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Leticia Priscilla Arantes

Caenorhabditis elegans COMO MODELO EXPERIMENTAL PARA ESTUDOS TOXICOLÓGICOS E FARMACOLÓGICOS DOS EXTRATOS DE *Luehea divaricata* e *Paullinia cupana*

Santa Maria, RS
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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 a obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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Aprovado em 16 de março de 2016:

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Santa Maria, RS
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Se quiser realmente buscar a verdade, é preciso que pelo menos uma vez em sua vida você duvide, ao máximo que puder, de todas as coisas.

(René Descartes)

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas envolvidos.

A metodologia realizada e os resultados obtidos neste trabalho estão apresentados sob a forma de um artigo e dois manuscritos redigidos em inglês, os quais se encontram no item ARTIGOS CIENTÍFICOS. Nesses, constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas.

O item DISCUSSÃO apresenta interpretações e comentários gerais sobre os trabalhos científicos aqui incluídos.

Os itens CONCLUSÕES e PERSPECTIVAS apresentam conclusões gerais sobre os resultados apresentados e as perspectivas para futuros trabalhos.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem nos itens INTRODUÇÃO e DISCUSSÃO.

RESUMO

Caenorhabditis elegans COMO MODELO EXPERIMENTAL PARA ESTUDOS TOXICOLÓGICOS E FARMACOLÓGICOS DOS EXTRATOS DE *Luehea divaricata* e *Paullinia cupana*

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O nematódeo *Caenorhabditis elegans* é uma valiosa ferramenta para estudos toxicológicos e farmacológicos. Apesar de seu corpo simples, a maior parte de seus genes e de suas vias de sinalização são similares às de humanos. Extratos de plantas podem ser úteis na Medicina, entretanto, poucas espécies foram estudadas. Assim, neste trabalho, foram investigados os possíveis efeitos tóxicos e farmacológicos do extrato hidroalcoólico das folhas de *Luehea divaricata* sobre o sistema nervoso e os possíveis efeitos do extrato hidroalcoólico das sementes de *Paullinia cupana* sobre o envelhecimento e a toxicidade induzida por metilmercúrio (MeHg) utilizando *C. elegans*. O extrato de *L. divaricata* demonstrou atividade antioxidante sobre diferentes prooxidantes *in vitro* e em *C. elegans* de maneira distinta. *C. elegans* permite estudos de atividade antioxidante em um organismo inteiro, em uma situação biológica de estresse oxidativo. Esse extrato também aumentou a taxa de batimentos faríngeos dos nematódeos através de atividade anticolinesterásica e demonstrou maior potencial farmacológico que seu principal constituinte (rutina), provavelmente devido a efeitos sinérgicos de seus constituintes. O extrato de *P. cupana* estendeu o tempo de vida e o tempo de vida saudável nos nematódeos através de efeito antioxidante e da ativação das vias de sinalização DAF-16/FOXO e HSF-1, envolvidas no aumento da expressão de genes relacionados à longevidade e à resistência ao estresse. O efeito foi acompanhado de uma redução no acúmulo de lipofuscina intestinal e no número de agregados proteicos. Demonstrou-se ainda que o sistema purinérgico pode ser um novo alvo terapêutico para a modulação do envelhecimento. O extrato de *P. cupana* também atenuou os efeitos tóxicos causados por MeHg aumentando a sobrevivência e diminuindo os distúrbios no desenvolvimento larval e no comportamento dos nematódeos da cepa *skn-1(ok2315)*, deficiente na enzima glutationa-S-transferase e consequentemente mais sensível ao estresse oxidativo. Esse efeito se deve, menos em parte, pelo aumento da expressão de genes relacionados ao transporte (*aat-2*) e detoxificação (*mtl-1* e *mtl-2*) do metal e à atividade antioxidante (*sir 2.1* e *sod-3*), resultando em reparos celulares mais rápidos e eficientes. *C. elegans* possibilitou análises simples dos mecanismos envolvidos nos efeitos dos extratos e direcionou pesquisas futuras.

Palavras-chave: açoita-cavalo, envelhecimento, estresse oxidativo, guaraná, mercúrio, neuroproteção

ABSTRACT

Caenorhabditis elegans AS EXPERIMENTAL MODEL FOR TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES OF *Luehea divaricata* AND *Paullinia cupana* EXTRACTS

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ADVISOR: Félix Alexandre Antunes Soares

The nematode *Caenorhabditis elegans* is a valuable tool for toxicological and pharmacological studies. Despite its simple body, the most part of its genes and signaling pathways are similar to humans, and alterations in behaviors may be related to specif neuronal circuits Plant extracts might be useful in Medicine, however few species were studied. Thus, Herein, toxicological and pharmacological effects of the ethanolic extract of *Luehea divaricata* and the potencial effects of the ethanolic extract of *Paullinia cupana* seeds on aging and methylmercury (MeHg)-induced toxicity were investigated in *C. elegans*. *L. divaricata* extract showed antioxidant activity against different prooxidants *in vitro* and in *C. elegans* in a different manner. *C. elegans allows* antioxidant studies in a whole organism with a biological situation of oxidative stress. *L. divaricata* extract also increased nematode pharynx pumping rate through anticholinesterasic activity and showed a higher pharmacological potencial when compared to its isolated compound (rutin), probably due to synergic effects of its compounds. *P. cupana* extract extended nematode's lifespan and healthspan in a DAF-16/FOXO and HSF-1 dependent manner, signaling pathways involved in upregulation of genes associated to longevity and stress resistance. This effect was accompanied by a reduction in intestinal lipofuscin and in the number of protein aggregates. Genes modulated by GEE included *gst-4*, *hsf-1* and *skn-1*. Furthermore, this study demonstrated that purinergic signaling might be a therapeutic target to modulate aging. *P. cupana* extract also exerted neuroprotective effects against MeHg, increasing worm survival and decreasing behavior impairments in *skn-1(ok2315)* strain, which is deficient in detoxification and oxidative stress response. The extract conducted *skn-1(ok2315)* worms to a faster recover and progress in development, at least in part, through upregulation of genes related to metal transport *(aat-2)* and detoxification *(mtl-1* e *mtl-2*), and antioxidant response (*sir-2.1* and *sod-3*), resulting in faster and more efficient cellular repairs. *C. elegans* allowed simple analyzes of the mechanisms involved in extract's effects and directed further studies.

Keywords: açoita-cavalo, aging, ,guaraná, mercury, neuroprotection, oxidative stress

SUMÁRIO

1 INTRODUÇÃO

1.1 *Caenorhabditis elegans* COMO MODELO EXPERIMENTAL

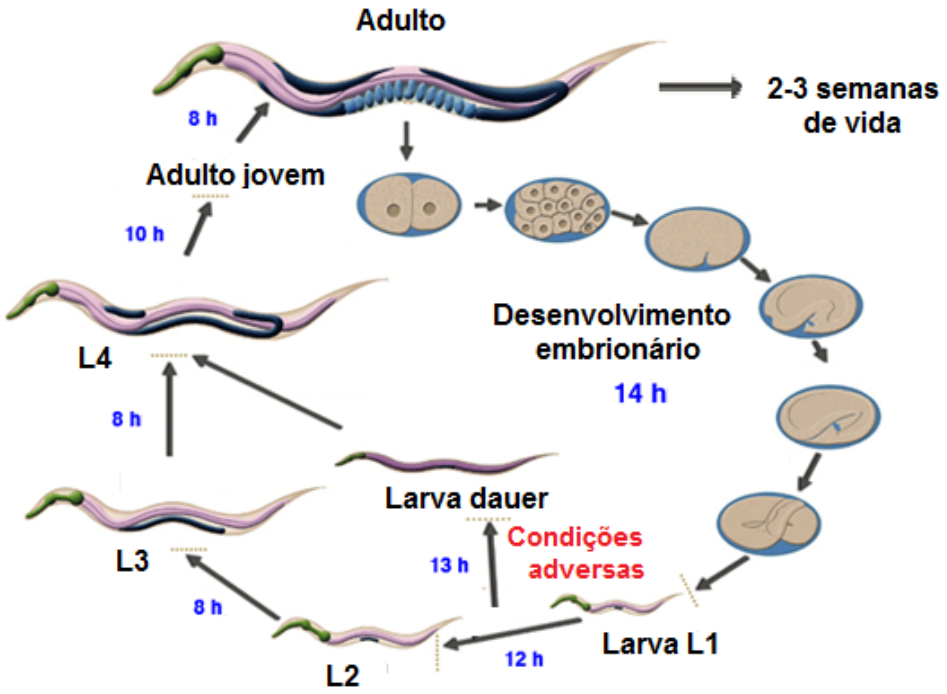
Caenorhabditis elegans é um nematódeo do solo, cilíndrico, não segmentado, não parasita, não patogênico e mede aproximadamente 1 mm de comprimento e 80 μm de diâmetro quando adulto (Figura 1) (Brenner, 1974; Riddle *et al.*, 1997). Possui células musculares, hipoderme, cutícula, sistema nervoso, intestino, gônadas e sistema excretor (Artal-Sanz *et al.*, 2006). A maioria dos nematódeos são hermafroditas que se auto fecundam, poucos são machos (~0,2%), e produzem um grande número de progênie (300 a 1000 ovos por animal) (Brenner, 1974; Riddle *et al.*, 1997).

Após a eclosão do ovo, os nematódeos passam por quatro estágios larvais (L1, L2, L3 e L4), pelo estágio adulto e adulto com ovos e a partir daí vivem em média 2 a 3 semanas (se mantidos a 20°C) (Figura 2) (Gruber *et al.*, 2009; Nelson e Raizen, 2013). O desenvolvimento é dependente da temperatura, variando de aproximadamente 2 dias a 25°C à 6 dias a 15°C de ovo até adulto (Corsi *et al.*, 2015). Em casos de aglomeração, falta de alimento ou outras condições adversas, o desenvolvimento é interrompido no final da segunda fase larval, com a formação da larva dauer, e pode ser retomado quando o ambiente se tornar favorável (Golden e Riddle, 1984).

Figura 1 – O nematódeo *Caenorhabditis elegans*

Adulto com ovos ao centro e duas larvas no estágio L4. À esquerda, alguns ovos.
Fonte: http://www.yourgenome.org/stories/sequencing-the-worm

Figura 2 - Ciclo de vida de *C. elegans* a 22°C



Fonte: http://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html (adaptado)

C. elegans é transparente, o que permite o emprego de técnicas não invasivas de visualização das estruturas celulares e de transcritos marcados com proteínas fluorescentes, mesmo que expressos em uma única célula. Dessa forma, é possível o acompanhamento de processos biológicos como embriogênese, metabolismo lipídico, interação proteica e degeneração de neurônios no animal vivo (Chalfie *et al.*, 1994; Boulin *et al.*, 2006; Corsi *et al.*, 2015).

Esse nematódeo possui 302 neurônios com sinapses que utilizam os mesmos neurotransmissores presentes em mamíferos: acetilcolina, ácido gama amino butírico, glutamato, dopamina, serotonina e numerosos neuropeptídeos, e foi o primeiro animal a ter seu sistema nervoso completamente mapeado (White *et al.*, 1986; Bargmann, 1998; Hobert, 2005). *C. elegans* sente a presença de diferentes substâncias e odorantes e as alterações de temperatura, do nível de oxigênio e da densidade de outros nematódeos, por exemplo (Bargmann, 2006; Hart e Chao, 2010). Uma vez que seu sistema nervoso é bem caracterizado, alterações em comportamentos específicos podem ser atribuídas a circuitos neuronais e genes específicos, direcionando investigações posteriores (White *et al.*, 1976). Análises

comportamentais podem ser facilmente realizadas com o auxílio de microscópio ou estereomicroscópio (Riddle *et al.*, 1997; Corsi *et al.*, 2015).

As similaridades entre os processos celulares e moleculares presentes em *C. elegans* e em mamíferos fazem desse nematódeo um excelente organismo para a pesquisa (Artal-Sanz *et al.*, 2006; Leung *et al.*, 2008). *C. elegans* foi introduzido como modelo experimental pelo biólogo Sydney Brenner em 1965 para o estudo do desenvolvimento celular e da neurobiologia (Brenner, 1974). O trabalho foi reconhecido com o prêmio Nobel em Fisiologia ou Medicina em 2002 e consistiu na compreensão da regulação genética do desenvolvimento dos órgãos e da apoptose (Brenner, 2003). O nematódeo foi também posteriormente utilizado em mais duas pesquisas reconhecidas: Andrew Fire e Craig C. Mello receberam o prêmio Nobel em Fisiologia ou Medicina em 2006 pelo descobrimento do RNA interferente em *C. elegans*, permitindo o silenciamento gênico (Fire, 2007; Mello, 2007), e Martin Chalfie, Osamu Shimomura e Roger Y. Tsien receberam o prêmio Nobel em Química em 2008 pela descoberta e desenvolvimento da proteína verde fluorescente (GFP), um marcador biológico, em *C. elegans* (Chalfie *et al.*, 1994).

C. elegans foi o primeiro entre os organismos multicelulares a ter o genoma completamente mapeado, em 1998 (*C. elegans* Genome Consortium, 1998). Estima-se que 60 a 80% dos genes humanos possuam um ortólogo no genoma de *C. elegans* e, ainda, que a maioria dos genes e das vias de sinalização envolvidos em doenças humanas estejam presentes nesse nematódeo (Culetto e Sattelle, 2000; Kaletta e Hengartner, 2006). Atualmente, *C. elegans* é uma poderosa ferramenta para diferentes estudos, incluindo aqueles relacionados à apoptose, à sinalização celular, ao estresse oxidativo, à regulação gênica, ao metabolismo, ao envelhecimento, à toxicologia e à modulação de doenças humanas (Riddle *et al.*, 1997; Murakami, 2007; Leung *et al.*, 2008; Zheng e Greenway, 2012; Corsi *et al.*, 2015).

Apesar de alguns aspectos do envelhecimento humano não estarem presentes em *C. elegans*, como o risco de desenvolvimento de doenças relacionadas à idade, alguns processos biológicos em comum podem ser observados, como dano oxidativo em macromoléculas (Harman, 1956), declínio da função mitocondrial (Harman, 1972), declínio nos movimentos resultantes de sarcopenia (Herndon *et al.*, 2002) e acúmulo de pigmentos, como a lipofuscina (Brunk e Terman, 2002). Ademais, embora não desenvolvam doenças relacionadas à idade, diversos nematódeos transgênicos já estão disponíveis para o estudo de doenças como Alzheimer e Parkinson, e modelos através de indução química também já foram descritos (Link, 2001; Braungart *et al.*, 2004).

Devido ao conhecimento detalhado sobre a arquitetura genômica e à facilidade nas manipulações genéticas, diversas cepas *knockout* e transgênicas foram produzidas em laboratório e também estão disponíveis para aquisição através do *Caenorhabditis Genetics Center* (CGC, Universidade de Minnesota, EUA) (Fraser *et al.*, 2000; Kamath *et al.*, 2001; Thompson *et al.*, 2013). Isso permite que análises genéticas simples e de baixo custo sejam realizadas e, como o verme predominantemente se auto fecunda, as mutações gênicas são mantidas através das gerações (Corsi *et al.*, 2015).

O pequeno tamanho, a simplicidade e o baixo custo facilitam o cultivo de *C. elegans* em laboratório (Brenner, 1974). Em condições controladas, o nematódeo é mantido em meio sólido (ágar) ou líquido, com *Escherichia coli* como fonte de alimento, geralmente a 20°C. Os animais podem também ser armazenados congelados a -80°C no estágio L1-L2 com posterior recuperação (Riddle *et al.*, 1997; Corsi *et al.*, 2015).

C. elegans é também um excelente modelo para ser utilizado em triagens de alto rendimento de substâncias através de processos automatizados (*High Throughput Screening*) devido a várias vantagens, principalmente: 1) é pequeno, pode ser economicamente cultivado em microplacas; 2) pode ser cultivado e manuseado em líquido, facilitando a automação; 3) pode ser usado como modelo de doenças humanas complexas que não podem ser reproduzidas *in vitro* ou em modelos unicelulares; 4) permite avaliar o efeito farmacológico e toxicológico de substâncias simultaneamente; 5) possui diversos fenótipos relacionados a circuitos neuronais, a genes ou a vias de sinalizacão específicos; 6) é um organismo inteiro e multicelular, aumentando a chance de identificação de substâncias eficazes em organismos multicelulares mais complexos, como humanos e 7) possui várias ferramentas genéticas e recursos genômicos disponíveis, como livrarias de RNA interferente (Leung *et al.*, 2011; O'reilly *et al.*, 2014). Alguns equipamentos, *softwares* e detectores sensíveis já estão disponíveis e outros estão em desenvolvimento (Burns *et al.*, 2006; Leung *et al.*, 2011; Likitlersuang *et al.*, 2012).

Não se espera que *C. elegans*, um sistema invertebrado, forneça total confiabilidade sobre a segurança e a eficácia de substâncias em humanos, do mesmo modo que modelos mamíferos também não fornecem. Todos modelos animais possuem limitações em consequência das diferenças anatômicas, metabólicas e genéticas próprias de cada espécie (Lin, 2008; Brehm *et al.*, 2013; Prussing *et al.*, 2013; Seth *et al.*, 2013). *C. elegans* está distante evolucionariamente dos humanos, não possui órgãos, não desenvolve patologias humanas e difere muito na farmacocinética. A presença de cutícula dificulta a absorção de substâncias, fazendo-se necessário o emprego de doses altas nos ensaios. Elas podem ainda

ser metabolizadas pela *E.coli* utilizada como alimento pelo nematódeo, afetando o crescimento microbiano e/ou causando efeitos secundários no animal. Portanto, os efeitos ou a ausência deles devem ser ponderados, uma vez que podem ser resultado de problemas na biodisponibilidade das substâncias, como a forma e o tempo de exposição. Ainda, por ser pequeno, análises bioquímicas requerem um grande número de animais (Johnson, 2003; Gruber *et al.*, 2009).

Entretanto, *C. elegans* possui processos bioquímicos e moleculares similares aos de humanos e, dentre os modelos animais, ele é o mais rápido, mais barato e mais propício a tecnologias eficazes automatizadas para a análise de um grande número de compostos em pesquisas preliminares (Burns *et al.*, 2006; Leung *et al.*, 2011; Likitlersuang *et al.*, 2012; O'reilly *et al.*, 2014). Dessa forma, a utilização desse nematódeo diminui os procedimentos laboriosos e o tempo gasto em análises e fornece resultados confiáveis para pesquisas posteriores (O'reilly *et al.*, 2014). Esse modelo se insere, portanto, no princípio dos 3Rs (reduction/redução, replacement/substituição e refinement/refinamento) especificados na "Diretriz Brasileira para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos" do Conselho Nacional de Controle de Experimentação Animal (Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos, 2013).

Não há custos relacionados à criação e à manutenção de biotérios, não há necessidade de cuidados a fim de preservar o bem-estar dos animais e não há tempo despendido até o fornecimento dos mesmos. O desenvolvimento dos nematódeos é rápido, um grande número de progênie é gerado e as cepas precisam ser adquiridas apenas uma vez, podendo ser mantidas indefinidamente (Riddle *et al.*, 1997; Corsi *et al.*, 2015). É possível então, analisar com rapidez e com baixo custo um grande número de substâncias, incluindo extratos vegetais, quanto à toxicologia e/ou ao possível efeito farmacológico e investigar moléculas que alterem a expressão de um gene específico, por exemplo.

1.2 UTILIZAÇÃO DE EXTRATOS VEGETAIS NA MEDICINA

Distúrbios em processos bioquímicos específicos que originam diversas doenças em particular ainda não são bem compreendidos (Bonomini et al. 2015; Farina et al. 2013; Halliwell 1994; Torgovnick et al. 2013). Entretanto, o estresse oxidativo tem sido implicado como a causa principal de danos celulares em uma diversidade de patologias, incluindo desordens metabólicas, doenças associadas à idade, doenças neurodegenerativas e câncer, por exemplo (Halliwell, 1994; Bonomini *et al.*, 2015).

