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AVALIAÇÃO DO EXTRATO AQUOSO DA ALFARROBA (*Ceratonia siliqua L.*) E SEUS POSSÍVEIS EFEITOS ANTIOXIDANTES E SOBRE O METABOLISMO LIPÍDICO EM *Caenorhabdits elegans*

DISSERTAÇÃO DE MESTRADO

Cristiane de Freitas Rodrigues

Uruguaiana, RS, Brasil

2015

CRISTIANE DE FREITAS RODRIGUES

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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: Prof[a]. Dr[a]. Daiana Silva de Ávila

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Área de concentração: Bioquímica Farmacológica e Toxicolóigica

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Dedico este trabalho

A minha mãe, Glacir Rodrigues, por me ensinar que o melhor do ser humano,

é o conhecimento;

e ao meu pai, José Carlos Rodrigues a onde estiver.

“Eu tenho a grandeza

dos pequenos sonhadores.

Já estou entre os menores do mundo.

Sonhei ser o dono das cores,

já fui rei do silêncio profundo.

No ranking dos pequenos sonhos,

Subi e primeiro para segundo.”

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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa

AVALIAÇÃO DO EXTRATO AQUOSO DA ALFARROBA (*Ceratonia siliqua L.*) E SEUS POSSÍVEIS EFEITOS ANTIOXIDANTES E SOBRE O METABOLISMO LIPÍDICO EM *Caenorhabdits elegans*

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Local e Data da Defesa: Uruguaiana, 26 de setembro, 2015

A *Ceratonia siliqua* L. popularmente conhecida como alfarroba, faz parte da classe dos diversos alimentos que apresentam uma grande quantidade de antioxidantes, principalmente pela composição dos compostos fenólicos. Além disso, este alimento tem sido utilizado como substituinte do cacau em diversos produtos alimentícios em função do sabor e cor similares, porém com reduzido conteúdo de lipídeos. Alguns estudos sugerem que o extrato aquoso de alfarroba apresenta uma ação protetora contra os radicais livres. Entretanto, são necessários testes *in vitro* e *in vivo* a fim de comprovar a eficácia deste alimento funcional. Para tanto, utilizamos o *Caenorhabditis elegans* como modelo experimental em prol de substituir o uso de mamíferos e oferecer novas possibilidades de ensaios para avaliar a eficácia de produtos naturais. Neste presente estudo observou que o extrato aquoso da alfarroba apresentou um excelente potencial sequestrador de espécies reativas *in vitro* pelos ensaios de redução do radical DPPH e de redução do Ferro. Tal efeito pode ser devido à presença dos compostos fenólicos, conforme verificado na análise bromatológica. A partir disso, no estudo percorreu as atividades *in vivo*. O extrato não apresentou toxicidade significativa até a concentração testada (62.0µg/mL), conforme observado nos ensaios de sobrevivência e longevidade. Também foi observado que o extrato de alfarroba protege contra a mortalidade induzida por paraquat em curto prazo, porém o mesmo não ocorreu ao longo prazo. Encontrou-se também redução da peroxidação lipídica e um aumento da atividade enzimática da catalase, sugerindo estar relacionada ao fator de transcrição DAF-16, importante para a defesa antioxidante nestes animais. Além disso, nosso estudo demonstrou que o extrato aquoso da alfarroba é mais eficiente do que o extrato de cacao na redução dos níveis de triglicerídeos em nematoides selvagens e em mutantes *tub-1* (mais obesos), não alterando a ingestão alimentar. Este efeito pode estar relacionado com elevado de teor de fibras presentes no extrato, conforme observado pelo ensaio bromatológico. Em

conclusão, nosso estudo sugere que a utilização de alfarroba é segura e pode ser de fato bastante benéfica ao consumidor, fornecendo compostos fenólicos e fibras que podem auxiliar no envelhecimento e na redução de peso.

Palavras-chave: *Ceratonia siliqua, C. elegans*, antioxidantes.

ABSTRACT

Dissertation of Master's Degree

Post-Graduation Program in Biochemistry

Federal University of Pampa

EXTRACT OF EVALUATION OF AQUEOUS CAROB (*Ceratonia siliqua L.*) AND ITS POSSIBLE EFFECTS AND ANTIOXIDANTS ON LIPID METABOLISM IN *Caenorhabdits elegans*

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ADVISOR: Daiana Silva de Ávila

Place and Date of Defense: Uruguaiana, September 26th, 2015

The *Ceratonia siliqua* L. popularly known as Carob, part of the class of many foods contain a large number of antioxidants, especially phenolic compounds of the composition. Moreover, this food has been used as cocoa substituent in many food products depending on the flavor and color similar, but with reduced lipid content. Some studies suggest that the aqueous extract of carob has a protective effect against free radicals. However, they are necessary in vitro and in vivo in order to prove the effectiveness of this functional food. Therefore, *Caenorhabditis.elegans* used as an experimental model towards replace the use of mammalian and offer new opportunities assays to assess the efficacy of natural products. Our study has shown that the aqueous extract of carob showed excellent potential kidnapper of reactive species by in vitro assays to reduce the DPPH radical and reduction of iron. This effect may be due to the presence of phenolic compounds as found in chemical analysis. From this, our study ran activities in vivo. The extract showed no significant toxicity to the tested concentration (62.0μg / ml), as noted in survival and longevity tests. It was also noted that the carob extract protects against mortality induced by paraquat in the short term, but the same was not true in the long run. It was found also reducing lipid peroxidation and an increase in the enzymatic activity of catalase, suggesting be related to the transcription factor DAF-16 important for the antioxidant defense in these animals. Furthermore, our study has demonstrated that the aqueous extract of carob is more efficient than cacao extract in reducing triglyceride levels in wild nematodes and tub-1 mutants (most obese) without altering food intake. This effect may be related to high content of fiber present in the extract, as observed by bromatological assay. In conclusion, our study suggests that the use of carob is safe and can be very beneficial to the consumer fact, phenolic compounds and providing fibers which can assist in aging and weight reduction.

Keywords: *Ceratonia siliqua*, *C.elegans*, antioxidants.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA: Análise de Variância

ATP- Adenosina trifosfato

CAT: Catalase

CGC: Caenorhabditis Genetics Center

DCF: Dichlorofluorescein

DCF-DA: Dichlorofluorescein Diacetate

DAF-16 - Fator de transcrição ortólogo ao FOXO

DL50- Quantidade de uma determinada substância que é necessária para provocar a morte a pelo menos 50% da população

ERO - Espécies reativas de oxigênio

GAE: Equivalentes de Ácido Galico

GFP - Proteína verde fluorescente

GPx - Glutationa-peroxidase

H_2O_2 - Peróxido de hidrogênio

IGF-1 - Fator de crescimento semelhante à insulina

NGM: Nematode Growth Media

O2 - Oxigênio molecular

O2 •- - Ânion superóxido

OH• - Radical hidroxila

SOD - Superóxido dismutase

UNIPAMPA: Universidade Federal do Pampa

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Introdução

A crescente preocupação com hábitos alimentares mais saudáveis tornam os alimentos ricos em antioxidantes um forte aliado para a saúde. Neste contexto, diversos estudos têm demonstrado que o consumo diário destes alimentos pode atuar como agentes protetores contra os processos oxidativos, os quais ocorrem naturalmente no organismo, como durante o processo de envelhecimento (CERQUEIRA; MEDEIROS DE; AUGUSTO, 2007). Alguns estudos abordam a ação protetora dos antioxidantes contra danos causados por espécies reativas de oxigênio (EROS), os quais são capazes de interceptar os radicais livres gerados pelo metabolismo celular (BRENNA; PAGLIARINI, 2001; YILDRIM, MAVI, KARA; 2002).

Neste contexto, existem duas fontes de antioxidantes, a endógena e a exógena; na endógena ou enzimática, os antioxidantes catalisam a neutralização de espécies reativas, com destaque para a superóxido dismutase, catalase e glutationa peroxidase. Já as fontes exógenas ou não enzimáticas são adquiridas através da ingestão de alimentos ricos em moléculas que são capazes de interagir quimicamente com as espécies reativas, reduzindo a reação destas com biomoléculas importantes. Também se destaca a glutationa de forma reduzida (GSH) por ser fonte endógena e não enzimática (YILDRIM, MAVI; KARA, 2002). Entre os antioxidantes obtidos pela dieta destacam-se as vitaminas C, E e A, os carotenóides e flavonóides. Os flavonóides são compostos fenólicos que estão presentes em diversos alimentos, como o vinho tinto, uva, maçã, chá, cacau e alfarroba, os quais vêm sendo amplamente estudados pelos seus efeitos benéficos à saúde (ROTHWELL *et al*., 2013; MANACH *et al*., 2004).

Dentre diversos alimentos ricos em antioxidantes está a alfarroba, fruto da *Ceratonia siliqua* L. (alfarrobeira), nativa da Bacia do Mediterrâneo e também das regiões da Arábia e Oman. É conhecida na medicina popular por suas ações diurética, antidiarreica e em tratamentos gástricos de bebês lactantes (HABER, 2002). A alfarroba também se destaca como um dos novos alimentos que proporciona diversos benefícios à saúde, pela sua composição fotoquímica dos compostos fenólicos, como flavonóides, ácidos fenólicos, antocianinas, vitaminas C, E e carotenóides (AYAZ *et al*., 2007). Perante a sua diversidade de compostos fenólicos, existem estudos que abordam os efeitos dos polifenóis naturais da alfarroba na redução de células cancerígenas. A alfarroba também pode ser utilizada como um adoçante natural, com sabor e aparência

semelhante ao cacau, e utilizada como um substituto do mesmo. Além do mais, sua utilização se expandiu por não apresentar glúten, cafeína e lactose (YOUSIF; ALGHZAWI, 2000).

Atualmente, o cacau é um dos alimentos mais populares e faz parte da ingestão diária de inúmeras pessoas, e muitas desta também o utilizam pelo seu caráter funcional (DILLINGER *et al.,* 2000). *A Theobroma cacao* conhecida como Cacaueiro, árvore de cacau, pertence à família das Malvaceae. Além do mais, o estudo apontou para os efeitos benéficos do chocolate amargo, rico em flavonóides, no sistema cardiovascular, atuando na melhora da disfunção endotelial (CORTI *et al.,* 2009). De modo que seus efeitos benéficos estão atrelados ao alto teor de flavonóides, metabólitos secundários amplamente presente no reino vegetal (CORTI *et al.,* 2009).

Desta forma, a tendência global muito divulgado em níveis crescentes de obesidade e diabetes tem reabastecido interesse na ingestão de macronutrientes (gorduras, proteínas e carboidratos) necessárias para manter o estado de normalidade de um indivíduo. Ambas as atenções científicas e públicas têm incidido sobre a intervenção dietética de síndromes crônicas de saúde, através do uso de suplementos dietéticos e alimentos com maior quantidade de antioxidantes (BRENNAN, 2005).

Em função dos poucos estudos com a alfarroba e sua capacidade de substituição ao cacau, em modelos *in vivo.* Em prol de verificar seus reais efeitos, o presente estudo tem como propósito avaliar seu potencial antioxidante *in vivo,* utilizando o modelo *Caenorhabditis elegans (C.elegans). O C.elegans* é um nematóide de vida livre na matéria orgânica e no solo, de fácil manipulação e cultivo de baixo custo. Nas últimas décadas, o *C.elegans* tem sido utilizado como organismo modelo para estudos de biologia do desenvolvimento, genética, envelhecimento, de ecotoxicologia e para *screening* de novos fármacos. Com tal característica, há muitos estudos sobre alguns alimentos funcionais e seus efeitos em *C.elegans* (PUN *et al.*, 2010).

Revisão Bibliográfica

1. Antioxidantes

O estilo de vida moderna faz com que as pessoas empenhem-se em encontrar alternativas alimentícias mais eficazes e principalmente, que tragam benefícios para todos os males. Assim, diversas pessoas buscam nos alimentos funcionais importantes aliados em amenizar certos danos ocasionados pelo envelhecimento e pelo estresse oxidativo (BAGCHI *et al.*, 2004). Os antioxidantes são uma categoria heterogênea de moléculas, cujas estruturas químicas possibilitam a interação com os radicais livres, impedindo a reação destes com biomoléculas. Os antioxidantes agem por diferentes mecanismos, como a neutralização direta dos radicais livres ou a quelação de íons metálicos de modo que eles não sejam capazes de gerar espécies reativas ou nem mesmo favorecer a decomposição de peróxidos (ASIMI; SAHU; PAL, 2013).

