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Daniele Coradini Zamberlan

EFEITO PROTETOR DE EXTRATOS NATURAIS NO ENVELHECIMENTO E EM MODELOS DE DOENÇA DE ALZHEIMER NO NEMATÓDEO *Caenorhabditis elegans*

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

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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meus pais Marli e Roberto e meu irmão Daniel,
por serem meu porto seguro e a luz que me orienta.
Com todo meu amor e gratidão!

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A GENTE ESTUDA, ESTUDA, ESTUDA...
...E APRENDE A DAR RESPOSTAS!
QUANDO VAMOS APRENDER A FAZER AS PERGUNTAS?

APRESENTAÇÃO

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

O DESENVOLVIMENTO da tese está apresentado sob a forma de dois artigos, os quais se encontram alocados no item ARTIGOS CIENTÍFICOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

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

Os itens CONCLUSÕES e PERSPECTIVAS são encontrados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e DISCUSSÃO, uma vez que o artigo científico contém as suas próprias referências.

RESUMO

EFEITO DE COMPOSTOS NATURAIS NO ENVELHECIMENTO E EM MODELOS DE DOENÇA DE ALZHEIMER NO NEMATÓDEO *Caernorhabditis elegans*

Autor: Daniele Coradini Zamberlan
Orientador: Félix Alexandre Antunes Soares

Durante o envelhecimento, os sistemas de reparo, incluindo a resposta adaptativa ao estresse oxidativo e a degradação de proteínas danificadas, diminuem. Nas últimas décadas, o aumento contínuo na expectativa de vida na população, somado a fatores genéticos e ambientais, tem aumentado significativamente a incidência de doenças relacionadas ao envelhecimento, em especial as neurodegenerativas, como a Doença de Alzheimer (DA). A neuropatologia da DA é caracterizada principalmente pela presença de placas amiloides no cérebro formadas pela agregação da proteína β-amiloide (βA). Compostos antioxidantes provindos de fontes naturais têm demostrados diversos efeitos benéficos, principalmente relacionados ao envelhecimento. Além disso, vias de sinalização que regulam a expressão gênica em resposta ao estresse têm sido alvos na pesquisa por genes envolvidos na longevidade. Desta forma, este trabalho tem como objetivos avaliar os efeitos *in vivo* do tratamento crônico com o extrato etanólico de *Rosmarinus officinalis* (eeRo) no envelhecimento e de *Paullinia cupana* (GEE) em modelos de DA no nematódeo *Caernorhabditis elegans*, bem como elucidar seus mecanismos. Para isso foi utilizada a cepa selvagem e cepas transgênicas com expressão da βA como modelos de DA. Foram realizados ensaios comportamentais, de resistência a diferentes tipos de estresse, longevidade, quantificação de espécies reativas de oxigênio (ERO) e dos níveis da proteína βA e silenciamento de genes envolvidos na resposta ao estresse. Os dados demonstraram que o tratamento com eeRo diminui os níveis de ERO e aumenta a longevidade e a resistência ao estresse, de forma dependente dos fatores de transcrição daf-16, hsf-1 e skn-1. Em relação ao GEE, os dados demonstraram que o tratamento reduziu os níveis da βA, retardando seus efeitos tóxicos através da ativação da HSF-1. Assim, neste estudo foram demonstrados compostos capazes de reforçar os sistemas de reparo celular, oferecendo novas alternativas contra o envelhecimento e, ainda, no caso do GEE, prevenção contra a agregação proteica observada na DA.

Palavras-chaves: Envelhecimento; Doenças Neurodegenerativas; Alzheimer; β-amilóide; *Caenorhabditis elegans*

ABSTRACT

EVALUATION OF NATURAL COMPOUNDS IN THE NEMATODE *Caernorhabditis elegans* AGING AND ALZHEIMER DISEASE MODELS

Author: Daniele Coradini Zamberlan
Advisor: Félix Alexandre Antunes Soares

During ageing, the cell repair system, including adaptative response to oxidative stress and unfolded protein degradation. In last decades, the increasing life expectancy, added to genetic and ambiental factors, has increasing neurodegenerative diseases incidence, as Alzheimer's Disease (AD). Amyloid plaques in brain formed by amyloid-β (Aβ) protein aggregation mainly characterize AD. Antioxidant compounds from natural sources have demonstrated beneficial effects, mainly related to aging. In this way, this study aims to evaluate in vivo effects of chronic treatment with the ethanolic extract of Rosmarinus officinalis (eeRo) on aging and Paullinia cupana (GEE) on AD models in the *Caernorhabditis elegans* nematode, as well as to elucidate its mechanisms. For this, the wild-type strain and transgenic strains with βA expression were used as DA models. Behavioral assays, resistance to different types of stress, longevity, quantification of reactive oxygen species (ROS) and levels of the βA protein and silencing of genes involved in the stress response were performed. The data demonstrated that eeRo treatment decreases ROS levels and increases longevity and stress resistance, in a daf-16, hsf-1 and skn-1 dependent way. Regarding GEE, data demonstrated that the treatment reduced βA levels, delaying its toxic effects through the activation of HSF-1. Thus, in this study, we demonstrated compounds capable of reinforcing the cellular repair systems, offering new alternatives against aging and, related to GEE, prevention against the protein aggregation observed in AD.

Key words: Ageing; Neurodegenerative Diseases; Alzheimer, Amilóide-β; *Caenorhabditis elegans*

LISTA DE ABREVIATURAS E SIGLAS

βA	beta-amilóide
DA	Doença de Alzheimer
DH	Doença de Huntington
DN	Doenças Neurodegenerativas
DP	Doença de Parkinson
eeRo	Extrato etanólico de Rosmarinus officinalis
ELA	Esclerose Amiotrófica Lateral
ERO	Espécies reativas de oxigênio
FOXO	Fator de transcrição *Forkhead Box*
GEE	Extrato etanólico de guaraná
HSF	Fator de choque térmico
HSP	Proteína de choque térmico
IGF-1	Fator de crescimento semelhante a insulina tipo 1
Nrf	Fator nuclear relacionado ao fator eritróide
PPA	Proteina precursora amilóide

SUMÁRIO

1 INTRODUÇÃO

1.1 PROBLEMA

O aumento do estresse oxidativo, uma das principais características do envelhecimento, tem sido implicado em uma variedade de patologias relacionadas à idade, como as neurodegenerativas. No envelhecimento, a produção de oxidantes de várias fontes aumenta, enquanto as enzimas antioxidantes, as principais linhas de defesa, diminuem. Sistemas de reparo, incluindo a resposta adaptativa ao estresse oxidativo e a degradação proteica de proteínas danificadas, também diminuem, desencadeando em patologias.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Avaliar os efeitos do tratamento crônico com extratos naturais *in vivo* no envelhecimento e em modelos de Doença de Alzheimer no nematódeo *Caernorhabditis elegans*, bem como os mecanismos envolvidos.

1.2.2 Objetivos específicos

- Investigar se o tratamento com extrato etanólico de *Rosmarinus officinalis* (eeRo) aumenta a resistência ao estresse e consequentemente, a longevidade em *C. elegans* e determinar vias envolvidas;

- Investigar se o tratamento com o extrato etanólico de guaraná (GEE) diminui a agregação e a toxicidade induzida pela proteína βA em *C. elegans*, bem como os mecanismos envolvidos na proteção frente a toxicidade da proteína βA.

1.3 JUSTIFICATIVA

Tendo em vista a projeção futura para o número de idosos e frequência da DA nesta população, somado ao fato do mecanismo desta patologia não estar bem elucidado, e assim, ainda não possuir cura ou mesmo tratamento eficaz, há a necessidade de estudos na finalidade de atender a esta carência. Somando-se a isso, produtos naturais com potencial antioxidante vêm demonstrando exercer um papel fundamental na prevenção e tratamento de doenças associadas ao envelhecimento e dano oxidativo.

Visando as propriedades farmacológicas já descritas da planta *Rosmarinus officinalis*, este estudo investiga seu possível efeito na proteção frente a diferentes tipos de estresse e consequente redução do estresse oxidativo e aumento do tempo e da qualidade de vida dos animais. Além disso, avalia-se o envolvimento da principal via envolvida nestes efeitos no nematódeo, a via da insulina.

Além disso, em virtude dos efeitos benéficos já descritos do guaraná na proteção frente ao estresse e aumento do tempo e qualidade de vida dos nematódeos, este estudo investiga seu possível efeito frente a toxicidade do peptídio βA em modelos de DA. Ainda, pesquisa-se uma possível modulação da resposta ao estresse mediada por HSF pelo guaraná.

2 REVISÃO DE LITERATURA

2.1 ESTRESSE OXIDATIVO E ENVELHECIMENTO

O envelhecimento é um processo caracterizado pela perda progressiva da função de tecidos e órgãos ao longo do tempo (Flatt, 2012). A homeostase celular é regulada por vários processos biológicos na célula, os quais estão interligados e abrangem uma ampla gama de escalas. Cada um deles está ligado a regulação do envelhecimento a nível celular, afetando o controle do envelhecimento de todo o organismo (Diloreto e Murphy, 2015). O entendimento do processo regulatório global que controla a homeostase celular, proporciona um melhor entendimento do processo de envelhecimento, o que pode nos permitir retardá-lo, bem como melhor tratar e prevenir doenças degenerativas relacionadas ao envelhecimento, melhorando assim a qualidade de vida dos indivíduos (Diloreto e Murphy, 2015).

O controle de qualidade de proteínas é fundamental para a sobrevivência celular tanto em condições fisiológicas quanto patológicas (Falcone e Mazzoni, 2018). Durante o envelhecimento, a habilidade da célula de conservar a funcionalidade do proteoma diminui progressivamente, ocasionando uma agregação de proteínas ao longo do tempo (Koga *et al.*, 2011). Vários sistemas celulares estão ativamente envolvidos no controle de qualidade de proteínas a fim de garantir que estas sejam devidamente sintetizadas, enoveladas e compartimentalizadas. Assim, ele pode atuar pela regulação da expressão gênica, pela degradação de proteínas, ou ainda controlando a estabilidade e tradução do mRNA (Diloreto e Murphy, 2015). Alterações nesse controle podem levar a proteotoxicidade e consequente disfunção da homeostase celular e tem se mostrado como bases fundamentadoras de doenças relacionadas a conformação de proteínas, como as neurodegenerativas (Esser *et al.*, 2004; Morimoto, 2008).

A teoria do envelhecimento do estresse oxidativo é baseada na hipótese do dano estrutural, onde o acúmulo de dano oxidativo a macromoléculas (DNA, lipídeos e proteínas) pelos radicais livres ao longo do tempo acarreta em perdas funcionais (Sohal e Weindruch, 1996). A produção de radicais livres pode ser de origem endógena, relacionada às interações metabólicas, ou exógena, como fatores ambientais. O oxigênio é a principal fonte de radicais livres em sistemas biológicos, porém, é fundamental para os mecanismos celulares e para a geração de energia. Dessa forma, a principal fonte endógena geradora são as mitocôndrias, onde o oxigênio é reduzido em etapas sequenciais para produzir água. Ou seja, ele participa da cadeia de transporte de elétrons da mitocôndria, na qual é reduzido pela citocromo oxidase em água e o NADH é oxidado a NAD+, para que haja a produção de ATP (Sastre *et al.*, 2003).

O aumento das espécies reativas de oxigênio (ERO) conduz a senescência celular, comprometendo a resposta celular a danos durante a replicação. Este mecanismo ocorre através da modulação de componentes secretórios da célula. Entre eles: a inibição da atividade do fator de transcrição Forhead box (FOXO), o qual está inserido na via do tipo insulina mediando proteção frente ao estresse, bem como a indução da expressão de enzimas degradativas, como as metaloproteases, as quais estão associadas ao desenvolvimento de doenças crônicas relacionadas a idade, como a Doença de Alzheimer (DA) (Diloreto e Murphy, 2015). Além disso,

acredita-se que as ERO podem ter função sinalizadora, ativando processos de proteção e adaptação (Ristow e Schmeisser, 2011).

Em contrapartida, as evidências para esta teoria são relativas e inconclusivas (Wickens, 2001), com um número crescente de estudos a contradizendo (Blagosklonny, 2008; Perez *et al.*, 2009; Van Raamsdonk e Hekimi, 2009; Speakman e Selman, 2011). Segundo estes estudos, o dano oxidativo, como qualquer outro dano observado isoladamente ou combinado, não representa a causa do envelhecimento. Viña e colaboradores (2013) propuseram a 'teoria do envelhecimento da alteração da sinalização celular', a qual postula que se a célula não for capaz de lidar e se adaptar ao estresse causado pelas ERO, o dano se instalará induzindo o envelhecimento (Vina *et al.*, 2013). Vadin e Gladyshev (2014) propõem que a heterogeneidade e a imperfeição biológica, geram um inevitável acúmulo de danos nas células, o qual conduzirá as células a senescência (Gladyshev, 2014).

Entretanto, a teoria dos radicais livres ainda é a mais aceita na tentativa de explicar os mecanismos que regem o envelhecimento, pelo fato de possuir argumentos concretos de que sua ação é parte inevitável deste processo. Apesar disso, ela não sustenta a senescência em sua totalidade, demonstrando que o envelhecimento não pode ser compreendido a partir de uma única visão, mas sim como um agregado de múltiplos fatores intrínsecos em diversas teorias já descritas.

Diversos estudos têm demonstrado que alterações nutricionais e manipulações genéticas podem modular o envelhecimento (Katewa e Kapahi, 2010; Kenyon, 2010; Barzilai *et al.*, 2012; Hadem *et al.*, 2017). Ainda, intervenções que induzem aumento do tempo de vida de um organismo, geralmente podem ser extrapolados para outros (Braeckman *et al.*, 2001). Acredita-se que esta modulação da longevidade ocorre, além de outros mecanismos, através da hormese. Ou seja, os tratamentos regulam os níveis de dano, visando as moléculas que os produzem (Anderson e Weindruch, 2010).

2.2 VIAS DE SINALIZAÇÃO DA INSULINA E A RESPOSTA AO ESTRESSE

A resposta dos antioxidantes ao estresse oxidativo evolui como um mecanismo crítico de defesa para combater os efeitos nocivos dos insultos oxidativos intrínsecos e extrínsecos e é preservado em todos os organismos. Nas

últimas décadas, estudos tem elucidado vias de sinalização pelas quais tais respostas são reguladas.

Os fatores de transcrição da família *forkhead box*, classe *O* (FoxO), são importante reguladores da resposta celular ao estresse e promovem a defesa antioxidante celular. FoxO estimula a transcrição de genes codificadores de proteínas antioxidantes localizadas em diferentes compartimentos celulares e extracelulares (Kaestner *et al.*, 2000). As ERO, bem como outros estímulos estressantes, modulam a atividade da FoxO, alterando a síntese e estabilidade proteica (Klotz *et al.*, 2015). FoxO foi primeiramente relacionado a resistência ao estresse através de estudos em *Caenorhabditis elegans* mutantes analisados quanto a traços genéticos que interferiam na longevidade. Foi demonstrado que o gene *daf-16* é especificamente um ortólogo da família FoxO, cuja ativação promove a extensão da vida útil (Kenyon, 2011).

A sinalização através do fator nuclear eritróide tipo 2 (Nrf2) também participa da regulação da expressão basal e induzível de muitas enzimas antioxidantes e do proteassoma (Slocum e Kensler, 2011). A atividade de Nrf2 é regulada em vários níveis, incluindo transcrição, pós-tradução e interações com outras proteínas. O sistema de sinalização Nrf2 emergiu como uma das mais importantes vias de defesa e sobrevivência celular contra toxicidade e estresse oxidativo (Zhang *et al.*, 2015). O bloqueio desta via está associada a uma maior suscetibilidade a insultos oxidativos em seres humanos e organismos modelo (Sykiotis e Bohmann, 2010). O *C. elegans* possui um gene ortologo ao Nrf2 em mamíferos, o *skn-1*, também responsável por mediar respostas adaptativas ao estresse (Blackwell *et al.*, 2015).

O fator de transcrição HSF-1 regula a expressão de genes de resposta ao estresse, como as proteínas de choque térmico (HSP), exibindo diversas propriedades funcionais durante o desenvolvimento e longevidade (Wu, 1995). O HSF-1 é altamente conservado entre humanos e diferentes organismos modelo, como o *C. elegans*. Sua ativação é induzida por estresse agudo e crônico, alterações fisiológicas, envelhecimento e patologias (Morimoto, 2008). A resposta ao choque térmico é uma resposta molecular altamente conservada frente a alterações da homeostase protéica (Morimoto 2008, 2011; Åkerfelt et al. 2010). A estabilidade de cada proteína dentro do proteoma de cada célula e tecido é alcançada através de interações com componentes da rede de proteostase, incluindo chaperonas moleculares, que influenciam o enovelamento, a conformação e a estabilidade, e as

atividades autofágica responsáveis por eliminar proteínas danificadas (Balch et al. 2008; Powers et al. 2009).

Assim, alterações no controle de qualidade de proteínas podem levar a proteotoxicidade e disfunção da homeostase celular, e estão relacionados ao desenvolvimento de doenças relacionadas a conformação de proteínas, como as neurodegenerativas.

2.3 DOENÇAS NEURODEGENERATIVAS

O aumento contínuo na expectativa de vida na população, somado a fatores genéticos e ambientais, tem aumentado significativamente a incidência de doenças relacionadas ao envelhecimento, em especial as neurodegenerativas.

As doenças neurodegenerativas são caracterizadas pela perda progressiva e seletiva de neurônios do sistema nervoso central e consequente declínio das funções cerebrais. Dentre elas, as mais comuns são a doença de Alzheimer (DA), a doença de Parkinson (DP), a doença de Huntington (DH) e a Esclerose Lateral Amiotrófica (ELA). Embora não bem elucidados, os mecanismos envolvidos na morte neuronal envolvem eventos em comum, incluindo agregação de proteínas mal-formadas (como α-sinucleína na DP, β-amilóide na DA, huntingtina na DH, tau nas tauopatias, entre outras), inflamação e estresse oxidativo (Ross e Poirier, 2004).

Entretanto, a gama de sintomas, bem como a classificação da doença, varia dependendo da área afetada. Problemas no movimento, como ataxia, estão presentes na DP, DH e ELA. O declínio progressivo da função cognitiva, o qual afeta a memória, o comportamento, a aprendizagem, a emoção, entre outros, originam a demência, com a DA. Esta pletora de fenômenos patológicos parece preceder as manifestações tardias das doenças (ataxias/demências), fato que oferece esperanças de avanços terapêuticos, uma vez que a neurodegeneração pode encontrar-se em diferentes níveis e ser mediada por diferentes fatores.

2.3.1 Doença de Alzheimer

A DA é a principal causa de demência em idosos. De acordo com o relatório DEMENTIA – a Public Health Priority, no mundo inteiro, em 2010, o número ascendia 35,6 milhões. Anualmente, calcula-se que o número de novos casos de

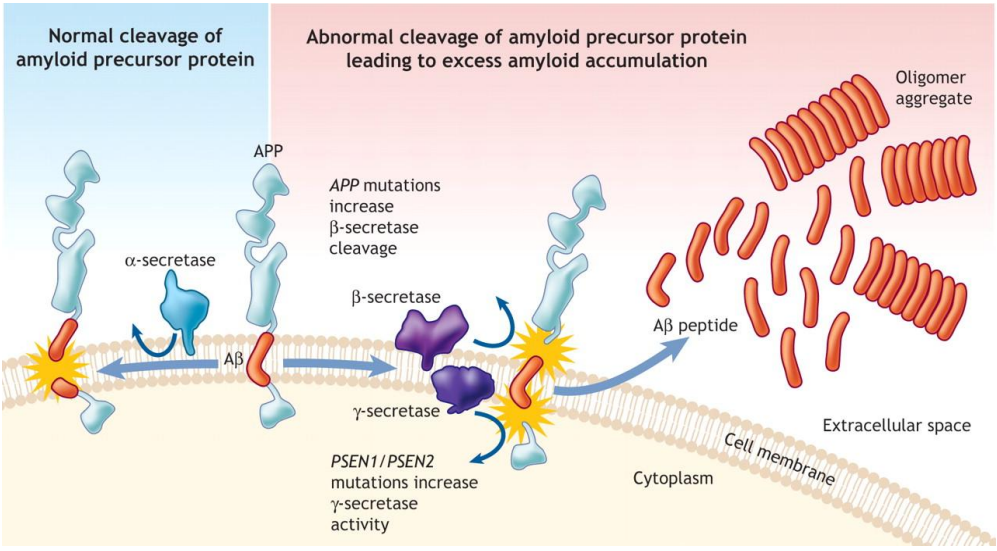
pessoas com demência seja de 7,7 milhões, ou seja, um novo caso a cada 4 segundos. As previsões são para que, em 2050, o número de pessoas que sofrem de demência no mundo inteiro triplique, situando-se nos 115,4 milhões de pessoas (Organização Mundial da Saúde). No Brasil, o número chega a 1,2 milhão de pessoas e apenas metade destas se tratam (Instituto Brasileiro de Geografia e Estatística – censo de 2014). Estima-se ainda que na DA a neurodegeneração comece cerca de 20 a 30 anos antes do aparecimento dos sintomas clínicos (Goedert e Spillantini, 2006).