A formação de espécies reativas de oxigênio (EROs) é necessária para a função celular normal (Finkel, 2011). Entretanto, o aumento de fatores oxidantes no organismo (alterações genéticas, contaminantes ambientais e xenobióticos, por exemplo) ou a diminuição de fatores antioxidantes (diminuição na produção de enzimas antioxidantes e carência de nutrientes, por exemplo) geram quantidades excessivas de EROs e podem conduzir ao estresse oxidativo (Halliwell, 1994; John *et al.*, 2002; Balk Rde *et al.*, 2010; Chin-Chan *et al.*, 2015). Esse é caracterizado por danos irreversíveis a componentes celulares, incluindo proteínas, lipídios, DNA e RNA, que originam diversas patologias (Halliwell, 1994). O acúmulo de danos em macromoléculas parece estar relacionado também às alterações progressivas degenerativas nas funções do organismo que caracterizam o envelhecimento de todas as espécies (Harman, 1956; Collins *et al.*, 2008).

Postula-se, portanto, que antioxidantes possam prevenir o surgimento ou auxiliar no tratamento de doenças e retardar a morte (Chatzianagnostou *et al.*, 2015). Extratos vegetais, no geral, possuem ampla atividade antioxidante, atribuída principalmente à presença de flavonoides e outros polifenóis, além de outras atividades farmacológicas (Pereira *et al.*, 2009; Bonomo Lde *et al.*, 2014). Além disso, extratos vegetais possuem uma diversidade de componentes com atividades biológicas distintas (Clardy e Walsh, 2004; Koehn e Carter, 2005; Cragg e Newman, 2013). O uso de plantas na prevenção e no tratamento de doenças tem origem milenar. Os primeiros documentos escritos reportando o emprego de produtos naturais na medicina datam de 2600 a.C. e mostram um sistema medicinal sofisticado na Mesopotâmia, incluindo aproximadamente 1000 compostos medicinais derivados de plantas (Borchardt, 2002; Cragg e Newman, 2013; Atanasov *et al.*, 2015).

Durante muito tempo, as plantas medicinais foram aplicadas tradicionalmente, sem o conhecimento de seus mecanismos de ação farmacológica e de seus constituintes ativos. A descoberta racional de medicamentos a partir de plantas aconteceu somente no início do século XIX, com o isolamento da morfina, de ação analgésica e indutora do sono, a partir do ópio (*Papaver somniferum*), o que impulsionou a análise de outras plantas medicinais nos anos seguintes (Cragg e Newman, 2013; Atanasov *et al.*, 2015). Exemplos de importantes descobertas incluem a quinina, isolada das espécies *Cinchona*, com efeito antimalárico, e o ácido salicílico, isolado das espécies *Salix*, com efeito analgésico e antipirético (Butler, 2004; Cragg e Newman, 2013).

Apesar dos avanços nos processos de síntese de substâncias em laboratório, os produtos naturais e seus derivados representam, atualmente, mais da metade dos compostos aprovados pela FDA (Food and Drug Administration), sendo que grande parte destes são

originários de plantas (Harvey, 2008; Kinghorn *et al.*, 2011; Cragg e Newman, 2013). Dessa forma, ainda hoje, extratos vegetais representam fonte importante para a descoberta de novos medicamentos (Harvey, 2008; Cragg e Newman, 2013). Dentre os produtos obtidos ou derivados de plantas que foram aprovados pela FDA nos últimos anos, pode-se citar a galantamina, para tratamento da Doença de Alzheimer; a colchicina para o tratamento da gota e a capsaicina, para tratamento da dor neuropática, por exemplo (Kinghorn *et al.*, 2011).

O interesse em extratos vegetais para o desenvolvimento de novos medicamentos pode ser justificado pela complexidade e pela diversidade estrutural de seus componentes. Outro fator importante é que como seus constituintes são produzidos por organismos vivos, eles possuem propriedades otimizadas pela evolução para obter diferentes funções específicas (Molinari, 2009). Além disso, produtos naturais demonstram menos efeitos colaterais e muitos já foram utilizados na medicina popular, sugerindo efeitos farmacológicos em potencial (Butler, 2004; Atanasov *et al.*, 2015).

Dados da literatura mostram que as propriedades farmacológicas de extratos brutos de plantas podem ser perdidas quando componentes específicos são isolados. Isto indica que grande parte destas propriedades podem estar relacionadas a efeitos sinérgicos de diferentes compostos. Assim, extratos brutos podem oferecer maiores vantagens em relação a compostos isolados, uma vez que apresentam melhor atividade farmacológica, oferecem um menor custo, possuem toxicidade mais baixa e são a forma mais utilizada tradicionalmente pela população (Carlini, 2003; Pietrovski *et al.*, 2006; Pereira *et al.*, 2009).

Apesar da comprovada eficiência terapêutica de muitos extratos vegetais, possíveis efeitos tóxicos são muitas vezes ignorados, uma vez que, tradicionalmente, é disseminado que o uso de produtos naturais é somente benéfico à saúde. Alguns efeitos tóxicos podem estar relacionados à dose, a variações genéticas na população ou a condições patológicas pré existentes, por exemplo (Zhou *et al.*, 2013; Chedea *et al.*, 2014; De Oliveira e De Aguiar, 2015). De acordo com dados do Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX), no ano de 2012, foram registrados 1185 casos de intoxicação humana e 113 casos de intoxicacão animal por plantas no Brasil (Ministério da Saúde, Fiocruz, SINITOX).

Diversas pesquisas tem se direcionado ao estudo da flora terrestre. Entretanto, é estimado que apenas 6% das espécies de plantas superiores (estimadas em 300 a 500 mil espécies) tenham sido analisadas farmacologicamente e apenas 15%, fitoquimicamente (Fabricant e Farnsworth, 2001; Cragg e Newman, 2013). Infelizmente, devido a mudanças climáticas e a fatores antropogênicos, o número global de espécies vegetais tem diminuído,

colocando em risco novos medicamentos em potencial derivados da natureza (Sen e Samanta, 2015).

Luehea divaricata Mart., é um exemplo de espécie vegetal ainda pouco estudada. Pertencente à família *Tiliaceae*, essa árvore de grande porte é natural do Brasil, Argentina e Paraguai. Também apresenta as sinonímias de *Luehea speciosa* Wild., *Brotera mediterranea* Vell. e *Luehea parvifolia* Mart. No Brasil, pode ser encontrada em diversos estados, desde o Rio Grande do Norte até o Rio Grande do Sul e é popularmente conhecida como açoita-cavalo (Alice *et a*l., 1995; Lorenzi, 1998). As folhas são usadas tradicionalmente para tratar disenteria, leucorréia, gonorréia, reumatismo e tumores; a infusão das flores é usada contra bronquite; o caule em feridas de pele e a raiz é depurativa (Tanaka *et al.,* 2003; Tanaka *et al.,* 2005).

A análise fitoquímica das folhas de *L. divaricata*, mostrou a presença de flavonoides, taninos catéquicos, saponinas e mucilagem, em menor quantidade alcalóides, óleos fixos, antocianidinas, carotenóides e polissacarídeos (Bortoluzzi *et al*., 2002) e a ausência de antraquinonas livres, antracenosídeos, taninos gálicos, cumarinas e óleos voláteis (Alice *et al*., 1995). O estudo químico do extrato bruto metanólico das folhas revelou a presença de ácido 3b-*p*-hidroxibenzoil tormêntico, ácido maslínico, vitexina e glicopiranosilsitosterol (Tanaka *et al*., 2005).

Poucos trabalhos estão disponíveis na literatura sobre o potencial farmacológico dessa planta. Foi relatada uma atividade genotóxica do extrato aquososo das folhas de *L. divaricata* no teste de Ames (*Salmonella*/microssomo) com ativação microssomal (Vargas *et al.,* 1991) e efeito citostático sem seletividade e efeito citocida seletivo do extrato metanólico das folhas em diferentes linhagens de células tumorais (Tanaka *et al*., 2005). Entretanto, em um estudo de toxicidade com administração oral do bruto hidroalcoólico de *L. divaricata* em camundongos, não foram observados sinais de toxicidade (Bighetti *et al*., 2004).

Outra espécie ainda pouco estudada é *Paullinia cupana*, o guaraná, planta originária da região amazônica, principalmente do Brasil (Krewer Cda *et al.*, 2011). O pó de suas sementes é ingerido habitualmente pela população local (Schimpl *et al.*, 2013) e é composto principalmente por metilxantinas: cafeína, teobromina e teofilina (Costa, 1972). O extrato das sementes possui algumas funções descritas, como redução do peso corporal (Andersen e Fogh, 2001; Boozer *et al.*, 2001), estimulação do sistema nervoso central (Espinola *et al.*, 1997), melhora da memória (Espinola *et al.*, 1997), redução na síntese de tromboxanos nas plaquetas (Bydlowski *et al.*, 1991), proteção contra lesões gástricas induzidas por etanol (Campos *et al.*, 2003) e atividade antioxidante *in vitro* (Mattei *et al.*, 1998). Alguns estudos

também mostraram ação protetora contra cádmio em testículos de ratos adultos (Leite *et al.*, 2011; Leite *et al.*, 2013) e contra rotenona em uma linhagem de células dopaminérgicas de neuroblastoma (De Oliveira *et al.*, 2011).

Além disso, o consumo habitual de guaraná tem sido associado à uma extensão na longevidade da população da cidade de Maués (Amazonas, Brasil), uma das principais regiões produtoras e consumidoras desse fruto (Ribeiro *et al*., 2012). De acordo com o Instituto Brasileiro de Geografia e Estatística (IBGE), a cidade possui o dobro de idosos acima dos 80 anos que a média nacional. Um estudo epidemiológico associou a ingestão habitual de guaraná ao baixo índice de prevalência de doenças crônicas relacionadas à idade naquela população (Costa Krewer *et al.*, 2011), entretanto não há estudos sobre os efeitos do guaraná no tempo de vida e os possíveis mecanismos envolvidos.

O envelhecimento é inerente a todas as espécies e é caracterizado por alterações degenerativas progressivas nos tecidos e declínio das funções do organismo (Tissenbaum, 2012). Dessa forma, o surgimento de doenças metabólicas, neurodegenerativas e câncer está diretamente relacionado ao avanço da idade (Ames *et al.*, 1981; Bonomini *et al.*, 2015). Compostos que possam melhorar a qualidade de vida durante o envelhecimento ou retardar esse processo têm sido então, o objetivo de muitas pesquisas (Ayyadevara *et al.*, 2013; Torgovnick *et al.*, 2013; Carretero *et al.*, 2015).

O consumo habitual de guaraná também foi associado a efeitos protetores em relação à toxicidade do mercúrio (Hg), um metal pesado sem funções biológicas no organismo (Farina *et al.*, 2013). A ocorrência desse metal na Amazônia é alta no solo e na água como resultado de processos geológicos e da mineração (Wasserman *et al.*, 2003) e a maior fonte de exposição do Hg às pessoas é o consumo de peixes contaminados com metilmercúrio (MeHg) (Clarkson, 2002; Clarkson e Magos, 2006).

O MeHg é mais tóxico que a forma inorgânica do metal. É formado através da metilação do mercúrio inorgânico causada principalmente por microorganismos aquáticos (Compeau e Bartha, 1985) e sofre bioacumulação e biomagnificação, ou seja, como não é biodegradável, se acumula progressivamente ao longo da cadeia alimentar, gerando efeitos tóxicos principalmente no homem (Hintelmann, 2010). Além disso, o MeHg possui maior taxa de entrada no sistema nervoso central que o Hg, provocando efeitos tóxicos variados, incluindo distúrbios na função motora e cognitiva, dependendo da idade, dose e duração da exposição, podendo levar à morte (Clarkson, 2002; Clarkson e Magos, 2006). Apesar do MeHg possuir efeitos que incluem neurodegeneração dopaminérgica, alterações na homeostase do cálcio, indução de estresse oxidativo e alta afinidade por cisteína, alvos

específicos continuam desconhecidos (Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002; Helmcke *et al.*, 2009; Martinez-Finley e Aschner, 2011).

A concentração de mercúrio em amostras biológicas da população amazônica excede os valores máximos recomendados pela Organização Mundial da Saúde (De Castro e Lima Mde, 2014). Porém, não são observados sinais significativos de toxicidade. Assim, a aparente tolerância à intoxicação pelo mercúrio observada na população nativa, incluindo a influência de fatores nutricionais como potenciais modificadores da toxicidade do mercúrio, incluindo o guaraná, uma vez que é habitualmente consumido (Chapman e Chan, 2000; Schimpl *et al.*, 2013).

Extratos vegetais, incluindo os das espécies *L. divaricata* e *P. cupana*, podem exibir uma grande variedade de efeitos tóxicos e farmacológicos que merecem ser investigados. A compreensão dos mecanismos envolvidos nesses efeitos pode contribuir para a criação de novos e melhores medicamentos ou até para o descobrimento de novos alvos terapêuticos. O sucesso no desenvolvimento de novos tratamentos para distúrbios no organismo depende de metodologias apropriadas e bem escolhidas, que combinem simplicidade, rapidez e baixo custo com boa sensibilidade e reprodutibilidade. Esses fatores estão diretamente relacionados a resultados mais eficazes em ensaios clínicos posteriores (Kola e Landis, 2004; Schuster *et al.*, 2005; Atanasov *et al.*, 2015). *C. elegans* parece se encaixar nesses requisitos e ser um bom modelo para o estudo de extratos vegetais *in vivo*.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar possíveis efeitos toxicológicos e farmacológicos dos extratos de *Luehea divaricata* e *Paullinia* cupana utilizando o nematódeo *Caenorhabditis elegans* como modelo experimental.

2.2 OBJETIVOS ESPECÍFICOS

- Investigar possíveis efeitos tóxicos e farmacológicos do extrato hidroalcoólico das folhas de *Luehea divaricata* sobre o sistema nervoso;

- Investigar possíveis efeitos do extrato hidroalcoólico das sementes de *Paullinia cupana* sobre o envelhecimento e sobre a toxicidade induzida por metilmercúrio.

3 ARTIGOS CIENTÍFICOS

Os resultados deste trabalho estão apresentados sob a forma de um artigo científico e dois manuscritos. Os itens Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas, encontram-se nos próprios artigos. O artigo está disposto na forma que foi publicado na revista “Industrial Products and Crops”. Os manuscritos em preparação estão dispostos na forma em que serão submetidos para publicação.

3.1 ARTIGO

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Luehea divaricata Mart. anticholinesterase and antioxidant activity in a *Caenorhabditis elegans* model system

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ABSTRACT

Oxidative stress has been implicated as a major cause of a number of clinical abnormalities. This effect of oxidative stress on human health has led many research groups to search for new antioxidant compounds. Because there are only small number of studies that have examined the pharmacological effects of *Luehea divaricata*, the antioxidant potential of leaf extract against different pro-oxidants *in vitro* and in the nematode *Caenorhabditis elegans* was investigated. The scavenger activity of the extract was evaluated *in vitro* against $NO^{\bullet}$, $DPPH^{\bullet}$, and H_2O_2. The iron chelating and reducing properties of the extract were determined using the ortho-phenantroline method. In the *in vivo* assays, the nematodes were scored for viability after pretreatment with extract and exposure to juglone, sodium nitroprusside, H_2O_2, or Fe^{2+}. The effects of the extract on behavioral parameters were also evaluated. Although the extract was able to scavenge $NO^{\bullet}$ and $DPPH^{\bullet}$, and exhibited Fe^{3+} reducing power *in vitro*, it was not able to protect deoxyribose against H_2O_2 or to chelate Fe^{2+}. Furthermore, a protective effect of the extract against juglone and Fe^{2+} was demonstrated by a 23% increase in nematode survival *in vivo*. The antioxidant effects depicted *in vitro* were not directly correlated to the results *in vivo*, which may be due to metabolism of the extract or to other effects besides polyphenol scavenger activity. Behavior studies revealed a 13% increase in the pharynx pumping rate in the treated larvae, which was associated with the inhibition of acetylcholinesterase activity. Our study clearly indicated that the *L. divaricata* leaf extract was able to increase the resistance of a whole organism to oxidative stress and to increase cholinergic tonus. Thus, the *L. divaricata* antioxidant and anticholinergic activities that were presented in this study for the first time *in vivo* indicate that the extract or its component substances may be useful in preventing or treating neurodegenerative diseases, such as Alzheimer's disease.

1. Introduction

Reactive oxygen species (ROS) are produced during normal cellular metabolism and by abnormal biological processes that are impacted by endogenous or exogenous factors (Balk et al., 2010; Tyrrell, 2012; Wei et al., 2001). Although normal cellular functions lead to the formation of physiological levels of ROS, an imbalance between the production of reactive species and the antioxidant defense system can lead to oxidative stress that causes damage to cellular components (Halliwell, 2008). Oxidative stress has therefore been implicated as a major cause of cellular injuries associated with the pathophysiology of a plethora of genetic and acquired disorders (Halliwell, 2006, 2012; Wei et al., 2001).

In an attempt to protect against ROS and disease development, research has been directed toward the screening of new antioxidant substances, including those obtained from natural sources (Wagner et al., 2006; Wang et al., 2008). However, due to the wide diversity of species and the cost and duration of research, many products have not been studied. Furthermore, little information is often available concerning the effects of these products on whole organisms.

One example of a natural antioxidant substance is from *Luehea divaricata* Mart. (*Tiliaceae*), which is a plant that grows in South America (Lorenzi, 1998). The leaves of the plant are traditionally used in the treatment of dysentery, leucorrhea, rheumatism, blennorrhoea, and tumors. A phytochemical screening of *L. divaricata* leaves identified the presence of flavonoids, tannins, saponins,

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and mucilage. Smaller quantities of alkaloids, fixed oils, antocianidins, carotenoids, and polysaccharides were also present. However, there have been only a small number of studies that have investigated the pharmacological properties of this species (Felício et al., 2001; Tanaka et al., 2005; Vargas et al., 1991).

Despite the simple body scheme of the nematode *Caenorhabditis elegans*, this species has been shown to share a number of vital biological pathways with mammals. This model has become a valuable animal system for understanding organismal reactions to various forms of environmental and physiological stress. The organism also allows for high-throughput screening of compounds (Boyd et al., 2007). We therefore decided to evaluate the antioxidant potential of the leaf extract of *L. divaricata* against different pro-oxidants *in vitro* and *in vivo* using the animal model *C. elegans*. We also searched for possible pharmacological activities of the extract.

2. Materials and methods

2.1. Plant material and extract preparation

L. divaricata leaves were collected in Santa Maria (Rio Grande do Sul, Brazil) during the flowering period. The taxonomic identification was confirmed by the Department of Industrial Pharmacy at the Federal University of Santa Maria. Leaves were macerated three times with 70% ethanol (EtOH) at room temperature during a 3-week period. The extracts were filtered under vacuum and then concentrated to dryness using a reduced pressure rotary evaporator at 37 °C. The material was dissolved in 70% EtOH to prepare a series of concentrations of the extract.

2.2. Determination of total phenolic content

The total phenolic content was determined by the method of Folin–Ciocalteu using gallic acid as a standard phenol (Singleton et al., 1999). The total phenolic content was calculated as milligrams of gallic acid equivalent per gram of extract.

2.3. Qualitative and quantitative HPLC-DAD analyses

High performance liquid chromatography (HPLC-DAD) was performed with a Prominence Auto-Sampler (SIL-20A) that was equipped with Shimadzu LC-20AT (Shimadzu, Kyoto, Japan) reciprocating pumps that were connected to a DGU-20A5 degasser and a CBM-20A integrator. The UV-VIS detector DAD SPD-M20A and software LC Solution 1.22 SP1 were also used. Reverse phase chromatography was carried out with a Thermal Scientific C-18 column (4.6 mm × 250 mm) packed with 5 μm diameter particles. The injection volume was 40 μl, and the gradient elution was conducted as previously described (Evaristo and Leitão, 2001). UV absorption spectra were recorded in the 230–400 nm range.

The *L. divaricata* leaf extract was screened for the presence of the following polyphenolic compounds: pyrogallic acid, gallic acid, catechin, chlorogenic acid, caffeic acid, coumarin, 4-hydroxycoumarin, rosmarinic acid, quercetin, rutin, chrysin and kaempferol. Identification of the compounds was achieved by comparing their HPLC retention time and UV absorption spectrum with those of commercial standards. Quantification was performed by integrating the peaks using the external standard method at 365 nm for rutin. Chromatographic operations were carried out at ambient temperature and in triplicate.

