UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE

Perfil cromatográfico e potencial tóxico-farmacológico do óleo da polpa de *Attalea phalerata* Mart. ex Spreng. (bacuri)

FERNANDO FREITAS DE LIMA

DOURADOS/MS
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Área de concentração: Farmacologia e Toxicologia

Orientadora: Drª Maria do Carmo Vieira
Co-orientadoras: Drª Claudia Andrea Lima Cardoso e Drª Eliana Janet Sanjinez-Argandoña

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ATA DA DEFESA DE **TESE DE DOUTORADO** APRESENTADA PELA CANDIDATA **FERNANDO FREITAS DE LIMA**, ALUNO DO PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM CIÊNCIAS DA SAÚDE ÁREA DE CONCENTRAÇÃO "**FARMACOLOGIA**".

Ao vigésimo quinto dia do mês de agosto do ano de dois mil e dezessete (25/08/2017), às 09h00min, em sessão pública, realizou-se, no Auditório da Faculdade de Ciências da Saúde, Universidade Federal Grande Dourados, a Defesa de Tese de Doutorado intitulada **"Perfil cromatográfico e potencial tóxico-farmacológico do óleo da polpa dos frutos de *Attalea phalerata* Mart. ex Spreng. (bacuri)."** apresentada pelo doutorando FERNANDO FREITAS DE LIMA, do Programa de Pós-Graduação Doutorado em Ciências da Saúde, à Banca Examinadora constituída pelos professores **Dra. Maria do Carmo Vieira** (Presidente/orientadora), **Dra. Priscila Neder Morato** (membro titular/externo), **Dra. Caroline Alves Breda** (membro titular/externo), **Dr. Virgínia Demarchi Kappel Trichez** (membro titular/programa) e **Dra. Ariany Carvalho dos Santos** (membro titular/externo). Iniciada sessão, a presidência deu a conhecer ao candidato e aos integrantes da Banca as normas a serem observadas na apresentação da Tese. Após o candidato ter apresentado a sua Tese, os componentes da Banca Examinadora fizeram suas arguições, que foram intercaladas pela defesa do candidato. Terminadas as arguições, a Banca Examinadora, em sessão secreta, passou ao julgamento, tendo sido o candidato considerado **APROVADO**, fazendo *jus* ao título de **DOUTOR EM CIÊNCIAS DA SAÚDE**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelos membros da Banca Examinadora.

Dourados, 25 de agosto de 2017.

Dra. Maria do Carmo Vieira MCVieira

Dra. Priscila Neder Morado Priscila Neder Morato

Dra. Caroline Alves Breda Caroline Alves Breda

Dra. Virgínia Demarchi Kappel Trichez Virgínia Demarchi Trichez

Dra. Ariany Carvalho dos Santos Ariany Carvalho dos Santos

ATA HOMOLOGADA EM: ___/___/_____, PELA PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA / UFGD.

Profa. Kely de Picoli Souza
Pró-Reitora de Ensino de Pós-Graduação e Pesquisa

''Que de bom gosto a vida me mande tudo que meu coração estiver precisando para caminhar em paz. E que de bom grado eu saiba receber e, então, devolver o meu melhor. ''

Vanessa Haas

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Dedico este trabalho à minha família e amigos por representarem tudo em minha vida.

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Lista de abreviaturas e símbolos

A. phalerata	*Attalea phalerata* Mart. ex Spreng.
ANVISA	Agência Nacional de Vigilância Sanitária
DL50	Dose letal 50%
DNA	Ácido desoxirribonucleico
IL-1	Interleucina-1
IL-6	Interleucina-6
MCN	Micronúcleo
MTT	(3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina)
OECD	Organization for Economic Co-operation and Development
RNS	Espécie reativa de Nitrogênio
ROS	Espécie reativa de Oxigênio
TNF-α	Fator α de necrose tumoral
α-caroteno	Alfa-caroteno
β-caroteno	Beta-caroteno
β-zeacaroteno	Beta-zeacaroteno
γ-caroteno	Gama-caroteno
ζ-caroteno	Zeta-caroteno
ω-3	Ácido linolênico
ω-6	Ácido linoleico

Resumo

A *Attalea phalerata* Mart. ex Spreng. (bacuri ou acuri, Arecaceae) é utilizada na medicina popular. O óleo do fruto é utilizado para o tratamento cosmético anticaspa e contra queda de cabelos, em forma oral para o tratamento de congestão pulmonar e anti-inflamatório para articulações. Este trabalho descreve o estudo cromatográfico dos carotenoides e ácidos graxos presentes no óleo da polpa de frutos de *A. phalerata* e sua atividade antioxidante, bem como a avaliação da toxicidade aguda, subaguda e genética (citotoxicidade, genotoxicidade e clastogenicidade), atividade anti-inflamatória *in vitro/in vivo*, antiproliferativa e citoprotetora *in vitro*. Após avaliação por cromatografia, o óleo apresentou como carotenoides majoritários α-caroteno, β-caroteno e ζ-caroteno, e ácidos graxos insaturados. Na atividade antioxidante pelo sistema β-caroteno/ácido linoleico, esses carotenoides podem ter apresentado influência direta na atividade, que foi de 47,33% de inibição de oxidação após exposição de 120 min aos radicais peróxido linoleico formados. A avaliação da toxicidade aguda não apresentou alteração macroscópica e no peso dos órgãos nas ratas avaliadas e comprovou que a DL50 do óleo é maior que 2000 mg/kg. No teste de toxicidade subaguda, o óleo apresentou baixa toxicidade nas doses de 125 mg/kg, 250 mg/kg, 500 mg/kg e 1000 mg/kg após exposição de 28 dias consecutivos. Os parâmetros bioquímicos e hematológicos não apresentaram alteração negativa quando comparados com os valores de referência na literatura, porém, valores de glicemia, colesterol e triglicérides foram reduzidos em alguns grupos tratados com o óleo de *A. phalerata*. As análises histopatológicas na toxicidade subaguda não mostraram quaisquer alterações que indiquem sinais de toxicidade nos tecidos dos órgãos avaliados. A toxicidade genética serviu para avaliar os possíveis efeitos citotóxicos, genotóxicos e mutagênicos (clastogênicos) do óleo da polpa. Os experimentos de citotoxicidade *in vitro* demonstraram que o óleo não apresenta toxicidade frente aos náuplios de *Artemia salina* e em células avaliadas pelo método de MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina). O ensaio de cometa demonstrou que o óleo não é genotóxico e o ensaio de micronúcleo confirmou a ausência de citotoxicidade e clastogenicidade. Ao avaliarmos a atividade anti-inflamatória do óleo pelos métodos *in vitro,* os resultados demonstraram que o óleo em dose de 1 mg/mL apresenta capacidade de inibição do óxido nítrico produzido pelos macrófagos, induz os macrófagos ao espraiamento e em dose de 10 µg/mL é um inibidor não seletivo da COX. Nos modelos *in vivo,* o óleo na dose de 700 mg/kg reduziu significativamente o edema de pata e impediu a migração leucocitária pelo modelo de pleurisia. O óleo apresentou-se inativo frente as células de carcinoma de rim e de próstata, porém nas doses 250 µg/mL e 500 µg/mL desenvolveu capacidade citoprotetora contra a Doxorrubicina. Após os experimentos realizados, concluímos que o óleo pode ser considerado seguro para o consumo nas doses testadas e é um promissor anti-inflamatório natural devido à presença de compostos bioativos e atividade antioxidante.

Palavras-chave: bacuri, carotenoides, antioxidante, toxicidade, anti-inflamatório, citoproteção.

Abstract

Attalea phalerata Mart. ex Spreng. (bacuri or acuri, Arecaceae) is used in folk medicine. The fruit oil is used in anti-dandruff treatment and against hair loss, orally for the treatment of pulmonary congestion and as an anti-inflammatory for joints. This work describes the chromatographic study of carotenoids and fatty acids present in *A. phalerata* fruit pulp oil and its antioxidant activity, as well as the evaluation of acute, subacute and genetic toxicity (cytotoxicity, genotoxicity and clastogenicity), *in vitro/in vivo* anti-inflammatory activity, and *in vitro* antiproliferative and cytoprotective. The oil presented α-carotene, β-carotene and ζ-carotene, and unsaturated fatty acids as major compounds after evaluation by chromatography. In the β-carotene/linoleic acid system, these carotenoids may have had a direct influence on the activity, which was 47.33% inhibition of oxidation after 120 min exposure to linoleic peroxide radicals formed. The acute toxicity assessment did not show any macroscopic or organ weight alteration in rats evaluated and the LD50 of *A. phalerata* oil was established as greater than 2000 mg/kg. In the subacute toxicity test, the oil presented low toxicity at the doses of 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg after 28-day exposure. The biochemical and hematological parameters did not present any negative changes when compared to the normal range for the species according to the literature, however glycemia, cholesterol and triglyceride values were reduced in some groups treated with *A. phalerata* oil. Histopathological analysis in the subacute toxicity experiment did not present any changes that indicate signs of toxicity in all organs evaluated. Genetic toxicity served to evaluate the possible cytotoxic, genotoxic and mutagenic (clastogenic) effects of pulp oil. *In vitro* cytotoxicity experiments demonstrated that the oil had no toxicity against *Artemia salina* nauplii and in cells evaluated by the MTT (3-(4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazoline bromide) method. The comet assay demonstrated that the oil is not genotoxic and the micronucleus assay confirmed the absence of cytotoxicity and clastogenicity. When we evaluated the anti-inflammatory activity of the oil through *in vitro* methods, the results demonstrated that the oil at a dose of 1 mg/mL has the ability to inhibit nitric oxide produced by macrophages, induces macrophage spreading and is a non-selective COX inhibitor at a dose of 10 μg/mL. In *in vivo* models, the oil at the dose of 700 mg/kg significantly reduced paw edema and prevented leukocyte migration by the pleurisy model. The oil was inactive against kidney and prostate carcinoma cells. However, at 250 μg/mL and 500 μg/mL, it developed cytoprotective capacity against Doxorubicin. After the experiments, we conclude that the oil can be considered safe for consumption at the doses tested and is a promising natural anti-inflammatory due to the presence of bioactive compounds and antioxidant activity.

Keywords: bacuri; carotenoids; antioxidant activity; toxicity; anti-inflammatory; cytoprotection.

Sumário

1. INTRODUÇÃO

O Cerrado brasileiro está distribuído em aproximadamente 23% do território nacional, onde podem ser encontradas diversas espécies frutíferas que ainda não foram devidamente estudadas (RATTER et al., 1997; ROCHA et al., 2013; MORZELLE et al., 2015). Os frutos do Cerrado apresentam alto valor nutricional e compostos bioativos (VERA et al., 2009; LESCANO et al., 2015), sendo de suma importância a inserção destes no estudo científico e no desenvolvimento de novos produtos alimentícios e farmacológicos.

A presença de compostos bioativos nos frutos do Cerrado, como, por exemplo, carotenoides e ácidos graxos, podem trazer benefícios à saúde humana. Alguns carotenoides, quando ingeridos, são metabolizados e convertidos em vitamina A, e também podem atuar como um potente antioxidante com capacidade preventora de doenças crônicas como câncer, doenças cardiovasculares, processos inflamatórios e no fortalecimento do sistema imunológico (HIANE et al., 2003; CANUTO et al., 2010; RODRIGUEZ-AMAYA, 2010). Ácidos graxos benéficos, como ácido alfa-linolênico e linoleico, atuam na redução do colesterol total, melhoramento da memória e diminuição da gordura corporal (NAVARRO; VILLANUEVA, 2003; NAVARRO et al., 2006; NGUEMENI et al., 2010).

A *Attalea phalerata* Mart. ex Spreng. (bacurizeiro, Arecaceae) ocorre no Brasil, mais especificamente, nos Estados de Mato Grosso do Sul e Mato Grosso. O seu fruto é constituído de casca (exocarpo), polpa (mesocarpo) e amêndoa (endocarpo mais semente). O óleo extraído da polpa do fruto é usado pelas populações locais para aliviar dores nas articulações e como tônico capilar (BALSLEV; MORAES, 1989; NEGRELLE, 2015). A polpa é utilizada na culinária devido ao alto valor nutricional e atributos sensoriais. O fruto possui em sua composição elevadas quantidades de carotenoides, como β-caroteno, β-zeacaroteno e α-caroteno, além de ácidos graxos insaturados, tanto na polpa como amêndoa (HIANE et al., 2003; DE LIMA et al., 2017).

Apesar de o óleo do fruto do bacurizeiro apresentar compostos considerados benéficos e possuir utilização popular, é necessário validar a segurança de seu uso e estimar as doses seguras para seu consumo. Os testes de toxicidade aguda e subaguda são métodos utilizados para estudos da segurança de produtos, nos quais são avaliados a mortalidade em ratos através da dose letal, DL50, parâmetros hematobioquímicos e alterações histopatológicas (OECD, 2008ab; ANVISA, 2013). Além dos testes de

toxicidade aguda e subaguda, atualmente os órgãos mundiais têm aumentado as exigências de testes que avaliem o potencial citotóxico, genotóxico e clastogênicos dos produtos naturais, os quais validam a segurança nas células e DNA humano (OECD, 1997; TRAESEL et al., 2014).

Diante do exposto, o objetivo deste trabalho foi determinar a composição química dos carotenoides, ácidos graxos e atividade antioxidante do óleo da polpa de *Attalea phalerata* Mart. ex Spreng., validar a segurança do seu uso através de testes de toxicidade aguda, subaguda e genética, bem como, avaliar seu potencial anti-inflamatório e antiproliferativo.

2. REVISÃO DA LITERATURA

2.1. Cerrado

O Cerrado corresponde a 23% do território brasileiro, sendo o segundo maior bioma; suas áreas são difundidas pelos estados de Goiás, Tocantins, Mato Grosso, Mato Grosso do Sul, Bahia, Minas Gerais, São Paulo, Maranhão, Piauí, Paraná, Rondônia, Distrito Federal e Pará (RATTER et al., 1997). Caracteriza-se por um complexo bioma e diferentes fisionomias, como por exemplo, formações florestais (Mata Ciliar, Mata de Galeria, Mata Seca e Cerradão), savânicas (Cerrado Denso, Cerrado Típico, Cerrado Ralo, Parque de Cerrado, Veredas e Palmeiral) e campestres (Campo Rupestre, Campo Sujo e Campo Limpo) (RIBEIRO et al., 1998; QUEIROZ, 2009; RUFINI et al., 2015).

O Cerrado brasileiro apresenta rica biodiversidade de espécies frutíferas, que oferecem alto valor nutricional, além de atrativos como sabor, cor, aroma peculiar e intenso, ainda pouco explorados (ROCHA et al., 2013). Estudos dos compostos nutricionais e antioxidantes presentes em frutos nativos contribuem para a inclusão destes em dietas saudáveis e na prevenção de doenças crônicas, assim como a valorização do bioma (LIMA et al., 2007; VERA et al., 2009).

Os frutos do Cerrado apresentam propriedades funcionais que são atribuídas à presença de substâncias bioativas. As principais são os ácidos graxos, compostos fenólicos e os carotenoides, sendo esses últimos caracterizados como um pigmento de cor amarelo claro ao vermelho, podendo ser convertidos em vitamina A no organismo (RODRIGUEZ-AMAYA, 1997; PENTEADO 2003; RODRIGUEZ-AMAYA, 2010). Esses compostos com atividade antioxidante também podem atuar sobre o estresse oxidativo, prevenindo distúrbios crônicos, como o diabetes, o câncer, problemas cardiovasculares e processos inflamatórios (ROCHA et al., 2011).

2.2. *Attalea phalerata* Mart. ex Spreng.

A *Arecaceae* é difundida principalmente em regiões tropicais e subtropicais da Terra, com poucas espécies nas zonas temperadas quentes (LORENZI et al., 1996; HENDERSON et al., 1997; NEGRELLE, 2015), apresentando cerca de 1.500 espécies distribuídas em mais de 200 gêneros, popularmente conhecidas como palmeiras. *Attalea* Kunth é considerado o gênero tropical mais importante da família

Arecaceae, ocorrendo desde o Caribe, do México à Bolívia, Brasil e Paraguai, podendo englobar pelo menos 20 espécies validadas (FAVA et al., 2011; NEGRELLE, 2015).

O gênero *Attalea* se apresenta complexo e incompletamente compreendido, tendo como característica a dificuldade no entendimento taxonômico pela presença de hibridização entre suas espécies. A *Attalea phalerata* (Figura 1) foi identificada por Martius e, por conseguinte registrada por Sprengel em 1825. Foi reordenada em 1929 por Burret, como pertencente ao gênero *Scheelea.* Glassman, em 1977, explicitou que a espécie dispõe de problema na alocação taxonômica, dificuldade na determinação da espécie tipo, sendo assim, estabelece como lectótipo *Attalea phalerata* de acordo com publicação de Martius em 1845 (NEGRELLE, 2015).

Figura 1 - Palmeira *Attalea phalerata* Mart. ex Spreng com frutos
(Fonte: O próprio autor, 2016)

A palmeira *Attalea phalerata* Mart. ex Spreng (bacurizeiro, *Arecaceae*) é encontrada no Brasil, nos estados de Mato Grosso do Sul e Mato Grosso. Seu fruto (Figura 2), denominado bacuri ou acuri (POTT; POTT, 1994; HIANE et al., 2003), apresenta polpa carnosa com cor que varia do amarelo ao laranja, tem forma oval achatada e sementes (amêndoas) envolvidas por um endocarpo rígido (HIANE et al., 2003; LIMA et al., 2014). É utilizado no preparo de sucos, geleias e sorvetes, bem como no tratamento de caspas, problemas pulmonares e, até mesmo, para dores nas articulações (BALSLEV; MORAES, 1989; NEGRELLE, 2015).

Figura 2 – Fruto de *Attalea phalerata Mart.* ex Spreng (bacuri ou acuri) sem exocarpo.
(Fonte: O próprio autor, 2016)

A polpa e a amêndoa apresentam minerais em sua composição; já, a polpa possui grande quantidade de carboidratos, enquanto a amêndoa, lipídios. São encontrados ácidos graxos benéficos no óleo da polpa, tendo como os principais, ácidos graxos, o oleico, palmítico, linoleico e láurico. Sua polpa apresenta elevado teor de carotenoides, destacando-se o β-caroteno e β-zeacaroteno (HIANE et al., 2003; SIQUEIRA et al., 2016). Os compostos presentes no fruto apresentam elevado potencial farmacológico, sendo as possíveis ações anti-inflamatória e citoprotetora atribuídas aos carotenoides contidos ou um promissor hipocolesterolêmico devido à presença dos ácidos graxos insaturados (TOLEDO et al., 2003; SCHMITT et al., 2012; DE OLIVEIRA et al., 2015; LESCANO et al., 2015).

2.3. Ácidos graxos

Os ácidos graxos ocorrem no organismo principalmente como ésteres em óleos e gorduras naturais, também são encontrados na forma não esterificada como ácidos graxos livres, sendo assim transportados no plasma. Os ácidos graxos, em sua maioria, presentes nas gorduras naturais contém um número uniforme de átomos de carbono (BOTHAM; MAYES, 2006). A cadeia pode ser saturada (não contem ligações duplas) ou insaturada (contém uma ou mais ligações duplas), sendo este último subdividido em monoinsaturados e poli-insaturados (Figura 3). Os ácidos graxos insaturados, quando ingeridos em quantidades desejáveis, desempenham importantes funções no organismo, sendo elas, a manutenção do sistema imunológico em processos inflamatórios e diminuição da gordura corporal (CINTRA et al., 2012; ITARIU et al., 2012; PEREIRA et al., 2014).

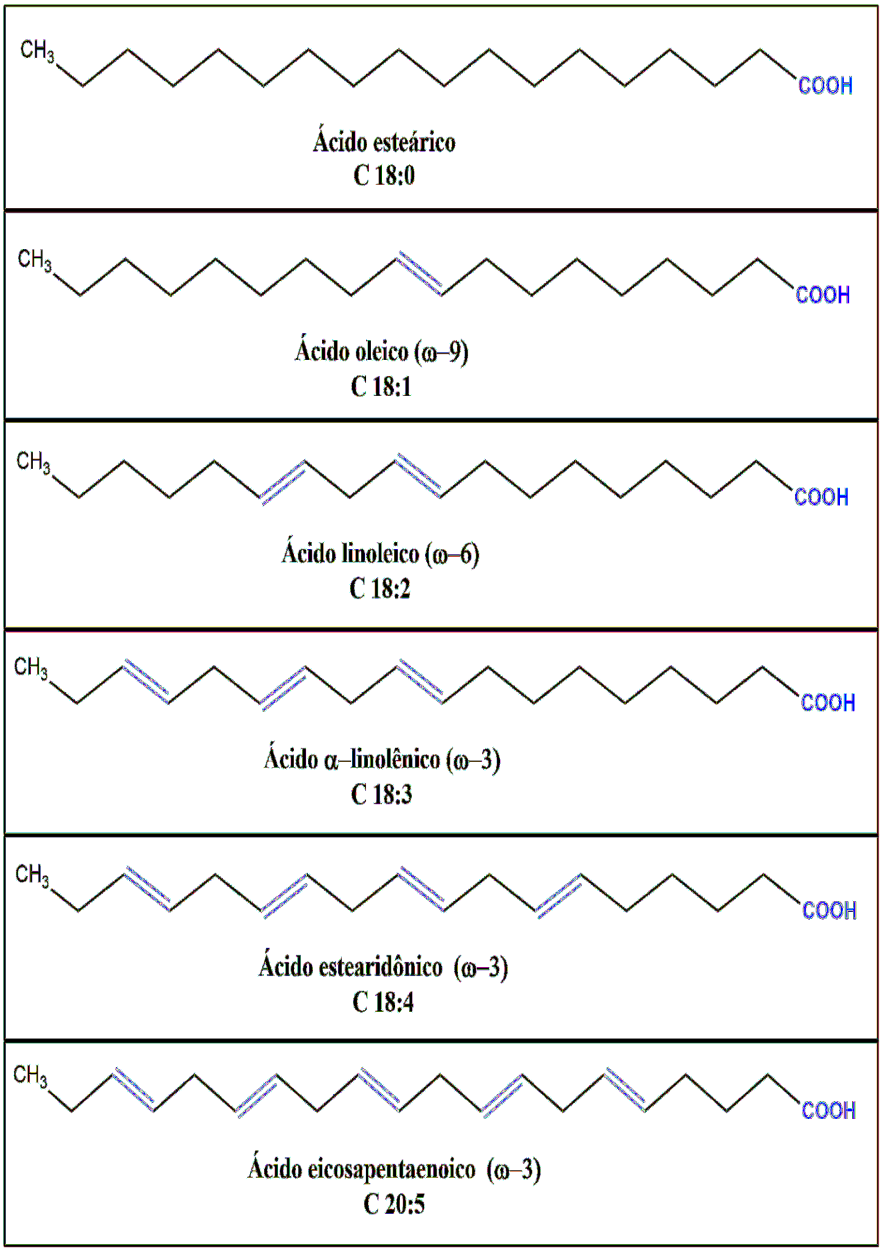


Figura 3 – Estrutura química dos ácidos graxos saturados e insaturados.

