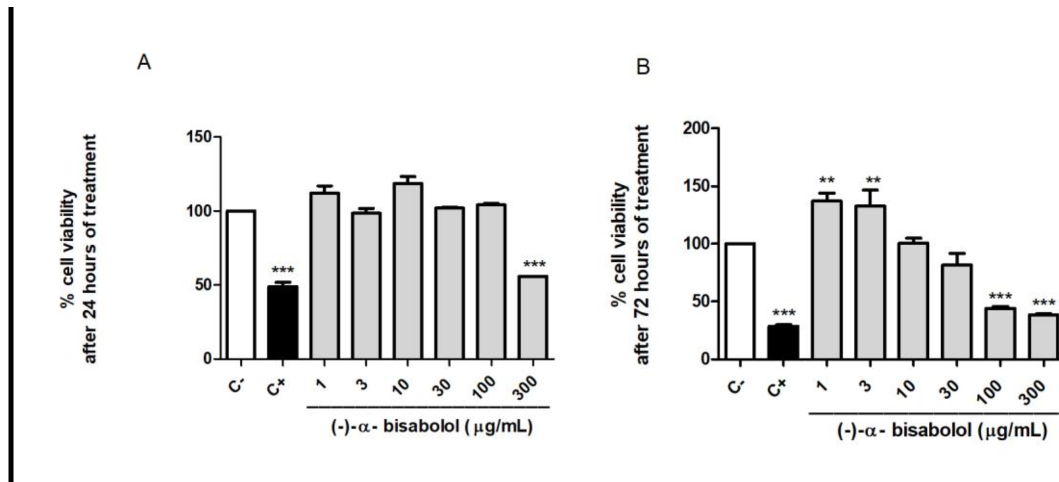
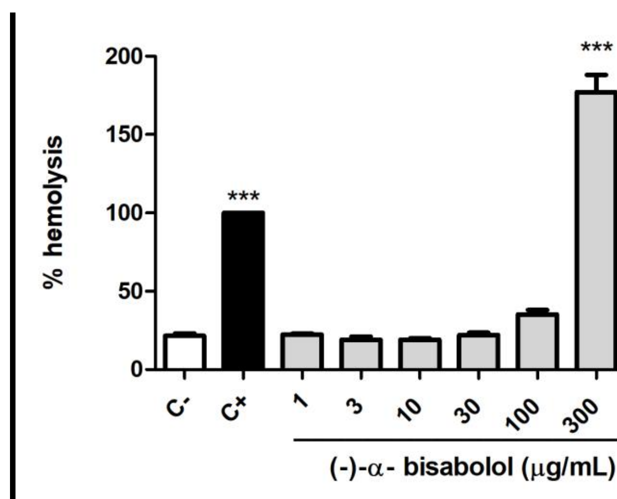



**Figure 6** – Effect of (-)-α-Bisabolol on the hemolysis test in total peripheral blood. Data were expressed as percentage of positive control ($H_2O_2$-100 mM). C-: negative control. The hemolysis percentage values are expressed as mean ± SD, analyzed by one-way ANOVA, followed by Dunnett's post hoc test. *** (p <0.001) represents statistical difference when compared with the negative control.



## 3.3. ARTIGO 3

**Protective effect of (-)-α-Bisabolol on rotenone-induced toxicity in *Drosophila melanogaster***

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**Abstract**

(-)-α-Bisabolol (BISA) is a sesquiterpene alcohol, which has with several recognized biological activities, including anti-inflammatory, anti-irritant, and antibacterial properties. In the present study, we investigated the influence of BISA (5, 25, and 250 μM) on rotenone (500 μM)-induced toxicity in *Drosophila melanogaster* for 7 days. BISA supplementation significantly decreased rotenone-induced mortality and locomotor deficits. The loss of motor function induced by rotenone correlated with a significant change in stress response factors; it decreased thiol levels, inhibited mitochondria

complex I, and increased the mRNA expression of antioxidant marker proteins such as superoxide dismutase (SOD), catalase (CAT), and the *keap1* gene product. Taken together, our findings indicate that the toxicity of rotenone is likely due to the direct inhibition of complex I activity, resulting in a high level of oxidative stress. Dietary supplementation with BISA affected the expression of SOD mRNA only at a concentration of 250 μM, and did not affect any other parameter measured. In conclusion, our results showed a protective effect of BISA on rotenone-induced mortality and locomotor deficits in Drosophila; this effect did not correlate with mitochondrial complex I activity, but may be related to the antioxidant protection afforded by eliminating superoxide generated as a result of rotenone-induced mitochondrial dysfunction.

**Keywords**: *Drosophila melanogaster,* (-)-α-bisabolol, oxidative stress, rotenone.

## 1. Introduction

(-)-α-Bisabolol (BISA), a volatile monocyclic sesquiterpene alcohol, is a naturally occurring constituent of the essential oil of several plants of the Asteraceae family. BISA is widely used in dermatological and cosmetic formulations (Gomes-Carneiro et al. 2005). BISA also displays various biological activities, such as antitumor activity (Darra et al. 2008; Silva et al., 2010), inhibition of peripheral nerve conduction (Alves et al. 2010), blockade of $Ca^{2+}$ (Siqueira et al. 2012) and $K^{+}$ channels (Bezerra et al. 2009), gastroprotection (Bezerra et al. 2009; Rocha et al. 2010, 2011a), anti-nociceptive and ant-inflammatory activities (Leite et al. 2011, 2012; Rocha et al. 2011b), leishmanicidal activity (Morales et al. 2010), antioxidant activity (Braga et al. 2009; Rocha et al. 2011a; Leite et al. 2016), antimutagenic activity (Gomes-Carneiro et al. 2005), wound healing properties (Villegas et al. 2001), and inhibitory actions on α7-nicotinic acetylcholine

a complex ketone derived from the roots of *Lonchocarpus* species, and is a high affinity specific inhibitor of mitochondrial NADH dehydrogenase (complex I) (Uversky 2004). Exposure of adult *Drosophila* to sublethal doses of rotenone in the diet over 7 days causes concentration-dependent locomotor deficits, dopaminergic specific neuronal loss, and a reduction in dopamine levels in adult flies (Coulom and Birman 2004).

The rotenone-based model has been broadly and successfully used by researchers to screen putative neuroprotective phytochemicals (Chaudhuri et al. 2007; Ravikumar et al. 2009; 2010; Sudati et al. 2013). In addition to disrupting electron transport by inhibiting mitochondrial complex I, rotenone causes oxidative damage in mitochondria that correlates with reduced SOD activity (Sherer et al. 2003; Cannon et al. 2009).