2.4. In vitro assays

2.4.1. Scavenging activity of NO•

Nitrite content was determined by using the Griess reagent (Green et al., 1981). NO• scavenging activity was calculated as the percentage of free radical inhibition by the sample by using the following equation: % inhibition = $(A_0 - A_{sample})/A_0 \times 100$, where A_0 was the absorbance of the control and A_{sample} was the absorbance in the presence of different extract concentrations.

2.4.2. DPPH• scavenging activity

The DPPH• scavenging assay was performed according to Cho et al. (2002). DPPH• scavenging activity was calculated as the percentage of free radical inhibition by the sample.

2.4.3. Determination of oxidative damage to deoxyribose

The deoxyribose oxidation assay was performed as previously described (Puntel et al., 2005). Oxidation was induced by 500 μM H_2O_2 and 50 μM $FeSO_4$ alone and in combination. The values were calculated as the percentage of control.

2.4.4. Iron chelating and reducing properties

The ortho-phenantroline method was used to examine the iron chelating and reducing properties of the *L. divaricata* extract (Minotti and Aust, 1987). Iron chelation is based on the formation of complexes between Fe^{2+} (150 μM $FeSO_4$) and the samples. Iron reducing activity was based on the power of the samples to reduce free Fe^{3+} (150 μM $FeCl_3$). The results were compared in absorbance values.

2.5. In vivo assays

2.5.1. C. elegans strain and growth conditions

A wild-type *C. elegans* strain was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C as previously described (Brenner, 1974). Nematodes and bacteria were provided by the *C. elegans* Genetics Center (CGC) at the University of Minnesota (Minneapolis, USA). Gravid hermaphrodites were washed off the plates and lysed with a bleaching mixture (0.45 mol/L NaOH and 2% HOCl) to obtain a synchronous population (Brenner, 1974).

2.5.2. Acute oxidative stress exposure protocol

To perform acute oxidative stress studies, young adult nematodes were exposed to a series of concentrations of the following pro-oxidants: juglone, fresh sodium nitroprusside (SNP), H_2O_2 and Fe^{2+} in M9 solution or a 0.5% NaCl solution (for Fe^{2+}). Juglone was prepared in EtOH (1% final concentration), and the other reagents were dissolved in water. After 1 h at 20 °C, approximately 100 nematodes per treatment were examined for viability using a microscope. Animals that reacted to a mechanical stimulus were scored as alive, and non-responding animals were considered to be dead. The assay was repeated at least four times. The concentration of compound that killed approximately 50% of the nematodes (LD_{50}) was determined for use in further analyses.

2.5.3. Assessment of resistance to oxidative stress

Nematodes at the young adult stage were incubated with the *L. divaricata* leaf extract (100, 200, 400 and 800 μg/ml) or with vehicle (0.7% EtOH) in M9 medium (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl and 1 mM $MgSO_4$) without bacteria for 1 h at 20 °C. The nematodes were washed three times and then exposed to the estimated LD_{50} of the different pro-oxidants. After 1 h at 20 °C, 100 nematodes per treatment were scored for viability using a microscope. The assay was repeated four times.

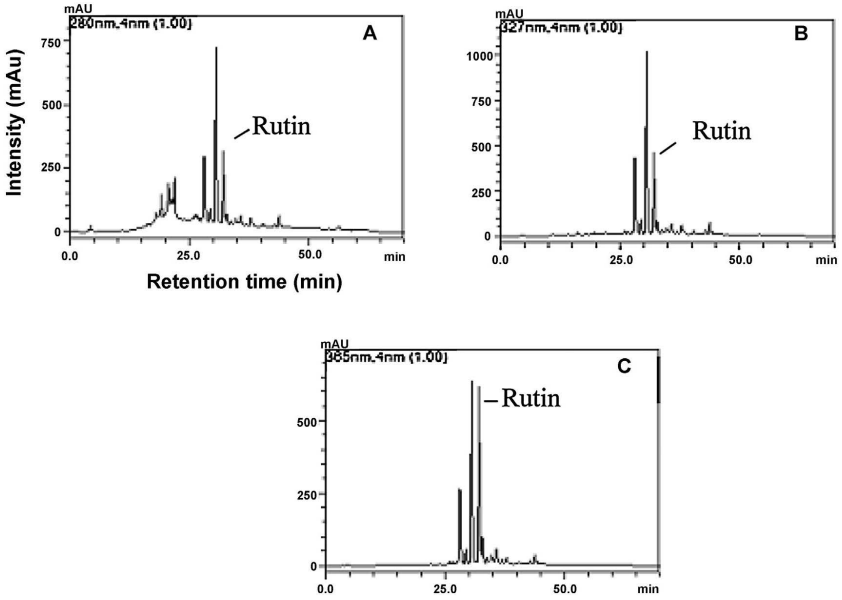

Fig. 1. HPLC analysis of the *L. divaricata* leaf extract reveals the presence of rutin (retention time ~32.10 min), and two major unidentified peaks. Similar results were obtained in three independent experiments. Chromatograms are shown in 280 (A), 327 (B) and 365 nm (C). Two unidentified peaks were also detected in 28.06 and 30.52 min. Similar results were obtained in three independent experiments.

2.5.4. Effects of the L. divaricata leaf extract on C. elegans behavior

To analyze the effects of the *L. divaricata* leaf extract on *C. elegans* behavior, nematodes exposed to the extract were washed with M9 solution and then transferred to agar plates seeded with *E. coli* OP50. The animals were observed, and ten nematodes per treatment were analyzed for the following behavior parameters: body bending, defecation cycle length and pharyngeal pumping rate (Huang et al., 2004; Migliori et al., 2011; Thomas and Lockery, 1999). The assays were repeated three times.

2.5.5. Acetylcholinesterase activity assay

Control and extract-treated nematodes were sonicated in M9 solution on ice and then centrifuged. The supernatants (lysates) were collected and used for acetylcholinesterase activity measurements as previously described (Cole et al., 2004). The protein contents of the nematode homogenates were determined using the Bradford method (Bradford, 1976).

2.6. Statistical analyses

All data are presented as the mean ± SD. A one-way ANOVA that was followed by a Newman–Keuls multiple comparison test was used to evaluate the differences among the concentrations of the extract. A two-way ANOVA that was followed by a Bonferroni post-hoc test was used to compare the results of *L. divaricata* and the standard compound rutin. Results were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Total phenolic content and HPLC analysis

The total phenolic content of the *L. divaricata* leaf extract was determined to be equivalent to 647.4 ± 0.005 mg of gallic acid per g of extract. HPLC analysis allowed the identification of rutin at a concentration of 15.0575 ± 0.41 mg per g of extract (Figs. 1A–C and 2C).

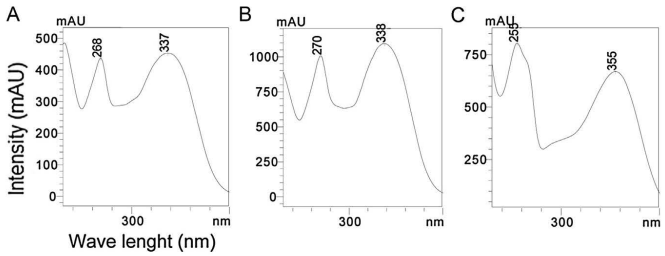

Fig. 2. UV absorption spectrum (230–400 nm) of the three major peaks detected in *L. divaricata* leaf extract. Unidentified peaks in 28.06 (A) and 30.52 min (B), and rutin peak in 32.10 min of retention (C).

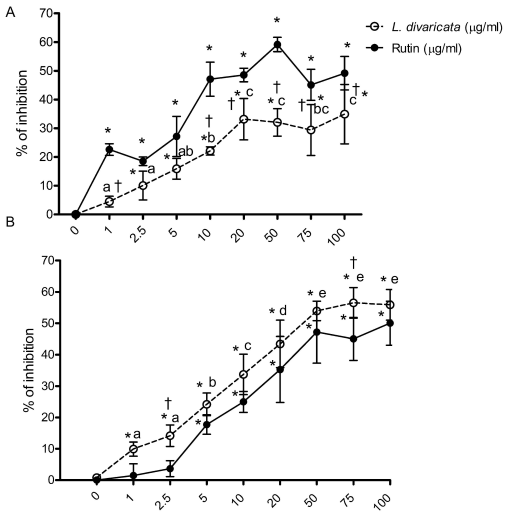

Fig. 3. The scavenging activity of the *L. divaricata* leaf extract on the NO• (A) and DPPH• radicals (B). Results are expressed as the percent inhibition relative to a control sample (±SD). The mean control value is 21.07 ± 0.089 μM of nitrite (A) and 0.7859 ± 0.0228 absorbance (B). (a–e) indicate significant differences among the different concentrations and (*) indicates a significant difference from the control sample using a one-way ANOVA that was followed by the Newman–Keuls Multiple Comparison Test ($p < 0.05$). (†) indicates a significant difference between the *L. divaricata* leaf extract and rutin at the same concentrations by a two-way ANOVA that was followed by Bonferroni post-tests ($p < 0.05$). The vehicle was not different from the control treatment in all of the experiments and is not shown. All experiments were performed in duplicate ($n = 4$).

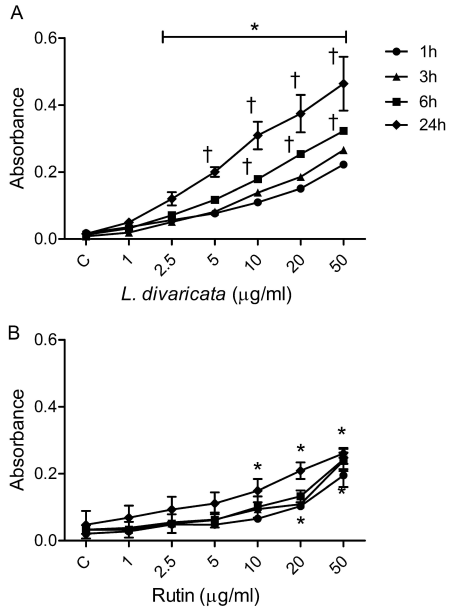

Fig. 4. The reducing power of (A) the *L. divaricata* leaf extract and (B) rutin on Fe^{3+}. Reduction was expressed as absorbance ±SD at different time points: 1 (●); 3 (▲); 6 (■) and 24 h (♦). The mean control value is 0.0137 ± 0.0024. (*) indicates a significant difference from the control treatment by a one-way ANOVA that was followed by a Newman–Keuls multiple comparison test ($p < 0.05$). (†) indicates significant differences among the same concentrations of the *L. divaricata* leaf extract and rutin by a two-way ANOVA that was followed by Bonferroni post-tests ($p < 0.05$). The vehicle was not different from the control treatment in all of the experiments and is not shown. All experiments were performed in duplicate ($n = 4$).

Rutin was therefore used as a standard compound for comparison in all of the assays. Two unidentified peaks were also detected (Fig. 2A and B). However, pyrogallic acid, gallic acid, catechin, chlorogenic acid, caffeic acid, coumarin, 4-hydroxycoumarin, rosmarinic acid, quercetin, chrysin and kaempferol were not identified in the extract using this assay.

3.2. In vitro assays

3.2.1. Free radical scavenging activity

The inhibitory effect of the *L. divaricata* leaf extract on NO• radicals was concentration-dependent, started at 5 μg/ml (15.86% inhibition, $p < 0.05$) and showed a maximum inhibition of 33.14% at 20 μg/ml ($p < 0.05$, Fig. 3A). Rutin had a greater effect with a 27% inhibition at 5 μg/ml and a maximum inhibition of 59% at 50 μg/ml ($p < 0.05$, Fig. 3A).

The extract also significantly reduced the levels of the DPPH• radical in a concentration dependent manner ($p < 0.05$, Fig. 2B). The extract showed an inhibition of 9.92% at 1 μg/ml and a maximum 53.93% inhibition at 50 μg/ml. Rutin showed an inhibition of 1.47% at 1 μg/ml, which was not significantly different than the control, and it reached a maximum inhibition of 47.22% at 50 μg/ml. *L. divaricata* and rutin had similar scavenging activities on the DPPH• radical.

3.2.2. Inhibition of deoxyribose oxidation

The oxidant species H_2O_2 and Fe^{2+} alone and together through induction of the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^•$) were able to stimulate the oxidative degradation of deoxyribose and to increase malondialdehyde (MDA) formation (approximately 140, 400 and 550% of control, respectively). However, the effects of the *L. divaricata* leaf extract and rutin at concentrations of 1, 2.5, 5, and 10 μg/ml were not significantly different from the control (data not shown). Thus, the extract did not protect against deoxyribose degradation induced by any oxidant species that was tested.

3.2.3. Iron chelating and reducing properties

Fig. 4A shows the reducing power of the *L. divaricata* leaf extract in a concentration- and time-dependent manner, which was determined to be significant in a two-way ANOVA [$F = 12.04$, $p < 0.001$]. Moreover, a one-way ANOVA showed a significant effect of the extract beginning at a concentration of 2.5 μg/ml ($p < 0.05$) after one hour of incubation. Fig. 4B shows the reducing power of rutin. The effects of time and concentration were not significant as demonstrated by a two-way ANOVA followed by a Bonferroni post-test [$F = 0.81$, $p = 0.6718$]. A one-way ANOVA showed a significant effect of rutin only at 20 μg/ml ($p < 0.05$) after one hour of incubation. A two-way ANOVA also showed that the extract possesses a more powerful reducing property than does rutin. Neither the extract nor rutin showed significant chelating effects on Fe^{2+} (data not shown).

3.3. In vivo assays

3.3.1. Assessment of resistance to different pro-oxidants

Table 1 shows the survival of the *C. elegans* young adults after a 1 h exposure to different concentrations of the following pro-oxidants: juglone (100 μM), fresh SNP (50 mM), H_2O_2 (100 mM) and Fe^{2+} (200 mM). The concentration of the compound that killed 50% of nematodes was used in other assays to test the

Table 1
Survival of *C. elegans* young adults after 1 h of exposure to different concentrations of pro-oxidants.

Pro-oxidant	Concentration	Percentage of nematodes alive (mean ± SD)
Juglone	50 μM	94.73 ± 3.09
	100 μM	49.48 ± 12.03
	150 μM	30.46 ± 7.27
	200 μM	20.08 ± 3.57
SNP	25 mM	87.57 ± 6.01
	50 mM	47.02 ± 9.76
	100 mM	15.29 ± 0.53
	150 mM	0.90 ± 1.05
H_2O_2	50 mM	79.75 ± 9.27
	100 mM	54.18 ± 8.79
	150 mM	39.31 ± 14.16
	200 mM	27.77 ± 16.52
Fe^{2+}	50 mM	80.28 ± 6.94
	100 mM	72.18 ± 6.63
	200 mM	56.18 ± 4.53
	400 mM	32.64 ± 6.79

$n = 4$ independent experiments, approximately 100 nematodes scored per group in each experiment.

antioxidant activity of the *L. divaricata* leaf extract *in vivo* and to compare the effect to rutin. Pretreatment with extract increased nematode survival after juglone and Fe^{2+} exposures by 22.45 and 23%, respectively, in comparison to the treatment in the absence of the *L. divaricata* leaf extract. This effect was similar to rutin (Fig. 5A–C). Rutin increased nematode survival by 40% after SNP exposure. In contrast, the *L. divaricata* leaf extract had no effect on SNP exposure (Fig. 5B). Neither the *L. divaricata* leaf extract nor rutin were able to protect the nematodes against H_2O_2 at the concentrations that were tested here (data not shown).

3.3.2. Effects of the *L. divaricata* leaf extract on *C. elegans*

After exposure to the extract or rutin, several *C. elegans* behavioral parameters were analyzed. Body bending and defecation cycle length were not altered when compared to the control treatment (data not shown). In contrast, the *L. divaricata* leaf extract at the concentrations of 400 and 800 μg/ml and rutin at all tested concentrations increased the pharynx pumping rate when compared to the control treatment (Fig. 6A, $p < 0.05$). Because acetylcholine is involved in the control of this behavior in *C. elegans*, acetylcholinesterase activity was tested in the treated nematodes. The *L. divaricata* leaf extract caused a 28 and 43% inhibition of the enzyme at 100 and 800 μg/ml, respectively. Rutin inhibited the enzyme by approximately 53% at both concentrations (Fig. 5B, $p < 0.05$).

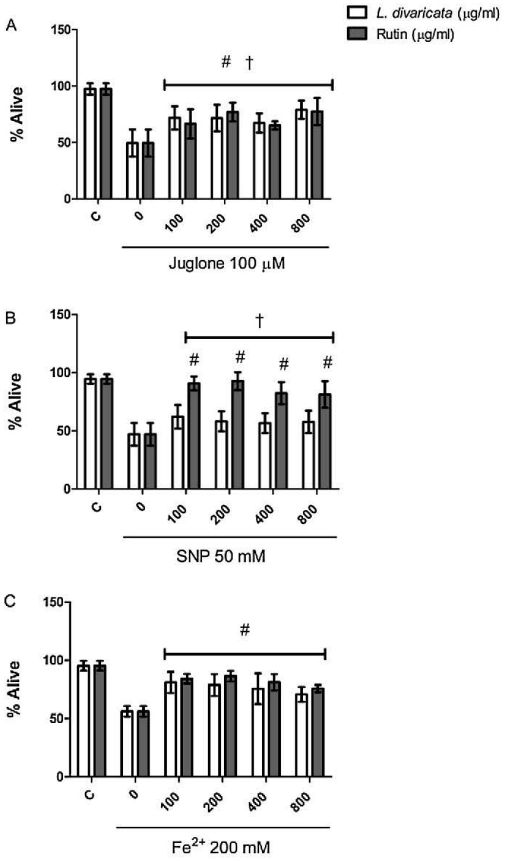

Fig. 5. Effects of the *L. divaricata* leaf extract and rutin 1 h pretreatment on the wild-type *C. elegans* L4-larval stage exposed for 1 h to the following oxidative stressors: (A) 100 μM juglone, (B) 50 mM SNP and (C) 200 mM Fe^{2+}. Values are displayed as the mean ± SD ($n = 4$ independent experiments, with approximately 100 nematodes were scored per group in each experiment). (#) indicates a significant difference from the induced group by a one-way ANOVA that was followed by a Newman–Keuls Multiple Comparison Test ($p < 0.05$). The *L. divaricata* group was compared to rutin by a two-way ANOVA that was followed by Bonferroni post-tests, and (†) indicates a significant difference ($p < 0.05$). The vehicle was not different from the control treatment in all experiments and is not shown.

4. Discussion

Epidemiological studies have shown an inverse association between the daily consumption of natural products and the risk of degenerative and chronic diseases. These effects have been attributed to antioxidant compounds, such as polyphenols, in the natural products (John et al., 2002). These findings have directed considerable attention toward the identification of natural substances that can be used for therapeutic purposes. In this study, we investigated the antioxidant activity of *L. divaricata* against several pro-oxidants that are implicated in various disorders.

The *L. divaricata* leaf extract that was used in this study showed a high content of phenolic compounds. HPLC analysis identified the presence of rutin, which is a flavonoid with the potential to function as an antioxidant (Alía et al., 2006; Kamalakkannan and Prince, 2006). *In vitro*, the *L. divaricata* leaf extract effectively reduced the amount of nitrite that reacted with the Griess reagent, which indicated the presence of NO• scavenger activity. In addition, the extract exhibited a significant concentration-dependent scavenger activity against the DPPH• radical. Hydrogen donation to the free radical and its reduction to non-reactive species are mechanisms by which the extract can remove the free radical (Wang et al., 2008). The extract did not show significant Fe^{2+}-chelating activity and did not protect deoxyribose from Fe^{2+}- and H_2O_2-induced oxidation or hydroxyl radicals generated by the Fenton reaction at the concentrations that were tested here. However, the extract was able to reduce Fe^{3+} to Fe^{2+}. This property may be due to the presence of reducing compounds besides rutin that exert antioxidant activities through the formation of inactive Fe^{2+}–polyphenol complexes that cannot react with oxygen and/or can scavenge reactive oxygen species (Alía et al., 2006; Yoshino and Murakami, 1998).