(Fonte: O próprio autor, 2017)

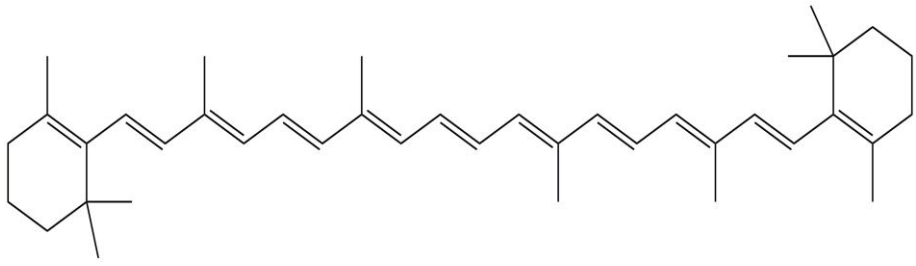
Os ácidos graxos linolênico (ω-3) e linoleico (ω-6), representantes da família ômega (ω), exercem ação efetiva na saúde humana, porém não são produzidos pelo organismo (BRENNA et al., 2009) e necessitam ser ingeridos em alimentos que os contenham. O restrito consumo de ácido linolênico (ω-3) pode resultar em alterações de humor, perda de memória e dificuldade de aprendizado; por outro lado, seu consumo regular está ligado à proteção neuronal (NGUEMENI et al., 2010) e à prevenção de

doenças inflamatórias (SIRIWARDHANA et al., 2012; SKULAS RAY, 2015). O ácido linoleico (ω-6) auxilia na redução do colesterol (NAVARRO et al., 2006), da gordura corporal (NAVARRO; VILLANUEVA, 2003) e melhora o funcionamento do sistema imunológico (HARO et al., 2006).

2.4. Carotenoides

Os carotenoides são corantes naturais que estão presentes nos produtos naturais em geral (RAMOS et al., 2008; KIOKIAS et al., 2016), sendo considerados compostos bioativos com capacidade de proteger o organismo humano contra o estresse oxidativo e prevenindo distúrbios crônicos (CANUTO et al., 2010). Aos carotenoides estão atribuídas ações que promovem a saúde, como diminuição do risco de desenvolvimento de câncer, melhora do sistema imunológico e prevenção de doença cardiovascular (HIANE et al., 2003; RODRIGUEZ-AMAYA, 2010).

A estrutura dos carotenoides é constituída de um extenso sistema de ligações duplas conjugadas, que consiste em alternar uma ligação dupla e uma simples através das ligações carbono-carbono (RODRIGUEZ-AMAYA, 1997; RODRIGUEZ-AMAYA, 2010). O longo sistema de ligações duplas conjugadas dos carotenoides é denominado como cadeia polieno (Figura 4), essa parte da molécula, conhecida como cromóforo, é responsável pela capacidade antioxidante dos carotenoides e preventora de doenças crônicas (BELLIZI et al., 1994; RODRIGUEZ-AMAYA, 1997; SILVA et al., 2010).



β–caroteno

Figura 4 – Estrutura química do carotenoide β-caroteno. (Fonte: O próprio autor, 2017)

Grande parte dos carotenoides são hidrofóbico-lipofílicos e englobam uma grande quantidade de moléculas, como β-caroteno, α-caroteno, luteína, zeaxantina e licopeno (GOMES, 2007), e são conhecidos pela capacidade de gerar provitamina A; porém, nem todos podem ser convertidos em vitamina A (RODRIGUEZ-AMAYA, 1997;

DAMODARAN et al., 2008). Os principais carotenoides provitamina A apresentam estrutura cíclica β ionona em sua molécula; por isso, são considerados precursores da vitamina A, sendo eles: α, β e γ-caroteno e β-criptoxantina (SILVA et al., 2010). O β-caroteno é o principal precursor da vitamina A devido à sua elevada bioatividade e ocorrência nas frutas e hortaliças (RODRIGUEZ-AMAYA, 1997; DAMODARAN et al., 2008). Também apresentam alta eficiência durante a conversão em retinol, devido à presença de dois anéis beta-ionona em sua estrutura, diferente dos demais carotenoides que possuem apenas um anel beta-ionona (RODRIGUEZ-AMAYA, 1997; NAVES, 1998; RODRIGUEZ-AMAYA, 2010).

2.5. Toxicidade aguda e subaguda

Apesar da presença de compostos bioativos nos produtos naturais, incluindo as frutas, seu consumo em doses desconhecidas pode levar a danos à saúde. A validação das doses seguras das substâncias através de testes de toxicidade é de suma importância, o que é justificado por trabalhos que destacam os efeitos tóxicos devido ao uso indiscriminado desses produtos (VEIGA JÚNIOR et al., 2005; TAVARES et al., 2006; MONTEIRO, COUTO, 2008; DOS SANTOS MEDEIROS et al., 2009).

O estudo do perfil toxicológico de produtos naturais com propriedades funcionais, sejam de prevenção ou ação curativa, é necessário para validar sua segurança e impulsionar o uso como novos fitoterápicos pelas indústrias farmacêuticas (TRAESEL et al., 2014; PACÍFICO et al., 2016). Estes produtos podem conter algumas substâncias que, quando administradas em doses elevadas, podem desempenhar graves sintomas como hepatotoxicidade, insuficiência renal e outras complicações, comprometendo o funcionamento do organismo e podendo levar a óbito (MEIRA et al., 2013; TERRES, 2016).

Experimentos de toxicidade aguda compreendem um dos métodos mais utilizados para avaliar substâncias que são utilizadas em dose única ou repetidas, durante períodos não superiores a 24 h (ANVISA, 2004; ANVISA, 2013). O teste consiste em avaliar sinais de toxicidade por meio do controle de consumo diário de água, ração, peso corporal, análise macroscópica dos órgãos (OECD, 2008a; ANVISA, 2013) e *screening* hipocrático sugerido por Malone e Robichaud (1962). Nesse teste, avalia-se o comportamento dos animais pelo estado consciente, atividade e coordenação do sistema motor e tônus

muscular, reflexos, atividades sobre o sistema nervoso central e atividades sobre o sistema nervoso autônomo.

Caso a substância teste apresente baixa toxicidade, constatada por meio da composição química ou de trabalhos semelhantes, utiliza-se como dose limite 2000 mg/kg. Os animais são expostos ao tratamento com a substância teste por 14 dias, sendo que no primeiro dia de administração, devem ser observados no mínimo duas vezes e, posteriormente, uma vez ao dia (OECD, 2008a; ANVISA, 2013). A primeira dose é administrada no animal; caso o mesmo sobreviva, administra-se a mesma dose sequencialmente nos outros animais com intervalos de 48 horas (OECD, 2008a; TRAESEL et al., 2014). Ao final do experimento, é determinada a dose letal para 50% dos animais (DL50) (ANVISA, 1995; ANVISA, 2013).

O teste de toxicidade subaguda, ao contrário da aguda, avalia sinais tóxicos em animais expostos a doses repetidas (28 dias); essa avaliação é fundamental para substâncias testes que serão utilizadas continuamente. São avaliados parâmetros como o consumo de água e ração, peso corporal, análise macroscópica dos órgãos, *screening* hipocrático, análise hematobioquímica e análise histopatológica (OECD, 2008b; ANVISA, 2013). O experimento deve ser realizado com animais de ambos os sexos, respeitando a dose limite de 1000 mg/kg diários e as demais doses são estabelecidas de forma decrescente em intervalos de 2 a 4 vezes. Os grupos experimentais devem ser constituídos de no mínimo 10 animais por dose e utiliza-se um grupo satélite que é exposto à maior dose por 28 dias e mantem-se por no mínimo 14 dias sem administração da substância teste a fim de avaliar possíveis sinais tardios ou de reversibilidade (ANVISA, 2013; OECD, 2008b; TRAESEL et al., 2014)

2.6. Bioensaio com *Artemia salina* e citotoxicidade

A avalição da citotoxicidade em agentes com potencial citotóxico com novos mecanismos de ação é necessária para a condução de estudos de segurança farmacológica (ANVISA, 2004). Os estudos de compostos citotóxicos desenvolvem um importante papel para avaliação da morte celular ou até mesmo auxiliando no desenvolvimento de fármacos com ação antiproliferativa e antitumoral (ANVISA, 2013).

O ensaio de *Artemia salina* – MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) é considerado uma das ferramentas mais úteis para ensaios preliminares de avaliação da toxicidade geral, pois, além de ter baixo custo, mostra boa correlação com a

atividade citotóxica (MEYER et al., 1982; SHARIFIFAR et al., 2009). O ensaio de MTT torna possível avaliar a citotoxicidade, sendo utilizado com grande sucesso para estimar o número de células viáveis em um "*screening*" inicial para novas drogas (SADEGHI et al., 2013).

2.7. Toxicidade Genética

A validação da segurança genotóxica e mutagênica de fármacos tornou-se uma das exigências dos órgãos mundiais, antes de os mesmos serem inseridos no mercado. O estudo da genotoxicidade de fármacos ou produtos naturais abrange as áreas de toxicologia e a genética, sendo avaliados os possíveis efeitos genotóxicos (QUEIROZ et al., 2013). A genotoxicidade é caracterizada pela mutação gênica, dano cromossômico ou lesão no DNA e a associação desses indicadores biológicos fortalece a importância dos testes de genotoxicidade (BARBISAN et al., 2003; RIBEIRO et al., 2003). Como exemplo, os ensaios cometa e micronúcleo são comumente utilizados para avaliar desordens genéticas e mutagênicas no organismo humano, tendo o ensaio micronúcleo a particularidade de também avaliar a capacidade citotóxica (ARALDI et al., 2015).

A versão *in vivo* do ensaio cometa avalia os danos ao DNA através da exposição do núcleo da célula (OECD, 2014), por apresentar largo espectro na detecção dos níveis de danos de DNA, tais como a desnaturação do DNA e detecção de sítios alcalino-lábil (SINGH et al., 1988; HARTMANN et al., 2003; COLLINS et al., 2008). Essa técnica foi introduzida em 1988 por Singh e desde então vem sendo adaptada por diversos autores (ROJAS et al., 1999).

O ensaio cometa não é aplicado na detecção de mutações, mas sim em lesões genômicas que podem resultar em mutação, após iniciadas. As lesões detectadas pelo ensaio cometa são ainda reversíveis, possibilitando o uso do teste em estudos de reparo do DNA (RIBEIRO et al., 2003; TRASEL et al., 2014). Os danos às células são classificados em danos 0 (zero), 1, 2 e 3, sendo essa classificação em função da extensão da ''cauda'' formada pela exposição do DNA após rompimento da célula danificada (DA SILVA et al., 2010; BARBISAN et al., 2003).

O ensaio de micronúcleo em sua versão *in vivo* possibilita avaliar a presença ou ausência de danos causados por compostos químicos nos cromossomos e/ou aparelho mitótico dos eritroblastos (OECD, 1997). Este ensaio *in vivo* é realizado para a nidificação de agentes clastogênicos (quebram cromossomos) e agentes aneugênicos (que induzem a

segregação cromossômica anormal), fornecendo resultados citotóxicos e mutagênicos, sendo por isso recomendado por órgãos reguladores mundiais (VINOD et al., 2011). Essa detecção é possível por meio da análise dos eritrócitos na medula óssea ou dos eritrócitos policromáticos no sangue periférico de roedores (OECD, 1997; VINOD et al., 2011). O eritroblasto presente na medula converte-se em eritrócito policromático através de sua maturação. Nesse processo, o núcleo da célula é expulso, mas qualquer micronúcleo (MCN) que tenha sido formado pode permanecer no citoplasma, possibilitando sua visualização. Um aumento na frequência de eritrócitos policromáticos com MCN é indicativo de dano cromossômico induzido (OECD, 1997; TRAESEL et al., 2014).

Após a elucidação da composição química, realização dos testes de toxicidade aguda e subaguda por doses repetidas e testes de citotoxicidade e toxicidade genética, pode-se então, iniciar a avaliação de possíveis atividades farmacológicas para o desenvolvimento de fitoterápicos (TOLEDO et al., 2003; OLIVEIRA; FRUTUOSO, 2009).

2.8. Inflamação

O processo inflamatório pode ser denominado como uma resposta fisiológica do organismo a uma infecção ou dano tecidual (LIMA et al., 2007; ORTEGA-GÓMEZ et al., 2013). Os processos inflamatórios são caracterizados pelos sinais cardinais, sendo estes, calor, tumor, rubor e dor; também se caracteriza pela migração leucocitária e mudanças na fisiologia vascular na inflamação aguda (BECHARA; SZABÓ, 2005; MEDZHITOV, 2010).

A resposta inflamatória pode seguir diversas vias, isto é, depende do indutor do processo inflamatório. Existem diversos indutores do processo inflamatório, como as bactérias, agentes físicos, químicos, reações imunológicas e/ou necrose tecidual (LIMA et al., 2007). O processo denominado como cascata inflamatória associa-se com a liberação de diferentes tipos celulares de mediadores pró-inflamatórios e células teciduais, tais como os neutrófilos, macrófagos, linfócitos, mastócitos e fibroblastos (WALZOG; GAEHTGENS, 2000; LIMA et al., 2007). A resposta imune inata ou adaptativa só ocorre quando os leucócitos migram ao endotélio, processo esse denominado diapedese e também se caracteriza pela ligação dos leucócitos com o endotélio (CARVALHO et al., 1998).

A interação dos leucócitos recrutados com a lesão celular (células endoteliais) desempenha um papel fundamental no desenvolvimento do processo inflamatório mediante

a liberação de fatores solúveis de regulação denominados citocinas, como por exemplo, a interleucina-1 (IL-1), a interleucina-6 (IL-6) e fator α de necrose tumoral (TNF-α). Além das citocinas, uma série de mediadores pró-inflamatórios são produzidos durante o processo inflamatório, como a bradicina, histamina, serotonina, produtos da cascata do ácido araquidônico e adenosina. Estes últimos são responsáveis pela redução no limiar de percepção do estímulo doloroso, exagerada resposta a estímulos nociceptivos supralimiares (hiperalgesia) e dor espontânea (alodinia) (CARVALHO; LEMÔNICA, 1998).

A produção exacerbada de macrófagos e neutrófilos, bem como as células teciduais lesadas, liberam uma variedade de substâncias oxidantes e enzimas criando estresse oxidativo, no qual espécies reativas de oxigênio (ROS) e nitrogênio (RNS) são produzidas em abundância, promovendo perda dos estoques energéticos celulares, rompimento de mitocôndrias com liberação de enzimas líticas, peroxidação e destruição de membranas e danos em DNA (DRAY, 1995; CARVALHO; LEMÔNICA, 1998).

2.9. Tumorigênese

O desequilíbrio entre espécies reativas de oxigênio e nitrogênio (moléculas oxidativas) – ROS/RNS com antioxidantes ocasionam processos inflamatórias e ou danos celulares, que levam à desordem no organismo e consequentemente ao desenvolvimento da carcinogênese (CARVALHO; LEMÔNICA, 1998; BIANCHI; ANTUNES, 1999; ROESSNER et al., 2008). Células cancerígenas utilizam as ROS/RNS para estimular a proliferação, invasão, migração e angiogênese, inibindo os mecanismos de apoptose (ROESSNER et al., 2008; KWEE, 2014). Os danos causados ao DNA pelos radicais livres desempenham importante papel nos processos de mutagênese e carcinogênese (POULSEN et al., 1998; BIANCHI; ANTUNES, 1999).

A exposição dos seres humanos aos agentes nocivos como alimentos processados, metais pesados, radiação, poluição, entre outros, presentes no meio ambiente podem levar ao desenvolvimento de câncer. O processo denominado carcinogênese ocorre pela conversão de uma célula normal em uma maligna alterada por agentes indutores do processo carcinogênico (LOUREIRO et al., 2002). Para que haja o desenvolvimento de tumores, a exposição do indivíduo aos agentes deve ser contínua (LOUREIRO et al., 2002; CEBALLOS et al., 2014).

A carcinogênese apresenta-se de forma complexa e ocorre muito lentamente, sendo então dividida em três fases: a iniciação, a promoção e a progressão. Seguido das

alterações iniciais, aparecem novos conjuntos de células que crescem desordenadamente resultando em células pré-neoplásicas, pré-malignas e malignas (CÂNDIDO et al., 2016; MUNHOZ et al., 2016). A primeira etapa da carcinogênese é denominada como iniciação, quando as células sofrem ação de fatores carcinogênicos e iniciam-se os danos ao DNA celular. As células "iniciadas" permanecem latentes, até que sofram a ação dos agentes promotores (SHUKLA; KALRA, 2007; CÂNDIDO et al., 2016; MUNHOZ et al., 2016).

A segunda fase, chamada de promoção, indica o período em que a célula iniciada acumula novas alterações e adquire vantagens proliferativas com capacidade de não responder aos mecanismos de controle do organismo. A promoção só ocorre após as células terem sido iniciadas. A fase de progressão representa a etapa em que as células alteradas desenvolvem mudanças irreversíveis, maior agressividade, crescimento rápido e potencial de invasão e disseminação, através da proliferação descontrolada (SHUKLA; KALRA, 2007; CÂNDIDO et al., 2016; MUNHOZ et al., 2016).

Recentemente, muitas pesquisas demonstraram que frutas e hortaliças apresentam atividade antioxidante, anti-inflamatória e antiproliferativa/antitumoral, sendo estes benefícios à saúde atribuídos, principalmente, à presença dos carotenoides e ácidos graxos (BATISTA et al., 2010; LESCANO et al., 2015; LINNEWIEL-HERMONI et al., 2015). Com base na composição do óleo do fruto da *A. phalerata*, rico em carotenoides e ácidos graxos benéficos à saúde, despertou-se o interesse do grupo em avaliar sua possível ação farmacológica, e, além disso, assegurar seu consumo em doses seguras, através de testes de toxicidade aguda, subaguda, citotoxicidade e genotoxicidade.

Considerando que:

1) O Cerrado brasileiro vem sendo degradado e a existente necessidade de valorizar o bioma. 2) A polpa e óleo do fruto Attalea phalerata utilizado na medicina popular deve ter seu uso seguro comprovado. 3) A importância da aplicação de testes de toxicidade para fornecer a segurança de produtos naturais. 4) Alternativas naturais com capacidade anti-inflamatória e menor efeito colateral. 5) Alternativas naturais com capacidade antiproliferativa com menor efeito colateral e citoprotetora. 6) Uso da medicina preventiva e curativa através de produtos naturais.

Justifica-se esse estudo.

3. OBJETIVOS

- Objetivo Geral
  - Avaliar o perfil cromatográfico e potencial toxico-farmacológico do óleo da polpa de *Attalea phalerata* Mart. ex Spreng.

- Objetivos Específicos
  - Determinar a composição de carotenoides, ácidos graxos e atividade antioxidante do óleo da polpa de *A. phalerata*.
  - Certificar a segurança do uso do óleo da polpa de *A. phalerata* em modelos de toxicidade aguda, subaguda e genética.
  - Avaliar o potencial anti-inflamatório, antiproliferativo e citoprotetor do óleo da polpa de *A. phalerata*.

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5. APÊNDICES

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Acute and subacute oral toxicity assessment of the oil extracted from *Attalea phalerata* Mart ex Spreng. pulp fruit in rats

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ABSTRACT

Attalea phalerata Mart. ex Spreng., popularly known as "bacuri", is a native plant from the brazilian Cerrado and used in folk medicine as a pulmonary decongestant, an anti-inflammatory for joints and antipyretic. There is an expectation about the use in chronic disease of the *Attalea phalerata* oil since its composition is high in carotenoids and beneficial fatty acids. The aim of the study was to evaluate the toxicological profile of the oil extracted from *Attalea phalerata* Mart. ex Spreng. pulp (APO). Acute and subacute toxicity studies were performed in male and female *Wistar* rats according to the OECD - Guidelines 425 and 407. For the acute toxicity, one single dose of the APO (2000 mg/kg) was administered by gavage to five female rats. In the subacute toxicity, four different doses (125, 250, 500 and 1000 mg/kg) of the APO were administered to male and female rats for 28 consecutive days. No deaths or behavioral changes were observed during both experiments as well as no changes in organ weights, hematological, histopathological parameters. The biochemical parameters showed changes in phosphatase alkaline and albumin levels, however these values are within the normal range for the species. A significant reduction in cholesterol and triglycerides was also observed in some of the animals treated with the APO. Therefore, the LD50 is higher than 2000 mg/kg and the APO oil can be considered safe at the doses tested in rats. However, further assessments are required in order to proceed to clinical studies in humans.