*Drosophila melanogaster* is the invertebrate organism closest to humans, based on gene sequence similarity and conservation (Rubin et al. 2000; Bier et al.2005). *Drosophila* are highly sensitive to toxic substances and are considered a useful model for identifying pollutants as well as for evaluating the biological action of pharmacological agents. In fact, *Drosophila* have been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) as promoting the 3Rs (reduction, refinement, and replacement) of laboratory animal use in toxicity and testing studies (Benford et al. 2000). *D. melanogaster* have been effectively used to investigate the underlying mechanisms in the pathophysiology of numerous neurological and non-

neurological human diseases (Nichols 2006; Pandley et al. 2011; Adedara et al. 2015) and are widely used to assess the therapeutic potential of phytochemicals.

Based on our findings on the antioxidant activity of BISA (Leite et al. 2016), we hypothesized that BISA could prove to be protective in neurotoxicity models. Therefore, we used rotenone-induced toxicity in a *D. melanogaster* as our test model. In the present investigation, we examined the ability of BISA to modulate rotenone-induced death, locomotor deficits, induction of antioxidant biomarkers, and mitochondrial dysfunction in *D. melanogaster*.

## 2. Materials and Methods

### 2.1. *Drosophila* stock

*D. melanogaster* (Harwich strain) used in the present investigation were obtained from the National Species Stock Center (Bowling Green, OH, USA). The flies were reared in vials containing 3 mL agar medium (2%, w/v sucrose; 1%, w/v brewer's yeast; 1%, w/v powdered milk; 1%, w/v agar; 0.08%, v/w nipagin) at constant temperature and humidity (23 ± 1 °C and 60% relative humidity, respectively) with a 12-hour light and dark cycle. All experiments were performed using the same strain.

### 2.2. Experimental Procedure

#### 2.2.1. Rotenone Exposure and (-)-*α*-Bisabolol Treatment

Flies (1–2 days old, male and female) were divided into eight groups: (1) control; (2) rotenone (500 µM); (3, 4, and 5) BISA (5, 25, and 250 µM, respectively); and (6, 7, and 8) BISA (5, 25, and 250 µM, respectively) plus rotenone (500 µM). Rotenone (dissolved in ethanol 98%) and BISA were added into the fly food. The volume of ethanol in the

food was limited to 1%. Two controls were used (with and without ethanol). However, only the ethanol control is shown in the results because there was no statistical difference among the groups for any of the parameters evaluated. The flies were exposed to the treated food for 7 days (Sudati et al. 2013) and the vials maintained in an incubator at 23 ± 1 °C until being processed for various assays. The BISA concentrations were based on previous observations showing that BISA in the range of 5–500 μM did not cause overt signs of toxicity to flies (data not shown).

**2.2.2. Survival Rate**

The vials were scored daily for mortality. The survival rate was based on the living fly count each day over the 7-day experimental period, and the survivors were transferred to freshly prepared food. The number of flies in the final calculation represents the sum of three independent experiments (25 flies/treatment/repetition).

**2.2.3. Negative Geotaxis Assay**

The locomotor activity of flies was determined based on negative geotaxis behavioral assay (climbing) as previously described by Feany and Bender (2000) with some modifications. The flies (both sexes) were immobilized by brief cold exposure, sorted, and placed in a vertical glass column tube (15 cm long and 1.5 cm in diameter). After they recovered (approximately 20 min), the flies were gently tapped to the bottom of the column. The flies that reached the top portion of the column (6 cm) and the flies that remained at the bottom were counted separately over 6 s. The scores represent the mean of the numbers of flies at the top ($n_{top}$) as percentage of the total number of flies ($n_{tot}$).

About 10 flies per group from three independent experiments were included in the negative geotaxis assay (30 flies).

### 2.2.4. Quantitative Real-Time RT-PCR and Gene Expression Analysis

Approximately 2 µg of total RNA from 25 young flies was extracted using TRIzol® Reagent (Invitrogen™), according to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (Invitrogen™) and cDNA amplified with M-MLV reverse transcriptase enzyme and random primers according to the manufacturer's suggested protocol (Invitrogen™). Quantitative real-time polymerase chain reaction was performed in 20 µL reaction volumes containing 1x PCR buffer, 25 µM dNTPs, 0.2 µM of each primer (described in Table I), 1.5–2.5 mM $MgCl_2$, 0.1x SYBR Green I (Invitrogen™, Molecular Probes™), and 1 U Platinum® Taq DNA polymerase (Invitrogen™) by using StepOnePlus real time PCR systems (Applied Biosystems). The following parameters were used in the qPCR protocol: activation of the Taq DNA polymerase at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 15s at 60 °C, and 25 s at 72 °C. All samples were analyzed in triplicate as both technical and biological replicates and a negative control was included. Threshold and baselines were manually determined using the StepOne Software 2.0 (Applied Biosystems). SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The actin genes were used as endogenous reference genes presenting no alteration in response to the treatment. For each well, analyzed in triplicate, a $\Delta C_T$ value was obtained by subtracting the actin CT value from the CT value for the gene of interest (sequences of tested genes are represented in Table I). The mean $\Delta C_T$

value obtained from the control group for each gene was used to calculate the ΔΔC of the respective gene ($2^{-\Delta\Delta CT}$).

### 2.2.6. Thiol Determination

The total thiol content was determined based on a spectrophotometric method using Ellman's reagent, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid).

### 2.2.7. Mitochondrial Complex I

#### 2.2.7.1. Preparation of *Drosophila melanogaster* Mitochondria

The treated *D. melanogaster* were homogenized in a Potter homogenizer in homogenization medium (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 0.1% albumin pH 7.4). The homogenate was centrifuged at 1,000g for 3 min at 4 °C, and the supernatant fraction was then centrifuged at 12,000 g for 10 min to pellet the mitochondria, which were washed once by resuspension and centrifugation under identical conditions (Miwa et al. 2003). Total protein content was adjusted to 20 mg/mL (Peterson, 1977), and the samples were immediately frozen and kept at -80°C.

#### 2.2.7.2. Mitochondrial Complex Activity Assay

The activity of complex I was determined spectrophotometrically at 30 °C using mitochondria prepared as above and suspended in 100 mM phosphate buffer (pH 7.4) as previously described (Navarro et al. 2002, 2004). The reaction was initiated by addition of NADH at a final concentration of 100 µM, and the enzymatic activity was determined by following the decrease in absorbance at 340 nm (Puntel et al. 2013).