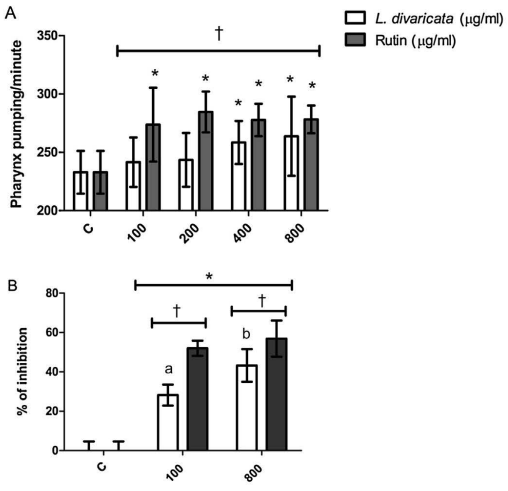

Fig. 6. (A) Pharynx pumping rate and (B) acetylcholinesterase activity of the wild-type *C. elegans* adults after a 1 h of treatment with the *L. divaricata* leaf extract or with rutin. Values are displayed as the mean ± SD ($n = 3$ independent experiments, with 10 nematodes scored per group in the behavior assay). (*) indicates a significant difference from the control treatment and (a–b) indicate significant differences among the different concentrations, which was determined by a one-way ANOVA that was followed by a Newman–Keuls Multiple Comparison Test ($p < 0.05$). The *L. divaricata* leaf extract group was compared to the rutin treatment by using a two-way ANOVA that was followed by Bonferroni post-tests, and (†) indicates a significant difference ($p < 0.05$). The vehicle was not different from the control treatment in all of the experiments and is not shown.

Nevertheless, the antioxidant effects of the substances *in vitro* are not directly correlated to their effectiveness *in vivo* (Halliwell, 2008). We therefore used several pro-oxidants, including juglone, fresh SNP, H_2O_2 and Fe^{2+}, to evaluate the potential antioxidant effects of the *L. divaricata* extract in the nematode *C. elegans*. Juglone is reduced by enzymes and results in the formation of a semiquinone radical. These semiquinones can then react with molecular oxygen to generate O_2^- and H_2O_2 (Inbaraj and Chignell, 2004). SNP is a pro-oxidant agent that acts through the release of NO•. Furthermore, ferricyanide anions $[(CN)_5Fe]^{3-}$ can react with H_2O_2 *via* the Fenton reaction to generate hydroxyl radicals (Rauhala et al., 1998). Our results clearly demonstrate that the *L. divaricata* leaf extract protected the *C. elegans* young adults against some of the pro-oxidant agents. These findings indicate that the extract was ingested or absorbed through the cuticle of the nematodes from the medium and most likely exerted its pharmacological effects at low concentrations *in vivo*.

In contrast to the results obtained in the *in vitro* studies, the *L. divaricata* extract improved nematode survival in response to juglone and Fe^{2+} but not in response to SNP or H_2O_2. One possible explanation for this difference may be due to metabolism of the extract by the worms. This metabolism may generate active and inactive compounds. Metabolism may also decrease the concentration of the compounds that reach the organism, which would impact the ability of the compounds to act effectively against the ROS (Evason et al., 2005; Leung et al., 2010). In addition to the ROS scavenger activity, polyphenols can have many other biological antioxidant effects, including the ability to interact with enzymes and DNA and to modulate cellular signaling processes (Halliwell, 2008; Yu et al., 2010).

Superoxide anion is readily produced through the one-electron reduction of oxygen by the ferrous ion and is largely dismutated into hydrogen peroxide by enzymatic and non-enzymatic mechanisms. Hydrogen peroxide can be further converted into the hydroxyl radical by the Fenton reaction, which requires reduced iron or copper. In our experiments, the *L. divaricata* extract protected *C. elegans* against juglone and Fe^{2+} most likely by reacting with superoxide formed by juglone oxidation and chelating reduced iron. Scavenging of superoxide and iron chelation prevents the formation of hydroxyl radicals, which are highly reactive and act as the initiating species for cellular lipid peroxidation (Yoshino and Murakami, 1998).

With the exception of the NO• scavenger activity, the antioxidant capacity of the extract was similar to that of rutin. Thus, the effect of the extract may be due to other compounds and not only due to rutin. The pharmacological properties of plant crude extracts may be lost when specific components are isolated, which indicates that some of these properties may be related to the synergistic effects of many compounds (Carlini, 2003). Crude extracts may therefore offer a number of advantages when compared to isolated compounds, including cost, conservation of pharmacological properties, low toxicity and the presence of forms that are used popularly worldwide (Pereira et al., 2009; Pietrovski et al., 2006).

After acute treatment with extract or rutin, nematode behavior was also analyzed to evaluate the effects on the nervous system and on toxicity. Although body bending and the defecation cycle length were not altered, we found that the pharyngeal pumping rate was increased. The pharynx is responsible for *C. elegans* feeding and is regulated by the cholinergic, glutamatergic, and serotoninergic neurotransmitter systems (Avery and You, 2012). Indeed, rutin has been reported to be an acetylcholinesterase (AChE) inhibitor in human plasma *in vitro* (Katalinic et al., 2010). In this study, we demonstrated for the first time that the *L. divaricata* leaf extract and rutin can exert this activity in an *in vivo* model. Thus, the extract and its isolated compound may be useful in treatments where acetylcholinesterase inhibition is used, including in neurological disorders such as Alzheimer's disease (AD).

Despite advances in the field, AD remains a devastating neurodegenerative disease with limited therapeutic options. One of the most useful approaches for the treatment of AD is based on the development of AChE inhibitors to mediate a deficit of cerebral acetylcholine levels (Ballard et al., 2011). Only a minority of drugs that are tested in clinical trials become successfully licensed treatments, which emphasizes the urgent need for an increased number of clinical trials (Ballard et al., 2011). In addition to its anticholinesterasic activity, the *L. divaricata* extract may also be helpful in AD because of some of the activities of the compound rutin, which possesses antioxidant activity, inhibits β-amyloid aggregation *in vitro*, ameliorates neuroinflammation and enhances neuronal function in a rat model of AD (Javed et al., 2012; Jiménez-Aliaga et al., 2011; Wang et al., 2012).

5. Conclusions

Our experiments demonstrated that the *L. divaricata* leaf extract and rutin were able to increase the resistance of a whole organism to stress and to increase cholinergic tonus. These results contribute to the body of knowledge concerning plant extracts and their various components that may be used as medicines. These findings also merit further investigation for the prevention and treatment of several health disorders, including Alzheimer's disease.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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3.2 MANUSCRITO 1

Guarana (_Paullinia cupana_ Mart) extends lifespan and healthspan of _Caenorhabditis elegans_

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Abstract

Guarana (*Paullinia cupana* Mart) is a native plant to Amazon region, and is a key ingredient in various energy drinks consumed worldwide. Guarana has several reported beneficial effects on health, although there are no studies related to its effects on lifespan. Thus, our study investigated the effects of guarana ethanolic extract (GEE) on aging in *Caenorhabditis elegans*. The analyses included evaluation of guarana's effects on parameters related to toxicity, antioxidant activity and longevity. We also examined if guarana's ability to increase longevity is mediated via the adenosine receptor, since caffeine is one of the main components of guarana. Lifespan in GEE-treated worms was extended and this effect was associated to antioxidant activity and DAF-16 and HSF-1 pathways, accompanied by a reduction in intestinal lipofuscin and in the number of protein aggregates. Genes modulated by GEE included *sir-2.1*, *hsf-1*, *gst-4*, *gcs-1* and *hsp-16.2*. Furthermore, was demonstrated that ADOR-1 (adenosine receptor homolog) might be necessary for GEE-mediated lifespan extension, demonstrating a possible involvement of the purinergic system in longevity. Combined, our results not only contribute to the study of natural products' effects *in vivo*, but also provide a novel and plausible therapeutic target against aging-associated diseases.

Keywords: aging; natural products; neurodegenerative diseases; purinergic signaling; xanthines

1 Introduction

Paullinia cupana Mart var. *Sorbilis*, also referred to as guarana, is a native plant to the Amazon basin and especially common in Brazil. The powder of its seeds are habitually ingested by people of all ages in the Amazon region (Schimpl *et al.*, 2013). Moreover, guarana is a key ingredient in various energy drinks consumed in many countries (Smith e Atroch, 2010). The guarana extract contains methylxanthines, such as caffeine, theobromine and theophylline, as well as tannins, saponins, catechins, epicatechins, proanthocyanidols and other compounds in trace concentrations (Schimpl *et al.*, 2013). Guarana has several reported effects, including weight loss (Boozer *et al.*, 2001), actions within the central nervous system (Espinola *et al.*, 1997; Da Silva Bittencourt *et al.*, 2014), lowering platelet thromboxane synthesis (Bydlowski *et al.*, 1991), protecting against gastric lesions (Campos *et al.*, 2003) and antioxidant activity (Mattei *et al.*, 1998; Bittencourt *et al.*, 2013; Portella Rde *et al.*, 2013). Moreover, consumption of guarana has been linked to an extension in longevity in people living in Maués, a specific Amazon region in Brazil (Costa Krewer *et al.*, 2011).

Despite an epidemiological study associating guarana ingestion with low prevalence of chronic age-related diseases in the Amazonian population (Costa Krewer *et al.*, 2011), there are no studies related to guarana's effects on lifespan. With increased lifespan, the percentage of older people is growing worldwide, resulting in increased frequency of age-related diseases (Drago *et al.*, 2011; Castorina *et al.*, 2015). Accordingly, research emphasis has been placed on identifying efficacious compounds and therapies that attenuate age-related diseases, yet preserve the quality of life (Joseph *et al.*, 2005; Kennedy e Pennypacker, 2014; Kerch, 2015).

Herein, *Caenorhabditis elegans* was used as an experimental model to study the effects of guarana on aging process. This nematode has been an invaluable model system for understanding organismal responses to various synthetic and natural compounds and their influence on aging and lifespan (Sutphin *et al.*, 2012; Carretero *et al.*, 2015; Golegaonkar *et al.*, 2015), because vital biological pathways and numerous aspects of aging are analogous in nematodes and mammals, including humans (Guarente e Kenyon, 2000; Herndon *et al.*, 2002; Kaletsky e Murphy, 2010). In addition, *C. elegans* offers unique advantages over conventional mammalian models, including the ease of maintenance, short lifespan, rapid generation time, experimental flexibility and high-throughput capability (Guarente e Kenyon, 2000; Boyd *et al.*, 2007).

The aim of the present study was to investigate the effects of guarana on aging and putative pathways that regulate its physiological effects. Since in rodents, caffeine, one of the main components of guarana, has beneficial effects against cognitive impairments through the adenosine receptors (Arendash *et al.*, 2009; Cunha e Agostinho, 2010; Espinosa *et al.*, 2013), we also determined if guarana might affect aging in *C. elegans* by mediating adenosine receptor activity.

2 Materials and methods

2.1 Strains and maintenance

Strains used in this study included: Bristol N2 (wild-type); AM141, *rmIs133 [unc-54p::Q40::YFP]*; CF1038, *daf-16(mu86) I*; EU1, *skn-1(zu67)*; PS3551, *hsf-1(sy441) I*; TK22, *mev-1(kn1)*; VC199, *sir-2.1(ok434)*; as well as the *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, MN, USA. The EG6890 strain, *ador-1(ox489)*, was kindly supplied from Dr. Erik Jorgensen laboratory (University of Utah, USA). This strain has a deletion from 1kb upstream and the first three exons of the ador-1 gene, and was outcrossed 6 times. Ador-1 gene encodes an ortholog of human adenosine receptor (WormBase).

Nematodes were maintained and assayed at 20°C on nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 (Brenner, 1974). Synchronization of nematode cultures was achieved by bleaching treatment of gravid hermaphrodites (Sulston and Hodgkin 1988). Eggs were allowed to hatch overnight in M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl and 1 mM $MgSO_4$).

2.2 Plant material and extract preparation

The powder of toasted seeds of guarana was isolated and supplied by EMBRAPA Oriental (Agropecuary Research Brazilian Enterprise) located in the Western Amazon in Maués, Amazonas-Brazil. The hydro-alcoholic extract was obtained as described (Bittencourt *et al.*, 2013). Briefly, the extract was produced using 70% ethanol. After 24 hours, the resulting solution was filtered, the ethanol was removed and the extract was lyophilized. The predominant xanthines and catechins presented in the guarana extract were analyzed by

means of HPLC (Andrews *et al.*, 2007), showing the following concentrations: caffeine = 12.240 mg/g, theobromine = 6.733 mg/g and total catechins = 4.336 mg/g.

2.3 Treatment of the worms

Liophilyzed guarana ethanolic extract (GEE) was dissolved in distilled autoclaved water and spread over NGM plates with *E. coli* as food source at final concentrations of 100, 500 and 1,000 µg/ml of agar. Plates were incubated at 37°C overnight to allow bacterial growth. Synchronized L1 larvae were transferred to treatment plates and cultured to adulthood at 20°C.

2.4 Development and behavioral assays

2.4.1 Development and body length

Thirty wild type worms per treatment were mounted on 2% agarose pads (in M9 buffer) 46 hours after hatch, paralyzed with 50 mM sodium azide and photographed under a microscope (Olympus IX81). The development stage was analyzed and length and width (ventral segment) of the animals were measured with the ImageJ software (NIH Image) (Bonomo Lde *et al.*, 2014). Analyses were carried out in three independent assays.

2.4.2 Pharynx pumping rate

Pharyngeal pumping was assessed with a Nikon E200 microscope by observing the number of pharyngeal contractions during a 60 second interval in wild-type young adults (Thomas and Lockery 1999). Analyses were carried out in three independent assays. Thirty worms were analyzed per group.

2.4.3 Locomotor activity

Trash frequency was selected for analysis of locomotion. Wild-type young adults from control or GEE treatments were individually picked and placed in a drop of M9. The worms were allowed to adapt for 1 minute and then the number of trashes were quantified with a

Nikon E200 microscope during a 20 second interval. A trash was defined as a change in the direction of bending at the middle of body (Ju *et al.*, 2013). Analyses were carried out in three independent assays. Thirty nematodes were examined per group.

2.5 Aging assays

2.5.1 Lifespan

Lifespan analyses started at L4 larvae in NGM plates seeded with *E. coli* OP50 in the absence or presence of GEE (day 0). Animals were transferred to fresh plates with or without GEE every other day to avoid confounding of generations, and scored at the same time until death (Kenyon *et al.*, 1993; Apfeld e Kenyon, 1999). Absence of response to a mechanical stimulus was scored as death. Worms were censored if they crawled off the plate, displayed extruded internal organs, or died because of hatching progeny inside the uterus. Lifespan assays were repeated three times with 60-120 worms per assay. Through mutant strains, the major known longevity pathways were analyzed: (1) *daf-16*, the insulin/IGF-1 signaling, which the DAF-2 receptor signals through a conserved PI3-kinase/AKT pathway and down regulates DAF-16/FOXO, responsible for promoting expression of genes that confer extended longevity and enhanced stress resistance (Lee *et al.*, 2003; Murphy *et al.*, 2003); (2) *skn-1*, which is related to vertebrate Nrf family proteins and promotes expression of detoxification enzymes in response to oxidative stress, like glutathione-S-transferase (An e Blackwell, 2003); (3) *hsf-1*, which encodes heat-shock transcription factor-1 (HSF-1) and induces activation of various heat-shock genes or chaperones involved in maintaining the conformational homeostasis of proteins, among other important functions (Tatar *et al.*, 1997; Putics *et al.*, 2008) and (4) *sir-2.1*, which encodes a histone deacetylase-like protein that integrates metabolic status with lifespan, and is associated to caloric restriction (Tissenbaum e Guarente, 2001; Wood *et al.*, 2004). A possible relationship between longevity and purinergic signaling was also investigated through *ador-1(ox489)* strain (Shaye e Greenwald, 2011).

2.5.2 Lipofuscin

Lipofuscin is an autofluorescent material that accumulates in aging cells in many organisms (Hosokawa *et al.*, 1994; Brunk e Terman, 2002) and was examined in 9-days-old adult worms. GEE-treated and control worms were mounted on 2% agarose pads, paralyzed

with 50 mM sodium azide, and photographed under a fluorescence microscope (Olympus IX81). Lipofuscin levels were measured with the ImageJ software (NIH Image) by determining average pixel intensity in each animal's intestine (Wilson *et al.*, 2006). Analyses were carried out in three independent assays. Thirty worms were analyzed per group.

2.5.3 Protein aggregation

Protein aggregation was assessed in 9-day-old adult AM141 worms expressing a muscle-specific fusion protein, polyglutamine (Q40)::YFP. Epifluorescence images were captured as above. Each worm was analyzed with ImageJ software, and aggregates were counted in thirty animals per group (Ayyadevara *et al.*, 2013). Analyses were carried out in three independent assays.

2.6 RNA isolation and real-time polymerase chain reaction (RT-qPCR)

Wild type worms in L4 and 9-day-old adult stage were analyzed for gene expression related to longevity and oxidative stress response. After adulthood, worms were transferred every other day to plates containing 150 mM of FUDR (5-fluoro-2'-deoxyuridine) to inhibit reproduction, in the presence or absence of GEE. RNA from 20,000 worms per condition was isolated using Trizol followed by chloroform extraction, as previously described (Chomczynski e Mackey, 1995)1 ug of input RNA was reverse transcribed to cDNA by Applied Biosystems' high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Expression analysis was performed by Custom TaqMan® Array Analysis utilizing the corresponding TaqMan® Gene Expression Assays for mitochondrial superoxide dismutase *sod-3* (Ce02404515_g1), glutathione-S-transferase *gst-4* (Ce02458730_g1), gamma glutamylcysteine synthetase *gcs-1* (Ce02436726_g1), *daf-2* (Ce02422835_g1), *sir-2.1* (Ce02459018_g1), *hsf-1* (Ce02423758_m1), heat shock protein *hsp-16.2* (Ce02506738_s1) and *skn-1* (Ce02407445_g1) (Applied Biosystems). Target gene expression was normalized to the expression values of actin *afd-1* (Ce02414573_m1). The relative quantification was determined with the $2^{-\Delta\Delta Ct}$ method (Livak e Schmittgen, 2001) and data were expressed as fold change in mRNA levels compared to control worms. This experiment was carried out in three independent worm preparations, each in triplicate.

2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). The results were plotted as the mean ± SD (standard deviation of the mean) of at least three individual experiments. A student's *t* test was used to compare pairs of groups, whereas a one or two-way ANOVA followed by Bonferroni's *post-hoc* test was used to compare three or more groups. All survival curves were analyzed by the Log-rank (Mantel-Cox) test. The statistical significance was determined as $p<0.05$.

3 Results

Treatment with GEE did not affect larval growth and 46 hours after hatch all worms reached young adult stage. Photographs were taken and length and width of the treated animals were indistinguishable from the control group: 817±34.24 μμm length and 36±1.68 μμm width (data not shown).

Feeding behavior and locomotion were analyzed throughout the lifespan. GEE at all concentrations tested did not influence these behavioral parameters in young adults (Figure 1 A and B). However, the extract delayed the age-related decline in pharyngeal pumping (100, 500 and 1,000 μg/ml) and trashes (1,000 μg/ml) (Figure 1 A and B).

Control wild-type animals had a mean lifespan of 11 days and average maximum lifespan of 14 days. In media containing GEE, mean lifespan of wild-type animals was extended by 18% at 100 μg/ml and 36% at 500 and 1,000 μg/ml and maximum lifespan was extended by an average of 28% at the three tested concentrations (Table 1). GEE extract (1,000 μg/ml) also extended mean lifespan of *mev-1* mutants by 44% and maximum lifespan by 77.8% (Table 1).

GEE treatment extended mean lifespan in *skn-1* and *sir 2.1* mutants, establishing that the extract did not act through these pathways to promote lifespan extension, and in contrast, the treatment did not prolong lifespan in *daf-16, hsf-1* and *ador-1* mutants (Table 1).

As bacteria play a role in *C. elegans* mortality (Gems e Riddle, 2000), we evaluated *E.coli* OP50 growth in the presence or absence of GEE to investigate if beneficial effects could be a response to a possible antimicrobial property. The optical density of the bacteria was measured over 4 hours in liquid medium (Bonomo Lde *et al.*, 2014), but there were no differences between media with presence or absence of 1000 μg/mL GEE (data not shown).