1. Introduction

The study of medicinal plants has been very important in the development of new drugs. In this sense, the pharmaceutical industry has invested in the study of secondary metabolites derived from plants and vegetables, and these metabolites assist in the prevention of numerous chronic diseases (Konan, Bacchi, Lincopan, Varela, & Varanda, 2007; Pacifico et al., 2016.). However, tests to determine the safety of using medicinal plants are required, demonstrating the need to evaluate the toxicological profile.

Attalea phalerata Mart. ex Spreng. is commonly found in Brazil, especially in the states of Mato Grosso do Sul and Mato Grosso, typically in the Cerrado (Pott & Pott, 1994; Fava, Covre, & Sigrist, 2011). The palm tree belongs to the Arecaceae family and its fruit, popularly known as "bacuri", is composed of yellow-orange pulp and edible almonds (Pott & Pott, 1994; Hiane, Bogo, Ramos, & Ramos-Filho, 2003; Barreto, Benassi, & Mercadante, 2009). In folk medicine, the juice of the fruit is used as eye drops (Laturner, Rassolin, Poleto, Neto, & Macedo, 2010) and the pulp oil is used orally to relief pulmonary congestion, joint pain and fever reduction (Balslev & Moraes, 1989; Moraes, Borchsenius, & Blicher-Mathiesen, 1996; Negrelle, 2015).

The oil extracted from *Attalea phalerata* Mart. ex Spreng. pulp is rich in carotenoids (Lima, Kassuya and Sanjinez-Argandoña, 2014) and beneficial fatty acids, which have beneficial effects on human health. Among

Abbreviations: APO, *Attalea phalerata* Mart. ex Spreng. oil; LD50, oral lethal dose; ALT, alanine aminotransferase; AST, aspartate aminotransferase; RDW, red cell distribution width.

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these effects are boosting the immune system (Gomes, 2007) and the prevention of chronic diseases such as diabetes, cardiovascular disease and inflammatory processes (Lima, Kassuya and Sanjinez-Argandoña, 2014; Rodriguez-Amaya, 2010).

There are few nutritional, chemical and pharmacological informations regarding the use of the pulp and oil of *Attalea phalerata* Mart. ex Spreng. fruits (Lima, Kassuya and Sanjinez-Argandoña, 2014); therefore, a more specific toxicological knowledge for possible future studies is necessary. The aim of the study was to identify the toxicological profile of the oil extracted from the pulp of *Attalea phalerata* Mart. ex Spreng. fruits after a single oral administration (acute toxicity) and 28 consecutive daily administrations (subacute toxicity).

2. Material and methods

2.1. Material and sample preparation

Fresh, healthy and ripe selected *Attalea phalerata* Mart. ex Spreng. fruits, fit for consumption and defined by the yellow coloring of the fruit, were collected in the municipality of Rio Brilhante – MS (21°48′ 07″ S 54°32′47″ W). The plant name is in accordance with the on-line database published by "The Plant List", accessed on May 02, 2016. A voucher specimen was deposited in the herbarium of the Federal University of Grande Dourados (UFGD), under the number 5033.

After selection, the healthy fruits were washed in tap water and sanitized in sodium dichloroisocianurate 0.66% (active chlorine 3%). The fruits were peeled and pulped and the pulp was dried in an oven at 40 °C with an air flow of 0.5 m/s for 72 h. The dried material was crushed, sieved using a 20 mesh sieve (for content uniformity), subsequently packaged in flexible polyethylene packages and stored at room temperature.

2.2. Oil extraction

The *Attalea phalerata* oil (APO) was obtained by solid-liquid extraction with hexane solvent PA (Vetec) at a ratio of one part of dewatered pulp powder to 3 parts of solvent 1:3 (w/v) under continuous stirring (150 rpm) for 3 days. The product was filtered and the solvent removed on a rotary evaporator IKA (Model RV 0.5 basic) under reduced pressure at 50 °C. The oil obtained is of vegetable origin, yellow-orange color, density 0.921 g/L and characteristic odor. The oil was then packaged in an amber bottle and stored at 9 ± 2 °C.

2.3. Analysis of fatty acid profile

The analyses were performed using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) equipped with a mass spectrometer detector (GC–MS Ultra 2010) employing a fused silica DBWax column (30 m length × 0.25 mm inside diameter, J & Scientific, USA). Helium was used as the carrier gas (99.999%) at a flow rate of 1.0 mL/min, and 1 μL sample volumes were injected in split mode 1:20. The initial oven temperature was 200 °C, followed by a ramp to 280 °C at a rate of 1 °C/min. The injector, detector, and transfer line temperatures were kept at 280 °C. The detector was operated in scanning mode using an electron ionization voltage of 70 eV and a mass range of 45–800 *m/z*. The identification of the compounds were performed using standards of the compounds with 98% of purity (Sigma) and the interpretation of mass spectra obtained for the samples were compared to the NIST21 and WILEY229 databases. The analysis was based on the methodology described by Fernandes et al. (2009).

2.3.1. Carotenoids HPLC analysis

The sample was analyzed using an analytical HPLC (LC-6AD, Shimadzu, Kyoto, Japão) system with a binary solvent. A photodiode array detector (PAD) was monitored at *λ * = 200–800 nm and column ODS HYPERSIL (C-18, 150 mm length × 4.6 mm inside diameter, particle size, 5 μm Thermo Electron Corporation). The elution was carried using acetonitrile 90% and ethyl acetate 10%, after 15 min reached acetonitrile 50% and ethyl acetate 50% and returned to the initial condition after 25 min. The flow rate and injected volume were 0.7 mL min-1 and 20 μL, respectively. All chromatographic analyses were performed at 22 °C. The analysis was based on the methodology described by Rodriguez-Amaya (1999).

2.4. Animals

Seventy *Wistar* rats (*Rattus norvegicus*, 8–9 weeks, weighing between 235 and 243 g for females and 413–434 g for males) from the bioterium of the State University of Maringa were maintained at the bioterium of the Federal University of Grande Dourados. The rats were housed in polypropylene cages at 23 °C, 12 h light and dark cycle and had *ad libitum* access to water and standard commercial rodent feed. From the seventy rats used in the whole experiment, 10 were destined for the acute toxicity test and 60 for the subacute toxicity test (OECD, 2008a, 2008b). The experiments were performed in accordance with the Ethical Principles in Animal Research and approved by the Ethics Committee in Animal Experimentation from the Federal University of Grande Dourados (protocol: 21/2015).

2.5. Acute toxicity

The oil extracted from the pulp of *Attalea phalerata* (APO) was administered at a dose of 2000 mg/kg, by gavage, to one female (OECD, 2008a) under an 8-hour period of fasting. Sequentially, at intervals of 48 h, the same dose was given to four adult female rats, totaling five treated animals (APO group 2000 mg/kg). A control group was treated with saline + Tween® 80 in order to establish a comparative negative control group (OECD, 2008a).

The animals were observed periodically during the first 24 h after the administration of the oil and thereafter, daily for 14 days. The five parameters of the Hippocratic screening suggested by Malone and Robichaud (1962) were evaluated during the experiment, as follows: conscious state (general activity); activity and coordination of motor system and muscle toning (response to tail touch and grip, straightening, strength to grab); reflexes (corneal and headset); activities on the central nervous system (tremors, convulsions, straub, sedation, anesthesia and ataxia) and activities on the autonomic nervous system (lacrimation, cyanosis, ptosis, salivation and piloerection). Body weight, water and food intake were also recorded daily (OECD, 2008a).

At the end of the observation period, all animals were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination. The organs (heart, lung, spleen, liver, kidney, uterus and right ovary) were removed, weighed and examined macroscopically.

2.6. Subacute toxicity

The animals were divided into five experimental groups ($n = 10$ animals/group, 5 males and 5 females). Four different doses of the APO were established according to the LD50 obtained in the acute toxicity test: APO group 125 mg/kg; APO group 250 mg/kg; APO group 500 mg/kg and APO group 1000 mg/kg. All doses of the APO were administered per group by gavage, daily, for 28 consecutive days. The control group (negative control group) received only the vehicle (saline + Tween 80) and another group entitled Satellite (Satellite Group) received the maximum dose of the APO (1000 mg/kg) for 28 days and remained untreated for 14 more days. The Satellite group is important in order to observe reversibility, persistence, or delayed occurrence of toxic effects related to the administration of substance analyzed (OECD, 2008b).

During the treatment, body weight, water intake, food consumption, behavioral parameters and possible signs of toxicity according to the Hippocratic screening were observed and recorded daily. Clinical

examination was performed once a day, individually and with the animal out of the cage. Blood samples were collected by cardiac puncture immediately after euthanasia and taken for hematological and biochemical analysis.

The following biochemical parameters were analyzed: total bilirubin, total cholesterol, triglycerides, glucose, electrolytes (sodium, potassium and calcium), liver function markers (alanine aminotransferase - ALT, aspartate aminotransferase - AST, gamma glutamyltransferase and alkaline phosphatase), renal (urea, creatinine and uric acid) and protein profile (albumin and total protein) using Cobas C111 Plus sing commercial kits (Roche). Hematological analysis measured total and differential leukocyte counts, erythrocytes, platelets, levels of hemoglobin, hematocrit and red cell distribution width, with the unit KX-21 (Sysmex).

After the blood collection process, the vital organs (heart, lung, kidney, liver and spleen) and reproductive organs (testes, epididymis, uterus and ovary) were weighed and dissected. Samples of vital organs were fixed in 10% buffered formalin. Subsequent to fixation, the fragments were cleaved, dehydrated with increasing absolute ethanol concentrations, diaphanized in xylene and embedded in paraffin. The sections were cut at a thickness of 5 μm, stained with hematoxylin and eosin (H & E) and mounted on glass slides for examination by light microscopy (Martey, Armah, & Okine, 2010).

2.7. Statistical analysis

The results were expressed as mean ± SEM. Student's *t*-test was used to compare the two groups in the acute toxicity test. The differences between groups of subacute toxicity test were determined by analysis of variance (one-way ANOVA) followed by Dunnett's test. *P*-values < 0.05 were set as the level of significance.

3. Results and discussion

3.1. Chemical composition

The knowledge of the chemical composition of natural products to be tested is necessary in order to identify the constituents performing certain activities, be it toxic or not (Berenguer-Rivas et al., 2013; Traesel et al., 2014) and certifies the equivalent quality (Marakhova, 2015). Table 1 presents the fatty acids values found in the APO analyzed by gas chromatography coupled to mass spectrometry.

Table 1
Fatty acid composition of oil extract of *A. phalerata* pulp.

Fatty acids	Crude oil (%)
Caprylic acid (C 8:0)	0.25 ± 0.01
Capric acid (C 10:0)	0.35 ± 0.02
Lauric acid (C 12:0)	2.19 ± 0.05
Myristic acid (C 14:0)	1.81 ± 0.01
Palmitic acid (C 16:0)	14.53 ± 0.09
Palmitoleic acid (C 16:1)	0.31 ± 0.02
Margaric acid (C 17:0)	0.05 ± 0.03
Stearic acid (C 18:0)	1.03 ± 0.04
Oleic acid (C 18:1)	57.18 ± 0.11
Linoleic acid (C 18:2)	20.08 ± 0.02
Alpha-linolenic (C 18:3)	0.79 ± 0.01
Arachidonic acid (C 20:0)	0.18 ± 0.01
Elaidic acid (C 20:1)	0.17 ± 0.01
Behenic acid (C 22:0)	0.14 ± 0.01
Lignoceric acid (C 24:0)	0.16 ± 0.02
Saturated fatty acids	20.69
Mono-unsaturated fatty acids	57.66
Poly-unsaturated fatty acids	20.87
Not identified	0.78

The results represent the means ± standard deviation of the analysis performed in triplicate.

The APO is mainly composed of saturated (20.69%) and unsaturated fatty acids (78.53%), in which 57.66% are monounsaturated and 20.87% are polyunsaturated. Oleic acid, linoleic acid and palmitic acid are predominant. Unsaturated fatty acids are predominant in the chemical composition of Cerrado fruits (Hiane et al., 2003; Traesel et al., 2014; Lescano, Iwamoto, Sanjinez-Argandoña, & Kassuya, 2015) and carry out important functions such as enhancing the immune system in specific cases, in inflammatory processes (Itariu et al., 2012; Pereira, Correia-da-Silva, Valentão, Teixeira, & Andrade, 2014) and in body weight reduction (Jump, 2002; Itariu et al., 2012). In specific cases such as ω-6 (linoleic acid), studies indicate its contribution to the reduction of total cholesterol (Navarro, Fernandez-Quintela, Churruca, & Portillo, 2006)

Although carotenoids have not been quantified, Fig. 1 shows qualitatively the presence of carotenoids in the APO, especially the β-carotene. The presence of α-carotene can be stated, as well as other unidentified carotenoids. Studies carried out with the oil and the flour of *Attalea phalerata* pulp present high levels of carotenoids (Hiane et al., 2003; Lima, Kassuya and Sanjinez-Argandoña, 2014) and corroborate the findings in this study. The presence of carotenoids in Cerrado fruits play an important role in inflammatory processes. Recent studies have shown its anti-edematogenic activity associated with inflammatory markers (Lima, Kassuya and Sanjinez-Argandoña, 2014; Lescano, Oliveira, et al., 2015).

3.2. Acute toxicology

In the acute toxicity test, the dose of 2000 mg/kg of the APO did not cause any death or changes in the parameters evaluated when compared to the control group. Animals exposed showed no statistical changes in weight gain, food and water consumption (Table 2). Macroscopic analysis and relative organ weight did not show any changes that indicate toxicity (Table 3).

The five parameters of the Hippocratic screening in the acute toxicity showed no behavioral changes. It is extremely important to measure the acute clinical signs in an attempt to establish a lethal dose that can cause the death of 50% of the animals tested (LD50) in order to obtain parameters for further clinical trials of toxicity, specifically subacute, subchronic and chronic toxicity (OECD, 2008a; ANVISA, 2013). In this study, therefore, it is assumed that the APO LD50 is above 2000 mg/kg.

3.3. Subacute toxicology

Subacute oral toxicity is an important factor to characterize dose-response studies after repeated administrations (28 days) (OECD, 2008b; ANVISA, 2013). Animals treated orally with the APO showed no changes

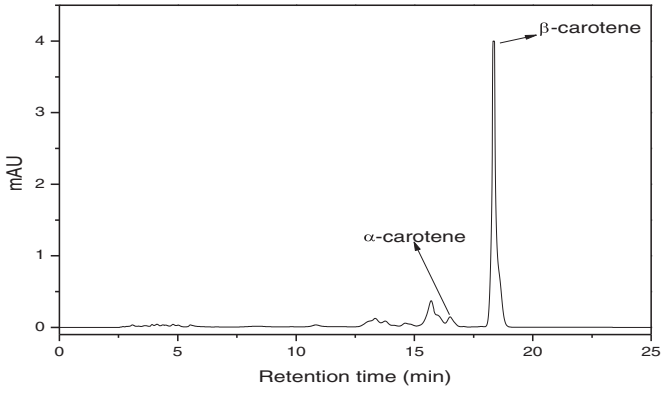

Fig. 1. Composition of carotenoids (α-carotene and β-carotene) in the oil extract of *A. phalerata* pulp.

Table 2
Body weight gain, food and water intake of rats observed for 14 days after a single exposition to the oil of *A. phalerata* pulp in acute toxicity assay and treated orally for 28 days in subacute toxicity assay.

	Acute toxicity		Subacute toxicity					
	Control	2000 mg/kg	Control	125 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	Satellite
Female								
Initial weight (g)	192.00 ± 7.92	180.00 ± 5.92	239.80 ± 20.86	243.20 ± 13.31	240.00 ± 27.95	235.40 ± 12.46	245.00 ± 9.90	241.40 ± 18.40
Final weight (g)	213.00 ± 12.93	208.00 ± 7.52	273.60 ± 24.40	251.20 ± 11.69	257.00 ± 21.44	252.20 ± 8.20	253.20 ± 6.42	275.60 ± 17.33
Body weight gain (%)	15.45 ± 4.07	11.14 ± 2.91	8.68 ± 2.06	3.18 ± 2.90	6.82 ± 3.67	6.68 ± 3.22	3.21 ± 3.99	7.08 ± 5.12
Food intake (g/day)	107.85 ± 10.65	104.15 ± 6.91	108.67 ± 10.33	95.00 ± 5.00	88.75 ± 13.75	92.50 ± 7.25	93.00 ± 8.50	96.33 ± 7.78
Water intake (ml/day)	168.46 ± 13.49	171.54 ± 13.43	195.83 ± 5.55	153.75 ± 10.63	177.50 ± 22.50	155.00 ± 10.00	185.00 ± 12.50	180.00 ± 20.00
Male								
Initial weight (g)	–	–	428.00 ± 42.17	434.20 ± 60.57	433.60 ± 30.09	413.60 ± 24.06	429.60 ± 13.46	421.75 ± 34.77
Final weight (g)	–	–	458.40 ± 45.63	456.80 ± 75.50	458.60 ± 29.13	447.60 ± 29,13	435.80 ± 24.22	458.25 ± 37.29
Body weight gain (%)	–	–	5.07 ± 1.95	4.63 ± 3.12	5.47 ± 1.78	7.43 ± 2.97	3,73 ± 2.18	6.23 ± 1.52
Food intake (g/day)	–	–	129.83 ± 16.22	118.50 ± 6.00	127.00 ± 6.00	133.50 ± 3.00	124.00 ± 8.00	130.83 ± 1.94
Water intake (ml/day)	–	–	206.67 ± 15.56	195.00 ± 10.00	195.00 ± 7.50	182.50 ± 22.50	193.75 ± 16.25	193.33 ± 2.78

Values expressed in mean ± standard deviation. $n = 5$ animals/group for acute toxicity and $n = 10$ animals/group for subacute toxicity.

in body weight gain, food and water consumption in both females and males (Table 2).

The relative organ weight of the animals submitted to subacute exposure are shown in Table 3. The male rats showed no changes and therefore, are not statistically different from the control group. However, the female rats showed a statistical difference only in the relative weight of the ovaries. Changes in the relative weights of the ovaries were expected since the control of the estrous cycle was not performed. These variations are due to ovarian activity which changes abruptly consequent to alterations in estradiol levels (Vilela, Santos Júnior, & Castro e Silva, 2007).

In subacute tests, other variables such as biochemical and hematological are considered. In particular, the knowledge of these parameters is of paramount importance and are presented in Tables 4 and 5, respectively.

The biochemical parameters are used to identify possible toxic effects of the substance tested within the organism and in isolated organs, since the toxic effects may be systemic or occur in target organs. In addition, the hematopoietic system is the most sensitive target to toxic substances, constituting an important parameter for assessing the physiological and pathological state of human beings and animals (Li et al., 2010).

Regarding the biochemical parameters of the female rats, statistical changes were identified for aspartate aminotransferase (APO group 500 mg/kg), total bilirubin (APO group 500 mg/kg), total protein (APO group 125 mg/kg), urea (satellite group), potassium (APO groups 500 and 1000 mg/kg), calcium (satellite group) and uric acid (APO group 500 mg/kg). In particular, alkaline phosphatase and albumin presented statistical differences in all doses tested (APO groups 125, 250, 500 and 1000 mg/kg) when compared to the control group. However, the differences observed are within the normal range for the species (Dantas, Ambiel, Cuman, Baroni, & Bersani-Amado, 2006; Lapchik, Mattaraia, & Ko, 2009; Lima, Lima, et al., 2014).

In male rats treated with the APO, the biochemical parameters that differed statistically from the control group were: aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, total bilirubin, total protein, urea (APO group 1000 mg/kg) and sodium (APO group 250 mg/kg). However, the differences observed are not biologically meaningful for the male and female rats treated with the APO, since the values are within the normal range for the species (Dantas et al., 2006; Lapchik et al., 2009; Lima, Lima, et al., 2014), showing thereby that the changes found should not be attributed to toxic effects of the plant, but rather to the physiological variability among the animals.

Table 3
Organ weight (g/100 g body weight) of rats observed for 14 days after a single exposition to the oil of *A. phalerata* pulp in acute toxicity assay and treated orally for 28 days in subacute toxicity assay.