### 2.2.8. Western Blot Analysis of tyrosine hydroxylase

Flies were homogenized at 4 °C in 500 µL of lysis buffer (4 % sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, 0.5 mM sodium orthovanadate, 2 µg/mL aprotinin, 0.1 mM benzamidine, 0.1 mM PMSF). Samples were boiled for 6 min and centrifuged at 1.000g at 4 °C for 10 min. The protein concentration was determined in the supernatant fraction using the method of Lowry. The samples were then mixed with 25% glycerol and 8% 2-mercaptoethanol and resolved by 10% SDS-PAGE. Aliquots of the samples were transferred onto a nitrocellulose membrane (Millipore, USA). The membrane was stained for protein using Ponceau solution (0.5 % Ponceau plus 5 % glacial acetic acid in water), to monitor loading (Romero-Calvo et al. 2010). After staining, the membranes were dried and protein quantified by scanning. The membranes were then processed, blocked with 1% bovine serum albumin, and incubated overnight with an anti tyrosine hydroxylaxe antibody (1:10000; Millipore). Thereafter, the membranes were incubated with alkaline phosphatase-coupled secondary antibody (1:10000; Millipore, USA). The amount of enzyme present was determined by following the reaction colorimetrically using nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate (Trevisan et al. 2013) and the values were normalized to the protein concentration based on that determined by Ponceau staining.

### 2.2.9. Statistical Analysis

Statistical analysis was performed using statistical analysis for censored data: Log - rank (Mantel - Cox) test to survival curve, or one-way ANOVA, followed by Newman-Keuls Multiple test for another results. The difference among the groups was considered to be significant when $p < 0.05$.

### 3. Results

**Effect of (-)-α-Bisabolol on the Survival of Flies Exposed to Rotenone**

Log - rank Test demonstrated that survival curves are significant different, with $p < 0.001$. Acute exposure to rotenone caused an increase in the mortality of flies when compared to the no treatment (control) group. BISA treatment offered protection, at all concentrations tested (Figure 1). Further, the BISA (5, 25, and 250 µM) only treated groups did not show any difference from the no treatment control group.

**Effects of (-)-α-Bisabolol on Rotenone-induced Locomotor Deficits**

Rotenone treated flies exhibited severe motor impairment, and BISA significantly improved the performances of flies in the negative geotaxis test. In general, flies treated with added BISA appeared to be more active than rotenone alone treated flies. Further, BISA alone had no significant effect on motor behavior among the groups of flies (Figure 2).

**Effect of Rotenone and (-)-α-Bisabolol on CAT, SOD, and KEAP1 mRNA Expression**

Rotenone exposure caused a significant increase in the mRNA expression of genes for catalase (2 fold) (Figure 3A), superoxide dismutase (2.6 fold) (Figure 3B), and Keap-1 (1.7 fold) (Figure 3C). BISA significantly reduced SOD mRNA expression only at the 250 µM concentration (Figure 3B).

**Effect of (-)-α-Bisabolol on Thiol Content in Homogenates of Flies Exposed to Rotenone**

Rotenone exposure significantly reduced the total thiol content in flies as compared to the control group (Figure 4). The presence of BISA was not found to ameliorate the effect of rotenone on thiol content.

**Effect of (-)-α-Bisabolol on Complex I Activity of Flies Exposed to Rotenone**

BISA alone had no effect on complex I activity as compared to that in the control group. However, among the groups of rotenone treated flies, significant inhibition was evident. Furthermore, the activity level of complex I was not different in groups of flies concurrently treated with BISA than that in groups treated with rotenone alone (Figure 5).

**Effect of (-)-α-Bisabolol on Cell Signaling**

We evaluated the expression of the target protein involved in dopaminergic system signaling, tyrosine hydroxylase (TH), as a possible mechanism of BISA action. As demonstrated in Figure 6, BISA produced no change in the expression of TH compared to that in the control group. Furthermore, the basal levels of TH remained unaltered in groups of flies exposed to the rotenone and treated concurrently with BISA (Figure 6).

**4. Discussion**

Using *Drosophila* as a model system of neurological damage, our data show significant protective effects of BISA against mortality and locomotor deficits elicited by rotenone. Our previous findings had led us to hypothesize that BISA was potentially

counteracting the well documented toxicity mechanism related to rotenone, namely inhibition of complex I activity followed by oxidative stress-mediated cell death (Uversky 2004; Sherer et al. 2003; Cannon et al. 2009).

There are a large number of articles in the literature that have used this animal model for rotenone toxicity testing, which reinforces that the rotenone toxicity test model in Drosophila is widely accepted and use (Rao et al.  2016; Vargas et al. 2014; Hwang et al. 2014; Ravikumar et al. 2010; Lawal et al. 2010). In adittion, Drosophila has a short generation time (10 days) and life span (60–80 days). The complete sequence of the Drosophila genome has revealed that 77% of human disease genes are conserved in the fly (Rubin 2000; Bier 2005). These features make flies an excellent model system in which to study the function of disease genes including those involved in neurodegenerative diseases (Lessing and Bonini 2009; Lu and Vogel 2009). It is a well known for its high sensitivity to toxic substances and is being considered a model for detection of pollutants and widely employed as a model to assess the therapeutic potential of phytochemicals.

To follow up, we focused the present investigation on specific molecular/biochemical pathways in an attempt to better elucidate the mechanism by which BISA may block the deleterious effects of the pesticide. As expected, rotenone strongly inhibited complex I activity. Moreover, we found evidence that flies treated with rotenone mounted a response to oxidative stress, as evidenced by the increase of catalase, SOD and Keap I mRNA expression. Unfortunately, despite the initial observation that dietary BISA is protective against rotenone-induced toxicity in flies, we found no clear correlation with complex I inhibition or oxidative stress when flies were given rotenone and BISA concurrently. On the contrary, these findings may indicate an unknown

property of BISA for future investigation. We are currently exploring several possibilities.

Rotenone is a lipophilic compound that freely crosses cell membranes and can access both the cytoplasmic and mitochondrial compartments. Its research use has grown exponentially over the last few years largely due to the discovery of its ability to reproduce many features of PD in rats, including development of α-synuclein-positive cytoplasmic inclusions, which are similar to Lewy bodies found in nigral neurons in human PD patients (Betarbet et al. 2000). Inhibition of mitochondrial respiratory chain complex I by rotenone has been found to induce cell death in a variety of cells, as well as cause reactive oxygen species (ROS) generation.

The present research demonstrated that rotenone exposure significantly increased the mortality of flies. This observation is supported by findings of previous studies where rotenone caused high mortality among flies during a 7-day exposure period (Ravikumar et al. 2009, 2010; Sudati et al. 2013). However, supplementation of BISA in the diet reversed the rotenone-mediated toxicity and decreased mortality.