Demência é um termo geral o qual refere-se a um grave declínio cognitivo, suficiente para interferir nas atividades da vida diária. De acordo com a área do cérebro afetada e, consequentemente, o grau dos sintomas, a DA divide-se nos estágios pré-clínico, leve, moderado e tardio (Kumar e Tsao, 2018). Na DA típica ocorre perda de neurónios colinérgicos e atrofia cerebral, com início no hipocampo, sendo o lobo temporal medial o primeiro local de atrofia (Scahill *et al.*, 2002). A acetilcolina é o principal neurotransmissor utilizado na formação e recordação da memória. Desta forma, o primeiro e principal sintoma observado é a perda da memória recente, seguido do comprometimento progressivo das funções cognitivas, como aprendizagem, raciocínio, atenção e julgamento (Johnson *et al.*, 2008; Weintraub *et al.*, 2012). Nos estágios iniciais estes déficits podem ser sutis, mas se agravam com a progressão da doença. As alterações neuropatológicas subjacentes a este processo evoluem progressivamente no lobo temporal medial. Ao mesmo tempo, a neurodegeneração segue um processo de disseminação regional, com afetação gradual de outras partes do córtex cerebral (Weintraub *et al.*, 2012). Nos estágio mais avançados são observados também sintomas neuropsiquiátricos (como apatia, afastamento social, agitação, psicose) e comportamentais (dispraxia, disfunção olfatória, distúrbios do sono, distonia) (Kicherova e Reikhert, 2018).

A neuropatologia da DA é caracterizada principalmente pela presença no cérebro de emaranhados neurofibrilares intraneuronais e placas senis no meio extracelular, além de ativação da glia e inflamação, o que acarreta em morte neuronal e, consequentemente, comprometimento da neurotransmissão (Lublin e Gandy, 2010). Os emaranhados neurofibrilares são característicos de tauopatias e não específicos da DA. A sua formação está associada a presença de mutações e a hiperfosforilação anormal da proteína tau associada ao microtúbulo, a qual é responsável por dar estabilidade ao citoesqueleto dos neurônios (Goedert e

Spillantini, 2006). As placas senis são formadas por agregados extracelulares do peptídio tóxico beta-amilóide (βA), o qual é formado pela clivagem errônea da proteína precursora amilóide (PPA) (Selkoe, 2000). A PPA é uma proteína integral de membrana cuja função (não totalmente elucidada) esta implicada na regulação da formação das sinapses (Priller *et al.*, 2006), neuroplasticidade (Turner *et al.*, 2003) e exportação de ferro (Duce *et al.*, 2010). Quando a PPA não é normalmente clivada pela enzima α-secretase, esta pode ser internalizadas em compartimentos endocíticos e consequentemente clivada pelas enzimas β-secretase e γ-secretase, formando o peptídeo βA (Figura 1) (Selkoe, 2000). A βA também pode ser formada no Complexo de Golgi ou no retículo endoplasmático (Greenfield *et al.*, 1999). A βA apresenta-se predominantemente na forma de um peptídio de 40 resíduos de aminoácidos ($A\beta_{1\text{-}40}$) e menos comumente, porém mais predisposta a formar oligômeros tóxicos, com 42 resíduos ($A\beta_{1\text{–}42}$) (Haass e Selkoe, 2007). A PPA é produzida normalmente por diferentes células e a presença de mutações genéticas em formas familiares da DA, acarretam em um aumento da produção da βA ou da proporção da forma $\beta A_{1\text{-}42}$ (Citron *et al.*, 1992), evidenciando a hipótese da cascata amilóide nesta patologia (Selkoe, 1996) (Figura 1).

Figura 1. Representação esquemática da clivagem da proteina precursora amilóide (PPA). A PPA é normalmente clivada pela enzima α-secretase (esquerda). Em condições patológicas, a PPA é clivada pelas enzimas β/γ secretase liberando peptídio β-amilóide, o qual se agrega formando oligômeros tóxicos e placas no meio extracelular (direita). Fonte: (Patterson *et al.*, 2008)



Entretanto, o mecanismo pelo qual tau e βA atuam como agentes tóxicos na DA ainda não está totalmente elucidado e não há cura para esta patologia. Desta forma, os tratamentos disponíveis são eficazes apenas no alívio dos sintomas da doença, aumentando a qualidade de vida dos pacientes. Os fármacos aprovados para uso dividem-se em: inibidores da acetilcolinesterase, e antagonistas dos receptores N-metil D-aspartato NMDA, as quais aumentam a sinalização colinérgica e reduzem o acúmulo de cálcio, respectivamente (Kim e Factora, 2018). Entretanto, estes não alteram o curso da doença.

Estudos mais recentes demonstraram que a vacinação pode ser uma nova terapia modificadora da DA (Wiessner *et al.*, 2011). Além de reduzir a carga de placa, os anticorpos específicos também podem atenuar alguns efeitos tóxicos atribuídos à βA_{1-42} (Graf, 2010). Entretanto, o desenvolvimento clínico tem mostrado resultados contraditório. Nenhuma das vacinas testadas atualmente demonstrou atraso na progressão da doença. Outra desvantagem dessas vacinas de segunda geração é que elas não são otimizadas para os idosos e, portanto, espera-se que induzam respostas moderadas de anticorpos (Fettelschoss *et al.*, 2014).

2.4 COMPOSTOS NATURAIS

Muitas DN são multifatoriais e o estresse oxidativo esta entrelaçado aos seus mecanismos (BARNHAM; MASTERS; BUSH, 2004). Em virtude disto, a busca por antioxidantes tem sido alvo de muitos estudos (CASETTA; GOVONI; GRANIERI, 2005). Compostos antioxidantes provindos de fontes naturais, como polifenóis, carotenóides e vitaminas, têm demostrado diversos efeitos benéficos, principalmente relacionados ao envelhecimento (ABBAS; WINK, 2009; POWOLNY et al., 2011; WILSON et al., 2006). Consequentemente, o interesse mundial no uso de plantas medicinais vem crescendo e seus efeitos benéficos são redescobertos para o desenvolvimento de novos medicamentos. Com base em suas vastas aplicações etnofarmacológicas, que inspiraram pesquisas atuais na descoberta de medicamentos, os produtos naturais podem fornecer novas e importantes pistas contra vários alvos farmacológicos.

As plantas proporcionam um efeito terapêutico desejável com risco reduzido de complicações iatrogênicas, como os efeitos colaterais frequentemente associados à medicação convencional (Atanasov *et al.*, 2015). O tratamento

combinado de medicamentos fitoterápicos e drogas sintéticas pode reduzir alguns efeitos adversos de drogas altamente potentes (Butler, 2004). Entretanto, devido ao custo e à duração de pesquisas, pouco se sabe sobre o efeito desses compostos em organismos íntegros.

2.4.1 *Rosmarinus officinalis* L. (Lamiaceae)

Rosmarinus officinalis L. (Lamiaceae), conhecido popularmente como alecrim ou *Rosemary*, é uma planta doméstica cultivada em diversos países. Além do uso culinário devido ao aroma característico, esta planta é amplamente utilizada pelas populações indígenas. Em relação aos extratos, os fitoquímicos presentes são o ácido rosmarínico, a cânfora, o ácido cafeico, o ácido ursólico, o ácido betulínico, o ácido carnósico e o carnosol (Begum *et al.*, 2013).

O alecrim tem sido amplamente utilizado não só na culinária, especialmente para modificar e realçar os sabores, mas também na medicina tradicional, sendo uma planta medicinal altamente apreciada para prevenir e curar resfriados, reumatismo, dores musculares e articulares (Calvo *et al.*, 2011; Chen, H. *et al.*, 2015). Os extratos de *R. officinalis* são hoje em dia uma das fontes mais populares de compostos bioativos naturais e, de fato, esta planta exerce várias atividades farmacológicas, tais como antioxidante (Amaral *et al.*, 2018), antibacteriana (Bozin *et al.*, 2007), antidiabética (Bakirel *et al.*, 2008), antiulcerogenica (Amaral *et al.*, 2013); anti-inflamatória {Yu, 2013 #234}{Amaral, 2018 #232}, antinociceptiva (Takaki *et al.*, 2008) e antitumoral (Cheung e Tai, 2007; Tai *et al.*, 2012).

2.4.2 *Paullinia cupana* M.

Paullinia cupana Kunth var*, Sorbilis (*MART), popularmente conhecido como guaraná, é um fruto originário da Amazônia, usado popularmente pela comunidade indígena como estimulante, afrodisíaco, entre outras propriedades medicinais (Henman, 1982). O guaraná é produzido principalmente nos estados brasileiros do Amazonas e da Bahia, e aproximadamente 70% da produção é utilizada pela indústria de bebidas leves e energéticas. Os outros 30% se tornam pó de guaraná para consumo direto em cápsulas ou diluição em água, ou serve como matéria-prima para as indústrias farmacêutica e cosmética (Schimpl *et al.*, 2013).

O guaraná apresenta principalmente metilxantinas, taninos, catequinas, epicatequinas e proantocianidinas em sua composição (COSTA, 1972). Além de sua propriedade estimulante, o guaraná possui outras propriedades terapêuticas, o que despertou o interesse da comunidade científica, entre elas: antiagregante (Bydlowski *et al.*, 1991), ergogênica (Espinola *et al.*, 1997), emagrecedora (Boozer *et al.*, 2001), antioxidante (Mattei *et al.*, 1998; Basile *et al.*, 2005), antidepressiva (Campos *et al.*, 2005), melhora na memória e cognição (Espinola *et al.*, 1997; Kennedy *et al.*, 2008), antitumoral (Fukumasu *et al.*, 2008; Cadona *et al.*, 2017), anti-envelhecimento (Arantes *et al.*, 2018), neuroprotetora (Veloso *et al.*, 2018), entre outras revisadas em (Schimpl *et al.*, 2013).

Muitas propriedades do guaraná ainda podem ser exploradas. Até agora, a cafeína tem sido a principal razão para estudar o guaraná e ainda vai liderar as pesquisas devido a demanda por este alcalóide pela indústria alimentícia e farmacêutica, além de um mercado em forte crescimento relacionado a produtos de beleza. No entanto, o guaraná possui outros componentes e há grande interesse em estudos destinados a elucidar os efeitos dos componentes bioativos do guaraná e suas potenciais aplicações farmacológicas. Parte significativa da produção de guaraná no Brasil ainda vem de tribos indígenas no Estado do Amazonas, e qualquer melhoria nesta planta, em qualquer aspecto, pode propiciar um impacto econômico positivo em suas vidas.

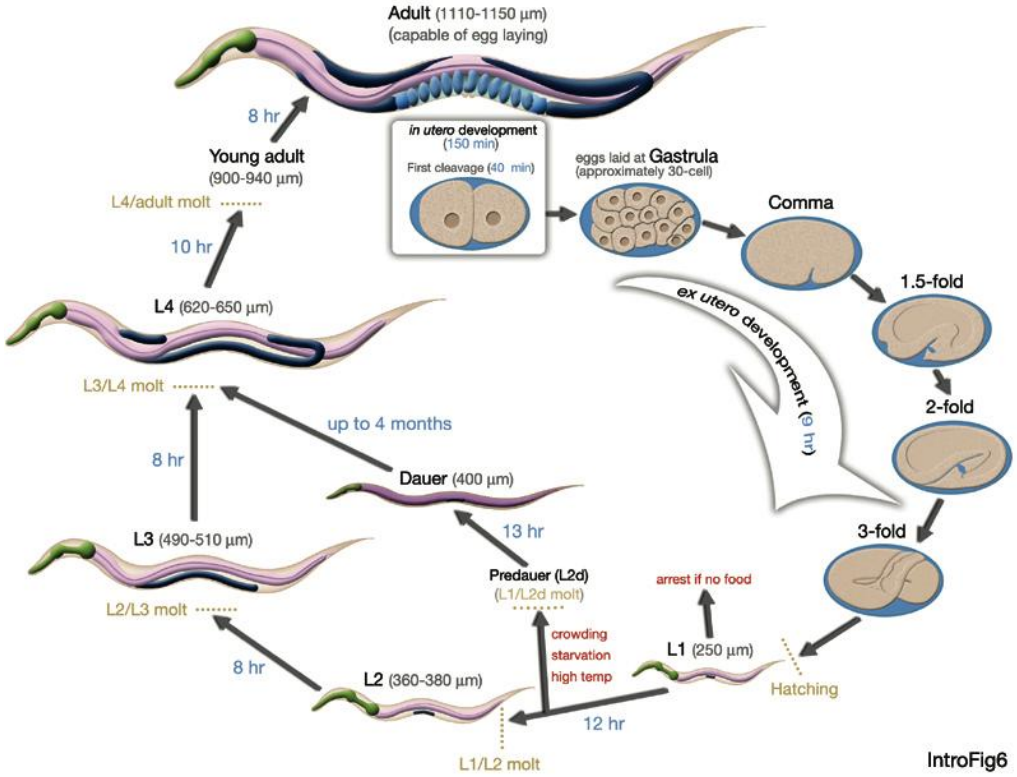
2.5 O NEMATÓDEO *Caenorhabditis elegans*

Em 1965, Sydney Brenner deu início a utilização do nematódeo *C. elegans* (Figura 2) como um potencial modelo experimental nas pesquisas relacionadas a biologia celular e molecular, principalmente como um organismo favorável para análises genéticas (Brenner, 1974).

Figura 2. O nematódeo *Caenorhabditis elegans a*dulto com ovos ao centro e duas larvas no estágio L4. À esquerda, alguns ovos. Fonte: http://www.yourgenome.org/stories/sequencing-the-worm

O *C. elegans* é um nematódeo inofensivo de vida livre, hermafrodita em sua quase totalidade, com machos representando apenas 0.2% da população total (Brenner, 1974). Possui 1 mm de comprimento, ciclo de vida rápido de aproximadamente 3,5 dias, bem como um tempo de vida relativamente curto de cerca de 20 dias a temperatura de 20ºC (Figura 3) (Riddle *et al.*, 1997a). Estas características o tornam vantajoso também em pesquisas relacionadas ao envelhecimento. Atualmente, este modelo é amplamente utilizado em laboratórios ao redor do mundo principalmente devido sua a facilidade de manutenção, cultivo e manipulação (Girard *et al.*, 2007).

Figura 3. Ciclo de vida do nematódeo *C. elegans* a 22°C. O ciclo de vida consiste de quatro estágios larvais. No primeiro estagio larval o ciclo de vida pode ser interrompido pela formação da larva "dauer", a qual é desencadeada pelo aumento nos níveis de ferormônio como resultado de escassez de alimento, temperatura inadequada, entre outras situações de estresse. Fonte: Introduction to *C. elegans* anatomy, Handbook, Life cycle (http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm#Lifecycle)



C. elegans foi o primeiro organismo multicelular a ter seu genoma totalmente sequenciado (*C. elegans* Sequencing Consortium 1998), o que conduziu a identificação molecular de genes-chave em processos biológicos e do desenvolvimento. Além disso, suas vias metabólicas e biossintéticas são altamente conservadas em mamíferos (aproximadamente 60% de homologia), incluindo vias envolvidas no metabolismo celular, manutenção do sistema nervoso e apoptose. *C. elegans* possui um sistema nervoso simples composto de 302 neurônios (em um hermafrodita adulto), com mapa de conectividade neural completo (White *et al.*, 1986) e sistema de neurotransmissores conservado, incluindo dopaminérgico, colinérgico, serotoninérgico, glutamatérgico e gabaérgico, os quais controlam seu comportamento (Riddle *et al.*, 1997b).

O fato deste nematodeo ser transparente o torna também um modelo excelente para o estudo da regulação transcripcional. A regulação gênica pode ser visualizada em namatódeos vivos através da fusão com proteinas reporteres fluorescentes (Chalfie *et al.*, 1994). A facilidade de manipulação genética também permite a construção de cepas transgênicas com deleções e superexpressões de genes de interesse através de diferentes métodos (Mello *et al.*, 1991; Mello e Fire, 1995; Praitis *et al.*, 2001; Kaymak *et al.*, 2016). Existem atualmente milhares de mutantes

produzidos disponíveis online no Centro de Genética de *Caenorhabditis elegans* (cgc.umn.edu) e as sequencias gênicas depositadas no banco de dados online de *C. elegans WormBase* (wormbase.org). De uma forma ainda mais simples, silenciamento gênico pode ser facilmente induzido através de RNA de interferência (RNAi) em qualquer estágio de vida do nematódeo (Fire *et al.*, 1998).

Além disso, devido a linhagem selvagem invariante e a neuroanatomia do *C. elegans*, mutações que originam defeitos no desenvolvimento e comportamento podem ser facilmente identificadas através de triagens genéticas. A triagem de populações com mutações em genes específicos tem sido realizada por laboratórios individuais e sistematicamente pelo *C. elegans Deletion Mutant Consortium* (Thompson *et al.*, 2013).

Desta forma, tendo em vista o tempo e o grande número de animais requeridos em estudos em mamíferos, modelos invertebrados como o nematódeo *C. elegans* proporcionam uma ponte entre estudos em culturas celulares e mamíferos.

2.5.1 *C. elegans* e envelhecimento

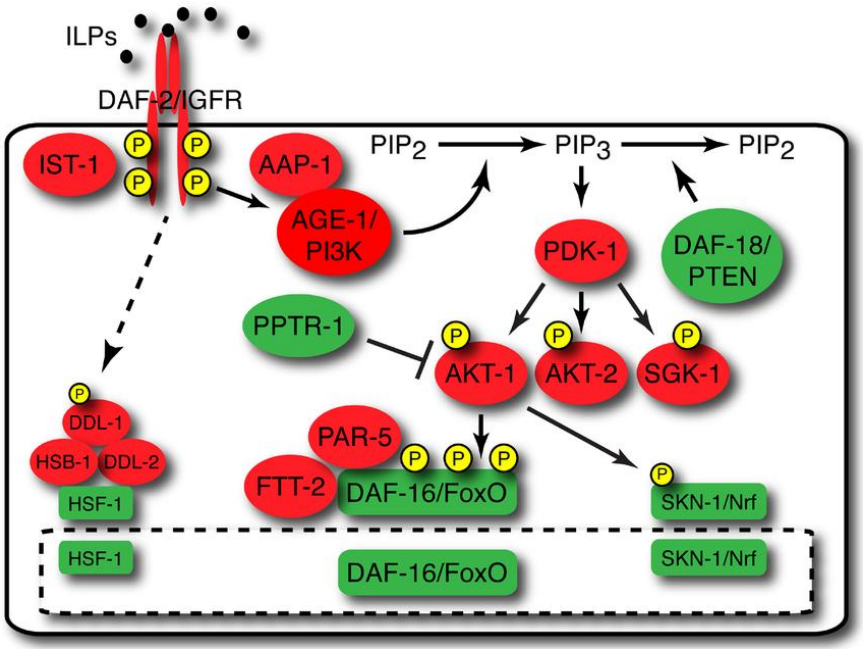
O envelhecimento é afetado por diversos fatores genéticos (Guarente e Kenyon, 2000). Vias de sinalização que regulam a expressão gênica em resposta ao estresse têm sido alvos na pesquisa por genes envolvidos na longevidade (Koubova e Guarente, 2003). Estudos em *C. elegans* tem elucidado importantes mecanismos na modulação do envelhecimento. Em *C. elegans*, a via de sinalização do tipo insulina/IGF-1 regula o crescimento, a longevidade e as funções anabólicas através da modulação da resistência ao estresse (Kuningas *et al.*, 2008).

