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

Francisco Assis Bezerra da Cunha

TOXICIDADE DE *Eugenia uniflora* L. (MYRTACEAE) EM MODELOS DE *Drosophila melanogaster* E CÉLULAS SANGUÍNEAS HUMANAS

Santa Maria, RS, Brasil

2015

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

Orientador: Prof. Dr. Jeferson Luis Franco
Co-orientador: Prof. Dr. Henrique Douglas Melo Coutinho

Santa Maria, RS, Brasil

2015

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

TOXICIDADE DE *Eugenia uniflora* L. (Myrtaceae)
EM MODELOS DE *Drosophila melanogaster*
E CÉLULAS SANGUÍNEAS HUMANAS

elaborada por
Francisco Assis Bezerra da Cunha

como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 17 de dezembro de 2015.

DEDICATÓRIA

Dedico este trabalho *in memoriam* as minhas avós: Dona Maroca que me apresentou o livro As Plantas Curam e Dona Adelaide pela firmeza de suas convicções e aos meus pais Seu Antônio e Dona Marina. Dedico, também, de uma forma especial a minha esposa Advanda e aos meus filhos Frederico Haeckel, Frances Marina, Cunha-Neto e Leandro, por tudo que representam para mim no ontem, no hoje e no amanhã.

AGRADECIMENTOS

A Deus, a quem devo somente agradecer pela sua infinita bondade.

Ao Pe. Cícero, por ter colocado uma centelha de luz em meu coração.

A minha família: Tios, primos, irmã, sobrinhos, esposa e filhos.

Ao Prof. Dr. Jeferson Luis Franco pela orientação deste trabalho, pela sua clarividência e pela sua compreensão para com minhas limitações.

Ao Prof. Dr. Henrique Douglas Melo Coutinho pela co-orientação, incentivo e amizade.

Ao Prof. Dr. João Batista Teixeira da Rocha, Coordenador do DINTER URCA-UFSM, pela persistência e tenacidade para que este Projeto se tornasse uma realidade.

Ao Prof. Dr. Antonio Álamo Feitosa Saraiva, Coordenador acadêmico do Dinter, que sempre acreditou neste Projeto.

A Profa. Vera Maria Melchiors Morsch, coordenadora deste Programa de Pós-graduação.

Aos professores do Programa PPGBTox que ministraram aulas no Dinter.

Ao Prof. Dr. José Galberto Martins da Costa pela orientação na preparação e concentração do óleo e extrato utilizados neste trabalho.

Aos colegas Audilene, Saulo Relison, Elba Sobral-Souza, Nadghia Leite e bolsitas do LPPN pelo auxílio nos trabalhos preliminares, durante a primeira fase deste trabalho na URCA.

A Universidade Federal do Pampa – UNIPAMPA, *campus* de São Gabriel especialmente a Profa. Thaís Posser, Gabriel Luz Walau, Litiele Cezar da Cruz e Mauro Eugênio Medina Nunes pela dedicação durante a segunda fase deste projeto, especialmente na experimentação e redação do Artigo sobre o óleo essencial de *Eugenia uniflora.*

A Universidade Federal de Santa Maria – UFSM, na terceira fase deste projeto, agradeço especialmente a Emily Waczuc, Aline Boligon e Jean Paul Kamdem pela colaboração na fase experimental do extrato de *E. uniflora* e redação final e submissão do Manuscrito.

A todos os colegas do PPGBTox, principalmente aqueles a quem aprendi a admirar: Ivanildo, Eliene, Marivando e Cleide Correia.

Aos colegas Conselheiros e funcionários do Conselho Estadual de Educação do Ceará, cujas atividades de Conselheiro, realizei simultaneamente com as deste Doutorado, obrigado por compreenderem minhas eventuais ausências.

A todos os colegas do Departamento de Ciências Biológicas.

Aos colegas professores: Waltécio, Robson, Alisson, Samuel, Samuel Brito Felipe, Marta Roseli, Imeuda e Hênio.

Aos colegas decanos de Departamento, especialmente: Ivone Pequeno (*in memorian*), Abraão Batista, Ivonete Louro, Terezinha Batista, Margarida Angélica, Rubens Cabral, José de Souza Filho (careca) e Jorge Barros.

Aos meus ex alunos e atuais alunos pela compreensão e bondade que tem para comigo, quer seja nas minhas atividades administrativas, quer sejam acadêmicas.

Um agradecimento e uma menção especial aos meus bolsistas de iniciação científica: Dalva, Lays, Zildene, Pedro, Guilherme, Ana Lyvia, Lara, Lucas, Bia e Nair e aos meus estagiários: Joycy 2y, Joyce, Jayze.

Aos funcionários da URCA, meus amigos de primeira hora.

Aos professores e bolsistas do Laboratório de Pesquisa de Produtos Naturais – LPPN, Laboratório de Microbiologia e Biologia Molecular – LMBM, Laboratório de Eletrofisiologia e Laboratório de Farmacologia.

Aos professores que compõem minha Banca de Defesa: Profa. Dra. Maribel Antonello Rubin, Profa. Dra. Sara Marchesan de Oliveira, Profa. Dra. Adriana Rolim Campos Barros e Prof. Dr. Irwin Rose Alencar de Menezes.

Aos meus amigos do Santa Luzia: Cortez, Rui, Newton, Adriano, Cícero, Lula, Luis Alberto, Gérson, Frei Balinha, Seu Isaías, Seu Sebastião, Seu João e Jojó pela sua amizade e estímulo.

As instituições que tornaram possível este Dinter: Universidade Federal de Santa Maria – UFSM, Universidade Regional do Cariri – URCA e a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

Ainda que eu falasse as línguas dos homens e dos anjos, e não tivesse caridade, seria como o metal que soa ou como o sino que tine.
E ainda que tivesse o dom de profecia, e conhecesse todos os mistérios e toda a ciência, e ainda que tivesse toda a fé, de maneira tal que transportasse os montes, e não tivesse caridade, nada seria.
E ainda que distribuísse toda a minha fortuna para sustento dos pobres, e ainda que entregasse o meu corpo para ser queimado, e não tivesse caridade, nada disso me aproveitaria.
A caridade é sofredora, é benigna; a caridade não é invejosa; a caridade não trata com leviandade, não se ensoberbece.
Não se porta com indecência, não busca os seus interesses, não se irrita, não suspeita mal;
Não folga com a injustiça, mas folga com a verdade;
Tudo sofre, tudo crê, tudo espera, tudo suporta.
A caridade nunca falha; mas havendo profecias, serão aniquiladas; havendo línguas, cessarão; havendo ciência, desaparecerá;
Porque, em parte, conhecemos, e em parte profetizamos;
Mas, quando vier o que é perfeito, então o que o é em parte será aniquilado.
Quando eu era menino, falava como menino, sentia como menino, discorria como menino, mas, logo que cheguei a ser homem, acabei com as coisas de menino.
Porque agora vemos por espelho em enigma, mas então veremos face a face; agora conheço em parte, mas então conhecerei como também sou conhecido.
Agora, pois, permanecem a fé, a esperança e a caridade, estes três, mas o maior destes é a caridade.

Primeira carta de São Paulo aos coríntios
Capítulo 13
Versículos de 1 a 13

RESUMO

TOXICIDADE DE *Eugenia uniflora* L. (MYRTACEAE) EM MODELOS DE *Drosophila melanogaster* E CÉLULAS SANGUÍNEAS HUMANAS

AUTOR: Francisco Assis Bezerra da Cunha
ORIENTADOR: Prof. Dr. Jeferson Luis Franco
CO-ORIENTADOR: Henrique Douglas Melo Coutinho

A crescente utilização de produtos naturais torna imperativa a investigação do potencial toxicológico dos metabólitos secundários de plantas. Neste contexto insere-se o estudo toxicológico de *Eugenia uniflora*, espécie pertencente à família Myrtaceae, a maior família das Angiospermas. Este trabalho investigou a toxicidade do óleo essencial desta planta em modelo *in vivo* de *Drosophila melanogaster* e do extrato etanólico em células sanguíneas humanas. O perfil fitoquímico do óleo essencial foi analisado por GC-MS e GC-FID e apresentou como constituintes majoritários: curzereno (48,06%), γ-elemeno (13,49%), atractilone (11,78%) e trans-β-elemenone (8,94%). Os fitoconstituintes majoritários do extrato etanólico, identificados por HPLC, foram: ácido elágico (1,19%), cianidina (0,56%), quercetina (1,58%), quercitrina (1,34%), isoquercitrina (1,01%) e luteolina (1,01%). O extrato, nas concentrações (1-480 µg⁄mL) não apresentou citotoxicidade pelos modelos de viabilidade celular em leucócitos humanos e de fragilidade osmótica em eritrócitos, nem genotoxicidade avaliada pelo Ensaio Cometa. Tendo apresentado uma atividade concentração dependente em modelo de DPPH, quando comparado ao ácido ascórbico. A sua atividade antioxidante em condições basais, nas concentrações de (30-480 µg/mL) inibiu a formação de TBARS. Com inibição máxima (30 µg/mL) no cérebro e (120 µg/mL) no fígado. O extrato também atenuou a formação de TBARS induzida por Fe^{2+} (10 µM) nas concentrações de (120 µg/mL) para o cérebro e (240 µg/mL) para o fígado. O óleo essencial induziu mortalidade em *D. melanogaster* com CL_{50} (5,56 µg⁄mL) em 12 h e déficit na capacidade locomotora. Foi escolhida uma concentração subletal de (3 µg⁄mL) em intervalos de tempo de 3, 6 e 12 horas para realização dos testes. Em paralelo, as moscas também apresentaram sinais de estresse oxidativo, incluindo formação de ERO's em 3 h de exposição o que se manteve em 6 e 12 h. Apresentando aumento nos níveis de TBARS em 6 e 12 h de exposição. Um aumento na atividade da enzima GST às 6 e 12 h e da SOD às 12 h foram significantes. Apresentou, também, um aumento significativo na expressão de NQO-1 a 3 h de exposição. O nível da proteína HSP70 apresentou um aumento significativo as 12 h. Os níveis do fator de transcrição Nrf2 permaneceram inalterados. A co-exposição do óleo essencial com o Paraquat (20 mM) e Ferro (10 mM), aumentou a toxicidade em (104,4% e 98,82%) respectivamente. De forma similar, Paraquat e Ferro aumentou, respectivamente, o déficit locomotor em (35,94% e 103,1%) em relação ao controle. Em resumo, o extrato etanólico não apresentou toxicidade nas concentrações e modelos testados e o óleo essencial apresentou toxicidade em *D. melanogaster*, tendo o estresse oxidativo como importante mecanismo de ação. A partir dos estudos realizados, sugere-se uma potencial aplicação do óleo essencial como inseticida de origem natural. Novos estudos necessitam ser realizados para melhor compreender os mecanismos toxicológicos envolvidos.

Palavras-chave: Sinalização Celular. Geotaxia negativa, Cromatografia.

ABSTRACT

TOXICITY OF *Eugenia uniflora* L. (MYRTACEAE) IN *Drosophila melanogaster* AND HUMAN BLOOD CELLS

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SUPERVISOR: JEFERSON LUIS FRANCO
CO-SUPERVISOR: HENRIQUE DOUGLAS MELO COUTINHO

The increasing use of natural products has led to the imperative need of investigating the toxicological potential of secondary plant metabolites. In this context, toxicological studies were carried on *Eugenia uniflora* (family: Myrtaceae), a species used in Ceara state, Brazil, for medicinal purpose. Particularly, this study investigated the toxicity of *E. uniflora* leaf essential oil in *Drosophila melanogaster* as well as the toxicity of ethanolic extract of *E. uniflora* in human cells. The phytochemical profile of the essential oil analyzed by GC-MS and GC-FID showed curzerene (48.06%), γ-elemene (13.49%) atractilone (11.78%) and trans-β-elemenone (8.94%) as the major constituents. Polyphenolic constituents of E. uniflora ethanolic extract analysed by high performance liquid chromatography (HPLC) revealed the presence of ellagic acid (1.19%), cyanidin (0.56%), quercetin (1.58%), quercitrin (1.34%), isoquercitrin (1.01%) and Luteolin (1.01%) as the major components. The extract at the concentrations tested (1-480 µg/mL) showed no cytotoxic effect to leukocytes and erythrocytes. In addition, the extract did not have any genotoxic effect, suggesting that the extract can be consumed safely at relatively high concentrations. The extract exhibited lower DPPH radical scavenging activity in comparison to ascorbic acid, but strongly inhibited (30-480 µg/mL) lipid Fe^{2+} (10 µM)-induced lipid peroxidation (LPO) in rat brain and liver homogenates The results obtained with *E. uniflora* leaf essential oil indicate that it induces mortality in *D. melanogaster* (LC_{50} = 5.56 µg/mL) and locomotor deficit after 12 h. Based on this observation, the flies were exposed to 3 µg/mL of essential oil for 3, 6 and 12 h. Exposure of flies to 3 h caused a significant increase in reactive species production which remained stable from 6 to 12 h. There was an increase in TBARS formation following exposure of flies to *E. uniflora* leaf essential oil after 6 and 12 h, and this was associated with a significant increase in GST and SOD activities. A significant increase in the expression of NQO-1 was noted after 3 h of exposure, and this was associated with a significant increase in the protein level of HSP70 after 12 h. Basal levels of Nrf2 transcription factor remained unchanged. Co-exposure of the essential oil with Paraquat (20 mM) and Fe^{2+} (10 mM) increased the mortality (104.4% and 98.82%) compared to the control. Similarly, Paraquat and Fe^{2+} increased the locomotor deficit (35.94% and 103.1%) respectively. These results indicate the toxic effects of *E. uniflora* leaf essential oil on *D. melanogaster* and points oxidative stress as an importante mechanism of toxicity. Nevertheless, further studies should be conducted to better understand the mechanism(s) underlying its toxicity.

Keywords: Cell Signaling, Negative Geotaxia, Chromatography.

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INTRODUÇÃO

ARTIGO

MANUSCRITO

LISTA DE ABREVIATURAS

Nrf2	Fator de transcrição
ROS	Espécies reativas de oxigênio
L.	Linnaeus
GC-FID	Cromatografia gasosa acoplada a detector por ionização de chama
GC-MS	Cromatografia gasosa acoplada a Espectrometria de Massa
IR	Índice de retenção
PET	Politereftalato de etileno
SD	Desvio padrão
Fe	Ferro
PQ	Paraquat
et al.	E outros ou e outras
NPSH	Tióis não proteicos
PSH	Tióis proteicos
TBARS	Ácido tiobarbitúrico
DCF	Diclorofluoresceína
DCFDA	Diclorofluoresceína diacetato
GST	Glutationa s-transferase
CAT	Catalase
SOD	Superóxido dismutase
CDNB	1-cloro-2,4-dinitrobenzeno
GSH	Glutationa
EDTA	Ácido etilenodiamino tetra-acético
TEMED	Tetrametiletilenodiamina
Nrf2	Fator de transcrição nuclear
NQO-1	Quinona oxidoredutase 1
HSP70	Proteina do choque térmico
Anti-Nrf2	Anticorpo do fator de transcrição Nrf2
Anti-NQO-1	Anticorpo anti-Quinona oxidoredutase 1
Anti-HSP70	Anticorpo anti-HSP70
Anti-β-actin	Anticorpo anti-β-actin
TBST	Solução Salina Tamponada Tris-Tween
Keap1	Proteina quinase 1
PKC	Proteina quinase C
MAPK	Proteinas quinases ativadas por mitógeno
NAD(P)H	Nicotinamida adenina dinucleotídeo fostato
GCL	Glutamato cisteína ligase

LISTA DE SÍMBOLOS

L.	Linnaeus
cm^3	Centímetro cúbico
mM	Milimolar
Fe	Ferro
PQ	Paraquat
pH	Potencial hidrogeniônico
rpm	Rotações por minuto
H_20_2	Peróxido de hidrogênio

SUMÁRIO

APRESENTAÇÃO

Esta Tese está redigida sob a forma de artigos, conforme preceitua norma da Universidade Federal de Santa Maria. Na INTRODUÇÃO consta uma revisão sucinta da literatura, enfocando especialmente a toxicidade do óleo essencial e do extrato etanólico de *Eugenia uniflora*.

No item RESULTADOS estão apresentadas as seções: Materiais e Métodos, Resultados, Discussão, Conclusão e Referências do Artigo: *Eugenia uniflora* leaves essential oil induces toxicity in *Drosophila melanogaster:* involvement of oxidative stress mechanisms. Como também do Manuscrito: Ethanolic Extract from the Leaves of *Eugenia uniflora* L. (Myrtaceae): Antioxidant and Cytotoxicity Evaluation in Human Blood Cells.

A DISCUSSÃO E CONCLUSÃO apresentam uma síntese das interpretações e comentários sobre o artigo e o manuscrito, os quais são partes integrantes desta Tese. Ao final estão apresentadas as Perspectivas de estudo, como também: Outros trabalhos publicados e aceitos durante o Doutorado.

As REFERÊNCIAS apresentadas ao final do trabalho correspondem as citações apresentadas nos itens: INTRODUÇÃO E DISCUSSÃO. As citações constantes do Artigo e Manuscrito encontram-se referenciadas ao final dos mesmos.

Quanto aos aspectos éticos, o acesso ao óleo essencial e ao extrato da planta estavam isentas de autorização, nos termos do Art. 1º da Resolução 29, de 6 de dezembro de 2007, do Conselho de Gestão do Patrimônio Histórico. O uso de ratos Wistar recebeu parecer favorável da Comissão de Ética no Uso de Animais, da Universidade Federal de Santa Maria. Protocolo Nº (076-2012-2). O uso de células sanguíneas humanas de doadores voluntários do Hospital da Universidade Federal de Santa Maria – UFSM, recebeu parecer favorável do Comitê de Ética em Pesquisa da UFSM. Protocolo Nº (0089.0.243-07).

1. INTRODUÇÃO

A família Myrtaceae possui 145 gêneros válidos, dos quais o gênero *Eugenia* apresenta 1.058 espécies descritas (THEPLANTLIST, 2014). As folhas de *Eugenia uniflora* são perenes, enquanto os frutos são sazonais. As folhas possuem diversos metabólitos secundários: monoterpenos, triterpenos, flavonóides, taninos e leucoantocianidinas (AMORIM, 2009). *Eugenia uniflora* L. (Figura 1) conhecida popularmente por pitanga é nativa da região Neotropical, encontra-se amplamente distribuída na porção atlântica que vai do Ceará ao Rio Grande do Sul (LORENZI & MATOS, 2002). Trabalhos têm relatado as suas propriedades farmacológicas, mas são escassos os estudos toxicológicos sobre esta planta.

