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ANDRÉIA LIMANA TAMBARA

AVALIAÇÃO DO POTENCIAL ANTIOXIDANTE DO EXTRATO ETANÓLICO DA FRUTA PITANGA ROXA (*Eugenia uniflora* L.) NO MODELO EXPERIMENTAL *Caenorhabditis elegans*

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Dissertação apresentada ao Programa de Pós-graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do título de Mestre em Bioquímica.

Orientadora: Cristiane Casagrande Denardin

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“Você pode encarar um erro como uma besteira a ser esquecida, ou como um resultado que aponta uma nova direção.”

Steve Jobs

RESUMO

O consumo mundial de frutas tem aumentado principalmente em decorrência de seu valor nutritivo e dos possíveis efeitos terapêuticos. A pitanga, fruto da pitangueira (*Eugenia uniflora* L.), pertence à família botânica *Myrtaceae,* é uma planta frutífera nativa do Brasil que apresenta uma elevada capacidade antioxidante e riqueza em antocianinas. O presente estudo tem como objetivo investigar a composição do extrato etanólico de pitanga roxa e avaliar seu efeito antioxidante no nematoide *Caenorhabditis elegans*. As antocianinas e compostos fenólicos presentes no extrato foram identificadas e quantificadas por HPLC-DAD-MS. Os tratamentos foram realizados com diferentes concentrações do extrato de pitanga roxa (5, 50, 100, 250 e 500 µg de equivalentes de Ácido Clorogênico/ml) aplicados ao *C. elegans*. Após o tratamento foram realizados os ensaios de sobrevivência, longevidade, reprodução, resistência ao estresse oxidativo com os agentes estressores peróxido de hidrogênio (H_2O_2 0,6mM) e juglone (75µM), determinação de espécies reativas de oxigênio, expressão de enzimas antioxidantes (SOD e CAT) e expressão da proteína de choque térmico HSP-16.2. Foi observado que o extrato de pitanga roxa *per se* não alterou significativamente a sobrevivência e a postura de ovos dos vermes, porém aumentou a longevidade dos vermes nas concentrações de 100, 250 e 500 µg EAC/ml. Algumas concentrações de extrato também foram capazes de proteger ou reverter o estresse oxidativo induzido pelo H_2O_2 ou juglone. No ensaio de determinação de espécies reativas de oxigênio com o agente estressor H_2O_2, a pitanga roxa foi capaz de diminuir significativamente as espécies reativas. O extrato de pitanga roxa também aumentou significativamente a expressão da SOD e da proteína HSP-16.2. Esses resultados sugerem que a pitanga roxa não apresentou efeitos prejudiciais nos vermes e protegeu significativamente contra o estresse oxidativo induzido, indicando um possível efeito antioxidante *in vivo*.

Palavras-chave: *C. elegans*; estresse oxidativo; envelhecimento; frutas.

ABSTRACT

World fruit consumption has increased mainly because of its nutritional value and possible therapeutic effects. The pitanga, fruit of the pitangueira (*Eugenia uniflora* L.), belongs to the botanic family Myrtaceae, is a fruit plant native to Brazil that has a high antioxidant capacity and richness in anthocyanins. The present study aims to investigate the composition of the ethanolic extract of purple pitanga and evaluate its antioxidant effect on the nematode *Caenorhabditis elegans*. The anthocyanins and phenolic compounds present in the extract were identified and quantified by HPLC-DAD-MS. The treatments were carried out with different concentrations of the purple pitanga extract (5, 50, 100, 250 and 500 µg of Chlorogenic Acid equivalents/ml) applied to *C. elegans*. After the treatment, survival, longevity, reproduction, resistance to oxidative stress with stressor agents hydrogen peroxide (H_2O_2 0.6mM) and juglone (75µM), determination of reactive oxygen species, expression of antioxidant enzymes (SOD and CLT) and expression of thermal shock protein HSP-16.2. It was observed that the extract of purple pitanga *per se* did not significantly alter the survival and egg laying of the worms, but increased the longevity of the worms at concentrations of 100, 250 and 500 µg CAE/ml. Some extract concentrations were also able to protect or reverse oxidative stress induced by H_2O_2 or juglone. In the assay for the determination of reactive oxygen species with the H_2O_2 stressor agent, the purple pitanga was able to significantly decrease the reactive species. The purple pitanga extract also significantly increased the expression of SOD and HSP-16.2 protein. These results suggest that purple pitanga has no deleterious effects on worms and significantly protects against induced oxidative stress, indicating a possible antioxidant effect *in vivo*.

Keywords: *C. elegans*; oxidative stress; aging; fruits.

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1 INTRODUÇÃO

A pitanga (também conhecida por “Brazilian cherry”), fruto da pitangueira (*Eugenia uniflora* L.) é originária do Brasil, espalhando-se desde o nordeste até o Rio Grande do Sul, ultrapassando fronteiras para chegar a algumas regiões do Uruguai e da Argentina (Bezerra, 2000). Com relação às conhecidas atividades terapêuticas da pitangueira, suas folhas, têm sido referenciadas como eficientes no tratamento de diversas enfermidades com ação antiinflamatória (Schapoval *et al.*, 1994), para tratamento da bronquite (Rivera e Obon, 1995), com ação calmante (Grainger, 1996), com atividade diurética (Consolini *et al.*, 1999), para tratamento da diabetes e obesidade (Arai *et al.*, 1999), com atividade cardiovascular (Lee *et al.*, 2000) e antioxidante (Velazquez *et al.*, 2003). Porém, são praticamente inexistentes os estudos farmacológicos avaliando o efeito dos frutos da pitangueira, principalmente no que diz respeito a sua capacidade antioxidante *in vivo*.

Considerado como um dos países com a mais rica biodiversidade do mundo, o Brasil vem utilizando de forma ineficiente seus recursos naturais (Calixto, 2005). Além disso, as plantas têm sido de importância crucial para a saúde humana há séculos (Balbani *et al.*, 2009). Os extratos de algumas plantas possuem diversas atividades biológicas como antioxidantes, antiúlcera, antimalária, anticâncer, anti-inflamatórias, antiobesidade entre outras. Além disso, o elevado consumo de frutas está associado com uma redução na incidência de doenças degenerativas, incluindo doenças do coração, artrite, depleção do sistema imunológico e disfunção cerebral (Youdim e Joseph, 2001; Hu e Willett, 2002; Arts e Hollman, 2005; Collins, 2005). As frutas contêm altos níveis de componentes biologicamente ativos que conferem benefícios à saúde além do valor nutricional básico. Dentre os componentes biologicamente ativos, os antioxidantes naturais têm atraído a atenção devido sua segurança e efeito terapêutico em potencial. Além disso, suas propriedades farmacológicas e terapêuticas têm sido atribuídas a diferentes constituintes químicos isolados de seus extratos, embora, alguns estudos demonstraram que os extratos integrais de frutas têm uma eficácia mais elevada, quando comparada com as moléculas isoladas (Surveswaran *et al.*, 2010).

Existe uma tendência mundial para reavaliar a utilização de animais nos experimentos, concretizada a partir de um programa denominado de 3Rs (Reduction, Refinement, Replacement), que objetiva além de diminuir o número de animais, minimizar a dor e o desconforto e buscar alternativas para a substituição dos testes *in vivo* (Cazarin *et al.*, 2004). Portanto, para avaliar os efeitos do extrato de pitanga roxa *in vivo*, foi utilizado o modelo experimental *Caenorhabditis elegans* que é um pequeno nematoide, de vida livre do solo, que tornou-se um organismo modelo proeminente para um grande número de estudos de desenvolvimento biológico, comportamento, envelhecimento, ecotoxicológicos e genéticos (Diogo e Mota, 2001). Esses fatores fazem com que este organismo seja a escolha ideal para nosso estudo.

Nos últimos anos, muitas pesquisas vêm sendo realizadas na busca por agentes antioxidantes naturais, com o objetivo de proteger as células e os órgãos da ação dos radicais livres, e com isso retardarem o processo de envelhecimento e a progressão de diversas doenças. Tendo em vista o exposto acima, a hipótese é que, devido a riqueza de compostos fenólicos com poder antioxidante presentes no extrato de pitanga roxa, este possa aumentar a expectativa de vida dos *C. elegans*, além de proteger ou reverter os danos causados por estresse oxidativo induzido por agentes externos.

2 REVISÃO DE LITERATURA

2.1 Estresse oxidativo e antioxidantes

A geração de radicais livres constitui um processo contínuo e fisiológico, cumprindo funções biológicas relevantes. Define-se radicais livres ou espécies reativas (RLs) espécies independentes que contêm um ou mais elétrons não pareados. A presença de elétrons não pareados no átomo ou na molécula aumenta a sua reatividade química. Além disso, essa característica confere-lhes grande instabilidade, por tenderem a acoplar o elétron não pareado com um outro que esteja presente em estruturas próximas à sua formação. Os mecanismos de geração de radicais livres ocorrem, normalmente, nas mitocôndrias, membranas celulares e no citoplasma. A mitocôndria, por meio da cadeia transportadora de elétrons, é a principal fonte geradora de radicais livres. Espécies reativas de oxigênio (EROs), tais como radical hidroxila (•OH), ânion radical superóxido (O2•–) e hidroperoxila (ROO•), causam danos ao DNA ou podem oxidar lipídios e proteínas (Leite e Sarni, 2003; Barbosa *et al.*, 2010).

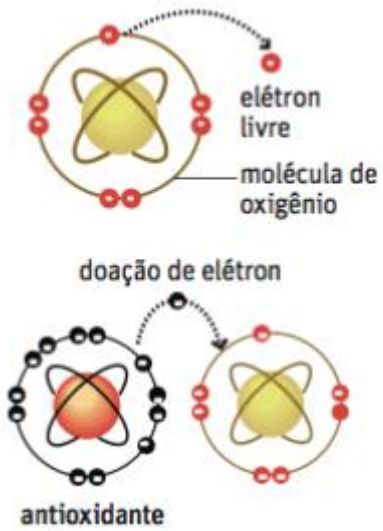
A instalação do processo de estresse oxidativo decorre da existência de um desequilíbrio entre compostos oxidantes e antioxidantes, em favor da geração excessiva de radicais livres ou em detrimento da velocidade de remoção desses. Tal processo conduz à oxidação de biomoléculas com consequente perda de suas funções biológicas e/ou desequilíbrio homeostático, cuja manifestação é o dano oxidativo potencial contra células e tecidos. A cronicidade do processo em questão tem relevantes implicações sobre o processo de numerosas enfermidades crônicas, entre elas a aterosclerose, diabetes, obesidade, transtornos neurodegenerativos e câncer (Barbosa *et al.*, 2010).

A produção contínua de radicais livres durante os processos metabólicos culminou no desenvolvimento de mecanismos de defesa antioxidante. Estes têm o objetivo de limitar os níveis intracelulares de tais espécies reativas e controlar a ocorrência de danos decorrentes. O sistema de defesa antioxidante tem a função de inibir e/ou reduzir os danos causados pela ação deletéria dos radicais livres ou das

espécies reativas não-radicais (Barbosa *et al.*, 2010). A produção de radicais livres é controlada nos seres vivos por diversos compostos antioxidantes, os quais podem ter origem endógena, ou serem provenientes da dieta alimentar e outras fontes (Sousa *et al.*, 2007).

Os antioxidantes são definidos como qualquer substância que, presente em menores concentrações que as do substrato oxidável, seja capaz de atrasar ou inibir a oxidação deste de maneira eficaz. Tais substâncias podem agir diretamente, neutralizando a ação dos radicais livres e espécies não-radicais como representado na Figura 1, ou indiretamente, participando dos sistemas enzimáticos com tal capacidade.