O *C. elegans* possui um receptor do tipo insulina/IGF-1, o DAF-2, que regula negativamente os fatores de transcrição DAF-16/FOXO (Kimura *et al.*, 1997), HSF-1/HSF-1 (Hsu *et al.*, 2003) e SKN-1/Nrf (Tullet *et al.*, 2008), responsáveis pela resposta de defesa do organismo. Em condições ambientais favoráveis (presença de alimento, temperatura adequada, etc), a sinalização através do receptor DAF-2 ativa uma cascata a qual induz a fosforização do DAF-16 e seu aprisionamento no citoplasma da célula (Gems *et al.*, 1998; Henderson e Johnson, 2001). Em condições desfavoráveis ao desenvolvimento e /ou redução da sinalização da via da insulina, a DAF-16 é desfosforilada e migra para o núcleo da célula ativando a transcrição de genes envolvidos na regulação da longevidade (Lin *et al.*, 1997),

resistência ao estresse (Mcelwee *et al.*, 2003) e formação da larva dauer (Vowels e Thomas, 1992) (Figura 4).

De forma semelhante, o fator de choque térmico tipo 1 (HSF-1), que regula a resposta ao estresse através da expressão das proteínas de choque térmico (HSP), também influencia na longevidade em *C. elegans* (Hsu *et al.*, 2003). Mutações que reduzem a sinalização via receptor DAF-2/IGF-1, aumentam a longevidade em *C. elegans* de forma dependente dos fatores de transcrição DAF-16/FOXO e HSF-1/HSF (Hsu *et al.*, 2003). DAF-2/IGF-1 também regula negativamente o fator de transcrição SKN-1/Nrf, o qual modula a resistência ao estresse oxidativo e a expressão de genes de detoxificação. Porém, a translocação para o núcleo e ativação de HSF-1/HSF e SKN-1/Nrf é regulada por vias independentes a DAF-16/FOXO (Inoue *et al.*, 2005; Chiang *et al.*, 2012). Ou seja, múltiplos fatores nucleares e vias de sinalização convergem na via do tipo insulina para a modulação da longevidade e resposta ao estresse em *C. elegans*.

Figura 4. Representação esquemática da via do tipo insulina/IGF-1 em *C. elegans*. Os fatores de transcrição HSF-1, DAF-16/FOXO e SKN-1/Nrf em *C. elegans* são regulados negativamente pelo receptor DAF-2/IGF-1. Quando ativados (desfosforilados), HSF-1, DAF-16/FOXO e SKN-1/Nrf migram do citoplasma para o núcleo da célula ativando as vias de detoxificação, de resistência ao estresse, a proteostase e a longevidade do nematódeo. Fonte: (Lapierre e Hansen, 2012).



Desta forma, o envolvimento da via da insulina na modulação do envelhecimento tem sido alvo de muitos estudos em *C. elegans*. Alguns genes alvo transcricionais são regulados por ambos DAF-16/FOXO e HSF-1/HSF, enquanto outros são controlados especificamente por um fator. Mais estudos são necessários para melhor elucidar os mecanismos genéticos, bem como contribuir para o desenvolvimento de novas terapias contra o envelhecimento e as doenças relacionadas a idade. O *C. elegans* emergiu como uma plataforma eficaz para a triagem de compostos e validação da sua bioatividade em diferentes modelos de patologias (Lapierre e Hansen, 2012).

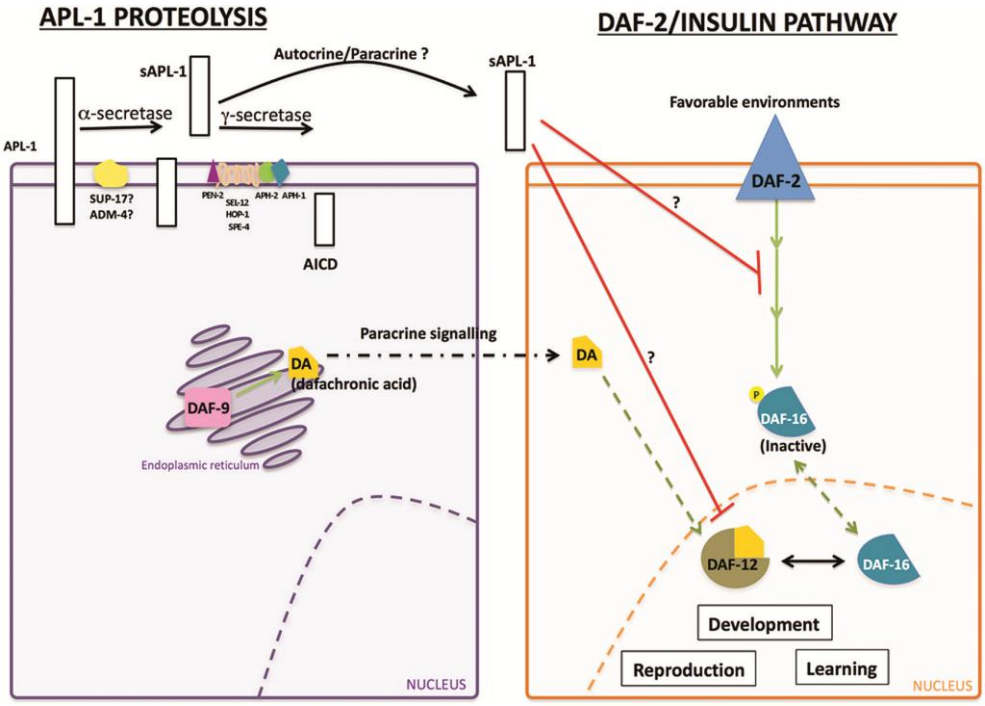
2.5.2 *C. elegans* como modelo de DA

Além das vias de sinalização altamente conservadas, o sistema nervoso simples do *C. elegans* permite o estudo das funções e circuitos neuronais em um sistema passível de tratamento. Assim, este nematódeo nos proporciona uma ferramenta para elucidar a patologia da DA, bem como de outras doenças neurodegenerativas e conceber estratégias eficazes para o seu tratamento e prevenção (Alexander, Adanna G. *et al.*, 2014; Moreno-Arriola *et al.*, 2014; Carretero *et al.*, 2017; Ma *et al.*, 2018).

O C. elegans possui um gene relacionado ao que codifica a PPA em mamíferos, o *apl-1*. A APL-1/PPA é expressa nos neurônios, músculos da cabeça, da vulva e em células tronco epidérmicas. A clivagem da APL-1/PPA pela α-secretase libera o fragmento extracelular sAPL-1. Ewald e colaboradores (2012) demonstraram que mutações no gene *apl-1* em *C. elegans* induzem atraso no desenvolvimento e redução do tamanho corporal e da reprodução (Ewald *et al.*, 2012). Esses fenótipos demonstraram ser dependentes da atividade da DAF-16/FOXO, sugerindo que a sinalização mediada por sAPL-1 atua em uma via paralela a via da insulina, modulando o receptor DAF-2/IGF-1, ativando a DAF-16/FOXO e interferindo no desenvolvimento e na reprodução (Figura 5) (Ewald *et al.*, 2012). Mais recentemente, foi demonstrado que a superexpressão de *apl-1* aumenta o tempo e a qualidade de vida via DAF-16/FOXO e HSF-1/HSF, propondo um elo entre a PPA e a modulação do envelhecimento (Ewald *et al.*, 2016).

Figura 5. Interação entre as vias APL-1 e DAF-2. Representação esquemática da via proteolítica da APL-1 e como ela modula a via de sinalização do receptor DAF-2. APL-1 é clivado pela via da α/ɣ-

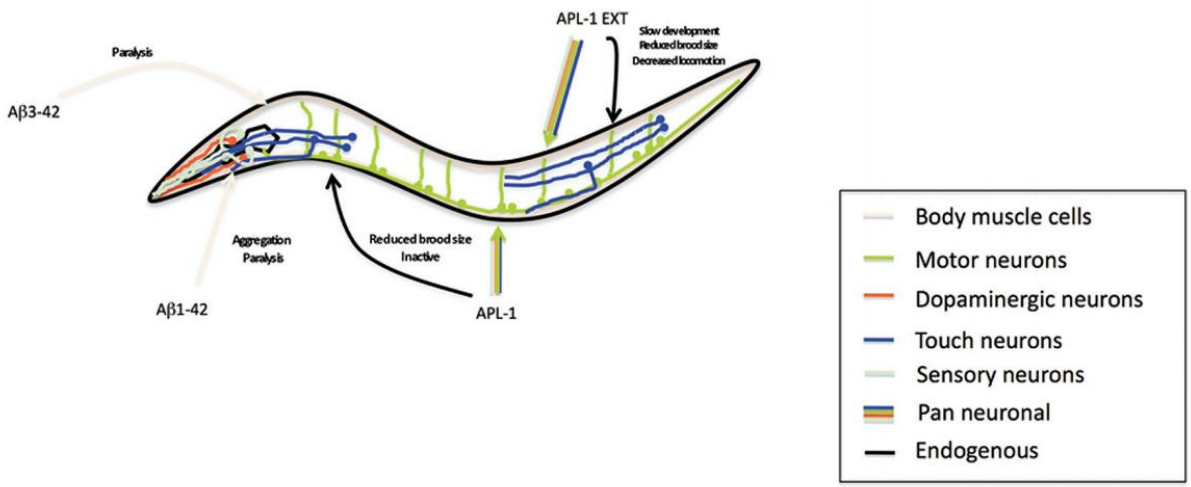
secretase em *C. elegans*. A sAPL-1 liberada pode atuar como uma molécula sinalizadora na mesma célula (regulação autócrina) ou em células vizinhas (regulação parácrina) para inibir o receptor DAF-2/IGF-1 e a via DAF-12/NHR, comprometendo o desenvolvimento e a viabilidade destes nematódeos. Fonte: (Alexander, A. G. *et al.*, 2014)



Entretanto, o *apl-1* não contempla a região codificante do peptídeo βA (Daigle e Li, 1993). Além disso, não há evidências de atividade de β-secretase na clivagem da APL-1/PPA em *C. elegans*. Desta forma, foram criadas cepas transgênicas que expressam as espécies tóxicas Aβ, as quais têm sido utilizadas como modelos de DA (Link, 1995; 2006; Dostal e Link, 2010) (Figura 6). Estes modelos são capazes de reproduzir os processos celulares que fundamentam a DA, apesar de não serem capazes de retratar a complexidade neuronal cognitiva de mamíferos (Wu e Luo, 2005).

Estas cepas apresentam fenótipos específicos em decorrência do acúmulo do peptídeo βA no nematódeo. Cepas com expressão neuronal deste peptídeo apresentam baixo número de progênie, parcial esterilidade, diminuição do tempo de vida e da memória associativa (Dosanjh *et al.*, 2010). A expressão muscular do peptidio βA ocasiona uma diminuição dos movimentos e paralisia progressiva, além de aumento na produção de ERO (Link *et al.*, 2003). A paralisia ocorre de forma mais severa e progressiva a temperaturas mais elevadas, principalmente quando a expressão da βA é induzida (Mccoll *et al.*, 2012) e não constitutiva.

Figura 6. Modelos de Doença de Alzheimer em C. elegans expressando o peptídeo βA ou APL-1. As setas coloridas representam o tecido onde a proteína é expressa e seu fenótipo. Fonte: (Alexander, A. G. *et al.*, 2014).



Estudos anteriores demonstraram que mutações na via da DAF-2/IGF-1 retardam o envelhecimento e diminuem a suscetibilidade a doenças relacionadas a idade através da ativação de DAF-16/FOXO e HSF-1/HSF e consequente aumento da expressão de HSPs (Hsu *et al.*, 2003), provavelmente devido a sua capacidade de reduzir a agregação de proteínas (Clark e Muchowski, 2000). De fato há evidências de que a expressão da βA em *C. elegans* induz a expressão de HSPs (Link *et al.*, 1999; Link *et al.*, 2003). Além disso, foi demonstrado que a proteína de choque térmico HSP-16.2 é capaz de se co-imunoprecipitar com a βA, promovendo o seu sequestro e degradação ou remodelamento (Fonte *et al.*, 2002). Ainda, que a superexpressão da HSP-16.2 reduz a agregação da βA, suprimindo sua toxicidade e consequente paralisia (Fonte *et al.*, 2008).

Corroborando com esses dados, outros estudos envolvendo modelos de DA em *C. elegans* apresentaram uma significativa diminuição da paralisia induzida pela βA com a redução da sinalização via DAF-2/IGF-1 (Cohen *et al.*, 2006). Este efeito foi revertido com a inibição de DAF-16/FOXO e HSF-1/HSF (Cohen *et al.*, 2006), indicando que a toxicidade mediada pela βA esta correlacionada com o envelhecimento e é dependente da via da insulina.

3 DESENVOLVIMENTO

O desenvolvimento que faz parte desta tese está apresentado sob a forma de dois artigos científicos. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos.

O artigo 1 foi publicado na revista *Brazilian Journal of Medical and Biological Research* e encontra-se no formato da mesma.

O artigo 2 foi aceito para publicação na revista *Nutritional Neuroscience* e esta apresentado na forma de manuscrito, na formatação para publicação desta revista.

3.1 ARTIGO CIENTÍFICO I

***Rosmarinus officinalis* L. increases *Caenorhabditis elegans* stress resistance and longevity in a DAF-16, HSF-1 and SKN-1-dependent manner**

Daniele C. Zamberlan, Guilherme P. Amaral, Leticia P. Arantes, Marina L. Machado, Caren R. Mizdal, Marli M. A. Campos and Felix A. A. Soares1

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Rosmarinus officinalis L. increases *Caenorhabditis elegans* stress resistance and longevity in a DAF-16, HSF-1 and SKN-1-dependent manner

D.C. Zamberlan[1], G.P. Amaral[1], L.P. Arantes[1], M.L. Machado[1], C.R. Mizdal[2], M.M.A. Campos[2] and F.A.A. Soares[1]

[1]Centro de Ciências Naturais e Exatas, Departamento de Bioquímica e Biologia Molecular, Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Departamento de Análises Clínicas Toxicológicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

Abstract

Improving overall health and quality of life, preventing diseases and increasing life expectancy are key concerns in the field of public health. The search for antioxidants that can inhibit oxidative damage in cells has received a lot of attention. *Rosmarinus officinalis* L. represents an exceptionally rich source of bioactive compounds with pharmacological properties. In the present study, we explored the effects of the ethanolic extract of *R. officinalis* (eeRo) on stress resistance and longevity using the non-parasitic nematode *Caenorhabditis elegans* as a model. We report for the first time that eeRo increased resistance against oxidative and thermal stress and extended *C. elegans* longevity in an insulin/IGF signaling pathway-dependent manner. These data emphasize the eeRo beneficial effects on *C. elegans* under stress.

Key words: *Caenorhabditis elegans*; Natural compounds; Rosemary; *daf-2*; Stress resistance; Aging

Introduction

Aging and lifespan of multicellular organisms are affected by several genetic factors. Signal transduction pathways that regulate gene expression in response to extracellular cues are common targets in the search for longevity genes (1). Insulin/IGF signaling is a conserved signal transduction pathway that regulates growth and anabolic functions of multicellular organisms at the expense of cellular stress defenses and repair by modulating stress resistance and longevity (2).

Rosmarinus officinalis L. (Labiatae), popularly known as rosemary, is a common household plant grown in many parts of the world. Aqueous and ethanolic extracts of *R. officinalis* have been shown to contain many substances with pharmacological properties. Health benefits include the following characteristics: antioxidant, antidiabetic (3), hepatoprotective (4), antithrombotic (5), antinociceptive (6), anti-inflammatory (7), antidepressant (8), and gastroprotective (9). Given that *R. officinalis* appears to have beneficial effects in diseases that are strongly linked to aging, we investigated the effects of the ethanolic extract of this plant (eeRo) on aging using the non-parasitic nematode *Caenorhabditis elegans* as a model.

C. elegans has been shown to be a valuable model in understanding the molecular mechanisms that modulate aging and stress responses. Its short lifespan, fully mapped genome and its application in genetic manipulations have enabled researchers to study the function, regulation and output of insulin/IGF-1 signaling (10). This pathway is highly conserved between worms and mammals. *Daf-2* encodes the only insulin/IGF-1 receptor expressed in *C. elegans*. Studies have demonstrated that mutations in *daf-2* increase *C. elegans* resistance to oxidative stress (11) and heat stress (12) and lead to an extended lifespan in a DAF16/FOXO-dependent manner (13). Tullet et al. (14) suggested that the transcription factor SKiNhead (SKN-1/Nrf2) directly integrates insulin/IGF signaling and the stress response. Recent studies have also implicated heat shock factor (HSF) as a regulator of longevity that interacts with the insulin pathway (15,16). MEV-1 is also involved in aging and sensitivity to oxidative stress. The expression patterns of the antioxidant enzymes genes *superoxide dismutase (sod)* and *catalase* (ctl) mirrored one another in the two mutants *daf-16* and *mev-1*. In addition, both strains were extremely sensitive

Correspondence: F.A.A. Soares: <felix@ufsm.br>

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to paraquat, a superoxide anion generator. However, the short life span and oxidative stress-hypersensitivity of *daf-16* mutant may result from suppression of anti-oxidant genes, such as *sod-1* or *sod-3*, rather than increase of ROS production from mitochondria as in *mev-1* (17).

Studies have proposed that integration of cytoprotective and stress-responsive signaling pathways is crucial for environmental adaptation and hence, control of longevity (18). Despite documentation of the many protective properties of the *R. officinalis* extract, there have been no studies on the signaling pathways that may be involved. *C. elegans* has the potential to bridge the gap between *in vitro* and *in vivo* approaches. This model complements genetic studies and helps in the search for a mechanism of action of the extract. In this study, we investigated the effect of eeRo on *C. elegans* stress resistance and longevity and evaluated the signaling pathways involved.

Material and Methods

Chemicals and reagents

Ethanol, 5-hydroxy-1,4-naphthoquinone (juglone) and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) were purchased from Sigma-Aldrich (USA).

The eeRo was obtained from the dried leaves (40°C) of this plant, which were collected in the botanical garden of Universidade Federal de Santa Maria, Brazil. The leaves were subjected to an alcoholic extraction (100% ethanol, 1.5 h, 60–70°C) in the Soxhlet apparatus with some modification in relation to the original technique (19). High performance liquid chromatography (HPLC–DAD) was previously performed and revealed the presence of the rosmarinic acid, carnosic acid, chlorogenic acid, caffeic acid, quercetin, rutin and kaempferol (12).

C. elegans strains, maintenance and treatment

The wild-type *C. elegans* strain N2 (Bristol) and mutant worms TK22 [mev-1(kn1)]; CB1370 [daf-2 (e1370) III]; CF1038 [daf-16(mu86)], PS3551 [hsf-1(sy441)]; EU-1 [skn-1(zu67) IV/nT1] and TJ356 [daf-16p::daf-16a/b::GFP + rol-6] were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN, USA).

For all worms, age-synchronized eggs were obtained by isolating embryos from gravid hermaphrodites using bleaching solution (1% NaOCl, 0.25 M NaOH). For EU-1 worms, we grew synchronized animals and plated young adults. *skn-1* worms are uncoordinated (Unc), while *skn-1* homozygotes are non-Unc, allowing us to enrich for *skn-1* homozygotes using a plate crawling assay. The L1 population was transferred to 10 mL NGM (nematode growth medium) plates seeded with *Escherichia. coli* OP50 as a food source and eeRo at 10, 25 or 50 μg/mL or vehicle (0.1% EtOH) and allowed to develop. The strain CB1370 was maintained at 16°C since it is sensitive to dauer formation at 20°C. The wild-type and the other mutant strains were maintained at 20°C.

Bacterial growth assay

The minimum inhibitory concentration (MIC) in *E. coli* OP50 was performed according to Clinical and Laboratory Standards Institute with few modifications (20). Bacteria was seeded onto plates with Mueller Hinton agar and allowed to grow for 24 h at 37°C. We then prepared suspensions of microorganisms in Mueller Hinton broth. Fifty microliters of the standardized microorganism suspension was placed in each well of a 96-well microliter plate, along with an equal volume of compound to be tested at different concentrations. We performed broth, growth, and compound vehicle controls to which the results were compared. The plates were incubated for 24 h at 37°C. The MIC was considered as the lowest concentration of the test product able to inhibit the growth of microorganisms evidenced by the use of 2,3,5 triphenyltetrazolium chloride 1%.

Oxidative stress resistance

Synchronized L1-larva N2 and mutant strains were transferred to treatment plates containing eeRo or vehicle (control) and allowed to develop at 20°C up to adulthood (approximately 2 days). The pretreated worms were collected, washed three times with M9 buffer and transferred into Eppendorf tubes. A volume of 10 μL of 10 mM juglone (stock solution freshly prepared) was added to the micro tubes containing 1000 worms in 990 μL of M9. The worms were exposed to 100 μM juglone (final concentration) during 1 h, washed three times with M9 buffer and transferred to NGM plates containing vehicle or eeRo. After 24 h, the number of living worms was counted and reported as percent of the control. The mutant assays were conducted using the concentration that was deemed most effective in increasing survival (in wild type).