Existe uma variedade de nomes científicos e intra específicos, como sinonímia para esta espécie: *Eugenia arechavaletae; E. costata; E. dasyblasta; E. decidua; E. indica; E. lacustres; E. michelii; E. microphylla; E. myrtifolia; E. oblongifolia; E. strigosa; E. uniflora* var. *atropurpurea; E. willdenovii* e *E. zeylanica*. Outros gêneros também apresentam sinonímia: *Luma, Myrtus, Plinia, Stenocalyx e Syzygium* (THEPLANTLIST, 2015).

Figura 1 – Aspecto geral de *Eugenia uniflora*

1.1 PERFIL FITOQUÍMICO DE *Eugenia uniflora*

A identificação do perfil fitoquímico de extratos e óleos voláteis de *Eugenia uniflora* é importante para se compreender a bioatividade destes compostos. As principais técnicas utilizadas para esta finalidade são os métodos cromatográficos, pela rapidez e nível de precisão. Especialmente, o uso de Cromatografia líquida de alta eficiência – CLAE para a identificação e quantificação de fitoconstituintes de extratos de plantas e de Cromatografia gasosa acoplada a espectrometria de massas – GC-MS, na identificação de compostos voláteis de óleos essenciais (BIASI & DESCHAMPS, 2009).

Diversos modelos são propostos para se investigar a toxicidade destes compostos. Um *screening in vitro* pode ser um caminho preliminar para se rastrear a citotoxicidade, podendo-se utilizar células sanguíneas humanas como modelos (SHIING, 1980; OHKAWA, et al., 1979). Outra alternativa, é se utilizar modelos *in vivo* de artrópode. A *Drosophila melanogaster* tem sido relatada na literatura como uma alternativa para estes ensaios, dadas as suas características de ciclo biológico de curta duração, facilidade de criação e pela homologia de seus genes para diversas doenças em humanos (SOBRAL-SOUZA, et al., 2014; POSSER, et al., 2009; FRANCO, et al., 2009). O uso de *D. melanogaster* em testes pilotos com óleos essenciais demonstrou ser eficaz nos testes por fumigação.

Os óleos essenciais ou óleos voláteis são derivados de fenilpropanóides ou compostos terpênicos, estes estão preponderantemente presentes e formados a partir de unidades isoprênicas (MATOS, 2009). Os óleos essenciais são de fácil extração que vai desde a hidrodestilação e destilação por arraste de vapor até métodos mais sofisticados de extração como é o caso do CO_2 supercrítico (GARMUS et. al., 2014). Estes óleos possuem uma infinidade de aplicações que vai da cosmética a farmacologia e suas propriedades estão associadas aos seus fitoconstituintes isolados ou agindo de forma sinérgica. Na Tabela 1 está representado um resumo dos principais trabalhos publicados com o óleo volátil da *E. uniflora*.

Tabela 1 – Trabalhos publicados de óleos essenciais de *E. uniflora*. Apresentando parte da planta, método de extração, técnica analítica e constituintes majoritários.

Parte da Planta	**Método de Extração**	**Técnica Analítica**	**Constituintes Majoritários**	**Referências**
Folhas e ramos finos	Hidrodestilação	GC-MS	Germacrone 32,8 % Curzerene 30,0 % Germacrene B 15,6 %	Maia et al. (1999)
Folhas Frutos	Hidrodestilação	GC-MS	Curzerene 19.7% Selina-1,3,7(11)-trien-8-one 17.8% Atractylone 16.9% Furanodiene 9.6% Germacrone 27.5% Selina-1,3,7(11)-trien-8-one 19.2% Curzerene 11.3% Selina-1,3,7(11)-trien-8-one 11.0%	Ogunwande et al. (2005)
Frutos	Coluna a vácuo	GC-MS	trans- β -Ocimene 36.2% β -Ocimene 15.4% cis-Ocimene 13.4% β -pinene 10.3%	Oliveira et al. (2006)
Folhas frescas	Aparelho de soxhlet e clevenger	GC-FID GC-MS	Cyclohexane, 1ethenyl-1-methyl; 1,5-Cycloundecadiene, 9-1-methylethyl; 5-Benzofuranacetic acid, 6-ethenyl; 1,2-butanediol, 1-phenyl.	Galhiane et al. (2006)
Folhas	Hidrodestilação	GC GC-MS	Furanodiene/furanoelemene ou Curzerene 50.2% β -elemene 5.9% α -cadinol 4,7%	Melo et al. (2007)
Partes aéreas	Hidrodestilação	GC-MS	Estação úmida Selina-1,3,7(11)-trien-8-one 43% Selina-1,3,7(11)-trien-8-one epoxide 29 % Spathulenol 7.5% Estação seca Selina-1,3,7(11)-trien-8-one 43% Selina-1,3,7(11)-trien-8-one epoxide 20% Spathulenol 10%	Costa et al. (2009)
Folhas frescas	Hidrodestilação	GC-MS GC-	Atractylone Furanoeudesmene	Amorim et al. (2009)
Folhas	Arraste de vapor	GC-MS GC-FID	Selina-1,3,7(11)-trien-8-one (34,0 %) Selina-1,3,7(11)-trien-8-one epoxide (17,0 %) Germacrene B (10.5 %)	Gallucci et al. (2010)
Folhas frescas	Hidrodestilação	GC-MS	Germacrene B (13,09%) Germacrene D (12,64%) α-Humulene (11,56%)	Yoshida et al. (2010)
Folhas	-	GC-MS	Grupo I germacrene B 11.1-30.7% germacrone 9.8-54% atractylone 0-19.9% Grupo II curzerene 42.0-43.2% germacrene D 8.7-9.0% germacrene A 5.9-8.9% Grupo III selina-1,3,7(11)-trien-8-one 40.3-55.4% Selina-1,3,7(11)-trien-8-one epoxide 12.7-24.4%	Costa et al. (2010)

Folhas frescas	Hidrodestilação	GC-FID-MS	Atractylone 26,78 % Curzerene 17,96 % Germacrene B 9,31 %	Lago et al. (2011)
Folhas	Hidrodestilação		Selina-1,3,7(11)-trien-8-one epoxide 25,4	Coitinho et al. (2011)
Folhas secas ao ar	Hidrodestilação	Cromatografia Gasosa acoplada a Espectrometria de Massa – GC-MS	Curzerene 85,1 % Furanodiene 1,2 %	Chang et al. (2011)
Ramos vegetativos	Hidrodestilação	GC MS GC-FID	(*E*)-β-ocimene 24.1% Germacrene D 20.8% Bicyclogermacrene 10.9% (*Z*)-β-ocimene 10.4%	Tucker et al. (2011)
-	-	(GC-MS)	Selina 1,3,7(11) trien-8-one (30.1%), Selina 1,3,7(11) trien-8-one-epoxido (21.89%), β cariofilene (6.51%)	Silva et al. (2012)
Folhas	Hidrodestilação	GC-MS	Germacrene B 21,2% Seline-1,3,7-trien-8-one oxide 19,3% β cariophyllene 12,6% Germacrene A 11,6% Germacrene D 11,4% Seline-1,3,7-trien-8-one 9,7%	Victoria et al. (2012)
Folhas	Hidrodestilação	GC-MS GC-FID	Curzerene 47,3% γ-elemene 14,25% Trans- β-elemene 10,4%	Rodrigues et al. (2013)
Folhas	CO_2 supercrítico	GC-MS	Selina-1,3,7(11)-trien-8-one (15.72%) Germacrene D (15%) Trans-caryophyllene 14.18% Germacrene B (13.49%)	Garmus et. al. (2014)
Folhas	Hidrodestilação	GC-MS GC-FID	Curzereno 48,06% γ-Elemeno 13,49% Atractilone 11,78%	Cunha et al. (2015)

1.2 ATIVIDADE BIOLÓGICA

O óleo essencial da fruta de *E. uniflora* foi relatado ter um forte efeito antibacteriano contra *Staphylococcus aureus*, enquanto que o óleo das folhas mostrou uma forte inibição do crescimento de *Bacillus cereus* (OGUNWANDE et al., 2005). Uma atividade bactericida frente a *Staphylococcus aureus* e *Salmonella typhimurium* foi descrita por Mazaro et. al 2008. Também foram relatadas atividades antibacterianas contra várias bactérias gram-positivas, principalmente *Streptococcus. equi* e *Staphylococcus epidermidis* e atividade antifúngica contra cepas de leveduras, principalmente para *Cryptococcus gattii* e *Cryptococcus neoformans* (LAGO, et al., 2011). Encontra-se relatado na literatura moderada atividade antifúngica contra *Candida albicans* (CASTRO & LIMA, 2011);

Atividades antidiarréica, diurética, antirreumática, antifebril e antidiabética foram investigadas por Schapoval et al. (1994) e atividades antinociceptiva e hipotérmica

(AMORIM et al., 2009). A forma de levedura do *Paracoccidioides brasiliensis* foi completamente inibida com uma concentração de 62,5 μg⁄mL (COSTA et al., 2010); atividade antioxidante e antimicrobiana foi demonstrada por Victoria et al. (2012), bem como propriedades antidepressivas e antioxidantes (VICTORIA, et al., 2013). A atividade antileishmania contra *Leishmania amazonenses* foi demonstrada por Rodrigues et al. (2013).

1.3 ATIVIDADE BIOINSETICIDA

Nos últimos anos tem crescido o número de estudos sobre as propriedades biológicas dos extratos e óleos essenciais de plantas. Trabalhos têm sido publicados associando sua aplicação no tratamento da dor, inflamação, doenças virais e câncer. Atividade antioxidante, assim como a sua utilização na permeabilidade de células e tecidos para aumentar a penetração de drogas, bem como o seu uso como repelentes de insetos (ADORJAN e BUCHBAUER, 2010).

Sobral-Souza e colaboradores (2013), demonstraram a toxicidade do óleo essencial de *Eugenia jambolana.* Esta toxicidade ficou evidenciada pelo índice de mortalidade e pelo dano ao aparelho locomotor em *D. melanogaster*.

Dentre as bioatividades, há relatos na literatura demonstrando também a atividade biocida do óleo essencial de *E. uniflora* contra artrópodos: Atividade bioinseticida contra *Sitophilus zeamais* em sementes de milho (COITINHO et al., 2010); inibição de 100% da viabilidade de larvas de *Aedes aegypti*, nas concentrações de (0,2, 0,4 e 0,8 μg/mL) (LEITE et al., 2009); Fraca atividade acaricida contra *Tyrophagus putrescentiae* (ASSIS et al., 2011).

Ao avaliar a toxicidade do óleo essencial, Cunha e colaboradores (2015) demonstraram que o óleo essencial de *E. uniflora*, por fumigação, possui toxicidade contra a mosca da fruta *D. melanogaster* e que os mecanismos de estresse oxidativo podem estar envolvidos no aumento da mortalidade e nos déficits de locomoção. Estes dados são consistentes com os achados para o óleo essencial de *Psidium guajava* (PINHO et al., 2014).

Cunha e colaboradores (2015), demonstraram também a expressão de enzimas de detoxificação de xenobióticos como a Glutatiosa-S-transferase (GST) e Superóxido dismutase (SOD) e avaliou os mecanismos de sinalização celular envolvidos, através de biomarcadores:

Nrf2, NQO-1 e HSP70. Ao analisar o aumento da toxicidade de Paraquat e Fe^{2+}, demonstrou que este óleo aumenta a mortalidade em torno de 100% quando em co-exposição com o PQ e Fe^{2+}. Fato semelhante ocorre com os danos ao aparelho locomotor. Este fato é animador pois estimula a realização de novas pesquisas visando a testar a co-exposição deste e de outros óleos essenciais ou de seus constituintes isolados.

Estudos de Pavela e colaboradores (2010), demostraram que substâncias terpênicas - cânfora e acetato de trans-chrysanthenyl, induziram toxicidade aguda em larvas de borboletas *Spodoptera littoralis* (Boisduval). Cânfora foi o constituinte majoritário do óleo essencial de *Tanacetum parthenium* L, com 46,2%. Sendo esta, uma substância biologicamente ativa no controle de bactérias, fungos e insetos.

O besouro da batata do colorado - *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) - é uma importante praga para a agricultura. Foram testados 30 monoterpenos contra as fases larvares e adultas deste inseto. Encontrou-se uma maior toxicidade dos hidrocarbonetos monoterpenos, quando comparados aos monoterpenos oxigenados. O estudo conclui que 1,8-cineol, fenchona, β-pineno ᵞ-terpineno apresentaram potencial atividade bioinseticida tanto contra as fases jovens, quanto a fase adulta deste Artrópodo.

1.4 TOXICIDADE DOS PRODUTOS NATURAIS

1.4.1 Modelos de toxicidade em células sanguíneas humanas

Diversos estudos têm sido realizados para se investigar a toxicidade de produtos naturais em células sanguíneas humanas. Kamdem e colaboradores (2013), demostrou que o extrato etanólico de *Melissa officinalis* possui baixa toxicidade em leucócitos e eritrócitos humanos. Possuindo atividade antioxidante significativa em diversos modelos, o que pode ser explicado em parte pelos compostos fenólicos presentes no extrato desta planta. Ao avaliar a genotoxicidade pelo Ensaio Cometa, também demonstrou que o extrato desta planta nas concentrações e modelo testado não induz dano ao DNA.

Em outro estudo com *Anacardium microcarpum*, uma planta da família Anacardiaceae, popularmente conhecida por cajuí, ficou demonstrado que o extrato etanólico e suas frações

possuem atividade antioxidante e não possui citotoxidade quando avaliada em modelos de viabilidade celular em leucócitos humanos e de fragilidade celular de eritrócitos em diferentes concentrações salinas. Este estudo também atribui a sua atividade antioxidante aos ácidos fenólicos e flavonoides identificados e quantificados por Cromatografia líquida de alta eficiência – CLAE (BARBOSA-FILHO et al., 2014).

Ao estudar a citotoxicidade e genotoxicidade de *Euphorbia tirucalli*, Waczuk e colaboradores (2015), demonstraram a citotoxicidade por um dos modelos estudados em células sanguíneas humanas e a sua genotoxicidade pelo ensaio Cometa. O perfil fitoquímico desta planta apresenta compostos fenólicos em sua composição química, os quais não foram suficientes para proteger as células de agentes tóxicos de outras classes químicas de compostos. Ao se avaliar a atividade antioxidante pelo modelo de DPPH, este apresentou um efeito concentração dependente mas não superior ao controle realizado com ácido ascórbico.

1.4.2 Modelo de toxicidade em *Drosophila melanogaster*

A mosca da fruta - *Drosophila melanogaster*, é um inseto da ordem Diptera, muito usado como artrópodo-modelo em estudos de biologia molecular e recentemente seu estudo tem sido ampliado em avalições toxicológicos em diferentes modelos. Por possuir ciclo de vida curto e ser de fácil criação, tem sido muito utilizado para se estudar a toxicidade e modelos de citoproteção com metais pesados, especialmente cloreto de mercúrio (FRANCO et al., 2009; POSSER et al, 2009)

O envolvimento dos mecanimos de estresse oxidativo podem ser avaliados através de biomarcadores, tais como: Produção de espécies reativas de oxigênio e nitrogênio; tióis protéicos (PSH) e tióis não protéicos (NPSH); atividade de enzimas do sistema antioxidante e sinalização celular. Bem como do aumento da toxicidade do Paraquat (PQ) e o Ferro (Fe^{2+}) (CUNHA et al., 2015).

Técnicas de marcadores de estresse oxidativo são relatadas na literatura. A toxicidade do extrato da alga *Prasiola crispa* foi avaliada em dois artrópodos: *D. melanogaster* e *Nauphoeta cinerea*, por Zemolim e colaboradores (2014). As evidências de toxicidade desta planta na mosca da fruta foram avaliadas pela mortalidade e alterações bioquímicas, tais como: níveis

de acetilcolinesterase e marcadores de estresse oxidativo. Moscas expostas a uma concentração de (2 mg⁄mL) durante 24 horas tiveram uma mortalidade substancialmente maior em relação ao controle. Este trabalho concluiu que a toxicidade desta planta pode estar relacionada com sistemas de detoxificação e antioxidantes (ZEMOLIM, et al., 2014).

1.5 JUSTIFICATIVA

O uso crescente de produtos naturais com diversas propriedades farmacológicas tem ensejado o estudo toxicológico de diversas plantas, visando especialmente a segurança da sua utilização. A caracterização do perfil fitoquímico destas plantas, por diferentes métodos cromatográficos tem permitido a identificação e quantificação de seus fitoconstituintes, ensejando um maior conhecimento das suas propriedades e sua toxicologia.

Neste contexto, *E. uniflora* popularmente conhecida como pitanga, uma espécie da família Myrtaceae, a maior família das angiospermas, necessita de estudos toxicológicos que venham a esclarecer a sua potencial toxicidade dos seus extrato e óleo essencial em diversos modelos animais.

Este trabalho justifica-se pelo uso etnofarmacológico do extrato para a cura de diversos males. A folha da pitanga é utilizada na medicina popular como: anti-hipertensivo, antipirético, antireumático, antimicrobiano, modulador da atividade antibiótica, diurético, nos distúrbios digestivos, contra bronquite e gripe. No entanto há escassos relatos na literatura sobre a sua toxicidade, especialmente em células sanguíneas humanas. Bem como o estudo de sua atividade antioxidante, *in vitro*, em modelos de mamíferos. Também não existem estudos que correlacionem a citotoxicidade deste óleo com o envolvimento de mecanismos de estresse oxidativo. Assim como não há estudos que avaliem o aumento da toxicidade do Paraquat (PQ) e o Ferro (Fe^{2+}) em có-exposição com o óleo essencial de *E. uniflora*.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a toxicidade das folhas de *Eugenia uniflora* L. (Myrtaceae) através do uso do óleo essencial em *Drosophila melanogaster* e do extrato etanólico em células sanguíneas humanas.

2.2 OBJETIVOS ESPECÍFICOS

Do óleo essencial

2.2.1 Identificar os fitoconstituintes do óleo essencial de *E. uniflora*;

2.2.2 Avaliar a toxicidade através da medição das taxas de sobrevivência e geotaxia negativa em modelo de mosca da fruta;

2.2.3 Quantificar a peroxidação lipídica em *D. melanogaster*. Avaliar os níveis de tióis protéicos (PSH) e tióis não protéicos (NPSH), bem como analisar a atividade das enzimas: Catalase - CAT, Superóxido dismutase - SOD e Glutationa s-transferase - GST;

2.2.4 Identificar as vias de sinalização celular como resposta ao estresse oxidativo e

2.2.5 Avaliar o aumento da toxicidade do óleo essencial em co-exposição com o Paraquat

e Fe^{2+}.