The “youthfulness” of the worms was also prolonged after GEE treatment. A decrease in intestinal lipofuscin accumulation (39.42%) and a decrease in the number of protein aggregates (20%) were detected in 9-day-old adults after GEE treatment, when compared to control worms (Figure 2).

PCR analyses assessed gene modulation by GEE (Table 2). GEE at 500 and 1000 µg/ml down regulated *daf-2*, *sir-2.1*, *hsf-1*, *gst-4*, *gcs-1* and *hsp-16.2* in L4-larval stage worms and down regulated *skn-1*, *daf-2*, *sir-2.1* and *hsp-16.2* in 9-days-old adults. No effects were observed on *sod-3* expression in both stages.

4 Discussion

The present work investigated the effects of chronic consumption of guarana ethanolic extract using *Caenorhabditis elegans*. Given that chronic consumption of guarana may cause harmful effects, our studies commenced by evaluating GEE’s toxicity in this organism. The results suggest that guarana at the tested concentrations failed to produce adverse effects in the whole organism once prolonged exposure to GEE did not affect worm development, feeding and locomotion behaviors. These parameters are exquisitely sensitive endpoints of toxicity assessment in nematodes (Qiao *et al.*, 2014).

Extensive research has focused on aging processes and compounds that are efficacious in promoting lifespan extension and in delaying age-associated diseases (Wilson *et al.*, 2006; Canuelo *et al.*, 2012; Regitz *et al.*, 2014). Our study showed that GEE extended lifespan in wild-type worms; accordingly, mechanisms that might be implicated in this effect were investigated. Oxidative stress appears to be a major factor limiting lifespan in both *C. elegans* and humans (Larsen, 1993; Finkel e Holbrook, 2000) and is associated to many age-related diseases (Halliwell, 2006; Bonomini *et al.*, 2015), which directs attention toward antioxidant compounds with effects *in vivo*. To further investigate whether GEE could extend lifespan through an antioxidant activity, its effect on *mev-1* lifespan was evaluated. This strain is characterized by superoxide overproduction and has a shorter lifespan compared to wild type strain (Ishii *et al*., 1998). Consistent with previously described antioxidant effects of guarana extract *in vitro* (Mattei *et al.*, 1998; Bittencourt *et al.*, 2013), GEE treatment significantly extended mean and maximum lifespan of *mev-1* worms. Besides that, DAF-16 and HSF-1 (heat shock transcription factor-1) appeared essential for GEE-mediated lifespan extension. Survival and adaptation to adverse environmental conditions requires the activity of HSF-1 (Tatar *et al.*, 1997; Putics *et al.*, 2008). The latter functions in cooperation with DAF-16 to

activate the expression of common target genes, including the family of *sHsp* (small heat shock proteins genes)(Hsu *et al.*, 2003).

Methylxanthines, as caffeine, are the main component of guarana and it is well known that these compounds can act through adenosine receptors in mammals (Chen, J. F. *et al.*, 2013). Caffeine has been associated with beneficial effects, including aging-related effects (Laurent *et al.*, 2014; Rivera-Oliver e Diaz-Rios, 2014) and improvement of cognitive impairment phenotypes by antagonizing the adenosine receptors A_1 and A_{2A} in rodents (Cunha e Agostinho, 2010). Thus, we tested if the GEE-induced extension of lifespan by GEE might also depend upon ADOR-1, an adenosine receptor homolog (Shaye e Greenwald, 2011). Our results indicate that *ador-1(ox489)* worms failed to show extended lifespan, demonstrating, for the first time, a possible role of the purinergic system in lifespan extension. Accordingly, purinergic signaling may be profitably studied in the future as a potential target for longevity modulation. Despite GEE is high in caffeine, and previous studies described caffeine's effects in worms (Lublin *et al.*, 2011; Sutphin *et al.*, 2012; Bridi *et al.*, 2015), the GEE's effects in increasing lifespan established in our study might be related to synergic effects of different methylxanthines, since the concentration of caffeine in the extract is much lower than the effective concentration previously demonstrated. Besides, data from literature shows that extracts could have greater pharmacological activities than isolated compounds (Pietrovski *et al.*, 2006; Adebajo *et al.*, 2009).

GEE might also act by prooxidant effects once the extract down regulated the expression of *sir-2.1*, *hsf-1*, *gst-4*, *gcs-1* and *hsp-16.2,* genes related to antioxidant defense, which might lead to a low-oxidative status in the early ages. Low-level stress early in life has been proposed to induce complex stress responses that involve changes in gene expression and metabolism, resulting in lifespan extension (Rattan *et al.*, 2007). In contrast, down regulation of *skn-1*, *sir-2.1* and *hsp-16.2* in 9-days-old adults treated with GEE might reflect less need to activate these pathways to repair cell damages during aging compared to untreated worms. Previous studies have already shown the efficacy of various compounds to act both as prooxidants or antioxidants via diverse mechanisms (Sautin e Johnson, 2008; Samec *et al.*, 2015).

Due to the costs associated with caring for the health of the aged population, it is intriguing that medicines not only extend lifespan, but also the quality of life. Thus, some parameters related to healthspan in worms were analyzed upon GEE treatment. The extract slowed the age-dependent decline in pharyngeal pumping and locomotion, reliable physiological biomarkers of *C. elegans* aging (Huang *et al.*, 2004). These results may be

related to a reduction in intestinal lipofuscin levels and in protein aggregates, factors that accumulate during aging. Protein aggregation due to oxidation, misfolding, or un-structured/nonpolar interactions between polyglutamine tracts, underlies aging-related neurodegenerative disorders and possibly many other age-dependent traits (Morley *et al.*, 2002).

The protective role of DAF-16 and HSF-1 on age-dependent proteotoxicity has been demonstrated (Cohen *et al.*, 2006). While necessary for lifespan extension by GEE, they also might mediate the healthspan extension and protein homeostasis. DAF-16 is involved in the formation of less toxic high-molecular weight protein aggregates (Ushikubo *et al.*, 2014), and although HSF-1 regulates protein disaggregation activity releasing small toxic aggregates, it might have a beneficial effect contributing to protein clearance through enzymatic metabolism (Iwata *et al.*, 2001; Leissring *et al.*, 2003).

Thus, GEE might act through antioxidant activity, DAF-16 and HSF-1 to improve longevity, and it might be helpful in treating neurodegenerative disorders related with oxidative stress, aging and protein aggregates, such as Alzheimer's and Parkinson's disease (Park e Kim, 2013; Das *et al.*, 2015). In addition, as ADOR-1 was also necessary for GEE effects on lifespan, more studies should be directed at evaluating the potential role of purinergic system in aging and protein homeostasis.

5 Conclusion

This study shows that exposure to GEE was safe and capable of extending lifespan and healthspan in the nematode *C. elegans*. GEE extended lifespan through antioxidant activity, gene modulation (*sir-2.1*, *hsf-1*, *gst-4*, *gcs-1* and *hsp-16.2)* and DAF-16 and HSF-1, likely linked to regulation of protein homeostasis during aging. Furthermore, was demonstrated ADOR-1 might be necessary for GEE-mediated lifespan extension, indicating a possible involvement of the purinergic system in the longevity pathway. Therefore, our results not only contribute to the study of natural products' effects *in vivo*, but also provide a novel and plausible therapeutic target against aging-associated diseases.

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Legend of Figures

Fig. 1. GEE effects on behavioral parameters. Pharyngeal pumping rate (A) and thrash frequency (B) during aging in wild-type worms. (*) represents a significant difference from the control by one-way ANOVA followed by Bonferroni Multiple Comparison Test ($p<0.05$) ($n= 30$ worms per group).

Fig. 2. Effect of GEE (1,000 μg/ml) treatment on biomarkers of aging in 9-day-old adults compared to the untreated group. (A) Representative images and (B) mean autofluorescence intensity of intestinal lipofuscin in wild-type worms in AFU (arbitrary fluorescent units); (C) representative images of protein aggregation in AM141 (Q40::YFP) worms and (D) number of protein aggregates per worm. (*) represents a significant difference from the control by *t* test ($p<0.05$) ($n= 30$ worms per group).

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Figure 1

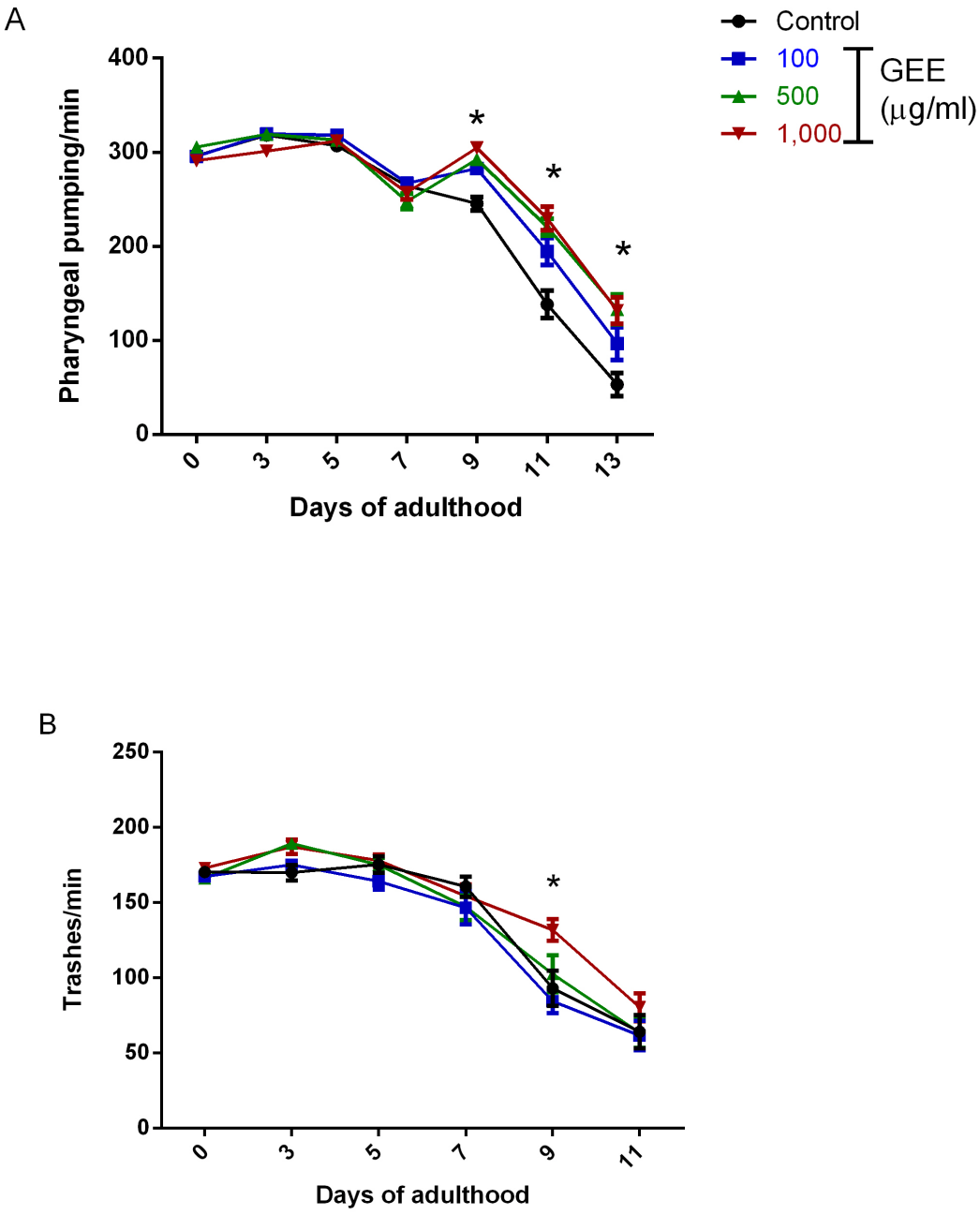

Fig. 1. GEE effects on behavioral parameters. Pharyngeal pumping rate (A) and thrash frequency (B) during aging in wild-type worms. (*) represents a significant difference from the control by one-way ANOVA followed by Bonferroni Multiple Comparison Test ($p<0.05$) (n= 30 worms per group).

Figure 2

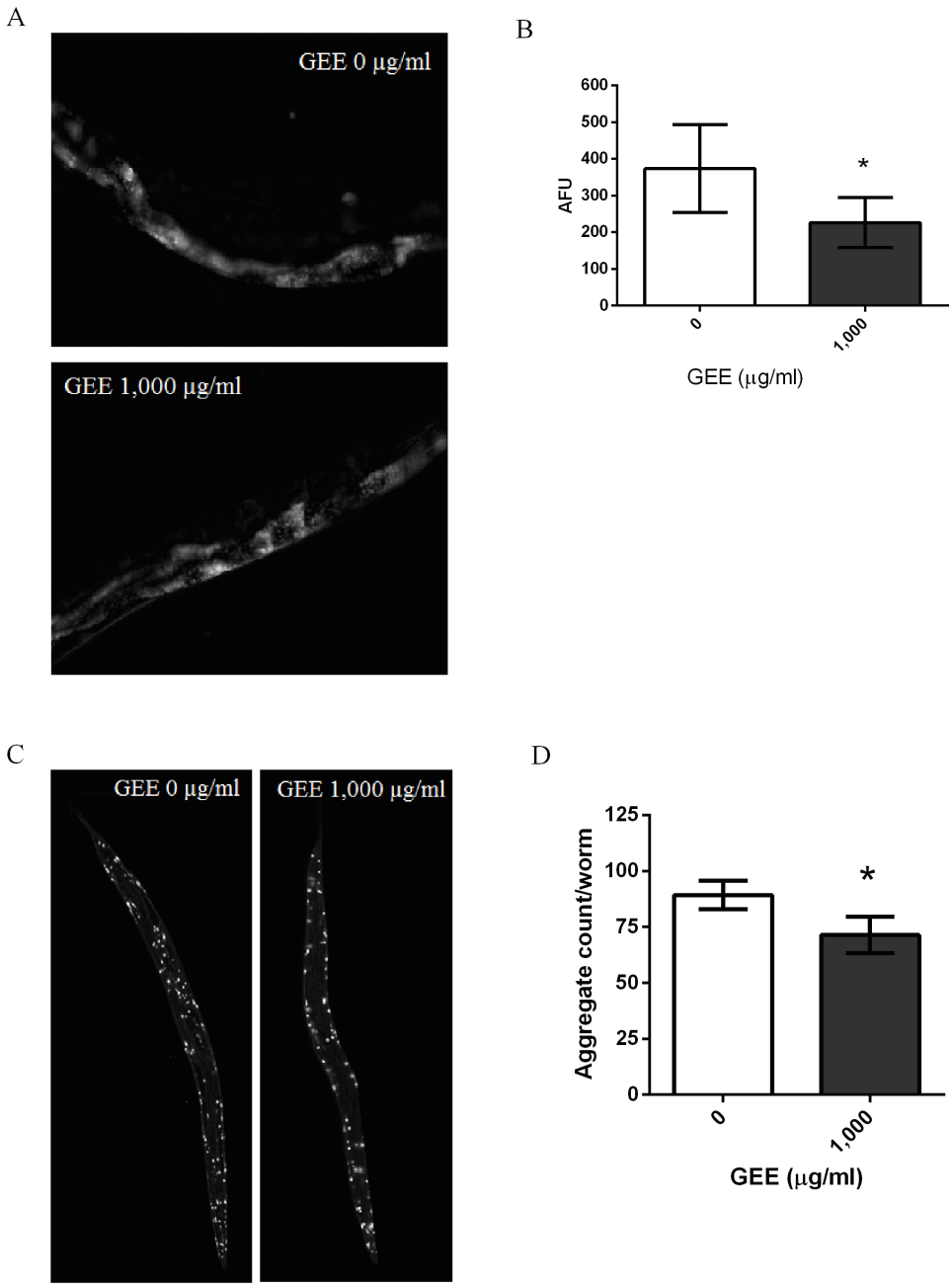

Fig. 2. Effect of GEE (1,000 µg/ml) treatment on biomarkers of aging in 9-day-old adults compared to the untreated group. (A) Representative images and (B) mean autofluorescence intensity of intestinal lipofuscin in wild-type worms in AFU (arbitrary fluorescent units); (C) representative images of protein aggregation in AM141 (Q40::YFP) worms and (D) number of protein aggregates per worm. (*) represents a significant difference from the control by *t* test ($p<0.05$) ($n= 30$ worms per group).

Table 1. Lifespan of untreated and GEE-treated *C. elegans*

Genotype	GEE (µg/ml)	Mean Lifespan ± SD (days)	Maximum Lifespan ± SD (days)
N2	0	11 ± 1.73	14 ± 1.81
	100	13 ± 2.00*	17 ± 1.91*
	500	15 ± 1.15*	18 ±1.07*
	1,000	15 ± 1.63*	18 ± 1.33*
mev-1	0	9 ± 1.02	12 ± 1.22
	1,000	13 ± 1.07*	15 ± 1.55*
daf-16	0	11 ± 1.53	13 ± 1.28
	1,000	11 ± 1.57	12 ± 1.44
skn-1	0	9 ± 1.21	9 ± 1.23
	1,000	9 ± 1.35	11 ± 1.38*
hsf-1	0	11±0.2	13±0.7
	1,000	11 ± 0.6	13±0.7
sir-2.1	0	15 ± 1.80	16 ± 1.42
	1,000	17 ± 2.10*	17 ± 1.66
ador-1	0	13 ± 1.74	17 ± 1.62
	1,000	15 ± 1.68	15 ± 1.33

Lifespan assays were performed at 20°C. Maximum lifespan is represented as the mean lifespan of the longest living 10% of the worm population.
Each experiment was repeated three times starting with at least 60 nematodes per group.
* means significant difference from untreated group by the Log-rank (Mantel-Cox) test, $p<0.05$

Table 2 – Fold change mRNA expression of genes related to longevity and oxidative stress response in wild type worms treated with GEE compared to untreated worms

Stage	Gene / GEE (µg/ml)	*skn-1*	*daf-2*	*sir-2.1*	*hsf-1*	*gst-4*	*gcs-1*	*sod-3*	*hsp-16.2*
L4	**0**	+ 0.002	+ 0.002	+ 0.004	+ 0.001	+ 0.002	+ 0.005	+ 0.003	+ 0.011
		(0.079)	(0.075)	(0.120)	(0.064)	(0.210)	(0.039)	(0.082)	(0.052)
	100	+ 0.056	- 0.084[a]	- 0.077[a]	+ 0.006[a]	- 0.146[a]	- 0.012[a]	- 0.030	- 0.176[a]
		(0.110)	(0.078)	(0.072)	(0.112)	(0.018)	(0.084)	(0.125)	(0.210)
	500	+ 0.041	**- 0.173[a]***	**- 0.437[b]***	**- 0.204[b]***	**- 0.490[b]***	+ 0.036[a]	+ 0.104	**- 0.556[b]***
		(0.031)	**(0.151)**	**(0.175)**	**(0.082)**	**(0.083)**	(0.042)	(0.186)	**(0.245)**
	1,000	- 0.042	**- 0.153[a]***	**- 0.531[b]***	**- 0.199[b]***	**- 0.694[c]***	**- 0.129[b]***	- 0.160	**- 0.809[b]***
		(0.108)	**(0.019)**	**(0.143)**	**(0.075)**	**(0.025)**	**(0.081)**	(0.168)	**(0.117)**
9-days-old-adults	**0**	- 0.017	- 0.026	+ 0.015	+ 0.001	+ 0.005	+ 0.006	+ 0.005	- 0.010
		(0.037)	(0.087)	(0.072)	(0.104)	(0.114)	(0.116)	(0.117)	(0.089)
	100	+ 0.052[a]	- 0.066[a]	+ 0.046[a]	+ 0.140	+ 0.180	- 0.141	+ 0.055	- 0.056
		(0.043)	(0.159)	(0.194)	(0.234)	(0.268)	(0.134)	(0.434)	(0.254)
	500	**- 0.267[b]***	**- 0.311[a]***	**- 0.658[b]***	- 0.147	- 0.016	- 0.037	- 0.391	**- 0.842[b]***
		(0.269)	**(0.276)**	**(0.158)**	(0.109)	(0.254)	(0.230)	(0.393)	**(0.053)**
	1,000	**- 0.613[c]***	**- 0.692[b]***	**- 0.708[b]***	- 0.154	- 0.083	- 0.059	- 0.388	**- 0.670[b]***
		(0.133)	**(0.133)**	**(0.164)**	(0.150)	(0.425)	(0.493)	(0.137)	**(0.228)**

This experiment was assessed by RT-qPCR and carried out in three independente worms preparations, each in triplicate. Data are expressed as means and ±SD in parentheses.