In addition, rotenone induced toxicity is accompanied by a high rate of locomotor deficits, and concurrent exposure to BISA significantly improved the performances of flies in the negative geotaxis test. Flies with locomotor deficits have the tendency to stay at the bottom of a glass column and appear not to be able to coordinate their legs in a normal fashion. This behavior has been explained by severe complex I inhibition. High energy expenditure is a requirement of ambulatory and flight muscles, but rotenone exposure can cause loss in complex I activity (Figure 5) leading to a decrease in the rate of energy production as well as causing increased oxidative stress at the mitochondrial membrane. Ravikumar (2009) attribute the locomotor deficits caused in rotenone-

exposed flies to uncoupling of the mitochondrial machinery (severe damage in complex I) and neurodegeneration. In the insect, rotenone induced neurotoxicity is attributed to the unique sensitivity of dopaminergic neurons to reactive oxygen species and oxidative damage. In our work, rotenone caused complex I activity loss, however, its effects on the dopaminergic system was not clear, and, although BISA protected against the effects of rotenone, its mechanism of action is also unclear. We suggest that it is related to its ability to counteract free radicals produced as a result of the damage to complex I.

In previous studies, flies exposed to rotenone had significantly elevated levels of antioxidant enzymes, and that correlated to the increased ROS generation and formation of toxic aldehydes (Ravikumar et al. 2009 and 2010). Excessive ROS generation can cause lipid peroxidation; mitochondrial dysfunction; and damage to proteins, lipids, and nucleic acids; thereby, altering normal functions of the cell (Valko et al. 2007) and lead to cell death and mortality of the host. Thus, excessive ROS generation can be related to rotenone-induced loss of complex I activity. Complex I, also known as NADH-ubiquinone oxidoreductase, is the first step in the transfer of electrons from NADH to the electron-transport chain during biochemical respiration. Rotenone uncouples electron transport at the complex I site leading to ROS production (Cassarino et al., 1997; Barrientos and Moraes, 1999; Kushnareva et al., 2002). Our data demonstrated that rotenone inhibited complex I, however, BISA failed to protect against loss of activity. Apparently, instead BISA counteracts the effects of ROS generation when complex I is inhibited by rotenone. Thus, BISA ameliorates the downstream damage through an antioxidant mechanism.

In additional experiments, we observed that rotenone increased CAT, SOD and *keap1* mRNA expression, which may represent a compensatory response to oxidative

insults. The Keap1-Nrf2 pathway is the major regulator of cytoprotective responses to endogenous and exogenous stress caused by various oxidants and electrophilic agents. Keap1 is a repressor protein that binds to Nrf2 and promotes its degradation. Furthermore, Nrf2-bound Keap1 is inactivated. Nrf2 augments a wide range of cell defense processes, thereby enhancing the overall capacity of cells to detoxify potentially harmful molecular assaults (Kansanen et al. 2012). Additionally, Satoh et al (2009) showed that cortical neurons with *keap1* gene-knockout, exhibited increased resistance to oxidative stress induced by high concentrations of glutamate and rotenone. In those studies, rotenone increased *keap1* mRNA expression (Figure 3C), leading to Nrf2 inactivation, and consequently decreasing the ability to resist oxidative stress, the inability to detoxify harmful molecules, and, generally, lowering cell defenses.

The elevated expression of SOD mRNAs by rotenone was normalized by BISA treatment. Our data also corroborate previous studies showing that BISA-induced gastroprotection is associated with increased SOD activity (Rocha et al. 2011a). The effects of BISA on the usual oxidative stress markers indicate that it works through an antioxidant mechanism. Thus, the decrease in SOD mRNA expression may be related to the ability of BISA to scavenge rotenone-induced superoxide thereby removing the signals leading to SOD expression. This further suggests that BISA exerts its protective effect by counteracting superoxide anion generated by rotenone-damaged mitochondria.

Another mechanism of toxicity of rotenone is dopaminergic neuronal loss and reduction in the dopamine levels in adult flies (Coulom and Birman 2004). Therefore, we evaluated the expression of the key protein involved in dopaminergic system signaling, tyrosine hydroxylase (TH). In our work, the levels of TH remained unaltered in flies exposed to the rotenone and with or without BISA treatment. This result is similar to that

observed by Navarro et al. (2014), showing that acute rotenone treatment (500 µM for 6 days) leads to a neurodegeneration, as evidenced by a decrease in a reporter green-fluorescent protein (GFP) signal in dopaminergic neurons, without affecting the total number of dopaminergic neurons, but suggesting an abnormal neuronal status.

**5. Conclusion**

In conclusion, the presence of BISA protects against rotenone-induced toxicity as evidenced by markedly decreasing mortality, preventing locomotor activity deficits, and normalizing expression of SOD mRNA. The protective effect of BISA was likely associated with its free radical scavenging and antioxidant actions. BISA can be used to counteract toxicity due to an environmental toxin such as rotenone. Additional experiments are necessary to clarify the mechanism of action of BISA.

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**FIGURE LEGENDS**

Fig. 1. Effect of (-)-α -bisabolol on survival rate of flies exposed to rotenone. Data were collected every 24 h for each group. The number of flies (75 per group) was the sum of three independent experiments. Flies treated with rotenone and rotenone + BISA were followed until death of all subjects, whereas the control, and BISA alone were followed until 20 days of treatment. Statistical analysis was performed using the Log - rank (Mantel -Cox) Test. Survival curves are significant different, with $p < 0.001$.

Fig. 2. Effect of (-)-α-bisabolol on geotaxic response (climbing of flies exposed to rotenone for 7 days. The total number of flies (30 per group) represents the sum of three independent experiments (10 flies/ assay). ***Significant difference in relation to the control group, ($p < 0.001$); # Significant difference between ROT and flies treated with rotenone plus BISA 5 µM and BISA 25 µM ($p < 0.05$), ## Significant difference between ROT and ROT + BISA 250 µM ($p < 0.01$).

Fig. 3. qPCR relative gene expression of catalase (CAT) (3a), superoxide dismutase (SOD) (3b), and *keap1* (3c). Results are expressed as mean ± S.E.M (n = 3). The data are from 3 independent homogenizations of 20 flies each, and qPCR quantification was performed in duplicate. *Significant difference in relation to control group ($p < 0.05$), ## Significant difference in relation to ROT treatment ($p < 0.01$).

Fig. 4. Effect of (-)-α -bisabolol on total thiol content in homogenates of flies exposed to rotenone. **Significant difference in relation to control group ($p < 0.01$).

Fig. 5. Effect of (-)-α-bisabolol on mitochondrial complex I activity assayed in homogenates of flies exposed to rotenone. ***Significant difference in relation to control group ($p< 0.001$).