Assim, o desempenho dos antioxidantes *in vivo* depende de fatores como os tipos de radicais livres formados; onde e como são gerados esses radicais; análise e métodos para a identificação dos danos; e doses ideais para obter proteção. Desta forma, a melhor definição para antioxidantes é que são substâncias as quais, mesmo em baixas concentrações em relação ao substrato oxidante, podem atrasar ou inibir as taxas de oxidação (HALLIWELL *et al*., 1995; SIES, 1993). Além disso, alguns antioxidantes desempenham um papel importante na conservação dos alimentos pela inibição dos processos oxidativos (TIWARI, 2001; CUI; DECKER, 2015).

A oxidação lipídica é uma das principais causas de deterioração da qualidade em alimentos naturais e processados e, portanto, uma das grandes preocupações da indústria de alimentos. De tal forma, que os fosfolipídios, em particular lecitinas, são amplamente utilizados como emulsionantes naturais, e têm emergindo para controlar a oxidação lipídica. No entanto, devido ao seu alto grau de insaturação e grande área de superfície, os fosfolipídios podem facilmente reagir com pró-oxidantes como, por exemplo, metais de transição. Este efeito pró-oxidante atua como um substrato na oxidação de lipídios e resulta na alteração dos sabores dos produtos alimentares, como carnes, óleos, leites e derivados. De outra forma, os fosfolipídios também podem atuar como antioxidantes por meio de quelantes de metais pro-oxidativos, formadores dos produtos da reação de Maillard (CUI; DECKER, 2015).

Em paralelo a isso, diversas pessoas procuram alternativas para reduzir danos associados à obesidade ou doenças não transmissíveis, encontrando nos produtos naturais uma maneira eficaz de reduzir estes. Atualmente, diversos estudos têm se enfatizando em averiguar as propriedades de certos alimentos na redução de peso. Um exemplo é a fruta da tamarindo como um importante aliado na redução de peso em ratos, uma vez que inibe a ação da tripsina e consequentemente reduz a ingestão alimentar, um efeito mediado pelo aumento da colecistoquinina (RIBEIRO *et al.*, 2015). Com tal característica, diferentes alimentos apresentam ações diretas nos adipócitos, os quais oferecem funções fisiológicas importantes em vários aspectos da homeostase de nutrientes, incluindo o apetite e o metabolismo. Consequentemente, estas funções são moduladas pela glicose conforme o tecido adiposo e o tamanho do adipócitos (DAS *et al.*, 2004; WEYER *et al*., 2001; ABEL *et al*., 2001).

Uma vez que é necessária a insulina para controlar os níveis de glicose, também se torna essencial no armazenamento de nutrientes. Proporcionado uma melhor compreensão dos mecanismos que controlam a absorção de nutrientes, seu armazenamento e sua utilização em humanos. Desta forma, com a amplitude de mecanismos e a sua biodiversidade de nutrientes e moléculas são inevitáveis à utilização de modelos complementares, mais simples, que apresentem similaridades genéticas dos mamíferos (HASHMI *et al*., 2013).

Do mesmo modo, diversos estudos sobre os antioxidantes têm resaltado o uso de nutrientes isolados no tratamento e prevenção de doenças (JACOB, 1995; NIKI *et al*., 1995; HERCBERG *et al.*, 1998). Entre várias moléculas que são conhecidas pela capacidade de inativar ou neutralizar radicais livres estão o D-tocoferol, o ascorbato, o beta-caroteno, o ácido úrico, a ubiquinona, a transferrina, a ceruloplasmina, entre outros (OLSZEWER, 1995; FLESCHIN, 2000; MATKOVICS, 2003). Do mesmo modo, muitos estudos têm demonstrado que o uso de frutos ou dos seus componentes ativos isolados atua como agente de neutralização de EROS ou melhorara o sistema de defesa antioxidante (MANOHARAN *et al*.; 2009). Nesse estudo, enfatizou-se uso da alfarroba na medicina tradicional.

2. Alfarroba

A alfarroba também chamada algarroba ou pão de Santo John é o fruto da *Ceratonia siliqua*, que cresce amplamente na região do Mediterrâneo e pertence ao gênero Leguminosae (Figura 1). Antigamente, sua vagem foi consumida pelo alto teor de açúcares, sendo utilizada tanto para doces de crianças como em situações de escassez, no caso das guerras (OWEN *et al*., 2003).

A semente de alfarroba (Figura 2) é composta por casca (30-33%), endosperma (42-46%) e embrião ou gérmem (23-25%). As sementes de alfarroba contêm um endosperma branco translúcido, chamado goma de alfarroba ou goma de semente de alfarroba, que são aproveitados pelas suas propriedades de geleificação. São estas sementes da alfarroba que serão utilizadas na indústria alimentar para obtenção da goma. Após a remoção da semente, a vagem de fruta produz o pó de alfarroba ou farinha de alfarroba, que servirá nas preparações culinárias de bebidas e nas confeitarias (BATE-SMITH, 1973). Por séculos, na Sicília, a vagem da alfarrobeira foi usada na alimentação humana em preparações de bolos. Apesar disso, não se tornou popular por causa da baixa disponibilidade e pelo excesso de adstringência (OWEN *et al.*, 2003).

Figura 1: A alfarroba também chamada algarroba ou pão de Santo John (fonte: http://www.wisegeek.org/what-is-carob.htm#).

Figura 2: A semente de alfarroba é composta por casca (30-33%), endosperma (42-46%) e embrião ou gérmem (23-25%) (fonte:http://www.wisegeek.org/what-is-carob.htm#carob-seeds).

A adstringência excessiva do fruto se deve a presença de altos níveis de taninos. Os taninos são compostos polifenólicos complexos que pertencem a uma ampla variedade de alimentos e rações de origem vegetal. Os taninos podem ser classificados em dois grupos, taninos hidrolisáveis e taninos condensados ou proantocianidinas. Os taninos hidrolisáveis são polímeros de ácido gálico ou ácido elágico esterificados a uma molécula de núcleo, comumente um açúcar ou um polifenol, tais como catequina. Já os taninos condensados são polímeros flavonóides, que contribuem para o sabor amargo e adstringente da fruta e podem interferir com o processo digestivo por reação com certas proteínas ou por inativação de enzimas proteolíticas digestivas (WURSCH *et al.*, 1984).

A polpa de alfarroba contém 40-60% de hidratos de carbono de baixo peso molecular, principalmente a sacarose (CALIXTO; CANELLAS, 1982). Além do mais, os hidratos de carbono e quantidades elevadas de fibra dietética são características presentes nesta polpa. Destas fibras, apenas a insolúvel da polpa da bainha mostra propriedades hipocolesterolêmicas, tanto em animais como em seres humanos. De tal modo, estudos demonstram sua eficácia na redução e manutenção de níveis recomendados de colesterol e triglicerídeos (MARTINEZ-RODRIGUES *et al.*, 2013). Outro estudo demonstrou que o consumo da alfarroba proporciona redução no índice glicêmico e melhora no controle da glicose sanguínea. Além de regularizar o perfil lipídico e as concentrações de lipoproteínas, com apenas a ingestão de tabletes de farinha de alfarroba num determinado período em humanos (SANTOS *et al.*, 2015).

A utilização da farinha de alfarroba como fonte de matéria-prima alternativa e incorporada como um ingrediente em novas formulações de alimentos tem sido aplicada tornando-a um potente alimento funcional. Durazzo e colaboradores (2014) observaram o efeito dos polifenóis totais, presente no extrato aquoso da alfarroba. Estas análises *in vitro*, destacaram que a farinha de alfarroba apresenta propriedades antioxidantes excelentes devido à fibra insolúvel, lignanas e teor elevado de polifenóis totais.

Inserra e colaboradores (2015) investigaram a inclusão de polpa de alfarroba em dieta de porcos, os quais demonstraram que até 15% da polpa de alfarroba pode ser incluída na dieta sem afetar o crescimento da prole. Além de favorecer outros efeitos nutricionais, como o aumento das concentrações de ácidos graxos monoinsaturados e de ácidos graxos poliinsaturados n-3, e principalmente a redução da percentagem dos ácidos graxos saturados. Outro estudo utilizou cordeiros como modelos experimentais, que igualmente utilizou uma dieta contendo a polpa da alfarroba, no entanto, enfatizou o caráter sensorial da carne. O estudo destacou-se pelo aumento das concentrações de ácidos graxos poli-insaturados (PUFA) na carne do animal e a redução dos ácidos graxos saturados, sem comprometer a estabilidade oxidativa da musculatura (GRAVADOR *et al*., 2015).

Em estudo de Castro e colaboradores (2015), também foi observado o beneficio da goma da alfarroba no tratamento para feridas de porcos, mostrando a utilização da goma da alfarroba em prol de modular positivamente os mediadores de citocinas inflamatórias. Apesar do estado das feridas proporcionarem efeitos adversos, pela elevada absorção, capacidade de retenção de líquido, ambiente úmido e com flexibilidade porosa. Mesmo assim houve uma melhora na organização dos tecidos e re-epitelização mais rápida em comparação com feridas de animais considerados controle. Do mesmo modo que em um modelo *in vitro*, na qual a lesão oxidativa, por esforço dos fibroblastos teve efeitos protetores relevantes na redução de espécies reativas de oxigénio (EROS), resgatando a viabilidade celular, e a regulação da expressão de genes relacionados com inflamação (COX-2, TNF-α, IL-1α e IL-1β).

A atividade antioxidante da fração do polifenol bruto derivada da vagem de alfarroba em outro estudo. Evidenciam, respectivamente, efeitos dos polifénois pelos ensaios de proantocianidinas, beta-caroteno, redução dos radicais livres de 1,1-difenil-2-picrilhidrazil (DPPH), inibição da peroxidação lipídica dos eritrócitos e sistemas

microssomais. Sugerindo que a alfarroba poderia ser utilizada como um ingrediente alimentar com caráter de um alimento funcional (KUMAZAWA *et al.*, 2002).

Da mesma maneira Klenow e colaboradores (2009), ao utilizarem um extrato aquoso da alfarroba a fim de avaliar possíveis mecanismos quimio preventivos e como um substituto do cacau. Observaram que o principal componente do extrato era o ácido gálico que por sua vez auxilia na modulação da expressão gênica e protege as células do cólon contra o peróxido de hidrogênio. Em resumo, os estudos com alfarroba demonstraram seu possível potencial farmacológico e uma probabilidade de ser utilizada como substituinte do cacau por apresentar coloração semelhante, pelo sabor naturalmente adocicado e por ser isento de cafeína e teobromina (DAKIA *et al.*, 2007; BENGOECHEA *et al.*, 2008; HAJAJI *et al.*, 2011).

3. Cacau

A Theobroma cacao conhecida como Cacaueiro, árvore de cacau, pertence a família das Malvaceae (Figura 3), nativa das regiões tropicais da América Central e do Sul. O cultivo da árvore ocorre em regiões de clima tropical e úmido, que anualmente produz cerca de 500 gramas a 2kg de sementes (BELITZ; GROSCH, 1999). Historicamente, a mais de 3000 anos atrás o consumo das sementes da *Theobroma cacao* foi introduzido pelas civilizações Maias e Astecas para fins nutricionais e medicinais. Atualmente, o cacau é um dos alimentos mais populares e faz parte da ingestão diária de inúmeras pessoas, e muitas desta também o utilizam pelo seu caráter funcional (DILLINGER *et al.*, 2000).

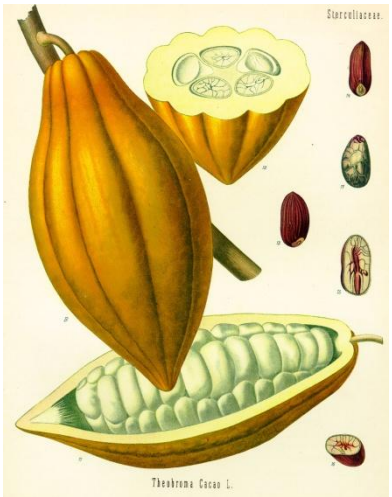

Figura 3: *Theobroma cacao* conhecida como Cacaueiro. Suas sementes e grãos de cacau são utilizados para fazer massa de cacau, cacau em pó e chocolate (fonte: http://dyingforchocolate.blogspot.com.br/2012/03/chocolate-theobroma-cacao-botanical.html).

Basicamente existem três variedades de cacau, o Forastero, o Criollo e o Trinidário (Figura 4). A variedade Forastero, comercialmente é a variedade mais abundante, apresentam cotilédones de cor púrpura, por causa das antocianinas. Já o cacau Criollo, foi classificado como mais suave, pois as antocianinas responsáveis pelo sabor 'forte' e adstringente estão ausentes. Também apresentam cotilédones, de coloração branca e é considerado de uma qualidade superior. A terceira variedade, o Trinitário, é um híbrido entre o Forastero e o Criollo, apresentam cotilédones de colorações variadas entre o branco e a púrpura (HANCOCK, 1994; BART-PLANGE; BARYEH, 2003).