3.3 MANUSCRITO 2

Guarana (*Paullinia cupana* Mart.) attenuates methylmercury-induced toxicity in *Caenorhabditis elegans*

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Abstract

The influence of routine guarana (*Paullinia cupana*) consumption on apparent tolerance to mercury intoxication has been proposed. The present study investigated this hypothesis in *Caenorhabditis elegans*, an optimal experimental model for studies in toxicology. Wild type (WT) and *skn-1 (ok2315)* worm strains were pre-treated with guarana ethanolic extract (GEE) from larvae 1 (L1) to L4 stage and then exposed for 6 hours to methylmercury (MeHg). The analyses included evaluation of GEE's effects on lethality, developmental delay, feeding, locomotion and gene expression (*sod-3*, *gst-4*, *sir-2.1*, *hsf-1*, *snn-1, mtl-1*, *mtl-2*, *aat-1, aat-2* and *aat-3*). GEE pre-treatment had no aberrant effects on WT worms exposed to MeHg, and protected *skn-1 (ok2315)* worms, which are known to produce higher levels of reactive oxygen species (ROS) compared to WT, and thus are more susceptible to environmental stresses. GEE might exert its protective effects against MeHg toxicity by increasing the expression of genes involved in metal transport (*aat-2*), metal detoxification (*mtl-1* and *mtl-2*) and antioxidant responses (*sir-2.1* and *sod-3*). Thus, routine consumption of guarana might be beneficial in protecting against MeHg-induced toxicity.

Keywords: mercury; natural products; neurological disorders; xanthines

1. Introduction

Mercury (Hg) is a heavy metal present in the environment due to both natural and anthropogenic sources (Farina *et al.*, 2013). A major route of mercury (Hg) exposure in humans is the consumption of seafood containing methylmercury (MeHg) due to water contamination from agriculture and industries (Clarkson, 2002; Clarkson e Magos, 2006). MeHg is more efficiently transported into the central nervous system (CNS) compared to inorganic mercurial, and has varying neurotoxic effects depending on age, dose and duration of exposure (Clarkson, 2002; Clarkson e Magos, 2006). Although MeHg has demonstrated effects, including dopaminergic neurodegeneration, alterations in calcium homeostasis, oxidative stress induction and high affinity for cysteine, the specific molecular targets of MeHg are still unknown (Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002; Helmcke *et al.*, 2009; Martinez-Finley e Aschner, 2011).

The Amazon region in Brazil has inherently high mercury levels in soil and water due to geological processes and gold mining (Wasserman *et al.*, 2003), and the concentration of this metal in biological samples of the native population exceeds the recommended value established by the World Health Organization (WHO) (De Castro e Lima Mde, 2014). Therefore, the reported tolerance to mercury in this population might be secondary to nutritional factors as potential modifiers of mercury toxicity (Chapman e Chan, 2000).

Paullinia cupana Mart var. *Sorbilis*, also referred to as guarana, is a native plant to the Amazon basin, especially in Brazil, with several reported effects including CNS stimulation (Espinola *et al.*, 1997), memory maintenance (Espinola *et al.*, 1997) and antioxidant activity (Mattei *et al.*, 1998). Previous studies also showed a protective effect against cadmium in adult rat testis (Leite *et al.*, 2011; Leite *et al.*, 2013), and against rotenone in human dopaminergic neuroblastoma SH-SY5Y cells (De Oliveira *et al.*, 2011). Guarana seed extracts contain several methylxanthines, such as caffeine, theobromine and theophylline, and tannins, saponins, catechins, epicatechins, proanthocyanidols as well as other compounds in trace concentrations (Costa, 1972).

As the powder of guarana seeds is habitually consumed in the Amazon region by people of all ages (Schimpl *et al.*, 2013), it might modulate mercury toxicity in this native population. The possible protective effects might contribute to the understanding of mechanisms of metal toxicity and to the development of new therapeutic strategies. The present study investigated this hypothesis using *Caenorhabditis elegans,* a valuable animal model for understanding organismal reactions to various environmental factors (Chen, P. *et*

al., 2013; Ye *et al.*, 2014) with high predictive value of toxicity to mammals (Williams e Dusenbery, 1988; Cole *et al.*, 2004; Leung *et al.*, 2008). Thus, the aim of this study was to investigate if guarana demonstrates protective effects against methylmercury-induced toxicity, as well as the mechanisms involved.

2. Materials and methods

2.1 *C. elegans* maintenance

The strains used in this study: Bristol N2 (wild-type; WT) and VC1772 (*skn-1(ok2315) IV/nT1 [qIs51] (IV;V)*), as well as the *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, MN, USA.

Nematodes were maintained on nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 (Brenner, 1974). Synchronization of nematode cultures was achieved by bleaching treatment of gravid hermaphrodites (Lewis e Fleming, 1995) and eggs were allowed to hatch overnight in M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mMNaCl and 1 mM $MgSO_4$).

2.2 Plant material and guarana extract preparation

The powder of toasted seeds of guarana was isolated and supplied by EMBRAPA Oriental (Agropecuary Research Brazilian Enterprise) located in the Western Amazon in Maués, Amazonas, Brazil. The hydro-alcoholic extract was obtained as previously described (Bittencourt *et al.*, 2013). Briefly, the extract was produced using 70% ethanol. After 24 hours, the resulting solution was filtered, the ethanol was removed and the extract was lyophilized. The predominant xanthines and catechins present in the guarana extract were analyzed by means of HPLC (Andrews *et al.*, 2007), showing the following concentrations: caffeine = 12.240 mg/g, theobromine = 6.733 mg/g and total catechins = 4.336 mg/g.

2.3 Guarana and MeHgCl treatments

Lyophilized guarana ethanolic extract (GEE) was dissolved in distilled autoclaved water and spread over NGM plates with *E. coli* as food source at final concentrations of 100,

500 and 1,000 µg/ml of agar. Plates were incubated at room temperature overnight to allow bacterial growth. Synchronized L1 larvae were transferred to treatment plates and cultured until L4 larvae stage at 20°C.

Methylmercury chloride (CH_3HgCl, MeHgCl) exposures were performed for 6 hours in L4 worms (Helmcke *et al.*, 2009) untreated and pretreated with GEE. Exposures were conducted in micro tubes by combining 500 L4 worms, 25 µl of concentrated *E. coli* OP50, 10 µl of MeHgCl dissolved in water at various concentrations, and M9 buffer to a volume of 500 µl. Following the exposure duration, animals were washed three times with M9 buffer by centrifugation and placed on OP50-containing NGM plates.

2.4 Lethality, developmental and behavioral parameters evaluation

A dose-response curve was generated to evaluate MeHg-induced lethality (0-150µM). Following MeHg exposure and washing, approximately 100 worms per treatment were transferred to NGM plates seeded with *E. coli* OP50, without extract, and allowed to recover for 24 hours. Nematodes were then scored as dead or alive using a stereomicroscope. Animals that reacted to a mechanical stimulus with a platinum wire were scored as alive, and non-responding animals were considered to be dead (Bischof *et al.*, 2006). The assay was repeated four times.

The surviving worms were analyzed using a microscope and scored through the larval stages observing vulva development and presence of eggs in the uterus (Hirsh *et al.*, 1976)

In order to evaluate behavioral parameters following MeHg exposure and washing, approximately 50 worms per treatment were transferred to NGM plates seeded with *E. coli* OP50 and analyzed immediately post MeHg exposure (0-hour post MeHg exposure) or 24-hour post exposure.

Pharyngeal pumping rate was assessed with a microscope by observing the number of pharyngeal contractions of worms during a 60 second interval in NGM plates seeded with *E. coli* OP50 (Huang *et al.*, 2004).

Thrash frequency was selected for analysis of locomotion. Worms were individually picked and placed in a drop of M9 and allowed to adapt for 1 minute and then the number of thrashes were quantified with stereomicroscope during a 60 second interval. A thrash was defined as a change in the direction of bending at the middle of body (Ju *et al.*, 2013).

Analyses were carried out in ten worms per group. The assays were repeated four times.

2.5 RNA isolation and real-time polymerase chain reaction (RT-qPCR)

Worms were analyzed 0- and 24-hour post the end of MeHg exposure for gene expression related to metal transport and response. For 24-hour post MeHg analyses, worms were transferred to plates containing 150 mM of FUDR (5-fluoro-2'-deoxyuridine) to inhibit reproduction. RNA from 20,000 worms per condition was isolated using Trizol followed by chloroform extraction, as previously described (Chomczynski e Mackey, 1995), 1 ug of input RNA was reverse transcribed to cDNA by Applied Biosystems' high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Expression analysis was performed by Custom TaqMan® Array Analysis utilizing the corresponding TaqMan® Gene Expression Assays for mitochondrial superoxide dismutase *sod-3* (Ce02404515_g1), glutathione-S-transferase *gst-4* (Ce02458730_g1), sirtuin *sir-2.1* (Ce02459018_g1), heat shock factor *hsf-1* (Ce02423758_m1), synapsin *snn-1* (Ce02407220_m1), metallothioneins *mtl-1* (Ce02551471_s1) and *mtl-2* (Ce02551627_s1) and amino acid transporters *aat-1* (Ce02458013_g1), *aat-2* (Ce02479487_g1) and *aat-3* (Ce02492836_g1) (Applied Biosystems). Target gene expression was normalized to the expression values of actin *afd-1* (Ce02414573_m1). The relative quantification was determined with the $2^{-\Delta\Delta Ct}$ method (Livak e Schmittgen, 2001) and data were expressed as fold change in mRNA levels compared to control worms. This experiment was carried out in three independent worm preparations, each in triplicate.

2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). The results were plotted as the mean ± SEM (standard error of the mean) of four individual experiments. Dose-response lethality curves and LD50 determination were generated using a sigmoidal dose-response model and analyzed with extra sum-of-squares F test method. Two-way ANOVA followed by Bonferroni's Multiple Comparison Test was used to compare groups. The statistical significance was determined as $p<0.05$.

3. Results

3.1 MeHg-induced lethality and developmental delay

A dose-response curve was generated to evaluate MeHg toxicity in L4- larvae stage WT worms after a 6-hour exposure and 24-hour recovery (Fig 1A). The LD50 was 66 μM for MeHg and pre treatment with GEE 100, 500 and 1,000 μg/ml did not affect lethality. A more sensitive strain to MeHg, *skn-1(ok2315)*, was also assessed, resulting in a LD50 of 38 μM of MeHg (Fig. 1B). GEE exerted a protective effect against MeHg-induced lethality in this strain. The extract at 100, 500 and 1,000 μg/ml significantly increased the dose required for LD50 in *skn-1 (ok2315)* worms to 45, 51 and 59 μM of MeHg, respectively. The highest GEE dose afforded a significant protection against MeHg-induced lethality compared to the other doses (Fig. 1B, $p<0.05$).

The living worms were also analyzed for developmental progress 24- and 48- hour post MeHg exposure. After 24 hours of recovery, WT and *skn-1 (ok2315)* control worms reached the egg laying adult-stage, while MeHg exposure induced a dose-dependent delay in worm development starting at 25 μM for both strains, with an accentuated effect in mutant worms (Fig 2 A and B). Pre-treatment with GEE did not protect WT worms against MeHg-induced developmental delay at any of the tested doses (data not shown), however, GEE at doses between 100 and 1,000 μg/ml had protective effects in *skn-1 (ok2315)* worms exposed to 25 and 50 μM of MeHg, decreasing the developmental delay (Fig. 2 C and D). After 48 hours of recovery, all worms from both strains reached the egg laying adult-stage (data not shown).

3.2 MeHg-induced behavioral alterations

Pharyngeal pumping rates were significantly decreased immediately after 6-hour MeHg exposure in WT and *skn-1 (ok2315)* worms (Fig. 3A and B, $p<0,05$). WT control worms pumped at a rate of 232.8 ± 6.8 pumps per minute while worms exposed to 10 μM MeHg pumped at a rate of 182.8 ± 6.5 pumps per minute. Mutant *skn-1 (ok2315)* worms pumped at a rate of 264.7 ± 8.3 pumps per minute, while *skn-1 (ok2315)* worms exposed to 10 μM MeHg pumped at a rate of 211.8 ± 7.2 pumps per minute. Pharyngeal pumping was inhibited at 25 μM MeHg in both strains. Worms were also analyzed 24-hour post metal exposure, and MeHg-exposed worms showed a recovery in pharyngeal pumping rate to

control levels, with the exception of the 50 μM MeHg group (Fig. 3C and D, $p<0,05$). Pre-treatment with GEE exerted difference only in *skn-1 (ok2315)* worms analyzed immediately after exposure to 25 μM MeHg, while pharyngeal pumping of untreated worms was inhibited (Fig. 3B $p<0,05$).

Thrash frequency was also altered in worms exposed to MeHg (Fig. 4). WT control worms showed a rate of 308.5 ± 6 thrashes per minute while MeHg induced a dose-dependent decrease in movements immediately post metal exposure starting at 5 μM (155.6 ± 6 thrashes per minute) and a total inhibition of movements at 50 μM. WT worms pre-treated with GEE showed a significantly higher thrash frequency of 251.9 ± 17 and 170.2 ± 21.9 thrashes per minute in comparison to GEE-untreated worms at 5 and 10 μM MeHg, respectively (Fig.4A). The *skn-1 (ok2315)* control worms showed a rate of 448 ± 26 thrashes per minute, while MeHg induced a dose-dependent decrease in movements immediately post exposure starting at 10 μM (327.5 ± 28.7 thrashes per minute) and completely inhibited movements at 50 μM (Fig. 4B, $p<0,05$). Thrash frequency was significantly higher in *skn-1 (ok2315)* worms treated with GEE in comparison to untreated worms. At 24-hour post MeHg exposure, thrash frequency remained decreased in WT worms treated with 25 and 50 μM MeHg (248 ± 19.7 and 270 ± 15.8 thrashes per minute, respectively) in comparison to controls (345 ± 5.2 thrashes per minute). Worms pre-treated with GEE at 500 and 1,000 μg/ml showed a significantly higher thrash frequency with approximately 300 ± 11 thrashes per minute in comparison to GEE-untreated worms at 25 μM MeHg (Fig. 4C, $p<0,05$). *Skn-1 (ok2315)* worms showed MeHg-induced decrease in thrashes after a 24-hour recovery period only at 50 μM (272.4 ± 9.5 thrashes per minute) compared to controls (424.2 ± 17 thrashes per minute). Pre-treatment with GEE had no effect in this strain 24-hour post MeHg exposure at any of the tested doses (Fig. 4D).

Only egg laying adults were analyzed for behaviors 24-hour post MeHg exposure to discard potential differences inherent to developmental delays.

3.3 MeHg-induced gene expression alterations

Gene expression was not altered by GEE pre-treatment in *skn-1 (ok2315)* worms immediately after MeHg exposure and thus was not analyzed in WT worms (data not shown).

Table 1 shows the fold change in mRNA expression compared to the control group in *skn-1* worms 24-hour post MeHg exposure. Expression levels of *mtl-1*, *mtl-2*, *aat-2*, *sod-3* and *sir-2.1* were significantly increased in *skn-1 (ok2315)* worms pre-treated with GEE in

comparison to GEE-untreated worms exposed to MeHg. Expression levels of *snn-1*, *aat-1*, *aat-3* and *hsf-1* after GEE pre treatment or MeHg exposure were not different from expression levels in control worms (data not shown). Gene expression levels that were altered in *skn-1 (ok2315)* post MeHg exposure were also investigated in WT worms (Table 2). Expression levels of *aat-2* and *sir-2.1* were significantly decreased in WT worms pre-treated with GEE in comparison to untreated worms, while *mtl-1* and *mtl-2* expression levels were significantly decreased after MeHg exposure, but were statistically indistinguishable between GEE-treated and –untreated worms. Expression levels of *gst-4* were significantly increased in WT worms pre-treated with GEE in comparison to untreated worms.

4. Discussion

The influence of routine guarana consumption on apparent tolerance to mercury intoxication has been proposed (Chapman e Chan, 2000; Krewer Cda *et al.*, 2011; De Castro e Lima Mde, 2014). The present study investigated the effects of pre-treatment with guarana ethanolic extract (GEE) on L4-larvae stage *C. elegans* exposed to MeHg for 6 hours. Our results show a protective effect of GEE against MeHg in *skn-1 (ok2315)* worms, but not in WT worms.

C. elegans is an optimal model for studying toxicity of many compounds, including metals (Williams e Dusenbery, 1988; Chen, P. *et al.*, 2013). Our data corroborated that 6-hour MeHg exposure induces lethality and behavior impairments in L4-larvae stage WT worms (Helmcke *et al.*, 2009). However, herein, these effects were shown at lower concentrations (LD50 of 66 *vs.* 570 μM MeHg as previously demonstrated). This discrepancy may be due to differences in media density secondary to bacterial concentration, or a result of natural drifting in genetic variations in the worms' population (Barriere e Felix, 2005).

Skn-1 (ok2315) worms were more sensitive to MeHg than WT worms, as previously described (Vanduyn *et al.*, 2010; Martinez-Finley *et al.*, 2013). SKN-1 is the ortholog of mammalian Nrf2, a leucine zipper class transcription factor that regulates a number of detoxifying enzymes, including glutathione-S-transferases, and is involved in cellular detoxification and stress response (Kensler *et al.*, 2007; Taylor *et al.*, 2008). The *skn-1 (ok2315)* mutant strain produces higher levels of ROS compared to WT and is more susceptible to environmental stresses (Kobayashi e Yamamoto, 2006; Martinez-Finley *et al.*, 2013). The LD50 of MeHg in WT worms treated with GEE were not significantly different compared to GEE-untreated worms. However, GEE afforded protection in *skn-1 (ok2315)*

worms against MeHg toxicity, increasing the LD50 values in comparison to *skn-1 (ok2315)* GEE-untreated worms.

After embryogenesis, the worm undergoes to 6 stages of development (L1, L2, L3, L4, young adults and egglaying adults) at specific time points, dependent upon the temperature (Hope 1999). Normal development in *C. elegans* is arrested by numerous toxicants, returning to a normal pattern, once more favorable conditions are re-encountered (Donohoe *et al.*, 2006; Bruinsma *et al.*, 2008). Exposure of WT and *skn-1 (ok2315)* L4-larvae stage worms to MeHg induced a developmental delay in a dose-dependent manner, which was accentuated in mutant worms. GEE had no effect on the development of WT worms, however, it decreased the percentage of *skn-1 (ok2315)* worms with developmental delay after MeHg exposure in comparison to GEE-untreated worms.

GEE effectively protected *skn-1 (ok2315)* worms from MeHg-induced decrease in pharyngeal pumping and thrashes. Although GEE- untreated *skn-1 (ok2315)* worms also recovered their movements 24-hour post MeHg exposure, pre-treatment with the extract, accelerated the worms recovery. Thus, GEE might decrease MeHg toxicity by increasing antioxidant defenses and/or increasing MeHg excretion, as well as the rate of cellular repair in damaged worms (Zhou *et al.*, 2012; Pohanka, 2015). However, GEE did not protect WT worms.

Analogous to behavior and development, gene expression was differentially affected by GEE exposure when compared to WT and *skn-1 (ok2315)* worms 24-hour post MeHg exposure. Expression of *aat-2* and *sir-2.1* was decreased in WT worms pre-treated with GEE, while expression of *mtl-1*, *mtl-2*, *aat-2*, *sod-3* and *sir-2.1* was increased in *skn-1 (ok2315)* worms pre-treated with GEE in comparison to untreated worms. Synthesis of metallothioneins, cysteine-rich metal biding proteins, are induced by metal exposure and other stressors, and they are involved in heavy metal detoxification and homeostasis (Aschner *et al.*, 2006; Martinez-Finley e Aschner, 2011). *C. elegans* has 2 isoforms of metallothioneins, independently encoded by *mtl-1* and *mtl-2* (Freedman *et al.*, 1993), which were increased by GEE in *skn-1 (ok2315)* worms exposed to MeHg. In *skn-1 (ok2315)* worms, GEE also increased the expression of sirtuins, which lead to activation and expression of antioxidant genes (Brunet *et al.*, 2004), such as *sod-3*, which plays a critical role in the defense of cells against the toxic effects of oxygen radicals.