Fig 6. Effect of BISA on Western blot analysis of TH in homogenates of flies exposed to rotenone

TABLE 1 - Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

| **Genes** | **Primer sequences** |
|---|---|
| Catalase | 5’ACCAGGGCATCAAGAATCTG 3’<br>5’AACTTCTTGGCCTGCTCGTA 3’ |
| Superoxide dismutase | 5’GGAGTCGGTGATGTTGACCT 3’<br>5’GTTCGGTGACAACACCAATG 3’ |
| KEAP 1 | 5’ CCAACTTCCTCAAGGAGCAG 3’<br>5”CGGCGACAAATATCATCCTT 3’ |
| | |

Fig. 1. Effect of (-)-α -bisabolol on survival rate of flies exposed to rotenone. Data were collected every 24 h for each group. The number of flies (75 per group) was the sum of three independent experiments. Flies treated with rotenone and rotenone + BISA were followed until death of all subjects, whereas the control, and BISA alone were followed until 20 days of treatment. Statistical analysis was performed using the Log - rank (Mantel - Cox) Test. Survival curves are significant different, with $p < 0.001$.

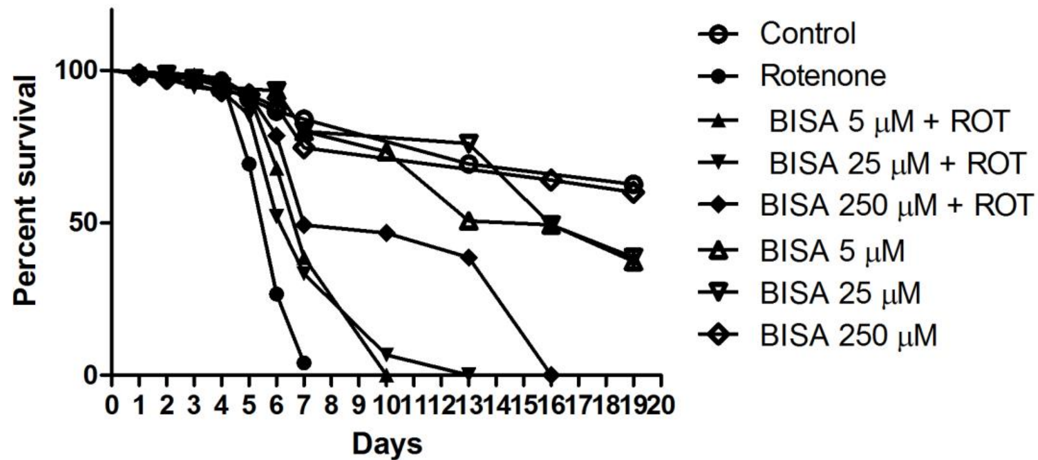
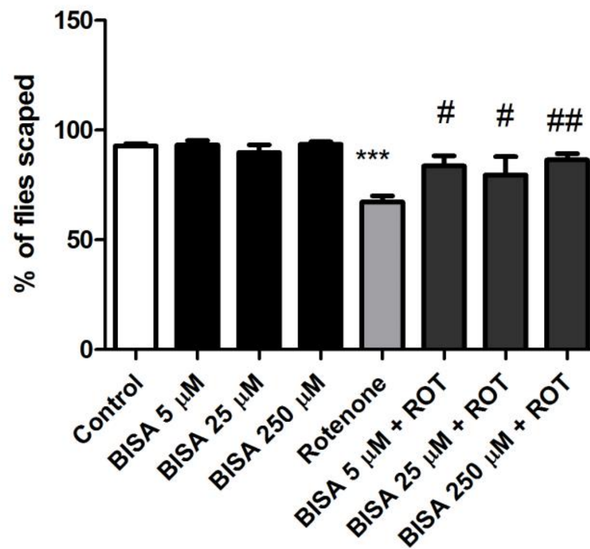



Fig. 2. Effect of (-)-α-bisabolol on geotaxic response (climbing of flies exposed to rotenone for 7 days. The total number of flies (30 per group) represents the sum of three independent experiments (10 flies/ assay). ***Significant difference in relation to the control group, ($p < 0.001$); # Significant difference between ROT and flies treated with rotenone plus BISA 5 μM and BISA 25 μM ($p < 0.05$), ## Significant difference between ROT and ROT + BISA 250 μM ($p < 0.01$).



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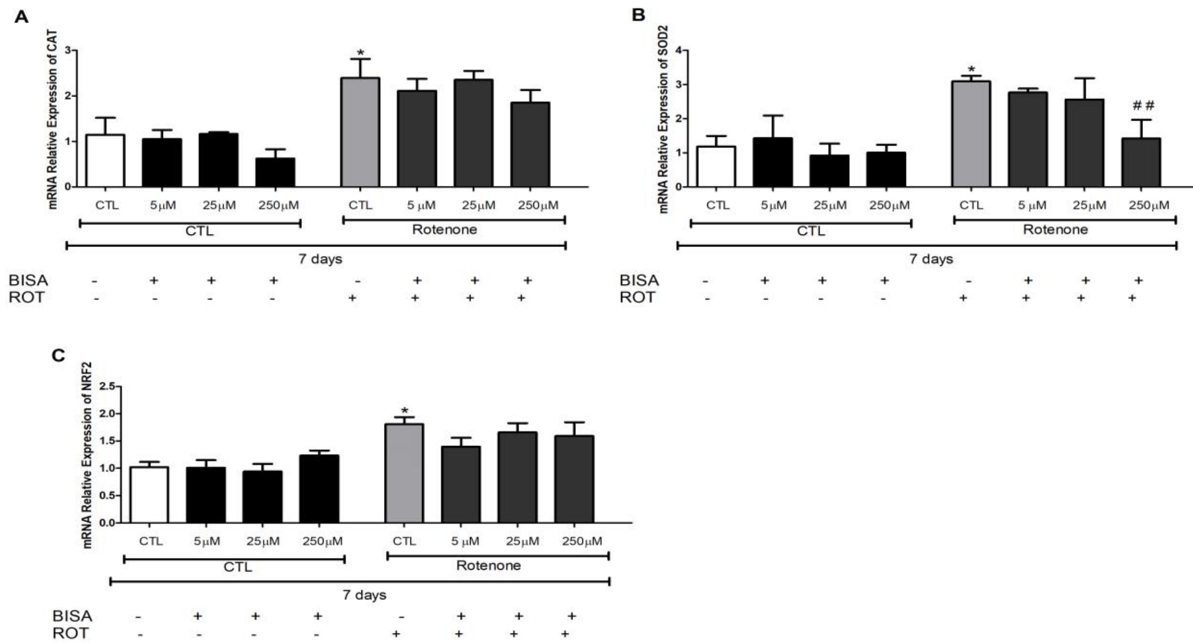



Fig. 4. Effect of (-)-α -bisabolol on total thiol content in homogenates of flies exposed to rotenone. **Significant difference in relation to control group (p < 0.01).