	Acute toxicity		Subacute toxicity					
	Control	2000 mg/kg	Control	125 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	Satellite
Female								
Liver	4.70 ± 0.22	4.80 ± 0.13	3.30 ± 0.27	3.34 ± 0.15	3.22 ± 0.22	3.22 ± 0.23	3.28 ± 0.11	3.19 ± 0.22
Kidney	0.50 ± 0.05	0.48 ± 0.01	0.39 ± 0.03	0.37 ± 0.03	0.36 ± 0.03	0.34 ± 0.02	0.36 ± 0.02	0.38 ± 0.03
Spleen	0.25 ± 0.02	0.23 ± 0.01	0.22 ± 0.03	0.22 ± 0.03	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.02	0.21 ± 0.01
Heart	0.51 ± 0.03	0.50 ± 0.05	0.46 ± 0.07	0.43 ± 0.02	0.46 ± 0.03	0.46 ± 0.03	0.45 ± 0.03	0.47 ± 0.04
Lung	0.63 ± 0.07	0.66 ± 0.07	0.63 ± 0.06	0.66 ± 0.02	0.71 ± 0.03	0.70 ± 0.04	0.67 ± 0.06	0.64 ± 0.03
Ovary	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.03 ± 0.00*	0.02 ± 0.00*	0.03 ± 0.00*	0.03 ± 0.00*	0.08 ± 0.02*
Uterus	0.27 ± 0.11	0.28 ± 0.11	0.28 ± 0.12	0.33 ± 0.16	0.22 ± 0.06	0.21 ± 0.02	0.27 ± 0.07	0.28 ± 0.03
Male								
Liver	–	–	3.17 ± 0.20	3.21 ± 0.20	3.15 ± 0.24	3.28 ± 0.28	3.26 ± 0.21	3.28 ± 0.31
Kidney	–	–	0.34 ± 0.03	0.33 ± 0.04	0.33 ± 0.04	0.33 ± 0.02	0.34 ± 0.01	0.34 ± 0.03
Spleen	–	–	0.14 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	0.15 ± 0.02
Heart	–	–	0.41 ± 0.03	0.41 ± 0.04	0.42 ± 0.02	0.42 ± 0.05	0.44 ± 0.01	0.41 ± 0.02
Lung	–	–	0.51 ± 0.01	0.50 ± 0.07	0.50 ± 0.05	0.52 ± 0.04	0.60 ± 0.14	0.58 ± 0.09
Testis	–	–	0.38 ± 0.03	0.36 ± 0.02	0.34 ± 0.02	0.37 ± 0.04	0.37 ± 0.01	0.38 ± 0.03
Epididymis	–	–	0.13 ± 0.02	0.12 ± 0.03	0.12 ± 0.02	0.13 ± 0.01	0.13 ± 0.02	0.14 ± 0.03

Values expressed in mean ± standard deviation. $n = 5$ animals/group for acute toxicity and $n = 10$ animals/group for subacute toxicity.
* $P < 0.05$ (ANOVA/Dunnett) compared with the control group.

Table 4
Biochemical parameters of rats treated orally for 28 days in subacute toxicity with the oil of *A. phalerata* pulp.

	Subacute toxicity						Reference value
	Control	125 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	Satellite	
Female							
Aspartate aminotransferase (U/L)	107.36 ± 33.12	86.28 ± 25.55	74.98 ± 11.71	54.12 ± 12.91*	76.86 ± 17.53	83.72 ± 3.29	51–211[a]
Alanine aminotransferase (U/L)	34.00 ± 5.18	39.44 ± 8.68	36.50 ± 11.25	22.38 ± 5.54	32.88 ± 9.33	40.24 ± 4.28	32–63[a]
Alkaline phosphatase (U/L)	52.70 ± 1.71	57.64 ± 1.53*	58.78 ± 0.91*	58.20 ± 1.19*	57.52 ± 1.20*	53.20 ± 1.52	51–116[a]
Gamma-glutamyl transpeptidase (U/L)	1.78 ± 1.10	4.92 ± 1.44	5.22 ± 1.02	2.50 ± 0.83	1.88 ± 1.05	0.76 ± 0.58	1–6[a]
Total bilirubin (mg/dL)	0.08 ± 0.02	0.08 ± 0.02	0.07 ± 0.03	0.04 ± 0.01*	0.07 ± 0.03	0.10 ± 0.02	0.02–0.18[a]
Total protein (g/dL)	6.28 ± 0.15	4.79 ± 1.01*	5.97 ± 0.91	5.95 ± 0.41	6.05 ± 0.33	6.05 ± 0.24	4–6.9[a]
Albumin (g/dL)	1.48 ± 0.18	2.78 ± 0.11*	2.98 ± 0.16*	2.40 ± 0.47*	2.94 ± 0.09*	1.42 ± 0.29	1.3–3.8[a]
Blood urea nitrogen (mg/dL)	35.35 ± 4.63	28.87 ± 2.18	27.97 ± 4.24	29.33 ± 5.55	32.13 ± 4.10	44.06 ± 6.90*	32–54[b]
Creatinine (mg/dL)	0.38 ± 0.08	0.32 ± 0.08	0.24 ± 0.09	0.20 ± 0.07*	0.20 ± 0.10*	0.40 ± 0.07	0.2–0.5[a]
Sodium (mmol/dL)	147.04 ± 4.35	145.60 ± 2.88	145.20 ± 3.56	146.60 ± 2.07	144.40 ± 3.78	150.20 ± 1.64	142–151[a]
Potassium (mmol/dL)	4.84 ± 0.17	4.74 ± 0.11	4.58 ± 0.29	4.32 ± 0.19*	4.42 ± 0.22*	5.12 ± 0.33	3.6–6.7[a]
Calcium (mg/dL)	10.12 ± 0.18	9.74 ± 0.09	9.96 ± 0.18	9.94 ± 0.21	9.82 ± 0.16	11.44 ± 0.99*	9.5–11.5[a]
Cholesterol (mg/dL)	74.62 ± 9.57	62.34 ± 12.18	58.02 ± 13.67	38.10 ± 8.51*	62.76 ± 16.20	60.67 ± 9.02	42–92[b]
Triglycerides (mg/dL)	62.01 ± 13.50	45.07 ± 6.57	36.84 ± 8.21*	29.98 ± 6.33*	42.09 ± 11.92	58.54 ± 21.57	23–138[a]
Uric acid (mg/dL)	2.36 ± 0.51	3.30 ± 1.17	2.48 ± 0.74	1.02 ± 0.41*	2.04 ± 0.59	2.20 ± 0.43	1–3.2[a]
Glucose (mg/dL)	163.70 ± 42.32	225.98 ± 43.05	210.68 ± 54.10	116.90 ± 31.41	162.02 ± 40.38	187.90 ± 42.32	72–193[a]
Male							
Aspartate aminotransferase (U/L)	73.32 ± 3.29	83.12 ± 9.13	70.64 ± 24.53	72.66 ± 14.22	34.28 ± 4.81*	73.55 ± 12.96	39–93[b]
Alanine aminotransferase (U/L)	41.88 ± 5.94	40.74 ± 2.82	37.80 ± 12.92	37.34 ± 6.56	18.66 ± 2.48*	42.93 ± 7.50	17–50[a]
Alkaline phosphatase (U/L)	60.50 ± 4.28	56.38 ± 1.62	56.28 ± 1.90	55.34 ± 1.39	55.36 ± 2.67	60.15 ± 5.22	56–153[a]
Gamma-glutamyl transpeptidase (U/L)	5.15 ± 1.55	4.76 ± 1.59	4.40 ± 1.70	4.66 ± 1.59	1.88 ± 1.15*	5.18 ± 1.69	1–6[a]
Total bilirubin (mg/dL)	0.58 ± 0.13	0.38 ± 0.20	0.32 ± 0.20	0.42 ± 0.13	0.22 ± 0.18*	0.37 ± 0.30	0.1–0.89[a]
Total protein (g/dL)	6.16 ± 0.21	4.81 ± 1.33	6.13 ± 0.52	6.80 ± 0.48	8.60 ± 2.34*	6.08 ± 0.17	5.5–10.4[a]
Albumin (g/dL)	2.18 ± 0.08	2.64 ± 0.99	2.20 ± 0.07	2.34 ± 0.15	2.24 ± 0.30	2.40 ± 0.08	2–3.5[a]
Blood urea nitrogen (mg/dL)	30.36 ± 1.92	28.92 ± 1.55	31.00 ± 8.77	32.68 ± 9.21	18.28 ± 7.28*	26.78 ± 2.89	12.3–24.6[a]
Creatinine (mg/dL)	0.28 ± 0.04	0.34 ± 0.05	0.32 ± 0.04	0.28 ± 0.11	0.20 ± 0.04	0.30 ± 0.04	0.24–1.2[a]
Sodium (mmol/dL)	146.42 ± 1.06	146.68 ± 3.11	141.62 ± 4.71*	145.70 ± 2.28	142.20 ± 1.64	148.13 ± 1.55	125–143[a]
Potassium (mmol/dL)	4.94 ± 0.05	4.72 ± 0.41	4.88 ± 0.44	4.80 ± 0.10	4.79 ± 0.15	5.03 ± 0.22	3.9–7.9[a]
Calcium (mg/dL)	9.26 ± 0.57	9.86 ± 0.88	9.64 ± 0.34	9.40 ± 0.46	9.48 ± 0.19	9.25 ± 0.34	4.9–9.8[a]
Cholesterol (mg/dL)	94.40 ± 40.17	91.32 ± 14.60	83.16 ± 30.86	87.17 ± 17.33	40.55 ± 19.78*	76.90 ± 6.17	68.9–105.1[c]
Triglycerides (mg/dL)	88.87 ± 36.21	99.09 ± 51.87	68.38 ± 27.86	70.99 ± 15.25	36.56 ± 18.28	101.34 ± 32.16	57.27–106.7[c]
Uric acid (mg/dL)	1.54 ± 0.18	2.54 ± 1.07	1.58 ± 0.63	1.82 ± 1.34	1.36 ± 1.19	1.15 ± 0.68	1–3.2[a]
Glucose (mg/dL)	219.64 ± 79.37	202.66 ± 35.79	154.42 ± 32.86	178.34 ± 66.77	101.56 ± 49.34*	212.64 ± 64.49	72–193[a]

Values expressed in mean ± standard deviation. $n = 10$ animals/group for subacute toxicity.
* $P < 0.05$ (ANOVA/Dunnett) compared with the control group.
[a] Lima, Lima, et al., 2014.
[b] Lapchik et al., 2009.
[c] Dantas et al., 2006.

The control of biochemical parameters such as glucose, cholesterol and triglycerides are of utmost importance as they are closely linked to chronic diseases such as diabetes mellitus and hypertension (Orsatti & Dellalibera-Joviliano, 2005). The results for these parameters were satisfactory since the glucose and cholesterol in male animals showed statistical reduction at a dosage of 1000 mg/kg and numerical reduction in triglycerides. The female rats showed a statistical reduction in cholesterol (APO group 500 mg/kg), triglycerides (APO groups 250 and 500 mg/kg) and numerical decrease for glucose (APO group 500 mg/kg). Since these changes did not happen with rats treated with the highest dose (1000 mg/kg) of the APO, we suggest that this is not a dose-dependent effect.

The significant reduction in total cholesterol values coincides with the study conducted by Schmitt, Tran, Peach, Bauter, and Marone (2012) in which the toxicology of the algae oil enriched with DHA (docosahexaenoic acid) was evaluated and also with a study performed by Navarro et al. (2006), in which the reduction of cholesterol due to ω-6 (linoleic acid) was presented. These values indicate that the APO may perform hypolipidemic and hypocholesterolemic activities. Therefore, further studies regarding these activities should be performed.

Regarding the hematological values of female rats treated with the APO, the leukocytes (APO groups 125 and 500 mg/kg), hemoglobin (APO group 125 mg/kg) and monocytes (APO group 250 mg/kg) were statistically different from the control group. The male rats showed statistical differences in band neutrophils (satellite group), lymphocytes (APO group 500 mg/kg) and monocytes (APO group 250 mg/kg). However, all the values are within the normal range for the species (Dantas et al., 2006; Lapchik et al., 2009; Lima, Lima, et al., 2014).

Macroscopic analysis in all tested doses of the APO showed no changes in vital and reproductive organs of the treated animals. Likewise, the histological assessment showed no signs suggestive of toxic effects (Fig. 2). These toxic signs would be characterized by congestion, leukocyte infiltration, extravasation of blood, degeneration, necrosis, apoptosis and fibrosis in the organ tissues analyzed histologically (Cunha et al., 2009).

These results corroborate the hematologic and biochemical analyzes, suggesting that the APO can be considered safe and presenting similar results to those found in previous toxicological studies of the oil extracted from *Acrocomia aculeata* pulp (Traesel et al., 2014) and *Dimocarpus longan* Lour. seed extract (Worasuttayangkurn et al., 2012).

This is the first study that presents the acute and subacute toxicological profile of *Attalea phalerata* and explains the absence of toxicity of the oil extracted from the pulp of its fruits. This suggests the safe use of the fruit and urges the scientific community to more specific studies, either in the development of food products or pharmaceutical applications.

4. Conclusion

The APO is chemically rich in fatty acids, carotenoids and fostered a considerable reduction in cholesterol and triglycerides levels of the

Table 5
Hematological parameters of rats treated orally for 28 days in subacute toxicity with the oil of *A. phalerata* pulp.

	Subacute toxicity						
	Control	125 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	Satellite	Reference value
Female							
Leukocytes (10^3/μL)	3.18 ± 0.74	5.04 ± 1.17*	4.58 ± 0.38	4.26 ± 1.01	5.42 ± 0.66*	4.92 ± 0.47	2.3–9.9[a]
Erythrocytes (10^6/μL)	8.07 ± 0.27	7.57 ± 0.21	7.80 ± 0.29	7.50 ± 0.18	7.86 ± 0.21	8.32 ± 0.47	5.4–8.5[b]
Hemoglobin (g/dL)	15.24 ± 0.87	14.08 ± 0.46*	14.68 ± 0.44	14.30 ± 0.57	15.00 ± 0.46	15.04 ± 0.57	11.1–17.1[a]
Hematocrit (%)	47.18 ± 3.35	45.96 ± 1.56	46.90 ± 1.81	45.16 ± 1.50	46.74 ± 1.17	47.14 ± 2.38	27.2–48.5[a]
Platelets (10^3/μL)	786.20 ± 162.15	914.20 ± 91.79	847.20 ± 155.09	886.40 ± 122.56	988.20 ± 67.86	909.00 ± 109.79	760–1313[a]
RDW (%)	12.54 ± 0.41	11.98 ± 0.46	12.92 ± 0.96	12.28 ± 0.40	12.66 ± 0.53	12.68 ± 0.21	12.7–18.2[c]
Segmented neutrophils (%)	23.40 ± 4.62	24.00 ± 4.90	21.64 ± 5.10	29.30 ± 5.56	20.18 ± 6.35	19.40 ± 5.46	NF
Band neutrophils (%)	1.60 ± 0.89	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.2 ± 0.84	NF
Lynphocytes (%)	71.80 ± 6.42	72.60 ± 5.64	60.54 ± 29.63	67.70 ± 5.56	76.42 ± 6.85	76.80 ± 6.53	30.1–95.0[a]
Monocytes (%)	1.40 ± 0.55	2.20 ± 0.45	2.40 ± 0.89*	2.00 ± 0.00	2.20 ± 0.45	1.80 ± 0.45	1–13.5[a]
Eosinophils (%)	1.40 ± 0.55	1.20 ± 0.45	1.20 ± 0.45	1.20 ± 0.45	1.20 ± 0.45	0.80 ± 0.84	0–2[a]
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0–0.1[a]
Male							
Leukocytes (10^3/μL)	5.16 ± 1.21	4.16 ± 2.48	3.86 ± 1.18	3.86 ± 0.79	4.20 ± 1.62	4.98 ± 1.23	3.41–13.7[a]
Erythrocytes (10^6/μL)	8.58 ± 0.23	8.02 ± 0.28	7.95 ± 0.22	8.03 ± 0.45	8.15 ± 0.74	8.71 ± 0.17	5.4–8.5[b]
Hemoglobin (g/dL)	14.80 ± 0.44	14.36 ± 0.49	14.02 ± 0.35	14.54 ± 0.93	14.70 ± 1.32	14.95 ± 0.57	10.2–17.8[a]
Hematocrit (%)	47.38 ± 1.81	44.86 ± 1.64	43.56 ± 1.24	44.90 ± 2.62	45.64 ± 4.42	48.10 ± 1.99	23.8–51.9[a]
Platelets (10^3/μL)	848.80 ± 99.09	828.40 ± 95.02	778.40 ± 78.95	829.80 ± 88.24	862.80 ± 87.29	875.00 ± 25.78	727–1351[a]
RDW (%)	14.10 ± 0.46	13.88 ± 0.41	14.22 ± 0.62	13.94 ± 0.43	14.40 ± 0.51	14.90 ± 1.07	13–18.4[c]
Segmented neutrophils (%)	25.80 ± 4.15	30.00 ± 2.45	33.20 ± 7.53	34.20 ± 6.46	33.40 ± 5.41	22.25 ± 6.75	NF
Band neutrophils (%)	3.55 ± 1.71	3.27 ± 1.00	2.98 ± 1.82	1.80 ± 0.84	1.80 ± 1.10	1.00 ± 0.00*	NF
Lynphocytes (%)	70.20 ± 4.15	61.20 ± 3.63	61.40 ± 8.14	59.00 ± 5.43*	60.00 ± 7.11	73.75 ± 6.75	43.1–93.7[a]
Monocytes (%)	2.00 ± 0.00	4.20 ± 1.10*	2.80 ± 1.48	3.40 ± 0.55	3.60 ± 1.52	2.00 ± 0.00	1.-15.2[a]
Eosinophils (%)	1.00 ± 0.00	1.60 ± 0.89	1.20 ± 0.45	1.60 ± 0.55	1.20 ± 0.45	1.00 ± 0.00	0–3.6
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0–3[a]

RDW: Red Cell Distribution Width. Values expressed in mean ± standard deviation. $n = 10$ animals/group for subacute toxicity.
NF – not found.
* $P < 0.05$ (ANOVA/Dunnett) compared with the control group.
[a] Lima, Lima, et al., 2014.
[b] Lapchik et al., 2009.
[c] Melo, Dória, Serafini, & Araújo, 2012.

treated rats. Acute and subacute toxicology experiments suggested that the APO has low toxicity since it did not cause any lethality as well as no changes in hematological and histological parameters. Therefore, other studies on chronic toxicity, reproductive toxicity, hypoglycemic potential, hypolipidemic potential and hypocholesterolemic potential are necessary in order to proceed to clinical studies of this plant.

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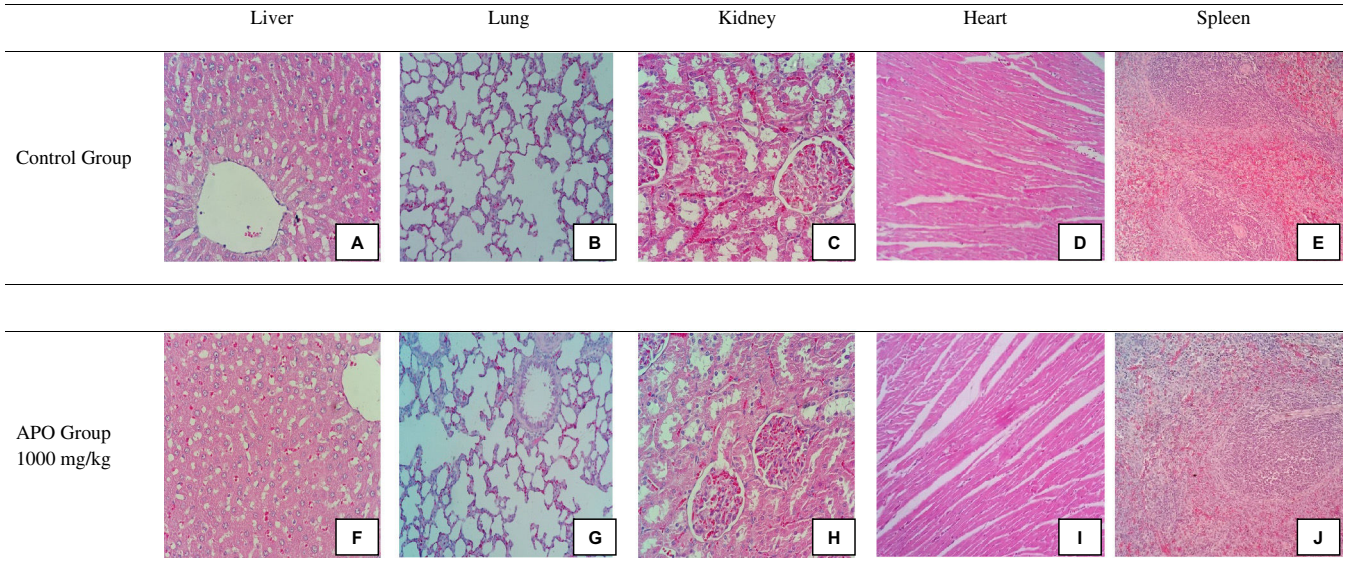

Fig. 2. Histopathological assessment of the organs of rats in the control and treated groups (1000 mg/kg of the APO) in the subacute toxicity test. HE. Zoom: 20× and 40×.

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5. ANEXOS

5.2. Artigo publicado no periódico Plos One

Artigo intitulado ‘Study on the cytotoxic, genotoxic and clastogenic potential of *Attalea phalerata* Mart. ex Spreng. oil pulp *in vitro* and *in vivo* experimental models’’, publicado em periódico com Fator de Impacto 3,052 e Qualis A2 em Medicina II.