Figura 1: Radical livre e antioxidante



Fonte: http://radicaislivreseantioxidantes.blogspot.com.br/2014/

O sistema de defesa enzimático inclui as enzimas Superóxido Dismutase (SOD), Catalase (CAT) e Glutationa Peroxidase (GPx) (Barbosa *et al.*, 2010). As células aeróbias são protegidas da ação do superóxido e do peróxido de hidrogênio pela ação da superóxido-dismutase (SOD), uma metaloenzima que converte o radical superóxido em peróxido de hidrogênio, e pela ação da catalase, que converte o peróxido de hidrogênio em água e oxigênio molecular. A SOD citoplasmática necessita da presença de cobre e zinco para agir e a mitocondrial, de manganês; já a catalase depende do ferro. Além da catalase, outra enzima importante no controle dos peróxidos é a glutationa peroxidase (GPx), que utiliza para sua ação a glutationa

(GSH), um tripeptídeo contendo cisteína, e representa o tiol não proteico mais abundante nas células de mamíferos. É substrato para as enzimas antioxidantes: *GSH transferases e peroxidases* (dependentes de selênio). As demais funções da GSH envolvem sua participação no estoque e transporte de cisteína, regulação do balanço "redox", metabolismo de prostaglandinas e leucotrienos, síntese de desoxirribonucleotídeos, função imune e proliferação celular (Leite e Sarni, 2003). Essas enzimas agem por meio de mecanismos de prevenção, impedindo e/ou controlando a formação de radicais livres e espécies não-radicais, envolvidos com a iniciação das reações em cadeia que culminam com propagação e amplificação do processo e, consequentemente, com a ocorrência de danos oxidativos (Barbosa *et al.*, 2010).

O sistema de defesa não enzimático inclui, especialmente, os compostos antioxidantes de origem dietética, entre os quais se destacam: vitaminas e compostos fenólicos (Barbosa *et al.*, 2010). A atividade antioxidante de compostos fenólicos deve-se principalmente às suas propriedades redutoras e estrutura química. Estas características desempenham um papel importante na neutralização ou sequestro de radicais livres e quelação de metais de transição, agindo tanto na etapa de iniciação como na propagação do processo oxidativo. Os carotenos protegem os lipídios dos danos oxidativo, através da reação com os radicais peroxila, hidroxila e superóxido. A atividade antioxidante dos carotenoides é decorrente da habilidade de sequestrar espécies reativas de oxigênio devido à presença de ligações duplas conjugadas em sua estrutura (Sousa *et al.*, 2007).

2.2 Consumo de frutas e hortaliças

A alimentação e a nutrição adequadas constituem-se em requisitos básicos para a promoção e a proteção da saúde e para o desenvolvimento sustentável (Haddad *et al.*, 2014). O consumo de frutas e hortaliças tem aumentado principalmente em decorrência do seu valor nutritivo e efeitos terapêuticos. Esse tem sido associado à diminuição do risco de mortalidade (Agudo *et al.*, 2007) e redução da ocorrência de doenças crônicas, tais como as doenças cardiovasculares

(Dauchet *et al.*, 2006), derrames (He *et al.*, 2006) e alguns tipos de câncer (Key *et al.*, 2002). A Organização Mundial da Saúde (OMS) afirma que existem evidências convincentes de que o consumo de frutas, legumes e verduras também diminui o risco de diabetes e obesidade. Eles também recomendam um consumo mínimo de pelo menos cinco porções diárias de frutas, legumes e verduras, o que equivale a 400g ou mais por dia (Who e Consultation, 2003), no Brasil, a ingestão desses alimentos não chega a um terço destes valores (Ibge, 2010).

Embora o Brasil seja um grande produtor mundial de frutas e hortaliças, com grande abundância de variedades nas diferentes regiões do país, o brasileiro ainda consume pouco esses alimentos, há várias pesquisas que demonstram o baixo consumo pela população. Segundo a mais recente pesquisa feita pelo Instituto Brasileiro de Geografia e Estatística - IBGE/2008-2009 (Ibge, 2010), frutas, verduras e legumes correspondem a apenas 2,3% das calorias totais ingeridas pela população. A Pesquisa Nacional de Saúde (2013) demonstrou que apenas 37,3% dos adultos consumiam cinco porções diárias de frutas e hortaliças (Ibge, 2014). Em 2014, a pesquisa do Sistema de Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico (VIGITEL) mostrou que 36,5% da população consumiam frutas e hortaliças cinco ou mais vezes por semana, enquanto apenas 24,1% ingeriam cinco ou mais porções diariamente (Brasil, 2015).

A inadequação do consumo de frutas e hortaliças possui abordagem complexa. Ocorre por questões ambientais, como sistemas de distribuição e comercialização; econômicos, como preços elevados comparados a outros alimentos; e individuais (Who, 2004). Entre os fatores individuais está a baixa renda, ser solteiro e possuir comportamentos não saudáveis, como tabagismo, sedentarismo e alimentação rica em açúcar e gorduras. Por outro lado, o consumo de frutas e hortaliças parece ser maior com o aumento da escolaridade e da idade dos indivíduos (Figueiredo *et al.*, 2008; Jaime *et al.*, 2009; Neutzling *et al.*, 2009; Campos *et al.*, 2010; Mondini *et al.*, 2010; Nepal *et al.*, 2012).

Portanto, o incentivo ao consumo e produção de frutas pode ser melhorado com pesquisas que promovam um maior conhecimento sobre os compostos com propriedades benéficas presentes nestes vegetais que podem atuar na prevenção ou tratamento de enfermidades. As frutas e vegetais contêm diferentes fitoquímicos,

muitos dos quais possuem propriedades antioxidantes que pode estar relacionada com o retardo do envelhecimento e a prevenção de certas doenças (Wang e Prior, 1996). A atividade antioxidante apresentada por vários vegetais, incluindo frutos, folhas, sementes e plantas medicinas, está correlacionada ao seu teor de compostos fenólicos totais (Velioglu *et al.*, 1998).

2.3 Compostos fenólicos

Os compostos fenólicos são substâncias amplamente distribuídas na natureza, mais de 8000 compostos fenólicos já foram detectados em plantas, eles são originados do metabolismo secundário das plantas, sendo essenciais para o seu crescimento e reprodução, além disso, se formam em condições de estresse como, infecções, ferimentos, radiações UV, dentre outros. Em alimentos, são responsáveis pela cor, adstringência, aroma9 e estabilidade oxidativa (Naczk e Shahidi, 2004).

Quimicamente, os fenólicos são definidos como substâncias que possuem anel aromático com um ou mais substituintes hidroxílicos, incluindo seus grupos funcionais. Possuem estrutura variável e com isso, são multifuncionais (Lee *et al.*, 2005). Os compostos fenólicos são classificados em três categorias: pouco distribuídos na natureza, polímeros e largamente distribuídos na natureza.

Na família dos compostos fenólicos pouco distribuídos na natureza, encontra-se um número bem reduzido, embora com certa frequência. Neste grupo estão os fenóis simples, o pirocatecol, a hidroquinona e o resorcinol. Pertencem ainda a esta família os aldeídos derivados dos ácidos benzoicos, que são constituintes dos óleos essenciais, como a vanilina (Soares, 2002). Os polímeros são alguns fenólicos que não se apresentam na forma livre nos tecidos vegetais, esta família engloba os taninos e as ligninas.

Na família dos compostos largamente distribuídos na natureza estão os fenólicos encontrados geralmente em todo reino vegetal, mas às vezes podem estar localizados em uma só planta. Este grupo pode-se dividir em flavonoides

(antocianinas, flavonóis e seus derivados) e ácidos fenólicos (ácido benzoico, cinâmico e seus derivados) e cumarinas (King e Young, 1999).

A diversidade estrutural dos compostos fenólicos devesse à grande variedade de combinações que acontece na natureza e os compostos resultantes são chamados de polifenóis. Estas combinações fenólicas podem ser categorizadas em várias classes como mostradas na Tabela 1 (Harborne, 1989; Baxter *et al.*, 1998). Dentre os fenólicos, destacam-se os flavonoides, os ácidos fenólicos, os taninos e os tocoferóis como os mais comuns antioxidantes fenólicos de fonte natural (King e Young, 1999).

Tabela 1: Classe de compostos fenólicos.

Classe	**Estrutura**
Fenólicos simples, benzoquinonas	C_6
Ácidos hidroxibenzóicos	C_6–C_1
Acetofenol, ácidos fenilacéticos	C_6–C_2
Ácidos hidroxicinâmicos, fenilpropanoides	C_6–C_3
Nafitoquinonas	C_6–C_4
Xantonas	C_6–C_1–C_6
Estilbenos, antraquinonas	C_6–C_2–C_6
Flavonoides, isoflavonoides	C_6–C_3–C_6
Lignanas, neolignanas	$(C_6$–$C_3)_2$
Biflavonoides	$(C_6$–C_3–$C_6)_2$
Ligninas	$(C_6$–$C_3)_n$
Taninos condensados	$(C_6$–C_3–$C_6)_n$

Fonte: (Angelo e Jorge, 2007)

Os antioxidantes fenólicos interagem, preferencialmente, com o radical peroxil por ser este mais prevalente na etapa da autoxidação e por possuir menor energia do que outros radicais, fato que favorece a abstração do seu hidrogênio (Decker, 1998). O radical fenoxil resultante, embora relativamente estável, pode interferir na reação de propagação ao reagir com um radical peroxil, via interação entre radicais.

O composto formado, por ação da luz ultravioleta e temperaturas elevadas, poderá originar novos radicais, comprometendo a eficiência do antioxidante, que é determinada pelos grupos funcionais presentes e pela posição que ocupam no anel aromático, bem como, pelo tamanho da cadeia desses grupos (Shahidi *et al.*, 1992; Madhavi *et al.*, 1995). Este mecanismo de ação dos antioxidantes, presentes em extratos de plantas, possui um papel importante na redução da oxidação lipídica em tecidos, vegetal e animal, pois quando incorporado na alimentação humana não conserva apenas a qualidade do alimento, mas também reduz o risco de desenvolvimento de patologias, como arteriosclerose e câncer (Namiki, 1990; Ramarathnam *et al.*, 1995).

2.4 Pitanga (*Eugenia uniflora* L.)

Segundo classificação de Cronquist (Cronquist, 1988) a pitangueira pertence à classe Magnoliopsida, subclasse Rosidae, ordem Myrtales, família Myrtaceae, gênero *Eugenia* e espécie *Eugenia uniflora* L. A pitangueira é uma frutífera de ampla distribuição geográfica. É nativa do Brasil e encontra-se disseminada, praticamente, por todo o território nacional. Em função da adaptação às diferentes condições de solo e clima, a pitangueira é encontrada em diversas partes do mundo. Há registros de cultivos em outros países da América do Sul e Central, no Caribe, nos Estados Unidos (Flórida, Califórnia, Havaí), China, Índia, Sri Lanka, México, Madagascar, África do Sul, Israel e vários países do Mediterrâneo (Popenoe, 1920; Moreuil, 1971; Sturrock, 1972; Correa e Penna, 1974; Campbell, 1977; Fouqué, 1981; Lahav e Slor, 1997).

A árvore é pequena, mede entre 2 a 4 m de altura, o tronco geralmente é tortuoso e com muitos galhos, possui folhas pequenas e verde-escuras, que exalam aroma característico, as flores são brancas e perfumadas. O fruto da pitanga como representado na Figura 2 é uma baga globosa, com sete a dez sulcos longitudinais de 1,5 a 5,0 cm de diâmetro, coroado com sépalas persistentes e aproximadamente 66% do fruto é constituído de polpa que possui aroma característico intenso e sabor doce e ácido, pode ser consumida fresca ou processada, na indústria os frutos são

utilizados na produção de suco, polpa congelada e sorvetes, assim como na indústria de cosméticos. Em decorrência de uma ampla diversidade genética, a pitanga apresenta cor que varia do laranja, passando pelo vermelho, e chegando ao roxo, ou quase preto (Bezerra, 2000; Silva, 2006). Durante a maturação, o epicarpo da fruta evolui de verde para laranja e vermelho, nas variedades laranja e vermelha e, do verde ao roxo profundo ou quase preto, na variedade roxa como representado na Figura 3.

Figura 2: Pitanga roxa

Fonte: http://stravaganzastravaganza.blogspot.com.br/2012/08/alimentos-que-curam.html

Na Tabela 2 encontram-se os valores referentes à composição média de 100g de polpa de pitanga.

Tabela 2: Valor nutricional de 100 g de polpa de frutos de pitangueira.