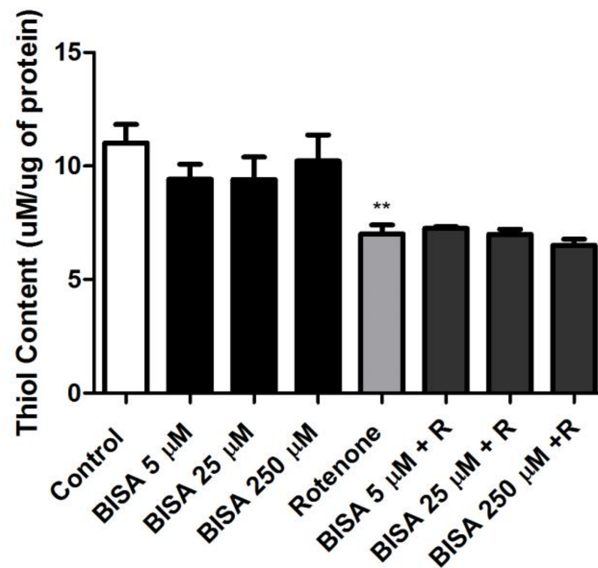


Fig. 5. Effect of (-)-α-bisabolol on mitochondrial complex I activity assayed in homogenates of flies exposed to rotenone. ***Significant difference in relation to control group ($p < 0.001$).

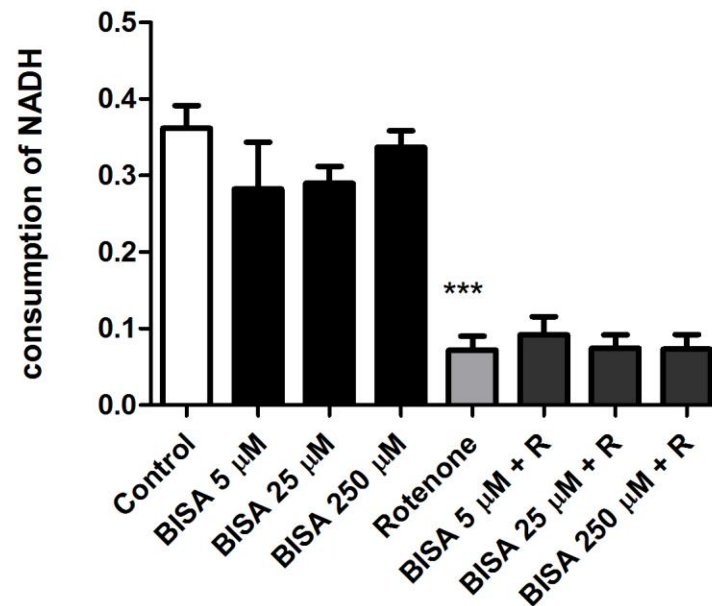
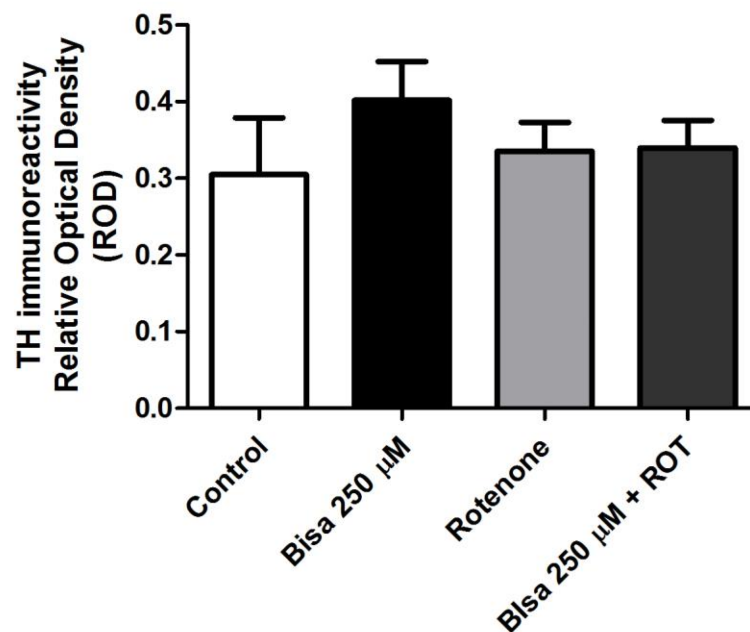



Fig 6. Effect of BISA on Western blot analysis of TH in homogenates of flies exposed to rotenone



# 4. DISCUSSÃO

A partir de um balanço entre as defesas antioxidantes e os efeitos tóxicos das EROs, os seres vivos conseguem manter o metabolismo e o funcionamento celular inalterados. Porém, em situações específicas, este panorama pode ser comprometido através do excesso de produção de EROs, falha das defesas antioxidantes ou ambos, gerando EO (TREVISAN, 2008). Muitas doenças neurodegenerativas têm sua causa, desenvolvimento ou complicações associadas ao EO. Dentre estas, podemos citar as doenças de Alzheimer, Parkinson e Huntington (SIMONIAN; COYLE, 1996). Então, nosso trabalho objetivou avaliar a atividade antioxidante *in vitro* do extrato aquoso e óleo essencial da *V. arborea* e BISA, bem como avaliar os possíveis efeitos terapêuticos, sobre alterações associadas à transcrição gênica, parâmetros bioquímicos e comportamentais em *D. melanogaster* expostas ao pesticida rotenona. Além, de avaliar o efeito antioxidante e toxicidade basal do BISA em modelo animal de *D. melanogaster* e os parâmetros de citotoxicidade e genotoxicidade em células monocucleares e hemáceas de sangue periféricos (CMSP) em humanos.