Thermotolerance assay

Synchronized 1-day-adult N2 and mutant worms, pretreated with eeRo or vehicle (control) since L1-larval stage, were exposed to 35°C for 4 h. After this procedure the plates were returned to 20°C for 24 additional hours. The number of survivors were scored. The mutant assays were conducted using the concentration that was deemed most effective in increasing survival (in wild type).

Lifespan assay

The lifespan assay of *C. elegans* was investigated as previously described (21). The pre-fertile period of adulthood was used as time zero (t=0). The worms were kept on NGM plates containing eeRo or vehicle (control) and *E. coli* just in the middle of the plate and transferred to new plates every two days. Nematodes were regarded as dead if they did not move after repeated stimulus. They were excluded if they crawled away from the plate.

The maximum lifespan was defined as the 10% of last survival population. The mutant assays were conducted using the concentration that was deemed most effective in inducing lifespan extension (in wild type). Blinding of studies was not possible due to the color of eeRo, which stains the NGM agar. Experiments were performed at least in triplicate with 100 nematodes each.

Quantification of ROS

Intra-worm ROS generation was measured in *C. elegans* wild-type and mutant strains using CM-H_2DCFDA, following a previously described method (22) with minor modifications. Briefly, L1 age-synchronized worms were transferred onto culture plates containing either vehicle or eeRo. The worms were maintained at 20°C until adulthood (~48 h). After, they were collected, washed with fresh M9 buffer three times and transferred to micro tubes. The worms were exposed to 25 mM H_2O_2 (final concentration, induced groups) or vehicle (basal groups) during 1 h and washed again with M9 buffer. After, 10 µL of 2 mM CM-H_2DCFDA were added to the Eppendorf tubes containing 1000 worms in 990 µL M9 (20 µM CM-H_2DCFDA final concentration) and incubated for 2 h. The mutant worms *mev-1* were just exposed to CM-H_2DCFDA. The worms were washed and transferred to 96-well plates (100 worms per well). The fluorescence intensity was measured with a plate reader (Excitation: 488 nm; Emission: 510 nm). The mutant assays were conducted using the concentration that was deemed most effective in decreasing ROS levels (in wild type).

DAF-16 localization

For each slide, a minimum of 20 worms were mounted on 4% agarose pads in M9 and anaesthetized with 10 mM sodium azide. Fluorescence was acquired with an epi-fluorescence microscope housed in air-conditioned rooms (20°C).

Statistical analyses

Statistical analyses were performed using GraphPad (Prism for Windows, Version 5.01 GraphPad Software, USA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test, or two-way ANOVA, followed by Bonferroni's test for *post hoc* comparison. Significance for survival analysis was assessed by the Kaplan-Meier curve followed by the log-rank test for trend. Values of $P<0.05$ were considered to be statistically significant.

Results

Effect of eeRo on resistance to oxidative stress

To test the antioxidant effect of eeRo *in vivo*, we monitored *C. elegans* survival under oxidative stress. Exposure to 100 µM juglone for 1 h induced an approximately 50% mortality rate in wild-type worms (Figure 1A). Treatment with 10 and 100 µg/mL eeRo did not have any

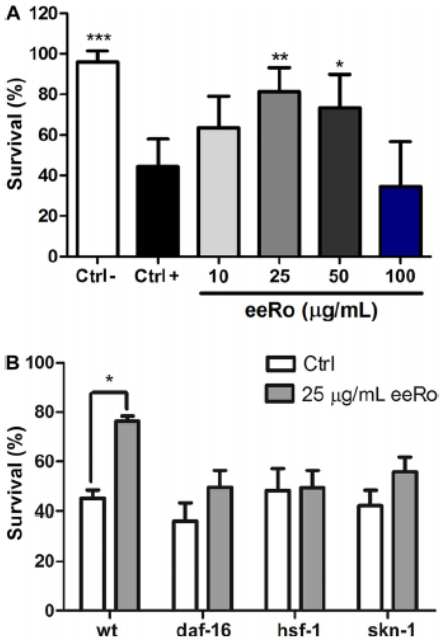

Figure 1. Effect of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) on juglone-induced mortality. Survival of wild-type (*A*) and mutant worms (*B*) treated with eeRo and exposed to 100 µM juglone for 1 h (Ctrl+). Data are reported as percentage of living worms of 100 worms per group in each experiment from 4 independent assays. *A*, *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to the Ctrl+ group (one-way ANOVA). *B*, *$P<0.05$ (two-way ANOVA).

effect on survival rate. In contrast, worms treated with 25 and 50 µg/mL eeRo had a significantly lower mortality rate compared to the untreated induced control group (Figure 1A, Ctrl+). The most effective eeRo concentration in decreasing mortality was 25 µg/mL ($P<0.01$). We investigated the effect of this dose on the survival rate against juglone exposure in *daf-16, hsf-1* and *skn-1* mutants. We found no significant differences in mortality among the strains evaluated. Furthermore, treatment with 25 µg/mL eeRo did not decrease mortality rate in the mutants, as observed in wild-type worms (Figure 1B).

Effect of eeRo on thermal tolerance

Worms were subjected to thermal stress at 35°C for 4 h, which induced a mortality of approximately 50% in wild-type worms (Figure 2A). Treatment with 25 and 50 µg/mL eeRo significantly decreased mortality in 28 and 24%, respectively, in wild-type worms compared to the control group. The most effective concentration of eeRo was 25 µg/mL ($P<0.01$). We performed the thermal tolerance assay with *daf-16, hsf-1* and *skn-1* mutants. No significant differences in thermal resistance were observed among the untreated strains, although increased mortality was observed in *daf-16*. No significant differences in

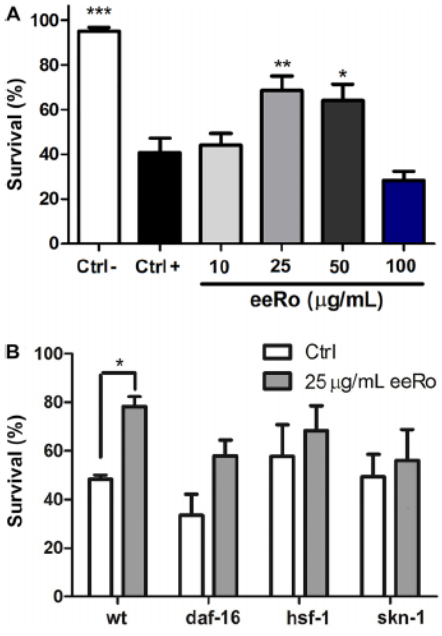

Figure 2. Effect of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) on thermal stress. Survival of wild-type (*A*) and mutant worms (*B*) treated with eeRo and exposed to thermal stress (35°C) for 4 h (Ctrl+). Data are reported as percentage of living worms of 100 worms in each experiment from 4 independent assays. *A*, *P<0.05, **P<0.01, ***P<0.001, compared to the Ctrl+ group (one-way ANOVA). *B*, *P< 0.05 (two-way ANOVA).

mortality were noted in *daf-16*, *hsf-1* and *skn-1* mutants treated with 25 µg/mL eeRo compared to the untreated group (Figure 2B).

Effect of eeRo on ROS production

We used the wild-type strain to investigate if eeRo decreased basal ROS production and ROS induced by H_2O_2 exposure. There was a significant increase in ROS levels induced by 1 h exposure to 25 mM H_2O_2 (Figure 3A). Worms treated with eeRo at 10, 25, and 50 µg/mL had significantly lower basal ROS production than untreated worms. Moreover, eeRo treatment also prevented an increase in ROS levels induced by H_2O_2 in the wild-type strain compared to the untreated induced control group ($P<0.05$).

We quantified ROS levels in TK22 (*mev-1*) mutant. Figure 3B shows significantly higher ROS levels in the transgenic worms compared to the wild-type strain ($P<0.001$). At all concentrations tested, the worms treated with eeRo had significantly lower ROS levels compared to the untreated worms ($P<0.001$). This decrease in ROS levels was most evident at an eeRo concentration of 25 µg/mL.

Measurement of ROS production in *daf-16, hsf-1* and *skn-1* mutants demonstrated that they have higher levels of reactive species than the wild type (Figure 3C). Treatment with 25 µg/mL eeRo significantly decreased ROS levels in these mutants compared to untreated worms ($P<0.01$). However, the effects of eeRo in the mutants were less apparent than in the wild-type strain ($P<0.001$).

Effect of eeRo on DAF-16 translocation

No differences in DAF-16 translocation were observed in worms treated with eeRo compared to untreated worms (data not shown).

Effect of eeRo on lifespan

To test the effects of eeRo on *C. elegans* longevity we monitored time-course survival in wild type and mutant worms. The mean survival time was extended by treatment with 25 µg/mL eeRo in the wild-type strain from 12- (control) to 15-day adults (treated). Similarly, an increase in maximum lifespan also was observed from 16- (control) to 20-day adults (treated) (Table 1). A possible explanation for the beneficial effects of eeRo on aging in *C. elegans* is that these compounds increased cellular stress resistance. To confirm this, we performed a lifespan assay in TK22 (*mev-1*) mutant. *mev-1* had a decreased lifespan compared to the wild type strain. EeRo treatment extended the *mev-1* mean survival-time from 10 to 12 days at a concentration of 25 µg/mL (Table 1). The maximum lifespan of *mev-1* was extended from 16 (control) to 18 days following treatment with 25 µg/mL eeRo (Figure 4B; Table 1).

We performed the lifespan assay in *daf-2*, *daf-16*, *hsf-1* and *skn-1* mutants with and without 25 µg/mL eeRo treatment (Figure 5). EeRo treatment increased the mean lifespan of *daf-16* from 10- (control) to 12-day adults (treated), but not the maximum lifespan. In *daf-2* mutant, we observed a slight increase in the maximum lifespan from 30 to 32 days in worms treated with the extract. EeRo treatment did not have an effect on lifespan in the *hsf-1* and *skn-1* mutants, which had mean survival times of 8 and 9 days, respectively, and maximum lifespans of 13 and 14 days, respectively (Table 2).

Effects of eeRo on *E. coli* OP50

To verify whether eeRo influenced *E. coli* OP50 growth, we assessed the minimum inhibitory concentration (MIC). EeRo did not have a significant antimicrobial effect on *E. coli* OP50 growth at a concentration range between 0.39–50 µg/mL (data not shown).

Discussion

The search for antioxidants from natural sources has received a lot of attention. Antioxidants can inhibit cellular oxidative damage and prevent development of related diseases. The plant *R. officinalis* represents an exceptionally rich source of different bioactive compounds (23).

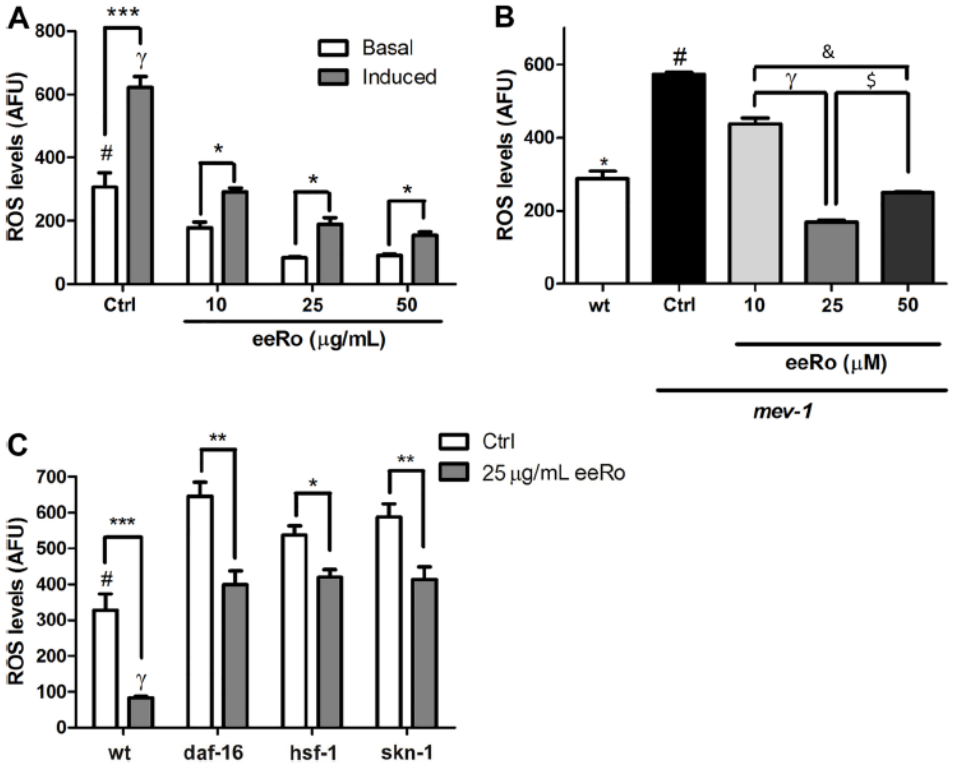

Figure 3. Effect of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) on reactive oxygen species (ROS) production. Data are reported in arbitrary fluorescence units (AFU) from 3 independent assays (n=3). *A*, Levels of basal and H_2O_2-induced (25 mM/1 h) ROS production in wild-type (N2) worms. *P<0.001 compared to eeRo-treated basal groups; #P<0.001 compared to eeRo-treated induced groups; γP<0.01 Ctrl basal compared to Ctrl induced groups. *B*, Levels of ROS production in wild-type (N2) and *mev-1* (TK22) worms. *P<0.001, compared to all other groups; #P<0.001, compared to all other groups; γP<0.001, 10 μM compared to the 25 μM eeRo treated group; $P<0.001, 25 μM compared to the 50 μM eeRo treated group; &P<0.001, 10 μM compared to the 50 μM eeRo treated group (one-way ANOVA). *C*, ROS levels in *daf-16, hsf-1 and skn-1* mutants treated or not with the extract. *P<0.05, **P<0.01, ***P<0.001, treated compared to untreated group; #P<0.01, compared to the other Ctrl groups; γP<0.01, compared to the other treated groups (two-way ANOVA).

Table 1. Lifespan of wild-type worms treated with eeRo (μg/mL).

	Median lifespan (days)	Maximum lifespan (days)
Control	12 ± 1.15	16 ± 0.57
10	12 ± 1.00	17 ± 1.00
25	15 ± 1.15*	20 ± 1.15*
50	13 ± 1.73	17 ± 1.52

Data are reported as means ± SD. Aging assays were performed at 20°C except for *daf-2*, which was carried out at 16°C. Experiments were repeated three times starting with 90 nematodes per group. *P<0.05, significantly different from untreated control group by the log-rank (Mantel-Cox) test.

The eeRo used in this study includes flavonoids (quercetin, rutin and kaempferol) and phenolic acids (chlorogenic, caffeic, rosmarinic and carnosic acids) as the most abundant components (9). *R. officinalis* appears to have beneficial effects in prevalent diseases that are strongly linked to aging, such as diabetes and cancer. In the present study, we explored the possibility that this plant can influence aging using the non-parasitic nematode *C. elegans* as a model. We report for the first time that the eeRo increased worm resistance against oxidative and thermal stress and extended *C. elegans* longevity in an insulin/IGF signaling-dependent manner.

Juglone is a naphthoquinone that induces superoxide anion radicals overproduction (24). Juglone can cause premature death at concentrations that overload the organism's protective capacity (24). As we previously described, 1 h of 100 μM juglone-exposition induces a lethality of approximately 50% of wild-type worms (25). The present study demonstrated that the eeRo treatment was able to decrease the juglone-induced mortality by reducing ROS production and protecting against oxidative

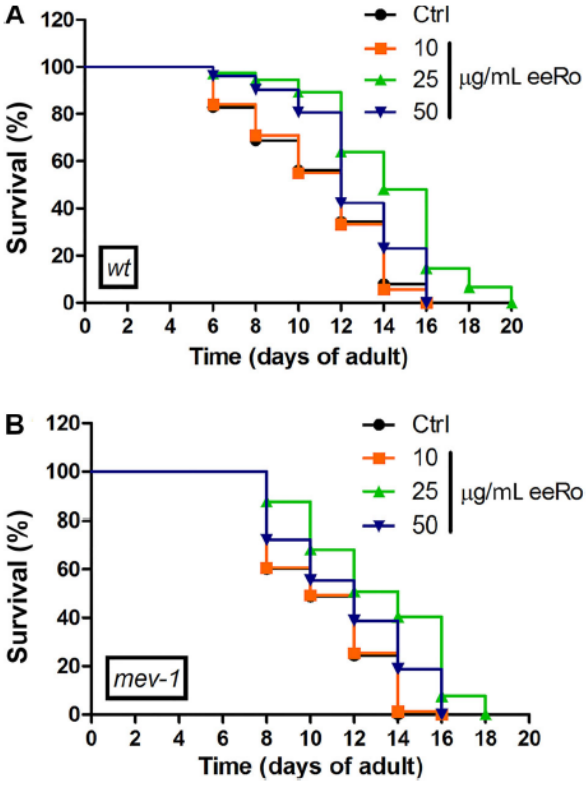

Figure 4. Effect of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) on *C. elegans* lifespan. Survival curves were significantly extended in (*A*) wild-type and (*B*) *mev-1* worms treated with 25 µg/mL eeRo ($P < 0.0001$, log-rank (Mantel-Cox) test).

damage. These data corroborate the extract antioxidant properties previously mentioned (3). In addition, thermal tolerance was significantly increased by eeRo treatment in wild-type worms (Figure 2A). Previous studies demonstrated that thermal stress causes an increase of ROS levels in the worms and therefore it is likely that the death of worms was at least partially due to oxidative stress (26). These outcomes are known to depend partly on intracellular stress signaling pathways that are activated in response to oxidative stress and as a consequence of direct damage to DNA, proteins, and lipids. These cellular injuries and signaling mechanisms modulate transcription factor activities resulting in changes to gene expression profiles (27). In this way, given that both thermal and chemical stresses result in similar changes in gene expression, eeRo may modulate signaling pathways that are crucial to defense processes. These findings emphasize the potential of eeRo against environmental stress.

Active insulin/IGF signaling promotes phosphorylation-dependent cytoplasmic sequestration of the transcription factors DAF-16/FOXO, HSF-1 and SKN-1/Nrf2 (14,28). Under favorable environmental conditions, signaling through this pathway activates a conserved PI3-kinase/AKT cascade, which causes phosphorylation of DAF-16/FOXO, thereby allowing reproductive development (29). The transcription factor HSF-1 guides DAF-16/FOXO activity and cooperatively induces transcription of a subset of target genes, including heat shock proteins involved in proteostasis (1). SKN-1/Nrf regulates resistance to oxidative stress and expression of detoxification genes (2). We performed survival assays in *daf-16, hsf-1* and *skn-1* mutants and found no significant differences among treated and untreated worms. Moreover, we observed that these strains were more sensitive to oxidative and thermal stress compared to the wild type. These data indicate that DAF-16, HSP-1 and SKN-1 expression, involved in the insulin/IGF signaling network, and the activation of target genes, are essential for eeRo to exert its effect. However, the extract decreased ROS levels in the transgenic strains tested, although less efficiently than in the wild-type. This data suggests that the constituents of the extract may be acting as direct scavengers and then reducing ROS production in the mutants. Besides, more studies are necessary in order to evaluate whether the eeRo capacity to decrease ROS production occurs by modulating the antioxidant system.

In several studies, increased longevity has been closely associated with improved survival under conditions of heat or oxidative stress. In accordance with those studies, eeRo treatment extended mean and maximum lifespan of wild-type worms, likely by increasing cellular stress resistance. Many common stress-induced effects on physiology, gene expression and signaling pathways among animals have been reported (30).

Studies have also demonstrated single gene mutations that influence lifespan. *mev-1* encodes the *C. elegans* ortholog of the succinate dehydrogenase cytochrome *b* subunit, which is required for oxidative phosphorylation. Mutations in *mev-1* result in premature aging and increased sensitivity to oxidative stress (31). Attention has also focused on the insulin-like signaling pathway in *C. elegans* because of its pivotal role in lifespan determination and oxidative stress resistance (32,33). DAF-16 is a well-known regulator of longevity (32). Studies have also implicated heat shock factor (HSF) as an influence on longevity (15). Tullet et al. (14) suggested that SKN-1/Nrf2 directly integrates insulin/IGF signaling and the stress response.