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PLOS ONE

RESEARCH ARTICLE

Study on the Cytotoxic, Genotoxic and Clastogenic Potential of *Attalea phalerata* Mart. ex Spreng. Oil Pulp *In Vitro* and *In Vivo* Experimental Models

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Abstract

Attalea phalerata Mart. ex Spreng. (*Arecaceae*), popularly known as "bacuri", is used in Brazilian folk medicine. Its oil is used orally to relieve pulmonary congestion and joint pain. In topical applications, it is applied as an effective hair tonic and anti-dandruff. The *in natura* pulp and its nuts are used as food because of its nutritional value. Despite its use in folk medicine, there is a lack of data regarding its *in vivo/in vitro* cytotoxic/genotoxic and clastogenic effects. Therefore, in this study, we evaluated the cytotoxic, genotoxic and clastogenic effects of *Attalea phalerata* Mart. ex Spreng. oil (APMO) *in vitro* and *in vivo*. For the analysis of cytotoxic potential, the *Artemia salina* and MTT (3-(4,5-dimethizzol-zyl)-2,5-diphenyltetrazolium bromide) assays were performed. Possible cytotoxic, genotoxic and clastogenic effects of APMO intake were determined by performing the comet and micronucleus assays. Male and female *Wistar* rats were orally treated with doses of 125, 250, 500 or 1000 $mg.kg^{-1}$ of the APMO daily for 28 consecutive days (four weeks). The results showed that the APMO did not induce cell death in the experiments of *Artemia salina* and MTT, indicating that it has no cytotoxicity. The APMO did not cause significant damage to the DNA of the rats in the four doses used when compared to the negative control group (saline + Tween® 80). The APMO did not present any significant increase in micronucleated polychromatic erythrocytes (MNPCEs) for the four tested doses. When compared to the positive control group, all groups (comet and micronucleus tests) were statistically different. These data suggest that the administration of *Attalea phalerata* Mart oil. ex Spreng does not cause cytotoxicity, genotoxicity and clastogenicity in experimental models *in vitro* and *in vivo* following oral administration in this study.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The therapeutic use of natural products, including medicinal plants, has become increasingly common. Pharmacological investigations are performed to identify bioactive compounds with beneficial abilities to the human organism in order to develop new drugs with reduced side effects [1, 2]. Due to the biological activity of these compounds, the evaluation of the toxic potential is essential for the safe and effective use of medicinal plants [3, 4]. Besides that, the phytochemical study of plants and foods with medicinal properties are important in order to learn about the natural compounds and their mechanisms of action [5, 6].

Attalea phalerata Mart. ex Spreng. (*Arecaceae*) belongs to the genus Attalea Kunth and is popularly known as "bacuri" or "acuri". In folk medicine, the pulp oil is used as a hair tonic and anti-dandruff. Orally, the oil is applied to relieve pulmonary congestion, joint pain and studies indicate the anti-inflammatory properties of the pulp oil due to its chemical composition [7, 8, 9]. The almond and the fruit pulp are consumed by the local population as they present high nutritional value. Some fruits of the Cerrado have similar chemical constituents, such as carotenoids and fatty acids. These compounds are of paramount importance to the pharmaceutical industry because of its pharmacological potential. Among its activities are the maintenance of the immune system and prevention of chronic diseases [10–12]. Recently, studies with Cerrado fruits have demonstrated pharmacological activities in rats [7, 13, 14].

Early in the development of a pharmaceutical there are a number of preliminary tests that basis for validating the safety of the natural chemical compounds and potentially the development of new pharmaceutical products [15–17]. According to the literature, the *Artemia salina* test is considered one of the most useful tools for preliminary tests assessing general toxicity at low cost and shows good correlation with cytotoxic activity [18, 19]. With the MTT (3-(4,5-dimethizzol-zyl)-2,5-diphenyltetrazolium bromide) assay, it is possible to assess the cytotoxicity and this test is used with great success for estimating the number of viable cells in the initial screening for new drugs [20]. Through the comet assay it is possible to evaluate the genotoxic potential of a substance and the micronucleus assay provides information on cytotoxic and clastogenic effects. The use of both tests jointly is recognized by international regulatory agencies [21, 22], since the assays are very sensitive and detect breaks in the chromosomal and chromatid levels [23]. Therefore, the present study was designed to investigate the cytotoxic, genotoxic and clastogenic potentials of the pulp oil of *Attalea pha*lerata Mart. ex Spreng. in *in vitro* and *in vivo* experimental models.

Material and Methods

Material and sample preparation

The *Attalea phalerata* Mart. ex Spreng. fruit was collected from a public area of the municipality of Rio Brilhante—MS, 21° 55' 04.6"S and 54° 32' 06.8"W and altitude 6 m. No specific permissions were required to access the area in which the fruits were collected since it is a public area (highway). The species (*A. phalerata* Mart.) used in this study is not an endangered or protected species. The plant name is in accordance with the on-line database published by "The Plant List", accessed on May 02, 2016. A voucher specimen of the species was deposited in the UFGD DDMS Herbarium under the number 5033. After the collection, the healthy fruits were washed with tap water and immersed in a sanitized solution of sodium dichloroisocyanurate 0.66% (content of active chlorine of 3%) for 10 minutes. Afterwards, the fruits were peeled, pulped and the pulp was subsequently dried in an oven at 40°C with an air flow of 0.5 $m.s^{-1}$ for 72 hours. The dried material was crushed, sieved through a 20-mesh sieve for powder

uniformity, subsequently packaged in flexible polyethylene packages and stored at room temperature.

Oil extraction

The *Attalea phalerata* Mart. ex Spreng. oil (APMO) was obtained by Soxhlet extraction with hexane solvent PA (Vetec) at a ratio of 3 part dewatered pulp powder to 6 parts solvent 3:6 ($w.v^{-1}$) under continuous extraction until sample exhaustion. The product was filtered, the solvent removed and the oil stored in low temperature (3°C) until further analysis.

Chemicals

For the analysis of carotenoids by high-performance liquid chromatography, β-carotene (≥97% Sigma-Aldrich), α-carotene (≥98% Sigma-Aldrich), ethyl acetate UV/HPLC (Analitica) and acetonitrile UV/HPLC (Merck) were used.

For the *Artemia salina* assay, artificial sea water, brine shrimp eggs Maramar®, absolute methyl alcohol (Sigma-Aldrich) and potassium dichromate (Sigma-Aldrich) was used. For the MTT assay, cells of human colon carcinoma cell lines (T84) were purchased from the Institute of Molecular Medicine, University of Texas Health Science Center (one year before the onset of the experiments (with mycoplasma tests conducted)), medium DMEM-F12 (Sigma-Aldrich), fetal bovine serum (Gibco), penicillin antibiotic 50 $UI.mL^{-1}$ (Gibco), streptomycin 50 $\mu g.mL^{-1}$ (Gibco) and triton X-100 (Proquímios) were used.

For the comet assay, the following reagents were used: hydrochloric acid (CRQ), low melting point agarose (Agargen), standard agarose (Agargen), absolute ethanol (CRQ), ethidium bromide (Ludwig-Biotec), cyclophosphamide (Sigma-Aldrich) ethylenediaminetetracetic acid (Proquímios), heparin (Critália), potassium chloride (Vetec), monobasic anhydrous potassium phosphate (Scientific Exodus), pH kits (Impex), dibasic sodium phosphate (Dynamics), sodium chloride (Impex), sodium hydroxide (Vetec), saline (Arboretum), tris (Vetec) and triton X-100 (Proquímios).

For micronucleus test, hydrochloric acid (CRQ), absolute ethanol (CRQ), absolute methanol (Sigma-Aldrich), anhydrous monobasic potassium phosphate (Ex Scientific), dibasic sodium phosphate (dynamics), cyclophosphamide (Sigma- Aldrich), Giemsa (Laborclin), sodium hydroxide (Vetec), saline (Arboretum) and fetal bovine serum (Laborclin) were used. In order to prepare the oil used in the gavage administration, Tween® 80 and saline (Arboretum) were used. For the euthanasia procedure, the anaesthetic Isoflurane (Cristalia) was applied.

Carotenoids characterization: *High performance liquid chromatography*

The sample was analyzed using an analytical high performance liquid chromatography (HPLC) (LC-6AD, Shimadzu, Kyoto, Japan) system with a binary solvent. A photodiode array detector (PAD) was monitored at $^{*}\lambda^{*}$ = 200–800 nm and column ODS HYPERSIL (C-18, 150 mm length x 4,6 mm inside diameter, particle size, 5 μm Thermo Electron Corporation). The elution was carried out using 90% acetonitrile, 10% ethyl acetate, in 15 min 50% acetonitrile, 50% ethyl acetate, in 25 min returning to the initial condition. The flow rate and injected volume were 0.7 mL min^{-1} and 20 μL, respectively. All chromatographic analyses were performed at 22°C. The content estimation of the compounds in the *A*. *phalerata* oil was performed by external calibration employing HPLC. A linear least-square regression of the peak areas as a function of the concentrations was performed to determine the correlation coefficients. The equation parameters (slope and intercept) of the standard curve were used to obtain the concentration values for the samples.

Artemia salina assay

The lethality of APMO was evaluated using the brine shrimp lethality test (*Artemia salina*). The procedure was performed in accordance with Martin's methodology [24]. Standard and sample solutions of the APMO (0.5–50 $mg.mL^{-1}$ concentration) were prepared in methanol. After the solvent evaporation, 10 newborn *Artemia salina* nauplii were placed in test tubes containing 5 mL of artificial seawater. The assays were repeated four times for each concentration. Plates were observed after 24 hours of incubation using a Research Stereomicroscope System (Olympus SZX9) and the survival rate (%) was logged.

MTT cytotoxicity assay

MTT (3-(4,5-dimethizzol-zyl)-2,5-diphenyltetrazolium bromide) assay was performed in accordance with Quassinti's methodology [25] with some modifications. The T84 cells were incubated with DMEM-F12 with 10% fetal bovine serum in 96-well plate (well volume: 0.34 ml; growth surface: 0,31cm 2, inner diameter: 6.4 mm, dimensions (mm): 128 x 85 x 22) in a humidified incubator with 5% CO_2 at 37°C for 72 hours until reaching 80–100% confluence. The cells were cultured in accordance with the ATCC protocol. Different concentrations of the APMO were prepared (1, 2.5, 5, 10 and 20 $mg.mL^{-1}$) and incubated for 1, 24 and 48 hours. The medium was replaced by serum-free medium and 10 uL (5 $mg.mL^{-1}$) of the MTT volume was added to it. Then, plates were incubated for 1–3 hours at 37°C (until reaching purple coloration), the medium removed and replaced by the solution of isopropyl alcohol/HCl 0.04 M. The mixture was stirred vigorously in a shaker until the purple color was homogeneous. The optical density was measured by a microplate reader (SpectraMax-M2^e –software SPECTRAmax M2e ROM versão 2.1) at 570 nm. Triton (1%) was used as positive control for cell viability.

Animals and exposures

This study was carried out using 30 male and 30 female *Wistar* rats (*Rattus norvegicus*). The animals were at 8–10 weeks old, weighing between 235–243 g for females and 413–434 g for males. The animals obtained from the State University of Maringa were housed in polypropylene rodent cages under controlled temperature (23°C), humidity (40–60%) and 12h light/dark cycle with *ad libitum* access to water and standard commercial feed.

The rats were divided into six experimental groups of ten animals each (five males and five females). The APMO was diluted in saline + Tween 80® and administered by gavage, in doses of 125, 250, 500 or 1000 $mg.kg^{-1}$ body weight, daily for 28 consecutive days. The doses were chosen based on our subchronic toxicity studies in rats, and following the dose limit recommended by the OECD [26] for subacute treatments in toxicology assays. The negative control group received saline + Tween 80®. The positive control group received an intraperitoneal injection of cyclophosphamide of 20 $mg.kg^{-1}$ body weight. The animals were observed daily during the whole experiment. Signs related to animal health and welfare were evaluated according to the Hippocratic screening, assessment of body weight, feed and water intake. If any animal presented any signs that would compromise its welfare, it would be euthanized in order to prevent suffering.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the Ethics Committee on Animal Research of the Federal University of Grande Dourados, under the permit number 21/2015. The animals used in this study were euthanized with inhalational anesthetic (isoflurane) in gas chamber followed by exsanguination.

Comet assay

The comet assay (Single Cell Gel Electrophoresis—SCGE) was performed according to the protocol described by the OECD [18] with some modifications. Blood samples from all animals were obtained by caudal puncture with a needle soaked in Heparin and 40 uL of the blood was transferred to a micro-tube containing 120 uL of low melting point agarose (1.5%) at 37°C. The mixture was homogenized and transferred into pre-coated slides with 5% standard agarose. Two slides were prepared per animal. The slides were covered with coverslips and maintained in the dark at 3°C for 20 minutes.

After the waiting time, the coverslips were removed and the slides were immersed in lysis solution [89 mL stock lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0), and 89 mL distilled water], 1 mL Triton X-100, and 10 mL dimethyl sulfoxide for 1 hour at 3°C in the dark. After 1 hour, the slides were placed into the electrophoresis unit (Loccus) containing buffer solution at pH > 13 (300 Mm NaOH and 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) at 4°C for 20 min in the dark, in order to allow the denaturation of the DNA.

The electrophoresis step was performed at 4°C for 20 min at 300 mA and 25 V, protected from light. After the run, the slides were submerged in neutralization buffer using 0.4 M Tris-HCl at pH 7.5 for three cycles of 5 min each, dried, fixed with 100% ethanol for 10 min, and stored for later analysis. Finally, the slides were stained with ethidium bromide (20 $mg.mL^{-1}$) and covered with a coverslip. All material was analyzed by fluorescence microscopy (Nikon—H550S—40X magnification), equipped with an excitation filter (420–490 nm) and a barrier filter (520 nm).

Only individual nuclei were analyzed and the extent of damage in DNA was assessed by examining 100 cells per animal. The extent of DNA which migrated during electrophoresis and the migration distance reflects the amount and size of the DNA fragments. The comet findings were classified as follows: class 0—no damage; class 1—comet tail shorter than the diameter of the nucleoid; class 2—comet tail once or twice the diameter of the nucleoid and class 3—comet tail greater than twice the size of the nucleoid. Comets with a turbid aspect or a head that was too small were excluded from the analyses, as they could represent dead cells [27].

The damage index (DI) and damage frequency (DF) were calculated based on the readings. The DI was calculated by multiplying the number of cells damaged by the value assigned to this damage. Therefore, this value ranges from 0 (no damage: 0 x 100 cells) to 300 (maximum damage: 3 x 100 cells). DF was calculated by summing the number of damaged cells (class 1, 2 or 3) and ranged from 0 (no damage) to 100 (maximum damage).

Micronucleus assay

The assay was performed following the protocol recommended by the OECD [19] with some modifications. The same animals used in the comet assay were also used in this test. The femoral bone marrow was removed, washed using 1 mL of bovine fetal serum and centrifuged (Spinlab—SL-5AM) for 5 minutes at 1000 rpm. The supernate was discarded and the pellets were used to make smears on slides. The slides were coded for blind analysis, fixed with methanol for 10 minutes and stained with Giemsa for 15 minutes. For the analysis of cells, 2000 polychromatic erythrocytes (PCE) per animal were evaluated for the presence of micronuclei in order to assess the clastogenic capacity of the APMO. The average number of micronucleated polychromatic erythrocytes (MNPCEs) per individual was used as the experimental unit and the standard deviation was based on the differences among animals of the same group.

To detect any cytotoxic effects, the relationship between PCE/NCE (polychromatic erythrocytes/normochromatic erythrocytes) was determined by analyzing 200 random erythrocytes

per animal. The cells were analyzed by light microscope (Olympus—CX41) under magnification of 1000x.

Statistical Analysis

The results were expressed as mean ± SEM. The differences between groups were determined by analysis of variance (one-way ANOVA) followed by Tukey's test. P-values less than 0.05 were considered significant.

Results and Discussion

Chemical composition

The characterization of carotenoid by HPLC of APMO showed two major compounds (Table 1). The main compounds found in the oil are β-carotene and α-carotene, in which β-carotene is the major one. Due to the high concentration of the carotenoids, the results indicate that the oil will exhibit activities and act as a potential preventer of chronic diseases [1, 22]. Previous pharmacological studies performed with fruit pulp oils from the Cerrado with high levels of carotenoids, have shown great anti-edematogenicpotential in rats [7, 13] Quantitative values for β-carotene were evaluated in other fruits from the Cerrado, such as the *Caryocar brasiliense* Camb. (42.4 μg/g), *Annona crassiflora* Mart. (19.7 μg/g), *Eugenia dysenterica* DC. (3.96 μg/g), *Hymenaea stigonocarpa* Mart. (3.96 μg/g) e *Hancornia speciosa* Gomes (0.6 μg/g) [28].

Artemia salina assay

The results of the brine shrimp nauplii survival rates were not statistically different ($p < 0.05$) in all doses of the APMO when compared to the negative control group. As expected, the experimental groups (10, 50, 100, 250, and 500 $\mu g.mL^{-1}$ of oil) and the negative control group presented statistical differences when compared to the positive control group ($K_2Cr_2O_7$ 0.7%). These results demonstrate the low cytotoxicity of the APMO in microcrustaceans (Fig 1).

MTT assay

The MTT results (Fig 2) showed the absence of cytotoxicity of the APMO at doses of 1, 2.5, 5, 10 and 20 mg/mL^{-1} (dose and time dependent manner) where the experimental doses did not differ statistically ($p < 0.05$) when compared to the negative control group (medium). However, the experimental groups and the negative control group showed statistical differences when compared to the positive control group (Triton 1%).

Table 1. Characterization of the α-carotene and β-carotene values by chromatographic analysis in *Attalea phalerata* Mart. ex Spreng. oil (APMO).

	Standards	
	α carotene	β carotene
Retention time (min)	17.35	18.51
Linear Range (μg)	3–12	0.30–2
Intercept(a)	3995.30	4314.30
Slope (b)	4009.30	16411.00
Determination coefficient (R^2)	0.993	0.991
Carotenoids concentration (μg/g)	10.93	61.72

Linear regression, formula: y = a + bx, where y = ratio of peak areas; x = concentration (μg); a = intercept and b = slope.

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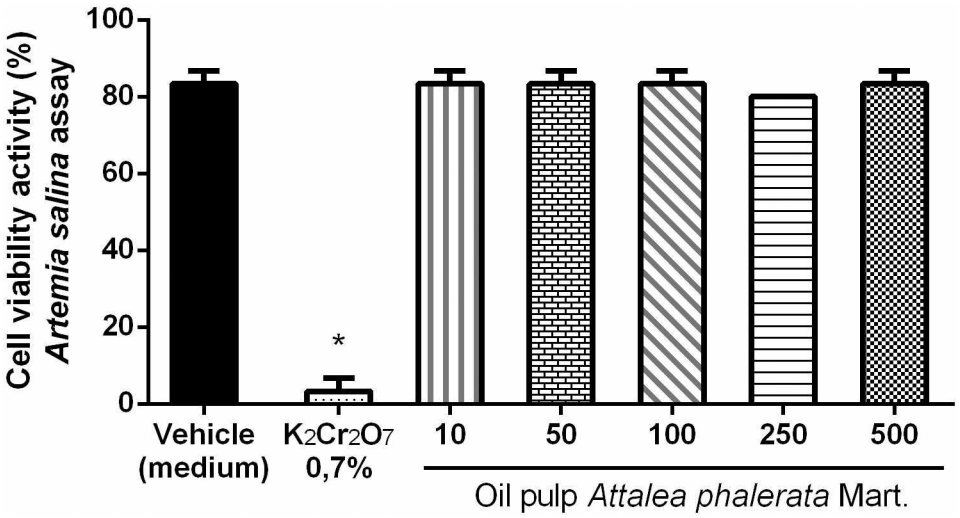

Fig 1. Effects of the APMO on *Artemia salina* assay. *Artemia salina* nauplii were treated with 10, 50, 100, 250 and 500 μg.mL^{-1} of oil or vehicle (medium) for 24 h. Results are presented as mean + SEM. n = 4; One-way ANOVA followed by Tukey´s test; *p<0.05 compared to vehicle (medium) and experimental group (10, 50, 100, 250 and 500 μg.mL^{-1} of oil).

doi:10.1371/journal.pone.0165258.g001

Comet and Micronuclei assays

Comet and micronuclei assays are effective tests to determine the cytotoxicity, genotoxicity and clastogenicity. The *in vivo* release from mammalian cells under alkaline conditions in the comet assay was used in our study, according to the OECD [19]. This test provides a broad-spectrum detection of the levels of DNA damage, such as DNA denaturation and detection of

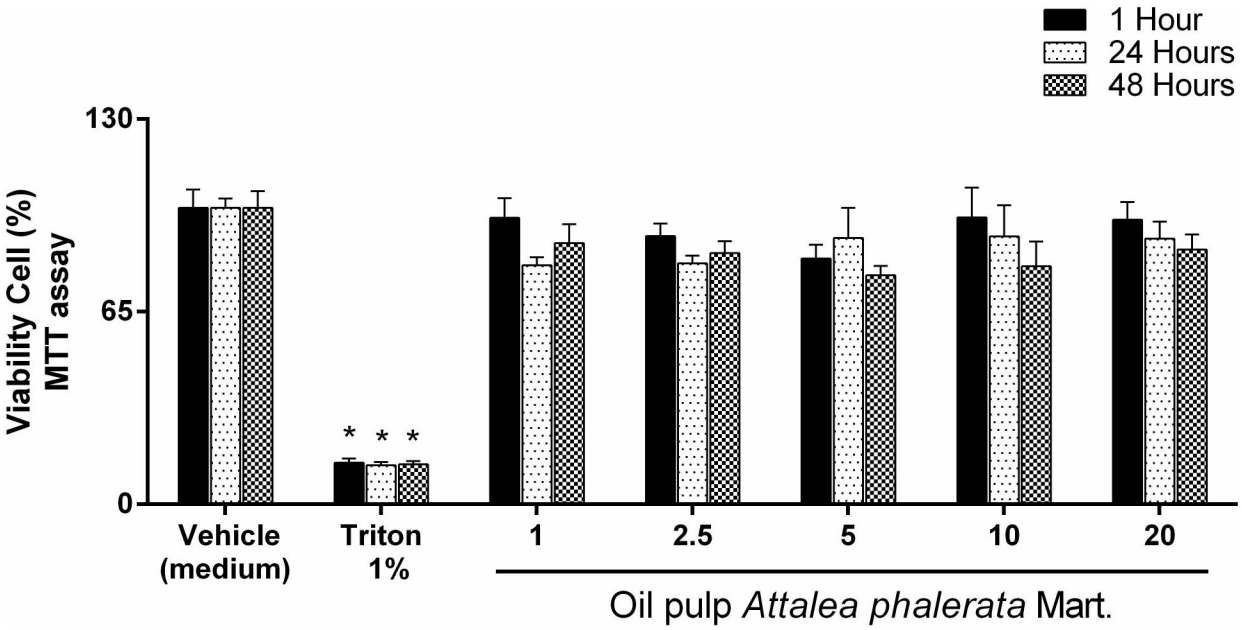

Fig 2. Effects of the APMO on cell metabolic activity by MTT assay. T84 cells were treated with 1, 2.5, 5, 10 and 1 mg.mL^{-1} of oil or vehicle (medium) for 1h, 24h and 48h. Results are presented as mean + SEM. n = 8; One-way ANOVA followed by Tukey´s test; *p<0.05 compared to vehicle (medium) and experimental group (1, 2.5, 5, 10 and 1 mg.mL^{-1} of oil).