Considerando a alta suscetibilidade do cérebro ao dano originado por radicais livres, a relação existente entre doenças neurodegenerativas e EO e o papel da *V. arborea* em nível central, o tecido cerebral foi escolhido para avaliar os efeitos do extrato aquoso da *V. arborea* frente aos insultos oxidativos induzidos pelo agente pró-oxidante ($FeSO_4$) através do ensaio de TBARS. A ação neurotóxica está ligada ao mecanismo: 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 ($OH^{·-}$) (DAUER; PRZEDBORSKI, 2003). Este por sua vez causa danos às membranas biológicas levando a geração de hidroperóxidos. Os resultados alcançados nesse parâmetro mostraram que o extrato aquoso das folhas da *V. arborea* foi eficaz em inibir a peroxidação lipídica causada pelo agente $FeSO_4$. Ainda neste trabalho, foi verificado que a *V. arborea* também exibiu efeito protetor no DPPH, indicando capacidade de eliminar radicais livres. O radical DPPH tem sido muito utilizado para testar a habilidade de vários produtos naturais em sequestrar radicais livres (BRAND-WILIAMS, 1995). Nenhum dos extratos de *V. arborea* apresentaram a capacidade de quelar Fe (II), resultados observados no teste da fenantrolina. A capacidade quelante de ferro da *V.*

*arborea* poderia aumentar as propriedades neuroprotetoras da planta particularmente pelo fato de que a sobrecarga de ferro está envolvida na patogênese de doenças cerebrais tais como a de Alzheimer (MALECKI et al., 2002). No entanto, não foi observado este efeito no seu extrato aquoso, então podemos concluir que seus efeitos antioxidantes estão relacionados a sua capacidade de sequestrar radicais livres e não por quelar moléculas de Fe.

Como parte das ações farmacológicas apresentadas pelo BISA e *V. arborea* estão relacionadas à sua ação gastroprotetora, analisamos o efeito destes compostos sobre a geração de espécies reativas em muco gástrico. As espécies reativas de oxigênio mostram que um dos fatores patogênicos prováveis nas lesões da mucosa gástrica estão associadas ao estresse oxidativo nas úlceras induzidas por etanol (BILICI et al., 2002). Este trabalho utilizou 2',7'-diacetato-diclorofluoresceína (DA-DCFH) para quantificar as EROs no muco, induzidas por $H_2O_2$ (KIM, 2010; ALI, 1992). O BISA e *V. arborea* diminuíram a formação de EROs, o que pode estar relacionado ao efeito gastroprotetor apresentada no modelo de úlcera induzida por etanol e indometacina relacionados com a capacidade de reduzir a quantidade de GSH na mucosa gástrica, mostrando uma atividade antioxidante (ROCHA et al., 2010; OLIVEIRA et al., 2011).

Os compostos naturais são amplamente utilizados pela indústria farmacêutica para a produção semissintética de medicamentos, além de serem empregados por várias populações como agentes terapêuticos (CRAGG; GROTHAUS; NEWMAN, 2014; PEREIRA et al., 2009). Dentre esses produtos naturais, destacam-se o BISA com uma ampla variedade de atividades biológicas. Nossos estudos mostram que existe um nível de citotoxicidade e genotoxicidade significativo quando as CMSP e as hemácias são expostas a altas concentrações deste composto, tornando a viabilidade celular reduzida, causando um dano severo a nível nuclear e apresentando atividade hemolítica elevada, sendo possível notar a necessidade de cautela frente a sua utilização. Cavalieri et al., (2004; 2009) demonstraram que o BISA apresenta um efeito citotóxico quando se correlaciona a sua dose e tempo de exposição sobre linhagens celulares malignas de glioma de rato e de humano, induzindo rapidamente estes a apoptose e também apontaram que a toxicidade se relaciona a concentração intracelular. Essa reação hemolítica do BISA pode ocorrer de duas maneiras. Uma é a ocorrência de solubilização da membrana plasmática do eritrócito

(APARICIO et al., 2005). O outro mecanismo compreende que os compostos xenobióticos reduzidos, como os compostos fenólicos, promovem a hemólise através da oxidação da hemoglobina, dando origem a meta-hemoglobina (BUKOWSKA; KOWALSKA, 2004)

*D. melanogaster* têm sido usadas para ensaios de toxicidade, como um método alternativo para o uso de animais, e tornou-se um excelente modelo alternativo para vários ensaios (ARAÚJO PINHO, et al., 2014; TIWARI et al., 2011; RAND, 2010). Os resultados obtidos no nosso trabalho não mostraram toxicidade do BISA nas concentrações testadas. As concentrações de BISA não são capazes de induzir mortalidade nem apresentaram sinais de estresse oxidativo, incluindo a formação de EROs, bem como mudanças nos importantes sistemas de resposta antioxidante. O papel protetor da catalase nas células está relacionado à eliminação de $H_2O_2$ (SANIOVA et al., 2006; ADEDARA et al., 2015); nenhuma alteração foi observada.

Então, nossas pesquisas foram direcionadas para um protocolo experimental de neurotoxicidade envolvendo o pesticida rotenona e a *D. melanogaster*, os quais vêm sendo amplamente utilizados (RAVIKUMAR et al., 2009; 2010; SUDATI et al., 2013). A relação entre a exposição à rotenona é relevante no estudo de doenças neurológicas. Com relação à modelo experimental *in vivo* utilizando a rotenona e terapia natural utilizando o composto BISA, não há dados na literatura. Neste trabalho foi observado danos motores, os danos oxidativos e a taxa de mortalidade induzidos pela exposição das moscas ao pesticida, estes resultados estão em acordo com estudos anteriores que retrataram que a rotenona causa elevada mortalidade e dano comportamental nas moscas durante uma exposição de 7 dias (RAVIKUMAR et al., 2009; 2010; SUDATI et al., 2013). Neste trabalho foi observado que a taxa de mortalidade induzida pela exposição das moscas ao pesticida foram significativamente reduzidos pelo tratamento concomitante com BISA. Na avaliação da função locomotora, a performance das moscas expostas à rotenona foi significativamente alterada no teste de escalada, quando comparada ao grupo controle. Essa alteração locomotora observada nas moscas foram normalizadas pelo tratamento com BISA. De acordo com os nossos resultados, o BISA não causou danos neurológicos ou déficits musculares em ratos (LEITE et al., 2012).

A geração excessiva de EROs pode causar a peroxidação lipídica, disfunção mitocondrial, e danos a proteínas, lípidos e ácidos nucleicos, alterando a função normal das

células (VALKO et al., 2007), levando à morte celular e mortalidade. Aparentemente o BISA não foi capaz de neutralizar a ação direta no complexo I causada pela rotenona.