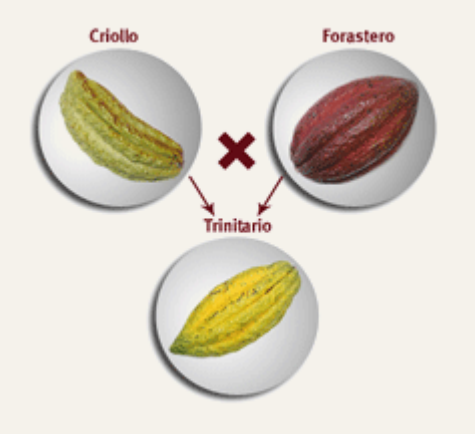

Figura 4: As três variedades do *Theobroma cacoa*, o Forastero, o Criollo e o Trinidário (fonte: http://avabayat.com/Products/CasaLuker/index.html)

Desta forma, o cultivo de cacau orgânico vem se expandindo bastante nos últimos anos. Após a colheita dos frutos, as sementes são removidas mecanicamente, e imediatamente, se inicia o processo de fermentação. Beckett (2000) enumera as etapas de beneficiamento das sementes da seguinte forma: fermentação, secagem, limpeza das sementes, torrefação, trituração. Nessa última etapa, se obtém a massa ou licor de cacau,

constituída com aproximadamente 55% de gordura, esta massa apresenta fluidez sob altas temperaturas.

O fruto do cacau apresenta uma grande variedade de componentes benéficos, porém o método de processamento e sua manipulação alteram suas propriedades e a de muitos compostos bioativos. Particularmente, estas alterações ocorrem durante a torrefação, fermentação e secagem. As condições de tempo e temperatura durante o processo de torra afetam o sabor, as características e perfil de nutrientes do produto final (HURST *et al.*, 2011).

Estudos observacionais e epidemiológicos apontaram para os efeitos benéficos do chocolate amargo, rico em flavonóides, no sistema cardiovascular, atuando na melhora da disfunção endotelial. A disfunção endotelial é um importante marcador do desenvolvimento de aterosclerose e um prognóstico importante de eventos cardiovasculares futuros. Contribuindo favoravelmente, a ingestão do chocolate amargo melhora o perfil lipídico, a resistência à insulina e o combate ao estresse oxidativo (CORTI *et al.*, 2009; BUITRAGO-LOPEZ *et al.*, 2011; HOOPER *et al.*, 2012; SHRIME *et al*., 2011). De modo que seus efeitos benéficos estão atrelados ao alto teor de flavonóides, metabólitos secundários amplamente presente no reino vegetal (CORTI *et al.*, 2009).

4. Caenorhabditis elegans

A partir de 1974, quando Brenner publicou o trabalho intitulado "A genética da *Caenorhabditis elegans*", abordando um novo modelo animal, se ascende um modelo complementar (Figura 5). Neste trabalho, Brenner abordou uma metodologia descrita para o isolamento, a complementação, e mapeamento de vermes mutantes, caracterizando o comportamento e morfologia do *C.elegans*.

A partir daquela época, muitas descobertas incluindo dissecção da morte celular programada, a clonagem sistemática do genoma, o decifrar da sequência inteira de DNA, microRANs, interferência de RNA e a utilização de GFP foram realizadas em *C.elegans* (COULSON *et al.*, 1986; ELLIS *et al.*, 1991; LEE *et al.*, 1993; CHALFIE *et al.*, 1994; CONSORTIUM, 1998; FIRE *et al.*, 1998; REINHART *et al.*, 2000; CRAWFORD, 2001; TISSENBAUM, 2015).

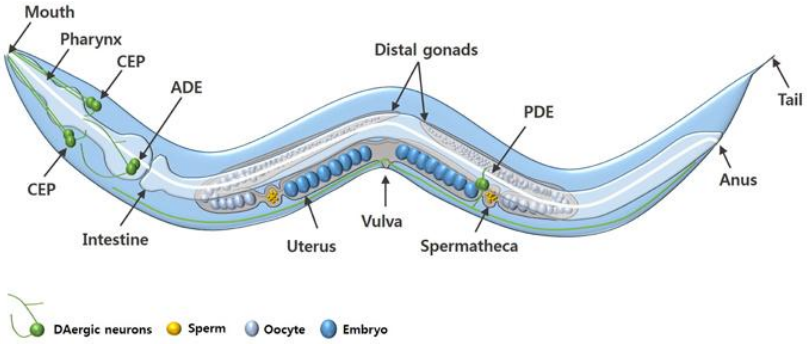

Figura 5: *Caenorhabditis elegans* (fonte: CHEGE; MCCOLL, 2014).

As crescentes descobertas se tornam relevante em função da grande semelhança que existe entre *C.elegans* e mamíferos, ou seja, cerca de 60-80% dos genes são homólogos aos dos mamíferos (KALETTA; HENGARTNER, 2006). Com esta similaridade aos humanos, se abriram inúmeras possibilidades para estudos relacionados a doenças humanas e os mecanismos dessas mesmas. De tal modo que as fáceis disponibilidades de diversos genes mutantes possibilitam elucidar os mecanismos moleculares e genéticos de xenobióticos (OISHI *et al*., 2005; SUE *et al*., 2008).

O desenvolvimento do *C. elegans* passa por quatro estágios larvais (L1, L2, L3, L4), antes de atingir a idade adulta (Figura 6). No primeiro estágio larval L1, se caso não encontrarem nenhum tipo de alimento disponível, os vermes entram num estágio larval denominado “dauer”, uma alternativa eficaz de sobrevivência. Esses dauers podem sobreviver a condições extremas, sendo dessecação ou falta de comida por longos períodos, retornando a normalidade até que as condições melhorem e comida esteja disponível (CASSADA; RUSSELL, 1975).

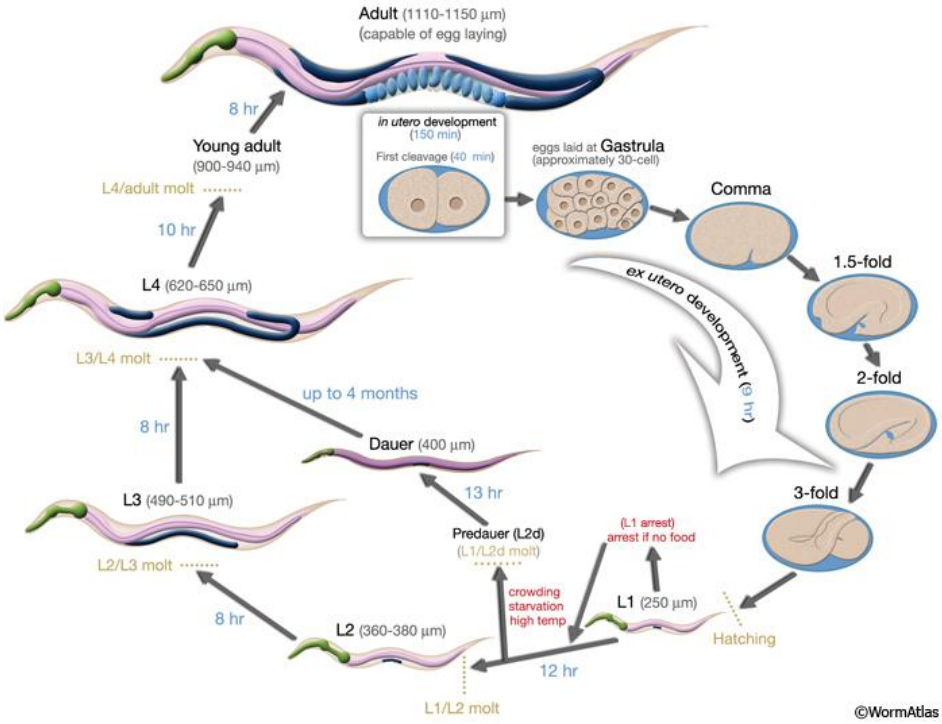

Figura 6: O desenvolvimento do *C.elegans* nos diferentes estágios larvais (fonte: http://wormatlas.org/hermaphrodite/introduction/Introframeset.html).

Com tais características, Spinder e colaboradores (2013) denotam a ideia que os antioxidantes e fitonutrientes são potenciais terapêuticos na longevidade em *C.elegans,* os quais foram testados extratos de mirtilo, romã, chá verde e preto, canela, gergelim e casca de pinheiro marítimo francês (Picnogenol e taxifolina), bem como a curcumina, morina, quercetina. Mostrando que outros fitonutrientes responsáveis por aumentarem a vida útil no *C.elegans*, também estão associados com maior extensão de saúde e expectativa de vida também em humanos.

Além do mais, o estudo de GUARENTE & KENYON (2000) tem mostrado que os fatores genéticos e ambientais específicos influenciam no envelhecimento e período de vida em *C.elegans* Tornando-se um modelo experimental de grande valia em estudos relacionados ao envelhecimento e longevidade, apesar do curto tempo de vida em torno de 3 a 4 semanas, apresentam uma excelente flexibilidade experimental num tempo de geração rápida.

Os *C.elegans* são um dos poucos invertebrados em que a fisiologia da ingestão de alimentos tem sido extensivamente estudada. Estes vermes se alimentam de bactérias

e deglutem nutrientes através de bombeamento da faringe. Logo, os nematoídes famintos bombeam mais rápido quando reexposto ao alimento, em comparação aos nematoídes bem alimentados. Além disso, os *C.elegans* se move ao redor no cultivo bacteriano, mas a velocidade e o padrão de seus movimentos são modulados conforme sua alimentação (HASHMI *et al*., 2013). Neste sentido, estes nematóides são capazes de acumular excesso de alimentos na forma de gordura, apresentando também vias de oxidação desta para produção de energia.

O *C.elegans* fornece uma importante ferramenta de investigação para estudos neuronais, pois este nematoide apresenta 302 neurônios. Esses neurônios compõem o sistema nervoso e seu mecanismo neural é responsável pela sua quimiotaxia, termotaxia e comportamento. Além de circuitos neuronais, existem várias chaves de transmissores químicos, incluindo a serotonina, a acetilcolina, o glutamato e a octopamina em prol de controlar a alimentação dos *C.elegans*. Adicionalmente, existem vários neuropeptídeos, abrangendo os peptídeos semelhantes à insulina, conhecidos por modificar o papel destes transmissores em *C.elegans* (LUEDTKE *et al*., 2010). Também, o sistema nervoso do verme regula o armazenamento de gordura, tanto em conjunto como em vias independente da alimentação e sua rede neuronal, coordenando as ações de transmissão de sinais entre partes diferentes do seu corpo (HASHMI *et al*., 2013).

A flexibilidade genética dos *C.elegans* revelou que os mecanismos de equilíbrio de energia apresentam uma gama de sinalizações endócrinas neuronais para absorção de nutrientes, transporte e mecanismos de armazenamento/utilização. De tal modo, ao se examinar vias reguladoras de gordura com diferentes condições ambientais, pode-se observar o potencial revelando vias fisiológicas coordenadamente moduladas à resposta de perturbação ambiental. Relacionando assim, com os estágios de desenvolvimento, idade, comportamento e ingestão alimentar dos mamíferos (ASHRAFI, 2007).

Por exemplo, em estudos com nematoides tratados com extrato de maçã foram observados o aumento tanto no tempo máximo, como na média de vida, além da redução do acúmulo da lipofuscina, um marcador de envelhecimento nos *C.elegans*. Os nematoides tratados com o extrato de maçã também apresentaram maior resistência ao calor, irradiação UV e ao estresse oxidativo induzido pelo paraquat. Desta forma, possibilitou-se esclarecer os mecanismos que auxiliam no anti-envelhecimento, por meio das funções de defesa celular e do sistema imune, tendo como vantagem os

nutrientes fitoquimicos dos antioxidantes do extrato de maçã no nematoide (VAYNDORF; LEE; LIU, 2013).

Da mesma forma, outro estudo com extrato de blueberry, mostrando a atividade antioxidante *in vitro* e benefícios *in vivo*, em *C.elegans*, demonstrou que o extrato de blueberry age unicamente em aliviar o estresse microbiano, uma hipótese razoável é que seu potencial antioxidante é ativado ao lidar com o estresse oxidativo de baixo nível, como por indução por estresse térmico ou durante o envelhecimento. Possivelmente, os polifenóis do extrato do blueberry protegem as células dos baixos níveis de radicais livres, possibilitando alterar a atividade das vias de sinalização necessárias para uma resposta ao estresse térmico (WILSON *et al.*, 2006).