Do extrato

2.2.6 Identificar os fitoconstituintes do extrato de *E. uniflora*;

2.2.7 Determinar o percentual de inibição da atividade antioxidante;

2.2.8 Avaliar a Viabilidade Celular de leucócitos humanos;

2.2.9 Avaliar a Fragilidade Osmótica em eritrócitos humanos;

2.2.10 Analisar a genotoxicidade e

2.2.11 Quantificar a peroxidação lipídica em cérebro e fígado de rato.

3 RESULTADOS

3.1 ARTIGO

Eugenia uniflora leaves essential oil induces toxicity in _Drosophila melanogaster_: involvement of oxidative stress mechanisms

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Toxicology Research
2015, 4, 634-644

Toxicology Research

PAPER

Cite this: *Toxicol. Res.*, 2015, **4**, 634

Eugenia uniflora leaves essential oil induces toxicity in *Drosophila melanogaster*: involvement of oxidative stress mechanisms†

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Eugenia uniflora L. (Myrtaceae family), also known as "pitanga", is a tree species widely used in popular medicine. Despite the well documented beneficial effects of the extracts and essential oils from this plant, little is known about its toxicity. We performed a phytochemical fingerprinting and evaluated the toxicity induced by the *Eugenia uniflora* leaves essential oil in a *Drosophila melanogaster* model. In order to understand the biochemical mechanisms involved in *E. uniflora* essential oil toxicity, changes in the Nrf2 signaling as well as the hallmarks of oxidative stress were measured. The exposure of adult flies to the essential oil *via* a fumigant method resulted in increased mortality and locomotor deficits. In parallel, an oxidative stress response signaling, evidenced by changes in ROS production, lipid peroxidation, alterations in the activity of antioxidant enzymes and expression of Nrf2 protein targets occurred. In the light of our findings, attention is drawn to the indiscriminate use of this plant for medicinal purposes. In addition, a potential bio-insecticidal activity of *Eugenia uniflora* volatile compounds is suggested, a fact that needs to be further explored.

Received 8th October 2014,
Accepted 12th December 2014

DOI: 10.1039/c4tx00162a

www.rsc.org/toxicology

1. Introduction

The use of plants in alternative medicine has been a common practice of the human population since ancient times, and a vast range of new drugs has been developed based on them.[1,2] The extensive use of botanical extracts and its derived compounds is favored because they are regarded as safe, easily accessible, and affordable and are culturally acceptable as a therapeutic alternative by large number of communities.[3–6] Despite the beneficial effects of plant extracts, there is substantial evidence suggesting their potential toxicity.[7–9] Consequently, studies on the toxic effects of plant derivatives are necessary since they are consumed without concern about their toxic effects.[10]

Eugenia uniflora (Myrtaceae family), popularly known as "pitanga" in Brazil, is an arboreal tree distributed all over the country.[11] This species has perennial leaves and produces seasonal fruits. Many secondary metabolites in its leaves are characterized as monoterpenes, triterpenes, flavonoids, tannins and leucoanthocyanins.[12] Plants of the genus *Eugenia* L. (Myrtaceae) are used in folk medicine as alternative therapies for diabetes, arthritis, rheumatism and stomach diseases.[13,14] In addition, a variety of biological activities is shown for *Eugenia uniflora* including antioxidant, anti-inflammatory, and antimicrobial activities as well as modulation of symptoms related to depression and mood disorders.[15–17] Despite the above mentioned beneficial effects, little is known about the potential toxicity induced by extracts and essential oils from this plant.

The fruit fly *Drosophila melanogaster* is a modern genetics premier model system, with an extensive literature ranging from classical and modern genetics, biochemistry, physiology and complex phenotypes. *D. melanogaster* has a rapid reproductive cycle and a distinct developmental stage and is easily maintained and handled in the lab with low operational costs. Moreover, *Drosophila* is not subject to ethical objection, unlike rodents and other vertebrates. Although humans and

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D. melanogaster are only distantly evolutionarily related, almost 75% of disease-related genes in humans have functional orthologs in flies,[18] making such an organism a valuable model system for the understanding of molecular mechanisms of humans diseases, the mode of action of toxic compounds and prospective studies focusing in new bioactive compounds.[19,20]

Our goal with the present study was to evaluate the toxicity of *E. uniflora* leaves essential oil using *D. melanogaster* as the model organism. We also searched for potential mechanisms of toxicity by measuring oxidative stress related parameters.

2. Material and methods

2.1 Chemical reagents

All chemicals were of analytical grade or higher and were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). Primary antibodies and the ECL chemiluminescent reagent were purchased from Santa Cruz Biotechnology (TX, USA). Secondary antibodies were from Sigma-Aldrich (São Paulo, SP, Brazil). Acrylamide, bis-acrylamide, hybond nitrocellulose were obtained from GE Life Sciences (São Paulo, Brazil).

2.2 Plant material

The botanical material from *Eugenia uniflora* L. was collected in June 2013 at 9:00 ± 00:30 h at the Botanical Garden of Crato, CE, Brazil (coordinates: 07°14′28.0″S and 39°24′56.7″W). The referred time of collection was chosen because it yielded a higher amount of oil compared to other collection times. After species identification one voucher specimen was deposited in the Herbarium Dárdano de Andrade Lima, Universidade Regional Cariri-URCA under number #3106.

2.3 *Drosophila* stock and culture

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in 2.5 × 6.5 cm^2 glass bottles containing 5 mL of standard medium (1% w/v brewer's yeast; 2% w/v sucrose; 1% w/v powdered milk; 1% w/v agar; 0.08% v/w nipagin) at constant temperature and humidity (25 ± 1 °C; 60% relative humidity, respectively). All experiments were performed with the same strain.

2.4 *E. uniflora* leaves essential oil

Leaves of *E. uniflora* L. were collected and perforated into pieces of about 1 cm^2. Subsequently, plant material was immersed in distilled water in a 5 liter glass flask, and subjected to extraction with Clevenger apparatus by hydro-distillation, according to the methodology described by Matos,[21] obtaining a yield of 0.136%. Such distillations were made in quadruplicates following the addition of anhydrous sodium sulfate. Then the essential oil was filtered using a pasteur pipette with cotton and stored in amber glass vials at −20 °C.

2.5 Phytochemical analysis

2.5.1 Gas chromatography (GC-FID). The gas chromatography (GC) analyses were performed with an Agilent Technologies 6890N GC-FID system equipped with a DB-5 capillary column (30 m × 0.32 mm; 0.50 mm) and connected to an FID detector. The thermal programmer was 60 °C (1 min) to 180 °C at 3 °C min^{-1}; injector temperature 220 °C; detector temperature 220 °C; split ratio 1 : 10; carrier gas helium; flow rate: 1.0 mL min^{-1}. The volume injected 1 μL diluted in chloroform (1 : 10). Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors.[22]

2.5.2 Gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed on an Agilent Technologies AutoSystem XL GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (220 °C). The transfer line temperature was 220 °C. Helium was used as a carrier gas (1.0 mL min^{-1}) and the capillary columns used were an HP 5MS (30 m × 0.35 mm; film thickness 0.50 mm) and an HP Innowax (30 m × 0.32 mm i.d., film thickness 0.50 mm). The temperature programmer was the same as that used for the GC analyses. The injected volume was 1 μL of the essential oil diluted in chloroform (1 : 10).

2.5.3 Identification of the components. Identification of the constituents was performed on the basis of retention index (RI), determined with reference of the homologous series of *n*-alkanes, C_7–C_{30}, under identical experimental conditions, and comparison was done with the mass spectra library search (NIST and Wiley), and with the mass spectra literature.[23] The relative amounts of individual components were calculated based on the CG peak area (FID response).

2.6 Essential oil exposure and *D. melanogaster* assays

The exposure of flies to the essential oil was performed by a fumigation protocol as described: 20 adult flies (males and females) were placed in 330 cm^3 flasks, containing a filter paper soaked with 1% sucrose in distilled water at the bottom. A counter-lid of polyethylene terephthalate (PET) was introduced on the screw cap of the flask, to which a filter paper was fixed at the inner side of the cap for application of the different doses of essential oil. By doing this, the flies feed and hydrate on sucrose solution at the bottom of the flasks and the essential oil is allowed to volatilize from the top in order to reach the fly's respiratory system. The flasks received the following treatments: 1% sucrose (control) and 3, 7.5, 15, 23.5 and 30 μg ml^{-1} of essential oil. Readings of flies' survivorship were taken at 3, 6, 12, 24 and 48 h. Results are presented as percentage (%) of live flies (mean ± SD) obtained from five independent experiments.

2.7 Locomotor assay

The locomotor capacity was evaluated by following the negative geotaxis behavior as described by Coulom and Birman[24] with some modifications. Twenty adult flies (1–4 days old; both genders) were exposed to essential oil as detailed above

(section 2.6). After completion of treatment, flies were immobilized on ice for 1–2 minutes and placed separately in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After 30 min recovery, flies were gently tapped to the bottom of the column and the number of flies that reached 8 cm of the column (top) and flies that remained below this mark (bottom) were registered. The assays were repeated five times for each fly. Results are presented as number of flies on top (mean ± SD) obtained from three independent experiments.

2.8 Oxidative challenge with iron (Fe) and paraquat (PQ)

In order to evaluate the toxicity of the *E. uniflora* essential oil in a comparative way with well known pro-oxidant compounds, twenty flies were exposed to Fe (10 mM) and PQ (20 mM) alone or in combination with the essential oil (3 μg ml^{-1}) and their survivorship and locomotor capacity were evaluated after 12 h. Fe and PQ were added to a filter paper with 1% sucrose and *E. uniflora* essential oil was administered to flies by the fumigation method described above. All assays were performed in five independent experiments and the results are presented as percentage of live flies (mean ± SD) and percentage of flies on the top (mean ± SD). The concentrations of iron and paraquat were chosen according to previous reports by our group, which were based on literature reports (Cruz *et al.*, 2014; Zemolin *et al.*, 2014).[66,67]

2.9 Oxidative stress markers

From then on, all experiments were performed at 3, 6 and 12 h of exposure to 3 μg ml^{-1} concentration of the *E. uniflora* essential oil. We used those time points and concentration based on the results from the mortality and locomotor deficit observed in *D. melanogaster* since we still have live flies after the treatment (Fig. 2), allowing us to evaluate oxidative stress related responses.

Oxidative stress was determined by measuring lipid peroxidation, reactive oxygen species formation (ROS), non-protein thiols (NPSH) and protein thiols (PSH). By-products of lipid peroxidation were quantified by the thiobarbituric acid reactive substances method (TBARS) following Ohkawa[25] with few modifications. Briefly, 20 flies from each treatment were homogenized in 0.5 ml of phosphate buffer 0.1 M pH 7.0 with the aid of a bead-based homogenizer (Powerlyzer, MO BIO, Carlsbad, CA) at 2000 rpm, over 20 seconds and centrifuged at 1000*g* over 10 min at 4 °C. After centrifugation, the supernatant was incubated in acetic acid 0.45 M/HCl buffer pH 3.4, containing thiobarbituric acid 0.28%, SDS 1.2%, at 95 °C over 60 min for color development, and then absorbance was measured at 532 nm. Malondialdehyde (0–3 nmol) was used as the standard. The 2,7-dichlorofluorescein diacetate (DCFDA) oxidation was used as a general index of ROS formation following Péres-Severiano.[26] The fluorescence emission of DCF resulting from DCFDA oxidation was monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm in a multi mode plate reader (EnsPire®PerkinElmer, USA). Protein and non-protein thiols were determined according to the method described by Ellman[27] and adapted to our lab conditions. In summary, after completion of treatment, flies were homogenized in 0.5 M perchloric acid and centrifuged at 5000*g* for 5 min at 4 °C. The NPSH content was determined in the supernatant while the pellet was used for PSH measurement. Total protein was quantified according to the Bradford assay.[28]

2.10 Enzymatic assays

For antioxidant enzymes activity, groups of 20 flies were homogenized in 1 mL 0.1 M phosphate buffer, pH 7.0, and centrifuged at 20 000*g* for 30 min. The resulted supernatant was used for determination of glutathione *S*-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) according to methods described earlier.[29] Glutathione *S*-transferase (GST; EC 2.5.1.18) activity was assayed following the procedure of Habig and Jakoby[30] using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mixture consisting of 100 mM phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase (CAT; EC 1.11.1.6) activity was assayed following the clearance of H_2O_2 at 240 nm in a reaction media containing 50 mM phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, 0.012% TRITON X100 according to the procedure of Aebi.[31] Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed following the procedure of Kostyuk and Potapovich.[32] The assay consists in the inhibition of superoxide-driven oxidation of quercetin by SOD at 406 nm. The complete reaction system consisted of 25 mM phosphate buffer, pH 10, 0.25 mM EDTA, 0.8 mM TEMED and 0.05 mM quercetin. All enzyme activities were performed at 25 ± 1 °C using a Cary60 spectrophotometer (Agilent Technologies, New Castle, DE) coupled to a Peltier-controlled circulating water bath. Total protein was quantified according to Bradford.[28]

2.11 Western blot analysis of Nrf2/NQO-1/HSP70 signaling pathway

Protein expression was determined by Western blotting according to Posser[33] with minor modifications. Thirty flies were homogenized at 4 °C in 300 μL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and phosphatase inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000*g* for 10 min at 4 °C and the supernatants (S1) collected. After protein determination (following Bradford[28]) using bovine serum albumin as standard, β-mercaptoethanol and glycerol were added to samples to a final concentration of 8 and 25%, respectively, and the samples frozen until further analysis. Proteins were separated using SDS-PAGE with 10% gels, and then electrotransferred to nitrocellulose membranes as previously described by Posser.[33] Membranes were washed in Tris-buffered saline with Tween (TBST; 100 mM Tris–HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4 °C) with different primary antibodies (Santa Cruz Biotechnology, Texas, USA), all produced in rabbit (anti-Nrf2, anti-NQO-1, anti-HSP70, anti-β-actin; 1 : 1000

dilution in TBST). Following incubation, membranes were washed in TBST and incubated for 1 h at 25 °C with HRP-linked anti rabbit-IgG secondary specific antibodies (Sigma, MO). Blottings were visualized in the Image Station 4000MM PRO using the ECL reagent (Santa Cruz Biotechnology, TX). Immunoreactive bands were quantified using the Scion Image software and expressed as a fold change of the mean relative to control group (treated only with sucrose).

2.12 Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test when necessary. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

3.1 Chemical compounds in the *E. uniflora* essential oil

The phytochemical fingerprinting of the *E. uniflora* essential oil was performed by GC-MS analysis. Fig. 1 shows a chromatogram in which at least 29 compounds could be identified. Regarding the composition and the proportion of the compounds found in the essential oil, the most abundant ones are curzerene (48.06%), γ-elemene (13.49%), atractylone (11.78%) and *trans*-β-elemenone (8.94%) (Table 1).

3.2 Mortality and locomotor behavior tests

Adult flies were exposed to varying concentrations of *E. uniflora* essential oil (control, 3, 7.5, 15, 23.5 and 30 μg ml^{-1}) through a fumigation method described earlier, and survivorship was evaluated at 3, 6, 12, 24 and 48 h after the initial exposure. We observed a time and concentration dependent increase in mortality (Fig. 2). According to the results, all oil concentrations tested were able to induce fifty percent or higher mortality rate at 12 h time point, except for 3 μg ml^{-1} concentration (Fig. 2). Besides a milder mortality induction observed at the 3 μg ml^{-1} concentration in the first 12 h of exposure, it induced around 60% and 75% mortality at 24 h and 48 h of exposure, respectively ($p < 0.05$; $F = 2288$). Concentrations higher than 7.5 μg ml^{-1} were able to induce 100% mortality between 12 and 24 h of exposure.

Similarly, a significant time and concentration dependent locomotor deficit was observed in flies exposed to essential oil (Fig. 3). At the first 3 h of treatment, significant decreases

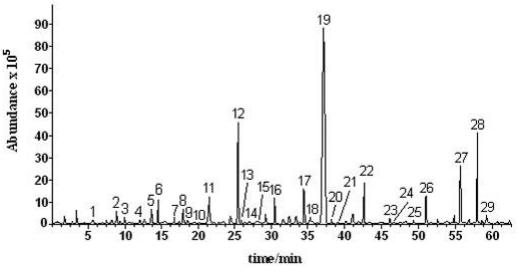

Fig. 1 Representative GC-MS chromatogram profile of *E. uniflora* leaves essential oil. The most abundant compounds are curzerene (peak 19), γ-elemene (peak 12), atractylone (peak 28) and *trans*-β-elemenone (peak 27).

Table 1 Composition of *Eugenia uniflora* essential oil[a]

No.	Compounds	RI[b]	RI[c]	Oil (%)
1	α-Pinene	937	939	0.05
2	β-Pinene	980	980	1.27
3	β-Myrcene	994	991	0.36
4	*p*-Cymene	1025	1029	0.18
5	Limonene	1033	1031	1.56
6	γ-Terpinene	1062	1061	2.09
7	Linalool	1098	1098	0.49
8	δ-Elemene	1335	1338	1.03
9	α-Cubebene	1349	1345	0.16
10	α-Copaene	1376	1376	0.08
11	β-Caryophyllene	1417	1418	2.57
12	γ-Elemene	1435	1433	13.49
13	Aromadendrene	1439	1439	0.51
14	α-Humulene	1451	1454	0.35
15	Alloaromadendrene	1465	1462	0.16
16	γ-Muurolene	1476	1477	2.85
17	Germacrene D	1483	1480	3.21
18	β-Selinene	1484	1485	0.78
19	Curzerene	1498	1496	48.06
20	γ-Cadinene	1509	1513	0.32
21	α-Cadinene	1540	1538	0.17
22	Germacrene B	1556	1556	4.93
23	Spathulenol	1577	1576	1.09
24	Caryophyllene oxide	1580	1581	0.08
25	Globulol	1585	1583	0.25
26	Viridiflorol	1590	1590	3.16
27	*trans*-β-Elemenone	1599	1601	8.94
28	Atractylone	1654	1653	11.78
29	Germacrone	1691	1693	1.24
Total identified (%)				**97.43**

[a] Relative proportions of the essential oil constituents were expressed as percentages. [b] Retention indices experimental (based on homologous series of *n*-alkane C_7–C_{30}). [c] Retention indices from the literature (Adams, 1995[23]).

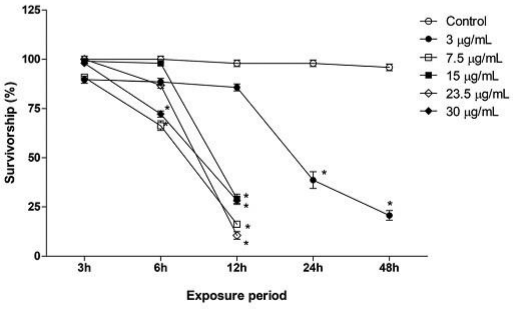

Fig. 2 Effect of the *E. uniflora* leaves essential oil on the survivorship of *D. melanogaster*. Flies were exposed through the fumigation method described in the Material and methods section. The survivorship was analyzed at the indicated time points. Results are expressed as mean ± SD of the percentage (%) of live flies after each exposure time. * $p < 0.05$ compared to control.