Verificamos que a exposição à rotenona causou aumento na expressão de mRNA das enzimas antioxidantes SOD, CAT e do Keap-1, os quais podem ter sido decorrentes de uma resposta compensatória aos insultos oxidativos induzidos pelo pesticida. A via Keap1-Nrf2 é o principal regulador das respostas citoprotetoras ao estresse endógeno e exógeno causado por vários agentes oxidantes e eletrofílicos. O Keap-1 é uma proteína repressora que se liga ao Nrf2 e promove sua degradação. Além disso, o Keap-1 quando ligado a Nrf2 mantem esse sinalizador celular inativado. O Nrf2 aumenta uma ampla gama de processos de defesa celular, aumentando assim a capacidade geral das células para desintoxicar danos moleculares potencialmente prejudiciais (KANSANEN et al., 2012). Além disso, Satoh et al (2009) mostraram que os neurônios corticais com os genes knockout do keap-1 exibiram maior resistência ao estresse oxidativo induzido por altas concentrações de glutamato e rotenona. Nesses estudos, a rotenona aumentou a expressão de mRNA de keap-1, sugerindo a inativação de Nrf2 e, conseqüentemente, diminuição da capacidade de resistir ao estresse oxidativo, a incapacidade de desintoxicar moléculas nocivas e, geralmente, diminuir as defesas celulares.

A expressão elevada de mRNA da SOD causada pela rotenona foi normalizada pelo tratamento com BISA. Nossos dados também corroboram com estudos anteriores mostrando que a gastroproteção foi regularizada pelo BISA na atividade SOD (ROCHA et al., 2011a). Os efeitos do BISA nos marcadores de estresse oxidativo habituais indicam que ele funciona através de um mecanismo antioxidante. Assim, a diminuição na expressão de mRNA da SOD pode estar relacionada com a capacidade do BISA em sequestrar o radical superóxido produzido pelo tratamento com rotenona, removendo assim os sinais que conduzem à expressão de SOD. Isso sugere ainda que o BISA exerce seu efeito protetor ao detoxificar o anion superóxido gerado por mitocôndrias danificadas pelo rotenona.

Além da expressão de mRNA, avaliamos os níveis de tióis total e a expressão de proteína alvo envolvidas na sinalização do sistema dopaminérgico, a tirosina hidroxilase (TH). Outro mecanismo de toxicidade da rotenona é a perda neuronal dopaminérgica e redução nos níveis de dopamina em moscas adultas (COULOM e BIRMAN, 2004). Portanto, avaliamos a expressão da proteína chave envolvida na síntese de dopamina em

neurônios dopaminérgicos, a tirosina hidroxilase (TH). Em nosso trabalho, os níveis de TH permaneceram inalterados nas moscas expostas à rotenona, com ou sem tratamento com BISA. Esse resultado é semelhante ao observado por Navarro et al. (2014), mostrando que o tratamento agudo com rotenona (500 μM, por 6 dias) leva a uma neurodegeneração, (como evidenciado por uma diminuição de um sinal de proteína fluorescente verde) em neurônios dopaminérgicos, sem afetar o número total de neurônios dopaminérgicos, mas sugerindo um estado neuronal anormal.

Esses dados sugerem que a ação tóxica da rotenona em *D. melanogaster,* está possivelmente associada com a geração de EROs através da inibição do complexo I mitocondrial e com os eventos moleculares provocados pela ação dos radicais livres nas células. Já o mecanismo de proteção exercido pelo BISA deve estar relacionado com a ação antioxidante, principalmente por sequestrar as EROs produzidas pela rotenona através do dano mitocondrial.

Nossos resultados comprovam a efetividade do uso *in vivo* do BISA como um agente antioxidante neste modelo de toxicidade. Conforme verificado pelos estudos anteriores, é provável que os efeitos exibidos pelo BISA sejam reconhecidos como agente antioxidante (ROCHA et al., 2011a; OLIVEIRA et al., 2011). Em conjunto, os dados dos trabalhos realizados com *V. arborea e* BISA, nos permitem sugerir que a ingestão destes podem ser considerada benéfica para proteger de insultos oxidantes, bem como, um promissor agente terapêutico para o tratamento de doenças neurodegenerativas relacionadas ao EO em humanos. No entanto, estudos em outros modelos experimentais são necessários para assegurar a segurança de uso da *V. arborea e* do BISA.

Em suma, os resultados obtidos no trabalho realizado mostram a atividade antioxidante da *V. arborea* em modelos de neurotoxicidade *in vitro* (tecido cerebral de rato) e outros parâmetros de estresse oxidativo, bem como, sugerem que o uso desta planta deve ser mais estudado quanto a esta linha de pesquisa. Com relação, ao uso do BISA para o tratamento da toxicidade induzida pela rotenona, é necessário a realização de mais experimentos *in vivo* e *ex vivo*, a fim de estabelecer o mecanismo deste composto.

# 5. CONCLUSÃO

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

- ✓ O extrato aquoso ou óleo essencial da *V. arborea* e o BISA apresentaram atividade antioxidante (*in vitro*) em homogeneizado de cérebro de rato, evidenciados pela diminuição no TBARS, capacidade de neutralizar o radical 2,2-difenil-1-picril-hidrazil – DPPH e reduziram a formação de EROs induzida pelo $H_2O_2$;

- ✓ O BISA não apresentou efeitos de toxicidade em *D. melanogaster;*

- ✓ O BISA apresentou efeito neuroprotetor *in vivo* em *D. melanogaster* expostas à rotenona, esses efeitos foram evidenciados pela proteção sobre a sobrevivência, e danos comportamentais e avaliação molecular;

- ✓ O BISA apresentou efeito toxicológico, através de ensaios de citotoxicidade, genotoxicidade e mutagênico, evidenciados pela viabilidade celular (teste MTT), danos nucleares (teste de cometas) em células mononucleares de sangue periférico (CMSPs) e atividade hemolítica de BISA para os glóbulos vermelhos obtidos a partir do sangue periférico.

Concluímos que o *V. arborea,* seu óleo essencial e principal constituinte: BISA tem amplo potencial farmacológico em diferentes modelos animais relacionados a atividade antioxidante, neuroproteção, possivelmente com baixo efeito tóxico. Esse estudo pode colaborar efetivamente para a busca por tratamentos a base de terapias naturais para patologias relacionadas ao estresse oxidativo e neurotoxicidade.

# 6. PERSPECTIVAS

- ✓ Quantificar os níveis de dopamina em *D. melanogaster* submetidas à intoxicação por rotenona e tratadas com o BISA;

- ✓ Avaliar os efeitos do BISA sobre parâmetros bioquímicos em *D. melanogaster* expostas à rotenona (Determinação da glutationa reduzida e/oxidada, Determinação da atividade da superóxido dismutase);

- ✓ Avaliar os efeitos do BISA sobre as atividades da acetilcolinesterase (AChE) e butirilcolinesterase (BChE);

- ✓ Avaliar a viabilidade celular em *D. melanogaster* expostas a rotenona e submetida ao tratamento com o BISA;

- ✓ Comparar os efeitos obtidos com BISA 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.

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