We performed survival assays using *mev-1, daf-2, daf-16*, *hsf-1* and *skn-1* transgenic strains. Activity of the DAF-2 insulin/IGF receptor regulates both L1 arrest and dauer formation in *C. elegans*. Complete loss of *daf-2* function leads to L1 arrest and lethality at 20°C (34). Due to this, we performed the *daf-2* mutant lifespan assay at 16°C. Because the eeRo concentration at 25 µg/mL was most efficient at decreasing the mortality rate and protecting the worms, we performed survival assays with mutants using this dose. We tested *daf-2* mutant in order

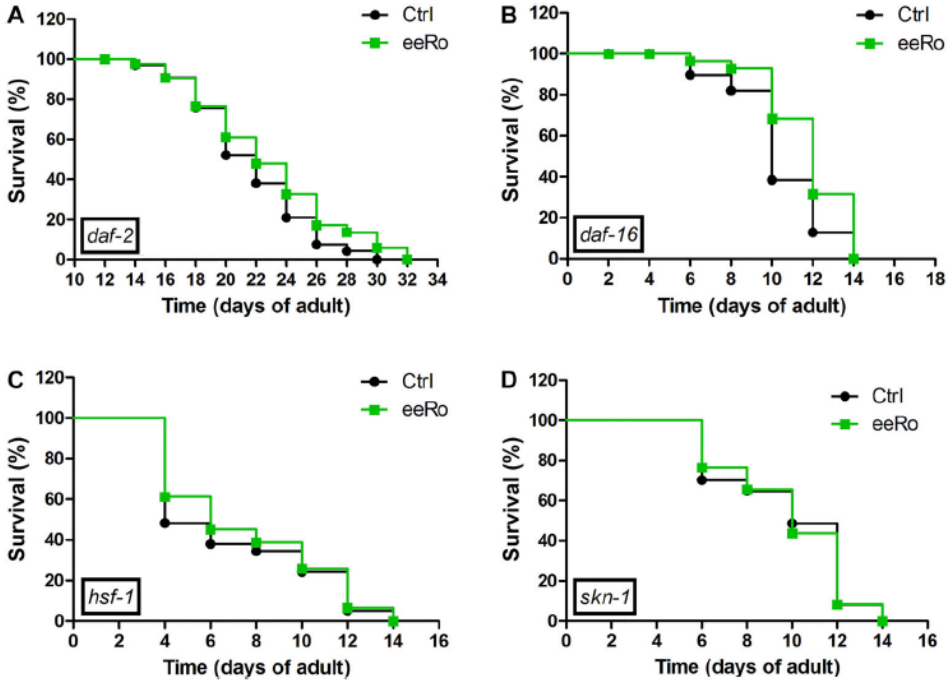

Figure 5. Effect of 25 μg/mL ethanolic extract of *Rosmarinus officinalis L.* (eeRo) in (*A*) *daf-2*, (*B*) *daf-16*, (*C*) *hsf-1* and (*D*) *skn-1* mutants lifespans. Survival curves were significantly different in *daf-16* (median lifespan) and in *daf-2* (maximum lifespan) ($P < 0.05$, log-rank (Mantel-Cox) test).

Table 2. Lifespan of mutant worms treated with 25 μg/mL eeRo.

Genotype	Median lifespan (days)		Maximum lifespan (days)	
	Control	eeRo	Control	eeRo
mev-1	10 ± 1.00	12 ± 0.57*	16 ± 0.57	18 ± 1.15*
daf-2	22 ± 1.75	22 ± 0.57	30 ± 1.15	32 ± 1.52*
daf-16	10 ± 0.57	12 ± 1.73*	14 ± 2.00	15 ± 1.15
hsf-1	8 ± 1.15	8 ± 2.00	13 ± 1.15	14 ± 0.57
skn-1	9 ± 0.86	10 ± 1.75	14 ± 0.57	14 ± 1.15

Data are reported as means ± SD. Aging assays were performed at 20°C except for *daf-2*, which was carried out at 16°C. Experiments were repeated three times starting with 90 nematodes per group. *$P < 0.05$, significantly different from untreated control group by the log-rank (Mantel-Cox) test.

to investigate if the eeRo effect on longevity was just through this pathway. In *daf-2* and *daf-16* survival curves, there was a slight difference between treated and untreated worms, in a less pronounced way compared to wild type. These data suggest a partial dependence of DAF-2 and DAF-16. These findings suggest that eeRo and *daf-2* mutants may extend adult lifespan through overlapping mechanisms that are not additive. Furthermore, our data demonstrated that DAF-16, HSF-1 and SKN-1 are required for the extract to exert its protective effect given that the treatment did not significantly decrease juglone- or thermal stress-induced mortality in *daf-16*, *hsf-1* and *skn-1* mutants, as observed in wild-type worms. Furthermore, the treatment did not extend *hsf-1* and *skn-1* lifespan, as observed in the wild type worms, emphasizing the need of these target genes for eeRo protective effect.

DAF-16 is crucial for many important processes, including development, stress resistance, thermal tolerance

and metabolism (35–38). Along with *hsf-1*, *daf-16* is part of the heat-shock response in *C. elegans* (28). The HSF plays essential and evolutionarily conserved roles in the activation of heat shock-inducible gene expression. HSFs are recognized as regulators of stress-induced gene expression, besides contributing to more complex organismal physiological processes such as development, growth, aging, immunity, and reproduction. We suggest that the beneficial effects of eeRo on aging in *C. elegans* are based on increased cellular stress resistance in a manner partially dependent on IIS pathway activation of target genes. We demonstrated that eeRo modulated the cellular response to oxidative stress in *mev-1* mutants, decreasing ROS levels and extending the lifespan. Previous studies have shown that treatment with natural compounds, such as *Ginkgo biloba* extract, increased *mev-1* mutant resistance to acute oxidative and thermal stress (39). Mutations in *daf-16* and *mev-1* resulted in similar patterns of hypersensitivity, with several interesting differences. The short lifespan and oxidative stress hypersensitivity of the *daf-16* mutant resulted from suppressed anti-oxidant gene expression rather than an increase in ROS production from the mitochondria, as in *mev-1*. In *mev-1* mutant, DAF-16 is present in the nuclei even under normal conditions (40). This observation leads to the prediction that eeRo affects both nematode ROS production and anti-oxidant gene expression.

This study demonstrates the potential protective effect of eeRo in *C. elegans,* as evidenced by an increase in tolerance against oxidative and thermal stress, a decrease in ROS production and extension of longevity in HSF-1- and SKN-1-dependent interactions. These findings suggest that eeRo triggers the signaling pathways that lead to transcriptional activation of downstream targets, which are essential for the effects described in *C. elegans*. These transcription factors play key roles in insulin/IGF-1 signaling, and several additional pathway components have been shown to modulate aging in flies, mice and possibly humans, implying that the effects of the pathway on aging and stress resistance are conserved.

We report for the first time that eeRo increased the resistance against oxidative and thermal stress and extended *C. elegans* longevity in a DAF-16, HSF-1 and SKN-1-dependent manner. These survival-enhancing effects of eeRo on *C. elegans* at both normal conditions and under stress emphasize the potential of *R. officinalis* to promote resistance against oxidative damage in these worms through the activation of related genes. As the death rate of a population is closely related to external stresses, it could be concluded that the survival-enhancing effects of eeRo on *C. elegans* under stress are very important for antiaging research.

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3.2 ARTIGO CIENTÍFICO II

***Paullinia cupana* MART prevents amyloid-β toxicity in *Caenorhabditis elegans* Alzheimer Disease model through heat shock response activation**

Daniele Coradini Zamberlan, Leticia Priscilla Arantes, Marina Lopes Machado, Tassia Limana da Silveira, Aline Franzen da Silva, Ivana Beatrice Mânica da Cruz, Félix Alexandre Antunes Soares

Guarana *(Paullinia cupana* Mart.) protects against amyloid-β toxicity in *Caenorhabditis elegans* through heat shock protein response activation

Daniele Coradini Zamberlan[1], Leticia Priscilla Arantes[1], Marina Lopes Machado[1], Tassia Limana da Silveira[1], Aline Franzen da Silva[1], Ivana Beatrice Mânica da Cruz[2], Claudia Pinto Figueiredo[3], Félix Alexandre Antunes Soares[1*]

[1]*Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas (CCNE), Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*

[2]*Laboratório de Biogenômica, Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*

[3]*Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, RJ, Brazil.*

[*]CORRESPONDING AUTHOR:

Félix Alexandre Antunes Soares

Departamento de Bioquímica e Biologia Molecular - CCNE – Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix@ufsm.br

Guarana *(Paullinia cupana* Mart.) protects against amyloid-β toxicity in *Caenorhabditis elegans* through heat shock protein response activation

Abstract

Alzheimer disease (AD) is a progressive neurodegenerative brain disorder that causes significant disruption in normal brain functioning, representing the most common cause of dementia in the elderly. The main hallmark of AD is the presence of amyloid plaques in the brain formed by the deposition of insoluble amyloid protein (Aβ) outside of neurons. Despite intensive investigation of the mechanisms of AD pathogenesis during the past three decades, little has been achieved in terms of effective treatments or ways to prevent the disease. *Paullinia cupana*, known as guarana, is a plant endemic to the Amazon region in Brazil with several beneficial effects reported, including delayed aging. In this study, we investigated the effects of chronic consumption of guarana ethanolic extract (GEE) on Aβ toxicity using a *C. elegans* model of AD. We analyzed the behavioral phenotype, oxidative damage and Aβ protein expression in worms treated with GEE. In addition, we investigated the possible role of the heat shock response on the beneficial effects induced by GEE. Overall, our data demonstrate that chronic GEE treatment decreased the formation of Aβ aggregates in *C. elegans*, preventing the behavioral deficits and the oxidative damage inducible by Aβ expression, due to activation of the heat shock protein (HSP) response. This finding provides a new alternative against amyloidogenic neurodegenerative diseases and other diseases caused by protein accumulation during aging.

Key words

Neurodegenerative Diseases, Alzheimer's, proteostase, natural compounds, heat shock factor-1, heat shock proteins.

Abbreviations

Aß, Amyloid-beta; AD, Alzheimer's Disease; Ach, Acetylcholine; AChE, Acetylcholinesterase; ASChI, acetylthiocholine iodide; DTNB, Dithiobis (2-nitrobenzoic acid); GEE, Guarana ethanolic extract; GFP, Green fluorescent protein; HSF, Heat Shock Factor; HSP, Heat Shock Protein; HSR, Heat Shock Response; ND, Neurodegenerative Disease; NIAD-4, -2-[5-[5-(4-hydroxyphenyl)thiophen-2-yl]thiophen-2-yl]methylidene] propanedinitrile; ROS, Reactive Oxigen Species; ThT, Thioflavin T.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder resulting in functional impairment and the most common cause of dementia in the elderly. Current estimates indicate that AD affects more than 35 million individuals worldwide and is a major psychosocial and economic burden to families, caregivers, and public health systems[1]. The main hallmarks of the disease are senile plaques comprised of aggregates of amyloid protein (Aβ),[2] and neurofibrillary tangles formed by hyperphosphorylation of tau protein, loss of synapses, and neurochemical changes.[3] Despite intensive investigation of the mechanisms of AD pathogenesis during the past three decades, little has been achieved in terms of effective treatments or ways to prevent the disease.

Mitochondrial dysfunction is an important component of disease and some evidence suggests that AD is a metabolic disease.[4] In addition, aberrant stimulation of excitatory N-methyl-D-aspartate receptors by Aβ has been proposed as a key mechanism leading to excessive production of reactive oxygen species (ROS),[5,6] which results in redox dyshomeostasis.[4] Antioxidants are important for maintaining the appropriate balance between oxidizing and reducing species in the body and thus delaying the aging process. Several natural compounds have been used to prevent or rescue symptoms of age-related diseases.[7]

Paullinia cupana MART, known popularly as guarana, is a Brazilian plant endemic to the Amazon region. Its seeds are rich in methylxanthines, such as, caffeine, theobromine, and theophylline, as well as tannins, saponins, catechins, epicatechins, proanthocyanidols, and other trace compounds. Ingesting guarana has been associated with beneficial effects, such as protecting against methylmercury toxicity,[8] stimulation of the central nervous system,[9] improved memory,[10] anticancer,[11] antioxidant and anti-aging.[12] Despite that guarana has been extensively used as a folk medicine for its beneficial effects on delaying age, little is known about the effects of these compounds in whole organisms or the molecular mechanisms underlying their effects.

Caenorhabditis elegans is a free living nematode that offers an alternative complementary system for deciphering neurodegenerative etiologies and aiding in drug discovery.[13] This powerful model organism presents advantages in relation to conventional models, including a rapid generation time, short lifespan, and a simple nervous system.[14] Human Aβ protein expression in *C. elegans* results in protein aggregation, leading to a progressive paralysis phenotype.[15] Previous studies have suggested that Aβ-induced toxicity in *C. elegans* can be blocked by the conserved heat-shock transcription factor orthologue *hsf-*

1, which functions as a transcriptional regulator of stress-induced gene expression and whose activity is required for the heat-shock and proteotoxicity responses.[16] In fact, dysfunctional chaperone activity has been related to age-dependent diseases, such as AD.[17] However, little is known about how to effectively control protein misfolding and prevent aggregation by targeting chaperone activity in neurodegenerative diseases.

Because AD incidence increases with age and the accumulation of Aβ in the brain is the primary influence driving AD pathogenesis,[18] we investigated the effects of chronic consumption of guarana ethanolic extract (GEE) on Aβ toxicity using a *C. elegans* model of AD. We hypothesized that GEE would maintain cellular redox control, decrease aging, prevent Aβ deposition and aggregation, and slow disease progression. In addition, we also investigated the possible role of the heat shock response on the beneficial effects induced by GEE.

1 Methods

2.1 Plant material and extract preparation

The powder of toasted seeds of *Paullinia cupana* Mart. **(**guarana) was produced and supplied by EMBRAPA Oriental (Agropecuary Research Brazilian Enterprise) located in the Western Amazon in Maués, Amazonas-Brazil. The hydro-alcoholic extract was obtained and characterized as described [19]. Briefly, the extract was produced using ethanol 70%. After 24 hours, the resulting solution was filtered, the ethanol was removed and further it was lyophilized. The predominant xanthines and catechins presented in the guarana ethanolic extract (GEE) were analyzed by means of HPLC [20] showing the following concentrations: caffeine = 12.240 mg/g, theobromine = 6.733 mg/g and total catechins = 4.336 mg/g [19]. The GEE was kept freezing to avoid auto-oxidation and dissolved in distilled autoclaved water at the time of used.

2.2 Caenorhabditis elegans Strain, Maintenance and Treatment

The strains used in the experiments were the transgenic GMC101 [unc-54p::$A\beta_{1-42}$::unc-54 3'-UTR + mtl-2p::GFP], CL2355 [pCL45 (*snb-1*/$A\beta_{1-42}$/long 3'-UTR) + mtl-2::GFP], their control strain CL2122 [unc-54(vector) + mtl-2::GFP] and CL2070 [hsp-16.2p::GFP + rol-6]. All strains were obtained from the *Caenorhabditis elegans* Genetics Center (University of Minnesota, Minneapolis, MN).

The nematodes were grown on solid nematode growth medium (NGM) seeded with live *Escherichia coli* OP50 ($O.D._{600}$ = 1.0) as a food source and maintained in a temperature-controlled incubator. 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, 8.6 mM NaCl and 1 mM $MgSO_4$), and the hatched worms at first larval stage (L1) were placed on NGM plates containing *E. coli* OP50 (excepted for the RNAi assays) and the treatment or vehicle.

Liophilyzed guarana ethanolic extract (GEE) was dissolved in distilled autoclaved water at the time of use and spread over NGM plates at final concentrations of 1 mg/ml of agar with *E. coli*. NGM plates containing *E. coli* and vehicle was used as control. Plates were incubated at 37°C overnight to allow bacterial growth. Synchronized L1 worms were transferred to treatment or control plates and cultured until adults, when the assays were performed.

The muscles specific $A\beta_{1-42}$-expressing worms GMC101 were maintained at 20ºC during 64-72h after hatching (time zero) and then submitted to a temperature upshift to 25°C to induce $A\beta_{1-42}$-muscles-expression until the assays were performed [15]. The panneuronal $A\beta_{1-42}$ expressing worms CL2355 worms were maintained at 16ºC during 36 h after hatching and then submitted to a temperature upshift to 23°C to induce $A\beta_{1-42}$ neurons-expression until the assays were performed.[21] The control strain CL2122 were maintained in the same conditions (Fig. 1). CL2070 worms were maintained at the permissive temperature 20°C.

1.3 Behavioral assays

2.3.1 Paralysis assay

After the temperature upshifting, GMC101 and CL2122 synchronous populations were assessed for body movement over time as indicated.[15] Nematodes were scored as paralyzed if they failed to complete full body movement either spontaneously or touch-provoked or if they were associated with a “halo” of ingested bacterial lawn, indicative of an inability to move to access food. Proportion of individuals not paralyzed was calculated and confidence intervals determined.

2.3.2 Pharyngeal pumping rate

The number of pharyngeal contractions during a 10-sec interval in triplicate was observed.[12] The assay was performed at three independent times with 10 worms per treatment and results are shown as pumping/minute.

2.3.3 Defecation cycle

The defecation cycle length was assessed by observing the duration between the pBoc steps (posterior body muscle contraction) of two consecutive defecations.[22] Ten animals were scored for three consecutive cycles (three successive pBoc steps) per assay. Experiments were performed at three independent times.

2.3.5 Egg laying assay

We determined the average number of progeny produced by each worm.[22] Individual synchronized young adult worms pre-treated with GEE or vehicle were transferred every day, at the same time, to fresh plates seeded with OP50 and allowed to lay eggs. The number of eggs released per day from each worm at room temperature was scored. The brood size of each worm during its whole fertile period also was scored.

1.4 Measurement of ROS

ROS generation was measured *in vivo* in *C. elegans* strains using CM-H_2DCFDA, following a previously described method [23] with minor modifications. Briefly, the worms were collected, washed with fresh M9 buffer three times and transferred to micro tubes. After, 10 µL of 2 mM CM-H_2DCFDA were added to the Eppendorf tubes containing 1000 worms in 990 µL M9 (20 µM CM-H_2DCFDA - final concentration) and incubated for 2 h. The worms were washed and transferred to 96-well plates (100 worms per well). The fluorescence intensity was measured with a SpectraMax® i3x Multi-Mode (Molecular Devices) microplate reader (Excitation: 488 nm; Emission: 510 nm).

1.5 Worms lysate samples preparation

Twenty-four hours after temperature shift, the worms were collected, washed 3 times with M9 and transferred to a microcentrifuge tube. The worms were frozen three times in liquid nitrogen and sonicated 5x for 15s (with 10s breaks) on ice at 30% amplitude, centrifuged for 30 min at 15,000 x g, and the supernatants (lysate) collected.

1.6 Acetylcholinesterase Activity assay

Acetylcholinesterase (AChE) activity in adult wild-type worms was analyzed with a colorimetric assay [24] with adaptations.[23] A 26 µl portion of the sample was mixed with 200 µl of 0.25 mM 5,5 Dithiobis (2-nitrobenzoic acid) (DTNB), and 7 µl of 156 mM acetylthiocholine iodide (ASChI) and incubated at 30°C for 5 min. The rate of change in absorbance was measured at 405 nm at 30s intervals for 4 min by spectrophotometry. Kinetic measurements were recorded and converted to total cholinesterase activity using the extinction coefficient for the colored product, 5-thio-c 2-nitro-benzoic acid (II).[24]

1.7 Aβ analysis with thioflavin-T

The abundance of Aβ was quantified with the thioflavin T dye according to a previously described method.[25] Equal amount of total protein from every worm lysate sample was used in each independent experiment. Each tube was mixed with 10 µl M9 and 2 µl of thioflavin-T 1mM (20 mM - final concentration) in a final volume of 100 µl. Fluorescence resulting from Aβ stained by thioflavin-T was measured with a SpectraMax® i3x Multi-Mode (Molecular Devices) microplate reader (Excitation: 440 nm; Emission: 482 nm).