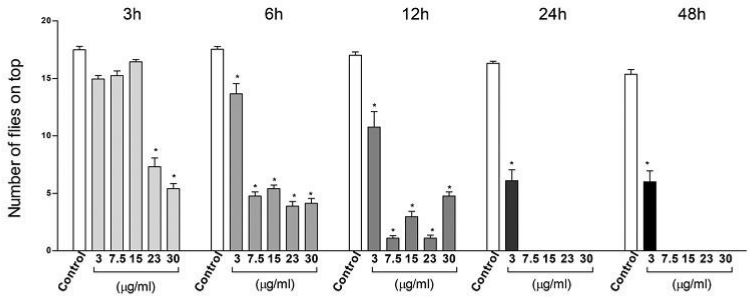

Fig. 3 Effect of the *E. uniflora* leaves essential oil on the locomotor ability of *D. melanogaster*. Flies were exposed through the fumigation method described in the Material and methods section. The locomotor activity was determined as negative geotaxis behavior. Results are expressed as mean ± SD of the number of flies able to climb a marked glass column as described earlier at each exposure time. * $p < 0.05$ compared to control.

($p < 0.05$; $F = 324.2$) in the locomotor ability of flies were evident at higher oil concentrations (23.5 and 30 μg ml^{-1}), while at 6–12 h time points all concentrations tested caused significant decreases in flies' locomotion. Oil concentrations higher than 7.5 μg ml^{-1} caused a complete loss of locomotor activity after 24 h of exposure (Fig. 2). Based on these results, the concentration of 3 μg ml^{-1} and time points up to 12 h were utilized for the next set of experiments.

3.3 Oxidative stress markers and antioxidant response

Aiming to clarify the potential mechanisms by which flies are affected by the *E. uniflora* essential oil, experimental animals were exposed to 3 μg ml^{-1} of oil over 3, 6 and 12 h. Then, oxidative stress markers and the activity of antioxidant enzymes were determined. This concentration is below the LC_{50} 12 h (5.56 μg ml^{-1}) for *D. melanogaster* as observed in Fig. 2. It was possible to observe a significant ($p > 0.05$; $F = 15.71$) increase in ROS production at 3 h exposure to the essential oil, a result that was maintained after 6 and 12 h as well (Fig. 4A). Our results also showed an increased ($p < 0.05$; $F = 17.65$) level of TBARS after 6 h and 12 h of exposure to essential oil (Fig. 4B). The levels of protein and non-protein thiols (NPSH and PSH) were not changed (Table 2).

We also evaluated the activity of three enzymes involved in the antioxidant cell defense: GST, SOD and CAT as well as the expression of protein targets involved in stress response and antioxidant signaling (Nrf2, NQO-1 and HSP70). When flies were exposed to essential oil, a significant increase ($p < 0.05$; $F = 42.18$) in the activity of GST was evident when compared to non-exposed controls at 6 and 12 h (Fig. 5A), whilst the activity of SOD was increased ($p < 0.05$; $F = 14.51$) at a 12 h time point (Fig. 5B). Catalase activity was unchanged at all time points evaluated (Fig. 5C). As demonstrated in Fig. 6, flies exposed to the essential oil presented a significant increase ($p < 0.05$; $F = 189.8$) in the expression of NQO-1 at 3 h of exposure (Fig. 6A and C). The protein levels of HSP70, a stress responsive chaperone, did not change after the first 3 and 6 h of treatment but had a significant increase ($p < 0.05$; $F = 16.4$) in flies

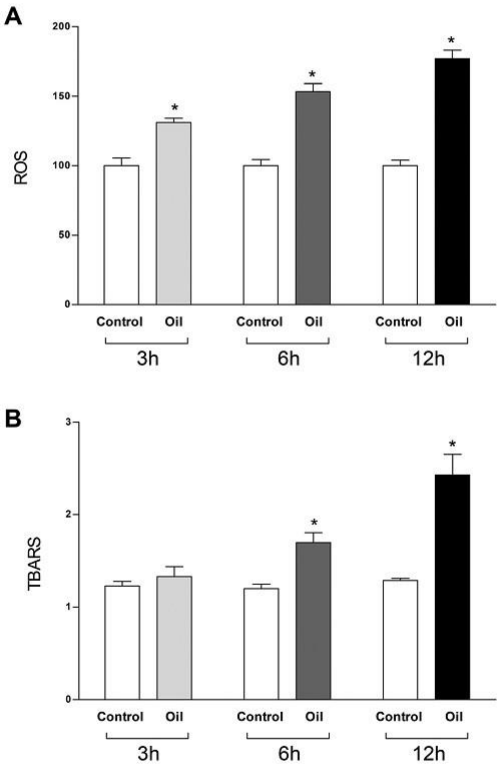

Fig. 4 Measurement of ROS (A) and TBARS (B) production in *D. melanogaster* exposed to *E. uniflora* leaves essential oil. Flies were exposed to essential oil at 3 μg ml^{-1} concentration for 3, 6 and 12 h through the fumigation method described in the Material and methods section. After completion of treatment, ROS and TBARS were determined while flies homogenates. ROS production is expressed as percentage of DCFDA oxidation (mean ± SD) in which control was considered 100%. TBARS levels are expressed as nmol TBARS per mg of total protein (mean ± SD). * $p < 0.05$ compared to control.

Table 2 Thiol status of *D. melanogaster* exposed to the *E. uniflora* leaves essential oil[a]

	PSH	NPSH
Control 3 h	6.0 ± 0.1	1.9 ± 0.3
Oil 3 h	5.8 ± 0.3	2.2 ± 0.3
Control 6 h	6.2 ± 0.9	1.9 ± 0.1
Oil 6 h	5.8 ± 0.4	2.0 ± 0.2
Control 12 h	5.6 ± 0.3	2.1 ± 0.1
Oil 12 h	5.5 ± 0.2	2.1 ± 0.1

[a] PSH and NPSH: μmol per mg protein. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test when necessary. Differences were considered to be statistically significant when $p < 0.05$.

exposed to essential oil at a 12 h time point (Fig. 6A, B and D). The basal levels of Nrf2 transcription factor remained unaltered in all time points tested (Fig. 6A and B).

3.4 Susceptibility to oxidative challenge with iron and paraquat

Since markers of oxidative stress were apparent in flies exposed to *E. uniflora* essential oil, we asked whether it would increase the susceptibility of flies to oxidative stress inducers: iron (Fe) and paraquat (PQ). These oxidative stressors are widely used to induce a pro-oxidative condition in a range of animal models including *Drosophila*.[34,35] The exposure of flies to 3 μg ml^{-1} of oil over 12 h did not cause significant changes in mortality, while PQ (20 mM) and Fe (10 mM) induced an increase in mortality of approximately 30% (Fig. 7A and 8A respectively). The combination of essential oil with PQ ($p < 0.05$; $F = 104.4$) or Fe ($p < 0.05$; $F = 98.82$) substantially potentiated the mortality effect (Fig. 7A and 8A). A similar result was observed in the locomotor behavior test in which both PQ or Fe alone had a mild potency in impairing flies locomotor ability, but when in combination with essential oil, had a potentiated effect (PQ: $p < 0.05$; $F = 35.94$; Fe: $p < 0.05$; $F = 103.1$) (Fig. 7B and 8B). In general, the administration of essential oil concomitantly to PQ or Fe caused a higher effect in the mortality of flies and locomotor performance when compared with PQ and Fe alone (Fig. 7B and 8B).

4. Discussion

Even though the use of plant extracts and essential oils have been reported to exert a variety of pharmacological actions, there is evidence that some botanical derivatives can cause toxicity.[30,32] Therefore, it is imperative to explore the toxicity potential of plant extracts and essential oils popularly used in folk medicine. Considering the crescent interest in the pharmacological properties of *E. uniflora* derived compounds,[15–17] the understanding of its potential toxicity is needed. In the present study, we investigated the potential toxicity of the essential oil extracted from the leaves of *E. uniflora* in a *D. melanogaster* model, and identified some phytochemicals present in the oil.

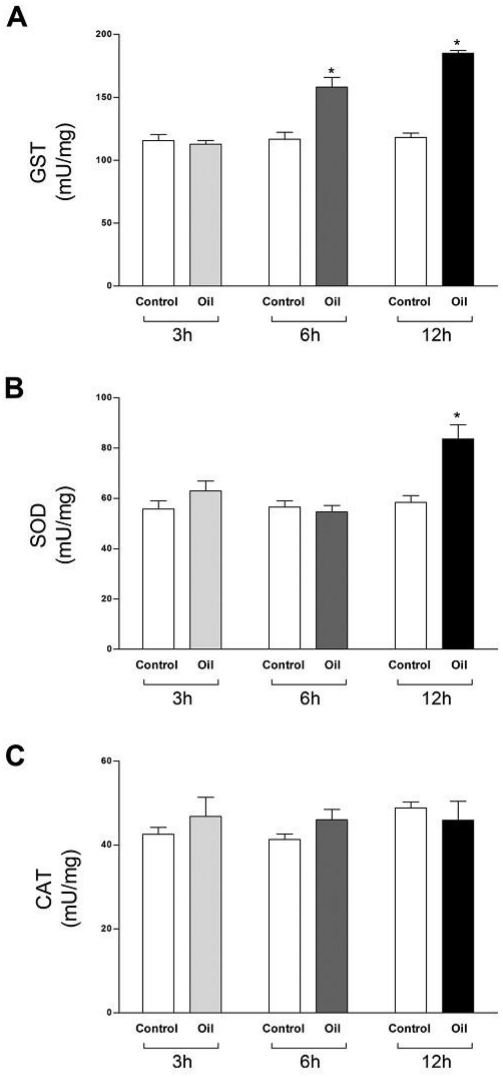

Fig. 5 Activity of antioxidant enzymes in *D. melanogaster* exposed to *E. uniflora* leaves essential oil. GST (A), SOD (B) and CAT (C) were determined in flies homogenates after exposure to essential oil at 3 μg ml^{-1} concentration for 3, 6 and 12 h, through the fumigation method described in the Material and methods section. Enzyme activity is expressed as mU per mg of total protein (mean ± SD). * $p < 0.05$ compared to control.

The beneficial effects of *E. uniflora* extracts and essential oils are well documented in the literature.[13–17] However, little is known about its potential toxicity. Here, we have demonstrated that in a short period of exposure, low concentrations of *E. uniflora* leaves essential oil are able to induce mortality and locomotor deficits in *Drosophila*. As a mechanism of the observed toxicity, it is suggested that a pro-oxidant condition

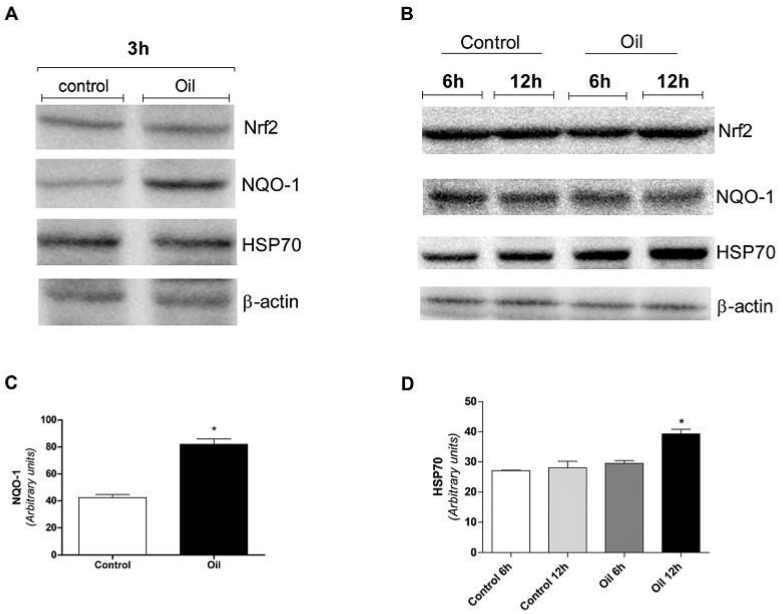

Fig. 6 Analysis of the Nrf2/NQO-1/HSP70 signaling pathway in *D. melanogaster* exposed to a 3 μg ml^{-1} *E. uniflora* leaves essential oil concentration. After treatment samples were collected at each time point indicated and processed for western blot evaluation of each protein target. (A) Representative immunoblots for protein targets at 3 (A), 6 and 12 h (B) of exposure to the essential oil. Optical densitometry of immunoreactive bands of NQO-1 (C) and HSP70 (D). Results are expressed as arbitrary units (mean ± SD). * $p < 0.05$ compared to control.

was established after the flies came in contact with oil derived volatile compounds. Such an effect is confirmed by increased production of reactive species and accumulation of lipid peroxidation end products. Additionally, a clear adaptive response to oxidative stress was apparent in the oil exposed flies, since it was possible to observe an activation of antioxidant signaling pathways and increased activity of key cellular antioxidant enzymes. Considering the rapid induction of mortality and locomotor deficit phenotypes in flies exposed to low concentrations of *E. uniflora* essential oil, we draw attention to the important toxicity imposed by the oil, which in turn, may be of interest in terms of a natural insecticide.

Plant derived compounds are reported to induce toxicity to a wide range of insects and may interfere directly with all developmental stages of the fruit flies and cockroaches.[36,37] Compounds such as terpenes, flavonoids, alkaloids, steroids and saponins are important phytochemicals when considering the insecticide activity of plant derivatives.[38] In addition to acute toxicity and mortality, terpenoids and flavonoids have been also studied for their insect repellent activity.[38,39] In this study, the major compounds found in the *E. uniflora* essential oil were mono and sesquiterpenoids (Table 1), an observation that is partially in agreement with previous reports.[15,40–43] The most abundant compound was curzerene, making approximately 48% of the oil's chemical constitution. Although we did not perform assays to evaluate the toxicity of each compound in our model, the presence and high abundance of curzerene suggests that it may present major importance in the toxicity of the essential oil. We also observed a significant impairment in the negative geotaxis behavior of flies treated with low concentrations of *E. uniflora* oil, which reflects a locomotor deficit. The locomotor effect of the oil occurred in a very short period of time, causing flies to present loss of locomotor performance at time points as early as 3 h of exposure. Compounds from essential oils such as terpenoids and phenylpropanoids can alter the insect neurotransmitters system, including the dopaminergic and cholinergic apparatus.[44–46] It has been shown that many terpenes are inhibitors of the acetylcholinesterase (AchE).[46] In general, the terpenoid compounds found in *E. uniflora* are suggested to be involved in the oil's observed biocide effect. Studies are ongoing in order to clarify the role of different compounds found in the essential oil tested here.

In parallel to the induced mortality and locomotor deficits, flies exposed to *E. uniflora* leaves essential oil also showed signs of oxidative stress, including ROS and TBARS formation as well as changes in important antioxidant response systems. The cellular response to oxidative stress is mostly regulated by the Nrf2 nuclear transcription factor.[47] ROS/xenobiotics-induced alterations in the cellular redox state constitute an important signal to promote adaptive responses mediated by Nrf2.[48,49] The up-regulation of detoxifying enzymes by natural compounds appears to be related to activation of the Nrf2-ARE pathway.[48,49] The Nrf2 nuclear translocation and subsequent

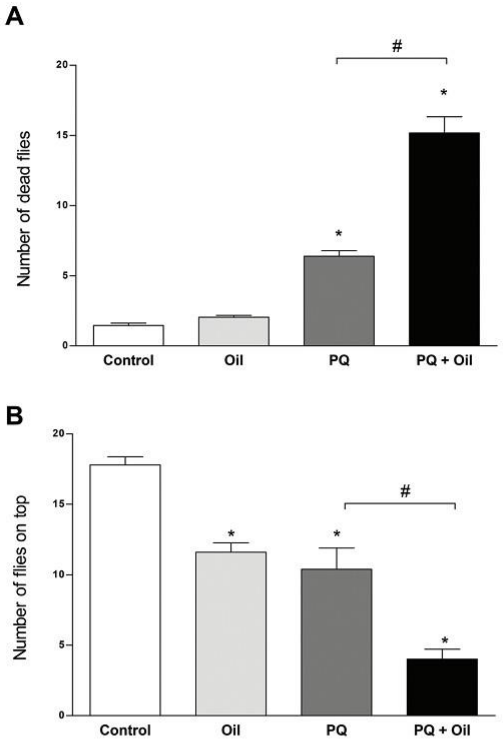

Fig. 7 Susceptibility of *D. melanogaster* exposed to *E. uniflora* leaves essential oil to paraquat (PQ), an oxidative stress inducer. Flies were co-exposed to essential oil (3 μg ml^{-1}) and PQ 20 mM for 12 h. After completion of treatment, survivorship and locomotor activity were determined. (A) Number of dead flies exposed to essential oil and PQ. (B) Negative Geotaxy behavior of flies exposed to essential oil and PQ 20 mM. Essential oil was administered by fumigation, as described earlier. PQ was given to flies as a solution prepared in 1% sucrose. Controls received only 1% sucrose. Results are expressed as mean ± SD. * $p < 0.05$ compared to control. # $p < 0.05$ comparing PQ and PQ + oil group.

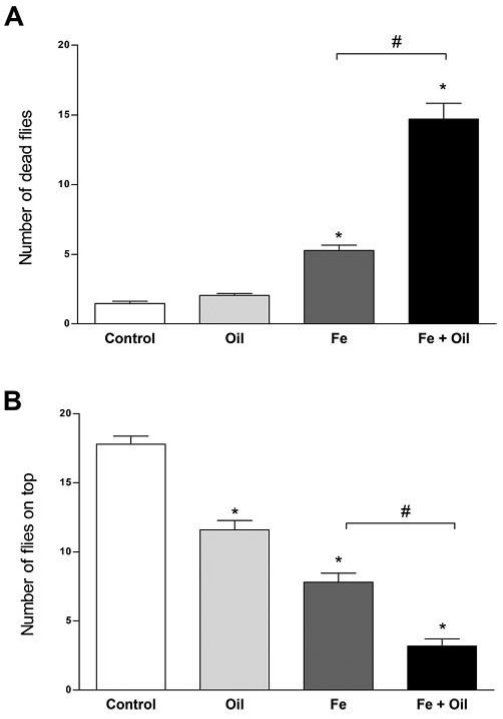

Fig. 8 Susceptibility of *D. melanogaster* exposed to *E. uniflora* leaves essential oil to iron (Fe), an oxidative stress inducer. Flies were co-exposed to essential oil (3 μg ml^{-1}) and Fe 10 mM for 12 h. After completion of treatment, survivorship and locomotor activity were determined. (A) Number of dead flies exposed to essential oil and PQ. (B) Negative Geotaxy behavior of flies exposed to essential oil and 10 mM Fe . Essential oil was administered by fumigation, as described earlier. Fe was given to flies as a solution prepared in 1% sucrose. Controls received only 1% sucrose. Results are expressed as mean ± SD. * $p < 0.05$ compared to control. # $p < 0.05$ comparing PQ and PQ + oil group.

binding to DNA sequence known as the "antioxidant response element; ARE" may be triggered by dissociation from the inhibitory protein Keap1 as well as by phosphorylation of serine residues at the Nrf2 protein by upstream kinases such as PKC and MAPK.[49] Among proteins that are usually involved in response to oxidative stress-driven Nrf2 activation, the NAD(P)H dehydrogenase, quinone 1 oxidoreductase (NQO-1), glutamate cysteine ligase (GCL), GST and CAT play central roles.[50] Our results showed a time dependent activation of key factors on the regulation of an antioxidant response. Since a high mortality rate was evident at all doses of essential oil at the first 24 h of exposure, we measured oxidative stress markers up to 12 h in order to obtain a profile of the antioxidant response in animals under *E. uniflora* oil treatment. Apparently, in response to the toxicity induced by oil compounds, flies presented increased ROS levels and a peak of NQO-1 expression at the first 3 h of treatment, a phenomenon that is consistent with an early activation of the Nrf2-ARE pathway.[51] While ROS continued to increase from 3 h up to 12 h, lipid peroxidation took place at 6 and 12 h time points (Fig. 4). The antioxidant enzyme GST was activated after 6 h of oil exposure and continued to increase up to 12 h of treatment. In addition, a later antioxidant response also can be observed by the increased activity of SOD at 12 h after essential oil treatment, a fact that occurred in parallel to an increase in the expression of HSP70. These results clearly suggest a two-phased adaptive response to oxidative stress induced by *E. uniflora* leaves essential oil: an early phase triggered by ROS induction, resulting in activation of the master regulator of cellular antioxidant response, the Nrf2 transcription factor, and a late phase, characterized by oxidative damage and increased ROS/xenobiotic detoxifying enzymes (CAT and GST) in parallel to increased HSP70 expression, a stress responsive chaperone. Later, the toxicity induced by the essential oil was

accomplished by the expression of mortality and locomotor deficits phenotypes.