De outro ponto de vista o armazenamento de gordura é um processo fisiológico complexo em diversos organismos, e tem sido explorado nos *C.elegans* como um modelo complementar para o estudo de acumulação lipídica, a qual seu corpo translúcido possibilita fazer marcações das partículas lipídicas ou dos ácidos graxos (KIM *et al*., 2009). Estas vias metabólicas para a síntese de ácidos graxos são quase idênticas às de outros organismos, e as funções de vários genes que codificam as principais enzimas de regulação. Pelo menos quatro vias centrais de regulação estão envolvidas no controle de armazenamento de gordura nos *C.elegans*, sendo as via de sinalização da insulina e TGF-beta, a via mediada PAS-1/MDT-15, a via mediana nhr-49 e TOR, e a via de hexosamina (BROCK *et al*., 2007).

Deste modo, os neurotransmissores 5-HT, dopamina e glutamato foram encontrados ao participar no controle da acumulação lipídica, as quais envolvem os gene tub-1 e BBS-1, no controle neuronal no armazenamento de gordura, sugerindo que as importantes estruturas neuronais, anfidios sensoriais, regulam a acúmulo dos lipídios, além de detectar melhor os movimentos ao seu redor. Assim, estudos auxiliam na contribuição para entender melhor as doenças associadas ao metabolismo lipídico e seu armazenamento, as quais estão diretamente interligadas à obesidade nos seres humanos (ASHRAFI, 2007).

Os *C.elegans* carecem da molécula de *leptin-like* e seus receptores, que participam da regulação e armazenamento de gordura. Proporcionando informações bioquímicas de armazenamento e utilização de gordura, as quais são independentes da inibição hormonal e da ingestão de alimentos. Tornando-se assim, um modelo bastante

atraente para muitos pesquisadores que utilizam vias anti-obesidade (COHEN; FRIEDMAN, 2004).

Assim, o acúmulo de lipídios envolve processos complexos de biossíntese de lipídios, transporte e armazenamento (BROCK et al., 2007). Essa biossíntese lipídica é controlada, principalmente por regulação de proteínas de ligação aos elementos relacionados aos esteróides (SREBPs). Desta forma, o gene TUB-1, está relacionado no gerenciamento do acúmulo de lipídios em cooperação com os KAT-1, que regem a beta-oxidação de ácidos graxos. Os mutantes *tub-1* mostram um fenótipo de obesidade excessiva, especialmente sob a ausêcia do gene *kat-1*- (TAUBERT *et al*., 2006).

No que diz respeito aos níveis de acil-CoA graxo no importante controle da taxa da beta-oxidação, seus níveis são rigorosamente controlados pela carnitina aciltranferase, que transporta acil-CoA e seus derivados para a mitocôndria, a atividade desta enzima é regulada ou inibida pelo malonil-CoA. Com aumento dos níveis de acetil-CoA e citrato, havendo a diminuição da beta-oxidação, como um *feedback* negativo (WANG *et al.,* 2003; DESVERGNE *et al.,* 2006). Como elucidado num estudo, o qual aborda a taurina como um agente anti-obesidade, mostra nos *C.elegans* tratados um efeito dose dependente na deposição dos lipídicos e redução do armazenamento lipídico nos vermes do tipo selvagem. Com isso, sob certas circunstâncias os *C.elegans* são de grande valia para estudar sistemas relacionados a obesidade e ao acúmulo de gordura (KIM *et al*. 2009).

Justificativa

A *Cerotonia siliqua* conhecida como alfarroba, tem atuado popularmente com um alimento rico em antioxidantes e também como substituinte do *Theobroma cacoa*, o cacau. Porém, existem poucos estudos que afirmam o seu potencial nutracêutico. Desta forma, o presente estudo justifica-se por averiguar os potenciais antioxidantes *in vitro* do extrato aquoso da alfarroba, além da quantidade já descrita nos estudos de compostos fenólicos.

Além do mais, também se justifica ao utilizar o modelo complementar *C.elegans* para a primeira análise *in vivo* sobre a eficácia do extrato aquoso da alfarroba. De tal

forma que possibilitaria transpassar os efeitos do extrato aquoso da alfarroba para o uso em humanos.

Ademais o uso demasiado da alfarroba como substituinte do cacau o torna cada vez mais comum, por isso comparou-se ambos os extratos aquosos, tanto a alfarroba como o cacau na modulação lipídica. Assim, o presente estudo tem como justificativa avaliar o extrato aquoso do fruto de *Ceratonia siliqua* quanto ao potencial antioxidante e à modulação do metabolismo lipídico *in vivo* em *Caenorhabditis elegans*.

Objetivo Geral

Avaliar os efeitos extrato aquoso do fruto de *Ceratonia siliqua* quanto ao potencial antioxidante e à modulação do metabolismo lipídico *in vivo* em *Caenorhabditis elegans*.

Objetivos Específicos

- Determinar *in vitro*, se os extratos aquosos da alfarroba e do cacau, apresentam efeitos antioxidantes;
- Averiguar os possíveis efeitos do extrato aquoso da alfarroba em parâmetros reprodutivos e longevidade;
- Analisar os possíveis efeitos do extrato aquoso da alfarroba frente aos agentes agressores, paraquat e peróxido de oxigênio, e estresse térmico em *Caenorhabditis elegans;*
- Aferir os possíveis efeitos do extrato aquoso da alfarroba em relação á enzimas e atividades antioxidantes em *Caenorhabditis elegans;*
- Avaliar os extratos aquosos da alfarroba e do cacau quanto as suas características bromatológicas, a fim de associar seus efeitos biológicos com as suas composições;
- Averiguar os efeitos protetores contra o estresse oxidativo nos vermes tratados com diferentes concentrações dos extratos aquosos da alfarroba;
- Verificar o metabolismo lipídico dos vermes tratados com o extrato aquoso da alfarroba e compará-la com a do cacau.

Manuscrito 1

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito.

As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio manuscrito, sendo que o mesmo encontra-se em fase final de elaboração para submissão.

Antioxidant activity of aqueous extract of carob (*Ceratonia siliqua* L.) in *C.elegans*

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Abstract

Nowadays, people care about food habits and seek antioxidant foods in an effective way of reducing harm caused by free radicals. In this case the carob (*Ceratonia siliqua* L), becomes important to present in folk medicine for its diuretic action, anti-diarrheal and gastric treatment of lactating babies and their photochemical composition of phenolic compounds such as flavonoids, phenolic acids, anthocyanins, vitamin C, E and carotenoids which provides broad benefits to health. For further preliminary studies of toxicity and efficacy of antioxidants in the aqueous extract of carob levels use the complementary models *Caenorhabditis elegans (C.elgans*). The nematode *C.elegans* is an alternative experimental model providing easy genetic manipulation, marking green fluorescent protein and *in vivo* efficacy analysis of various nutrients. In this study, we ascertain the potential antioxidant of aqueous extract of carob *in vitro* and *in vivo.* First, perform the antioxidants in vitro test, the methods of DPPH and FRAP, was determined after 50% lethal dose (DL50) and its effect on longevity and possible effects egg laying. This was followed by studies on the protective capacity or reversion to aggressive agents, paraquat, hydrogen peroxide and heat stress, showing that the concentrations used the extract not auxiliary in protecting these damages, nor interfering with the amount of reactive oxygen species (ROS) neither the expression of superoxide dismutase (SOD-3). Nevertheless, the increase in catalase activity, suggesting that the aqueous extract of carob may establish equilibrium in concentration gradient and in part by regulation of DAF-16 / FOXO signaling pathway.

Key-words: Carob, *C.elegans*, antioxidants

Introducion

The carob tree (*Ceratonia siliqua L.*) has been widely cultivated in Mediterranean countries for years. The carob fruit, a brown pod of 10–25 cm in length, contains several polyphenols, especially highly condensed tannins (AVALLONE *et al*., 1997).

Carob is a healthy and highly nutritional food, contains vitamin B1 necessary for the proper functioning of the nervous system, muscles and heart. In addition, it contains vitamin A that is essential for the growth of bones and teeth, skin vitality and healthy vision; vitamin B2, responsible for fat, proteins and carbohydrates metabolism, in addition to calcium, magnesium and iron. Likewise, the carob characterized by the presence of phenolic compounds such as catechins, gallic acid and quercetin, due to the presence of carotenoids such as lutein, lycopene, α-carotene and β-carotene (AVALLONE *et al.*, 2002).

Additionally, recent study discovered that carob leaf extract depicted some ameliorative effects against CCl_4-induced oxidative damage in rat's tissue (HSOUNA *et al*., 2011). Remarkably, the carob fruit is rich in important nutrients, however, little is known about whether its use is indeed beneficial to human health (ROUKAS, 1999; BINER *et al*., 2007). Leading into account that oxidative processes, as well as oxidative imbalance, may be linked to both longevity and heathspan we sought to investigate whether carob fruit extract would be capable to improve biochemical parameters by modulating antioxidant status. To investigate these parameters in an efficient mannerist would be required an organism with relatively short lifespan that could be assayed in a reproducible and robust manner, and for which genetic and environmental factors affecting lifespan are well defined (HERNDON *et al*., 2002).

The experimental organisms that best accommodate these requirements is the nematode *Caenorhabditis elegans*, which has become a popular model for studying aging and longevity, due to its short 3- to 5-weeks lifespan, rapid generation time and experimental flexibility (GUARENTE; KENYON, 2000). Studies have shown that specific genetic and environmental factors influence aging and lifespan of *C.elegans* (GEMS; RIDDLE, 2000; GARIGAN *et al*., 2002; HERNDON *et al*., 2002). In addition, aspects of aging are similar between nematodes and mammals, including humans. For instance, caloric restriction, the only known intervention that successfully extends

lifespan in mammals, can prolong significantly *C.elegans* lifespan. Finally, oxidative stress appears to be a major factor limiting lifespan in both *C.elegans* and humans. Notably, it has been shown that dietary antioxidants, as polyphenols, can extend worms lifespan (PENG *et al*., 2014; MINOR *et al*., 2010). These findings show that study of aging in *C.elegans* provide useful stepping-stones for identifying compounds that can prolong lifespan in humans (WILSON *et al*., 2006). Therefore, the present study aims to investigate the actual antioxidant effects of aqueous extract of carob fruit using *C.elegans* as an alternative model.

Materials and Methods

1. Preparation of Carob Extracts and Phenolic Content

The powder carob was acquired from a local supplier, as obtained by the population. 1g of powdered product was added to 10mL of distilled water, and then homogenized for 10 minutes, centrifuged for 2 minutes at 1000rpm, the supernatant removed and additional 10mL of water was added. The procedure was repeated once more, to then finally collect all the excess supernatant, which was submitted to a filtering process in porcelain crucibles powered by a vacuum pump. This method was carried out in order to get an equivalent extraction by population consumption.

Total phenolic content was determined according to the Folin-Ciocalteu colorimetric assay following the method described by Swain and Hillis (1959). An aliquot of diluted sample extract (0.5ml) was added to 0.9 ml of diluted Folin-Ciocalteu reagent and 3.6 ml sodium carbonate solution (75g/L). The test tubes were allowed to stand in the dark at room temperature for 30min. The absorbance at 725nm was read versus the prepared blank with spectrophotometer. Total phenolic content of samples were expressed as milligrams of gallic acid. All samples were analyzed in three replicates.

2. Antioxidant potential of the extracts *in vitro*

2.1 DPPH Radical Scavenging Assay

The capacity to scavengee the DPPH (2,2-diphenyl-1-picrydrazyl) radical was assessed according to Brand-Willians (1995) with some modifications. Carob Extracts

(100µL) were allowed to react with 3.9mL of DPPH solution for 90min in the dark. The blank sample consisted of 0.1mL of methanol added to 3.9mL of DPPH. Then the absorbance was taken at 515nm. The radical scavenging activity was calculated as follows: 1% = $[(Abs_0 – Abs_1)/Abs_0]$ x 100, were Abs0 the absorbance of the blank and Abs1 was the absorbance in the present of the test compound at different concentrations. The IC50 (concentration providing 50% inhibition of DPPH absorbance) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs. the corresponding scavenging effect.

2.2 FRAP assay

The ferric reducing antioxidant power of each ethanolic extract (three different dilution of the sample) was estimated according to the procedure describe by Pulido *et al.* (2000). Briefly, 2.7mL of FRAP reagent, prepared freshly and warmed at 37°C, was mixed with 270µL of Mili-Q water and 90µL of test sample, water, or methanol as appropriate for the reagent blank. The FRAP reagent contained 2.5mL of a 10mM TPTZ solution in 40mM HCL plus 2.5mL of 20mM $FeCl_3.6H_2O$ and 25mL of 0.3M acetate buffer, pH 3.6. The reaction mixture was incubated at 37°C for 30min and the absorption maximum was assessed at 595nm. An intense blue color is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form. Aqueous solutions of known Fe^{2+} concentration in the range of 500-1500µM ($FeSO_4.H_2O$) were used for calibration.