Componente	Unidade	Valor
Valor energético	cal	51,00
Umidade	g	85,80
Proteína	g	0,80
Gordura	g	0,40
Carboidratos	g	12,50
Fibra	g	0,60
Cinza	g	0,50
Vitamina A	mg	635,00
Tiamina	mg	0,30
Riboflavina	mg	0,60
Niacina	mg	0,30
Ácido ascórbico	mg	14,00
Cálcio	mg	9,00
Fósforo	mg	11,00
Ferro	mg	0,20

Fonte: (De Lira Júnior *et al.*, 2007)

O crescente interesse por estas frutas está relacionado com as grandes quantidades de catequinas, flavonoides, proantocianidinas e compostos fenólicos, conhecidos por sua atividade antioxidante, que elas podem apresentar (Bagetti *et al.*, 2011; Celli *et al.*, 2011). Além dos diversos efeitos na saúde humana, os compostos antioxidantes são também importantes na inibição e/ou prevenção da oxidação de produtos alimentares. As folhas da pitangueira têm sido utilizados a muito tempo na medicina popular, devido a suas diversas atividades biológicas, sendo geralmente preparadas como infusão para o tratamento da febre, reumatismo, bronquite, doenças do estômago, e distúrbios digestivos, bem como hipertensão, febre amarela e gota (Alice *et al.*, 1991; Velazquez *et al.*, 2003; Bagetti *et al.*, 2011). Também podem reduzir o peso corporal e a pressão arterial, servir como um diurético, além de sua comprovada atividade calmante e anti-inflamatória (Schmeda-Hirschmann *et al.*, 1987; Schapoval *et al.*, 1994). Recentemente, o extrato das folhas

da pitangueira apresentou atividade citotóxica e anti-*Trypanosoma cruzi* com baixa toxicidade em estudos *in vitro* (Santos *et al.*, 2012).

Figura 3: Todas as variedades da pitanga

Fonte: http://ciprest.blogspot.com.br/2016/09/pitanga-tutti-colore-eugenia-uniflora.html

A pitanga, ou seja, os frutos da pitangueira, também apresentam atividade antioxidante e atuam inibindo a peroxidação lipídica e na remoção de radicais livres (Velazquez *et al.*, 2003; Bagetti *et al.*, 2011; Celli *et al.*, 2011). Porém, ainda são poucos os trabalhos avaliando os efeitos dos extratos ou compostos isolados da pitanga, que apresentam diversos compostos com elevada capacidade antioxidante, sobre condições fisiológicas e patológicas. Portanto, este é um campo de pesquisa promissor que pode alavancar o consumo e a produção desta fruta nativa brasileira.

Devido ao seu sabor desejável e ao elevado conteúdo de carotenoides (Rodriguez-Amaya *et al.*, 2008), a pitangueira é uma das árvores frutíferas mais

promissoras para programas de exploração sustentável na Mata Atlântica Brasileira. No Brasil, as pitangueiras são cultivadas principalmente em hortas, pequenas propriedades agrícolas, ou de forma nativa. Atualmente, a Embrapa Clima Temperado localizada no município de Pelotas – RS possui um programa de melhoramento genético e expansão de produção desta árvore frutífera, o qual atua no fornecimento de mudas e programas informativos junto à comunidade. A região nordeste do Brasil é a única a explorar comercialmente esta fruta de alto potencial econômico. O valor comercial da pitanga resulta do seu elevado rendimento de polpa, valor nutritivo, sabor e aroma exóticos, atraindo, principalmente, os consumidores exigentes por produtos naturais e saudáveis (Donádio, 1983; Ferreira, 1987; Lederman *et al.*, 1992; Bezerra, 2000).

2.5 *Caenorhabditis elegans*

A avaliação da toxicidade de uma substância é realizada com o objetivo de predizer os efeitos nocivos que a mesma poderá desencadear quando da exposição humana pelas diversas vias. Para cumprir este propósito, o modelo animal é o mais utilizado nos estudos toxicológicos e requerido nos processos investigativos. Entretanto, a utilização de animais na pesquisa tem sido razão de diversas discussões em função do grande número necessário e do sofrimento causado, principalmente em relação aos estudos de toxicidade aguda. Existe uma tendência mundial para reavaliar a utilização de animais nos experimentos, concretizada a partir de um programa denominado de 3Rs que é assim denominado em função das iniciais, em inglês, de seus principais objetivos: 1) redução (*Reduction),* 2) refinamento *(Refinement)* e 3) substituição *(Replacement*), que de forma resumida significam a redução do número de animais utilizados na pesquisa, a melhora na condução dos estudos, no sentido de reduzir o sofrimento ao mínimo possível, e a busca de métodos alternativos que, por fim, substituam os testes *in vivo* (Cazarin *et al.*, 2004).

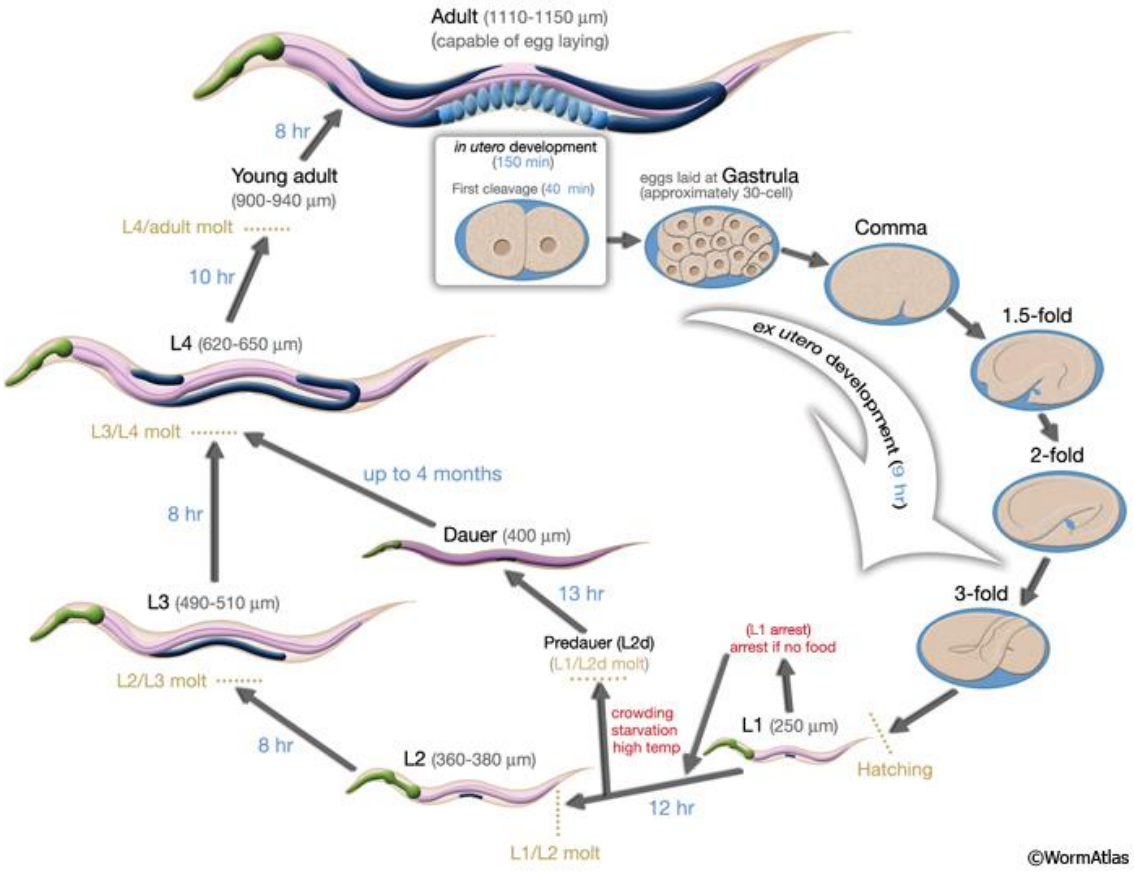
Diversas metodologias alternativas já foram implantadas, sendo este um processo complexo que abrange desde o seu desenvolvimento até sua aceitação e

adoção por diversas organizações. Sendo assim, o presente trabalho apresenta os nematoides que são organismos pertencentes ao filo *Nemata* (*Nematoda*), e constituem o mais numeroso grupo de metazoários existente no solo. Para além deste habitat, também se encontram em elevado número em ambientes aquáticos e parasitando animais e plantas (Politz e Philipp, 1992). O *Caenorhabditis elegans* pertence à família *Rhabditidae*, um diversificado grupo de nematoides com grande distribuição em habitat terrestres (Gilbert, 1988).

O *C. elegans* é um pequeno nematoide (cerca de 1 mm de comprimento) que tem sido utilizado como organismo modelo, por mais de 200 laboratórios mundiais. É um nematoide bacteriófago facilmente cultivado sob condições laboratoriais, normalmente são mantidos à temperatura de 20ºC em placas de Petri com meio NGM (nematode growth media) e como fonte de alimento *Escherichia coli*, sendo possível obter-se um elevado número de organismos, o que é fundamental para um bom modelo biológico (Gilbert, 1988). Os adultos são normalmente hermafroditas, sendo os machos raros (cerca de 0,2% da população total), cada hermafrodita produz uma descendência de cerca de 200-300 indivíduos (Bernt *et al.*, 1998). Após o desenvolvimento embrionário, o ovo eclode e liberta um jovem verme denominado larva L1, este se desenvolve por mais três sucessivas fases larvares (L2, L3 e L4) até chegar à fase adulta como representado na Figura 4 (Wood, 1988).

Este organismo possui uma série de características que o torna ideal para este tipo de estudos: possui um ciclo de vida curto, dimensões reduzidas, corpo transparente, um pequeno genoma e é fácil de cultivar (Diogo e Mota, 2001) e o genoma foi totalmente sequenciado em 1998 (Park, 2013). Este nematoide foi proposto como um organismo modelo por Sydney Brenner em 1965 (Garcia-Sancho, 2012). Desde então, tem sido utilizado em estudos biológicos, genéticos e neurobiológicos de células eucariotas superiores.

Figura 4: Ciclo de vida do *C. elegans* a 22°C



Fonte: WormAtlas

Diversos estudos utilizando a suplementação com antioxidantes presentes naturalmente em alimentos ou isolados têm demonstrado resultados positivos na redução da produção de radicais livres e aumento da longevidade em diversos modelos animais experimentais como o *C. elegans,* Entre eles podemos citar o extrato de café verde aumenta a resistência ao estresse oxidativo e atrasa o envelhecimento em *C. elegans* (Amigoni *et al.*, 2017)*,* extrato de maçã aumenta a vida útil, saúde e resistência ao estresse em *C. elegans (Vayndorf et al., 2013)* e um extrato rico em antocianinas do açaí aumenta a resistência ao estresse e retarda os marcadores relacionados ao envelhecimento em *C. elegans* (Peixoto *et al.*, 2016).

3 JUSTIFICATIVA

Em busca de qualidade de vida e longevidade os consumidores, cada vez mais, se tornam adeptos dos alimentos saudáveis. O valor nutricional é um dos principais fatores que conduzem ao crescente interesse pelo consumo de frutas, embora muitas frutas de menor expressão nacional ainda não tenham sido devidamente pesquisadas quanto a suas propriedades e atividades benéficas à saúde. Há uma variedade de compostos secundários, ou fitoquímicos, já identificados nas folhas da pitangueira, como flavonoides, terpenos, taninos, antraquinonas e óleos essenciais. No entanto, sobre a fruta da pitangueira existem poucos estudos, identificando somente algumas antocianinas e carotenoides, e nenhum mostra a relação do consumo de pitangas e prevenção ou combate de doenças. Neste contexto, o presente estudo se justifica pela importância das possíveis propriedades medicinais do fruto e por existirem poucos estudos sobre as funções da pitanga em ensaios *in vivo*.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar os efeitos antioxidantes do extrato de pitanga roxa no modelo *Caenorhabditis elegans.*

4.2 Objetivos específicos

- Realizar a extração etanólica dos compostos da pitanga roxa, determinar e caracterizar o conteúdo de fenólicos totais do extrato;

- Determinar os efeitos *per se* do extrato da fruta sobre o modelo experimental *C. elegans* através de ensaios toxicológicos e de expectativa de vida;

- Investigar o efeito do extrato sobre resistência ao estresse oxidativo induzido por peróxido de hidrogênio e juglone em *C. elegans*;

- Avaliar o potencial antioxidante do extrato através da determinação de espécies reativas de Oxigênio (EROS), modulação da expressão de enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT) e na expressão da proteína de choque térmico HSP-16.2.

5 ARTIGO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções Materiais e Métodos, Resultados e Discussão, conclusão e Referências Bibliográficas encontram-se no próprio manuscrito. O manuscrito está apresentado da mesma forma que será submetido à revista Food Chemistry.