Glutathione *S*-transferase is an important antioxidant enzyme involved in phase II detoxification systems.[52] GSTs belong to a family of multifunctional enzymes that catalyze the conjugation of GSH to various other molecules and play a role in the mechanisms of intracellular detoxification of endo and xenobiotic compounds.[53,54] The observed increase of GST activity in *Drosophila melanogaster* exposed to *E. uniflora* oil may be related to an adaptive response related to elimination of toxic plant derivatives.[55,56] Singh and co-workers demonstrated that natural compounds are able to increase the expression of GST that together with endogenous GSH favors the elimination of plant metabolites from organisms.[57] SOD plays a crucial role in the clearance of superoxide radical from cells as well as for oxidative stress defense.[31] Our results demonstrated a significant increase in SOD activity in flies treated with the essential oil (Fig. 5). This was concomitant to a rise in ROS production. The observed rise in GST and SOD activity by *E. uniflora* oil exposure in fruit flies may be explained by a potential activation of the Nrf2 signaling pathway. In fact, an early activation of this signaling pathway was noted in flies exposed to the essential oil, by means of increased NQO-1 expression, which is well documented as a main target of the Nrf2 transcription factor.[48,51] Corroborating with the results discussed above, we found that *E. uniflora* leaves essential oil increases the susceptibility of flies to oxidative challenge with the pro-oxidants Fe and PQ. Paraquat is a widely used nonselective broad spectrum herbicide with toxicological importance to animal and human health.[58] It has also been employed as a Parkinsonism inducer in animal models due to its structural similarities to 1-methyl-4-phenylpyridinium ion (MPP+), which is frequently used to induce Parkinson's disease-like[58] phenotypes in several models including *Drosophila*.[59] Oxidative stress induction is reported as the main mechanism of toxicity induced by PQ in *Drosophila*.[60] Exposure to high levels of Fe is also reported to increase oxidative stress in flies.[20] In light of our results and literature evidence, we can hypothesize that the increased susceptibility of *E. uniflora* leaves essential oil exposed flies to Fe and PQ is related to the presence of pro-oxidant compounds in the constitution of the essential oil.

The molecular mechanisms by which exposure to *E. uniflora* leaves essential oil induces oxidative stress in our model still need further clarification. Although we have not addressed this issue in the present study, literature reports have been published supporting our findings. Usually, natural compounds are studied for their antioxidant ability. However, depending on the concentration, hormetic-like effects may arise, mainly due to metabolizing by-products.[61] For instance, Martins and collaborators[62] observed an increased lipid peroxidation induced by high doses of quercetin, indicating a pro-oxidant effect of this natural compound. It was also shown that natural compounds can either induce or inhibit hydroxyl radical formation *via* Fenton's reaction.[63] In addition, interferences with mitochondrial function have also been shown as a mechanism involved in natural compound-induced toxicity.[9,64] Apparently, the anti or pro-oxidative fate of natural compounds depends on the concentration and model of study.[62,64,65]

5. Conclusion

In summary, our results showed that *E. uniflora* leaves essential oil induced a highly toxic effect in *D. melanogaster*, causing substantial mortality and locomotor deficits. As a mechanism associated with the essential oil induced-toxicity, oxidative stress appears to play a major role. The deleterious effects caused by the essential oil in flies were prominent; therefore, a potential application of the *E. uniflora* leaves essential oil as an alternative to synthetic insecticides is suggested. Furthermore, our findings point to the urgent need for additional toxicological evaluations of *E. uniflora* derived natural compounds before its pharmacological use.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge CNPq (482313/2013-7 and 311512/2011-9), FAPERGS (1954-2551/13-7), and PROPESQ-Unipampa for the financial support.

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3.2 MANUSCRITO

Ethanolic Extract from the Leaves of *Eugenia uniflora* L. (Myrtaceae): Antioxidant and Cytotoxicity Evaluation in Human Blood Cells

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Manuscrito submetido ao periódico: **Phytotherapy Research**

Phytotherapy Research

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Journal:	*Phytotherapy Research*
Manuscript ID	PTR-15-1422
Wiley - Manuscript type:	Full Paper
Date Submitted by the Author:	03-Dec-2015
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Keyword:	Eugenia uniflora, Pitanga, DPPH, TBARS, Comet assay

Phytotherapy Research

Ethanolic Extract from the Leaves of *Eugenia uniflora* L. (Myrtaceae): Antioxidant and Cytotoxicity Evaluation in Human Blood Cells

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Abstract

Eugenia uniflora is used in the Brazilian folk medicine in treat intestinal disorders and hypertension. However, no study on its potential toxicity to human cells has been reported so far, and little is known on its antioxidant activity in biological system. Hence, we investigated for the first time the potential toxic effects of ethanolic extract (EtOH) of *E. uniflora* (EEEU) in human leukocytes and erythrocytes, as well as its influence on membrane erythrocytes osmotic fragility. In addition, EEEU was chemically characterized and its antioxidant capacity was evaluated. We found that EEEU (1-480 μg/mL) caused neither cytotoxicity nor DNA damage evaluated by Trypan blue and Comet assay, respectively. EEEU (1-480 μg/mL) did not have any effect on membrane erythrocytes fragility. In addition, EEEU inhibited Fe^{2+}-induced lipid peroxidation in rat brain and liver homogenates, and scavenged the DPPH radical. EEEU presented polyphenolic compounds (quercetin, quercitrin, isoquercitrin and ellagic acid) that may be at least in part responsible for its beneficial effects. Our results suggest that consumption of EEEU at relatively higher concentrations may not result in toxicity. However, further *in vitro* and *in vivo* studies should be conducted to ascertain its safety.

Keywords: *Eugenia uniflora*, Pitanga, DPPH, TBARS, Comet assay

1. INTRODUCTION

Oxidative stress has been associated with the progression of several chronic diseases in humans. Scientific evidence from the literature has shown that elevated levels of reactive oxygen species (ROS) may induce oxidation of proteins, lipids and DNA (Rahman and Adcock, 2006), leading to the alteration of their normal functions. However, long term consumption of diets rich in plant polyphenols has been associated with reduced risk factor of cancer, cardiovascular diseases, diabetes and neurological disorders (Pandey and Rizvi, 2009; Lobo *et al*., 2010).

Currently, there is a growing demand for natural antioxidants especially from plant origin, not only to protect the organism against diseases associated with oxidative stress (Ozen *et al*., 2011), but also, in nutraceuticals, bio-pharmaceuticals and food industries (Braitwaite *et al*., 2014) as preservatives. To this end, isolation and characterization of bioactive phytochemicals are important so as give the scientific basis for the use of the plant/plant extracts in folklore (Kamdem *et al*., 2013; Barbosa-Filho *et al*., 2014).

The genus *Eugenia* has about 500 species native to South America and widely distributed in regions with tropical and subtropical climate. One of these species that has generated considerable interest in the Brazilian cosmetic industry is the Brazilian cherry, *Eugenia uniflora*. This is at least, in part, because of its astringent properties, which is associated with its pleasant smell (Silva, 2006; Amorim *et al*., 2009).

The leaves extract of *Eugenia uniflora,* popularly known in Brazil as "pitanga" or "pitangueira", are used in folk medicine to treat intestinal disorders and antihypertension (Consolini *et al*., 1999). Pharmacological studies have evidenced it anti-parasitic, anti-rheumatic and anti-inflammatory (da Franca Rodrigues *et al*., 2013) activities. However, to the best of our knowledge, there is no study on its potential

toxic effects in human blood cells, although we recently demonstrated the toxicity of its essential oil in *Drosophila melanogaster* (Cunha *et al.*, 2015). Phytochemical analysis from *E. uniflora* leaves extract has revealed the presence of tannins, steroids, cineol, phenols and flavonoids among others (Oliveira *et al*., 2014).

Considering the pharmacological potential of *E. uniflora* leaves extract and the fact that no study on its potential toxic effect to human blood cells has been reported so far, the present study was undertaken to investigate for the first time the cytotoxicity and genotoxicity (in human leukocytes) effects of *E. uniflora* ethanolic extract, as well as its osmotic fragility in human erythrocytes. Besides, its antioxidant activity in biological system is scanty in the literature. Therefore, we also investigated the antioxidant effect of ethanolic extract from the leaves of *E. uniflora* against Fe^{2+}-induced lipid peroxidation in rat brain and liver homogenates.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals used including solvents were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid, ellagic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, rutin, isoquercitrin, quercitrin, kaempferol, catechin and epicatechin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, malonaldehydebis-(dimethyl acetal) (MDA), thiobarbituric acid (TBA), sodium azide and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals compounds were purchased with analytical grade from local suppliers.

2.2. Plant collection

The botanical material (Leaves) from *Eugenia uniflora* L. was obtained from the Botanical Garden of Crato, Ceará, Brazil and a voucher specimen was deposited in the Herbarium Dárdano de Andrade Lima, at the Universidade Regional do Cariri-URCA under the number #3106.

2.3. Preparation of Extract and Identification of constituents by HPLC

2.3.1. Preparation of ethanolic extract from the leaves of E. uniflora

Six hundred and fifty (650) grams of fresh leaves was submerged in ethanol for 3 days at room temperature. Then, the mixture was filtered and concentrated on a rotary evaporator (model 344B-Q-Quimis, Brazil) to evaporate the ethanol. The concentrated extract (i.e., crude extract) was lyophilized and saved in a freezer for the experiments. The yield of the crude extract was 1.07%.

2.3.3. Qualitative and Quantitative analyses of compounds from E. uniflora leaves extract by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 250 mm) packed with 5µm diameter particles. The mobile phase with a flow rate of 0.7 mL/min consisted of a mixture of solvents: A (water/acetic acid, 98:2% v/v) and B (methanol/water/acetic acid, 70:28:2% v/v) using the following linear eluting gradient: 0-3 min: 0% B in A; 3-25 min: 30% B in A; 25-43 min: 50% B in A; 43-55 min: 60% B in A; 55-60 min: 80% B in A; 60-65 min: 50% B in A; and 65-69 min: 0% B in A; following the method described by Boligon *et al.* (2013) with some modifications. The wavelengths used were 254 nm for gallic acid; 327 for chlorogenic acid, caffeic acid and ellagic acid; 356 nm rutin, quercitrin, isoquercitrin, quercetin, kaempferol and luteolin; 520 nm for cyanidin and delphinidin 3-*0*-glucoside.

Eugenia uniflora ethanolic extract (15 mg/mL) and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.250 mg/mL. Chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve (Boligon *et al*., 2013).

2.4. DPPH radical scavenging assay of E. uniflora ethanolic extract

Scavenging activity on DPPH free radicals by the extract was assessed according to the method described by Kamdem *et al.* (2013). Twenty microliters of the extract (1–480 µg/mL) was mixed with 100 µL of 0.3 mM DPPH in ethanol. The mixture was allowed to stand at room temperature for 30 min in the dark. Blank solutions were prepared with each test sample (20 µL) and 100 µL of water. The negative control was 100 µL of 0.3 mM DPPH with 20 µL of water, while ascorbic acid (1–400 µg/mL) was used as positive control. The absorbance was measured at 518 nm against each blank using ELISA microplate reader (SpectraMax, USA) and the inhibition of DPPH free radical was expressed in percentage.

2.5. Preparation of human leukocytes

Heparinized venous blood was obtained from healthy volunteer donors from the hospital of the Federal University of Santa Maria (HUSM), Santa Maria, RS, Brazil (age 28 ± 10). This work was carried out in accordance with the Guidelines of the

Ethical Committee of UFSM and approved by the institutional review board of UFSM (0089.0.243.000-07).

Differential erythrocyte sedimentation with dextran was used to separate leukocytes of the blood as previously described (Kamdem *et al*., 2013). Two milliliters of dextran 5% was added to 8 mL of blood sample, mixed and allowed to stand at room temperature for 45 min. Subsequently, the supernatant was centrifuged (2000 rpm, 10 min) and the pellet was washed with a solution of erythrocyte lysis (NH_4Cl, 150 mM; $NaHCO_3$, 10 mM and disodium EDTA, 1 mM, pH 7.4), homogenized and left for 5 min. Then, the tube was centrifuged (2000 rpm, 2 min) and the pellet was washed again with lysis solution. The pellet was suspended in 2 mL Hank's buffer solution saline (HBSS) containing in mM: 5.4 KCl, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4.2 $NaHCO_3$, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.6 $MgSO_4$, 137 NaCl and 10 $_D$-glucose, 10 Tris–HCl, pH 7.4; and adjusted to 2×10^6 leukocytes/mL with HBSS buffer.

2.6. Preparation of human erythrocytes

Ten milliliters of blood was collected into heparinized tubes from healthy volunteers. The erythrocytes were separated by centrifuging the blood sample at 2000 rpm for 5 min at room temperature. The plasma was discarded and the cell pellet was washed three times with phosphate buffered saline (6.1 mM and pH 7.4, containing 150 mM NaCl) (Barbosa-Filho *et al*., 2014).

2.7. Cytotoxicity assay in human leukocytes

The potential toxic effect of leaf extracts of *E. uniflora* to human leukocytes was determined by the method described by Mischell and Shiingi (1980). Briefly, 2.5 µL of different concentrations of extract (1–480 µg/mL) was added to cell suspension (497.5 µL) and incubated in the presence or absence of hydrogen peroxide (2 mM) +

azide (1 mM), for 3 h at 37°C in a water bath. Hydrogen peroxide (2 mM) + azide (1 mM) was used as positive control, while distilled water was used as negative control. After the incubation, a volume of 50 µL of cells suspension was mixed with 50 µL of 0.4% Trypan blue solution and left for 5 min. The cell viability was determined microscopically (400× magnification) using a hemocytometer and was calculated as the number of living cells (*i.e.*, those not stained with Trypan blue) divided by the total number of cells multiplied by 100 (Barbosa-Filho *et al.*, 2014).

2.8. Osmotic fragility measurement of human erythrocytes

The osmotic fragility of red blood cells was determined by using a modified method of Barbosa-Filho *et al.* (2014). Five hundred microliters of erythrocytes, 100 µL of the extract (1–480 µg/mL) prepared in 1% PBS, pH 7.4, and 900 µL of phosphate buffer saline (6.1 mM and pH 7.4, containing 150 mM NaCl), pH 7.4 were pre-incubated for 3 h at 37°C. After incubation, the sample was mixed, centrifuged (2500 rpm for 10 min) and the supernatant was discarded. The erythrocytes were washed twice with phosphate buffered saline (6.1 mM and pH 7.4, containing 150 mM NaCl), pH 7.4, centrifuged at 2500 rpm for 2 min and the supernatant discarded. Two duplicate sets of assay tubes containing 1.5 mL of 0–0.9% (w/v) NaCl solution in distilled water were incubated with 7.5 µL of treated or untreated erythrocytes for 20 min. Then, the sample was homogenized and centrifuged at 2000 rpm for 5 min. The lysis of erythrocytes was followed by measuring the absorbance of hemoglobin content in the supernatants at 540 nm using microplate reader (SpectraMax, USA). A value of 100% lysis was assigned to the supernatant of the tube with erythrocytes and distilled water. Results are expressed as a percentage of PBS control group.

2.9. Comet assay

The comet assay was performed under alkaline conditions according to the procedure of Santos *et al*. (2009). Peripheral leukocytes were incubated for 3 h in the absence or presence of *E. uniflora* (1-480 µg/mL). Hydrogen peroxide (100 µM) was used as positive control while water was used as negative control. After the incubation, leukocytes were mixed with low-melting point agarose, and 90 µL of low melting point agarose (LMP) 0.75% was added to 15 µL of leukocytes solution and placed on a microscope slide pre-coated with normal melting point agarose 1.0%. A coverslip was added, and the slides immediately placed on ice (10 min). After solidification of the agarose , coverslips were removed, and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5) containing 1% Triton X-100 and 10% DMSO. The slides were kept frozen in lysis solution (4°C) and protected from the light for approximately 14 h. They were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13.5) for 20 min for DNA unwinding. Electrophoresis (20 min at 300 mA and 25 V) was performed in the same buffer. Every step was carried out under indirect yellow light. After electrophoresis, the slides were neutralized in 400 mM Tris (pH 7.5), rinsed three times in distilled water, and left to dry overnight at room temperature. The dry slides were re-hydrated for 10 min in distilled water and were then fixed for 10 min, rinsed three times in distilled water, and dried for at least 5 h. The dry slides were re-hydrated for 3 min in distilled water, stained (sodium carbonate 5% (w/v), ammonium nitrate 0.1% (w/v), silver nitrate 0.1% (w/v), tungstosilicic acid 0.25%, formaldehyde 0.15% (w/v) freshly prepared in the dark) at 37°C for 25 min, and constantly shaken for 25 min. The slides submerged in the stop solution (acetic acid 1%) were rinsed again and immediately tagged for analysis. The slides were analyzed under blind conditions by at least two individuals. One hundred cells per sample were randomly selected and visually scored according to

tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2 times the diameter of the head; (4) class 3: with a tail longer than 2 times the diameter of the head and (5) class 4: comets with no heads. Comets with no heads and images with nearly all DNA in the tail or with a very wide tail were excluded from the evaluation, because they probably represent dead cells (Hartmann and Speit, 1997). DNA damage was presented as DNA damage index (DI) and it is based on the length of migration. The DI was calculated from cells in different damage classes as follows: DI = n1 + 2n2 + 3n3 + 4n4. Where, n1 represents the number of cells with level 1 of damage; n2, number of cells with level 2 of damage; n3, number of cells with level 3 of damage; n4, number of cells with level 4 of damage (Kamdem et al., 2013).