1.8 Aß staining and microscopy in living C. elegans

The levels of Aβ peptide were quantified in nematodes using the Aβ-specific fluorescent probe NIAD-4 (2-[[5-[5-(4-hydroxyphenyl)thiophen-2-yl]thiophen-2-yl]methylidene] propanedinitrile) according to a previously describe method.[15] Live worms were incubated with 1 µM NIAD-4 (0.1% DMSO in M9 buffer) for 4 hours at room temperature. After staining, worms were allowed to recover o NGM plates for about 24 hours to allow destaining via normal metabolism. Stained animals were mounted on 2% agarose pads containing 40 mM NaN3 as anesthetic on glass microscope slides for imaging. Images were captures with an Olympus fluorescence microscope with a 20X objective and a *49004 ET-CY3/TRITC filter (Chroma Technology Corp).* Fluorescence intensity was calculated using ImageJ software (National Institutes of Health) and then normalized as the corrected total cell fluorescence. Only head region was considered because of the high background signal in the guts. All experiments were repeated at least three times and the data from one representative experiment are shown.

1.9 Confocal microscopy

GFP-expression in CL2070 was analyzed in adult worms treated with GEE or vehicle. As positive control, the worms were submitted to a heat shock of 30°C during 4h to induce *hsp-*

16.2 expression. Nematodes were anesthetized by the use of 10 mM levamisole and a drop of the worm pellet was put on a glass slide. Fluorescence was viewed under a confocal laser scanning microscope (fluoview KV10i Olympus) with excitations at 488 nm and emissions at 500-530 nm and fluorescence intensities was determinate using ImageJ software (National Institutes of Health). Only head region was considered because of the high background signal in the guts. All experiments were carried out in triplicate and ten animals per group were used in each experiment.

1.10 RNAi procedures

RNA interference (RNAi) was carried out using the feeding method described previously, with empty pL4440 as the control [26]. Briefly, RNAi clones were grown with 12.5 μg/mL tetracycline and 100 μg/mL ampicillin. On the following day, cultures were diluted in LB supplemented with 60 μg/mL ampicillin and grown to an OD600 of 1. This culture was used to seed plates containing ampicillin and 1 mM IPTG and incubated at room temperature for 2 days to allow bacteria growth and IPTG activation. Synchronized L1 larvae were then placed on the plates containing *E. coli* HT115 that expresses *hsf-1* or *hsp-16.2* or empty vector RNAi at 20°C for 48 h, until they reached the L4 stage and than submitted to a temperature upshift to 25°C to induce Aβ expression until all worms get paralised. *hsf-1* RNAi efficiency was verified by the absence of F1 larvae. For *hsp-16.2*, RNAi efficiency was confirmed by the suppression of GFP emission on Hsp-16.2::GFP transgenic line (CL2070).

1.11 Statistical analyses

Statistical analysis was performed using *GraphPad* (Version 5.0 for Macintosh OSX, *GraphPad* Software, San Diego, CA). Significance was assessed using t tests, or one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test, or two-way ANOVA, followed by Bonferroni Test for *post hoc* comparison. Values of $p<0.05$ were considered statistically significant.

2 Results

3.1 GEE decrease Aβ toxicity in C. elegans

Inducing the $A\beta_{1-42}$ muscle-expression in GMC101 worms we observed a progressive paralysis started about 30 h after the temperature increase, with a mid-paralysis (the time

when 50% of worms get paralyzed) of approximately 50 h for the Ctrl group (Fig. 2A). The worms treated with GEE in the concentration of 0.5 and 1.0 mg/mL demonstrated a significant delay in the paralysis phenotype ($p<0.01$ and $p<0.001$, respectively), with a mid-paralysis of 66 and 72 h, respectively. At 60 h after temperature up-shifting, almost 70% of GMC101 ctrl worms were paralyzed, against 60%, 40% and 20% of the worms treated with GEE at 0.1, 0.5 and 1.0 mg/mL, respectively (Fig. 2B).

We performed the pharyngeal pumping and defecation cycle behavioral assays in the worms at 24 and 48 h after the Aβ-induced expression. No difference in pharyngeal pumping rate or defecation cycle was observed among the groups 24 h after the temperature upshifting (Fig. 2C and Fig. 2D). However, we observed a significant decrease in the pharyngeal pumping rate 48 h after the temperature upshifting in the transgenic strain GMC101 when compared to its control strain CL2122 (Fig. 2E – $p<0.001$). We also observed an slower defecation cycle 48 h after the temperature upshifting in the transgenic strain GMC101 when compared to its control strain CL2122 (Fig. 2F – $p<0.001$). The treatment with GEE prevented both these effects induced by Aβ expression (Fig. 2D and E - $p<0.001$).

3.2 GEE increase transgenic C. elegans lifespan

The neuron expression of Aβ in *C. elegans* (CL2355) leads to an early mortality, presenting a median survival of 10 days comparing to 12 days in the control strain (CL2122 – Fig. 3A) A significant difference between CL2122 and GMC101 Ctrl groups can be observed since the day 8 of adult ($p<0.001$ – two-way ANOVA). The treatment with 1 mg/ml GEE increased lifespan in that transgenic strain, resembling the control with no Aβ expression ($p<0.001$). Still, GEE treatment increased lifespan in the control strain, which showed a median survival of 14 days ($p<0.001$). No difference in CL2355 worms lifespan was observed in the worms treated with lower GEE concentrations (data not show).

We analyzed the offspring during the fertile period of the worms expressing Aβ in neurons. Our results demonstrated an increase in egg position only in the first adult day in the worms expressing Aβ in neurons (CL2355) when compared to the control strain (CL2122 - Fig. 3B). The treatment with GEE prevented this increase. However, no difference in total progeny or egg production was detected between the groups (Fig. 3C and D).

2.3 GEE decrease the oxidative stress induced by Aβ

Aβ-muscles expression in *C. elegans* (GMC101) induced a significant increase in ROS levels when compared to the worms with no Aβ expression (CL2122) (Fig. 4 – $p<0.01$). However, no difference in ROS levels were induced by Aβ-neuron expression (CL2355).

After GEE treatment, worms expressing Aβ in both muscles and neurons demonstrated significant lower ROS levels when compared to untreated worms ($p<0.05$). In the control strain, this GEE effect was not observed (Fig. 4).

2.4 GEE decrease Ache activity

The worms expressing the Aβ peptide in either muscles (GMC101) or neurons (CL2355) demonstrated no difference in Ache activity when compared to the control strain (CL2122). Nevertheless, the treatment with GEE induced a significant decrease in Ache activity in Aβ-muscles expressing worms ($p<0.05$). In Aβ-neurons expressing control worms we can observe a similar tendency in reduce ache activity in treated worms, however, this effect was not significant (Fig. 5).

2.5 GEE decrease Aβ aggregates

We detected high Aβ fibrils levels in GMC101 transgenic worms using both ThT and NIAD-4 fluorescent dyes (Fig. 6A-E – $p<0.01$). After GEE treatment, these worms presented significant lower Aβ levels when compared to untreated worms ($p<0.05$). No Aβ fibrils were detected in CL2355 transgenic strain (data not show).

3.6 GEE induce hsp-16.2 expression

Transgenic worms expressing *hsp-16.2* linked to the reporter GFP demonstrated increased fluorescent levels after the chronic treatment with GEE, when compared to untreated groups (Fig. 7 D – $p<0.05$). Heat shock (30°C/4h) *hsp-16.2* expression was induced as a positive control.

3.7 GEE protective effects are dependent of hsf-1 and hsp-16.2

Feeding worms with *hsf-1* RNAi leads to an earlier paralysis in the worms expressing Aβ peptide in muscle (mid paralysis = 32 h) when compared to the worms feeding the empty vector (mid paralysis = 64 h). Curves are significantly different at all time points (Fig. 8A). The worms feeding *hsp-16.2* RNAi presented an accelerate paralysis at the time points between 30 h and 64 h (mid paralysis = 64 h) when compared to the control RNAi, less pronounced than *hsf-1* RNAi (Fig. 8A). The treatment with GEE postponed the paralysis

phenotype in control RNAi-feeding worms (mid paralysis = 96 h – Fig. 8B) similar to observed in OP50-feeding worms (Fig. 2A). This protective effect was observed in a less pronounced manner in *hsp-16.2* RNAi-feeding worms, (mid paralysis = 88 h – Fig. 8D) but not in *hsf-1* RNAi-feeding worms (mid paralysis = 32 h – Fig. 8C).

3 Discussion

In this study, we hypothesized that GEE would have preventive effects against the onset and progression of Aβ protein accumulation and toxicity. Overall, our data demonstrate that chronic GEE treatment decreased the formation of Aβ aggregates in *C. elegans*, preventing the behavioral deficits and the oxidative damage inducible by Aβ expression, due to activation of the heat shock response (HSR). Widespread protein misfolding and aggregation with aging could be critical for the initiation of pathogenesis, target of therapeutic strategies to alleviate ND.

Previous studies demonstrated that chronic treatment with guarana extract extends *C. elegans* lifespan and health span.[27] Data from our group demonstrated similar beneficial effects of GEE extract on *C. elegans* aging process[12] and modulation of genes involved in antioxidant response.[8] Among the tested concentrations, those beneficial effects of GEE were more pronounced at 1 mg/ml.[12] Accordingly, in the present study we observed a higher effect against Aβ toxicity at 1 mg/ml GEE, and performed the following experiments using that concentration.

Transgenic *C. elegans* strains in which the human Aβ sequence is expressed in neurons or muscles cells have been generated in the last few years allowing the screening for pathways or molecules that can prevent the formation or promote the disassembly of Aβ aggregates. Here, the 1 mg/ml GEE treatment induced a significant increase in the lifespan of *C. elegans* in worms expressing Aβ in neurons and in the control strain. Worms expressing Aβ in neurons treated with GEE lived a similar lifetime as the untreated control strain. However, it was not clear if the GEE effects were due to a protective effect against Aβ toxicity. Mc Coll et al. established *C. elegans* that express human $A\beta_{1-42}$ (predominant Aβ species in the brain) under control of the muscle specific promoter *unc-54*, resulting in age-dependent paralysis.[15] Our data demonstrate that expression of this full-length $A\beta_{1-42}$ in muscle induced an age-dependent impairment in the pharyngeal pumping rate and defecation cycle in worms, indicating damage to the motor control mechanism. A proper feeding rate and precise timing of pharyngeal movements is required for efficient feeding and survival in

nature. In addition, the animals that stopped feeding interrupted their defecation rhythms.[28] No impairment in the pumping activity of the pharynx was observed on CL2122 GEE treated worms, suggesting GEE lifespan extension is not due to a caloric restriction. Instead, here we show that chronic treatment with GEE prevented the behavioral impairments induced by $A\beta_{1-42}$-muscle expression. This was clearly demonstrated by diminished paralysis, increased pharyngeal pumping, and an accelerated defecation cycle observed in the Aβ-treated worms.

GEE protective effect against Aβ toxicity was also elucidated by diminished ROS levels observed in treated worms compared to those in untreated worms. Despite the previously demonstrated antioxidant effect of GEE,[8,12] we believe that this decrease in oxidative stress status was due to GEE preventing Aβ aggregation and toxicity, as no significant effect of the treatment was evidenced in no Aβ-expressing worms. Corroborating our data, other studies have demonstrated a decrease in ROS reflected by downregulation of Aβ expression [23], conferring protection to the worms against oxidative damage.

The ameliorated Aβ-phenotypes may be reflected by the GEE property to prevent Aβ expression and aggregation diminishing its toxicity, as we demonstrated using the fluorescent probes ThT and NIAD-4. The enhanced fluorescence exhibited by ThT on Aβ-muscle expressing worms indicates an increase in amyloid fibril formation. These effects were not observed in Aβ-neuron expressing worms. This unexpected result could be due to lower undetectable amyloid fibril levels in CL2355 transgenic worms, or even the presence of only Aβ monomers and oligomers, but not amyloid fibrils. In fact, there is no evidence of neuronal Aβ-deposits in this strain.[21] In order to confirm that this fibrils are formed by Aβ peptide, we use NIAD-4, which binds with high affinity to Aβ plaques.[29] Corroborating the ThT data, we observed the highest fluorescence intensity on Aβ-muscle expressing worms when compared to the other groups. In addition, worms treated with GEE demonstrated lower ThT and NIAD-4 fluorescence intensities compared to those of the untreated worms, indicating that GEE prevents Aβ aggregation. Peixoto et al. (2017) observed a lower number of poliQ40 aggregates in AM141 transgenic worms treated with a GEE extract, corroborating the putative mechanism of GEE in proteostasis [27].

Moreover, the GEE capacity to decrease acetylcholinesterase activity collaborate its protective effect against AD pathogenesis, as this probably increases acetylcholine (Ach) levels. In humans, Ach is involved in learning and memory and levels decrease with aging. Many researchers have presumed that progressive disruption of cholinergic function underlies much of the short-term memory loss seen in AD. Delaying or preventing cholinergic neurodegeneration or even minimizing its consequences is the main mechanism of action for

most currently available US FDA-approved drugs used to treat the cognitive dysfunction observed in AD.

Interestingly, we found induction of HSP-16.2 expression in response to the GEE treatment in transgenic *C. elegans*. HSP-16 is a family of small chaperone proteins, homologous to alphaB-crystalline that are induced as a protective response to heat shock or other environmental stressors. It has already been demonstrated that the HSP-16 protein co-immunoprecipitates with Aβ promoting its sequestration, degradation, or refolding and altering its toxicity.[30] Correspondingly, expression of HSP-16.2 reduces Aβ aggregation and suppresses paralysis *in vivo.*[31] These studies agree with our data, indicating HSP-16.2 overexpression is a putative mechanism for decreasing Aβ aggregation and the lower toxicity observed in worms treated with GEE. Another substantial finding in transgenic *C. elegans* was induction of the HSP-16 family by Aβ expression itself.[32,33] However, this seemed to be ineffective in preventing Aß aggregation, possibly because expression occurs at a later age when the Aß levels are already high. Our data indicate that inducing hsp-16.2 expression early in life, promoted Aß sequestration and degradation, and prevented late aggregation and proteotoxicity in *C. elegans*.

The expression of chaperones in *C. elegans* is induced by the heat-shock transcription factor orthologue HSF-1. HSF-1 functions as a transcriptional regulator of stress-induced gene expression whose activity is required for heat-shock and proteotoxicity responses. We performed a paralysis phenotypic analysis in transgenic worms expressing the Aß-peptide using *hsf-1* or *hsp-16.2* RNAi to confirm whether our GEE treatment influenced the heat shock response. Knocking down *hsf-1* in Aß muscle-expressing worms accelerated paralysis. This effect was less pronounced in *hsp-16.2* knockdown worms, indicating that HSF-1 function is essential in events of proteotoxicity, presumably by inducing release of the transcriptional target to perform their protein-folding role. Corroborating our data, Walker et al. (2003) reported that *hsf-1* (RNAi) worms are thermosensitive, display reduced *hsp16-2* expression, and have a reduced life span.[34] Furthermore, our data clearly demonstrate that the protective effect of GEE in Aß toxicity is nullified in *hsf-1* and *hsf-16.2* knockdown worms. These data support our hypothesis that the beneficial effects of GEE occur by activating HSPs. We speculate that GEE induced a hormetic response promoting activation of HSF-1 and inducing transcription of HSPs, which are able to detect unfolded proteins preventing their accumulation and aggregation. Focus on compounds that are efficacious in extending lifespan is substantial to delaying age-associated diseases, since aging affects energy metabolism, proteostasis, and cellular redox control.

Induction of the heat shock response has been suggested to be beneficial for diseases of protein conformation by preventing protein misfolding and aggregation, and as such has been proposed as a therapeutic target for age-associated neurodegenerative disorders. Our study demonstrates that GEE increased the ability of *C. elegans* to cope with proteotoxicity due to activation of the heat shock response and consequent transcription of target genes involved in detecting unfolded proteins, which prevented their accumulation and aggregation early in life. Wherefore, chronic GEE treatment decreased the formation of Aβ aggregates in *C. elegans*, preventing the behavioral deficits and the oxidative damage inducible by Aβ expression.

The predominant constituents found in the GEE extract were caffeine and polyphenols. Previous studies demonstrated caffeine enhance the heat shock response, promotes proteostasis[35] and delay age-associated pathology in *C. elegans*,[36] including protection against Aβ toxicity.[37] However, had already been demonstrated that high-dose caffeine is a developmental stressor to *C. elegans*[38] and humans[39]. Besides, Regitz et al. demonstrated a protective effect of polyphenols on Aβ-toxicity throw activation of protein degradation pathways[40].

We propose that guaraná consumption could be beneficial mainly in individuals with risk factors to prevent future protein aggregation, and proteotoxicity, and offers a new alternative against amyloidogenic ND and other diseases caused by protein accumulation during aging.

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Conflict of interest None

Ethics approval None

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Figures legends

Figure 1. Experimental design for the investigation of GEE effect in transgenic *C. elegans* expressing Aß peptide in muscles (GMC101) or neurons (CL2355) and the control transgenic strain (CL2122). GMC101 worms were maintained at 20°C during 64-72h post egg-laying (time 0) and then submitted to a temperature upshifting at 25°C to induce Aß muscles-expression. CL2355 worms were maintained at 16°C during 36h post egg-laying (time 0) and submitted to a temperature upshifting at 23°C for more 36h to induce Aß neuron-expression. The treatment with GEE was started at the first larval stage (L1) until the assays be performed.

Figure 2. Effect of GEE on Aß-muscles expressing *C. elegans* behavior. (A) GEE delayed worms paralysis induced by Aß-muscles expression in the temperature sensitive strain GMC101. *$p<0.01$ (compared to GMC101 Ctrl group). There were no difference in pharyngeal pumping rate (B) and defecation cycle (C) on transgenic worms 24 hours after temperature upshifting. GEE prevented pharyngeal pumping (D) and defecation cycle (E) deficits induced by Aß expression at 48h after the temperature upshifting. Data are average of three three independent assays with about 15 worms per group and are expressed as means ± SEM. Worm strain CL2122 was used as a transgenic control. *$p<0.001$ comparing CL2122 to GMC101 CTRL groups; $p<0.001$ comparing CTRL to GEE (Two-way ANOVA followed by Bonferroni post-hoc test).

Figure 3. Effect of GEE on Aß-neuron expressing *C. elegans* behavior. (A) Survival curve for lifespan experiment. GEE extended lifespan in both Aß-neuron expressing worms (CL2355) and its transgenic control strain (CL2122). Data are the average of three independent assays with 250-280 worms per group and are expressed as means ± SEM. *$p<0.001$ (Log-rank (Mantel-Cox test). (B) Viable eggs per worm per day on Aß transgenic worms (CL2355) and its control strain (CL2122). *$p<0.05$ comparing CL2122 and CL2355 Ctrl goups on day 1; #$p<0.05$ comparing CL2122 and CL2355 Ctrl goups on day 2; ɣ$p<0.05$ comparing Ctrl to GEE group (Two-way ANOVA followed by Bonferroni post-hoc test). (C) Total brood size laid per worm. (D) Number of eggs produced inside the worm. Data above are the average of three independent assays with 30 worms per group and are expressed as means ± SEM.

Figure 4. GEE effect on ROS levels in transgenic *C. elegans* expressing Aß peptide in muscles (GMC101) or neurons (CL2355). Worm strain CL2122 was used as a transgenic control. Data are the average of four independent assays with 400 worms per group and are expressed as means ± SEM.*$p<0.01$ compared to CL2122; #$p<0.05$ comparing Ctrl to GEE (Two-way ANOVA followed by bonferroni post-test).

Figure 5. GEE effect on Acetylcholinesterase (Ache) activity in transgenic *C. elegans* expressing Aß peptide in muscles (GMC101) or neurons (CL2355). Worm strain CL2122 was used as a transgenic control. Data are the average of four independent assays performed in duplicate (n=4) and are expressed as means ± SEM. *$p<0.05$ comparing Ctrl to GEE (Two-way ANOVA followed by bonferroni post-test).

Figure 6. GEE effect on Aß aggregates in transgenic *C. elegans* expressing Aß peptide in muscles (GMC101) or neurons (CL2355). Fluorescent images of Aß aggregates using NIAD-4 in (A) control transgenic strain (Ctrl -) and (B) Aß muscles expressing worms trated with vehicle (ctrl +) or (C) GEE. Scale bar: 100 μM. Quantification staining of Aß peptide using the fluorescent probes (D) NIAD-4 and (b) thioflavin T. Worm strain CL2122 was used as a transgenic control. Data are the average of three independent assays with (d) 30 or (e) 300 worms per group and are expressed as means ± SEM.*$p<0.05$; **$p<0.01$ compared to Ctrl -; #$p<0.05$ compared to Ctrl + (One-way ANOVA followed by bonferroni post-test).