The total antioxidant activity was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1mM $FeSO_4.H_2O$/g of fruit. Total antioxidant activity was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1mM $FeSO_4.H_2O$ solution determined using the corresponding regression equation.

3. *In vivo* assays

3.1 Strains And Exposure Protocol

The following strains were used in this study: Bristol N2 (wildtype; WT); GA800 (ctl-1+ctl-2+ctl-3+myo-2::GFP); TK22 [mev-1(kn1) III]; CF1553, muls84[pAD76(sod-3::GFP)]; TJ356 [pGP30(DAF-16::GFP)+pRF4(rol-6)]; CL2166(gst-4p::GFP::NLS). All *C.elegans* strains were obtained from the Caernorhabditis Genetics Center

(Minesotta, USA) and maintained at 20°C on solid nematode growth medium (NGM) seeded with E.coli (OP50) as a food source (BRENNER, 1974). The synchronization of worm cultures was achieved by hypochlorite treatment of gravid hermaphrodites. Following 14-16hs after synchronization, 2000 first staged larvae (L1) worms were exposed to the polyphenolic extracts at the concentrations of 3.1µg/mL, 31.0µg/mL and 62.0µg/mL GAE for 30 minutes. Then transferred onto NGM plates containing food (*E.coli* OP50). However, the concentrations vary depending on the amount referring to gallic acid found in aqueous extracts of carob.

3.1 Brood Size and Longevity Assays

Worms treated as above described were staged to the L4 stage and then transferred individually to new NGM plate in order to score their egg laying until the last reproductive day. Worms were transferred daily to a new plate until no new eggs were laid. The number of progeny and unhatched eggs were scored under a stereomicroscope 24 h after parents were transferred.

The longevity assay was performed in wild type N2 and *mev-1*mutants. Lifespan was scored every day after hermaphrodites completed the final larval molt, from the first day of adulthood (defined as d= 0) until death. Were observed 20 animals and were considered as dead if they displayed no spontaneous movement or failed to respond when touched. The longevity assay was conducted five times.

3.2. Stress Resistance Assays

To evaluate oxidative stress resistance, N2 wild-type animals were treated with M9 buffer (control) or different concentrations of carob fruit at L1 for 30minutes. Following extract removal, they were exposed to oxidant stressors Paraquat (0.5mM) or H_2O_2 (0.6mM) for 30 minutes, and survival was recorded after 48 hours of exposure to the stressor.

We also conducted another resistance assay using heat as a stressor, at which worms, were treated at L1 larval stage and then incubated at 37°C for 30 minutes. The mortality was recorded after heat exposure at time intervals of 30 minutes, 2 hours, 4 hours, and 15 hours.

3.3 Reactive oxygen species (ROS) determination

ROS was determined as described previously (GONZALEZ-MANZANO *et al.*, 2012) with modifications. The worms were treated in L1 stage with different concentrations of aqueous extract of carob and after the last wash H_2DCFDA (50µM) was added to the samples. Samples were incubated in the dark for 1h, and then washed 3 times with 1mL of M9 (3 minutes at 7000rpm) to remove the dye. To quantify the relative fluorescent intensity, 10.000 worms were transferred to each well of a Costar 96-well black clear bottom plate containing 100mL of M9 and were readied. The excitation/emission wavelengths were respectively 485/535nm (BURNAUGH *et al.*; 2007). The production of ROS was expressed as percentage of control corrected by measurement of protein.

3.5 Measurement of lipid peroxidation

Worms treated at the L1 stage were placed in NGM/OP50 and 48h after they were washed with M9 buffer and then transferred to microtubes. The *Escherichia coli* was washed until exhaustion, the worms were frozen 2x and sonicated 2x by 10s on ice. Was incubated 60µL of sample with 60µL of phosphoric acid (0,1%), 60µL of TBA (0,6%) and 60µL of SDS (0,6%) for 1.5h at 100ºC as described by Okawa (1979). Absorbance measurements were performed in 96-well plate (532nm).

3.6 Enzymatic Activities

For the quantification of the enzymatic activities of catalase, were treatments as L1 worms described above. After the end of treatment the worms were frozen sonicated and centrifuged (4°C to 12000rpm for 0.5 minutes), using the supernatant, were used for the assay in different amounts 15µL, 30µL and 45µL. Reacts catalase to hydrogen peroxide releasing peroxide to react with potassium phosphate buffer (TFK) in 50mM pH 7.0 and the sample, and read at 240nm as kinetic analysis for 2 minutes in every 15 seconds at 37°C, using 10µL of sample.

3.7 Epifluorescence microscopy

For each slide, at least 30 worms were mounted on 2% agarose pads in M9 andanaesthetized with 0.02% tetramisole in aqusoso. Image acquisitions and scoring

were carried out with an epifluorescence microscope housed in airconditioned room (20-22°C).

3.8 Protein determination

Determination of the total protein content in the samples was carried out according to Bradforf (1974).

4. Statistical analysis

The results were presented as mean ± standard error. Comparison was made using Anova followed by Tukey using GraphPad Prism version 6 (US Inc.) with model of program. The results were considered as statistically significant when p-value was less than 0.05 ($p < 0.05$).

Results and Discussion

The increased concerns with healthier eating habits make antioxidants a strong ally for health. In this context, several studies have shown that daily consumption of foods rich in antioxidants can act as protective agents against oxidative processes, highlighting the *Ceratonia siliqua* L. as an important ally in the fight against free radicals. Carob, as it has known in folk medicine has diuretic action, anti-diarrheal and gastric treatment of lactating babies (HABER, 2002). Also stands as one of the new foods that provide many health benefits for its photochemical composition of phenolic compounds such as flavonoids, phenolic acids, anthocyanins, vitamins C, E and carotenoids (AYAZ *et al.*, 2007).

Thus, this study examined in the phytochemical that the antioxidant properties in vitro, both in the elimination of DPPH assay and FRAP. In table 1 shows the antioxidant capacity that the aqueous extract of carob gives a fair amount of the reduction of DPPH scavenging radical by getting the EC50 2.58mg/ml. In addition to highlighting the carob is known by the quantities of phenolic compounds which have obtained 516.96µg GAE/ml extract.

Kim and collaborators (2015) also conducted an in vitro study in order to determine the antioxidant capacity of various extracts of A. koreanum senticosus by

testing 2,2-diphenyl-1-picrylhydrazyl (DPPH), [2,2'- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] (ABTS), by reduction of ferric antioxidant power (FRAP) and reducing the energy capacity and oxygen radical absorbance (ORAC) assay. Showing that the results of this study were founds with different antioxidant capacity of different reaction mechanisms. While the reduction of Fe^{3+} to Fe^{2+} 8.59mg/ml ferrous sulphate per gram fruit. The ability of the solution tested to deviate from the mechanism of the Fenton reaction by chelating metal ions (PSOTOVA et al., 2003). Such as Fe^{2+} and Cu^{2+}, which is responsible for converting the hydrogen peroxide to the hydroxyl radical (ROS) in the skin, can be measured using the FRAP assay, in extract showed a good amount of Fe capture (LIN *et al*., 2008).

In addition, study relate the amount of phenolic compounds and carob, this study demonstrates that even in aqueous solution exist a large amount of phenolic compounds and in this case, gallic acid (Table 1), and thus extracts were classified according with gallic acid concentrations captured as each extraction. In addition to suggesting, that the polyphenol present in the carob pods may have antioxidant properties *in vivo*. Thus, the antioxidant activity of crude polyphenols derived from locust especially strong effect was evident by against discoloration beta-carotene (KUMAZAWA *et al*.; 2002).

In another study, show that carob fiber not only has a high content of phenolic antioxidants, but according to Owen (2003) also contains a rich variety of individual components from several classes: phenol, polyphenols free flavones and flavonols, glycosylated flavonols, isoflavones, flavonols and gallotannins. Alltogether, 24 major individual structures were identified and quantified, with the amounts ranging from very low isoflavone genistein, to very high (gallic acid, $1.65 g.kg^{-1}$).

As much as the extracts showed a good antioxidant activity in vitro, was investigate whether the extract aqueous carob had activity in vivo, using *C.elegans* an experimental model. So that the preliminary study of toxicology the DL50 mortality showed up to a dose 613.84µL (fig.1A). Although the aqueous extract of carob showed a significant reduction in survival in the concentration 62.0ug/ml (Fig.1B) shows no reduction in the egg laying at this concentration, but the intermediate concentration 31.0ug/ml (fig.1C). But there was no change in the longevity of these nematodes (fig.1D). As can be seen in the study of Guha (2013) that despite reduce egg laying

there was an increase in the longevity in worms treated with aqueous extract of Cranberry.

As the extract does not present toxicological activity and has antioxidant activity *in vitro*, was investigate other criteria of activity in vivo, such as resistance to some external agent. So that were checked resistance to peroxide oxygen (fig.2A) did not improve, then when was change the offending agent, using the paraquat, was can see that there is a significant increase in all concentrations in survival (fig.2B). However, when the stress test performed to heat stress there was no change (fig.2C). Others studies discuss effects relating to thermal stress in which recently mammals have demonstrated that the cellular stress proteins are involved in the heat shock response and consequently the response of endoplasmic reticulum stress contributes to the regulation of immunity (MURALIDHARAN; MANDREKAR, 2013). Thus, some studies address that antioxidant activity of fruit extracts and aggressive agents such as paraquat and hydrogen peroxide act not always favorable longevity but in survival of nematodes (TRUONG et al., 2015; KUMSTA et al., 2011).

Unfortunately in this study showed that not all concentrations of aqueous extract of carob reduced the level of intracellular ROS in *C.elegans* (fig.3A). On the other hand, just as this shows that, the highest concentrations reduced lipid peroxidation levels significantly by the TBARS method (fig.3B). Thus suggest that there are significant increases in glutathione-s transferase-4 in worms treated with different concentrations of aqueous extract of carob, confirming the absence of toxicity (fig.3C). To correlate the *in vivo* antioxidant effect of extract aqueous carob treatment with a molecular response in *C.elegans*, which the glutathione-s transferase-4 is expressed primarily in the muscles and the hypodermis under normal conditions, and is, increased in response to a variety of oxidative stress treatments (HASEGAWA, 2010; HASEGAWA *et al*., 2008).

There was the tests reactive oxygen species (ROS) were measured with the aid of 2'-7'diclorodihidrofluoesceína probe diacetate (DCF-DA), which is permeable to the cell membrane and not fluorescent. Still elimination activity of free radicals in vivo intracellular C.elegans extract was measured by a non-fluorescent dye H2DCF-DA. This non-fluorescent dye interaction with intracellular ROS after converted into a fluorescent compound 2'7'-dichlorofluorescein (ROYALL, 1993).

According to Armstrong (1994) testing of 2-thiobarbituric acid (TBARS substances) are naturally biological species present, including lipid hydroperoxides and aldehydes in order to increase the concentration in response to oxidative stress. TBARS assays measuring the malondialdehyde (MDA) present in the sample, as well as malondialdehyde generated from lipid hydroperoxides by hydrolytic reaction conditions (PRYOR, 1991). In studies with aqueous extract of açai, both in humans and C.elegans demonstrated reducing effects in lipid peroxidation, in fruit antioxidant capacity (BONOMO et al., 2014; JENSEN et al., 2008).

Well as the aqueous extract of carob not helped in the analysis against stressor and thermal agent, could be involved with the electron transport chain in the mitochondrial. This case, Mev-1, is required for oxidative phosphorylation, and most mutations result in abnormal energy metabolism and increased sensitivity to oxidative stress and pathogen infection. Several study relate to mev-1 is a subunit of complex II in the electron transport chain and the mev-1 deletion mutant is hypersensitive to oxidative stress and displays a reduced lifespan, mostly due to mitochondrial ROS overproduction (ISHII et al., 1998). Things to be not seen to analyze the mev-1 observed that there was no forthcoming changes to the N2 control (fig.4A). Similarly can observe that haven't modification of Daf-16, because no change level cytoplasm to core (fig.4B).

On the other hand, will not be interconnected the Daf-16, because it is encodes the sole *C.elegans* forehead box O (FOXO) homologue. The daf-16 functions as a transcription factor that acts in the insulin/IGF-1-mediated signaling (IIS) pathway that regulates *dauer* formation, longevity, fat metabolism, stress response, and innate immunity. In addition, there is a significant correlation between longevity and tolerance to forced stress (KENYON, 2010).