2.10. Determination of Thiobarbituric acid reactive substances (TBARS) in rat brain and liver homogenates

Rats were decapitated; whole brain and liver were dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.4 (1:10, w/v for liver and 1:5, w/v for brain). The homogenates were centrifuged for 10 min at 3600 × g to yield a pellet which was discarded, and a low-speed supernatant (S1) was used for the determination of thiobarbituric acid reactive substances (TBARS) assay. Aliquots of the brain or liver homogenates (20 µL), and 10 µM of $FeSO_4$ were incubated for 1 h at 37°C in the presence or absence of *E. uniflora* (1–480 µg/mL) to induce lipid peroxidation. Thereafter, 40 µL of sodium dodecyl sulfate (8.1%), 100 µL of acetic acid/HCl (pH 3.4) and 100 µL of 0.6% thiobarbituric acid (TBA) were subsequently added to the reaction mixture and incubated at 100°C for 1 h. After cooling, samples were centrifuged for 2 min at 6000 rpm and the absorbance of supernatant was measured at 532 nm using an ELISA plate reader (SpectraMax, USA).

Standard curve of malondialdehyde (MDA) was used to quantify TBARS production in brain and liver homogenates (Ohkawa *et al*., 1979). It should be stressed that the usage of the animals for this study was approved by the institutional review board of UFSM (076-2012-2).

2.11. Statistical analysis

The results are shown as means ± SEM of three independent experiments performed in duplicate. Statistical significance was measured by one-way (DPPH, TBARS, Cytotoxicity e Comet assay) or two-way (osmotic fragility) analysis of variance (ANOVA), followed by Bonferroni post-hoc when appropriated. Differences between groups were considered to be significant when *$p < 0.05$.

3. RESULTS

3.1. Qualitative analysis of E. uniflora ethanolic extract by HPLC – DAD

High Performance Liquid Chromatography (HPLC) profile of *E. uniflora* ethanolic extract revealed the presence of the gallic acid (retention time - t_R = 10.13 min; peak 1), chlorogenic acid (t_R = 18.07 min; peak 2), caffeic acid (t_R = 23.15 min; peak 3), ellagic acid (t_R = 27.94 min; peak 4), cyanidin (t_R = 32.87 min; peak 5), delphinidin 3-*0*-glucoside (t_R = 35.49 min; peak 6), rutin (t_R = 37.26 min; peak 7), quercitrin (t_R = 43.38 min; peak 8), isoquercitrin (t_R = 47.01 min; peak 9), quercetin (t_R = 50.16 min; peak 10), kaempferol (t_R = 57.83 min; peak 11) and luteolin (t_R = 67.94 min; peak 12) (Fig. 1).

The chemical structure and the quantification of these compounds is represented in Fig. 2 and Table 1 respectively. It appears that quercetin (15.80 $\pm$ 0.02mg/g) and quercitrin (13.42 $\pm$ 0.01mg/g) were the major components present in *E. uniflora*

ethanolic extract , while chlorogenic acid (0.91 ± 0.03mg/g) and gallic acid (1.27 ± 0.01 mg/g) were the less abundant (Table 1).

3.2. Cell viability

The effect of ethanolic extract from the leaves of *E. uniflora* on human leukocytes is presented in Fig. 3. As expected, hydrogen peroxide (H_2O_2) used as positive control, caused a significant decrease in the cell viability in comparison to control cell ($p < 0.001$); whereas, the extract at the concentrations tested (1-480 µg/mL) did not have any effect (Fig. 3A, $p > 0.05$). In an attempt to investigate the potential anti-cytotoxic effect of *E. uniflora* ethanolic extract, we co-treated the cells with H_2O_2. The results showed that *E. uniflora* could not protect against H_2O_2-cytotoxicity (Fig. 3B, $p < 0.05$).

3.3. Osmotic fragility

The influence of *E. uniflora* ethanolic extract on human erythrocyte osmotic fragility is depicted on Fig. 4. The extract did not induce osmotic fragility at different salt concentrations (0-0.9%) when compared to control the their respective control ($p > 0.05$).

3.4. Genotoxicity

The genotoxic potential of *E. uniflora* ethanolic extract on human leukocytes evaluated under alkaline conditions is shown in Fig. 5. Methyl methanesulphonate (MMS) used as a positive control caused a significant increase in the DNA damage index when compared to the control group ($p < 0.001$). *E. uniflora* at all the concentration tested did not induce DNA Double-strand breaks in relation to the control group ($p > 0.05$).

288 *3.5. DPPH radical scavenging potential of E. uniflora ethanolic extract*

289 The DPPH radical scavenging ability of *E. uniflora* ethanolic extract and ascorbic acid
290 at different concentrations is depicted in Fig. 6. The extract and ascorbic acid scavenged
291 the DPPH radical in a concentration dependent-manner. However, the DPPH radical
292 scavenging potential of *E. uniflora* was lower than that of ascorbic acid used as standard
293 antioxidant.

294 *3.6. TBARS assay in rat and brain homogenates*

295 Figure 7 shows the effect of *E. uniflora ethanolic* extract on Fe^{2+}-induced lipid
296 peroxidation in rat brain (Fig. 7A) and liver (Fig. 7B) homogenates. Under basal
297 conditions, *E. uniflora* (30-480 µg/mL) significantly inhibited TBARS formation in the
298 brain (Fig. 7A) and liver (Fig. 7B) homogenates. The maximum inhibition was attained
299 at 30 µg/mL in the brain and 120 µg/mL in the liver. Fe^{2+}(10 µM) stimulated TBARS
300 formation in the homogenate of both homogenates in comparison to basal ($p < 0.05$),
301 and this was significantly attenuated by treatment with *E. uniflora* ethanolic extract
302 (Fig. 7A and B). The maximum reduction of TBARS formation was attained at 120
303 µg/mL (Fig. 7A) and 240 µg/mL (Fig. 7B) in the brain and liver homogenates
304 respectively.

305 **4. DISCUSSION**

306 Toxicological studies of plant extracts used in folk medicine are necessary to
307 evaluate their efficacy, quality and safety (Fang *et al*., 2015; Rodríguez-Chávez *et al*.,
308 2015). In this context, the present study aimed to evaluate the toxicity of ethanolic
309 extract from the leaves of *E. uniflora* in human leukocytes and erythrocytes. The results
310 demonstrated that *E. uniflora* ethanolic extract was not cytotoxic and genotoxic to

311 human leukocytes at relatively high concentrations, suggesting that its consumption
312 may possibly not result in any toxic effect.

313 It is well known that the antioxidant activity of chemical compound(s) or plant
314 extract(s) varies according to the model system used. Consequently, the use of only one
315 assay system for the determination of the antioxidant activity is not advisable (Tan and
316 Lim, 2015). Here, biological (TBARS assay) and chemical (DPPH assay) models were
317 used to access the antioxidant capacity of *E. uniflora* ethanolic extract. In the current
318 study, and *E. uniflora* ethanolic extract inhibited the DPPH radical to a lesser extent
319 compared with ascorbic acid used as standard, suggesting that the chemical constituents
320 in the plant extract reacted slowly with the DPPH radical. It has been postulated that
321 ascorbic acid can achieve a steady state with DPPH within minutes (Mishra *et al*.,
322 2012), while many polyphenolic compounds (which are generally responsible for the
323 antioxidant activity) founds in plant extracts requires more times of reaction with the
324 DPPH radical (Oliveira *et al*., 2016). Using similar assay system, Fortes *et al*. (2015)
325 demonstrated that hydrolysable tannins and flavonoid glycosides isolated from *E.*
326 *uniflora* using spectroscopic methods possess remarkable radical scavenging activity.

327 The antioxidant activity of *E. uniflora* was also in investigated by measuring the
328 amount of malondialdehyde (MDA) formed, which is considered as one of the
329 byproducts of lipid peroxidation (LPO), and widely used as an index of oxidative stress
330 (Michel *et al.*, 2008). Ethanolic extract from the leaves of *E. uniflora* demonstrated
331 antioxidant activity against Fe^{2+}- induced LPO in rat brain and liver homogenates.
332 Although, we have not investigated the compound(s) responsible for these effects, it is
333 possible that their phytochemicals including the polyphenols identified here, have acted
334 synergistically or alone to exert their antioxidant action (Kamdem *et al*., 2012).
335 Accordingly, phenolic compounds are considered strong antioxidants capable of

chelating transition metals ions, preventing free radical chain reactions and oxidative damage (Karaman *et al*., 2010). The phytochemical profile of *E. uniflora* ethanolic extract revealed the presence of phenolic acids and flavonoids, which is consistent with that obtained by Lima *et al.* (2002). However, not only polyphenolic compounds contribute to antioxidant activity (Franco *et al*., 2008). Some contribution may also come from other antioxidant secondary metabolites including essential oils, vitamins and carotenoids (Javanmardi *et al*., 2003). In line of this, we have recently shown that *E. uniflora* leaf essential oil contains some bioactive compounds such as curzerene (48.06%), γ-elemene (13.49%), atractylone (11.78%) and trans-β-elemenone (8.94%) that may have contributed to the antioxidant activity observed (Cunha *et al*., 2015).

Testing medicinal plant extracts for their safety assessment is of particular importance since approximately 80% of the population from Asian and African countries rely on traditional medicine for their primary healthcare. In addition, long-term consumption can result in genotoxicity and/or carcinogenicity. In this context, the potential toxic effects of *E. uniflora* ethanolic extract was evaluate using the comet assay (for genotoxicity) and trypan blue exclusion method (cytotoxicity assay). The ethanol extract of the leaves of *E. uniflora* (1-480 µg/mL) did not cause cytotoxicity to human leukocytes when compared with the control, and could not reverse H_2O_2 cytotoxicity, suggesting that it can be consumed safely at relatively higher concentrations. Our results are similar to that obtained by Santos *et al.* (2009) demonstrating that ethanolic extract of *E. uniflora* at 100 µg/mL exhibits low toxicity (8%) in macrophage J774. According to Nagarathna *et al*. (2013), genotoxic agents can act by different mechanisms of action including changes in the chromosomal structures, breaks formation, leading to cancer. Here, we found that the extract did not have any

effect on DNA damage index. Thus, it is possible to presume that the chemical constituents of the plant extract did not interact with the DNA of the cells.

Osmotic fragility can be affected by various factors including membrane composition and integrity, and its decrease has been found to be associated with chronic liver disease, iron deficiency anemia, thalassemia and hyponatremia (Kolanjiappan *et al*., 2002). Here, the influence of *E. uniflora* on human erythrocytes was assessed to verify its safety. Interestingly, the extract did not cause any abnormality to red blood cell membranes.

In conclusion, this study demonstrated that ethanolic extract from the leaves of *E. uniflora* is neither cytotoxic nor genotoxic to human leukocytes at the concentrations tested, and did not affect human erythrocytes osmotic fragility. On the other hand, its antioxidant activity may be at least in part, responsible for its therapeutic action in folk medicine, as evidenced by DPPH radical scavenging activity and inhibition of Fe^{2+}-induced lipid peroxidation in rat and liver homogenates. Further studies are being undertaken to isolate and identify the compound(s) underpinning this action.

Acknowledgement

The first author is grateful to CAPES-DINTER/URCA/UFSM Program. JPK acknowledges the financial support of CAPES, CNPq-TWAS, TWAS.

Conflict of Interest

The authors declare that they have no conflicts of interest regarding this study.

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536 **Tables**

537 **Table 1 –** Quantitative composition of *E. uniflora* ethanolic extract

Compounds	*Eugenia uniflora*		LOD	LOQ
	mg/g	%	µg/mL	µg/mL
Gallic acid	1.27 ± 0.01a	0.12	0.015	0.052
Chlorogenic acid	0.91 ± 0.03 b	0.09	0.032	0.107
Caffeic acid	4.72 ± 0.02 c	0.47	0.007	0.023
Ellagic acid	11.98 ± 0.03 d	1.19	0.026	0.086
Cyanidin	5.69 ± 0.01e	0.56	0.018	0.059
Delphinidin 3-*0*-glucoside	1.34 ± 0.01a	0.13	0.030	0.102
Rutin	4.59 ± 0.01 c	0.45	0.011	0.037
Quercitrin	13.42 ± 0.01 f	1.34	0.009	0.029
Isoquercitrin	10.17 ± 0.03 g	1.01	0.025	0.082
Quercetin	15.80 ± 0.02 h	1.58	0.017	0.055
Kaempferol	7.09 ± 0.01i	0.70	0.029	0.097
Luteolin	10.11 ± 0.03 g	1.01	0.012	0.040

538 Results are expressed as mean ± standard deviations (SD) of three determinations.
539 Averages followed by different letters differ by Tukey test at $p < 0.01$. LOD: limit of
540 detection and LOQ: limit of quantification.

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548 **Figures Caption**

549 **Figure 1.** Representative high performance liquid chromatography profile of *Eugenia*
550 *uniflora*extract. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3),
551 ellagic acid (peak 4), cyanidin (peak 5), delphinidin 3-*0*-glucoside (peak 6), rutin (peak
552 7), quercitrin (peak 8), isoquercitrin (peak 9), quercetin (peak 10), kaempferol (peak 11)
553 and luteolin (peak 12). Calibration curve for cyanidin: Y = 12678x + 1187.9 (r =
554 0.9995); delphinidin 3-*0*-glucoside: Y = 11845x + 1278.1 (r = 0.9999); gallic acid: Y =
555 13048x + 1236.7 (r = 0.9992); caffeic acid: Y = 12583x + 1359.0 (r = 0.9993);
556 chlorogenic acid: Y = 11738x + 1197.6 (r = 0.9997); ellagic acid: Y = 11893x + 1248.7
557 (r = 0.9998); isoquercitrin: Y = 13057x + 1329.1 (r = 0.9992); rutin: Y = 12652x +
558 1370.8 (r = 0.9999); quercitrin: Y = 12179x + 1197.5 (r = 0.9995); quercetin: Y =

559 13628x + 1327.2 (r = 0.9998); luteolin: Y = 11859x + 1327.8 (r = 0.9999) and
560 kaempferol: Y = 12658x + 1297.8 (r = 0.9996).

561 **Figure 2.** Chemical structure of identified compounds from *E. uniflora* ethanolic.
562 extract. Gallic acid, chlorogenic acid, caffeic acid, ellagic acid, cyanidin, delphinidin 3-
563 *0*-glucoside, rutin, quercitrin, isoquercitrin, quercetin, kaempferol and luteolin.

564 **Figure 3.** Effect of the ethanolic extract of *Eugenia uniflora* on human leukocytes in
565 the absence (**A**) or presence (**B**) of H_2O_2 (2mM) + Azide (1mM). EtOH extract did not
566 cause cytotoxicity (A) and could not prevent H_2O_2 toxicity (B). The results are
567 expressed as percentage of control. Values are the means of n = 3 independent
568 experiments performed in triplicate ± SEM. $p<0.05$ *vs.* control.

569 **Figure 4.** Osmotic fragility of erytrocytes treated with *E. uniflora* ethanolic extract.
570 Treated erythrocytes were added to various concentrations of salt solution (0-0.9%) and
571 incubated for 30 min. Absorbances of the supernatants were measured at 540 nm.
572 Hemolysis in each tube was expressed as percentage of control. The bars represent the
573 means of n = 3 independent experiments performed in triplicate ± SEM.

574 **Figure 5**. Damage index of human leukocytes exposed to *E. uniflora* ethanolic extract
575 for 3 h. Data are expressed as mean ± SEM of four independent experiments performed
576 in duplicate. Positive control: MMS (Methyl methanesulphonate) ($2x10^{-5}$M). One-way
577 ANOVA followed by Newman-Keuls (***$p < 0.001$ *vs.* control).

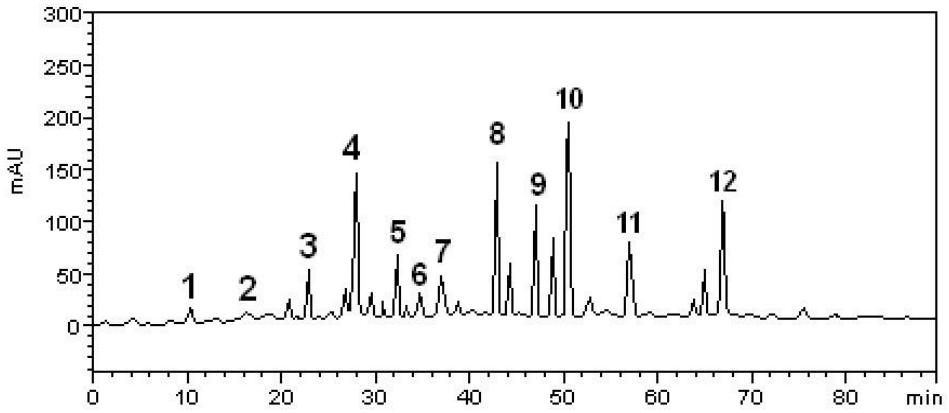
578 **Figure 6.** Percentage inhibition of DPPH radical by *E. uniflora* ethanolic extract.
579 Results are expressed as mean ± SEM (n = 4).