Figure 7. GEE effect on *hsp-16.2* expression. Fluorescent images of *hsp-16.2*::GFP worms (CL2070) in (A) basal conditions, (B) after thermal stress (30°C/4h) and (C) after GEE treatment. Scale bar: 100 μM. (D) Quantification of *hsp-16.2*::GFP expression. Data are the average of three independent assays with 30 worms per group and are expressed as means ± SEM. *$p<0.05$; **$p<0.01$ compared to Ctrl -; #$p<0.05$ compared to Ctrl + (One-way ANOVA followed by bonferroni post-test).

Figure 8. GEE effect on transgenic *C. elegans* is dependent of heat shock response. Paralysis behavioral on Aß-muscles expression worms (GMC101) feed with (a) RNAi without GEE treatmet and (b) empty vector (EV) RNAi, (c) *hsf-1* RNAi or (d) *hsp-16.2* RNAi, treated with GEE or vehicle. Data are the average of three independent assays with 150 worms per group. Curves are significant different at $p<0.001$ as indicated (Two-way ANOVA followed by bonferoni post test).

Graphical Abstract

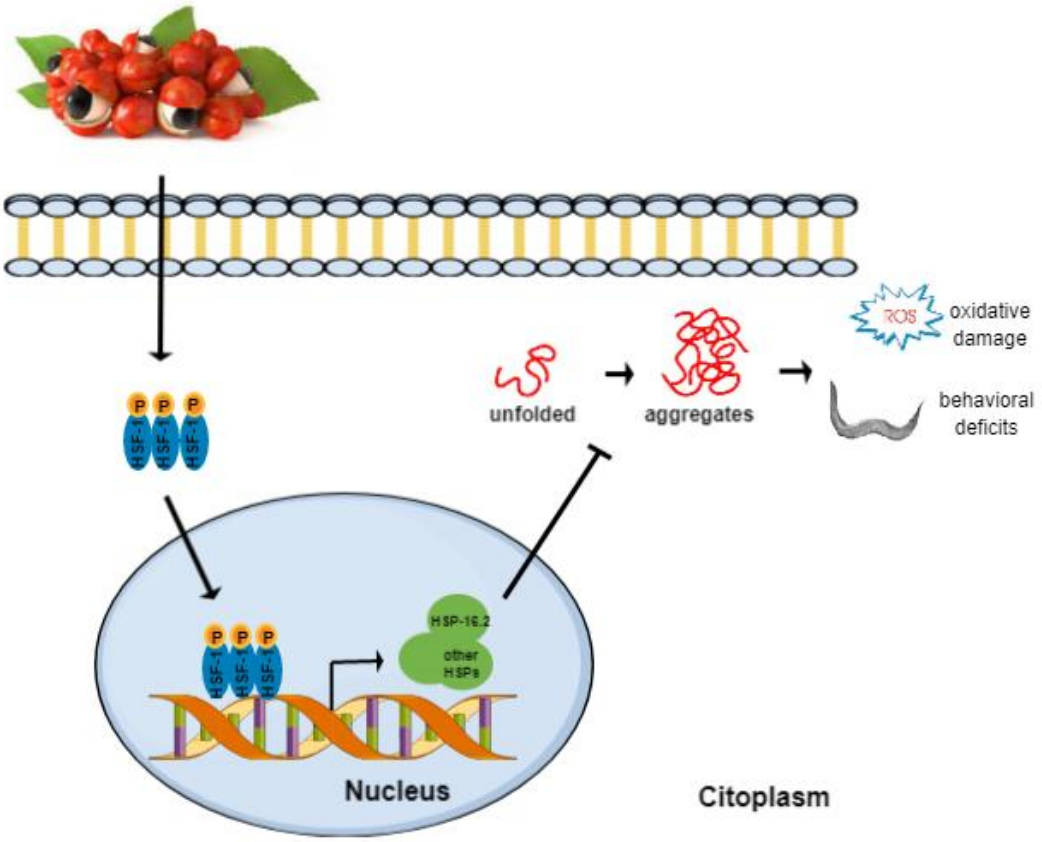


Figure 1

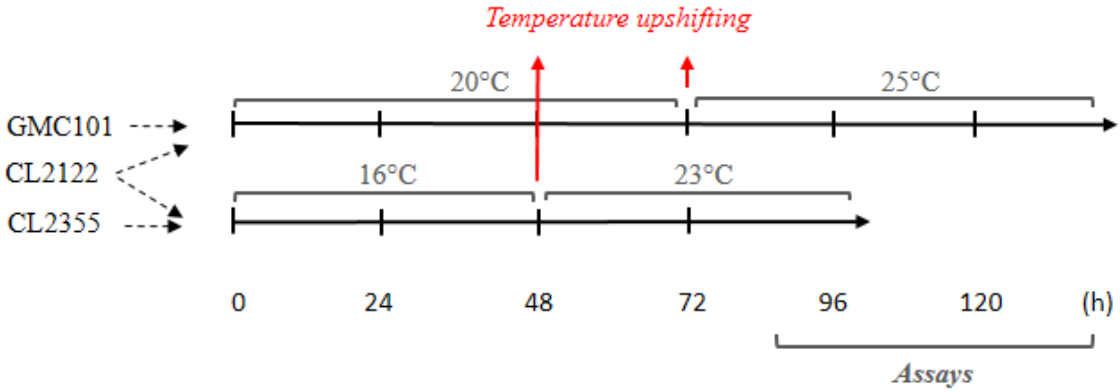


Figure 2

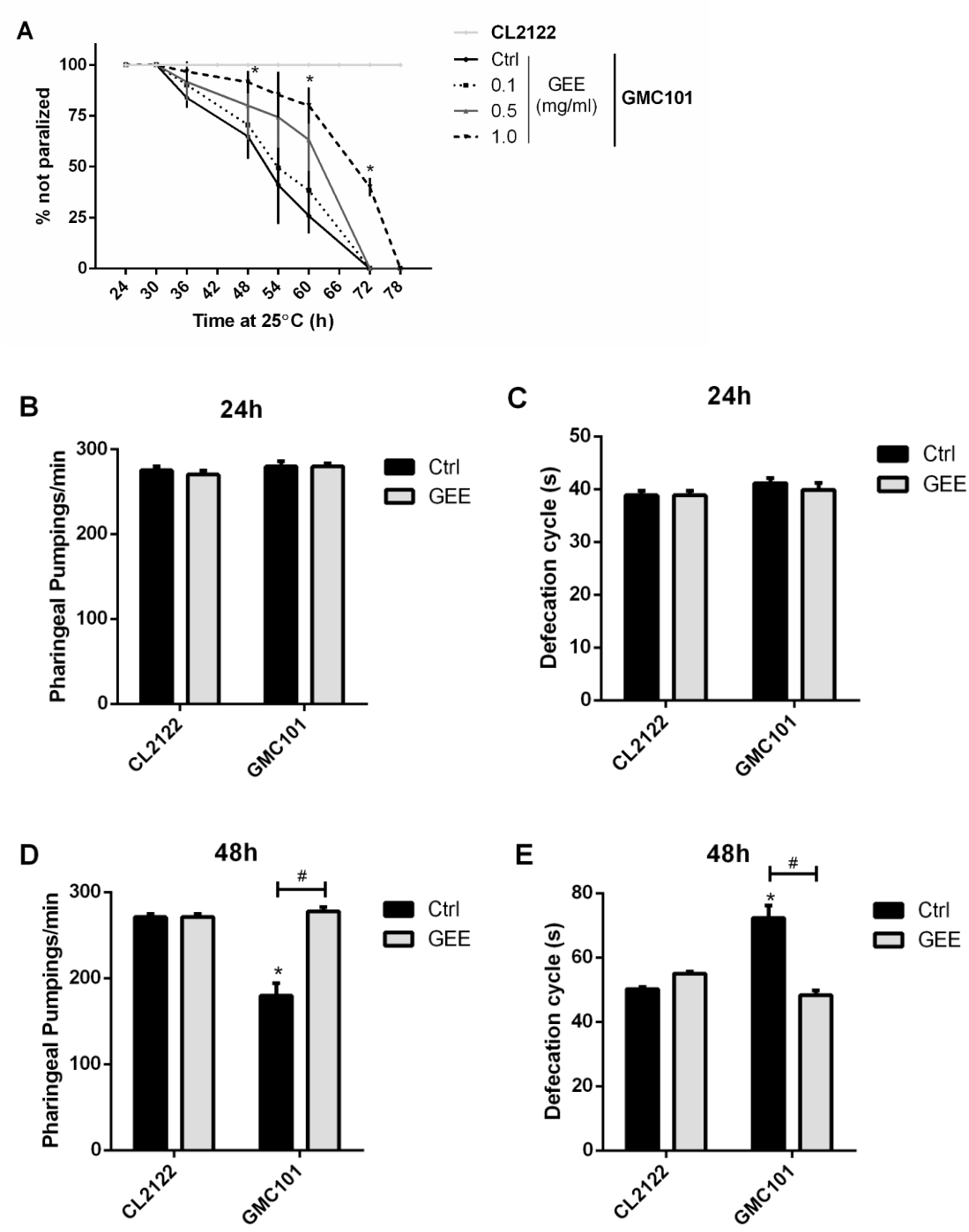

A
CL2122
Ctrl
0.1
0.5
1.0
GEE (mg/ml)
GMC101
% not paralized
Time at 25°C (h)
B
24h
Pharingeal Pumpings/min
Ctrl
GEE
CL2122
GMC101
C
24h
Defecation cycle (s)
D
48h
E
48h

Figure 3

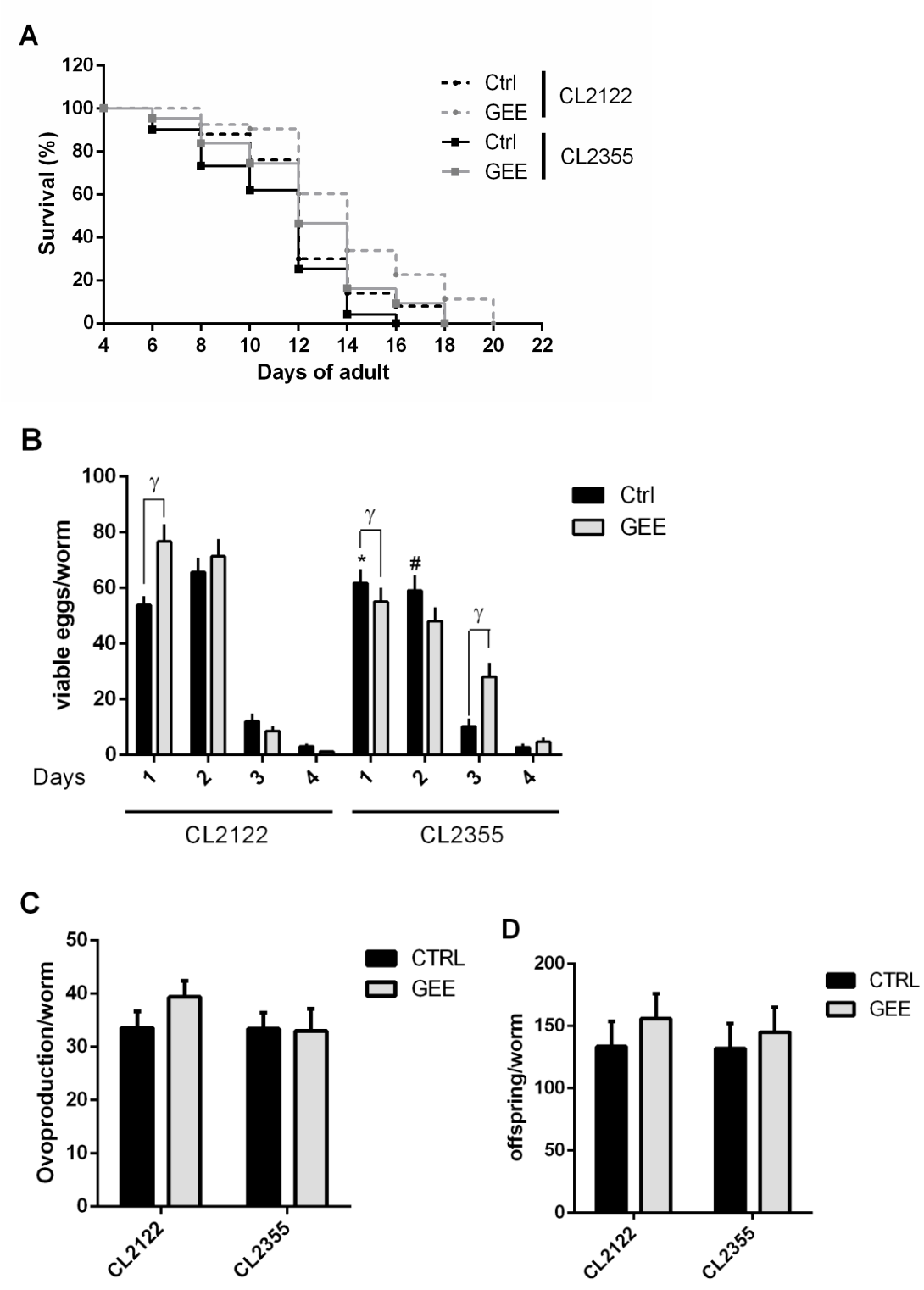

A
Survival (%)
Days of adult
Ctrl
GEE
Ctrl
GEE
CL2122
CL2355
B
viable eggs/worm
Days
Ctrl
GEE
CL2122
CL2355
C
Ovoproduction/worm
CTRL
GEE
CL2122
CL2355
D
offspring/worm
CTRL
GEE
CL2122
CL2355

Figure 4

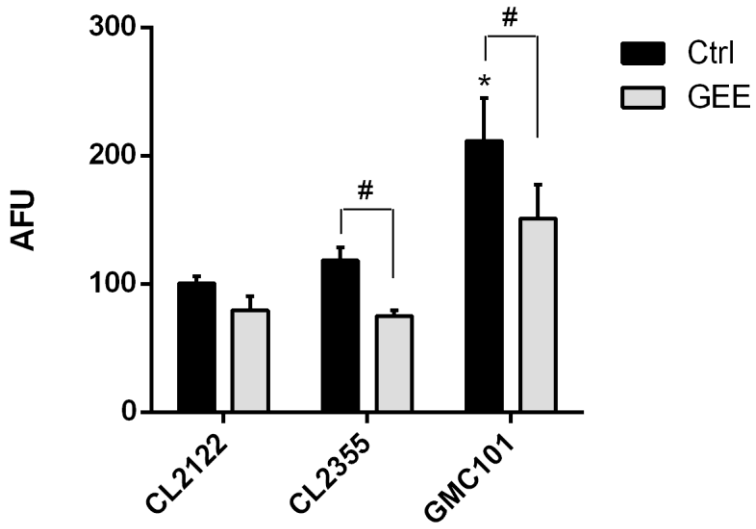


Figure 5

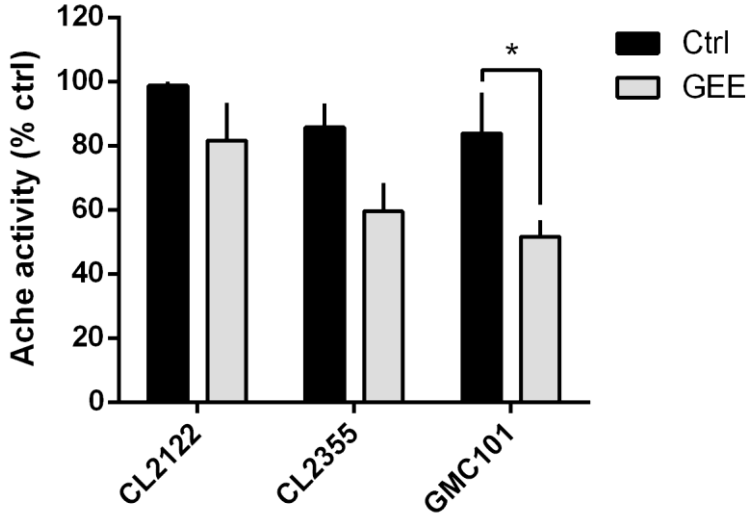


Figure 6

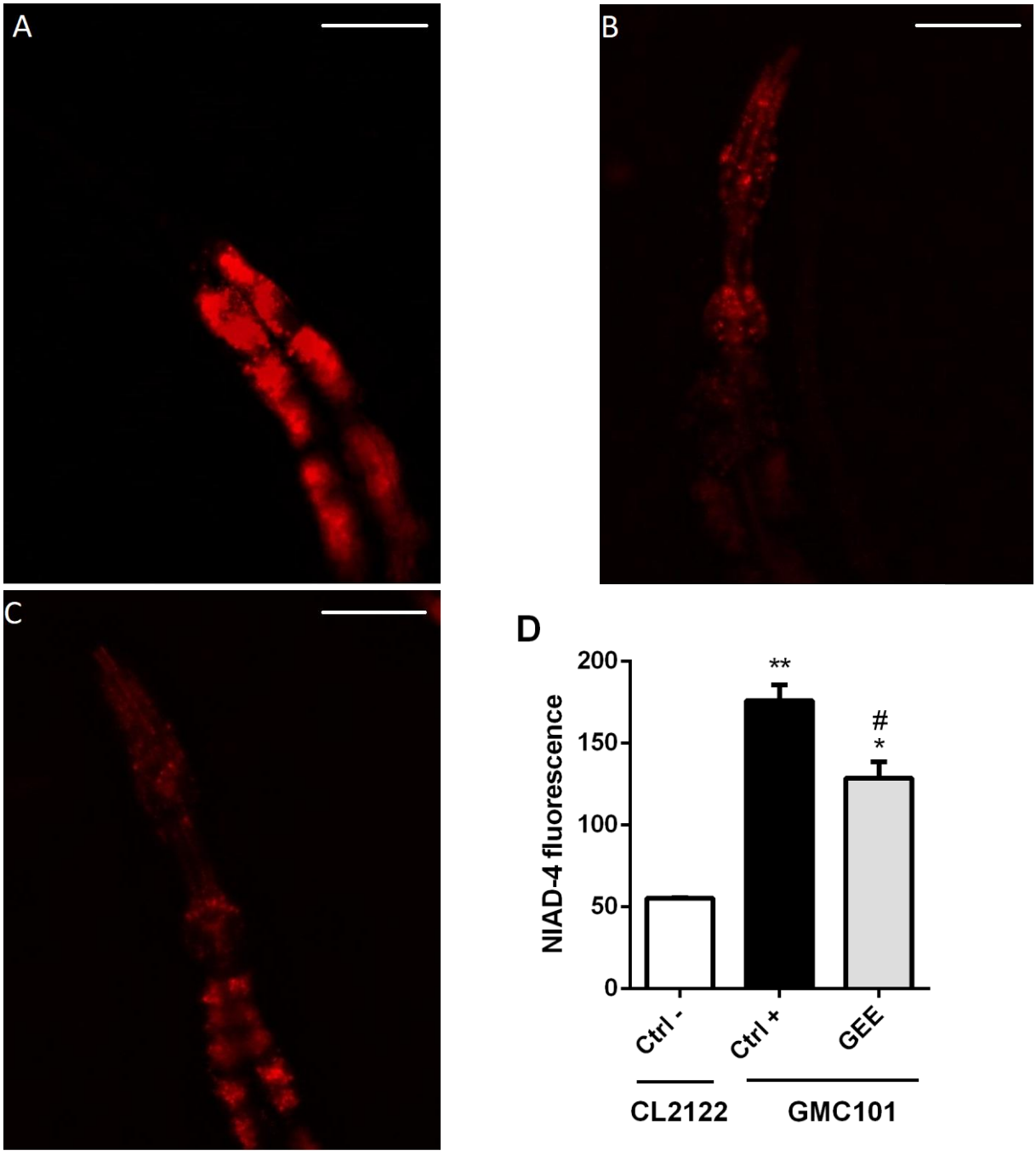

A
B
C
D
NIAD-4 fluorescence
200
150
100
50
0
**
#
*
Ctrl -
Ctrl +
GEE
CL2122
GMC101