The results showed da SOD did not have any change in the levels of green fluorescent protein (fig.5A). But when it was verified the protein levels of catalase apparently there was no modification, but there was a significant increase in the activity of catalase in concentrations 3.1 and 31.0µg/ml (fig.5C). Superoxide dismutase is an enzyme and most effective intracellular antioxidants present in all aerobic organisms and subcellular compartments prone to oxidative explosion due to an abiotic or biotic stress. Remember that SOD is a major enzyme that protects against oxidative stress by catalyzing the removal of O_2, which other studies have reported increased expression

sod-3 and enzyme activity were increased after juglone exposure related to other genes linked to DAF-16 (SHI *et al*., 2012).

Insomuch as exposing plants to biotic and abiotic stresses can increase the production of reactive oxygen species, such as $^1O_2$, $O_2^{\cdot-}$ and H_2O_2 and OH^-. For your own protection against these toxic intermediates of oxygen, the cells of plants and their organelles employ an antioxidant defense system (DAVAR *et al*., 2013). Corroborating this enzyme provides the first line of defense against the toxic effects of high levels of reactive oxygen species (GILL; TUTEJA, 2010). Already catalase the second Kampkotter and collaborators (2007) describe with one transcription was reduced in response to EGb761 treatment. The detoxing the reactive oxygen species H2O2 by catalase and it acts as an antioxidant enzyme (MATES, 2000) in *C.elegans* as well as in mammals (ROHRDANZ et al., 2001; ROHRDANZ; KAHL, 1998).

In conclusion, the aqueous extract of carob presented antioxidants significant property in vitro, compared to DPPH and FRAP methods. On the other hand, when using the *C.elegans* model data showed reduced brood size and there was nor increase in longevity perce neither in mutant compared to the control. In this way, it points out that the aqueous extract of carob does not act on the road to longevity at the concentrations tested. However, there is a significant increase in the activity of catalase, probably for the Fenton reaction, but of that, confirmation is necessary more tests. Considering that SOD and GST-4 are target genes of DAF-16, and that DAF-16 also upregulates lipases as LIPL-4, we will further investigate the role of these genes in worms exposed to *C.siliqua* extract.

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Table Legends

Table 1: Activity antioxidants in vitro by DPPH radical scavenging activity, FRAP activity and Phenolic Compounds.

DETERMINATIONS	CAROB
Antioxidant activity by DPPH radical scavenging **EC 50 (mg/ml)**	2.58
FRAP **µmol de ferrous sulphate/g fruits**	8.59 ± 1.19*
Total phenolic content **µg GAE*/ ml extract**	516.96 ± 76.63*

*GAE = gallic acid equivalents

*Medium standard deviation (SD)

Figure Legends

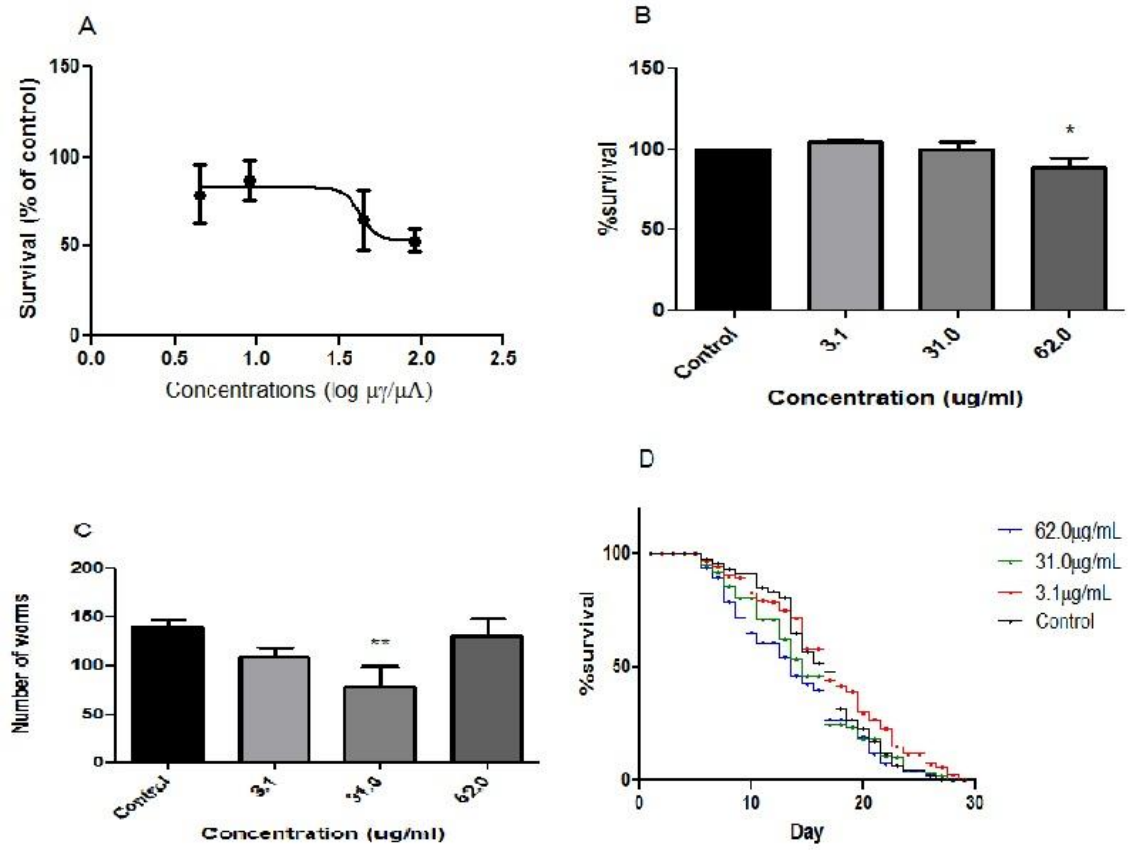

Figure 1 (A) DL50 of worms treated with different concentrations aqueous carob extract (B) Survival of worms treated with different concentrations aqueous carob extract (C) Brood Size of worms treated with different concentrations aqueous carob extract (D) Lifespan of worms treated with different concentrations aqueous carob extract. Data are expressed as mean ± SEM. * indicates $p \leq 0.05$ as compared to control with different concentrations of aqueous carob extract (n=5).

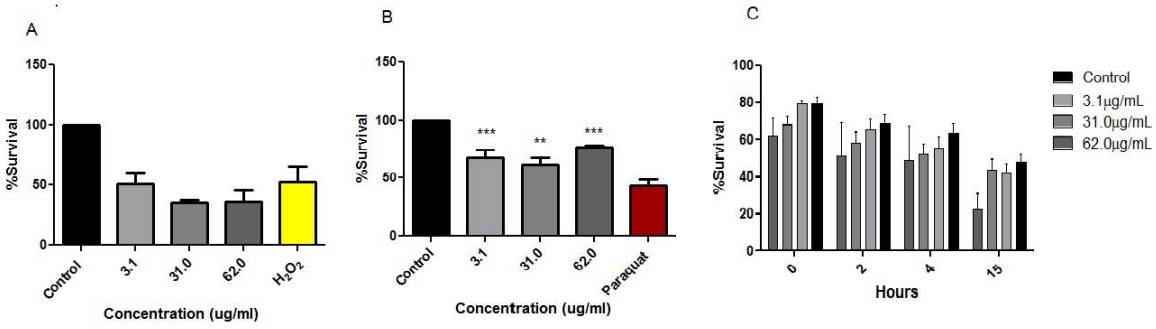

Figure 2 (A) Survival of worms treated with different concentrations aqueous carob extract and post treatment with peroxide oxygen (0.6mM) by 30 min. (B) Survival of worms treated with different concentrations aqueous carob extract and post treatment with paraquat (0.5mM) by 30 min. (C) Survival of worms treated with different concentrations aqueous carob extract and post incubated at 37°C for 30 minutes. The mortality was recorded after heat exposure at time intervals of 30 minutes, 2 hours, 4 hours, and 15 hours. Data are expressed as mean ± SEM. * indicates $p \leq 0.05$ as compared to control with different concentrations of aqueous carob extract (n=5).

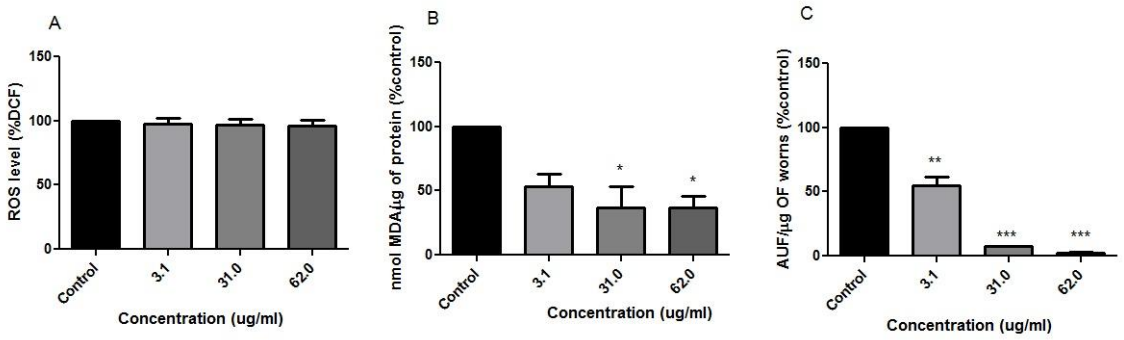

Figure 3 (A) Activity worms treated with different concentrations aqueous carob extract by ROS were measured by DCF-DA.DCF (50µM). (B) Lipid peroxidation assessed by TBARS in L1 worms. (C) Measurement of GST-4::GFP of worms treated with different concentrations of aqueous carob extract.

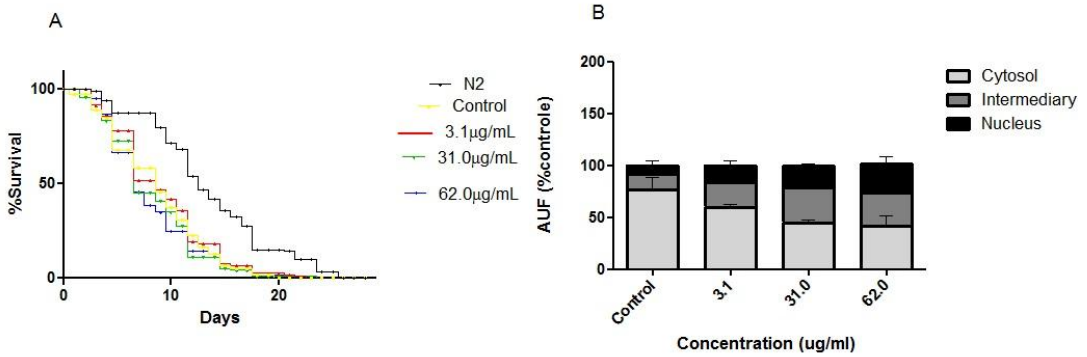

Figure 4: (D) Lifespan the MEV-1 of worms treated with different concentrations aqueous carob extract. (E) Measurement the migration of DAF-16 of worms treated with different concentrations aqueous carob extract. Data are expressed as mean ± SEM. * indicates p≤ 0.05 as compared to control with different concentrations of aqueous carob extract (n=5).

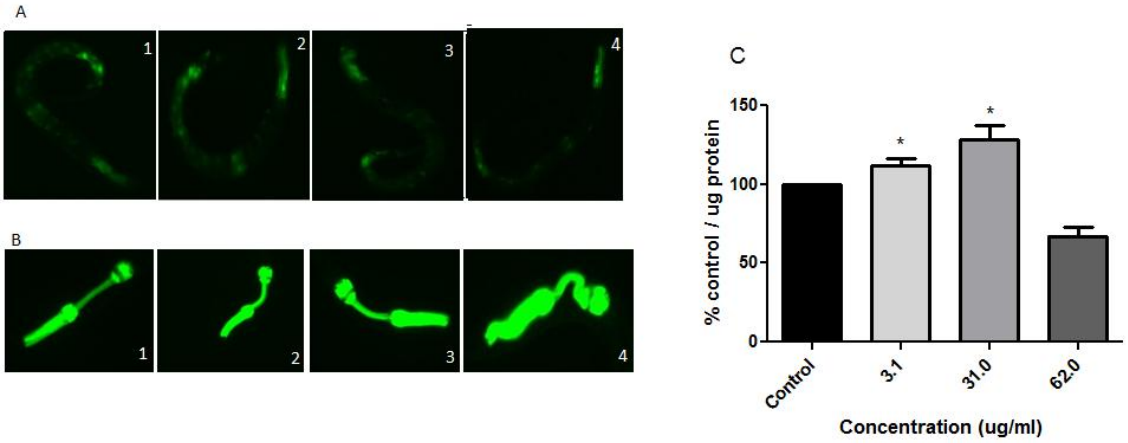

Figure 5: (A) Pictures of SOD-3::GFP strains of worms treated with different concentrations of aqueous carob extract. (1) Control, (2) 3.11µg/ml, (3) 31.0µg/ml and (4) 62.0 µg/ml. (B) Pictures of ctl-1,2,3::GFP worms treated with different concentrations of aqueous carob extract. (1) Control, (2) 3.11µg/ml, (3) 31.0µg/ml and (4) 62.0 µg/ml. (C) Catalase Enzymatic Activity. Data are expressed as mean ± SEM. * indicates p≤ 0.05 as compared to control with different concentrations of aqueous carob extract (n=5).

Manuscrito 2

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico.

As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio manuscrito, sendo o mesmo encontra-se em fase final de elaboração para submissão.

The reduction of lipid levels following treatments with aqueous extracts of carob and cacao using *C.elegans* as an experimental model

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Abstract

Increasingly people suffering from problems related to accumulation of lipids, and related diseases, searching for food in a strong ally to combat this disease. For many years the Cacao (*Theobroma cacao)*, became a strong ally in reducing problems in the cardiovascular system due to deposit fat. Thus, currently exists in the market a new food that is substituent cacao in this regard, besides presenting free lactose and caffeine, called carob (*Ceratonia siliqua)*, with characteristics still not as elucidated. So we will use the *C.elegans* model for being a model of easy manipulation, genetic mapping already pre described and its translucent enables fat markers. Thus, to determine whether there is a reduction in the aqueous lipid extracts and possibly the replacement of Cacao. First conducted in the bromatological trials and after we analyze the lipid levels of testing, both wild animals and mutants in order to ascertain if there were no problems in the intake observe the pharyngeal pumping. We therefore advise that aqueous extract carob makes an excellent reduction triglycerides due to its greater amount of fiber. But for such a statement will need further testing in order to consolidate such a result.

Keywords: *Ceratonia siliqua, Theobroma cacao, C.elegans***,** Lipid Levels

Introduction

Functional foods are defined as any substance or component of a food that provides health benefits, including the prevention and treatment of diseases. These products can range from isolated nutrients, biotechnological products, dietary supplements, genetically engineered foods to processed foods and derived from plants. Some parameters must be taken into account in relation to functional foods. Some studies suggest that they may exert a metabolic or physiological effect that contributes to reduce the risk of chronic diseases. Accordingly, they should be part of the usual diet and provide positive effects obtained with non-toxic amounts (GERMAN; DILLARD, 2000).

Carob is also part of this select group of functional foods. For this reason carob's extract has been studied for its putative beneficial effects on health such as cholesterol-lowering activity in humans suffering from hypercholesterolemia (ZUNFT *et al*., 2001) and also by its antioxidant properties in different *in vitro* and *in vivo* systems (HABER, 2002; KUMAZAWA *et al*., 2002)).

Carob powder is a natural sweetener that presents flavor and appearance similar to chocolate, being used as a chocolate or cocoa substitute. The advantage of using carob as a chocolate substitute resides in the fact that carob is an ingredient free from caffeine and theobromine. The kibble or locust bean is used directly after sun-drying and grinding to a fine powder in by the local population, and such product is called "household flour". Alternatively, carob kibble is industrially processed and sold in large stores and local markets (AYAZ *et al*., 2007).

On the other hand Cocoa polyphenols, particularly flavonols, delay the oxidation of LDLs *in vitro*, which is illustrated by the decrease in plasma concentration of F2-isoprostanes markers of lipid peroxidation (LOKE *et al*., 2008). The effects of cocoa on plasma lipids seem weak and studies on this point are sometimes discordant. It was reported a minor downward trend of LDL cholestérol (-5%) and an almost neutral effect on HDL cholesterol (JIA *et al*., 2010). Polyphenols exert an inhibitory effect on the intestinal absorption of cholesterol. Furthermore, LDL oxidation is reduced under the effect of flavonols (KOND *et al*., 1996).

Caenorhabditis elegans (*C.elegans*) is a small nematode that conserves 65% of the genes associated with human disease, has a 21-day lifespan, reproductive cycles of 3 days, large brood sizes, and lives in an agar dish and does not require committee approvals for experimentation. Studies using the *C.elegans* model have explored insulin signaling, response to dietary glucose, the influence of serotonin on obesity, satiety, feeding and hypoxia-associated illnesses. *C.elegans* has also been used as a model to evaluate potential obesity therapeutics, explore the mechanisms behind single gene mutations related to obesity and to define the mechanistic details of fat metabolism (ZHENG; GREENWAY, 2012).

Moreover, whereas mammals have adipocytes, *C.elegans* store fat in droplets in their intestinal cells and in their hypodermal cells. Because of their transparent bodies, these fat stores can be directly visualized in intact animals. Similarly to mammals when aging process is delayed, worms depict a modified lipid metabolism. Increased fat accumulation and altered metabolism are hallmarks of the long-lived and stress resistant dauers (BUNRELL *et al*., 2005; GEMS, 1999; LARSEN *et al*., 1995).

On the other hand, mutations in rodent tubby cause progressive degeneration in retinal and cochlear sensory receptor cells, infertility and adult-onset obesity with insulin resistance (CARROLL *et al*., 2004). Tubby is broadly expressed in mammals's central nervous system, mainly in the hypothalamus. It is suggested a specific role for vesicular transport in accumulation of excessive in tub-1 deficient worms. Moreover, tub-1 mutant animals have extended lifespan (MUKHOPADHAYAY *et al*., 2005). These adjustments favor energy conservation, fat storage, and utilization of stored reservoirs. Remarkably, *C.elegans* has homology concerning tubby protein, being successfully used as a model for obesity (HASHMI *et al*., 2013). Moreover mammals share genetic similarity in relation to the lipid metabolism, as the worms perform beta-oxidation and lipid biosynthesis too, accumulating lipids in droplets present under the cuticle and around the intestine (ASHRAFI, 2007). Thus, the present study aims to investigate whether the aqueous extract of carob have potential lowering of lipids using the *C.elegans* model.

Materials and Methods

1. Preparation of Carob and Cocoa extracts and Phenolic Content

Carob and cocoa in powder were acquired from a local supplier, as obtained by the population. 1g of powdered product was added to 10mL of distilled water, and then homogenized for 10 minutes, centrifuged for 2 minutes at 1000rpm, the supernatant removed and additional 10mL of water was added. The procedure was repeated once more, to then finally collect all the excess supernatant, which was submitted to a filtering process the filtering by a vacuum pump. This method was carried out in order to get an equivalent extraction by population consumption. Moreover the amount of cocoa extract was duplicated by using its concentration being 50% over the carob is 100%.

Total phenolic content was determined according to the Folin-Ciocalteu colorimetric assay following the method described by Swain and Hillis (1959). An aliquot of diluted sample extract (0.5ml) was added to 0.9 ml of diluted Folin-Ciocalteu reagent and 3.6 ml sodium carbonate solution (75g/L). The test tubes were allowed to stand in the dark at room temperature for 30min. The absorbance at 725nm was read versus the prepared blank with spectrophotometer. Total phenolic content of samples were expressed as milligrams of gallic acid. All samples were analyzed in three replicates.

2. Bromatological Analysis

This analysis was undertaken because we were aware of the considerable differences between the methods in which carob flour is prepared commercially around the world. Moisture of the samples was determined gravimetrically, according to the AOAC method (1990) for analysis of cocoa beans and products, using an oven at 105.0°C and vacuum desiccators to cool. Drying was effected to constant weight. In level of lipids; occur the quantification was carried out by Soxhlet extraction method according AOAC method (1990). The protein nitrogen and total nitrogen content of quantification was performed using a Micro-Kjeldahl method AOAC (1990) modified protein wherein the percentage of the samples was found by multiplying the percentage of nitrogen by the conversion factor 6.25. And the ash determination was carried out according to methodology of the Adolfo Lutz (1976).

3. Strains And Exposure Protocol

The following strains were used in this study: Bristol N2 (wildtype; WT); RB1600 [tub-1(ok1972)II]. All *C.elegans* strains were obtained from the Caernorhabditis Genetics Center (Minesotta, USA) and maintained at 20°C on solid nematode growth medium (NGM) seeded with E.coli (OP50) as a food source (Brenner, 1974). The synchronization of worm cultures was achieved by hypochlorite treatment of gravid hermaphrodites. Following 14-16hs after synchronization, 5000 first staged larvae (L1) worms were exposed to the polyphenolic extracts at the concentrations of 3.1μg/mL, 31.0μg/mL and 62.0μg/mL GAE for 30 minutes by both extracts. Then transferred onto NGM plates containing food (*E.coli* OP50) and incubated until the worms reach the larval stage L4 where the experiments were conducted.

4. Lipid Levels

In order to analyze the lipid accumulation in treated worms, we measured triglycerides and stained the lipids with AdipoRed Kit. For this porpoise, treated worms were aged to L4 stage, transferred to microtubes and Nile Red dye was added to the samples, which were incubated for 1 hour at room temperature and then washed to remove excessive dye.

To measure triglycerides, worms were frozen, sonicated and then pipetted on microplates (50ul of each sample). 150uL of the reagent, kit Labtest was added and the mixture was incubated for 10 minutes at 37°C. Absorbance was measured at 500nm in a microplate reader.

6. Pharyngeal pumping

In order to evaluate whether the effects of the carob extract were due to reduced food consumption, five L4 worms were transferred to a new NGM plate seeded with bacteria OP50 and then the pumping movement of the pharynx were scored for 1 minute. The assay was performed at least three times.

7. Protein determination

Determination of the total protein content in the samples was carried out according to Bradford (1974). All assays were repeated five times.

8. Statistical analysis

The results were presented as mean ± standard deviation determined at least in triplicates of two independent samples. Comparison was made using two samples T-test and Anova followed by Tukey using GraphPad Prism version 6 (US Inc.) with model of program. The results were considered as statistically significant when p-value was less than 0.05 ($p < 0.05$).

Results and Discussion

A nutraceutical is defined as any substance that is a food or a part of food that provides medical or health benefits, for the prevention and treatment of diseases (DE FELICE, 1995). Nutraceuticals include a broad range of categories such as dietary supplements, functional foods and herbal products (RADHIKA; SINGH; SIVAKUMAR, 2011). The active compounds or phytochemicals in plants, especially fruits, have been associated with numerous health benefits and are used as ingredients in many nutraceutical and pharmaceutical products (LACHANCE; DAS, 2007). In the present study, using this experimental design, we observed that the aqueous extract of carob depicted best effects than cacao, as it presented better reducing triglycerides levels activity in both the wild type as the *tub-1* mutant. The high amount of fibers presented in the extract may be contributing to this effect.

In this way, the bromatological analysis is important to verify the quality of the foods from the production to sale as natural or processed product. It is through it that is verified whether the food fits the legal specifications, detects the presence of adulterants, additives that are harmful to health if sterilization is adequate, whether there was contamination with type and size of packaging, labels, designs and types of letters and inks used. Lately, it has been compared carob as a substituent cacao, but its characteristics have still not been well elucidated. In order to provide more trustworthy

data we first analyzed the bromatological parameters by comparing both with their amounts of ashes, minerals, total fiber and total lipids. In table 1 we can determine that the aqueous extract of cocoa has a higher amount of fiber but also has larger amount of fat compared to the aqueous extract of carob.

The results of this study show that carob extract has a high content of phenolic antioxidants, when compared to cacao extract (table2). A previous study of Owen (2003) Alltogether, major individual structures were identified and quantified, with the amounts ranging from very low isoflavone genistein, to very high (gallic acid, 1.65g.kg^{-1}). Comparisons with other studies on the content of phenolic compounds in carob and cocoa have been conducted on the cocoa pod from which the fiber is obtained. Most previous studies have concentrated on the tannin content of carob pods with little in the way of definitive structure elucidation. In this way the current data can be compared to two recent publications on the content of phenolic compounds in carob and cocoa pods using *C.elegans* whith a complete model.

During the last years, *C.elegans* has been emerged as an important model to study the regulation of energy metabolism and lipid storage. As a great advantage, it enables the examination of the relationship of lipid metabolism, growth, reproduction and lifespan. Many of mammalian metabolic pathways, such as fatty acid synthesis, elongation and desaturation, mitochondrial and peroxisomal ß-oxidation of fatty acids are conserved in the nematode. A number of genes involved in pathways that regulate lipid homeostasis in mammals are assumed to control lipid storage as well in *C.elegans* (PALGUNOW *et al*., 2012).