MeHg is lipid soluble and may distribute throughout the organism by diffusion, but it also has a high affinity for thiol groups (Hughes, 1957). MeHg-L-cysteine conjugates are structurally similar to L-methionine, and thus by molecular mimicry enter cells through the L-

type large neutral amino acid transporter 1 (LAT1) (Aschner e Clarkson, 1988). *C. elegans* have nine genes encoding amino acid transporters and *aat-1* through *aat-3* have the highest homology to LAT1 (Veljkovic *et al.*, 2004). It was previously shown that knockdown of these three genes increased resistance of worms exposed to MeHg (Caito *et al.*, 2013). However, the function and localization of these transporters in worms has yet to be characterized. Herein, *aat-2* expression was increased in GEE-treated *skn-1 (ok2315)* worms exposed to MeHg, thus a potential corresponding increase in AAT-2 protein expression might hasten the extracellular transport of MeHg.

Combined, our data suggest that GEE might exert protective effects against MeHg-induced toxicity by modulating genes related to metal transport, detoxification and antioxidant responses. In individuals exposed to pollutants (Chin-Chan *et al.*, 2015), disease (Delmas-Beauvieux *et al.*, 1995; Rahal *et al.*, 2014) and nutritional deficiencies (John *et al.*, 2002), routine consumption of guarana might be helpful in restoring cellular protection, such as is shown herein for MeHg-induced toxicity.

5. Conclusion

The effects of guarana ethanolic extract (GEE) on MeHg-induced toxicity were investigated in *C. elegans*. GEE afforded a protective effect in *skn-1 (ok2315)* worms, an effect likely modulated by upregulation of genes involved in metal transport, detoxification and antioxidant response. These mutant worms (*skn-1 (ok2315))* produce high levels ROS and are more susceptible to environmental stresses, thus mimicking physiological situations of unbalanced ROS production commonly inherent to human populations exposed to a myriad of xenobiotics. Therefore, routine consumption of guarana might be helpful in protecting against MeHg-induced toxicity in organisms subject to harmful environmental stress conditions.

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Legend of figures

Fig. 1. Dose-response curves of *C. elegans* lethality upon 6-hour MeHg (0 - 150μM) exposure and a 24-hour recovery period ± pre-treatment with guarana ethanolic extract (GEE). GEE had no effect on wild type (WT) worms (LD50≈66 μM) (A) but significantly increased survival in *skn-1(ok2315)* worms (LD50=38, 45, 51 and 59 μM to untreated and GEE pre-treated worms at 100, 500 and 1,000 μg/ml, respectively) (B). Data were compared with extra sum-of-squares F test method, $p<0.05$, and are expressed as means ± SEM, *n=4,* approximately 100 worms analyzed per group in each assay.

Fig.2. *C. elegans* development in wild type (A) and *skn-1* (*ok2315*) (B) worms 24 hours post 6-hour MeHg-exposure. (#) represents a significantly difference between control and metal-exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$). Pre treatment with GEE significantly decreased the percentage of worms delayed in the mutant strain at 25 (C) and 50 μM of MeHg (D). ($^{a-b}$) represents significant difference between GEE doses and (*) represents significant difference from untreated group by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$). Data are expressed as means ± SEM, $n=4$, at least 30 worms analyzed per group in each assay.

Fig.3. Pharyngeal pumping rate in WT and *skn-1* (*ok2315*) worms at 0 hours (A and B) and 24 hours (C and D) post 6-hour MeHg exposure. (#) represents a significantly difference between control and metal exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$) and (*) represents a significantly difference between untreated and GEE-pre treated worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$). Data are expressed as means ± SEM, $n=4$, 10 worms analyzed per group in each assay.

Fig.4. Number of thrashes in WT and *skn-1*(*ok2315*) worms 0 hours (A and B) and 24 hours (C and D) post 6-hour MeHg exposure. (#) represents a significantly difference between control and metal exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$) and (*) represents a significantly difference between untreated and GEE-pre treated worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($p<0.05$). Data are expressed as means ± SEM, $n=4$, 10 worms analyzed per group in each assay.

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Figure 1

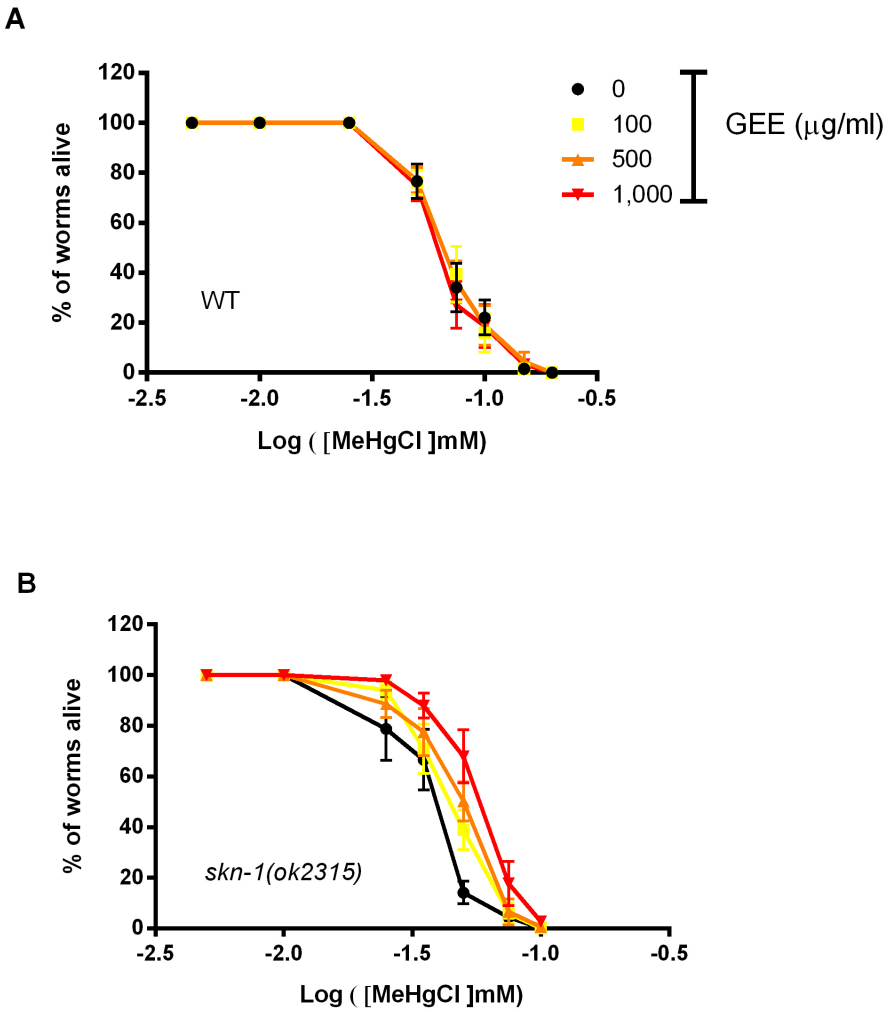

Fig. 1. Dose-response curves of *C. elegans* lethality upon 6-hour MeHg (0 - 150µM) exposure and a 24-hour recovery period ± pre-treatment with guarana ethanolic extract (GEE). GEE had no effect on wild type (WT) worms (LD50≈66 µM) (A) but significantly increased survival in *skn-1(ok2315)* worms (LD50=38, 45, 51 and 59 µM to untreated and GEE pre-treated worms at 100, 500 and 1,000 µg/ml, respectively) (B). Data were compared with extra sum-of-squares F test method, $p<0.05$, and are expressed as means ± SEM, $n=4$, approximately 100 worms analyzed per group in each assay.

Figure 2

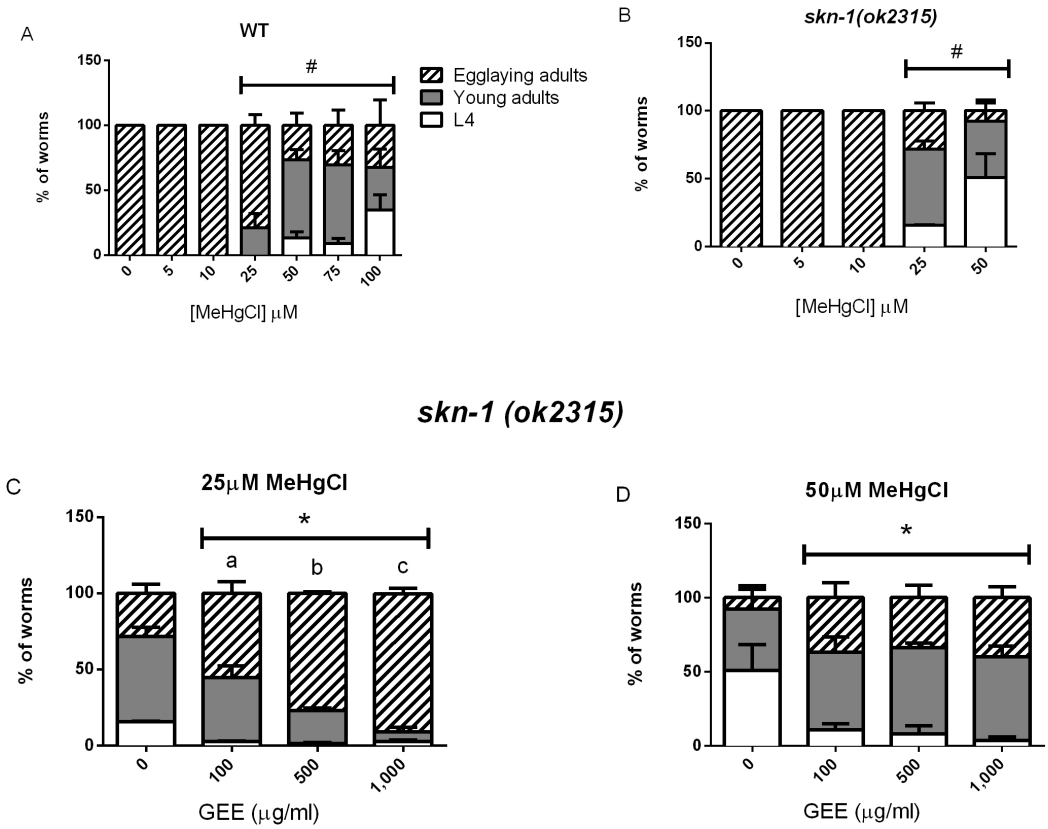

Fig.2. *C. elegans* development in wild type (A) and *skn-1* (*ok2315*) (B) worms 24 hours post 6-hour MeHg-exposure. (#) represents a significantly difference between control and metal-exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05). Pre treatment with GEE significantly decreased the percentage of worms delayed in the mutant strain at 25 (C) and 50 μM of MeHg (D). ($^{a-b}$) represents significant difference between GEE doses and (*) represents significant difference from untreated group by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05). Data are expressed as means ± SEM, n=4, at least 30 worms analyzed per group in each assay.

Figure 3

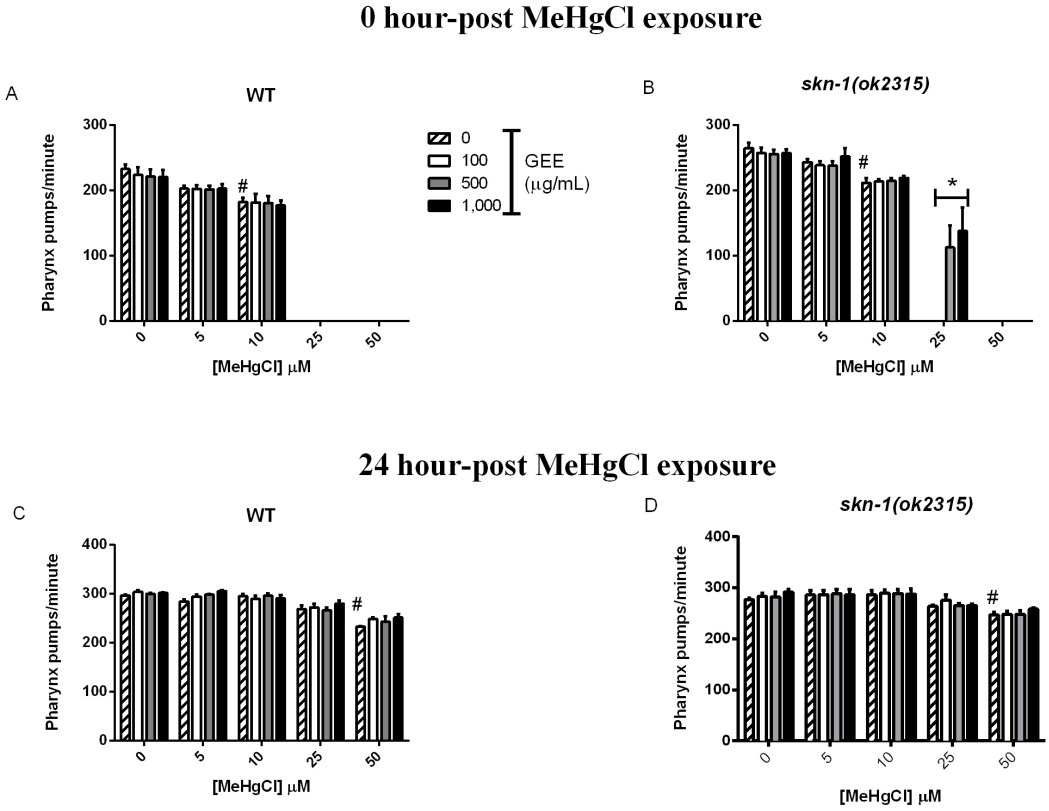

Fig.3. Pharyngeal pumping rate in WT and *skn-1* (*ok2315*) worms at 0 hours (A and B) and 24 hours (C and D) post 6-hour MeHg exposure. (#) represents a significantly difference between control and metal exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05) and (*) represents a significantly difference between untreated and GEE-pre treated worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05). Data are expressed as means ± SEM, n=4, 10 worms analyzed per group in each assay.

Figure 4

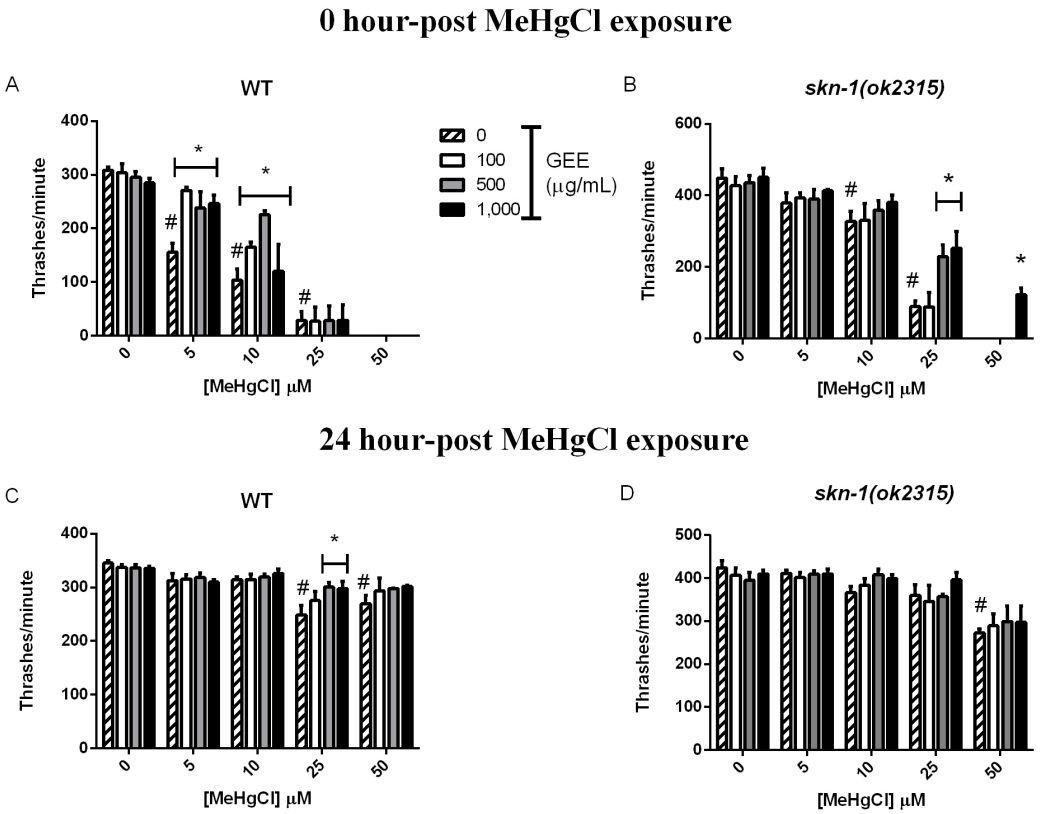

Fig.4. Number of thrashes in WT and *skn-1*(*ok2315*) worms 0 hours (A and B) and 24 hours (C and D) post 6-hour MeHg exposure. (#) represents a significantly difference between control and metal exposed worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05) and (*) represents a significantly difference between untreated and GEE-pre treated worms by two-way ANOVA followed by Bonferroni's Multiple Comparison Test (p<0.05). Data are expressed as means ± SEM, n=4, 10 worms analyzed per group in each assay.

Table 1. Fold change in mRNA expression of genes associated with metal transport, detoxyfication and antioxidant response in *skn-1*(*ok2315*) worms 24 hours post MeHg exposure

MeHgCl	0 µM		5 µM		25 µM		50 µM	
GEE / Gene	0 (µg/ml)	1000 (µg/ml)	0 (µg/ml)	1000 (µg/ml)	0 (µg/ml)	1000 (µg/ml)	0 (µg/ml)	1000 (µg/ml)
mtl-1	+0.01e-2 (0.04)	+0.24 (0.13)	-0.15 (0.33)	+0.07 (0.31)	+1.16 (0.79)	**+1.91 $** (0.60)	+0.32 (0.56)	**+5.25 *$** (0.69)
mtl-2	+0.06e-8 (0.03)	+0.31 (0.17)	-0.14 (0.20)	**+1.36 $*** (0.37)	-0.26 (0.10)	+0.01 (0.30)	-0.58 (0.08)	+0.08 (0.16)
aat-2	+0.09e-8 (0.04)	+0.01 (0.14)	+0.26 (0.15)	**+1.6 $*** (0.47)	-0.29 (0.09)	+0.31 (0.17)	-0.27 (0.08)	-0.30 (0.04)
sod-3	-0.01e-2 (0.03)	+0.24 (0.14)	-0.35 (0.04)	**+1.11 $*** (0.24)	-0.22 (0.08)	**+0.48 *** (0.26)	**-0.86 #** (0.02)	**-0.63 $** (0.08)
sir-2.1	+0.52e-9 (0.05)	+0.26 (0.08)	+0.02 (0.06)	**+1.26 $*** (0.21)	-0.10 (0.09)	**+0.35*** (0.01)	-0.27 (0.06)	-0.03 (0.08)

means significant difference between worms exposed and non-exposed to MeHgCl, $ means significant difference between GEE pre treated worms exposed and non exposed to MeHgCl and * means significant difference between GEE pre treated and untreated worms by two-way ANOVA followed by Bonferroni´s Multiple Comparison test ($p<0.05$)
This experiment was assessed by RT-qPCR. and carried out in three independent worm preparations, each in triplicate. Data are expressed as means ±SEM in parentheses.