Figure 7

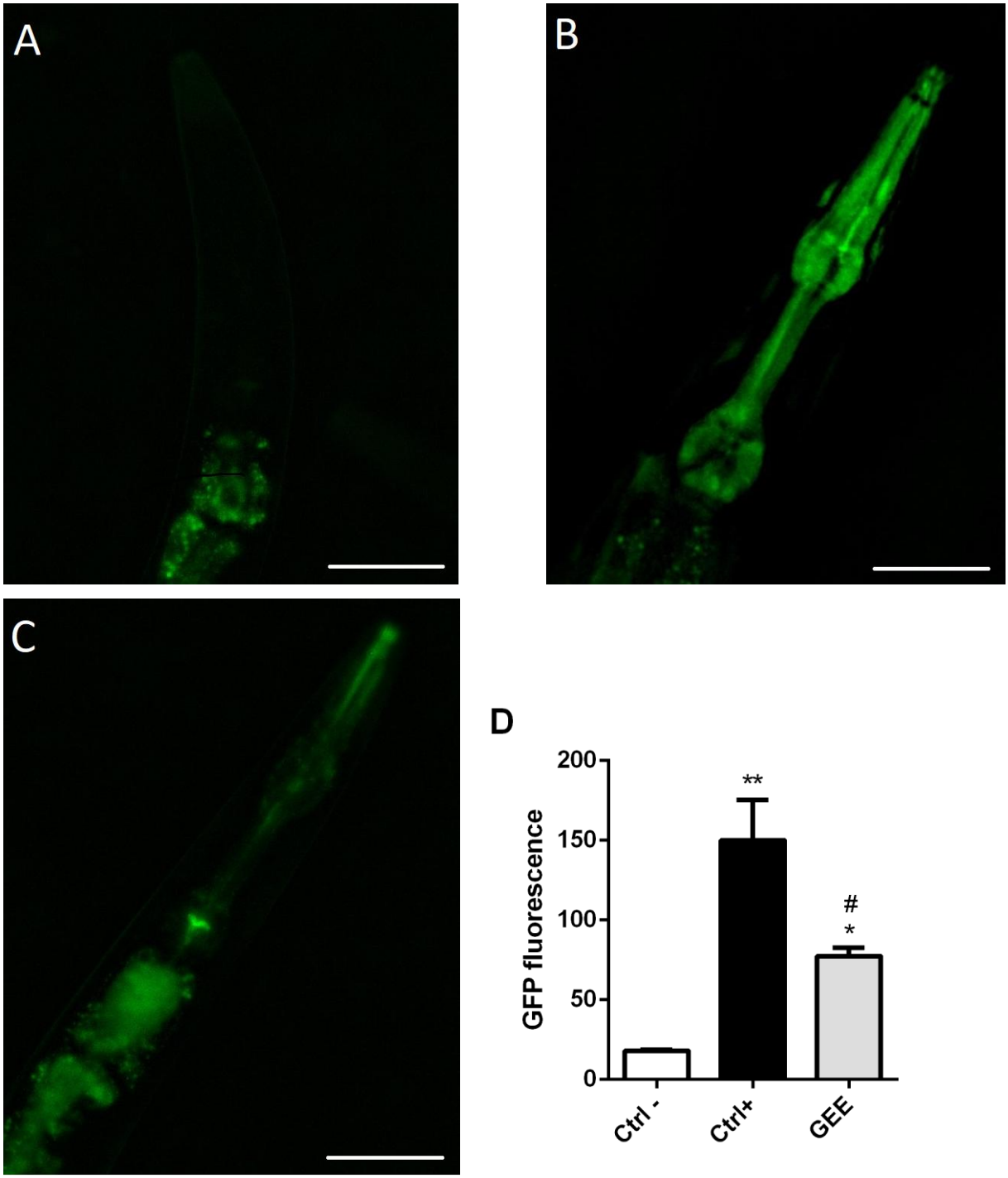
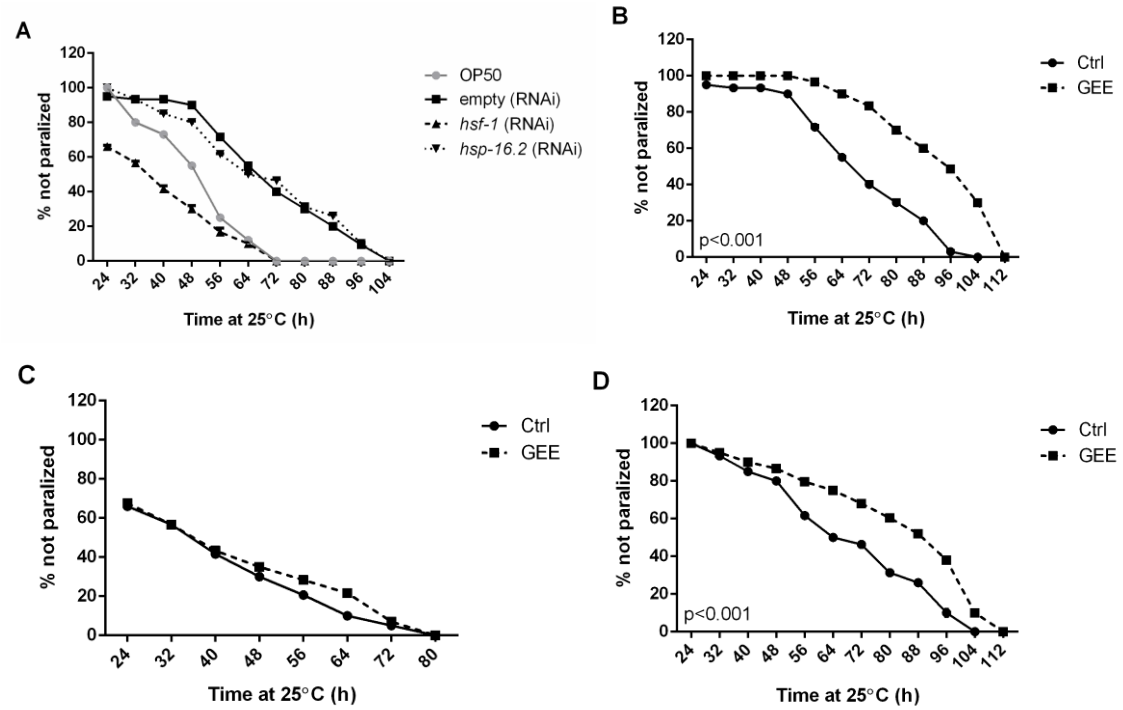


Figure 8


A
% not paralized
Time at 25°C (h)
OP50
empty (RNAi)
hsf-1 (RNAi)
hsp-16.2 (RNAi)
B
% not paralized
Time at 25°C (h)
Ctrl
GEE
p<0.001
C
% not paralized
Time at 25°C (h)
Ctrl
GEE
D
% not paralized
Time at 25°C (h)
Ctrl
GEE
p<0.001

4 DISCUSSÃO

As substâncias químicas presentes nas plantas, são uma das mais relevantes fontes de produtos farmacêuticos em diversas áreas (Harvey, 2008; Kinghorn *et al.*, 2011; Cragg e Newman, 2013). Extratos vegetais representam uma fonte importante para a descoberta de novos medicamentos, principalmente porque as plantas proporcionam um efeito terapêutico desejável com risco reduzido de complicações iatrogênicas (Andrade *et al.*, 2018). Neste trabalho foram analisados os efeitos da exposição crônica aos extratos das plantas *Rosmarinus officinalis* e *Paullinia cupana*, as quais possuem diversos efeitos farmacológicos descritos. Extratos brutos oferecem vantagens em relação a compostos isolados, por apresentarem muitas vezes atividade farmacológica mais pronunciada, toxicidade mais baixa, menor custo, além de serem a forma utilizada tradicionalmente pela população (Carlini, 2003). Ainda, dados da literatura mostram que as propriedades farmacológicas de extratos brutos de plantas podem ser perdidas quando componentes específicos são isolados (Pietrovski *et al.*, 2006).

Ensaios *in vitro* são amplamente utilizados para investigar possíveis efeitos tóxicos e farmacológicos de extratos vegetais, bem como outros compostos. Após identificação de compostos efetivos, estes são testados em um modelo animal (Misra, 1998; Artal-Sanz et al., 2006; Nile et al., 2012). Entretanto, ao serem 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). Desta forma, estudos em organismos íntegros e simples, permitem a avaliação simultânea de toxicidade e efeito de forma rápida e barata. Neste trabalho foi utilizado o nematódeo *Caenorhabditis elegans* como modelo experimental para estudos farmacológicos de ambos os extratos.

Os dados apresentados nesta tese abrangem dois trabalhos distintos, porém com um propósito em comum: explorar a capacidade de compostos naturais com elevado potencial terapêutico e baixo risco de complicações em aumentar a resistência a diferentes tipos de estresse, retardando o envelhecimento e ao mesmo tempo prevenindo o desenvolvimento de patologias relacionadas ao envelhecimento, aumentando a qualidade de vida dos indivíduos.

O tratamento de animais da cepa selvagem com eeRo reduziu significativamente a mortalidade induzida por estresse químico (Juglone e H_2O_2) e térmico (35°C), ambos os quais induzem toxicidade por aumentar os níveis de ERO (Blum e Fridovich, 1983; Michalski *et al.*, 2001). A atividade antioxidante de *R. officinalis* já havia sido previamente descrita (Bozin *et al.*, 2007). Corroborando com estes dados, o eeRo reduziu os níveis basais de ERO nos nematódeos. A diminuição nos níveis de ERO se reproduziu em animais “knockouts” para genes codificadores dos fatores de transcrição DAF-16, HSF-1 e SKN-1. Em contrapartida, a redução da mortalidade não se reproduziu nos animais “knockouts”. Estes dados indicam que o eeRo aumenta a resistência ao estresse através da ativação de genes envolvidos nas respostas de defesa do organismo frente a condições desfavoráveis e não simplesmente por reduzir os níveis de EROs. Por consequente, também pode-se sugerir que este efeito protetor se perpetua a longo prazo, de forma que foi observado um aumento significativo no tempo de vida dos nematódeos selvagens tratados com eeRo. Além disso, novamente este efeito não se reproduziu em animais “knockouts” para *daf-16, hsf-1 e skn-1*, indicando que a ativação destes é requerida para a proteção mediada pelo extrato. Estes fatores de transcrição são cruciais para diversos processos biológicos e sua modulação está relacionada a expressão de genes envolvidos na proteção frente a danos oxidativos, manutenção da proteostase e ativação sistema de detoxificação, conferindo efeitos benéficos no envelhecimento (Wu, 1995; Kenyon, 2011; Blackwell *et al.*, 2015).

O tratamento com eeRo nas concentrações utilizadas não surtiu efeito significativo na toxicidade mediada pela βA em modelos de DA em *C. elegans* (dados não mostrados). De fato ainda não há estudos evidenciando efeito protetor de *R. officinalis* ou de seus constituintes na agregação de proteínas mal formadas. Assim, neste estudo o efeito do eeRo no envelhecimento se restringiu a ativação de defesas relacionadas a danos oxidativos.

O efeito do GEE no envelhecimento já foi descrito previamente. Estudos em *C. elegans* demonstraram que o tratamento com extrato de guaraná aumenta a resistência ao estresse oxidativo, a longevidade e ainda melhora a qualidade de vida (Peixoto *et al.*, 2017; Arantes *et al.*, 2018). A concentração de GEE utilizada neste estudo foi a que previamente apresentou resultados mais significativos, considerando o mesmo tipo de extrato e meio de exposição (Arantes *et al.*, 2018).

Tendo em vista o efeito antienvelhecimento do GEE, neste trabalho foi a avaliado seu efeito frente toxicidade da proteína βA. A βA, juntamente com a tau, são proteínas cerebrais indicadoras de neurotoxicidade e desenvolvimento de DA, principal causa de demência em idosos. O acúmulo e a agregação da βA, formando oligômeros tóxicos e placas amiloides, induz a fosforilação da tau “in vivo” e “in vitro”, acarretando em instabilidade dos microtúbulos, inibição do transporte axonal e formação de emaranhados neurofibrilares, o que leva a toxicidade e neurodegeneração (Stancu *et al.*, 2014; Bane e Cole, 2015). O *C. elegans* fornece um eficaz sistema genético “in vivo” para estudar os efeitos e mecanismos da toxicidade da βA e tau através da análise de modelos transgênicos (Link, 2006; Brandt *et al.*, 2009). Recentemente foi desenvolvido um modelo com co-expressão de ambas, porém posterior ao desenvolvimento deste trabalho (Wang *et al.*, 2018).

Neste estudo foram utilizados nematódeos transgênicos nos quais uma sequência Aβ humana é expressa em todos os neurônios ou em células musculares (Dosanjh *et al.*, 2010; Mccoll *et al.*, 2012). O tratamento com GEE diminuiu os níveis da proteína βA muscular, bem como a sua toxicidade nos nematódeos, evidenciado pelo retardo no déficit motor e níveis de ERO. Este efeito demonstrou-se dependente da expressão do fator de transcrição HSF-1, o qual exibe diversas propriedades funcionais durante a longevidade principalmente no controle da homeostase proteica através da expressão de HSP (Wu, 1995). De fato o tratamento com GEE induziu a expressão da HSP-16.2 nos nematódeos. Corroborando com estes dados, o efeito antienvelhecimento do GEE também demonstrou ser dependente de HSF-1, além de DAF-16 e SKN-1 (Arantes *et al.*, 2018). A resposta ao choque térmico é uma resposta molecular altamente conservada frente a alterações da homeostase proteica (Morimoto 2008, 2011; Åkerfelt et al. 2010). Assim, sua indução tem sido relacionada a efeitos benéficos em doenças associadas a agregação de proteínas, tornando o HSF-1 um importante alvo terapêutico nas doenças neurodegenerativas. A indução de HSP promove o remodelamento adequado ou degradação proteossomal de proteínas mal-formadas evitando que estas se acumulem no cérebro e causem toxicidade (Penke *et al.*, 2018).

Apesar de estudados em diferentes modelos, ambos extratos demonstraram efeitos benéficos, aumentando a resistência dos nematódeos a diferentes tipos de estresse e aumentando a sua longevidade. Os dados sugerem que esse efeito

ocorre através da ativação de fatores de transcrição que modulam a expressão de genes envolvidos na resposta ao estresse e na detoxificação em *C. elegans*, como de enzimas antioxidantes, HSP, entre outros envolvidos nas linhas de defesa. Acredita-se que os efeitos visualizados neste estudo estão relacionadas a efeitos sinérgicos de vários dos constituintes dos extratos. Ambos eeRo e GEE são ricos em compostos químicos antioxidantes que possuem papel fundamental na defesa da planta, como os polifenóis. Sabe-se que a ingestão dietética de polifenóis atenua o estresse oxidativo e reduz o risco de doenças neurodegenerativas (Bhullar e Rupasinghe, 2013). Somando-se a isso, um estudo anterior demonstrou que a cafeína, principal constituinte do guaraná, reduziu a toxicidade mediada pela βA (Dostal *et al.*, 2010). Entretanto, a concentração de cafeína utilizada (3,6 mM) foi muito maior que a existente no GEE (0,06 μM) (Dostal *et al.*, 2010). Outros fitoquímicos como alcalóides, terpenos, flavonóides, taninos, saponinas e vitaminas também têm demonstrado efeitos contra os processos patológicos como as doenças neurodegenerativas (Auti e Kulkarni, 2018). Porém, mais estudos utilizando frações isoladas são necessários para confirmar esta hipótese.

O uso de produtos naturais na medicina ganhou popularidade nos últimos anos e vários compostos naturais com efeitos neuroprotetores vem sendo estudados. Fitoquímicos com potencial antioxidante vêm demonstrando exercer um papel fundamental na prevenção e tratamento de doenças associadas a dano oxidativo, incluindo as cardiovasculares e neurodegenerativas (Aherne et al. 2007; Leal et al. 2003). Sabe-se que as EROs são produzidas inevitavelmente pelo organismo como resultado de processos metabólicos (Botsoglou *et al.*, 2010). Durante o envelhecimento, os sistemas de reparo celular, incluindo a resposta adaptativa ao estresse oxidativo e a degradação de proteínas danificadas, diminuem. A exposição continua a radicais livres pode causar danos funcionais e estruturais, envelhecimento e morte celular (Gutierrez *et al.*, 2003). Os extratos utilizados neste estudo, além de serem fontes ricas em agentes antioxidantes, demonstraram efeitos benéficos no processo de envelhecimento através da modulação de sistemas de defesa, isto e, ativação de fatores de transcrição que induzem a expressão de genes envolvidos na resposta ao estresse oxidativo, vias de detoxificação e de reparo celular.

A DA, estudada neste trabalho, é o distúrbio neurodegenerativo mais comum e ainda sem cura ou terapia preventiva. Desta forma existe a necessidade de

procurar terapias alternativas e drogas mais eficazes. A exploração consciente da biodiversidade como fonte de compostos químicos permite o desenvolvimento de drogas novas, mais seguras e efetivas. Assim, essas drogas naturais podem ser propostas para estudos pré-clínicos e clínicos em diferentes doenças e condições patológicas. Considerando isto, mais estudos são necessários no futuro para avaliar a eficácia e segurança dos compostos ativos de *R. officinalis* e *P. cupana*, na proteção frente ao processo de envelhecimento e prevenção do desenvolvimento da DA, bem como outras patologias relacionadas a idade.

5 CONCLUSÕES

5.1 CONCLUSÃO GERAL

Durante o envelhecimento, os sistemas de reparo celular, incluindo a resposta adaptativa ao estresse oxidativo e a degradação de proteínas danificadas, diminuem. Neste estudo foram demonstrados compostos capazes de reforçar estes sistemas de reparo, oferecendo novas alternativas contra o envelhecimento e, ainda, no caso do GEE, prevenção contra a agregação proteica observada na DA.

5.2 CONCLUSÕES ESPECÍFICAS

- O extrato etanólico das folhas de *R. officinalis* (alecrim) aumenta a resistência ao estresse químico e térmico, bem como diminui o dano oxidativo, prolongando o tempo de vida em *C. elegans* de forma dependente dos fatores de transcrição DAF-16, HSF-1 e SKN-1;
- O extrato etanólico das sementes de *P. cupana* (guaraná) aumenta a longevidade em modelo de toxicidade neuronal da proteína βA em *C. elegans* e também em seu controle, demonstrando efeito antienvelhecimento;
- O extrato etanólico das sementes de *P. cupana r*eduz os níveis da proteína βA e sua toxicidade de forma dependente da ativação do fator de transcrição HSF-1 e consequente expressão de HSP, em modelo de toxicidade muscular da proteína βA.

4 PERSPECTIVAS

Tendo em vista os resultados obtidos nesta tese, as perspectivas para trabalhos posteriores em *C. elegans* são:

- Elucidar demais vias que possam estar envolvidas nos efeitos promovidos pelos extratos;
- Investigar o efeito do eeRo em modelos de DN;
- Investigar se o efeito do GEE se restringe a βA, ou se reproduz em outras proteínas envolvidas em proteinopatias, utilizando outros modelos de DN;
- Averiguar a diminuição na atividade da enzima Ache induzida pelo tratamento com GEE, a qual foi observada apenas na cepa transgênica que expressa a βA nas células musculares;
- Investigar possíveis efeitos tóxicos dos extratos em concentrações meiores das testadas;
- Comparar os efeitos dos extratos brutos com seus constituintes isolados.

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ANEXO 1

Date:	11 Aug 2018
To:	"Felix Antunes Soares" felix@ufsm.br
cc:	"DANIELE C ZAMBERLAN" dani_zamberlan@hotmail.com, "LETICIA P ARANTES" leprisca88@hotmail.com, "MARINA L MACHADO" marinamachado01@hotmail.com, "TASSIA L DA SILVEIRA" tassiasilli@yahoo.com.br, "ALINE F DA SILVA" alinefranzen@hotmail.com, "IVANA M DA CRUZ" ivanadacruz@gmail.com, "CLAUDIA P FIGUEIREDO" claufig@gmail.com
From:	"George E. Barreto" gesbarreto@gmail.com
Subject:	(Nutritional Neuroscience) Your submission has been accepted

Ref.: NNS1324R1
Guarana (Paullinia cupana Mart.) protects against amyloid-β toxicity in Caenorhabditis elegans through heat shock protein response activation
Nutritional Neuroscience

Dear Prof. Soares,

I am pleased to inform you that we have received favourable comments on the above submission, and that it is accepted for publication in Nutritional Neuroscience. It was accepted on 11 Aug 2018.

Thank you for submitting your work to Nutritional Neuroscience.

With kind regards
George E. Barreto, Ph.D.
Associate Editor
Nutritional Neuroscience

Comments from the Editors and Reviewers:

Reviewer #1: Although the paper has clear experimental procedures, discussion and it is complete and extremely interesting, authors should be to follow all the editorial instructions. In spite of that, they have been improve many of these points.