580 **Figure 7**. The inhibitory effect of *E. uniflora* extract (**A** and **B**) on Fe^{2+} (10μM) –
581 induced lipid peroxidation in rat brain (**A**) and liver (**B**) homogenates. Values are the

582 means of n = 3 independents performed in duplicate ± SEM. * p <0.05 *vs.* Basal, #

583 p<0.05 *vs.* Fe^{2+}

584 **Fig. 1**



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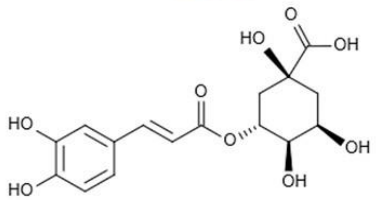
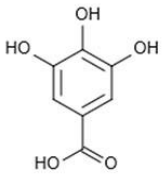
589 **Fig. 2**

Chlorogenic acid

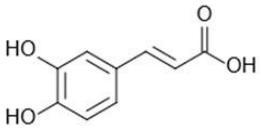


Gallic acid

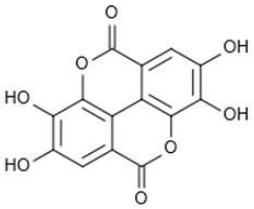


Caffeic acid

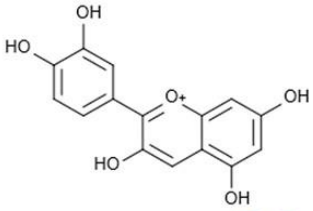
Ellagic acid



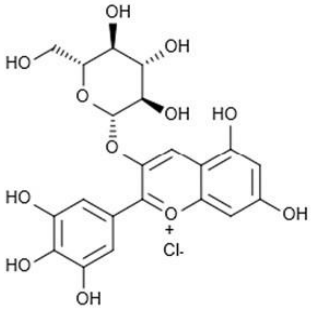
Cyanidin



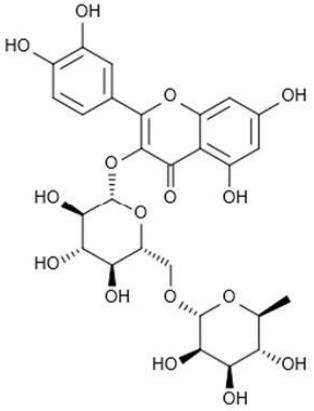
Delphinidin 3-0-glucoside



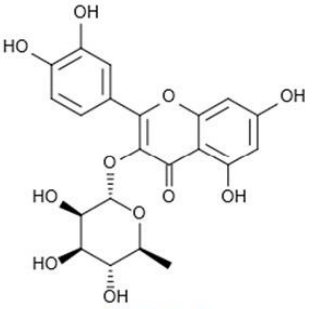
Rutin



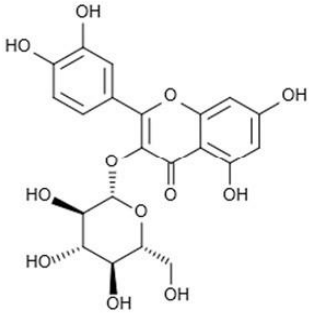
Quercitrin

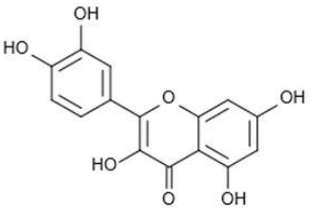


Isoquercitrin

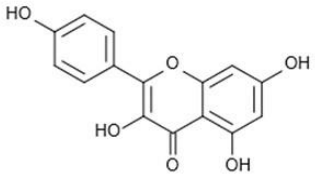


Quercetin

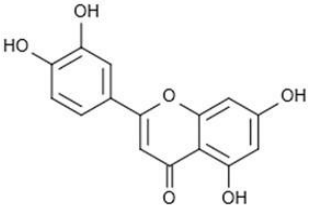




Kaempferol



Luteolin



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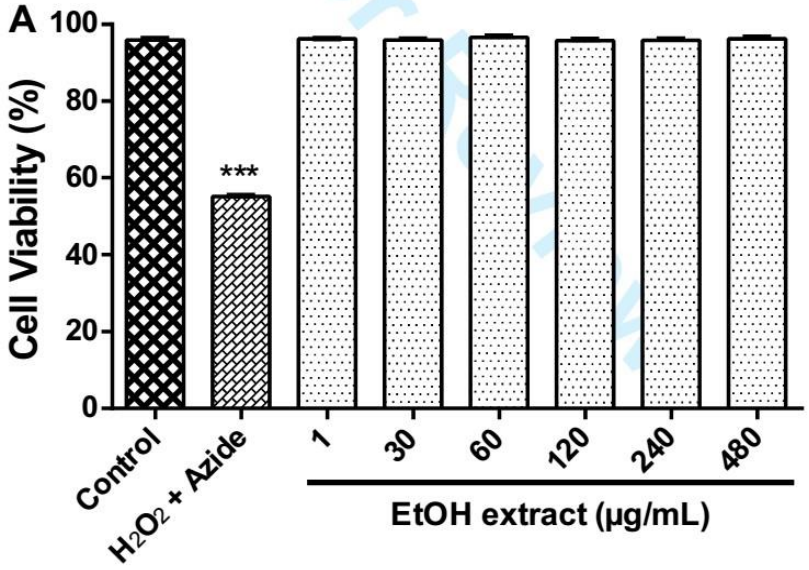
593 **Fig. 3**



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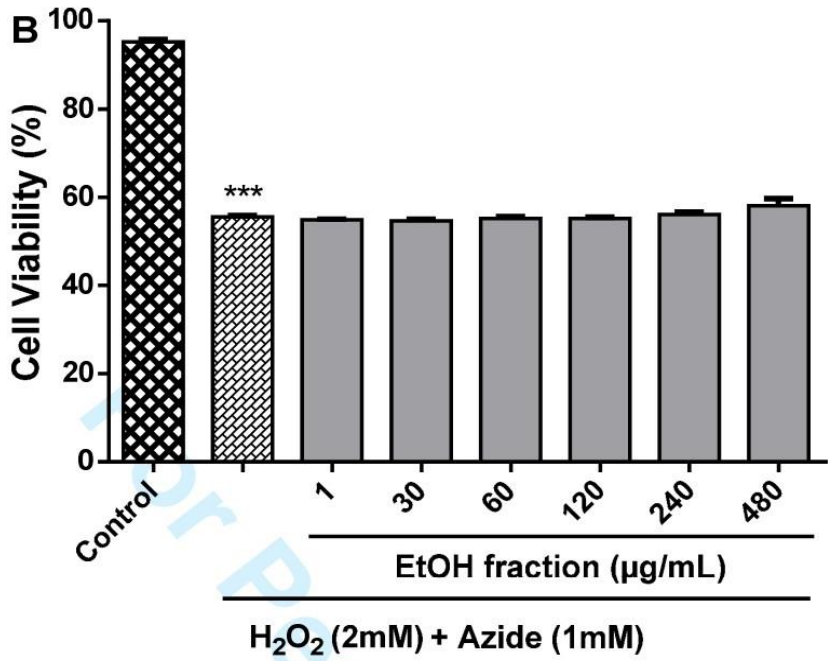

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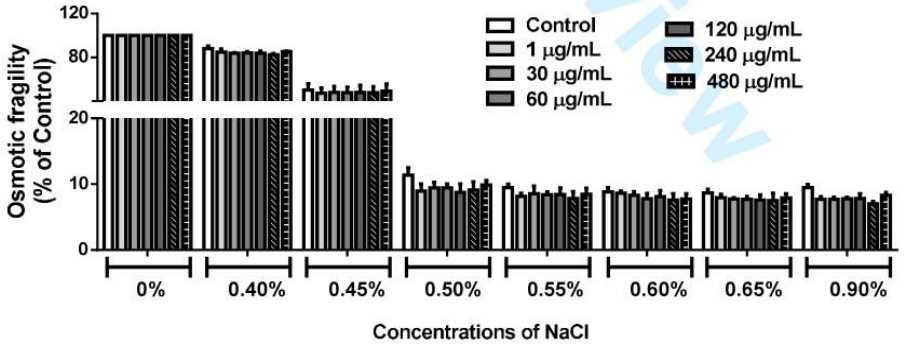
597 **Fig. 4**



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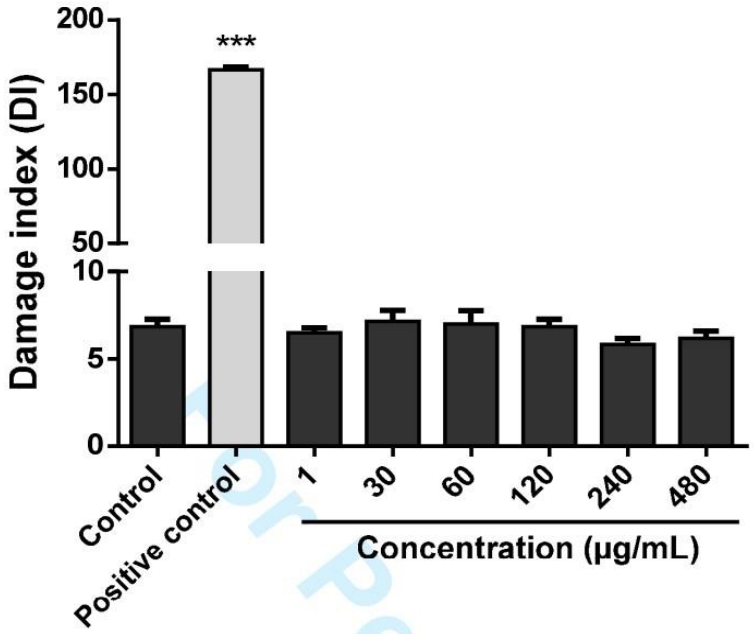
599 **Fig. 5**



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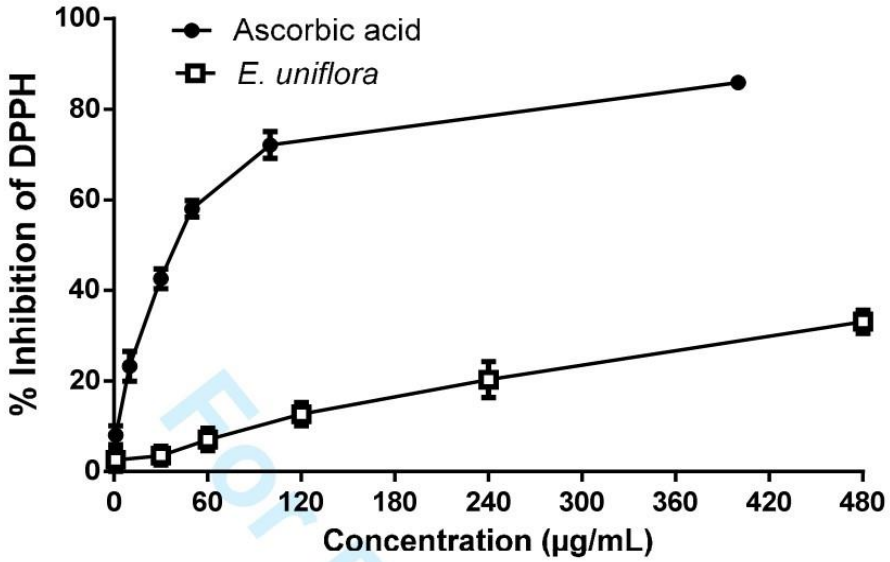
603 **Fig. 6**



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606 **Fig. 7**

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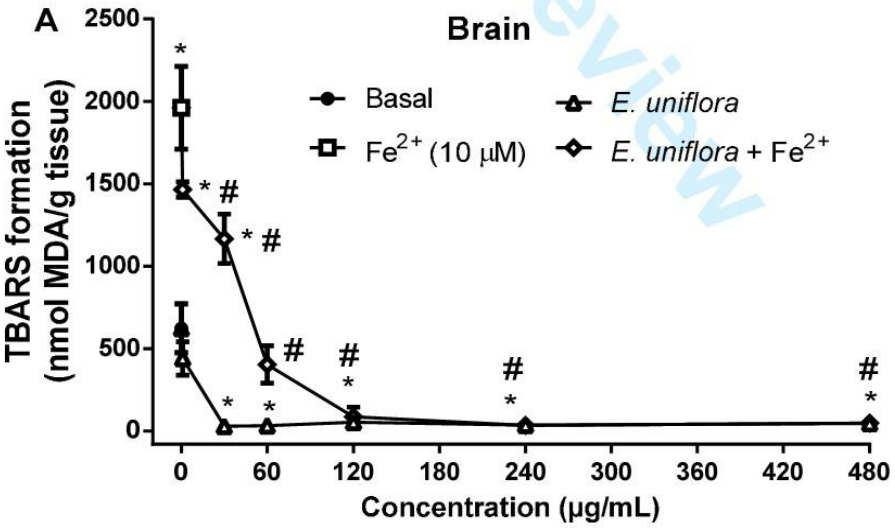

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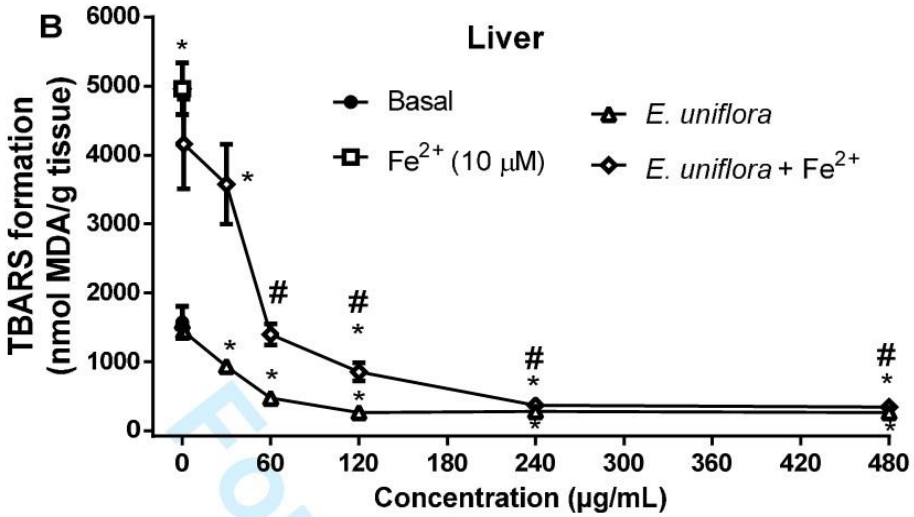

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4. DISCUSSÃO

Considerando o interesse crescente nas propriedades farmacológicas dos compostos derivados de *Eugenia uniflora* (VICTORIA et al., 2012; VICTORIA et al., 2013) e a necessidade de se compreender a sua potencial toxicidade, este trabalho avaliou a toxidade do extrato e do óleo essencial em diferentes modelos

Neste trabalho, o óleo essencial das folhas de *E. uniflora* foi extraído e teve seu perfil fitoquímico analisado por cromatografia gasosa acoplada a espectrometria de massa (BIASI & DESCHAMPS, 2009; MATOS, 2009; ADAMS, 1995). Os constituintes majoritários encontrados foram: curzereno (48,06%), γ-elemeno (13,49%), atractilone (11,78%) e trans-β-elemenone (8,94%) e estão de acordo com achados prévios na literatura (RODRIGUES et al., 2013; CHANG et al., 2011; LAGO, et al., 2011; OGUNWANDE et al., 2005). Outros constituintes majoritários foram encontrados, tais como Germacrone, Germacrene B, Selina-1,3,7(11)-trien-8-one, Selina-1,3,7(11)-trien-8-one epoxide, (*E*)-β-ocimene, trans-β-Ocimene (MAIA et al., 1999; OLIVEIRA et al., 2006; COSTA et al., 2009; COSTA et al., 2010; GALLUCCI et al., 2010; YOSHIDA et al., 2010; COITINHO et al., 2011; TUCKER et al., 2011; SILVA et al., 2012; VICTORIA et al., 2012; GARMUS et. al., 2014).

Os fitoconstituintes de um óleo essencial podem sofrer grandes variações. Estas variações podem estar relacionadas ao seu quimiotipo, parte da planta, idade das folhas, distribuição latitudinal, condições edafoclimáticas, método de extração, autoxidação, metodologia, temperatura, tipo do detector da cromatografia, dentre outras variáveis (SANTOS, 2015; COSTA et al., 2009; MELO et al., 2007). A variação encontrada no perfil fitoquímico deste óleo está de acordo com os achados da literatura.

O extrato etanólico das folhas de *E. uniflora* obtido por concentração em rotaevaporador e teve seu perfil fitoquímico identificado por Cromatografia líquida de alta eficiência – CLAE (BOLIGON et al., 2013). O ácido fenólico majoritário encontrado no extrato foi o: ácido elágico (1,19%) e dentre os flavonóides: quercetina (1,58%) e suas formas glicosiladas – quercitrina (1,34%) e isoquercitrina (1,01%). A luteolina também foi identificada com (1,01%). Estes compostos em baixas concentrações apresentam atividades antioxidantes (MARTINS et al., 2009; PEREIRA, et al., 2013; BARBOSA-FILHO, et al., 2014).

A atividade antioxidante deste extrato foi avaliada pela capacidade de sequestrar o radical DPPH em células sanguíneas humanas. As dosagens do extrato variaram de (1-480 µg⁄mL) apresentando uma atividade antioxidante de forma concentração dependente, mas não superior ao controle, usando como padrão o ácido ascórbico. Objetivando confirmar sua capacidade antioxidante, foi avaliada a peroxidação lipídica pelo método de TBARS em homogeneizados de cérebro e fígado de rato. Esta metodologia consiste em medir o malondialdeido (MDA) como produto final da peroxidação lipídica. Malondialdeído (MDA) é considerado como um dos produtos da peroxidação lipídica (LPO) sendo amplamente usado como um índice de estresse oxidativo (MICHEL et al., 2008).

Nas concentrações de (30-480 µg/mL) o extrato etanólico de *E. uniflora* inibiu significativamente a formação de TBARS no cérebro e fígado de rato. A inibição máxima foi atingida a (30 µg/mL) no cérebro e (120 µg/mL) no fígado. Fe^{2+} (10 mM) estimulou a formação de TBARS quando comparado ao controle e esta foi atenuada pelo uso do extrato. A redução máxima foi atingida com (120 µg/mL) para o cérebro e (240 µg/mL) para o fígado. Estes dados demonstram a capacidade antioxidante do extrato etanólico de *E. uniflora* em níveis basais e reverte a peroxidação lipídica quando induzida por Fe^{2+} (10 mM). Esta atividade antioxidante pode ser atribuída, pelo menos em parte, aos compostos fenólicos deste extrato e estão de acordo com trabalhos que atribuem aos ácidos fenólicos e flavonoides atividade antioxidante (KANDEM, et al., 2013; BARBOSA-FILHO, et al., 2014; MARTINS et al., 2009)

Embora, não tenham sido investigados os compostos isolados responsáveis por esses efeitos, é possível que os fitoconstituintes, incluindo os ácidos fenólicos e flavonoides aqui identificados, tenham de forma sinérgica ou isolada exercido ação antioxidante (KAMDEM et al., 2013). Os compostos fenólicos são considerados capazes de quelar fortemente íons de metais de transição, prevenindo reações em cadeia de radicais livres e danos oxidativos (KARAMAN et al., 2010). O perfil fitoquímico do extrato etanólico de *E. uniflora* revelou a presença de flavonóides e ácidos fenólicos, o que é consistente com os valores obtidos por Lima et al. (2002). No entanto, não só os compostos polifenólicos podem contribuir para a atividade antioxidante (FRANCO et al., 2009). Algumas contribuições também podem vir de outros metabólitos secundários antioxidantes, incluindo vitaminas e carotenóides (JAVANMARDI et al., 2003).