Antioxidant potential of extract from purple pitanga fruit (*Eugenia uniflora* L.) in the experimental model *Caenorhabditis elegans*

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Abstract

Pitanga, a fruit of the pitangueira (*Eugenia uniflora* L.), is a fruit plant native to Brazil that has a high antioxidant capacity and richness in anthocyanins. The present study aims to investigate the chemical composition of the ethanolic extract of purple pitanga and evaluate its antioxidant effect in the nematode *Caenorhabditis elegans*. We observed that the extract of purple pitanga *per se* did not significantly alter the survival and egg laying of the worms, although increased its longevity. The extract was also able to protect or reverse the oxidative stress induced by H_2O_2 and juglone. It significantly decreased the reactive oxygen species. The extract also increased the expression of the SOD enzyme and the expression of the heat shock protein HSP-16.2. Therefore these results suggest that the purple pitanga fruit extract did not exhibit detrimental effects on worms and significantly protected against oxidative stress induced.

Keywords: aging, berries, antioxidants, oxidative stress.

1. Introduction

Currently the attention of the researchers has been focused on the effects of nutraceutical bioactive compounds as polyphenols in the prevention of diseases related to oxidative stress, such as cardiovascular, oncological and aging related diseases (Huang *et al.*, 2005). Studies suggest that metabolites such as polyphenols, flavonoids, anthocyanins and others present in fruits and plants act in preventive nutrition, since they benefit the organism with antioxidant action attenuating oxidative damages generated by free radicals in cells (Hertog *et al.*, 1992). Polyphenols are organic compounds characterized by the presence of multiple structural units of phenol, found in foods of plant origin such as fruits, juices, teas, coffee, red wine, vegetables and cereals, these being the main antioxidants of our diet (Scalbert *et al.*, 2005).

Eugenia uniflora L. (Myrtaceae) is a Brazilian native tree, locally known as “pitangueira”, which produces edible fruits with the size of cherries that tastes sweet and sour at the same time (Costa *et al.*, 2013). Due to a wide genetic diversity, the pitanga fruit presents color that varies from orange, passing through the red, and reaching purple (Bezerra, 2000). *E. uniflora* leafs have been used in folk medicine for the treatment of a number of diseases such as diarrhea (Schapoval *et al.*, 1994; Almeida *et al.*, 1995), inflammation (Schapoval *et al.*, 1994), hyperglycemia, hyperlipidemia, hypertriglycemia (Ferro *et al.*, 1988; Arai *et al.*, 1999; Matsumura *et al.*, 2000) and hypertension (Auricchio e Bacchi, 2003). Moreover, these have been tested as anti-inflammatory and antidiabetic (Schumacher *et al.*, 2015), antimalarial (Morioka *et al.*, 2000), anti-Leishmania (Rodrigues, K. A. *et al.*, 2013) and as an inhibitor of DNA polymerase, maltase, sucrase and α-glucosidase (Lee *et al.*, 1997;

Lee *et al.*, 2000). The *in vitro* antioxidant capacity of the purple pitanga fruit has already been evaluated, presenting an excellent antioxidant potential (Denardin *et al.*, 2015). However, there are few studies evaluating the effect of the fruits of pitangueira, mainly with respect to its antioxidant capacity *in vivo*.

Aging is the progressive accumulation of changes over time that is associated with or responsible for the increasing susceptibility to disease and death. Although there are several theories that are though to contribute to the aging process, the free radical theory of aging seems to be a significant contributor in the process. Free radicals are highly reactive by-products of metabolism seeking stability by gaining an extra electron from any source. This very property leads to protein, lipid, and DNA damage. Once produced, free radicals are normally cleared by the body's naturally occurring antioxidants, but as organism age, the declining efficiency of this mechanism allows for the accumulation of free radicals in the body (Halliwell e Gutteridge, 1998; Brown *et al.*, 2006).

The soil nematode *Caenorhabditis elegans* is known as a model for studying the molecular mechanisms of aging process. Besides others, the reasons for using *C. elegans* as an *in vivo* model are the ease of handling, the strong conservation between *C. elegans* and mammals in biological principles and the multicellularity of this organism with the presence of important tissues and organ systems raising the possibility to consider interactions and metabolism. *C. elegans* is an excellent model to investigate aging because of its short lifespan, its susceptibility to oxidative stress and the similarities with the human aging process (Chatterjee *et al.*, 2013; Pang e Curran, 2014).

Therefore, our hypothesis is that the purple pitanga fruits have richness in phenolic antioxidant compounds, which can act by reducing oxidative stress and

increasing the lifespan of worms. Because pitanga is a plant widely used in folk medicine and its research performed so far is very small, this work aimed to evaluate the chemical composition and antioxidant activity of the ethanolic extract of the purple pitanga fruit in the *C. elegans* model.

2. Material and Methods

2.1. Extract preparation

The purple pitanga (*Eugenia uniflora* L.) was harvested in its mature stage at the Brazilian Agricultural Research Corporation - EMBRAPA-CPACT - Pelotas, RS/Brazil, washed in running water and frozen (-18°C) prior to transportation. For the extraction of the phenolic compounds, 100 g of frozen sample without seeds were homogenized with 300 ml of ethanolic solution (95%) in light-protected beaker using an ultra-turrax mixer for 5 minutes and then placed in a magnetic stirrer for 30 minutes. Samples were centrifuged for 5 minutes at 3000 rpm and the supernatant was recovered. The residue was subjected to the new extraction as described above, the supernatant being mixed to the above. The recovered supernatant was evaporated in a rotary evaporator using temperatures between 40-45°C and vacuum. The extract was resuspended in distilled water. The extract obtained was used for the determinations of total phenolic compounds quantified with Folin-Ciocalteu (Swain e Hillis, 1959). To evaluate the *in vivo* antioxidant activity of the purple pitanga extract, the concentrations tested on the nematode *C. elegans* were selected from the results of total phenolic compounds, which resulted in 96,537.68 µg of chlorogenic acid/ml (CAE/ml) and the concentrations used were 5, 50, 100, 250 and 500 µg CAE/ml.

2.2. Standards

Standards of cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside were acquired from Extrasynthèse (Genay, France). Quinic acid, gallic acid, quercetin and myricetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard purities were at least 95% as determined by HPLC.

2.3. Identification and quantification of anthocyanins and phenolic compounds by HPLC-DAD-MS^n

Anthocyanins were exhaustively extracted from 0.10±0.01 g of lyophilized sample with a 1% HCl solution (Faria *et al.*, 2011). Phenolic compounds were exhaustively extracted from 0.10±0.01 g of lyophilized sample with methanol/water (8:2, v/v) (Rodrigues, E. *et al.*, 2013). Both extractions were carried out in triplicate. Before injection into the HPLC-DAD-MS^n, the extracts were filtered through a 0.22 μm membrane (Millipore, USA). HPLC analyses for identification and quantification of the anthocyanins and non-anthocyanic phenolic compounds were carried out in a Shimadzu HPLC (Kyoto, Japan) coupled in series to a diode array (DAD, model, SPD-M20A, Shimadzu) and a mass spectrometer (Amazon Speed, Bruker Daltonics, Bremen, Germany) detectors. The chromatographic and mass spectrometer conditions used were those previously described for anthocyanins (Faria *et al.*, 2011) and phenolic compounds (Chiste e Mercadante, 2012). Anthocyanins and non-anthocyanic phenolic compounds were identified based on the elution order on C_{18} column and mass spectra features in comparison with authentic standards analyzed under the same conditions and data from the literature. Quantification was carried out

by HPLC–DAD using analytical curves of cyanidin 3-*O*-glucoside, gallic acid and quercetin.

2.4. Maintenance of *C. elegans* and synchronization

C. elegans were kept in incubator at 20°C in Petri plates containing NGM (nematode growth media) and *Escherichia coli* OP50 as food source (Brenner, 1974). The strains used were N2 (wild type), CF1553 muIs84 [(pAD76) *sod-3p*::GFP + *rol-6*(*su1006*)], GA800 wuIs151 [*ctl-1* + *ctl-2* + *ctl-3* + *myo-2*::GFP], CL2070 dvIs70 [*hsp-16.2p*::GFP + *rol-6*(*su1006*)] and TK22 [*mev-1*(kn1) III]. All strains were obtained from the Caenorhabditis Genetics Center (Minesotta, USA). L1 were obtained by a synchronization process, which consists in obtaining pregnant hermaphrodite eggs using a lysis solution (1% NaOCl, 2.4% NaOH) to break the worms. After 12-14 hours, the isolated eggs will hatch, releasing L1 larvae.

2.5. Survival assay with extract *per se*

2000 N2 L1 larvae obtained by the synchronization process were exposed to treatments with different concentrations of fruit extract for 30 minutes in a liquid medium containing 0.5% NaCl and in the absence of bacteria. At the end of treatment, worms were washed three times with 0.5% NaCl to remove the extract and then plated on NGM seeded with *E. coli* OP50. Survival parameter was evaluated by scoring the live worms at the plates 24h after the end of the treatment.

2.6. Oxidative stress resistance assays

Protection or reversal of induced oxidative damage was assessed using hydrogen peroxide (H_2O_2) and juglone (5-Hydroxy-1, 4-naphthoquinone) as a pro-

oxidant agent. For the effect of protection of extract was evaluated through a pre-exposure to the extract and a post-exposure to the pro-oxidant agent. The reversal effect was evaluated through a pre-exposure to the stressor followed by post-exposure to the extract. Tests using H_2O_2 were performed on N2 L1 worms that were treated for 30 minutes with the extract and exposed to H_2O_2 (0.6 mM) for 30 minutes. Tests using juglone were performed on N2 L4 worms which were treated for 30 minutes with the extract and exposed to juglone (75 µM) for 2h. Three additional washes were done on all assays, and then plated with medium NGM and *E. coli* OP50. Survival was evaluated after 24 h by counting live worms.

2.7. Reproduction assay

N2 (wild-type) worms treated as described above after 48 h (L4) were individually transferred to new mediated plaques seeded with *E. coli* OP50 bacteria. The egg laying was measured by monitoring the worms every 24 hours during the fertile cycle.

2.8. Lifespan

After 48 h of acute treatment exposure with different fruit extract concentration, twenty N2 worms (wild-type) and TK22 [*mev-1* (kn1) III] of each concentration were individually transferred to new plates with medium with *E. coli* OP50. The worms were monitored and transferred to new plaques until the last worm died.

2.9. ROS measurement

Synchronized wild-type L1 worms (5.000) were acutely treated with the extracts as previously described. A widely fluorescent probe for ROS detection,

H_2DCF-DA (2,7-dichlorofluorescein-diacetate) was added and allowed to stir for 1h, in the dark, at a final concentration of 500 µM, according to Bornhorst *et al*. (Bornhorst *et al.*, 2014) with some adaptations. The whole worms were transferred to a 96-well plate and added hydrogen peroxide (0.4mM). The fluorescence intensity of each sample was measured (excitation: 485 nm; emission: 535 nm) using a plate reader (SpectraMax M5 microplate reader, Molecular Devices®), read every 15 min for kinetics for 1h and normalized to time zero values. Results were expressed as Δ AUF/ Δ Time.

2.10. Fluorescence quantification

To analyze the expression of antioxidant superoxide dismutase (SOD) and catalase (CLT) enzymes, the strains marked with GFP (CF1553 and GA800) were exposed to the treatment as described above and the worms images were captured using a fluorescence microscope (EVOS FLoid ®). Worms CL2070 were treated with extracts for 30 min and then submitted to heat shock (2 h at 35°C), based on Strayer *et al*. (Strayer *et al.*, 2003) for thermal stress. Worms were collected and placed on NGM plates seeded with OP50 and allowed to recover overnight. The next day worms were washed and pictures were taken using a fluorescence microscope (EVOS FLoid®). The results were expressed as mean of pixels in comparison to the control group and later analysis performed with the ImageJ software.

2.11. Statistical analyses

Analyses were performed using GraphPad Prism version 7 (GraphPad Software). To analyze the results of longevity were applied ANOVA for repeated measures and post-hoc Tukey. The one-way ANOVA analysis was applied to all the

other tests, followed by post-hoc Tukey. All $p<0.05$ values were considered statistically significant. The values expressed in percentage (%) were normalized taking a value of 100% for the control. In all figures, error bars represent the standard error of the mean.