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Table 2. Effect of the APMO in exposed rats as measured by the comet assay. The groups were treated with 125, 250, 500 and 1000 mg.kg^{-1} of the *Attalea phalerata* Mart. ex Spreng. oil (APMO) daily for 28 consecutive days (four weeks) and the negative control group received the vehicle (saline + Tween 80®). The positive control group received intraperitoneal injection of cyclophosphamide at 20 mg.kg^{-1}.

Groups	Damage frequency	Classes of damage				Damage index
		0	1	2	3	
Female						
Negative Control	6.20 ± 0.73^{a}	93.80 ± 0.73	6.20 ± 0.73	0.00 ± 0.00	0.00 ± 0.00	6.20 ± 0.73^{a}
Cyclophosphamide	85.40 ± 1.75^{b}	14.60 ± 1.75	41.20 ± 1.07	38.80 ± 1.68	5.40 ± 1.83	135.00 ± 5.07^{b}
125 mg.kg^{-1} of APMO	8.40 ± 2.01^{a}	91.60 ± 2.01	8.00 ± 2.07	0.40 ± 0.24	0.00 ± 0.00	8.80 ± 1.98^{a}
250 mg.kg^{-1} of APMO	8.60 ± 2.80^{a}	89.25 ± 2.32	10.75 ± 2.32	0.00 ± 0.00	0.00 ± 0.00	8.60 ± 2.80^{a}
500 mg.kg^{-1} of APMO	6.20 ± 0.58^{a}	93.80 ± 0.58	5.80 ± 0.66	0.40 ± 0.24	0.00 ± 0.00	6.60 ± 0.60^{a}
1000 mg.kg^{-1} of APMO	7.80 ± 2.18^{a}	90.25 ± 1.25	8.75 ± 1.11	0.75 ± 0.48	0.25 ± 0.25	8.80 ± 2.56^{a}
Male						
Negative Control	6.60 ± 1.21^{a}	93.40 ± 1.21	6.00 ± 1.38	0.60 ± 0.40	0.00 ± 0.00	7.20 ± 1.16^{a}
Cyclophosphamide	89.60 ± 1.50^{b}	10.40 ± 1.50	41.80 ± 1.69	40.60 ± 1.50	7.20 ± 1.98	144.60 ± 2.87^{b}
125 mg.kg^{-1} of APMO	11.80 ± 0.97^{a}	88.20 ± 0.97	9.80 ± 1.16	2.00 ± 0.71	0.00 ± 0.00	13.80 ± 1.24^{a}
250 mg.kg^{-1} of APMO	13.20 ± 3.73^{a}	87.00 ± 3.79	12.80 ± 3.83	0.40 ± 0.40	0.00 ± 0.00	13.60 ± 3.67^{a}
500 mg.kg^{-1} of APMO	9.00 ± 1.52^{a}	91.00 ± 1.52	8.80 ± 1.50	0.20 ± 0.20	0.00 ± 0.00	9.20 ± 1.56^{a}
1000 mg.kg^{-1} of APMO	12.60 ± 2.52^{a}	87.40 ± 2.52	11.40 ± 2.54	1.00 ± 0.45	0.20 ± 0.20	14.00 ± 2.70^{a}

Negative control—saline solution + Tween 80®; Cyclophosphamide– 20 mg.kg^{-1}ip.; *Attalea phalerata* Mart. oil at doses of 125, 250, 500 e 1000 mg.kg^{-1} by gavage. Different letters (a and b) indicate statistically significant differences ($p < 0.05$) of positive group (cyclophosphamide) between negative control and experimental group (doses 125, 250, 500 e 1000 mg.kg^{-1}); ANOVA/Tukey

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alkali-labile sites [3–5]. Cells obtained by caudal puncture from rats were analyzed. The results obtained with the comet assay (Table 2) show DNA damage (according to the size of the tail) in peripheral blood leukocytes (harvested after daily treatment for four weeks). Cell viability for the negative control group (saline + Tween 80) was higher compared to the positive control group (cyclophosphamide 20 mg.kg^{-1}), confirming the low genotoxicity rate in healthy rats. As expected, cyclophosphamide significantly increased the DNA leukocyte migration ($p < 0.05$) when compared to the negative control group. At doses of 125, 250, 500 and 1000 mg.kg^{-1} of the APMO, there was no significant increase in DNA breaks ($p < 0.05$) when compared to the negative control group. Most cells exposed to four concentrations of test compound were examined in slides and showed no DNA damage (Class 0) followed by a few class 1 DNA damage events.

The in vivo version of the micronucleus test in mammalian cells was performed in our study [19] and enabled evaluation of the presence or absence of damage caused by the chemical compounds of the APMO on chromosomes and/or mitotic apparatus of erythroblasts [6, 19, 29]. This *in vivo* test is the main test which assess the genetic toxicology to provide cytotoxic and mutagenic results and is highly recommended by global regulators. In this study, cells obtained from the marrow bone of the animals were analyzed after daily treatment with the APMO, for four weeks, at four different doses, as showed in Fig 3.

There was no statistical difference ($p < 0.05$) in the frequency of micronuclei in polychromatic erythrocytes (MNPCEs) among the negative control group (saline + Tween 80) and the groups treated with four doses of the APMO, indicating no clastogenic effects of this oil. As expected, the treated animals in the positive control group (cyclophosphamide 20 mg.kg^{-1}) showed large numbers of micronucleated polychromatic erythrocytes in bone marrow cells when compared to the negative control and to the experimental groups ($p < 0.05$). The estimated ratio of PCE/NCE in bone marrow preparations showed no statistical significant

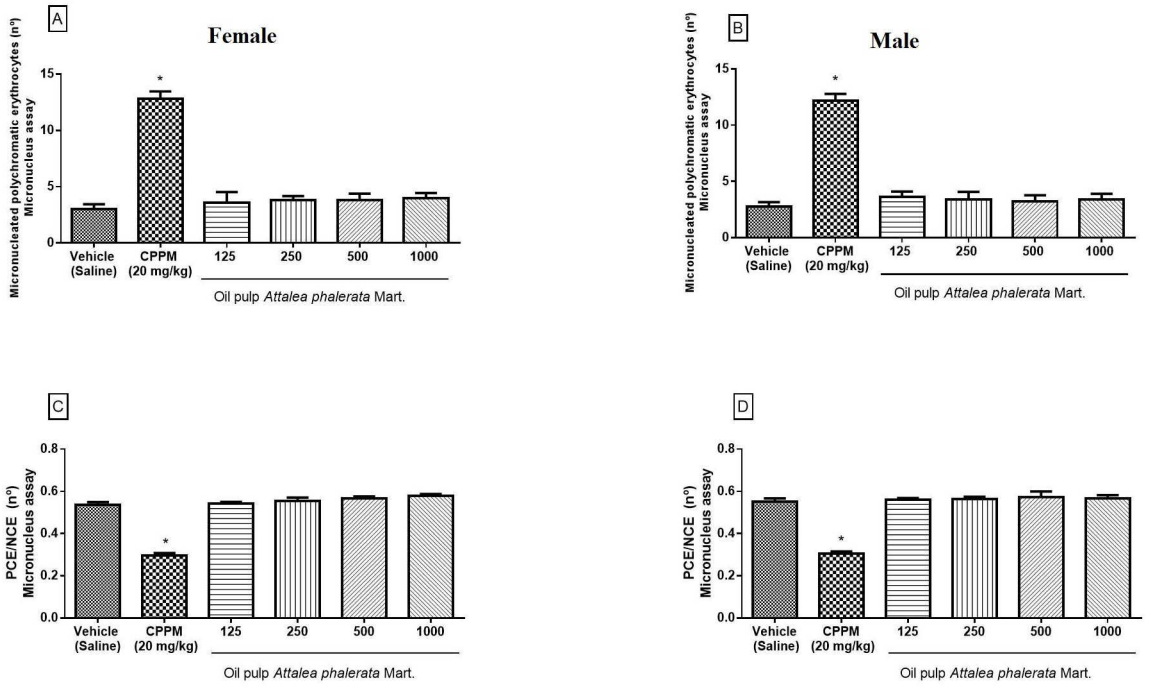

Fig 3. Effect of the APMO on Micronucleated Polychromatic Erythrocytes (Fig 3A and 3B) and the ratio between Polychromatic Erythrocytes/Normochromatic Erythrocytes—PCE/NCE (Fig 3C and 3D) micronucleus assay. Groups of female (Fig 3A and 3C) and male (Fig 3B and 3D) rats were treated with 125, 250, 500 and 1000 mg.kg^{-1} of the APMO daily for 28 consecutive days (four weeks) and the negative control group received the vehicle (saline + Tween 80®). The positive control group received intraperitoneal injection of cyclophosphamide (CPPM) 20mg.kg^{-1}. Results are presented as mean + SEM; One-way ANOVA followed by Tukey´s test; *p<0.05 compared to vehicle (saline) and experimental group (treated with 125, 250, 500 and 1000 mg.kg^{-1} of the oil).

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changes in hematopoiesis after the administration of the APMO, indicating no cytotoxic effects from the use of *A*. *phalerata* oil.

No published studies involving the evaluation of cytotoxic, genotoxic and clastogenic potential of *A*. *phalerata* oil were found. Thus, the results corroborate with studies conducted with Cerrado fruits, in which the antigenotoxic/genotoxic and antimutagenic/mutagenic activities were evaluated [30, 31], with satisfactory results for both parameters and driving interest in evaluating the possible antigenotoxic and antimutagenic activities of the APMO in future studies.

Conclusions

The results obtained in the micronucleus test are consistent with those observed by the comet assay, as well as *Artemia salina* and MTT assays. In conclusion, the results of this study demonstrate that the *A*. *phalerata* pulp oil has no cytotoxicity, genotoxicity and clastogenicity at doses tested in the experimental models used. The results demonstrated the safe use of the fruit pulp oil in folk medicine. In an ethnopharmacological context, the trials are considered important prerequisites for the identification of genetic diseases. Although the genotoxicity is not a direct measure of carcinogenicity, it is often used as an indicator for cancer, since the tests measure an initial or intermediate event in tumorigenesis.

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Writing – original draft: FFL SELTM GKT MCV SAO.

Writing – review & editing: FFL SELTM GKT MCV SAO.

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5. ANEXOS

5.3. Artigo em revisão no periódico Food Research International

Artigo intitulado ‘Anti-inflammatory, antiproliferative and cytoprotective potential of the bioactive compounds contained in the *Attalea phalerata* Mart. ex Spreng. pulp oil’’, em revisão no periódico com Fator de Impacto 3,086 e Qualis A2 em Medicina II.

Anti-inflammatory, antiproliferative and cytoprotective potential of the bioactive compounds contained in the *Attalea phalerata* Mart. ex Spreng. pulp oil

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Abstract

The anti-inflammatory, antiproliferative and cytoprotective activity of the *Attalea phalerata* Mart. ex Spreng pulp oil was evaluated by *in vitro* and *in vivo* methods. As for the chemical profile, the antiradical activity was performed by spectrophotometry, and the profile of carotenoids and amino acids by chromatography. Our data demonstrated that *A. phalerata* oil has high carotenoid content, antiradical activity and the presence of 5 essential amino acids. In the *in vitro* models of inflammation, the oil presented capacity to inhibit COX1 and COX2 enzymes, the production of nitric oxide and also induce macrophages to spreading. In the *in vivo* models of inflammation, the oil inhibited edema and leukocyte migration in the animals. In the *in vitro* model of antiproliferative and cytoprotective activity, the oil was shown inactive against the cells and with cytoprotective capacity, inhibiting the cytotoxic action of doxorubicin. Therefore, it is concluded that *A. phalerata* pulp oil has anti-inflammatory effects with functional properties due to the rich composition. Moreover, the oil also has cytoprotective activity because of their ability to inhibit the action of free radicals.

Keywords: bacuri; carotenoids; fatty acids; functional foods; inflammation; tumor.

1. Introduction

The fruits of *Attalea phalerata* Mart. ex Spreng. (Arecaceae) are known as bacuri or acuri. The pulp of "bacurizeiro" fruits are important foods for animals such as agouti, wild boars, macaws, parakeets, besides serving as raw material for starch development (Balslev & Moraes, 1989; Negrelle, 2015). The high nutritional value of the fruit presents a promising functional food due to the presence of beneficial minerals, high lipid content, most of which are short chain saturated fatty acids and unsaturated fatty acids and the presence of provitamin A carotenoids (de Lima et al., 2016; Negrelle, 2015).

The pulp oil is popularly used to relieve joint pain, pulmonary congestion (Balslev & Moraes, 1989; Negrelle, 2015) and antipyretic (Mónica, Borchsenius & Blicher-Mathiesen, 1996), additionally it is applied as hair tonic against hair loss and treatment

of dandruff (Balslev & Moraes, 1989). The fruit has a high content of carotenoids in its composition, predominantly β-carotene, β-zeacarotene and α-carotene and also high levels of fatty acids, mainly monounsaturated (de Lima et al., 2016; de Lima et al., 2017; Hiane, Danielle, Ramos, & Ramos Filho, 2003).

Carotenoids are antioxidants that assist in the maintenance of the immune system, in prevention of chronic diseases (Choo, Ishak, Simpson, Mueller, & Juenger, 2010; Hiane et al., 2003; Rodriguez-Amaya, 1997, 2010) and regularization of inflammatory processes by inhibiting the expression of nitric oxide synthase (iNOS) and cyclooxygenase (COX) (Lin et al., 2012; Soontornchaiboon, Joo, & Kim, 2012). In tumorigenesis, carotenoids are able to protect the human body from cellular damage caused by oxidative stress and decrease the risk of mutations (Rodriguez-Amaya, 1997, 2010; C. R. d. M. Silva & Naves, 2001). The prevention process takes place by direct action in free radicals in order to avoid oxidative stress and DNA damage in the human body (Ribeiro, 2010). Despite the occurrence of antioxidants endogenous production, it is recommended to complement with antioxidants from food sources, such as carotenoid supplementation from natural products (During & Harrison, 2004; Stahl & Sies, 2007).

According to the above, the objective of the present study was to evaluate the anti-inflammatory, antiproliferative and cytoprotection effect of the bioactive compounds present in *A. phalerata* Mart pulp oil.

2. Material and methods

2.1. Plant material and oil extraction

The fruits of *A. phalerata* were collected in the municipality of Rio Brilhante – MS (21 ° 48'07 "S 54 ° 32'47" W). The voucher specimen of the species was deposited in the Herbarium of the Universidade Federal da Grande Dourados (UFGD) No.

DDMS5033. The pulp of the fruit was dehydrated to obtain the oil, which was carried out with 1:3 hexane solvent (pulp: solvent, w/v) under continuous stirring (150 rpm) for 7 days at room temperature in the dark. After extraction, for complete removal of the solid residue, the material was filtered and the solvent was completely removed under reduced pressure at 40 °C on a rotary evaporator. The *A. phalerata* oil pulp (APOP) was packed in an amber bottle and refrigerated at 9 ºC until the beginning of the analysis.

2.2. Analytical evaluation

2.2.1. Analysis of carotenoids and amino acids by high performance liquid chromatography

The sample was analyzed in a high-performance liquid chromatography (HPLC) analytical system (LC-56AD, Shimadzu, Kyoto, Japan) with a binary solvent using ODSHYPERSIL column (C-18, 150 mm long x 4.6 mm of Internal diameter, 5 μm particle size, Thermo Electron Corporation) and diode array detector (PAD) monitored at *λ* = 200-800 nm. All chromatographic analyzes were performed at 22 ºC.

Carotenoids: Elution was started using 90% acetonitrile, 10% ethyl acetate, in 15 min 50% acetonitrile, 50% ethyl acetate and in 25 min returning to the initial condition. The flow rate and volume injected were 0.7 mL min^{-1} and 20 mL, respectively. The identification of the compounds was based on the absorption spectra in the UV region and the retention time (ζ-carotene) and associated with standards for α-carotene and β-carotene. Carotenoid quantification in the APOP was performed by external calibration curve. And concentration of α-carotene and β-carotene were determined with the respective standards. The amount of ζ-carotene was determined using the β-carotene curve.

Amino acids: Eluent A consists of a solution of 25 mM acetic acid and 0.02% sodium azide in ultrapure water, adjusted to pH 6 and the eluent B consists of acetonitrile. Elution was started using 96% of eluent A, 4% of eluent B, in 30 min 69% of eluent A, 31% eluent B and in 40 min returning to the initial condition. The flow rate and volume injected were 0.9 mL min^{-1} and 20 µL, respectively. The identification of the compounds was based on absorption spectra in the UV region and associated to standards for alanine, arginine, isoleucine, methionine, proline, serine, threonine, tryptophan and valine. The amino acid quantification in APOP was performed by external calibration curve and the concentration of alanine, arginine, isoleucine, methionine, proline, serine, threonine, tryptophan and valine were determined with respective standards.

A linear regression (or least squares) of the peak areas as a function of the concentrations was performed to determine the correlation coefficients. The equation parameters (slope and intercept) of the standard curve were used to obtain the concentration values for the samples.

2.2.2. Evaluation of the antioxidant capacity by the β-carotene / linoleic acid system

The antioxidant capacity of the β-carotene / linoleic acid system was adapted from Marco (1968) and dos Santos, Mamede, Rufino, de Brito, and Alves (2015). Firstly, the β-carotene / linoleic acid solution was prepared by dissolving 40 µL of linoleic acid, 530 µL of Tween 40, 50 µL of the β-carotene solution and, to complete the solubilization, 1 mL of chloroform. After complete solubilization of the compounds, the chloroform was evaporated and oxygenated water was added to the mixture. The samples were prepared using 5 mL of the solution of the β-carotene / linoleic acid system, 400 µL of Trolox (50 µg mL^{-1} to 800 µg mL^{-1}) and 400 µL of APOP extract at different concentrations (50 µg mL^{-1} to 800 µg mL^{-1}), homogenized and taken to the water bath at 40 ºC for 120 min.

129 Spectrophotometric readings (Biochrom® Libra S60) were performed every 15 min for
130 120 min at 470 nm. All determinations were performed in triplicate. The antioxidant
131 activity of the oil was expressed as % of inhibition of oxidation ± standard deviation.
132
133 2.3. Animals
134 Adult female *Wistar* rats (200-250 g) and adult male *Swiss* mice (30-50 g) were used
135 by the Central Biotério of the Universidade Federal da Grande Dourados (UFGD). The
136 animals were kept in collective cages (5 animals/cage) at controlled temperature (22 ± 1
137 °C), light cycle (12 h light / dark) and treated *ad libitum* water and commercial rodent
138 feed. At the end of the experiments, the euthanasia of the animals was performed with
139 isoflurane overdose followed by cardiac puncture exsanguination for the rats and CO_2
140 chamber for the mice. The experiments were conducted according to the standards of the
141 Conselho Nacional de Controle de Experimentação Animal (CONCEA) and the tests
142 previously approved by the Comissão de Ética no Uso de Animais (CEUA-UFGD)
143 (protocols 21-2013 and 22-2015).
144
145 *2.4. In vitro* anti-inflammatory evaluation
146 *2.4.1. Obtainment of murine peritoneal macrophages*
147 The mice were pretreated with 1.5 mL of a 3% thioglycolate solution,
148 intraperitoneally (ip), 96 h prior to collection of the cells. After this period, they were
149 submitted to euthanasia and macrophages obtained by washing the peritoneal cavity with
150 3 mL of buffered saline (PBS) in a sterile laminar flow chamber. The lavage from
151 peritoneal cavity was centrifuged (700 rpm/5 min) and the supernatant discarded. The
152 cells were resuspended in 1 mL of RPMI 1640 culture medium. Then, the total count was
153 carried out determining the number of mL^{-1} cells. From this, dilutions were performed to

obtain $2x10^6$ mL^{-1} cells. The peritoneal macrophages obtained were used in tests 2.4.2., 2.4.3. and 2.4.4.

2.4.2. Cell viability by the MTT assay.

Cell viability is determined by the MTT (3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazoline bromide) assay described by Mosmann (1983). Peritoneal macrophages were distributed in 96 well plates ($2x10^5$ cells/well) and incubated at 37 ºC in 5% CO_2 atmosphere. After 24 h the cells were treated with different concentrations of APOP (1, 5 and 10 mg mL^{-1}) for 16 h in RPMI 1640 culture medium. Then, the MTT (20 mL, 5 mg mL^{-1}) was added to each well and the cells incubated for 2 h. After formation of the resulting crystals from the cells, it was dissolved in DMSO (dimethylsulfoxide, 200 µL). The absorbances were determined using an ELISA microplate reader (Anthos Labtec® LP 400) at a wavelength of 540 nm.