O extrato etanólico das folhas de *E. uniflora* (1-480 µg/mL) não induziu citotoxicidade em leucócitos humanos quando comparado ao controle. No entanto, também não reverteu a citotoxicidade quando induzida por H_2O_2 + azida. Estes resultados estão em consonância com os dados obtidos por Santos et al. (2009). A fragilidade osmótica pode ser afetada por várias causas, provocando várias doenças, (KOLANJIAPPAN et al., 2002). Aqui, a influência do extrato etanólico de *E. uniflora* sobre eritrócitos humanos foi avaliada para verificar a sua segurança. O extrato nas concentrações de (1-480 µg/mL) não causou anormalidade nas membranas de glóbulos vermelhos, quando testada em diferentes concentrações salinas (0-0,9%) de cloreto de sódio. Por não apresentar citotoxidade em células sanguíneas humanas nos modelos e nas altas concentrações testadas, explica em parte, porque não há relatos de toxicidade pelo seu uso na medicina tradicional, mesmo ao ser consumido em doses altas.

O potencial genotóxico do extrato etanólico de *E. uniflora* foi testado em leucócitos humanos pelo Ensaio Cometa. Sob condições alcalinas o Metanossulfonato de metila (MMS), utilizado como controle positivo causou um aumento significativo no índice de dano ao DNA quando comparado com o grupo controle ($p < 0,001$). *E. uniflora* em todas as concentrações testadas (1-480 µg/mL), não induziu danos significativos na cadeia dupla de DNA quando comparada ao controle ($p > 0,05$).

Este estudo demonstrou que o extrato etanólico das folhas de *E. uniflora* não apresenta nem citototoxidade nem genotoxicidade para leucócitos humanos e não afetou eritrócitos humanos através da avaliação de sua fragilidade osmótica, nas metodologias e concentrações testadas. O que pode ser, pelo menos em parte, atribuída a sua atividade antioxidante, como evidenciado pela atividade de eliminação de radicais DPPH e da inibição de TBARS induzida por Fe^{2+} em homogeneizados de cérebro e fígado de ratos.

Compostos derivados de plantas são relatados na literatura como indutores de toxicidade para uma grande quantidade de insetos, podendo interferir diretamente em todos os estágios de desenvolvimento das moscas da fruta e baratas (RAVI & JANANRTHANAN, et al., 2007; MIYAZAWA et al., 1994). Os compostos tais como terpenos, flavonóides, alcalóides, esteroides e saponinas são fitoquímicos importantes quando se considera a atividade inseticida de derivados de plantas (BELO et al., 2009). Além de induzirem toxicidade aguda e mortalidade, terpenóides e flavonóides foram também estudados para determinar sua atividade repelente contra insetos (BELO et al., 2009; NDEMAH & SCHULTHESS, 2002).

Neste estudo, os principais fitocompostos encontrados no óleo essencial de *E. uniflora* foram mono e sesquiterpenos. Uma observação que está parcialmente de acordo com trabalhos reportados anteriormente (RODRIGUES et al., 2013; CHANG et al., 2011; LAGO, et al., 2011; OGUNWANDE et al., 2005). O composto mais abundante foi o curzereno, com aproximadamente 48% da constituição química do óleo. Embora não se tenha feito ensaios para avaliar a toxicidade de cada composto de forma isolada a presença e alta abundância de curzereno, sugere que este fitoconstituinte pode apresentar grande importância na toxicidade do óleo essencial.

As *D. melanogaster* expostas a fumigação do óleo essencial de *E uniflora* apresentaram uma mortalidade cuja LC_{50} foi de (5,56 µg⁄mL). Observou-se também um prejuízo significativo no comportamento da geotaxia negativa das moscas tratadas com baixas concentrações do óleo de *E. uniflora*, o que reflete um déficit locomotor. O efeito do óleo na capacidade locomotora ocorreu em um período de tempo curto, fazendo com que as moscas apresentassem perda de desempenho locomotor num curto espaço de tempo, 3 h de exposição. Compostos tais como os terpenóides e fenilpropanóides podem alterar o sistema de neurotransmissores de insetos, incluindo o sistema dopaminérgico e colinérgico (ENNAN et al., 1998; LOIZZO et al., 2010). Loizzo et al. (2010) demonstrou que muitos terpenos são inibidores da acetilcolinesterase. Em geral, os compostos terpenóides encontrados em *E. uniflora* são sugeridos por estar envolvido na toxicidade do óleo. Estudos estão em curso, a fim de esclarecer o papel dos diferentes compostos encontrados no óleo essencial aqui testado.

Em paralelo com os déficits de sobrevivência e capacidade locomotora induzida nas moscas pela exposição ao óleo essencial de *E. uniflora*, também mostrou sinais de estresse oxidativo, incluindo ERO e formação de TBARS bem como alterações em importantes sistemas de resposta antioxidantes. A resposta celular ao estresse oxidativo é principalmente regulada pelo fator de transcrição Nrf2 (NGUYEN et al., 2009).

D. melanogaster também apresentou sinais de estresse oxidativo, incluindo formação de ERO em 3 h de exposição o que se manteve em 6 e 12 h. Apresentando aumento nos níveis de TBARS após 6 e 12 h de exposição. Um aumento na atividade da enzima GST às 6 e 12 h e da SOD às 12 h foram significantes. Apresentou, também, um aumento significativo na expressão de NQO-1 a 3 h de exposição. O nível da proteína HSP70 apresentou um aumento significativo as 12 h. Os níveis do fator de transcrição Nrf2 permaneceram inalterados.

Glutationa S-transferase – GST é uma importante enzima antioxidante envolvida na fase II do sistema de detoxificação (SAU et al., 2010). Pertence a uma família de enzimas que catalisam a conjugação de GSH a várias outras moléculas e desempenham um papel nos mecanismos de detoxificação intracelular de compostos xenobióticos (CHELVANAYAGAM et al., 2001; WALTERS et al., 2009). O aumento observado da atividade de GST em *Drosophila melanogaster* exposta ao óleo de *E. uniflora* pode estar relacionada com uma resposta adaptativa relacionada com a eliminação de derivados de plantas tóxicas (AGIANIAN et al., 2003; YEI et al., 2001). Singh e colaboradores (2000), demonstraram que compostos naturais são capazes de aumentar a expressão de GST que, em conjunto com GSH endógena favorece a eliminação de metabólitos pela planta (SINGH et al., 2000).

A enzima Superóxido Dismutase - SOD exerce um papel crucial na depuração do radical superóxido bem como para a defesa celular contra o estresse oxidativo. Os resultados deste trabalho demonstraram um aumento significativo da atividade da SOD em moscas tratadas com o óleo essencial. Isto coincidiu com um aumento na produção de ERO. O aumento observado em GST e a atividade de SOD por exposição ao óleo de *E. uniflora* em moscas da fruta pode ser explicada por uma potencial ativação da via de sinalização do Nrf2. De fato, uma ativação precoce desta via de sinalização foi observada em moscas expostos ao óleo essencial, por meio do aumento da expressão do NQO-1, a qual está bem documentada como um dos principais alvos do fator de transcrição Nrf2 (NGUYEN, et al., 2003; JAISWAL, 2004).

Corroborando com os resultados discutido acima, descobriu-se que o óleo essencial de *E. uniflora* aumenta a susceptibilidade de moscas ao desafio oxidativo com os pró-oxidantes Fe^{2+} e PQ. O Paraquat é um herbicida de largo espectro, não seletivo, amplamente utilizado com importância toxicológica para a saúde humana e animal (UVERSKY, 2004). Ele também tem sido utilizado como um indutor de modelos animais de Parkinsonismo devido a suas semelhanças estruturais com íons de 1-metil-4-fenilpiridinium (MPP^{+}), que é frequentemente utilizado para induzir um modelo de doença de Parkinson em diversos modelos, incluindo *Drosophila* (CHAUDHURI, et al., 2007). O estresse oxidativo é relatado como o principal mecanismo de toxicidade induzida por PQ em *Drosophila* (HOSAMANI & MURALIDHARA, 2013).

A exposição a níveis elevados de Fe^{2+}, também é relatada por aumentar o estresse oxidativo em moscas (BONILLA-RAMIREZ et al., 2011) À luz destes resultados e

evidências da literatura, pode-se supor que o aumento da susceptibilidade das moscas ao óleo de *E. uniflora* em co-exposição com o Fe^{2+} e PQ está relacionado com a presença de compostos pró-oxidantes presentes na constituição do óleo essencial ou a um efeito sinérgico entre os fitoconstituintes do óleo em co-exposição com o Paraquat e ferro.

Os mecanismos moleculares pelos quais a exposição ao óleo essencial de *E. uniflora* induz estresse oxidativo ainda precisam de mais esclarecimentos. Apesar de não ter abordado esta questão no presente estudo, relatos na literatura têm sido publicados para apoiar estes achados. Normalmente, os compostos naturais são estudados por sua capacidade antioxidante. Contudo, dependendo da concentração, e outros fatores, uma capacidade pró oxidante poderá ser observada (AWAD, et al., 2002). Por exemplo, Martins e colaboradores (2009) observou um aumento da peroxidação lipídica induzida por doses elevadas de quercetina, indicando um efeito pró-oxidante deste composto natural. Sendo que a quercetina em baixas concentrações possui atividade antioxidante. Foi também demonstrado que compostos naturais podem induzir ou inibir a formação do radical hidroxil via reação de Fenton (PUPPO, 1992). Além disso, as interferências com a função mitocondrial, também têm sido mostradas como um mecanismo envolvido na toxicidade induzida por compostos naturais (SALEH et al., 2014; ROGALSKA et al., 2013). Aparentemente, o destino anti ou pró-oxidante dos compostos naturais depende de múltiplos fatores, dentre os quais: classe fitoquímica, concentração e do modelo estudado (MARTINS et al., 2009; ROGALSKA, et al., 2013; WATJEN et al., 2005).

Os efeitos benéficos dos extratos e óleo essencial de *E. uniflora* estão bem documentados na literatura (WEYERSTHAL, et al., 1988; VICTORIA et al., 2013). No entanto, pouco se sabe sobre sua potencial toxicidade. Este trabalho demonstra que num curto período de exposição e em baixas concentrações, o óleo essencial de *E. uniflora* é capaz de induzir mortalidade e deficiências locomotoras em *Drosophila*. Como mecanismo de toxicidade observada, sugere-se que uma condição pró-oxidante foi estabelecida após as moscas entrarem em contato com os compostos voláteis. Tal efeito é confirmado pelo aumento na produção de espécies reativas e de acumulação de produtos finais da peroxidação lipídica. Além disso, uma resposta ao estresse oxidativo foi aparente nas moscas expostas por fumigação ao óleo, tendo sido possível observar uma ativação da sinalização antioxidante, via aumento da atividade de enzimas antioxidante.

5. CONCLUSÃO

- ✓ O extrato etanólico apresentou os seguintes constituintes majoritários: ácido elágico, quercetina, quercitrina, isoquercitrina e luteolina;

- ✓ O óleo essencial teve como constituintes majoritários: preponderantemente o curzereno, seguido de γ-elemeno, atractilone e trans-β-elemenone;

- ✓ A atividade antioxidante do extrato medida pelo método do DPPH demonstrou uma atividade concentração dependente. Quando medida pelo método TBARS demonstrou atividade antioxidante significativa. Já o óleo essencial apresentou atividade pró-oxidante para *D. melanogaster* pelo método de TBARS;

- ✓ O extrato etanólico não apresentou citotoxidade, nem genotoxicidade em células sanguíneas humanas. O óleo essencial apresentou toxidade em modelo de *D. melanogaster*;

- ✓ As moscas apresentaram um aumento na produção de ROS. Tióis protéicos - PSH e Tióis não protéicos – NPSH permaneceram inalterados nas leituras. Quanto a atividade das enzimas: GST e SOD diferiu significativamente do controle e a CAT não apresentou atividade significante;

- ✓ Ao se analisar a sinalização celular, houve um aumento significativo na expressão de NQO-1 a 3 h de exposição. Os níveis de proteína HSP70 apresentaram um aumento significativo as 12 h. Os níveis do fator de transcrição Nrf2 permaneceram inalteradas;

- ✓ O óleo essencial aumentou a toxicidade em torno de 100% da mortalidade das moscas co-expostas ao (PQ) e ao (Fe^{2+}). A tendência se manteve sobre o comportamento locomotor, onde o óleo essencial concomitante com o (PQ) ou (Fe^{2+}) causou maior mortalidade e dano ao aparelho locomotor, quando comparado com o PQ e Fe^{2+} isolados.

Embora os dados ainda sejam preliminares, estudos de co-exposição do óleo essencial com outros biocidas, estão em curso. Visando a diminuir as concentrações de agrotóxicos, o que poderá resultar em menor toxicidade para seres humanos, organismos não alvos e resíduos tóxicos para o meio ambiente.

Em resumo, estes dados sugerem que o extrato não apresentou toxicidade para células sanguíneas humanas e o óleo essencial possui toxicidade contra *D. melanogaster* e aumenta a toxicidade do Paraquat e Fe^{2+}.

6. **PERSPECTIVAS**

Neste trabalho, alguns dos resultados são promissores e suscitam a realização de novos estudos:

- ✓ Os resultados do aumento da mortalidade e do dano ao aparelho locomotor de *D. melanogaster* quando em co-exposição com Paraquat e Fe^{2+}, ensejam a investigação do efeito modulatório deste óleo sobre biocidas;

- ✓ Novos testes com os fitoconstituintes isolados do óleo essencial, para investigar se a toxicidade se deve a algum constituinte isolado ou a ação sinérgica destes;

- ✓ Os testes de citotoxidade e genotoxicidade do extrato etanólico foram realizados *in vitro*, novos estudos utilizando modelos *in vivo* devem ser realizados visando a se determinar a toxicidade desta planta;

- ✓ A toxicidade da planta foi avaliada com seu extrato etanólico. No entanto, novos estudos utilizando frações dos extratos e os compostos fenólicos isolados podem ser testados, tanto em modelos *in vitro* como em modelos *in vivo*.

7. OUTROS TRABALHOS PUBLICADOS E ACEITOS DURANTE O DOUTORADO

Como primeiro autor

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Artigo Aceito em 11.11.2015 no periódico: **Pharmaceutical Biology.**

Como co-autor

SOBRAL-SOUZA, CELESTINA E.; LEITE, NADGHIA F.; **Cunha, Francisco A.B.**; PINHO, ANTONIO I.; ALBUQUERQUE, ROSIMEIRE S.; CARNEIRO, JOARA, N.P.; MENEZES, IRWIN R.A.; Costa, José G.M.; FRANCO, JEFERSON L. Coutinho, Henrique D.M. Cytoprotective effect against mercury chloride and bioinsecticidal activity of *Eugenia jambolana* Lam.. **ARAB J CHEM**, v. 7, p. 165-170, 2013.

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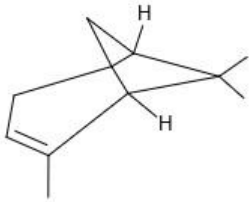
ANEXOS

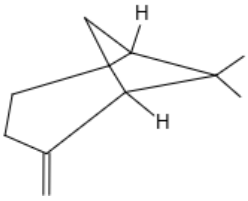
ANEXO 1

Estruturas Químicas dos fitoconstituintes do óleo essencial identificados por Cromatografia Gasosa acoplada a Espectrometria de Massa GC-MS e Cromatografia Gasosa acoplada a Detector por Ionização de Chama GC-FID.

α-Pinene



β-Pinene



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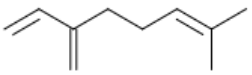
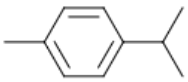


p-Cymene



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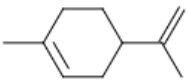
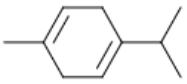


γ-Terpinene



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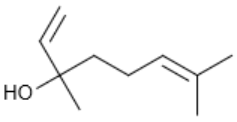
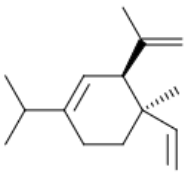
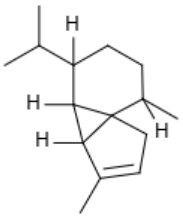


δ-Elemene



α-Cubebene



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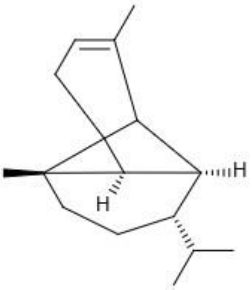

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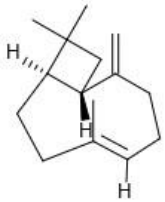
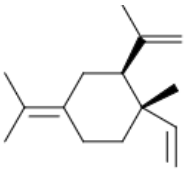
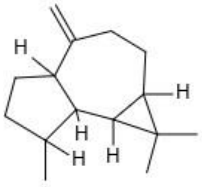


γ-Elemene



Aromadendrene



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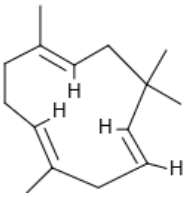
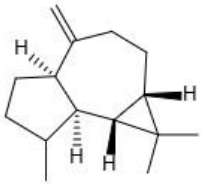
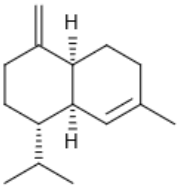


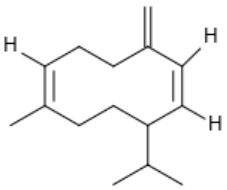
Alloaromadendrene



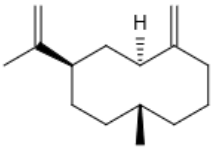
γ-Muurolene



Germacrene D



β-Selinene



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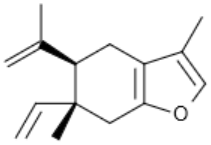

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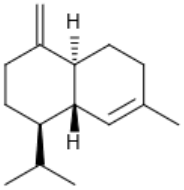

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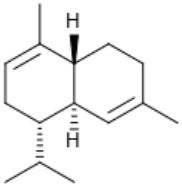
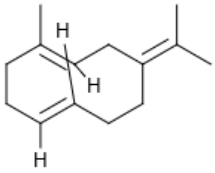
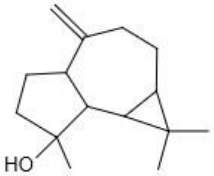


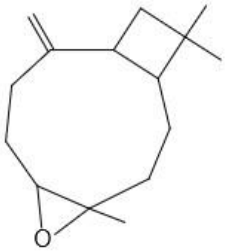
Germacrene B



Spathulenol



Caryophyllene oxide



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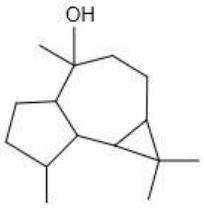
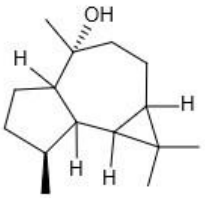


Viridiflorol



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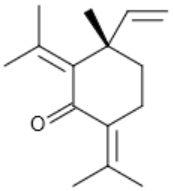

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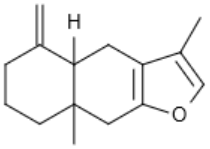

Germacrone