3. Results and Discussion

3.1. Identification and quantification of anthocyanins and phenolic compounds

Five anthocyanins were separated from purple pitanga (Figure 1, Table 1). Three anthocyanins were identified based on comparison with UV-vis, mass spectral characteristics and co-elution with authentic standards (Table 1), i.e delphinidin 3-*O*-glucoside (Figure 1, peak 1), cyanindin 3-*O*-glucoside (Figure 1, peak 2) and pelargonidin 3-*O*-glucoside (Figure 1, peak 3). The other two anthocyanins were tentatively identified (Table 1) as cyanidin 3-*O*-pentoside (Figure 1, peak 4), which showed molecular ion at *m/z* 419 and loss of one pentose (132 u) in the MS^2 spectrum, and a cyanidin derivative (Figure 1, peak 5), which showed a fragment ion at *m/z* 287 in the MS^2 spectrum corresponding to the cyanidin aglycone (Wu e Prior, 2005). As far as we are concerned, this is the first time that delphinidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside are reported in purple pitanga.

Cyanidin 3-*O*-glucoside was the major anthocyanin found in purple pitanga, followed by delphinidin 3-*O*-glucoside, corresponding respectively to 82.7% and 16.1% of the total anthocyanin content. Cyanidin 3-*O*-glucoside was previously identified in purple pitanga in amounts varying from 0.75 to 169.0 mg/100 g dry fruit during different maturity stages (Celli *et al.*, 2011). The content of cyanidin 3-*O*-glucoside found in the present study was about 3 times higher than the value

reported by Celli *et al.* (2011). This difference can probably be attributed to the different extraction procedures since in the present study an exhaustive extraction was carried out while in Celli *et al*. (2011), only one extraction using acetone/water/acetic acid (70:29:1, v/v/v) was performed.

Figure 2 and Table 2 shows the separation and characterization of 27 phenolic compounds of purple pitanga, being two anthocyanins (peaks 11 and 12), 20 non-anthocyanin phenolic compounds (peaks 1, 2, 4, 5, 10-27) and 5 non-identified compounds (peaks 3, 6-9). All the 20 non-anthocyanin phenolic compounds found in purple pitanga were derived from five aglycones: quinic acid (peaks 1 and 2), gallic acid (peaks 4 and 5), HHDP (hexahydroxydiphenoyl) (peak 10), myricetin (peaks 13-17, 24 and 25) and quercetin (peaks 18-23, 26 and 27).

Quinic acid (Figure 2, peak 1) and a mixture of quinic acid derivatives (Figure 2, peak 2) were detected only by MS, thus, these compounds were not quantified by HPLC-DAD. Quinic acid was identified based on comparison with MS and MS^2 characteristics and retention time with those of an authentic standard analyzed under the same conditions. MS spectra of peak 2 showed the presence of two deprotonated molecules $[M-H]^-$ at *m/z* 619 and 597 and diagnostic fragments at *m/z* 191 [quinic acid-H]$^-$ in the MS^2 and/or MS^3 spectra in both of them (Mariutti *et al.*, 2014). The deprotonated molecules $[M-H]^-$ at *m/z* 619 and 597 gave base peak fragments in the MS^2 spectrum, respectively, at *m/z* 427 $[M-H-192]^-$ and *m/z* 405 $[M-H-192]^-$, indicating the neutral loss of one quinic acid molecule. MS^2 spectrum of the ion at *m/z* 427 ($[M-H-192]^-$, fragmented in source) showed ion fragments at *m/z* 409 $[M-H-192-18]^-$ and at *m/z* 323 $[M-H-192-18-86]^-$, corresponding to the consecutive loss of one water molecule and one malonyl group, while the MS^3 spectrum of the ion

at *m/z* 405 showed ion fragments at *m/z* 387 [M-H-192-18]$^-$, corresponding to the loss of one water molecule.

Two gallic acid derivatives were tentatively identified, a gallic acid hexoside (Figure 2, peak 4) and galloyl quinic acid (Figure 2, peak 5). The presence of diagnostic fragments at *m/z* 169 [gallic acid-H]$^-$ and at *m/z* 191 [quinic acid-H]$^-$ in the MS^2 spectra along with UV-vis characteristic of gallic acid derivatives allowed the tentative identification of these compounds (Cuyckens e Claeys, 2004; Mariutti *et al.*, 2014).

Peak 10 was assigned as HHDP hexoside since its MS spectrum presented deprotonated molecule [M-H]$^-$ at *m/z* 481 and MS^2 fragment ions at *m/z* 319 [M-H-162]$^-$ and *m/z* 301 [M-H-18-162]$^-$, corresponding to the neutral loss of one hexose and to the HHDP moiety, respectively (Sandhu e Gu, 2010; Chiste e Mercadante, 2012). Myricetin (Figure 2, peak 25) and quercetin (Figure 2, peak 27) were identified based on comparison with UV-vis and MS and MS^2 spectra features of authentic standards (Table 2) and co-chromatography.

The myricetin derivatives (Figure 2, peaks 13-17 and 24) showed characteristic fragment ions of myricetin at *m/z* 317, 179 and 151 in the MS^2 or MS^3 spectra, while the quercetin derivatives (Figure 2, peaks 18-23 and 26) showed characteristic fragment ions of quercetin at *m/z* 301, 179 and 151 in the MS^2 or MS^3 spectra.

The fragment ion at *m/z* 317 of myricetin glycosides (Figure 2, peaks 13-17) and at *m/z* 301 of quercetin glycosides (Figure 2, peaks 18-23) in the MS^2 spectra resulted from the loss of one hexose (162 u), one pentose (132 u) or one deoxyhexose (146 u) from their deprotonated molecules (Cuyckens e Claeys, 2004; Lin e Harnly, 2007; Celli *et al.*, 2011; Mariutti *et al.*, 2014). All the glycosides were

assigned at position 3 because 3-glycosilated flavones and flavonols show a hypsochromic shift of 12-17 nm of UV absorption band in comparison to the respective aglycone (Lin e Harnly, 2007). Moreover, the absence of the fragment [M-H-18]$^-$ in the MS^2 spectra of myricetin and quercetin glycosides, corresponding to the loss of one water molecule, suggests *O*-glycosilation on phenolic hydroxyl (Cuyckens e Claeys, 2004).

The most common sugar moieties attached to phenolic compounds are glucose and galactose (hexoses), xylose and arabinose (pentoses) and rhamnose (deoxyhexose) (Cuyckens e Claeys, 2004); however, the general designations hexoside, pentoside and deoxyhexoside (Table 2) were used because MS technique alone does not allow to elucidate the sugar molecules conjugated phenolic compounds in the absence of authentic standards. Peak 17 (Figure 2, Table 2) was assigned as myricetin deoxyhexoside and peak 23 (Figure 2, Table 2) as quercetin deoxyhexoside and it is highly probable that this glycoside is a rhamnose (Celli *et al.*, 2011); therefore, these compounds could be tentatively identified, respectively, as myricetrin and quercitrin.

Peak 24 and 26 (Figure 2) were assigned as myricetin galloyl-deoxyhexoside (MW 616 g/mol) and quercetin galloyl-deoxyhexoside (MW 600 g/mol), respectively. The MS^2 spectra of the deprotonated molecule of both compounds showed the fragments at *m/z* 317 (myricetin) and at *m/z* 301 (quercetin), corresponding to the loss of one deoxyhexose and one gallic acid molecules [M-H-146-152] (Celli *et al.*, 2011; Saldanha *et al.*, 2013).

Celli *et al.* (2011) identified 7 non-anthocyanin phenolic compounds in purple pitanga: a myricetin hexoside, a myricetin pentoside, myricetin rhamnoside, a quercetin hexoside, a quercetin pentoside, quercetin rhamnoside and myricetin

galloyl-deoxyhexoside. Considering the chromatograms at 350 nm (Celli *et al.*, 2011) and ours at 360 nm (Figure 2C), the major non-anthocyanin phenolic compounds were myricetin rhamnoside, quercetin hexoside and quercetin rhamnoside in both sudies.

Aside from the same 7 non-anthocyanin phenolic compounds previously reported (Celli *et al.*, 2011), in this study we have identified 13 new non-anthocyanin phenolic compounds in purple pitanga, among them myricetin, quercetin, quercitrin, quercetin galloyl-deoxyhexoside, quinic acid and derivatives, a galloyl hexose, galloyl quinic acid, a HHDP hexoside and other myricetin glycosides and quercetin glycosides.

3.2. Effects of purple pitanga extract *per se* on *C. elegans*

To evaluate the *in vivo* antioxidant activity of the purple pitanga extract, we tested the crescent concentrations on the nematode *C. elegans*. We observed that the purple pitanga extract did not significantly alter the survival of the worms (Figure 3A). From this result, it can be observed that the purple pitanga extract did not present toxic effects on the nematode *C. elegans*. In the longevity with the N2 wild strain (Figure 3C) the concentrations of 100, 250 and 500 µg CAE/ml increased the life span of the nematode relative to the control and the lowest concentrations analyzed. An increase in life span is often correlated with a decrease in fecundity. To test whether the purple pitanga has a negative effect on fecundity, we measured egg laying on *C. elegans*. Egg laying did not show significant difference between treatments and control (Figure 3B). Similarly, a study with whole apple extracts also increased longevity per se and did not affect the reproduction of *C. elegans* (Vayndorf *et al.*, 2013). In addition, several studies have already observed that the

life span increase due to several antioxidants in *C. elegans*, e.g. resveratrol, blueberry extract and EGb 761, an extract derived from leaves of the *Ginkgo biloba* tree (Wilson *et al.*, 2006; Wu *et al.*, 2006). These results indicate that life span modulation may be achieved pharmacologically and not just for caloric restriction, and may be partially mediated through antioxidant mechanisms.

In order to verify if purple pitanga extract could have an effect on longevity against oxidative stress, we used the strain TK22 (*mev-1*), which shows increased sensitivity to oxidative stress and reduced life span, and we observed that the two largest concentrations of purple pitanga, 250 and 500 µg CAE/ml were able to increase the life span of the mutant TK22 (Figure 3D). Thus, the treatment with the extract prolonged the life span of *mev-1*, a mutant hypersensitive to oxidative stress that reduces the useful life due to the excessive production of mitochondrial ROS due to a mutation in the complex II mitochondrial cytochrome b subunit (Ishii *et al.*, 1998). Similarly, study using polyherbal extract (PHE) containing six herbs also observed prolongation of the life span of the mutant *mev-1* (Rathor *et al.*, 2017). Flavonoids such as quercetin and catechins were shown to increase lifespan, the resistance against stressors such as heat or oxidative stress in *C. elegans* (Grünz *et al.*, 2012). Among the processes that could affect the aging trajectory, especially their high antioxidant activity has been frequently mentioned. In flavonoids, the catechol function at the B-ring (3', 4' – OH) as well as hydroxylation (3-OH) and a double bond at the heterocyclic C-ring have been demonstrated to be important prerequisites for a high antioxidant action *in vivo* (Rice-Evans *et al.*, 1995; Silva *et al.*, 2002).

Moreover, these results may be due to the richness in anthocyanins of the purple cherry extract, such as delphinidin 3-*O*-glucoside (Figure 1, peak 1), cyanindin 3-*O*-glucoside (Figure 1, peak 2) and pelargonidin 3-*O*-glucoside, mainly with respect

to cyanindin 3-*O*-glucoside which was the majority anthocyanin in the extract. Cyanindin 3-*O*-glucoside (Cy3G) already has proven health benefits in several studies. The bioactivity of Cy3G includes DNA-RSC, gastro-protective, anti-inflammatory, antithrombotic, insulinotropic, antimicrobial, chemopreventive and epigenetic effects. In turn, these actions can help prevent infection by *H. pylory*, age-related diseases, type 2 diabetes mellitus, metabolic syndrome and oral cancer (either pure Cy3G or within plant matrices) (Olivas-Aguirre *et al.*, 2016).

Aging is an inevitable natural process accompanied by a progressive accumulation of damage in all constituent macromolecules (Chondrogianni *et al.*, 2015). Although the determined mechanisms of the aging process are not fully identified, evidence suggests that aging is strongly associated with reactive oxygen species (ROS) (Si e Liu, 2014). Therefore, the consumption of fruits rich in phenolic compounds such as anthocyanins, could improve the redox state of the cells, prolonging life, as observed in our work.

3.3. Purple pitanga extract presents antioxidant activity against oxidative stress The installation of the process of oxidative stress arises from the existence of an imbalance between oxidant compounds and antioxidants, in favor of excessive generation of free radicals or to the detriment of the speed of removal of these in aging. Therefore, we used two stressors, hydrogen peroxide (H_2O_2) and Juglone in order to observe the antioxidant capacity of the purple pitanga extract against induced oxidative stress.

In the oxidative stress protection assay of the tested concentrations of purple pitanga extract, only two (100 and 500 µg CAE/ml) partially protected *C. elegans* against oxidative damage induced by H_2O_2 (Figure 4A). In the reversal assay the

concentrations 100 and 500 µg CAE/ml are able to reverse the damage partially, whereas 250 µg CAE/ml reverted completely to control levels (Figure 4B). Aan *et al*. (2013) also demonstrated that a tocotrienol rich fraction restored the lifespan of *C. elegans* with oxidative stress induced by H_2O_2. Hydrogen peroxide is one of the most abundant reactive oxygen species (ROS) in living cells and has been implicated in the apoptosis pathway, induction of intracellular oxidative stress and acceleration of aging (Aan *et al.*, 2013).

As an evaluation to investigate the impact of oxidative stress induced by H_2O_2, we monitor the egg laying of *C. elegans*. In the reproduction test H_2O_2 significantly decreased *C. elegans* egg laying and only 100, 250 and 500 µg CAE/ml partially protected (Figure 4C), however, all other concentrations tested were able to reverse the damage partially (Figure 4D). This physiological behavior is very similar to the changes observed by Kumsta *et. al* (2011), demonstrating that H_2O_2 causes severe behavioral and physiological symptoms, including limited movement, decline in progeny production, pumping of the pharynx, decreased rate of growth, and ATP levels (Kumsta *et al.*, 2011). In addition, it is suggested that at least some of these changes may be due to accumulation of intracellular peroxide promoting oxidative stress. Overall these results indicate that purple pitanga extract is capable of improving resistance during oxidative stress conditions *in vivo*. Furthermore, we observed that the purple pitanga extract had a reversal effect greater than protection, demonstrating that the phytochemicals compounds present in purple pitanga fruits have a more effective antioxidant activity after de oxidative damage being installed, having the lesser effect on the damage protection.

To evaluate the effect of purple cherry extract on the accumulation of reactive oxygen species (ROS) induced by H_2O_2, the reagent H_2DCF-DA (2,7-

dichlorofluorescein-diacetate). This compound becomes deacetylated by intracellular esterases and remains within the cells. Inside the cells, H_2DCF-DA is subjected to oxidation in the presence of intracellular ROS. The oxidized compound emits fluorescence, the intensity of which correlates with intracellular ROS levels. Significantly lower fluorescence intensity were observed between treated with nematodes purple pitanga compared to the untreated group. Concentrations of 5, 50 and 500 µg CAE/ml decrease the production of reactive species (Figure 4E), indicating the antioxidant effect of the extract and its bioavailability to the worms. Similarly, açaí extract also decreased the accumulation of intracellular ROS in *C. elegans* (Peixoto *et al.*, 2016) and treatments with lipoic acid and EGCG also observed the dose-dependent attenuation of H_2O_2 levels in H_2DCF-DA assay (Brown *et al.*, 2006).

In the test of resistance to oxidative stress induced by Juglone (5-hydroxy-1,4-naphthoquinone), a natural *Juglans regia* quinine with toxic pro-oxidant activity (Saling *et al.*, 2011), concentrations of 50, 100, 250 and 500 µg CAE/ml protected against oxidative damage in *C. elegans* (Figure 5A). However, only the two highest concentrations (250 and 500 µg CAE/ml) are able to partially reverse the damage induced by juglone (Figure 5B). Other antioxidants such as Ginkgo biloba extract EGb 761 and EGCG have also been reported for attenuating the oxidative damage induced by juglone in *C. elegans* (Strayer *et al.*, 2003; Abbas e Wink, 2009). The intracellular redox cycler juglone enters the cells, is reduced with NAD(P)H by a diaphorase and reduces oxygen to superoxide anion so that it generates an intracellular oxidative stress (Jason F. Cooper *et al.*, 2016). Thus, we observed that the extract of purple pitanga was more effective in the protection than in the reversal of the oxidative stress caused by juglone, since when the superoxide anion was

formed it was already eliminated by the antioxidant compounds present in the purple pitanga extract.

Reactive oxygen species (ROS) are a byproduct of ATP generation and are usually eliminated by the cellular defense antioxidant system. The decrease in the activity of the cellular antioxidant system leads to an increase of ROS (Sohal *et al.*, 1994). Oxidative stress caused by ROS can lead to the oxidation of biomolecules, such as protein, DNA and lipids, which is considered to be one of the main factors of aging. Several previous studies have shown a correlation between antioxidants and aging (Kimoto-Kinoshita *et al.*, 1999; Sinha *et al.*, 2010; Gruber *et al.*, 2013). Thus, we observed that the purple pitanga extract was antioxidant against the two types of stressors used, hydrogen peroxide, a possible superoxide anion precursor, and juglone an intracellular anion superoxide generating compound, which is considered to be the most reactive of free radicals of oxygen. Therefore, our results suggest that purple pitanga extract can act in the antioxidant defense and cellular repair system, eliminating excessive ROS and repairing oxidative damage.

3.4 Purple pitanga extract alters the expression of enzymes with GFP: SOD, CLT and HSP

To verify the possible mechanism of extension of life mediated by purple pitanga extract and high stress resistance in nematodes, expression of oxidative stress resistance superoxide dismutase (SOD) and catalase (CLT) enzymes were investigated. In the quantification of the antioxidant enzymes, we did not observe significant differences in the expression of the enzyme catalase (strain GA800) (Figure 6A). However, superoxide dismutase (strain CF1553) increased its expression at concentrations of 50, 100, 250 and 500 µg CAE/ml (Figure 6B). All

tested concentrations of purple pitanga extract increased expression of the heat shock protein HSP-16.2 (strain CL2070) (Figure 6C). Similarly, dietary supplementation with antioxidants, such as N-acetyl-L-cysteine extracts and *Tenebrio molitor*, also led to the extended life span and induced up regulation of HSP-16.2 and SOD-3 in *C. elegans* (Won *et al.*, 2016).

SOD-3 and HSP-16.2 proteins can activate an enhanced maintenance program of life in response to environmental inputs (Wu *et al.*, 2012). Superoxide dismutase (SOD) has the ability to decrease the concentration of superoxide anion radicals. SOD-3 is an antioxidant enzyme that is induced in response to stress (Darr e Fridovich, 1995) and expressed high in many long-lived *C. elegans* mutants (Honda e Honda, 1999; Kenyon, 2005). Strong regulation of HSP-16 transcription is commonly observed in response to stress, which can later serve as a stress-sensitive reporter to predict longevity in *C. elegans* (Hsu *et al.*, 2003). In that paper, the transgenic strain CL2070 was used containing a construct in which the gene encoding GFP was coupled to the HSP-16 gene promoter (Link *et al.*, 1999). This transgene animal allows visualization of the stress response *in vivo* in which the amount of synthesized GFP is used as an experienced stress measure. Our results demonstrate that the extract was able to induce significantly GFP synthesis, indicating the ability to activate the heat shock promoter.

Therefore, our results suggest that the extract of pitanga presents a strong regulation in the expression of the antioxidant enzyme SOD-3 and in the heat shock protein HSP-16.2. Increased stress resistance could be mediated by regulated expression of longevity genes to a large content. SOD-3 and HSP-16.2, two targets regulated by DAF-16, can activate an enhanced life maintenance program in response to environmental and gonadal inputs (Wu, et al, 2012). Over-expression of

HSP-16.2 can enhance oxidative-stress resistance and increase lifespan, and is being used as a stress-sensitive reporter to predict longevity (Hsu *et al.*, 2003; Rea *et al.*, 2005). The FoxO transcription factor DAF-16 has been show to be a crucial factor in the control of stress response and its nuclear localization is a necessary prerequisite for the transcriptional activation of its target genes. Among the targets of DAF-16 are genes for antioxidant enzymes like manganese superoxide dismutase (*sod-3*) and catalases (*ctl-1, ctl-2*) (Kampkötter *et al.*, 2007). Thus we suggest that the antioxidant compounds from purple pitanga extract can be increase the lifespan and increase the resistance against thermal and antioxidant stresses due to antioxidant properties of the polyphenols or to alterations of signaling pathways.

4. Conclusion

The purple pitanga extract is rich in phenolic compounds, mainly in anthocyanin cyanindin 3-*O*-glucoside. In conclusion, the results of this work indicate that the extract is able to improve survival during oxidative stress and is also able to extend the survival time of N2 (wild type) and *mev-1* mutants, modulating the expression of SOD-3 and HSP-16.2. Therefore, this study shows that dietary supplementation of extracts with antioxidant properties may be promising candidates for the treatment of diseases related to aging.

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Table 1: Chromatographic and spectroscopic characteristics, obtained by HPLC-DAD-MS/MS of anthocyanins from purple pitanga.

Peak[a]	Compound	Concentration (mg cyanidin 3-*O*-glucoside/100 g lyophilized fruit)	t_R (min)[b]	λ_{max} (nm)[c]	$[M]^+$ (*m/z*)	MS/MS fragment ions (*m/z*)
1	delphinidin 3-*O*-glucoside[d]	99.65 ± 1.77	12.9-13.0	525	465	303 $[M-162]^+$
2	cyanidin 3-*O*-glucoside[d]	512.01 ± 11.18	14.8	517	449	287 $[M-162]^+$
3	pelargonidin 3-*O*-glucoside[d]	2.16 ± 0.13	16.7-16,8	502	433	271 $[M-162]^+$
4	cyanindin 3-*O*-pentoside[e]	0.83 ± 0.07	19.4	521	419	287 $[M-132]^+$
5	cyanidin derivative[e]	5.16 ± 1.23	21.1	518	477	287 $[M-190]^+$
	Total	**618.88 ± 12.50**				

[a] Peaks are numbered according to the chromatogram on Figure 1.
[b] Retention time on column C_{18} (Luna, 5 µm, 250 mm x 4.6 mm, Phenomenex, Torrance, USA).
[c] Linear gradient of water/methanol, both with 5% formic acid.
[d] Identified (standard available).
[e] Tentative identified.

Table 2: Chromatographic and spectroscopic characteristics of phenolic compounds from purple pitanga.

Peaks[a]	Compound	Concentration (mg/100 g lyophilized fruit)	t_R (min)[b]	λ_{max} (nm)[c]	[M-H]$^-$ (*m/z*)	MS^n (*m/z*)[d]	Identity confirmation
1	quinic acid	nc[e]	3.5	nd[f]	191	MS^2 [191]:173, 155, **127**, 111	standard
2	mixture of quinic acid derivatives	nc	6.8	Nd	619	MS^2 [619]: **427** MS^2 [427]: **409**, 381, 351, 323, 289, 261, 235, 191	
					597	MS^2 [597]: **405**, 191 MS^3 [405]: 387, 173, **191**, 111	
3	not identified 1[1]	13.27±1.32	7.2	291	Nd		
4	galloyl hexoside[1]	11.47±1.10	8.5	276	331	MS^2 [331]: 271, 211, **169**, 125 MS^3 [169]: **125**	Chisté and Mercadante, 2012; Sandhu and Gu, 2010
5	galloylquinic acid [1]	5.48±0.52	10.4	273	343	MS^2 [343]: 277, **191**, 169	
6	not identified 2[1]	29.56±0.65	14.0	267	Nd		
7	not identified 3[1]	18.36±0.001	14.7	270	Nd		
8	not identified 4[1]	12.07±0.31	15.3	270	Nd		
9	not identified 5[1]	19.41±0.26	16.2	268	Nd		
10	HHDP hexoside[2]	12.83±0.72	16.6	361	481	MS^2 [481]: 319, **301**, 175	Chisté and Mercadante, 2012; Sandhu and Gu, 2010
11	delphinidin 3-*O*-glucoside	Table 1	17.8	275, 525	463	MS^2 [463]: **301**	standard
12	cyanidin 3-*O*-glucoside	Table 1	19.2	279, 517	447	MS^2 [447]: **285**	standard
13	myricetin 3-*O*-hexoside 1[2]	15.90±0.53	23.6	357	479	MS^2 [479]: **317**, 271, 179, 151 MS^3 [479→317]: 271, **179**, 151	Lin and Harnly, 2007; Celli et al., 2011
14	myricetin 3-*O*-hexoside 2[2]	13.23±0.31	23.8	356	479	MS^2 [479]: **317,** 179, 151 MS^3 [479→317]: 271, **179**	Lin and Harnly, 2007; Celli et al., 2011
15	myricetin 3-*O*-pentoside 1[2]	11.37±0.46	24.6	358	449	MS^2 [449]: **317**, 179	Lin and Harnly, 2007; Celli et al., 2011
16	myricetin 3-*O*-pentoside 2[2]	11.79±0.50	25.4	356	449	MS^2 [449]: **317,** 179, 151	Lin and Harnly, 2007; Celli et al., 2011
17	myricetin 3-*O*-deoxyhexoside[2]	22.84±0.75	25.7	349	463	MS^2 [463]: **317**, 179 MS^3 [463→317]: 287, 271, 193, **179**, 151 MS^3 [463→179]: 151	Lin and Harnly, 2007; Celli et al., 2011
18	quercetin 3-*O*-hexoside 1[2]	18.85±0.75	26.2	354	463	MS^2 [463]: **301**, 179, 151 MS^3 [463→301]: 271, 193, **179**, 151, 121, 107	Lin and Harnly, 2007; Mariutti et al., 2014; Celli et al., 2011
19	quercetin 3-*O*-hexoside 2[2]	17.29±0.67	26.5	353	463	MS^2 [463]: **301**, 179, 151 MS^3 [463→301]: **179**, 151, 121	Lin and Harnly, 2007; Mariutti et al., 2014; Celli et al., 2011
20	quercetin 3-*O*-pentoside 1[2]	11.59±0.51	27.5	349	433	MS^2 [433]: **301**, 179, 151	Lin and Harnly, 2007; Celli et al., 2011

Peaks[a]	Compound	Concentration (mg/100 g lyophilized fruit)	t_R (min)[b]	λ_{max} (nm)[c]	$[M-H]^-$ (*m/z*)	MS^n *(m/z)*[d]	Identity confirmation
21	quercetin 3-*O*-pentoside 2[2]	12.71±0.54	28.2	352	433	MS^2 [433]: **301**, 179, 151	Lin and Harnly, 2007, Mariutti et al., 2014; Celli et al., 2011
22	quercetin 3-*O*-pentoside 3[2]	13.75±0.50	28.5	349	433	MS^2 [433]: **301**, 179, 151	Lin and Harnly, 2007; Celli et al., 2011
23	quercetin 3-*O*-deoxyhexoside[2]	19.68±1.04	29.0	349	447	MS^2 [447]: **301,** 179, 151 MS^3 [447→301]: 271, 193, **179**, 151, 121, 107	Lin and Harnly, 2007; Celli et al., 2011
24	myricetin galloyl-deoxyhexoside[2]	11.56±0.46	30.8	356	615	MS^2 [615]: 463, **317**	Celli et al., 2011; Saldanha et al., 2013
25	myricetin[2]	12.28±0.48	31.2	372	317	MS^2 [317]: 299, 287, **249**, 179, 151, 137	standard
26	quercetin galloyl-deoxyhexoside[2]	11.48±0.47	33.8	359	599	MS^2 [599]: **301**, 271, 179	
27	quercetin[2]	11.19±0.49	36.6	367	301	MS^2 [301]: 257, **231**, 179, 151	standard
total non-anthocyanin phenolic compounds		329.22±24.89					

[a] Peaks are numbered according to the chromatogram on Figure 2.
[b] Retention time on the C_{18} Synergi Hydro (4μm) column.
[c] Linear gradient of 0.5% formic acid in water and acetonitrile with 0.5% formic acid.
[d] In the MS^2 and MS^3, the most abundant ion is shown in boldface.
[e] nc= not calculated.
[f] nd = not detected.
Peaks were quantified as equivalents of gallic acid[1] and quercetin[2].

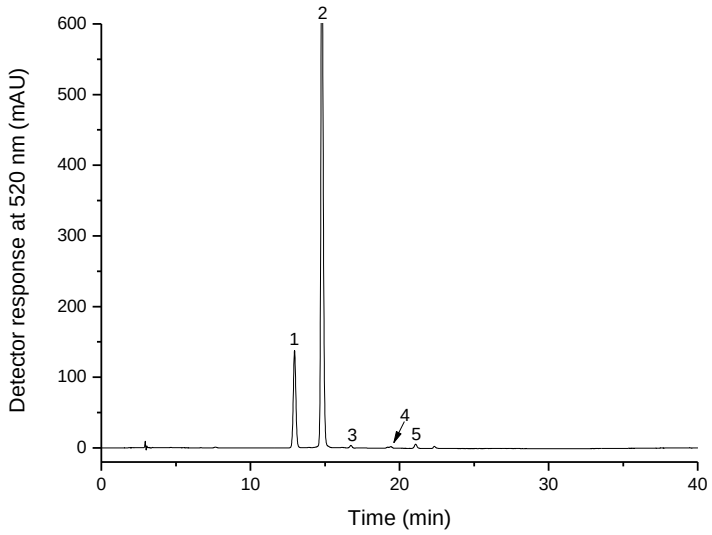
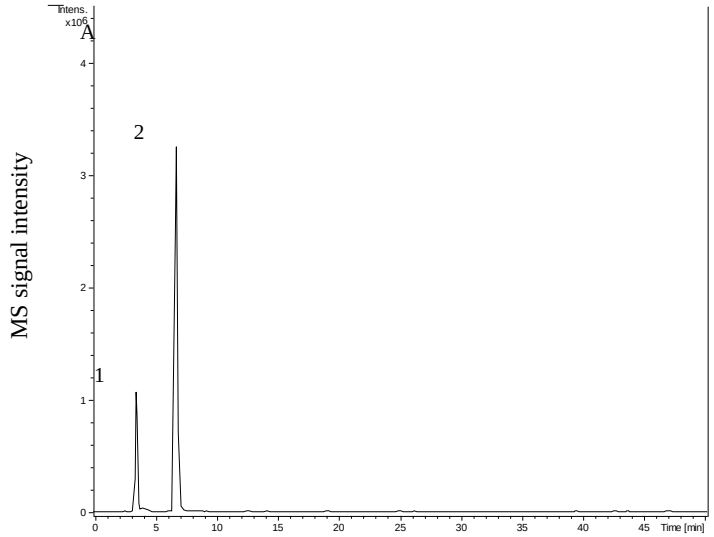
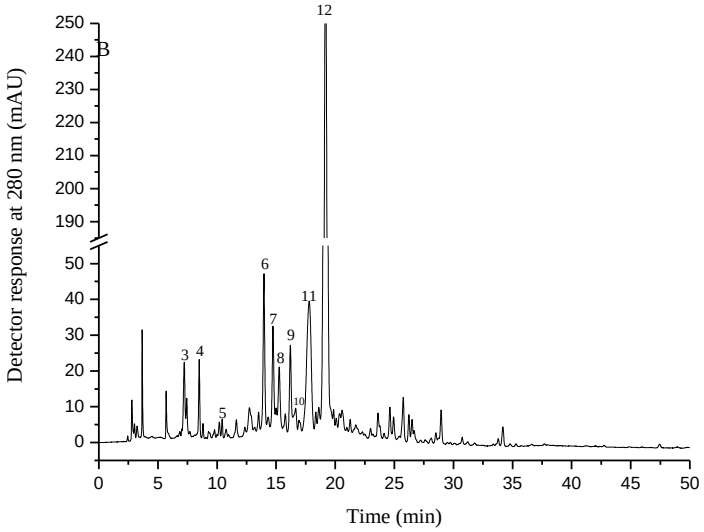


Figure 1: Chromatogram of the anthocyanins from purple pitanga fruit obtained by HPLC-DAD and processed at 520 nm. Peak characterization is given in Table 1. Chromatographic conditions according to Faria et al. (2011).


Intens.
x10^6
A
MS signal intensity
1
2
Time [min]


B
Detector response at 280 nm (mAU)
Time (min)

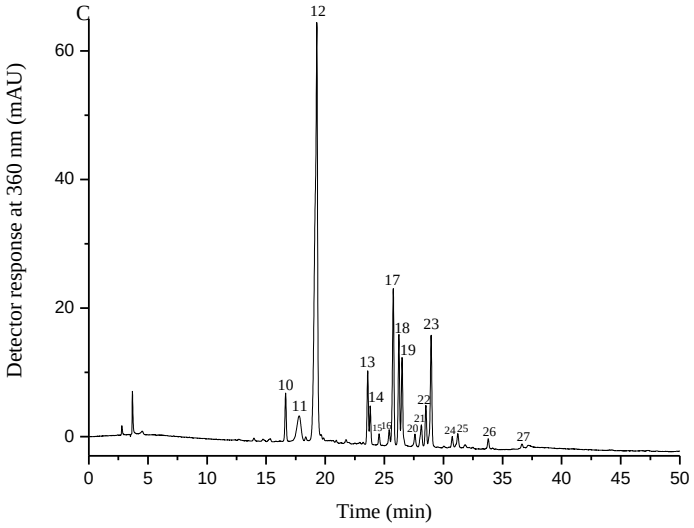

C
Detector response at 360 nm (mAU)
Time (min)

Figure 2: Chromatograms of phenolic compounds from purple pitanga: (A) HPLC-MS extracted ion chromatogram at *m/z* 191, (B) HPLC-DAD processed at 280 nm and (C) HPLC-DAD processed at 360 nm. Peak characterization is given in Table 2. Chromatographic conditions according to Chisté and Mercadante (2012).

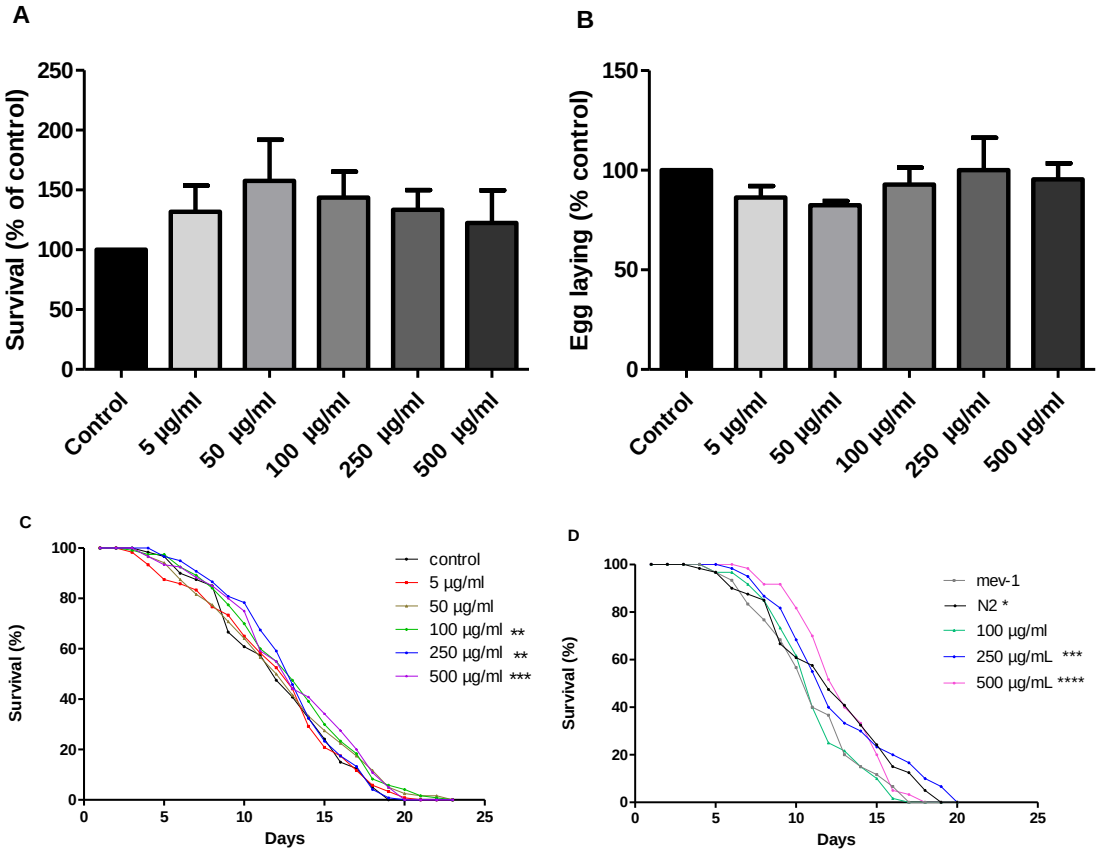


Figure 3: Effect per se of different concentrations of purple pitanga extract on survival (A); Egg laying (B); Longevity with N2 (C) and Longevity with TK22 (D) of the nematode *C. elegans*. Values are mean ± standard error of 3 independent experiments. * Different control.