Table 2. Fold change in mRNA expression of genes associated with metal transport, detoxyfication and antioxidant response in WT worms 24 hours post MeHg exposure

MeHgCl	0 µM		5 µM		25 µM		50 µM	
GEE / Gene	0 (µg/ml)	1000 (µg/ml)	0 (µg/ml)	1000 (µg/ml)	0 (µg/ml)	100 (µg/ml)	0 (µg/ml)	1000 (µg/ml)
mtl-1	+0.02e-4 (0.07)	+0.23 (0.26)	-0.03 (0.29)	-0.43 (0.06)	+0.05 (0.30)	-0.17 (0.08)	-0.49 (0.13)	**-0.65 $** 0.06
mtl-2	-0.02 (0.03)	+0.18 (0.26)	+0.04 (0.25)	-0.37 (0.10)	-0.54 (0.18)	-0.41 (0.12)	**-0.79 #** (0.07)	-0.79 0.03
aat-2	+0.13 (0.04)	-0.07 (0.14)	**+1.3 #** (0.14)	**+0.67 *$** (0.12)	+0.33 (0.19)	+0.11 (0.07)	+0.52 (0.10)	**-0.16 *** (0.11)
sir-2.1	+0.01 (0.02)	-0.40 (0.02)	-0.27 0.15	-0.21 0.04	+0.02 (0.25)	-0.19 (0.05)	-0.58 (0.06)	**-0.11 *** (0,19)
gst-4	+0.03e-2 (0.05)	+0.26 (0.29)	+0.82 (0.44)	**+3.6 $*** (0.54)	+0.40 (0.33)	+0.57 (0.44)	+0.32 (0.35)	+0.34 (0.45)

means significant difference between worms exposed and non-exposed to MeHg, $ means significant difference between GEE pre treated worms exposed and non exposed to MeHg and * means significant difference between GEE pre treated and untreated worms by two-way ANOVA followed by Bonferroni´s Multiple Comparison test ($p<0.05$).
This experiment was assessed by RT-qPCR carried out in three independent worm preparations, each in triplicate. Data are expressed as means ±SEM in parentheses.

4 DISCUSSÃO

Há algumas décadas, os possíveis efeitos tóxicos e farmacológicos de extratos vegetais e de outros compostos eram investigados com base em resultados de estudos *in vitro*. Reações químicas específicas, tecidos homogeneizados ou proteínas purificadas eram utilizados e, quando compostos efetivos fossem identificados, seriam, então, testados em um animal inteiro (Misra, 1998; Artal-Sanz *et al.*, 2006; Nile *et al.*, 2012). Esses ensaios ainda são bastante úteis, porém, quando analisados *in vivo*, os compostos podem exibir toxicidade, falta de especificidade ou até nenhum efeito, resultando em perda de tempo e de dinheiro (Artal-Sanz *et al.*, 2006). Outro fator negativo é que pesquisas em alvos específicos direcionam o descobrimento de moduladores moleculares específicos. Dificilmente revelam novos alvos terapêuticos e, ainda, a hierarquia de processos celulares e efeitos sinérgicos em diferentes sítios não são considerados (Artal-Sanz *et al.*, 2006).

Neste trabalho, o nematódeo *Caenorhabditis elegans* foi utilizado como modelo experimental para estudos toxicológicos e farmacológicos dos extratos de *Luehea divaricata* e *Paullinia cupana*. Esse modelo é bastante conveniente, pois une as vantagens de ensaios *in vitro* e *in vivo,* permitindo a avaliação simultânea de efeito e toxicidade de forma rápida e barata (Artal-Sanz *et al.*, 2006; Leung *et al.*, 2008).

O extrato de *L. divaricata* apresentou atividade antioxidante sobre diferentes prooxidantes, entretanto seu efeito *in vitro* e em *C. elegans* não foi diretamente relacionado. Ao contrário dos resultados obtidos *in vitro,* onde o extrato neutralizou os radicais óxido nítrico (NO□) liberados pelo nitroprussiato de sódio (NPS) e não quelou o Fe^{2+}, a pré exposição ao extrato, aumentou a sobrevivência dos nematódeos adultos expostos à juglone (5-hidroxi-1,4-naftalenodiona) e ao Fe^{2+} mas não ao NPS e ao peróxido de hidrogênio (H_2O_2). Ensaios *in vitro* consideram somente a reação direta entre as moléculas do extrato e do prooxidante e não a ação do extrato sobre uma situação de estresse oxidativo, que envolve a alteração de diversas reações bioquímicas e resulta em danos a macromoléculas (Halliwell, 2008; Yu *et al.*, 2010). Também não são considerados o metabolismo dos constituintes do extrato e a possível ativação ou inativação de moléculas, bem como a modulação de processos celulares através de interações com enzimas e DNA, por exemplo (Artal-Sanz *et al.*, 2006; Leung *et al.*, 2010).

Como demonstrado em análises de outras espécies vegetais (Carlini, 2003; Pietrovski *et al.*, 2006; Pereira *et al.*, 2009), o extrato bruto de *L. divaricata* também exibiu maior potencial farmacológico quando comparado a um dos seus principais constituintes ativos

isolado, a rutina. Grande parte das propriedades de extratos vegetais estão relacionadas a efeitos sinérgicos de diversos compostos. Assim, extratos brutos oferecem maiores vantagens em relação a compostos isolados, pois apresentam maior atividade farmacológica, oferecem um menor custo, possuem toxicidade mais baixa e são a forma utilizada tradicionalmente pela população (Carlini, 2003; Pietrovski *et al.*, 2006; Pereira *et al.*, 2009).

Neste estudo, optou-se por utilizar a rutina nas mesmas concentrações do extrato em vez da concentração presente no mesmo, Isso porque compostos em concentrações muito baixas dificilmente são absorvidos em *C. elegans.* A pequena absorção pode ser resultado da baixa permeabilidade da cutícula e da baixa ingestão como uma forma de defesa do animal (Johnson, 2003; Gruber *et al.*, 2009). Por exemplo, foi mostrado que a concentração interna de etosuximida (anticonvulsivante) em nematódeos expostos a 2 mg/mL foi de 30 μg/mL, ou seja, apenas 1,5% da concentração externa (Ballard *et al.*, 2011).

Os extratos de *L. divaricata* e *P. cupana* não demonstraram efeitos sobre a sobrevivência e sobre o desenvolvimento larval de *C. elegans*. Os nematódeos foram também avaliados quanto à taxa de batimentos faríngeos, à locomoção e à duração da defecação. Esses ensaios foram escolhidos por serem simples e permitirem a análise toxicológica em diferentes sistemas neuronais (Qiao *et al.*, 2014). A faringe é responsável por sugar a bactéria do meio externo para o intestino e é regulada por acetilcolina, glutamato e serotonina (Avery e You, 2012). A locomoção do nematódeo se dá através de movimentos sinusóides e pode ser analisada em ágar, através da observação do número curvaturas do corpo (*body bends*), ou em líquido, também conhecido como nado (*thrashe*s), sendo que o primeiro está mais relacionado ao sistema colinérgico e o segundo ao sistema gabaérgico. A defecação é regulada pelo ácido gama amino butírico (GABA) e acetilcolina. Uma série de outros comportamentos já foram descritos, incluindo ensaios de quimiotaxia, resposta ao toque e decréscimo da locomoção na presença de alimento, por exemplo (Jorgensen, 2005; Rand, 2007; Hart, 2006).

O extrato de *P. cupana* não alterou nenhum desses comportamentos no nematódeo jovem, enquanto o extrato de *L. divaricata* aumentou a taxa de batimentos faríngeos. A acetilcolina é o principal neurotransmissor excitatório nas junções neuromusculares dos nematódeos e é liberada por mais de um terço das células do sistema nervoso de *C. elegans* (Hart, 2006). A atividade da enzima acetilcolinesterase foi então dosada nos nematódeos expostos ao extrato, mostrando, pela primeira vez, um efeito anticolinesterásico causado pelo extrato de *L. divaricata in vivo*. De fato, a rutina foi reportada anteriormente como inibidora da acetilcolinesterase em plasma humano *in vitro* (Katalinic *et al.*, 2010). Além disso, em um estudo posterior realizado em nosso laboratório, demonstrou-se o efeito antioxidante e

anticolinesterásico do extrato aquoso das folhas de *L. divaricata* em modelo de doença de Huntington em cérebro de ratos (Courtes *et al.*, 2015). Isso mostra que há relação entre os efeitos observados em *C. elegans* neste trabalho e os resultados em mamíferos já descritos. E, ainda, que o extrato pode vir a ser benéfico no tratamento de doenças relacionadas ao estresse oxidativo e à perda do tônus colinérgico como, por exemplo, a Doença de Alzheimer (Ballard *et al.*, 2011).

Os possíveis efeitos do extrato de *P. cupana* foram investigados também durante o envelhecimento de *C. elegans*. Como previamente descrito em roedores (Mattei *et al.*, 1998), o uso prolongado de guaraná parece ser atóxico e seguro para um organismo inteiro. O extrato também estendeu o tempo de vida médio (tempo onde 50% da população está morta) e máximo de *C. elegans* (tempo onde 90% da população está morta). Para investigar possíveis mecanismos envolvidos nesse efeito, foram utilizadas cepas transgênicas.

Observou-se que o extrato age através de efeito antioxidante e da modulação das vias de sinalização DAF-16/FOXO (*forkhead transcription factor*) e HSF-1 (*heat shock transcription factor-1*), que atuam em cooperação para ativar genes específicos, incluindo os da família de proteínas de choque térmico *sHsp* (*small heat shock proteins genes*), envolvidos na regulação da longevidade e conservados entre espécies animais (Hsu *et al.*, 2003). DAF-16 de *C. elegans* é ortólogo da FOXO de mamíferos e é um fator de transcrição abaixo do receptor DAF-2, ortólogo do receptor de insulina/IGF-1 (*Insulin-like growth factor 1*). DAF-16/FOXO é ativado por fosforilação quando o receptor não está ocupado e desloca-se do citosol para o núcleo das células, induzindo a expressão de genes relacionados à longevidade e à resistência ao estresse (Torgovnick *et al.*, 2013). O possível efeito antimicrobiano de *P. cupana* foi também investigado, uma vez que a diminuição na disponibilidade de *E.coli* pode resultar em restrição calórica. Essa situação ativa diferentes vias de sinalização que resulta na extensão da longevidade em diferentes espécies animais (Baur, 2010; Torgovnick *et al.*, 2013). Entretanto, o extrato não causou efeito sobre a bactéria.

A análise da expressão gênica demonstrou diminuição da expressão dos genes antioxidantes *gst-4* (glutationa-S-transferase), *sir-2.1* (sirtuína, proteína deacetilase dependente de nicotinamida adenina dinucleotídeo - NAD) e *hsf-1* nos nematódeos no estágio L4 após exposição ao extrato de *P. cupana*. Um baixo nível de estresse oxidativo pode ter sido induzido pelo extrato nos estágios iniciais da vida do animal, o que tem sido associado a alterações na expressão de genes e no metabolismo que resultam em extensão do tempo de vida (Rattan *et al.*, 2007). Por outro lado, a diminuição na expressão de *skn-1*, *sir-2.1* e *hsp-16.2* em adultos velhos de 9 dias tratados com o extrato pode ser resultado da menor

necessidade de ativação de defesas antioxidantes para reparar danos celulares ocorridos durante o envelhecimento, já que os animais tratados são mais saudáveis que os não tratados. Alguns estudos já mostraram a capacidade de compostos agirem como antioxidantes e prooxidantes por mecanismos distintos (Sautin e Johnson, 2008; Samec *et al.*, 2015).

Metilxantinas, como a cafeína, são os principais componentes do guaraná e atuam em receptores de adenosina em mamíferos (Chen, J. F. *et al.*, 2013). A cafeína tem sido associada a diversos efeitos benéficos relacionados ao envelhecimento (Laurent *et al.*, 2014; Rivera-Oliver e Diaz-Rios, 2014) (Cunha e Agostinho, 2010) e alguns estudos já mostraram o seu efeito em *C. elegans,* inclusive na extensão do tempo de vida (Lublin *et al.*, 2011; Sutphin *et al.*, 2012; Bridi *et al.*, 2015). Porém, o sistema purinérgico em *C. elegans* não está bem descrito (Burnstock e Verkhratsky, 2009). Dessa forma, uma cepa *knockout* para um receptor ortólogo de adenosina (ADOR-1) (Shaye e Greenwald, 2011) foi utilizada e foi possível observar o envolvimento da modulação do sistema purinérgico na extensão do tempo de vida. Porém, apesar do extrato ser rico em cafeína, os efeitos do guaraná parecem estar relacionados ao efeito sinérgico de diferentes metilxantinas ou outros componentes, uma vez que a concentração de cafeína no extrato é muito menor que as concentrações efetivas previamente demonstradas (Pietrovski *et al.*, 2006; Adebajo *et al.*, 2009; Bridi *et al.*, 2015).

Devido aos custos associados à manutenção da saúde e ao bem-estar de idosos, é mais interessante que compostos não apenas estendam o tempo de vida, mas também melhorem a qualidade de vida, estendendo o tempo de vida saudável. O extrato retardou o declínio dos batimentos faríngeos e da locomoção durante o envelhecimento, marcadores fisiológicos sensíveis do envelhecimento de *C. elegans* (Huang *et al.*, 2004). Como os nematódeos são transparentes, foi possível relacionar esses resultados à observação da redução nos níveis de lipofuscina intestinal e no número de agregados proteicos, que se acumulam durante o envelhecimento.

A lipofuscina consiste em proteínas e lipídios oxidados originados de materiais celulares não digeridos após processos de fagocitose e autofagia que se acumulam como grânulos citoplasmáticos fluorescentes em condições fisiológicas e patológicas. Como não é degradada, acumula-se nas células e é conhecida como um "pigmento do envelhecimento" em invertebrados e vertebrados, incluindo humanos (Hosokawa *et al.*, 1994; Gerstbrein *et al.*, 2005). A agregação proteica resulta da oxidação de proteínas ou de alterações em sua estrutura e tem sido relacionada ao desenvolvimento de doenças neurodegenerativas associadas à idade em mamíferos (Morley *et al.*, 2002; Ballard *et al.*, 2011; Park e Kim, 2013).

O efeito do extrato de guaraná sobre a longevidade parece estar relacionado à diminuição do número de agregados protéicos através da modulação de DAF-16/FOXO e HSF-1. DAF-16/FOXO está envolvida na formação de agregados proteicos menos tóxicos (Ushikubo *et al.*, 2014) e, apesar de HSF-1 regular a atividade de desagregação proteica liberando agregados pequenos e tóxicos, parece exercer efeito benéfico contribuindo para a metabolização das proteínas através de metabolismo enzimático (Iwata *et al.*, 2001; Leissring *et al.*, 2003). Um estudo utilizando células neuronais humanas também descreveu um possível efeito benéfico do guaraná na Doença de Alzheimer pela prevenção da agregação da proteína beta-amiloide (Bittencourt Lda *et al.*, 2014).

A toxicidade de contaminantes ambientais também pode ser pesquisada em *C. elegans* (Roh *et al.*, 2006; Leung *et al.*, 2008). Estudos mostram que o grau de toxicidade nesses nematódeos correlaciona-se com o grau de toxicidade em roedores (Cole *et al.*, 2004; Leung *et al.*, 2008). Apesar do MeHg exercer efeitos tóxicos em *C. elegans*, em parte pela indução de estresse oxidativo (Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002; Helmcke *et al.*, 2009; Martinez-Finley e Aschner, 2011; Farina *et al.*, 2013), os nematódeos adultos são mais resistentes comparados aos mamíferos (Helmcke *et al.*, 2009; Sumathi e Christinal, 2015). Neste trabalho, o MeHg causou diminuição da locomoção e dos batimentos faríngeos de forma dose dependente até à inibicão total. Entretanto, após 24 horas de recuperação, os nematódeos retomaram quase totalmente seus movimentos. Esse fenômeno parece envolver diferentes conjuntos de neurônios que atuam modulando a robustez das sinapses, a excreção de metabólitos, a conservação de energia e a biossíntese de macromoléculas (Hill *et al.*, 2014; Trojanowski *et al.*, 2015; Trojanowski e Raizen, 2016) resultando no reparo dos danos e com consequente recuperação dos nematódeos.

O extrato de *P. cupana* aumentou a resistência de nematódeos da cepa *skn-1(ok2315)* frente à toxicidade do MeHg, mas não da cepa selvagem. A cepa *skn-1(ok2315)* possui níveis mais elevados de espécies reativas e é mais suscetível a estressores externos quando comparada à cepa selvagem, pois tem expressão de *skn-1* diminuída (Kobayashi e Yamamoto, 2006; Martinez-Finley *et al.*, 2013). SKN-1 é ortólogo do Nrf2 (*nuclear factor erythroid 2-related factor 2*) de mamíferos, um fator de transcrição que regula a expressão de uma série de enzimas de detoxificação, incluindo a glutationa-S-transferase, envolvida na resposta ao estresse oxidativo (Kensler *et al.*, 2007; Taylor *et al.*, 2008).

A pré exposição ao extrato de *P. cupana* atenuou os efeitos tóxicos do MeHg na cepa *skn-1(ok2315)*, observado pelo menor dano aos movimentos dos nematódeos e uma recuperação mais rápida em comparação aos nematódeos não expostos ao extrato. Alguns

mecanismos pelos quais o extrato de *P. cupana* pode exercer seus efeitos foram investigados através da expressão de alguns genes. A expressão de *mtl-1*, *mtl-2* (metalotioneínas isoformas 1 e 2), *aat-2* (ortólogo do transportador de aminoácidos neutros de mamíferos - LAT1), *sir-2.1* e consequentemente *sod-3* (superóxido dismutase 3, mitocondrial), que é um dos alvos de regulação de *sir-2.1* foi aumentada somente nos nematódeos da cepa *skn-1(ok2315)* pré tratados com *P.cupana* em relação aos nematódeos não tratados e aos nematódeos da cepa selvagem. Portanto, o extrato exerce seus efeitos protetores, pelo menos em parte, através da diminuição do estresse oxidativo induzido por MeHg, aumento da detoxificação do metal pela adição de metalotioneínas e alteração do transporte do metal transmembrana. Uma vez que as pessoas estão constantemente expostas a situações que podem causar desequilíbrios na homeostase do organismo, como poluentes (Chin-Chan *et al.*, 2015), doenças (Delmas-Beauvieux *et al.*, 1995; Rahal *et al.*, 2014) e deficiências nutricionais (John *et al.*, 2002), o consumo de guaraná pode ser benéfico frente à toxicidade induzida por MeHg.

Assim, o nematódeo *C. elegans* possibilitou o estudo de efeitos toxicológicos e farmacológicos dos extratos de *L. divaricata* e *P. cupana,* a pesquisa simples dos mecanismos de ação e o direcionamento para pesquisas futuras. Foi possível também especular o envolvimento do sistema purinérgico como um novo alvo terapêutico para a modulação do envelhecimento.

5 CONCLUSÕES

Baseando-se nos resultados obtidos neste trabalho, pode-se concluir que:

- O extrato hidroalcoólico das folhas de *L. divaricata* (açoita-cavalo) aumenta a resistência ao estresse oxidativo e o tônus colinérgico em um organismo inteiro;

- O extrato hidroalcoólico das sementes de *Paullinia cupana* (guaraná) estende a longevidade e melhora a qualidade de vida durante o envelhecimento através de atividade antioxidante e da modulação das vias DAF-16/FOXO e HSF-1;

- A sinalização purinérgica parece estar envolvida no processo de envelhecimento;

- O extrato hidroalcoólico das sementes de *P. cupana* protege da toxicidade induzida pelo metilmercúrio através do aumento da expressão de genes relacionados ao transporte (*aat-2*) e detoxificação (*mtl-1* e *mtl-2)* do metal e à resposta antioxidante (*sir-2.1* e *sod-3);*

- Há correlação com os resultados de estudos utilizando animais superiores, demonstrando que *C. elegans* é um excelente organismo para a investigação de efeitos tóxicos e farmacológicos de extratos vegetais.

6 PERSPECTIVAS

De acordo com os resultados obtidos, tem-se como perspectivas:

- Investigar o possível efeito do extrato de *Luehea divaricata* e de seus constituintes em modelo de Doença de Alzheimer em *C. elegans*;

- Investigar os efeitos de *Paullinia cupana* e de seus constituintes sobre o metabolismo de *C. elegans* durante o envelhecimento e após a exposição ao metilmercúrio;

- Investigar a participação do sistema purinérgico na modulação do envelhecimento em *C. elegans* através da quantificação da atividade de enzimas purinérgicas durante o envelhecimento;

- Investigar os processos bioquímicos envolvidos na recuperação de *C. elegans* após danos causados por metilmercúrio.

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