2.4.3. Production of nitric oxide (NO) by peritoneal macrophages

For determination of NO production the macrophage suspension ($2x10^5$ cells 100 μL^{-1}) was incubated for 60 min in the 96-well plate in RPMI 1640 medium at 37 ºC in 5% CO_2 atmosphere for adhesion. The non-adherent cells were removed after this period. LPS (E. coli lipopolysaccharide, 1 µg mL^{-1}, 10 µL) and/or APOP (1 mg mL^{-1}, 10 µL) were diluted in RPMI 1640 and added to the plate. Cells were incubated for 48 h, and at the end of this period, the production of NO was determinated by the accumulation of nitrite in the supernatants of the cell culture, which was quantified by the Griess method (Ding, Nathan, & Stuehr, 1988). Aliquots of the supernatants were added to an equal volume of Griess reagent [1% sulfanilamide / 0.1% n-(1-naphthyl) in 2.5% phosphoric acid) and incubated for 10 min at room temperature. Then, the absorbance was determined

on an ELISA sensor (Anthos Labtec® LP 400) at wavelength of 540 nm. The nitrite concentration values were extrapolated from a calibration curve of sodium nitrite as standard and expressed as μM of NO_{-2}.

2.4.4. Spreading of macrophages

The macrophage spreading assay was performed according to the methodology described by Rabinovitch and DeStefano (1973). Peritoneal macrophages ($2x10^5$ cells, 100 μL^{-1}) were dispersed on glass coverslips in a 24-well plate and incubated with 1 mL of RPMI 1640 medium in the presence or absence of APOP (10 μL, 1 mg mL^{-1}) and/or LPS (10 μL, 1 μg mL^{-1}) at 37 ºC in 5% CO_2 atmosphere. Subsequently, the coverslips were washed with PBS and the cells adhered to the glass were fixed with 2.5% glutaraldehyde and examined in a phase contrast microscope (Optphase®) at magnification of 400x. One hundred macrophages were counted and classified as spread or not, according to morphological criteria. Spread macrophages index was presented as percentage of spread macrophages related to the count of 100 cells.

2.4.5. COX1 and COX2 inhibition

The inhibition assay of cyclooxygenase 1 (COX1) and cyclooxygenase (COX2) was performed using three distinct groups, with diclofenac sodium as the reference compound. The assay included both ovine COX1 enzymes and recombinant human COX2 enzymes to perform the screening of specific inhibitors using diclofenac sodium as standard. The compounds tested were added to both COX1 and COX2 with the substrates and buffers to perform the COX reaction according to instructions from the COX Inhibition Assay Kit (Item No. 560131, Cayman Chemicals Company, USA).

Compounds were incubated with the enzymes for 10 min at 37 °C and absorbance was read using a plate reader (Synergy H1 Hibrid Reader-Biotek) at 412 nm.

2.5. *In vivo* anti-inflammatory evaluation

2.5.1. Carrageenan-induced paw edema

In the *in viv*o evaluation for carrageenan-induced paw edema *Wistar* rats were randomly distributed in five groups, 5 animals/group. Group 1 (negative control) was treated by oral means with vehicle (0.9% saline); Groups 2, 3 and 4 were given 300, 500 and 700 mg kg^{-1} of APOP, respectively. After one hour, the animals received 50 µL of 0.9% saline containing 300 µg of carrageenan in the right hind paw. The same volume of saline solution was given to the left hind paw. The edema was evaluated in both paws 0.5; 1; 2 and 4 h after the injection of carrageenan, with the aid of a digital plethysmometer (Formagio et al., 2013; Winter, Risley, & Nuss, 1962).

2.5.2. Pleurisy induced by carrageenan

In the *in vivo* evaluation by the experimental model of pleurisy induced by carrageenan, *Wistar* rats were randomly assigned to four groups, 5 animals/group. Group 1 (naive), which did not receive intrapleural carrageenan injection; Group 2 (negative control) was treated with vehicle (0.9% saline solution); In group 3, dexamethasone (1 mg kg^{-1}, subcutaneously) was applied and group 4 was treated with 700 mg kg^{-1} of APOP orally. After 1h, pleurisy was induced in the experimental groups by intrapleural injection of 100 µL of carrageenan in 1% saline solution. The naive group received 100 µL of sterile saline by intrapleural injection. After 4 h, the animals were euthanized and the pleural cavity was washed with 1 mL PBS. A 20 µL aliquot of the lavage (exudate) was

collected from the pleural cavity and diluted in Turk's solution (1: 20) and the total leukocyte count was performed in a Neubauer chamber (Kassuya et al., 2009).

2.6. Antiproliferative and cytoprotective evaluation

2.6.1. Antiproliferative evaluation

For the antiproliferative activity evaluation, two human neoplastic cell lines, 786-0 (ATCC-CRL-1932, kidney carcinoma), PC-03 (ATCC-CRL-1435, prostate carcinoma) and a normal cell line, NIH/3T3 (murine fibroblast) were used. Cells were incubated at 37 ºC (5% CO_2) for extension and increase in cell density (Freshney, 2005). Samples (APOP and β-carotene) were resuspended in DMSO at the concentration of 0.1 g mL^{-1}, diluted in complete culture medium and the highest dilution had a DMSO concentration of less than 0.4%. The test was performed with sulforhodamine B dye (SRB), which is based on its affinity for the basic proteins present in the intact cells fixed by trichloroacetic acid (Skehan et al., 1990). The test was performed with a T0 plate, where a complete medium and cell were placed, and test plate with triplicate of cell suspension, where the test samples were placed in concentrations of 0.25, 2.5, 25 and 250 μg mL^{-1}. The T0 plate was incubated for 24 h at 37 ºC (5% CO_2) and the test plate was incubated under the same conditions for 48 h after placing the test samples. The positive control was doxorubicin at concentrations of 0.025, 0.25, 2.5 and 25 μg mL^{-1}. The result was obtained in absorbance at 540 nm and the dose for inhibition of 50% growth was determined.

2.6.2. Cytoprotective evaluation

NIH / 3T3 cells ($7.5x10^3$cells/well) were grown in 96-well plates and treated with different concentrations of APOP (250, 500, 1000 and 2000 μg mL^{-1}) in triplicate for 24 h at 37 ºC, 5% CO_2 in a humid environment. After 24 h, doxorubicin at the concentrations

252 of 0.025, 0.25, 2.5 and 25 μg mL^{-1} was added to the wells with cell suspension and APOP,
253 in increasing order of concentration, respectively for further 24 h. The percentages of
254 growth were obtained as mentioned in the item antiproliferative activity.
255
256 2.7. Statistical analysis
257 The results were expressed as mean ± standard deviation (SD) for the analytical
258 evaluation experiments and standard error of the mean (SEM) for the biological
259 experiments. The analysis of variance was performed by one-way ANOVA followed by
260 the Tukey test to evaluate the possible differences between groups.
261
262 **3. Results e discussion**
263 3.1. Carotenoids, antioxidant activity and amino acids
264 Natural products are composed of numerous secondary metabolites, which
265 perform particular actions in the human body, and can be beneficial or harmful. The main
266 compounds are terpenes, carotenoids and phenolic compounds, which are associated with
267 the fight against chronic diseases such as cancer, diabetes, heart disease, chronic
268 inflammation and the fight against reactive oxygen and nitrogen species (ROS/RNS)
269 (Carey et al., 2015; De Oliveira et al., 2010; N. A. d. Silva, Rodrigues, Mercadante, & de
270 Rosso, 2014).
271 The elucidation of the compounds contained in the natural products is of extreme
272 importance for the knowledge of the mechanism of action of herbal medicines. APOP is
273 mainly composed of carotenoids, which are α-carotene, β-carotene and ζ-carotene (Table
274 1). Studies point out that the different carotenoids play an important role in capturing free
275 radicals by connecting their structural chains (Padhi, Liu, Hernandez, Tsao, & Ramdath,
276 2016; Sarada et al., 2002) these compounds bind to radicals so as to render them stable.

277 The carotenoids found in the oil may have had an influence on antioxidant activity
278 by the β-carotene/linoleic acid method, with 47.33 ± 1% inhibition of oxidation after
279 exposure of 120 min at a concentration of 400 μg mL^{-1}, whereas Trolox (standard) showed
280 inhibition of 61.64 ± 1% at a concentration of 200 μg mL^{-1} after the same exposure time.
281 The method allowed to estimate the relative capacity of the antioxidant compounds
282 contained in the APOP to inhibit the linoleic peroxide radicals from oxidizing the β-
283 carotene contained in the system (β-carotene/linoleic acid emulsion) (Othman, Ismail,
284 Hassan, Yusof, & Khatib, 2016).
285 The oil is also composed of beneficial fatty acids, consisting of 20.61% of
286 saturated fatty acids and 78.53% of unsaturated fatty acids; among these acids, 57.65%
287 are monounsaturated and 20.88% polyunsaturated, predominantly oleic, linoleic and
288 palmitic acids (de Lima et al., 2016). The unsaturated fatty acids, predominant in the oil
289 of the fruit, when consumed of appropriate form exert important function in the human
290 organism, like the maintenance of the immune system in inflammatory processes (Pereira,
291 Correia-da-Silva, Valentão, Teixeira, & Andrade, 2014; Skulas-Ray, 2015) and reduction
292 of corporal fat (Cintra et al., 2012; Itariu et al., 2012).
293 Complementary analysis by high-performance liquid chromatography identified
294 the presence of nine amino acids (Table 1), which include five essential amino acids
295 (isoleucine, methionine, threonine, tryptophan and valine). Serine was the amino acid
296 found in highest concentration, followed in decreasing order of concentration by alanine,
297 proline, threonine, tryptophan, valine, isoleucine, arginine and methionine. In the attempt
298 to extract the carotenoids contained in the APOP, it is noted that the extraction method
299 allowed the transition of the amino acids contained in the pulp to the oil, even in small
300 concentrations. Researchers have shown the presence of amino acids in Brazilian Cerrado
301 fruits almonds and highlighted their nutritional importance in food (de Oliveira Sousa,

302 Fernandes, Alves, De Freitas, & Naves, 2011; e Silva, Barbosa, Hiane, Braga Neto, &
303 Macedo, 2014). Amino acids have an important functional role, as they perform the
304 production of proteins, digestive enzymes and maintenance of muscles. They are
305 responsible for the production of hormones and neurotransmitters (Calbet & MacLean,
306 2002; Haraguchi, Abreu, & Paula, 2006; Ivy et al., 2002).
307
308 3.2. *In vitro* anti-inflammatory evaluation
309 Cell experiments have proven to be an important tool in elucidating the
310 mechanism of action of natural substances and also serve as a prelude to *in vivo* testing.
311 The MTT assay evaluates cell viability through the respiration of cells. APOP at 1 mg
312 mL^{-1} did not influence cell viability, but doses of 5 mg mL -1 and 10 mg mL^{-1} were
313 cytotoxic, reducing viability to approximately 50% (Figure 1).
314 A previous study with APOP in the same experimental model (de Lima et al.,
315 2016) did not present cytotoxicity in doses higher than that used in this study; However,
316 the cells used were T84 (colon carcinoma). Thus, the difference between the results
317 obtained can be attributed to the particularity of the cells in each study. Primary cells
318 (macrophages from this study) present limited division capability and reach their
319 senescence state more rapidly than immortalized cells, which are those obtained from
320 collections of cell cultures and have their cell death capability deactivated (Galler,
321 Schweikl, Thonemann, D'souza, & Schmalz, 2006; Kudo et al., 2002; Shay, Wright, &
322 Werbin, 1991).
323 Although important in the control of infectious diseases, due to its cytotoxicity,
324 NO, when produced in an exacerbated way, presents clinical correlation with septic
325 shock, autoimmune diseases, arteriosclerosis, tumorigenesis, genotoxicity and
326 inflammation (Bogdan, 2001; Moncada, Palmer, & Higgs, 1991; Schmidt & Walter,

1994; Wink et al., 1998). In this study, macrophages incubated with APOP (1 mg mL^{-1}) did not show increase in NO release (Figure 2). Macrophages, when stimulated with LPS, produce NO, but this did not occur in the treatment of macrophages with APOP+LPS. Therefore, it is suggested that APOP, when acting in the production of NO, can act as an important cytoprotective/chemoprotective agent, modulator of tumorigenesis and acute inflammatory processes.

The result of the macrophage spreading assay showed that APOP (1 mg mL^{-1}) induced macrophage spreading. However, treatment with APOP did not affect LPS-induced spreading (Figure 3), while LPS induced macrophages to spreading as expected. The ability of the cells to spread can be considered a cellular activation index (Rabinovitch & DeStefano, 1973), predisposing the cells to the phagocytosis process (Russo et al., 1989; Xia & Triffitt, 2006).

The production of COX1 and COX2 enzymes occurs during inflammatory processes in the organism, where COX1 is found in several tissue cells and modulates physiological processes (Crofford, 1997) and COX2 is stimulated by invading organisms and proinflammatory cytokines (Klein & Eliakim, 2010), with the prostaglandins as final product. With the inhibition of the COX1 and COX2 enzymes, the production of prostaglandins is consequently inhibited, thus reducing the characteristic signs of inflammatory processes.

Results for enzymatic inhibition of COX1 and COX2 (Figure 4) showed the inhibitory ability of APOP in both isoforms, which was 35% COX1 and 70% COX2, and did not differ statistically from the reference drug (diclofenac sodium). Therefore, the efficacy of inhibition of APOP (10 μg mL^{-1}), used in folk medicine can be attributed to the suppression of the inflammatory response due to the ability to inhibit the activity of COX enzymes.

352 The high content of carotenoids present in the APOP encouraged us to investigate
353 the potential effect on the inhibition of COX; and production of nitric oxide, however,
354 there are no data in the literature about its inhibition. The results found in the experiment
355 of inhibition of COX1, COX2 and in the production of NO by macrophages corroborate
356 with data published in the literature, where it is assumed that carotenoids act in the
357 production of prostaglandins (inhibiting COX) and nitric oxide production, by
358 inactivation of the NF-κB pathways (Lin et al., 2012; Soontornchaiboon et al., 2012)
359
360 3.3. *In vivo* anti-inflammatory evaluation
361 The use of carrageenan is applied in several experimental models of inflammation
362 and help in the development of anti-inflammatory drugs. The model of paw edema
363 induced by carrageenan is a classic model of vascular inflammatory response related to
364 the formation of edema (F. Silva et al., 2010). The inflammatory response of carrageenan
365 consists of three phases. The primary phase is mediated by histamine and serotonin,
366 followed by the secondary phase mediated by endogenous non-peptidic bradykinin and
367 the final phase mediated mainly by the production of prostaglandin E2 and induction of
368 COX2 (Di Rosa, Giroud, & Willoughby, 1971; Guay, Bateman, Gordon, Mancini, &
369 Riendeau, 2004; Morris, 2003). The results of the present study demonstrate that
370 intraplantar injection increases the edema in the paw of the animals at times 0.5, 1, 2 and
371 4 h after the injection of carrageenan, corroborating with the results shown in the
372 literature.
373 Oral administration of the 700 mg kg^{-1} APOP dose significantly reduced edema
374 after 0.5, 1 and 2 h of carrageenan injection. Treatment with APOP (700 mg kg-1) resulted
375 in a 68 ± 7% reduction of edema after 0.5 h of treatment (Figure 5A) and inhibition of
376 edema remained significant after 1 h (Figure 5B) and after 2 h (Figure 5C). It is suggested

377 that APOP performs activity at all stages of carrageenan-induced edema formation, and
378 therefore has action under the chemical mediators histamine, serotonin, bradykinin,
379 prostaglandin E2 and COX, and non-specific effect.

380 The experimental model of carrageenan-induced pleurisy has the ability to
381 represent the main events of acute inflammation in a manner similar to that in humans;
382 Thus, this model can be considered very efficient in investigating anti-inflammatory
383 effects on potential substances (Jantz & Antony, 2008). Oral administration of APOP at
384 a dose of 700 mg kg-1 significantly inhibited the inflammatory process (Figure 6),
385 evidenced by the reduction in leukocyte migration to the pleural cavity. There was
386 inhibition of 86 ± 4% of APOP in 4 h after administration of the phlogistic agent. The
387 method evaluated only the leukocyte migration, without differentiation, but it can be
388 assumed that the majority of the quantified cells are polymorphonucleated, with
389 predominance of neutrophils. Neutrophils are the first cells to migrate to the inflammatory
390 region and are present in greater amounts in acute inflammatory processes (Cassatella,
391 1995).

392 Thus, it is demonstrated that the compounds present in APOP have an action
393 against the leukocyte migration, besides anti-edematogenic action, evidenced in the paw
394 edema model. Our results corroborate with those presented by Batista et al. (2010), which
395 observed a decrease in the inflammatory process of tissue edema in the healing of
396 cutaneous wound in rats after application of the oil of the pulp of *Caryocar coriaceum*
397 Wittm, and Lescano, Iwamoto, Sanjinez-Argandoña, and Kassuya (2015) that found anti-
398 edematogenic activity of the oil of the pulp of *Acrocomia aculeata* (Jacq.) Lodd. in
399 experimental models of pleurisy and paw edema in rats, both attributing the action to the
400 presence of carotenoids and unsaturated fatty acids.

401

3.4. Antiproliferative and cytoprotective evaluation

The role of carotenoids on inflammation processes is directly associated with the inhibition of the generation of reactive oxygen and nitrogen species (Hadad & Levy, 2012; Yoshihisa & Shimizu, 2014), that is, protection against cellular damage caused by oxidative stress (Rodriguez-Amaya, 2010). Thus, it can be assumed that natural products, with high carotenoid content, have protective action in non-tumor cells exposed to chemotherapy. The results showed that APOP exhibited inhibitory action on cell proliferation in kidney carcinoma (786-0), prostate carcinoma (PC-03) lineage, without demonstrating toxicity (Table 2). This protective effect was observed during pre-treatment with APOP of non-tumor cells (NIH / 3T3 - murine fibroblast) in different concentrations (250 to 2000 μg mL^{-1}), following treatment with classic chemotherapeutic doxorubicin at concentrations of 0.025 to 25 μg mL^{-1} (Figure 7).

The effect of prior exposure to APOP suggests a significant protective effect on doxorubicin-treated murine fibroblast cells when compared to cell viability of non-oil exposed cells (Figure 7). The increasing combinations (A, B, C, and D) of doxorubicin and APOP were evaluated in association, and an oil-dependent protective effect on increasing concentrations of the chemotherapeutic agent (Figure 7, combinations A-C) was observed. However, in combination D the protective effect of the oil was proportionally lower, where we detected a low cell growth rate of 15 and 66% for cells treated alone with doxorubicin (25 μg mL^{-1}) and APOP (2000 μg mL^{-1}), respectively, suggesting an oil toxicity at very high concentrations as observed in combination D.

Conclusions

The fruit pulp oil of *Attalea phalerata* Mart. ex Spreng. in *in vitro* models of inflammation has action on the production of oxide nitric, inhibition of the COX1, COX2

enzymes and induces macrophage to spreading. In the *in vivo* models of inflammation, the oil performed anti-inflammatory activity, reducing paw edema during the action of carrageenan and positively influenced leukocyte migration by the pleurisy model induced by carrageenan. In the evaluation of the antiproliferative and cytoprotective activity *in vitro*, the oil did not influence the cellular growth and presented cytoprotective capacity at the evaluated low doses, inhibiting the cytotoxic action of doxorubicin in the studied cells.

The fruit of *A. phalerata* is a promising species as a functional food and herbal medicine, that is, due to its rich chemical composition and proven beneficial pharmacological capacity. However, additional studies are needed to elucidate their functional capacity, complete composition and mechanism of action.

Acknowledgement

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641 # Tables

642 Table 1. Analysis of carotenoids and amino acids in *A. phalerata* oil employing HPLC

	Retention time (min)	Linear range (μg mL^{-1})	Intercept (a)	Slope (b)	Determination coefficient (R^2)	Compound concentration (μg g^{-1} ± SD)
Carotenoids						
α-carotene	16.26	100-1000	3879.78	2980.99	0.994	11.02 ± 0.3
ζ-carotene	16.86	---	---	---	---	1.94 ± 0.1
β-carotene	18.15	100-1000	4237.56	9589.78	0.996	62.33 ± 0.7
Amino acids						
Serine	8.87	8-100	77158.69	2.70	0.997	1.18 ± 0.04
Threonine	10.98	8-100	10511.66	2.91	0.999	0.60 ± 0.01
Arginine	13.49	8-100	49917.22	2.61	0.998	0.25 ± 0.01
Alanine	14.26	8-100	42139.23	2.47	0.970	0.74 ± 0.02
Proline	16.66	8-100	12380.67	2.41	0.958	0.62 ± 0.01
Valine	24.99	8-100	29532.31	1.92	0.985	0.44 ±0.01
Methionine	25.87	8-100	6363.44	2.40	0.999	0.15± 0.01
Isoleucine	28.29	8-100	47234.52	2.01	0.983	0.28 ± 0.01
Tryptophan	28.88	8-100	10714.55	2.80	0.997	0.46 ± 0.01

643 ζ-carotene was determined employing curve of β-carotene. Values expressed in mean ± standard deviation (SD).
644 Formule: y = a + bx, where y = ratio of peak areas, x = concentration (μg g^{-1}), a = intercept and b = slope.
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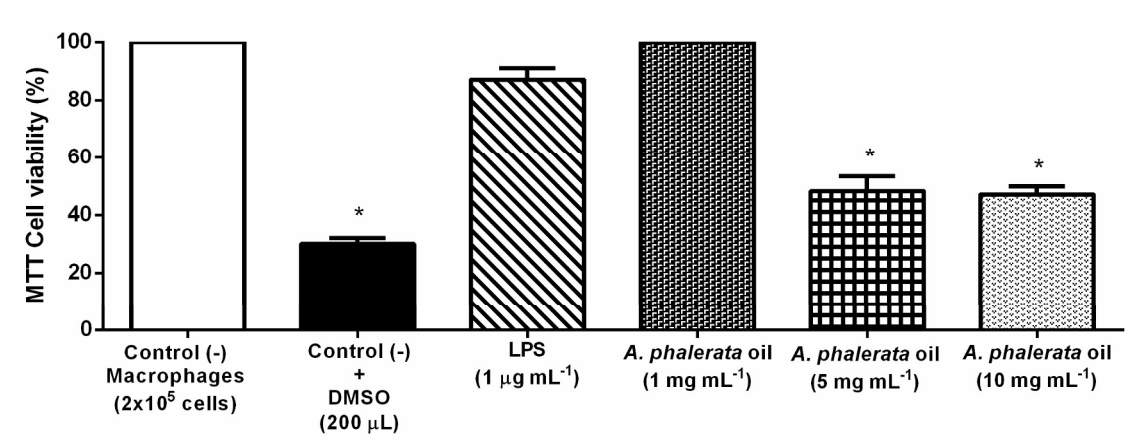
Table 2. Effect of the *A. phalerata* oil in antiproliferative activity.