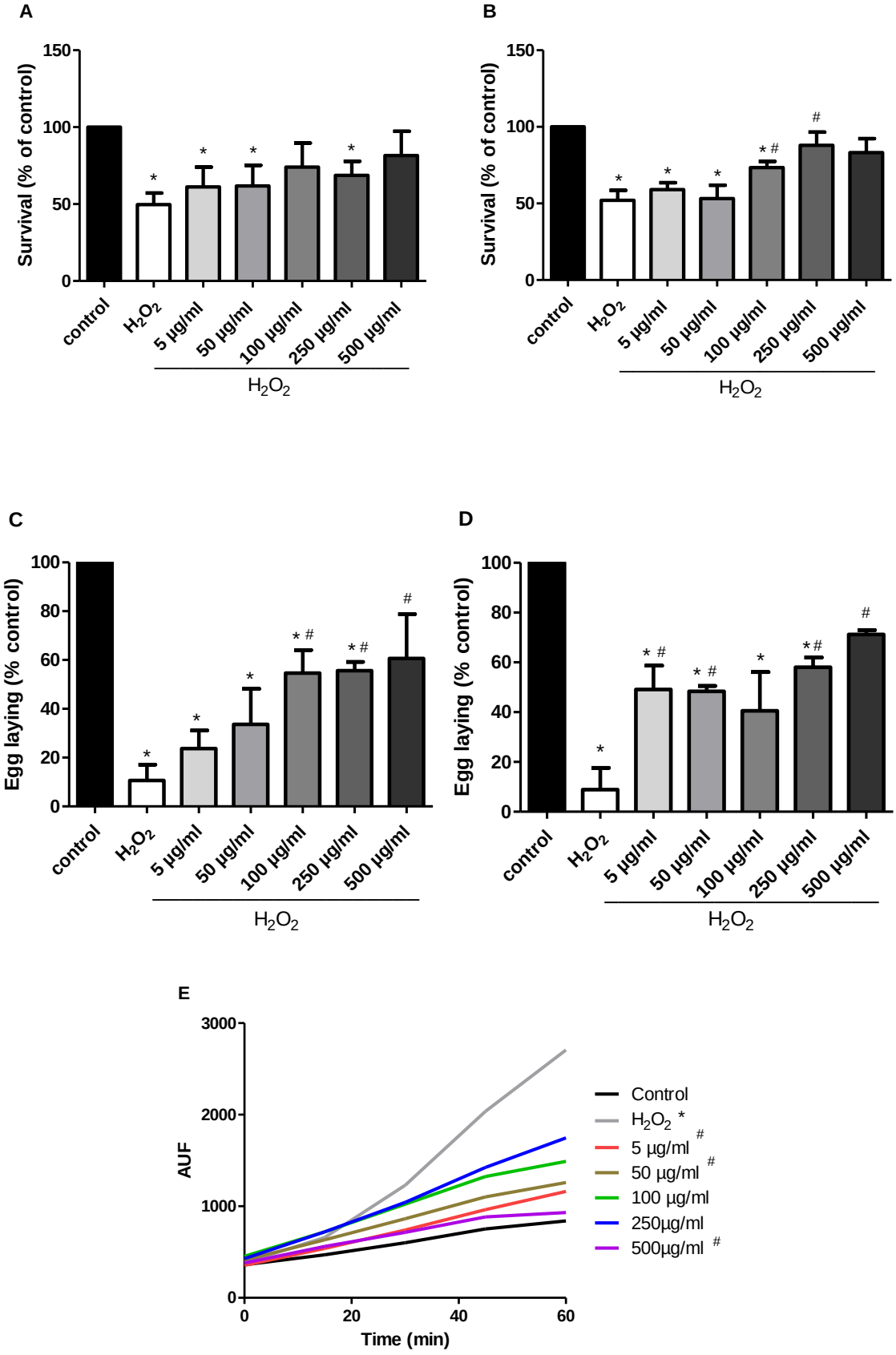

A
Survival (% of control)
150
100
50
0
control
H2O2
5 µg/ml
50 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml
H2O2
B
Survival (% of control)
150
100
50
0
control
H2O2
5 µg/ml
50 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml
H2O2
C
Egg laying (% control)
100
80
60
40
20
0
control
H2O2
5 µg/ml
50 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml
H2O2
D
Egg laying (% control)
100
80
60
40
20
0
control
H2O2
5 µg/ml
50 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml
H2O2
E
AUF
3000
2000
1000
0
0
20
40
60
Time (min)
Control
H2O2 *
5 µg/ml #
50 µg/ml #
100 µg/ml
250µg/ml
500µg/ml #

Figure 4: Effects of different concentrations of purple pitanga extract on survival in the protection (A) test and reversion (B) to oxidative damage induced by hydrogen peroxide. Effect on egg laying in the protection test (C) and reversion (D) in *C. elegans*. Analysis of reactive oxygen species (E). Values are mean ± standard error of 3 independent experiments. * Different control. # Different H_2O_2.

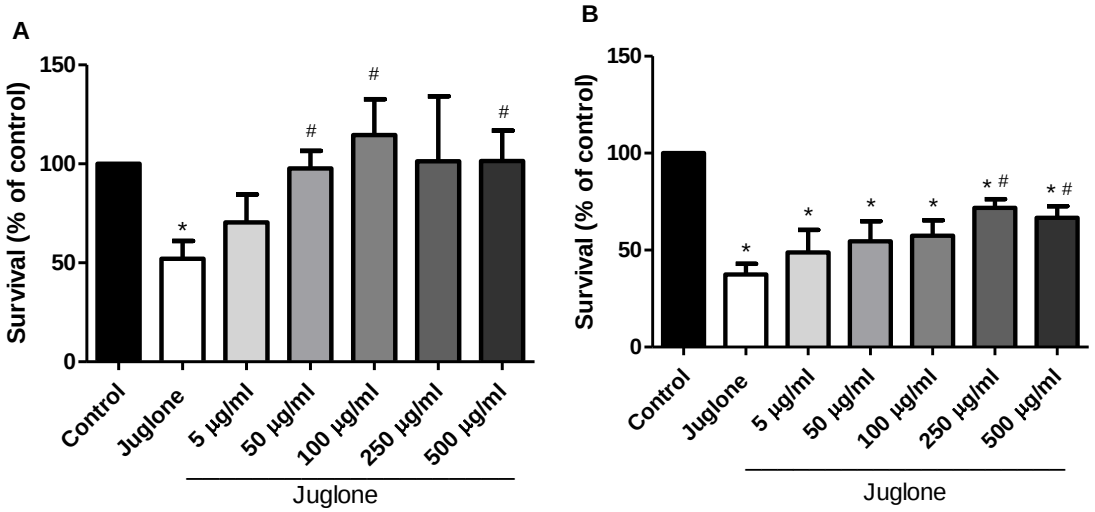


Figure 5: Effects of different concentrations of purple pitanga extract on the survival protection assay (A) and reverse (B) to oxidative damage induced by juglone in *C. elegans*. Values are mean ± standard error of 3 independent experiments. * Different control. # Different juglone.

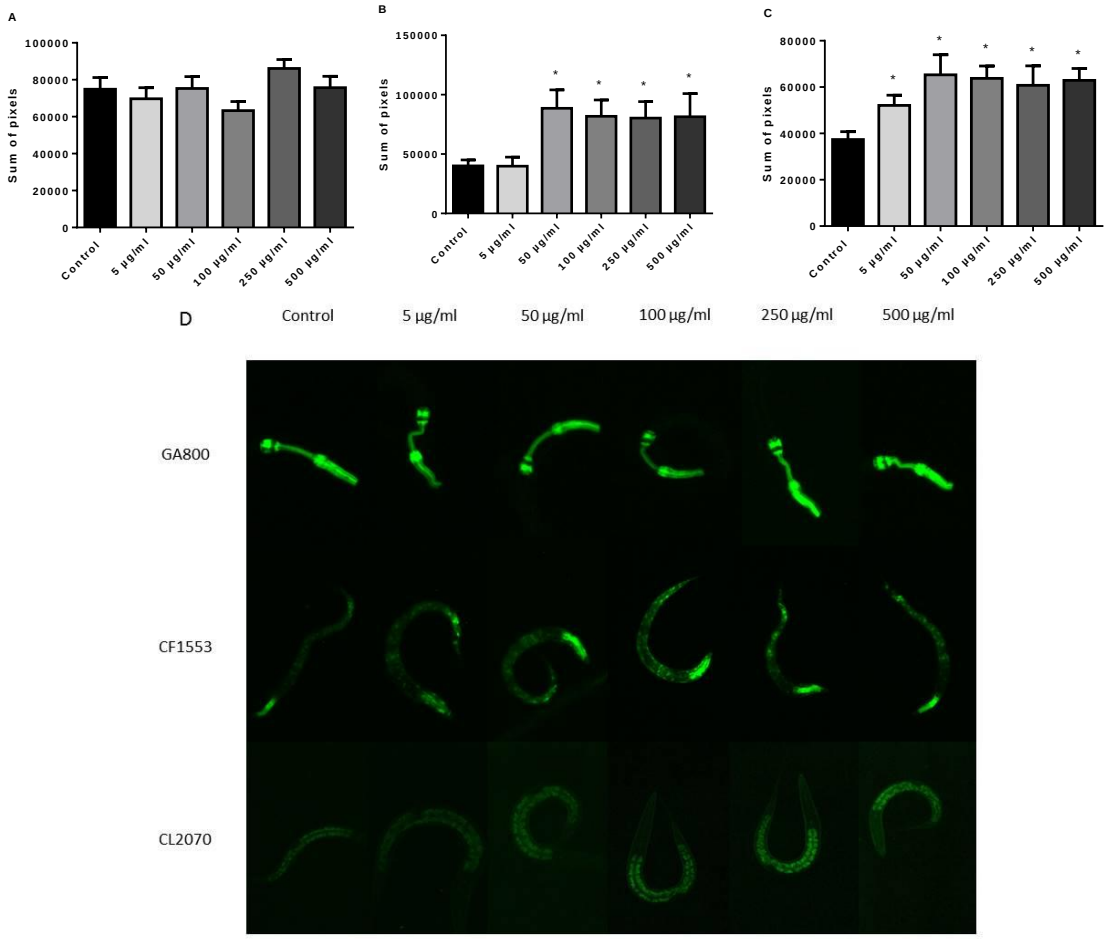


Figure 6: Quantification of fluorescence of GFP strains. (A) GA800 [*ctl-1* + *ctl-2* + *ctl-3*::GFP], (B) CF1553 [*sod-3p*::GFP], (C) CL2070 [*hsp-16.2p*::GFP]. (D) Representative photos. Values are mean ± standard error of 3 independent experiments. * Different control.

6 CONCLUSÕES

Muitas pesquisas vêm sendo realizadas na busca por agentes antioxidantes naturais, com o objetivo de proteger as células e os órgãos da ação dos radicais livres, e com isso retardarem o processo de envelhecimento e a progressão de diversas doenças. O extrato de pitanga roxa apresentou ser rico em antocianinas, principalmente na cyanindin 3-*O*-glucoside. Em conclusão, os resultados desse trabalho indicam que o extrato é capaz de melhorar a sobrevida durante o estresse oxidativo e também é capaz de estender o tempo de sobrevivência do N2 (tipo selvagem) e mutantes *mev-1,* modulando a expressão de SOD-3 e HSP-16.2. Portanto, este estudo mostra que a suplementação dietética de extratos com propriedades antioxidantes podem ser candidatos promissores para o auxilio do tratamento de doenças relacionadas ao envelhecimento.

7 PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são: avaliar possíveis efeitos farmacológicos do extrato de pitanga roxa, como efeitos na adipogênese e obesidade, utilizando cepas mutantes de *C. elegans* e cultura de células animais.

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