Building on this, we tested whether both extracts would change total lipids levels. We observed that when the worms were treated with the aqueous carob or cocoa extract they had no reductions in fat droplet (fig.1A and fig.1B). In addition, the carob extract reduced the triglycerides levels (fig.1D) whereas cocoa extract did not change this parameter (fig.1E) Yet when comparing the extracts including only 31.0ug/ml concentration of the aqueous extract of carob was lower compared to the aqueous cocoa extract (fig.1C), but not different to control. Some authors relate the importance of reducing triglycerides because they are associated with much pathology independently such as cardiovascular and acute pancreatitis. The study demonstrated that the aqueous

extract of carob caused a significant decrease of triglycerides levels when compared to aqueous extract of cocoa without changing the pumping pharyngeal (fig.1E).

The regulation of energy homeostasis integrates diverse biological processes ranging from behavior to metabolism and is linked fundamentally to numerous disease states. To identify new molecules that can bypass homeostatic compensatory mechanisms of energy balance in intact animals, its selects some kind of small-molecule modulators of *C.elegans* fat content. One example is the *tub-1*, which encodes a TUBBY homolog that affects fat storage across species including *C.elegans*, and then causing higher lipid accumulation in these worms (LEMIEUX *et al*., 2011).

We have observed that neither extracts caused reduction in lipid levels in *tub-1* worms (fig.2A and fig.2B). However, when we determined the triglycerides assay both extracts caused significant reduction in their levels (fig.2C and fig.2D). In addition, there were no significant differences on the pumping of the pharynx following treatment with each extract (fig.2E and fig.2F). This indicates that the reduction in triglycerides levels was not due to dietary restriction. Complex cellular mechanisms coordinate the equilibrium between lipid utilization and accumulation (WANG *et al*., 2008; LARSEN *et al.,* 2006). Tub-1 is a gene required for beta-oxidation, which is important in the mobilization of triglycerides and the conversion of fatty acids to acetyl-coA (RESH *et al.,* 2006; WARE *et al.,* 1975). Our results suggest that the carob extract may act modulating lipases in order to reduce the lipid accumulation induced by *tub-1* mutation.

The main finding of this study is that extract aqueous cocoa had only anti-obesity effect the tub-1 mutant, unlike on the extract aqueous carob that presenting reduction in both strains. Unlike the wild type, the tubby mutants are known to store more fat due to their inability to control excessive transport to fat storage tissues or to react properly to metabolic signals from ciliated neurons (LEWIS; HODGKIN, 1977; DOUGLAS *et al.,* 2005). Therefore, we suggest that the aqueous extract of carob has a better lipid profile when compared to reducing aqueous extract of cacao when using the genetic models of obesity. Unfortunately its regulation of fat metabolism is not pathway nhr-49 and consequently not activated via by the peroxisome proliferators (PPARs).

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Tables:

Table 1: Chemical analysis of aqueous extracts of carob and cacao

	%Dry Matter	%Ash	%Crude Protein	%EE
Carob	81.83 ± 1.26*	4,01 ± 0,64*	12.81 ± 0.87*	0.75 ± 0.07*
Cocoa	95.00 ± 0.31*	4,23 ± 0,04*	6.02 ± 0.76*	7.13 ± 0.21*

EE = ether extract

*Medium standard deviation (SD)

Table 2: Characterization of aqueous extracts of carob and cacao of total phenolic

Determinations	**Carob**	**Cocoa**
Total phenolic content		
µg GAE*/ ml extract	516.96 ± 76.63*	317.27 ± 97.64*

*GAE = gallic acid equivalents

*Medium standard deviation (SD)

Figures:

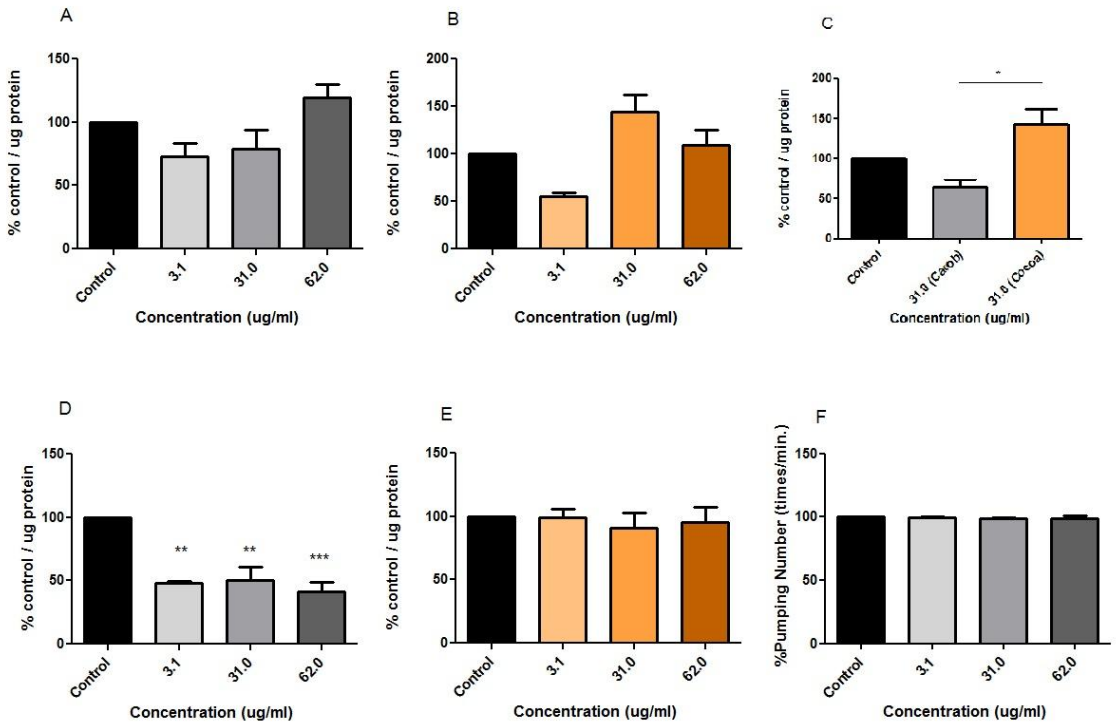

Figure 1 (A) AdipoRed assay of wild type worms treated with different concentrations of aqueous carob extract and aqueous cocoa extract (B). (C) Comparison of extracts carob and cocoa. (D) Analysis of triglycerides levels of wild type worms treated with different concentrations of aqueous carob extract. (E) Analysis of triglycerides levels of wild type worms treated with different concentrations of aqueous cocoa extract. (F) Analysis of pumping number of wild type worms treated with different concentrations of aqueous carob extract. Data are expressed as mean ± SEM. * indicates $p \leq 0.05$ as compared to control with different concentrations of aqueous carob extract (n=5).

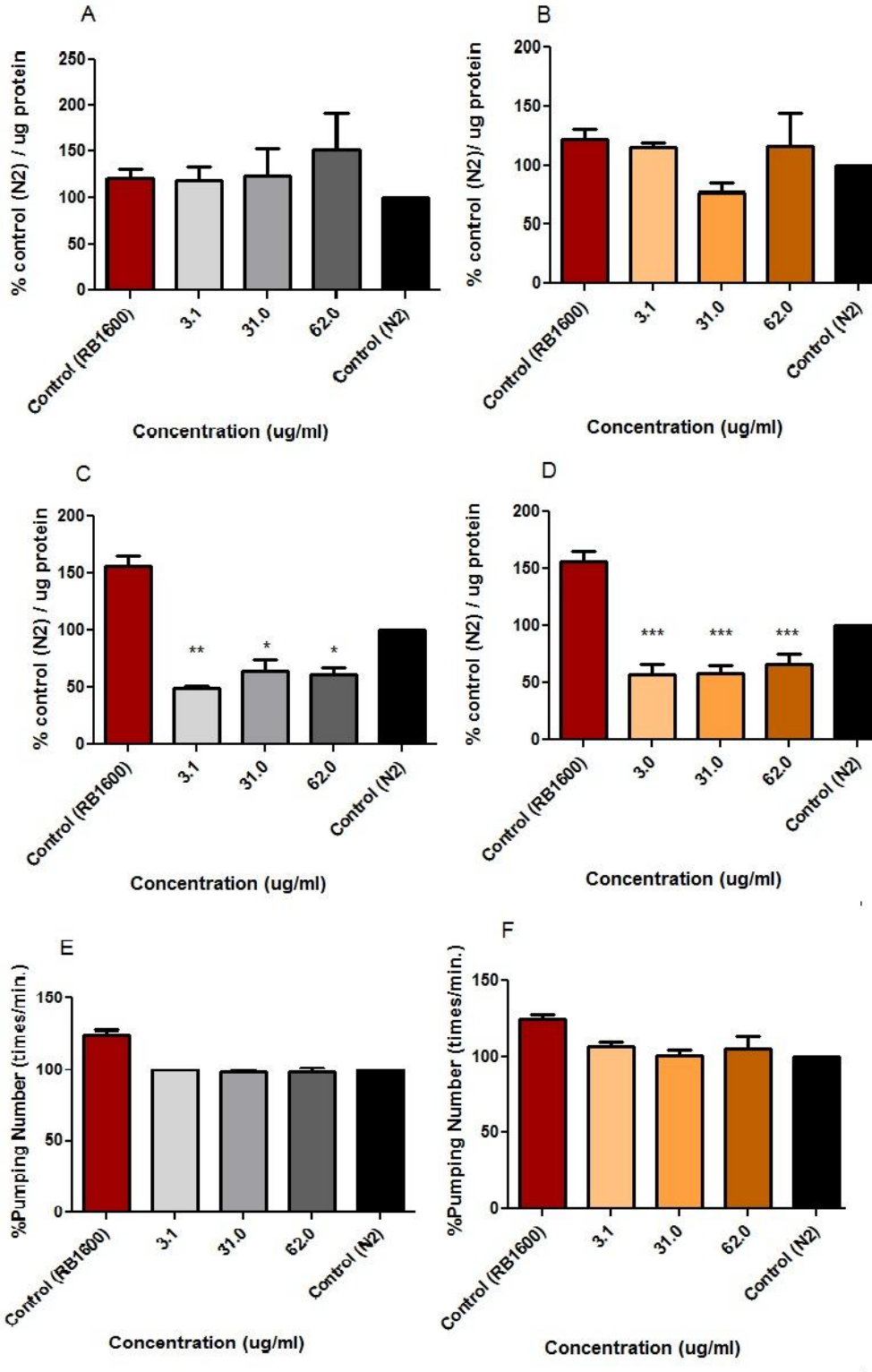

Figure 2 (A) Lipid levels of wild type and tub-1 worms treated with different concentrations of aqueous carob extract. (B) Lipid Levels of wild type worms tub-1 treated with different concentrations of aqueous cocoa extract. (C) Analysis of triglycerides levels of wild type worms tub-1 treated with different concentrations of aqueous carob extract. (D) Analysis of triglycerides levels of wild type worms tub-1 treated with different concentrations of aqueous cocoa extract. (E) Analysis of pumping number of wild type worms tub-1 treated with different concentrations of aqueous carob extract. (F) Analysis of pumping number of wild type worms tub-1 treated with different concentrations of aqueous cocoa extract. Data are expressed as mean ± SEM. * indicates $p \leq 0.05$ as compared to control with different concentrations of aqueous carob extract (n=3).

CONCLUSÕES

Com o presente estudo concluiu-se que o extrato aquoso da alfarroba (*Ceratonia siliqua* L.) apresenta excelentes atividades *in vitro*, pelos métodos DPPH e FRAP. No modelo experimental testado, *C.elegans*, o extrato aquosos da alfarroba nas doses testadas não apresentou mortalidade 50%, não alterou a longevidade dos nematoides, apesar de reduzir a postura de ovos, além de proteger contra agente agressor (paraquat) em curto prazo. O extrato aquoso da alfarroba por meio de análise bromatólogica apresentou uma maior quantidade de fibras e uma menor quantidade de lipídios, quando comparado ao extrato aquoso do cacao. Esta alta quantidade de fibras proporcionou uma redução dos níveis de triglicerídeos, tanto em nematoides selvagens como mutante. De tal maneira, podemos sugerir que o extrato aquoso da alfarroba pode ser um excelente substituinte ao extrato aquoso do cacao, em pequenas proporções, modulando positivamente o metabolismo lipídio e assim transmitindo para a população.

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PERSPECTIVAS

O presente trabalho é um passo inicial para entendimento da eficácia da alfarroba (*Ceratonia siliqua*), porém são necessários mais estudos para melhor validar o alimento como um nutracêutico. Preconizando realizar a atividade enzimática da SOD e da GST-4. Além de quantificar os tipos de fibras existências em ambos os extratos e por fim investigar outras vias do metabolismo lipídico, como os genes lipl-4, bbs-1, kat-1 para possíveis mecanismos.

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