Compounds	IC_{50} µg mL^{-1}		
	786-0[a]	PC-03[b]	NIH/3T3[c]
A. phalerata oil	> 250	> 250	> 250
β-carotene	> 250	> 250	> 250
Doxorubicin[d]	< 25	< 25	< 25

The results are expressed as µg mL^{-1} concentration of sample necessary to inhibit 50% of the proliferation cells. [a]Kidney carcinoma, [b]Prostate carcinoma, [c]Murine fibroblast and [d]Positive control.

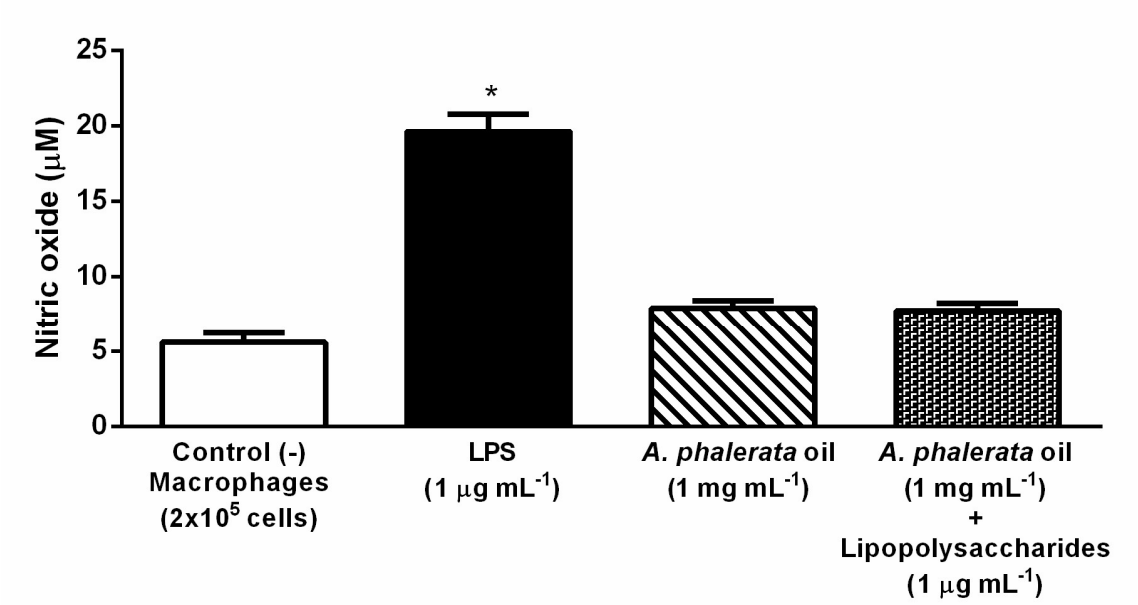
Figures

Figure 1. Effect of the *A. phalerata* oil in cell viability in macrophages cells by MTT assay.



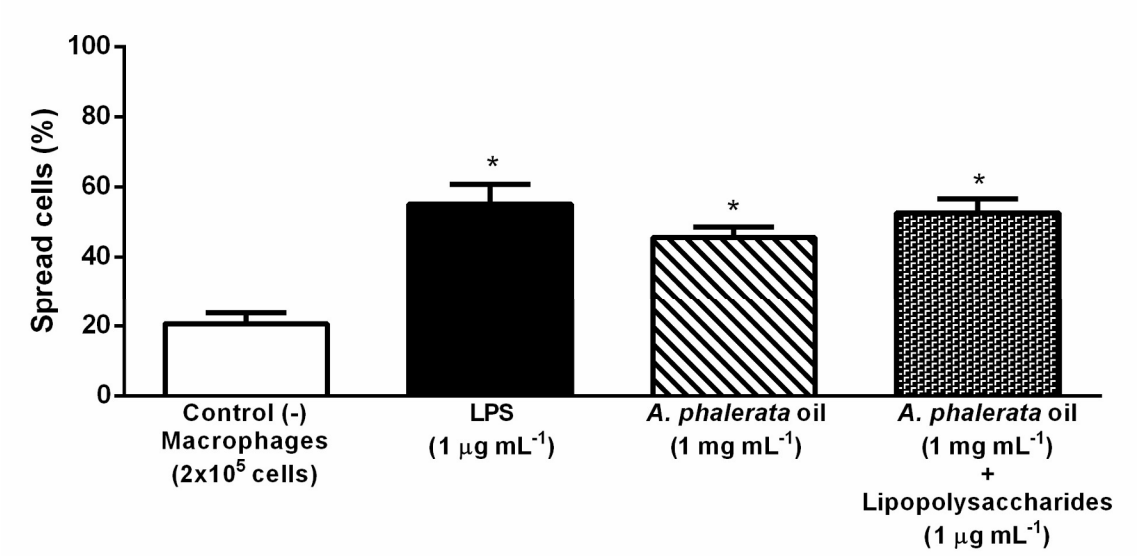
Values expressed in mean ± standard error of the mean. n = 3. * P <0.05 (ANOVA / Tukey) compared with the negative control (-). LPS – Lipopolysaccharide. DMSO - Dimethylsulfoxide

Figure 2. Effect of the *A. phalerata* oil in nitric oxide (NO) production.



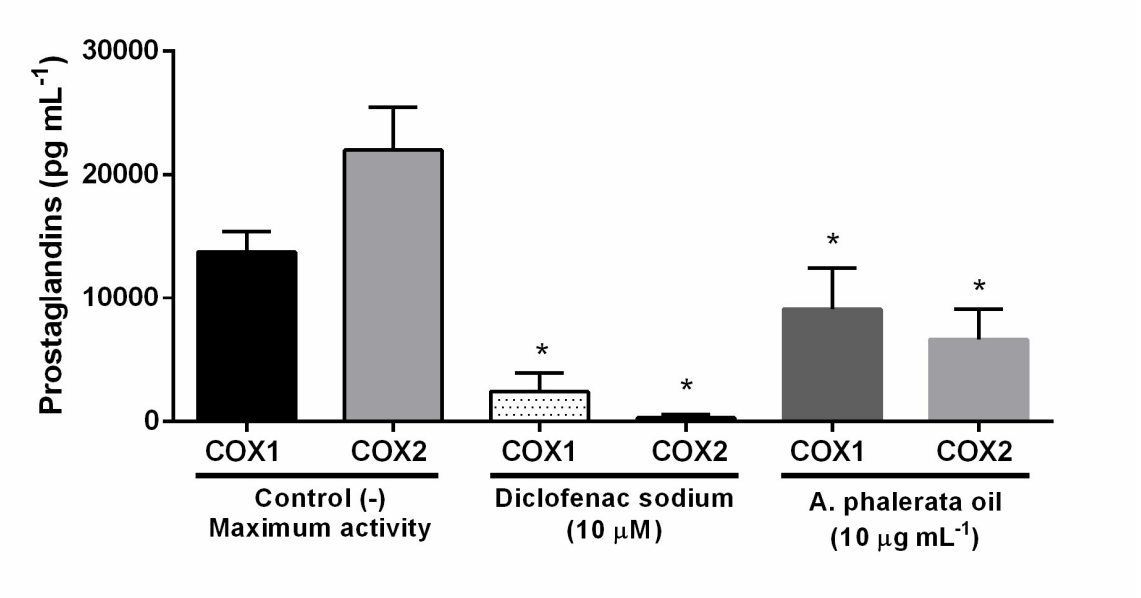
Values expressed in mean ± standard error of the mean. n = 33. * P <0.05 (ANOVA / Tukey) compared with the negative control (-). LPS – Lipopolysaccharide.

Figure 3. Effect of the *A. phalerata* oil in spreading of macrophages.



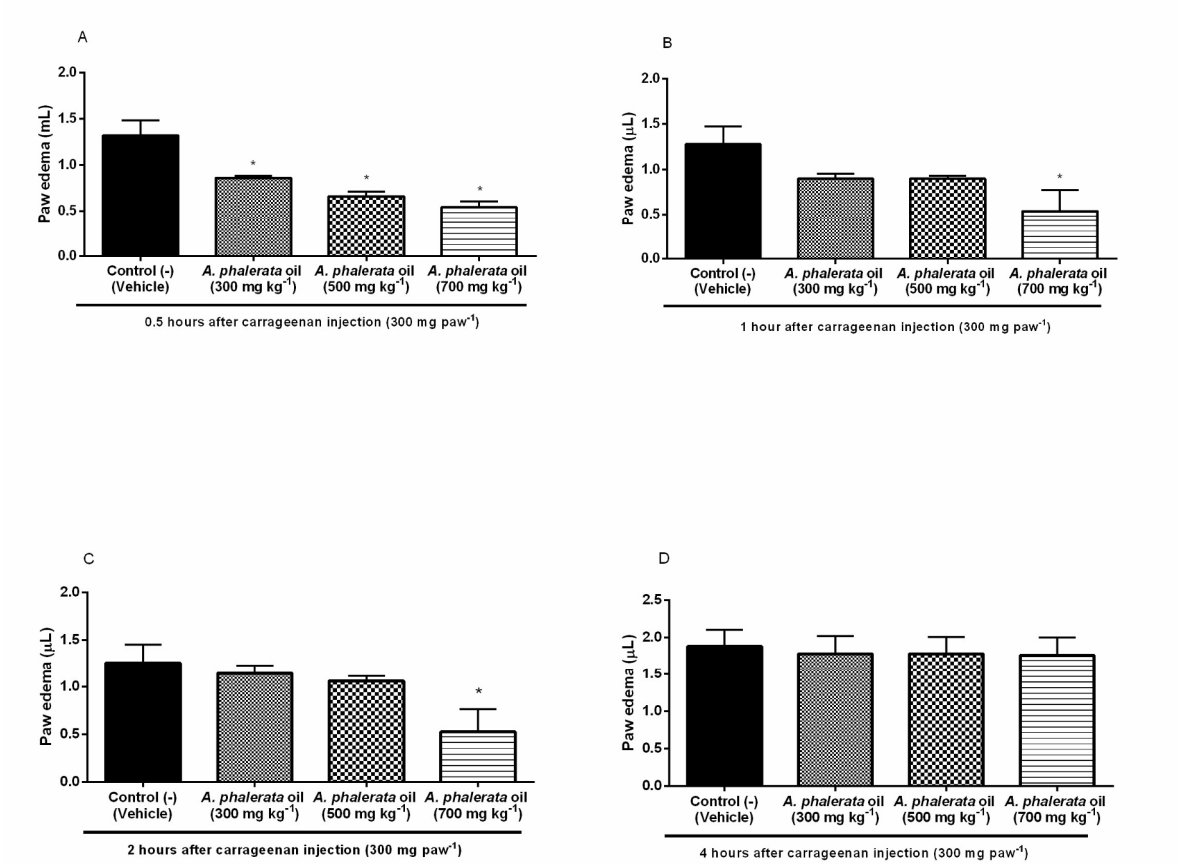
Values expressed in mean ± standard error of the mean. n = 3. * P <0.05 (ANOVA / Tukey) compared with the negative control (-). LPS – Lipopolysaccharide.

Figure 4. Effect of the *A. phalerata* oil in COX1 and COX2 inhibition.



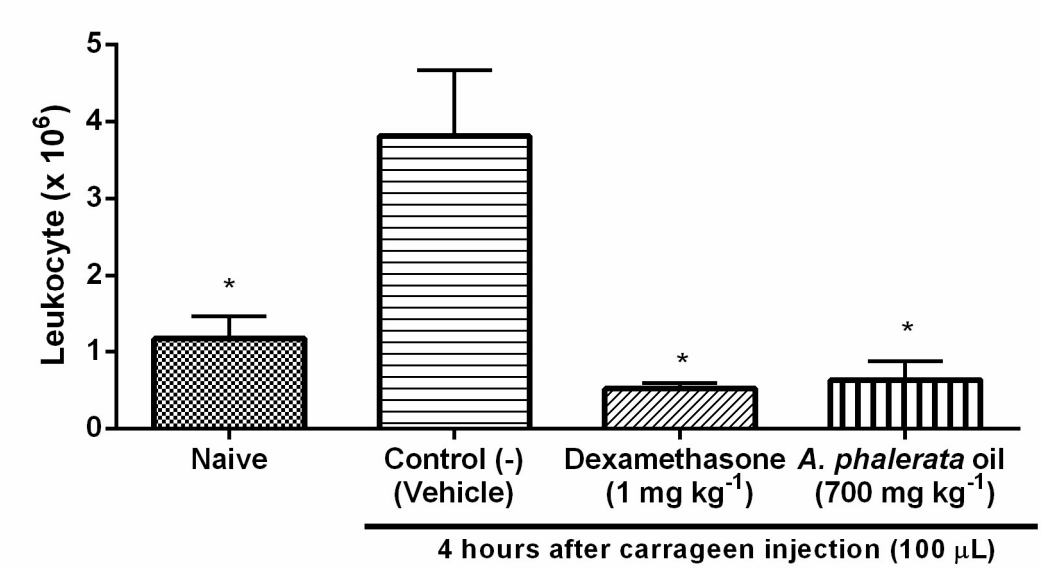
Values expressed in mean ± standard error of the mean. n = 4. * P <0.05 (ANOVA / Tukey) compared with the negative control (-).

Figure 5. Effect of the *A. phalerata* oil on the paw edema after 0.5h (A), 1h (B), 2h (C) and 4h (D) of the intraplantar carrageenan injection.



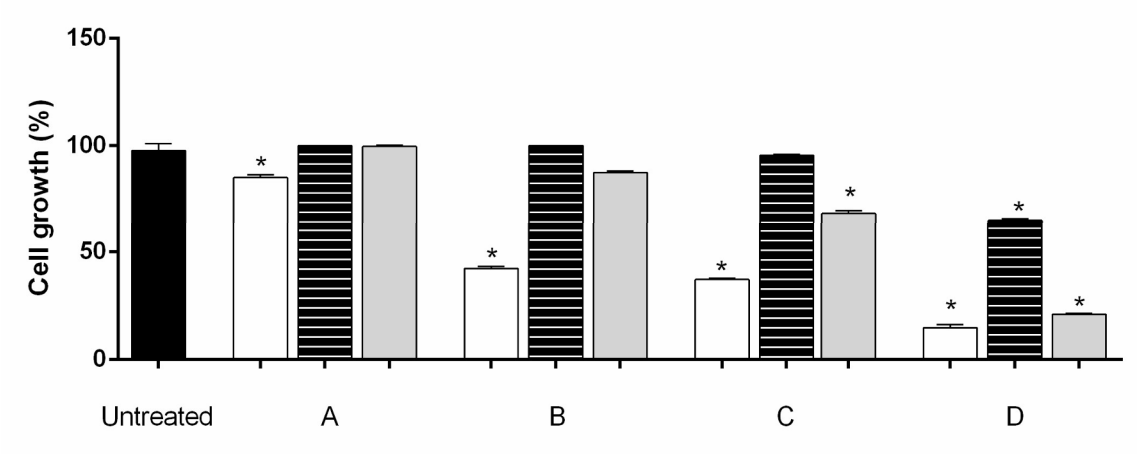
Values expressed in mean ± standard error of the mean. n = 5. * P <0.05 (ANOVA / Tukey) compared with the negative control (-).

Figure 6. Effect of the *A. phalerata* oil on the leukocyte migration induced by carrageenan in the pleurisy model in rats.



Values expressed in mean ± standard error of the mean. n = 5. * P <0.05 (ANOVA / Tukey) compared with the negative control (-).

867 Figure 7. In vitro protective effect of *A. phalerata* oil under murine fibroblast cells (NIH / 3T3).



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869 The control represents untreated cells. A-D are evaluated treatment combinations: (A) used doxorrubicina on 0.025 µg
870 mL^{-1} and *A. phalerata* oil on 250 µg mL^{-1}; (B) doxorrubicina on 0.25 µg mL^{-1} and *A. phalerata* oil on 500 µg mL^{-1}; (C)
871 doxorrubicina on 2.5 µg mL^{-1} and *A. phalerata* oil on 1000 µg mL^{-1} (D) doxorrubicina on 25 µg mL^{-1} and *A. phalerata*
872 oil on 2000 µg mL^{-1}. Values expressed in mean ± standard error of the mean. n = 3. * P <0.05 (ANOVA / Tukey)
873 compared with the untreated cells.
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6. CONCLUSÃO

O óleo da polpa de *Attalea phalerata* Mart. ex Spreng. apresenta em sua composição ácidos graxos benéficos, com predominância dos insaturados e elevada quantidade de carotenoides, principalmente o β-caroteno. Nos experimentos de toxicidade aguda e subaguda, o óleo apresenta DL50 maior que 2000 mg/kg e ausência de alterações deletérias nos parâmetros hematobioquímicas, histológicas e fisiológicas. Nos modelos experimentais de citotoxicidade, genotoxicidade e clastogenicidade nas doses e modelos testados, o óleo não apresentou toxicidade.

Na avaliação do potencial anti-inflamatório, o óleo apresentou ação anti-inflamatória nos modelos *in vitro* e *in vivo*, sugerindo ação por inibição da COX1, COX2 e produção de óxido nítrico. O óleo não apresentou atividade antiproliferativa nas células tumorais testadas, porém exerceu atividade citoprotetora nas células expostas a Doxorrubicina. Com isso, podemos concluir que o fruto de *A. phalerata* é um promissor alimento funcional, assim como o óleo exibe propriedades fitoterápicas isentas de toxicidade.

7. ANEXOS

7.1. Protocolo de aprovação pela Comissão de Ética no Uso de Animais

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 5 de setembro de 2013

Senhora Pesquisadora:

Eliana Janet Sanjinez Argandoña

O Projeto de sua responsabilidade – Protocolo nº. **021/2013 – CEUA/UFGD** - intitulado **"Microencapsulação do óleo de bacuri por coacervação complexa: obtenção, caracterização e avaliação biológica das microcápsulas"** foi integralmente **APROVADO** e poderá ser conduzido.

Ressaltamos que é de responsabilidade do (a) pesquisador (a) envio de notificação à CEUA sobre o término do projeto.

Prof. Dr. Fernando Miranda de Vargas Junior

Coordenador/CEUA

Comissão de Ética no Uso de Animais - CEUA/UFGD - Rua João Rosa Góes, 1761 - CEP 79.825-070, Cx. Postal 322, Dourados (MS) Fone/Fax: (67) 3410-2328 – e-mail: ceua@ufgd.edu.br

7. ANEXOS

7.2. Protocolo de aprovação pela Comissão de Ética no Uso de Animais

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 5 de abril de 2016.

CERTIFICADO

Certificamos que a proposta intitulada **"Avaliação da toxicidade aguda, subaguda, ensaio cometa e teste de micronúcleo do óleo da** *Attalea phalerata Mart* **em ratos.",** registrada sob o protocolo de nº 21/2015, sob a responsabilidade de Silvia Aparecida Oesterreich e Fernando Freitas de Lima – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 29/11/2015.

Finalidade	*(X) Ensino ( ) Pesquisa Científica*
Vigência da autorização	*30/04/2016 a 20/06/2016*
Espécie/linhagem/raça	*Rattus norvegicus/* Wistar
Nº de animais	80
Peso/idade	250 g/ 50 dias
Sexo	35 Machos e 45 Fêmeas
Origem	Biotério da Faculdade de Ciências da Saude/UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA

Comissão de Ética no Uso de Animais – CEUA/UFGD – Rua João Rosa Góes, 1761 – Vila Progresso. Dourados/MS. E-mail: ceua@ufgd.edu.br

7. ANEXOS

7.3. Protocolo de aprovação pela Comissão de Ética no Uso de Animais

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 30 de maio de 2016.

CERTIFICADO

Certificamos que o projeto intitulado **"Efeito ansiolítico e antidepressivo do óleo de** *Attalea phalerata Mart*.**",** protocolo nº 22/2015, foi adicionado o teste "**Efeito anti-inflamatório do óleo de** *Attalea phalerata Mart*." sob responsabilidade d*e Fernando Freitas de Lima* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados.

Vigência do Projeto	*20/06/2016 – 20/06/2016*
Espécie/linhagem	*Mus musculus* / Swiss
Nº de animais	40
Peso/idade	18-25 g
Sexo	Machos
Origem	Biotério Central da UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA