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DANILO CABRAL DE SÁ HYACIENTH

PREPARAÇÃO DE NANOEMULSÕES A BASE DA FRAÇÃO LIPOFÍLICA OBTIDA DE FRUTOS DE AÇAÍ (*EUTERPE OLERACEAE* MART.)

Macapá
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá, como parte dos requisitos para obtenção do Título de Mestre em Ciências Farmacêuticas, na área de concentração de Tecnologias Aplicadas a Fármacos.

Orientador: Prof. Dr. Caio Pinho Fernandes

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Salmos 19:7-11

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RESUMO

Preparação de nanoemulsões a base da fração lipofílica obtida de frutos de Açaí (*Euterpe oleraceae* Mart.)

Introdução: *Euterpe oleracea* é uma espécie de palmeira brasileira, os seus frutos são comumente conhecidos como açaí. O óleo é extraído a partir dos seus frutos e é quimicamente comparável ao azeite de oliva, sendo o ácido oleico considerado seu principal constituinte. Apesar do grande potencial do açaí, permanece quase que inexplorado a obtenção de nanoemulsões a partir da sua fração lipofílica. **Objetivo:** Portanto, o objetivo desse estudo é desenvolver nanoemulsões do tipo óleo em água a partir dos frutos de açaí. **Metodologia:** A reação de transesterificação foi catalisada pela lipase de Candida antarctica (CAL-B) e utilizada para a caracterização dos ácidos graxos presentes na amostra, onde o ácido oleico foi o principal composto. As antocianinas também foram encontradas na fração lipofílica. As nanoemulsões foram preparadas utilizando diferentes métodos, como solvente orgânico, métodos de aquecimento, ou isentos de solventes orgânicos e sem aquecimento. **Resultados e discussões:** Formulações foram obtidas mudando-se a natureza do tensoativo. Foi observado um tamanho médio de partículas inferior (184,7 ± 0,92 nm) e distribuição estreita (índice de polidispersão = 0,218 ± 0,003) utilizando dioleato de polietilenoglicol 400 1% (p / p, expresso como teor de tensoativo na fase aquosa anterior) no método do solvente orgânico. Não foi observada separação de fases, houve uma distribuição satisfatória do tamanho de partícula (tamanho médio de partícula = 313,3 ± 8,84 nm, índice de polidispersão = 0,400 ± 0,036) e potencial zeta (-37,4 ± 0,551) para a nanoemulsão preparada com polissorbato 80 / monooleato de sorbitano (EHL 11) no método isento de solventes / sem aquecimento. **Conclusões:** O presente estudo permitiu uma compreensão crítica de alguns aspectos da nanoemulsificação da fração lipofílica da nanoemulsão O/A de *Euterpe oleracea*. Portanto, esse estudo contribui para a valorização da fração lipofílica do açaí e o desenvolvimento de potenciais nanoprodutos farmacêuticos com esta matéria-prima natural da Amazônia.

Palavras-Chave: Arecaceae; Sistema coloidal; Dispersão de luz dinâmica.

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PREPARAÇÃO DE NANOEMULSÕES A BASE DA FRAÇÃO LIPOFÍLICA OBTIDA DE FRUTOS DE AÇAÍ (*Euterpe oleraceae* Mart.)

Introduction: *Euterpe oleracea* is a Brazilian palm species and its fruits are commonly known as açaí. The oil extracted from açaí fruits is chemically comparable to olive oil, being oleic acid considered its main constituent. Despite the great potential of açaí, to our knowledge, it remains almost unexplored concerning obtainment of nanoemulsions with its lipophilic fraction. **Objectives:** Therefore, the purpose of this study is to develop oil-in-water nanoemulsions from açaí fruits. **Methodology:** The transesterification reaction catalyzed by lipase from Candida antarctica (CAL-B) was used for characterization of the fatty acids present in the sample and oleic acid was the main compound. Anthocyanins were also found in the lipophilic fraction. Nanoemulsions were prepared using different methods, using organic solvent or heating or organic solvent-free/non-heating methods. **Results and discussion:** Several formulations were obtained by changing surfactant nature. Lower mean droplet size (184.7 ± 0.92 nm) and narrow distribution (polydispersity index = 0.218 ± 0.003) was observed using polyethylene glycol 400 dioleate 1% (w/w, expressed as surfactant content at former aqueous phase) in the organic solvent method. No phase separation, satisfactorily particle size distribution (mean droplet size = 313.3 ± 8.84 nm and polydispersity index = 0,400 ± 0,036) and zeta potential (-37.4 ± 0.551) were observed for the nanoemulsion prepared with polysorbate 80/sorbitan monooleate (HLB 11) in the organic solvent-free/non-heating method. **Conclusions:** The present study allowed a critical understanding of some aspects of nanoemulsification of the lipophilic fraction from *Euterpe oleracea* O/A nanoemulsion. Thus, this study it contributes to valorization of acai oil and development of potential phytopharmaceuticals innovative nanoproducts with this natural Amazon raw material.

Keywords: Arecaceae; colloidal system; dynamic light scattering.

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1 INTRODUÇÃO

1.1 *Euterpe oleracea* Mart.

A espécie *Euterpe oleracea* Mart., pertence à divisão Magnoliophyta, classe Liliopsida Principes, família Arecaceae e ao gênero Euterpe. A família Arecaceae contêm 38 gêneros e 277 espécies, sendo que o gênero Euterpe está distribuído por quase todo território brasileiro, sendo considerado ausente somente nos estados de Roraima, Ceará, Piauí e Mato Grosso do Sul. Este gênero conta com cinco espécies aceitas, incluindo *E. oleracea* (ARECACEAE IN FLORA DO BRASIL 2020 EM CONSTRUÇÃO, 2017). Esta espécie é popularmente conhecida como açaí, juçara, açaí de touceira, açaí-do-Pará e açaí-verdadeiro, encontrando-se distribuída ainda em cinco estados brasileiros (Amapá, Pará, Tocantins, Maranhão e Goiás) (INPA, 2008; ARECACEAE IN FLORA DO BRASIL 2020 EM CONSTRUÇÃO, 2017). O açaí pode ser encontrado também em alguns países da América do Sul e América Central, como Colômbia, Equador, Suriname, Guiana, Peru, Venezuela, Costa Rica e Panamá (TROPICOS, 2017). É encontrada ainda em dois tipos de vegetações, denominadas floresta de terra firme e floresta de várzea, sendo encontrada com maior predominância na segunda (INPA, 2008; ARECACEAE IN FLORA DO BRASIL 2020 EM CONSTRUÇÃO, 2017).

A palmeira de açaí possui cerca de 25 estipes, sendo que algumas chegam até 20 m de altura e 18 cm de diâmetro. A inflorescência é do tipo intrafoliar e envolvida por brácteas que quando se abrem expõem o cacho de açaí e consequentemente os seus frutos. A coloração dos frutos de açaí varia conforme a maturação, apresentando-se verdes quando imaturos e roxos quando maduros. Esses frutos possuem uma morfologia globosa com diâmetro variando de 1 a 2 cm, peso em torno de 1,5 g e mesocarpo com aproximadamente 1 mm de espessura que abrange o endocarpo, além de uma semente no interior (MAPA, 2002; INPA, 2008).

O açaí depois de dois anos e meio de plantio inicia o seu processo de floração, sendo esse mais comum nos períodos que possuem maior índice pluviométrico (MAPA, 2002; INPA, 2008). Segundo o Instituto Brasileiro de Geografia e Estatística - IBGE (2016), no Brasil foram extraídos 216.071 toneladas de frutos açaí no ano de 2015. Grande parte da colheita do açaí se encontra na região norte,

principalmente no estado do Pará, que concentrou a maior parte da extração (126.027 toneladas), já no estado do Amapá foram extraídas 2.413 toneladas. Além disso, essa espécie também é cultivada para a produção de palmito, sendo considerada de alto valor econômico (INPA, 2008).

Tradicionalmente, o açaí é utilizado para produção de polpa, utilizada ao longo das refeições e se constituindo como parte fundamental da dieta em determinadas regiões ribeirinhas (SERGIO et al., 1999; POMPEU; SILVA; ROGEZ, 2009).

O açaí é conhecido mundialmente por sua elevada atividade antioxidante, o que caracteriza sua principal atividade farmacológica, principalmente relacionados aos frutos maduros (GORDON et al. 2012). Também são atribuídos a derivados dos frutos do açaí a inibição de células cancerosas da mama, colo do útero e pulmão (SILVA et al., 2014; BARROS et al., 2015). Outra importante ação farmacológica exercida pelas substâncias ativas mais conhecidas do açaí que são as antocianinas, é na inflamação, onde atua como um potente inibidor da síntese de óxido nítrico e como possível inibidor do aumento de interleucinas e fator de necrose tumoral (TNF alfa) (MATHEUS et al., 2006; MACHADO et al., 2015). Outras atividades terapêuticas que se destacam são na prevenção da obesidade, esteatose hepática e como hipoglicemiante (UDANI et al., 2011; SOUZA et al., 2012; OLIVEIRA et al., 2015), prevenção de doenças cardiovasculares (COSTA et al., 2012), inclusive em casos de infarto do miocárdio (ZAPATA-SUDO et al., 2014). Previne ou diminui a progressão de doenças neurodegenerativas (SPADA et al., 2009), possivelmente reduz a inflamação pulmonar acarretada pela inalação da fumaça de cigarro (DE MOURA; FERREIRA; LOPES, 2012), além de prevenir lesões renais (MORSY; AHMED; AHMED, 2015) e convulsões (SOUZA-MONTEIRO et al., 2015).

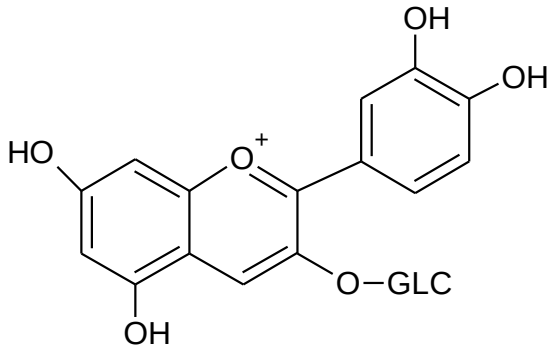
Diversas substâncias oriundas dos frutos de açaí foram identificadas, incluindo antocianinas, principalmente a cianidina 3-O- glucosídica (figura 1) e cianidina 3-O-rutinosídica (figura 2). As antocianinas, possuem em sua estrutura um cátion flavílio e anéis aromáticos, constituindo um subgrupo dos flavonoides e são as responsáveis pela pigmentação roxa do fruto de açaí (SIMÕES et al., 2010). Outros flavonoides encontrados são a homorientina, orientina e isovitexina, porém, encontradas em quantidades menores (GALORI et al., 2004). Além dessas

substâncias, um estudo conduzido por Darnet et al. (2011), evidenciou a presença de α-tocoferol (vitamina E) na polpa obtida dos frutos de açaí.

A fração lipofílica dessa espécie não varia significativamente em relação as partes do fruto (pericarpo, endocarpo e fruto íntegro). Suas substâncias majoritárias, são ácidos graxos insaturados, incluindo o ácido oleico e ácido palmitoleico, além de ácidos graxos saturados, como o ácido palmítico. Quantidades inferiores de ácidos mono e poli-insaturados também são observados (MANTOVANI et al., 2003; OKADA et al., 2011). O alto teor de ácido oleico e palmitoleico, que juntos constituem mais da metade da composição apolar, resulta em uma maior fluidez da fração lipofílica obtida de frutos de açaí (MANTOVANI et al., 2003). Estão presentes ainda na fração lipofílica do açaí os polifenóis, entre eles os dímeros e trímeros de procianidinas, ácido vanílico e síringico (PACHECO; PALENCIA, 2008). O ácido oleico, é o principal ácido graxo presente na fração lipofílica do açaí, o mesmo é um ácido graxo monoinsaturado (C 18:1) e tem como principais propriedades biológicas o efeito anti-inflamatório, hipocolesterolêmico e na prevenção de doenças cardiovasculares (REAVEN; GRASSE; TRIBBLE, 1994; CARRILLO et al., 2016; CARLUCCIO et al., 1998).

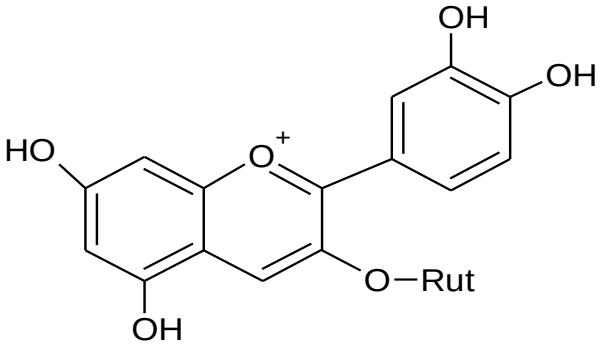
Esse produto possui um grande potencial nutricional e farmacológico, se assemelhando muito ao azeite de oliva (RUFINO et al., 2011). No entanto, ele ainda é considerado um produto secundário do processamento dos seus frutos (SILVA; ROGEZ, 2013).

Figura 1. Cianidina 3-O- glucosídica.



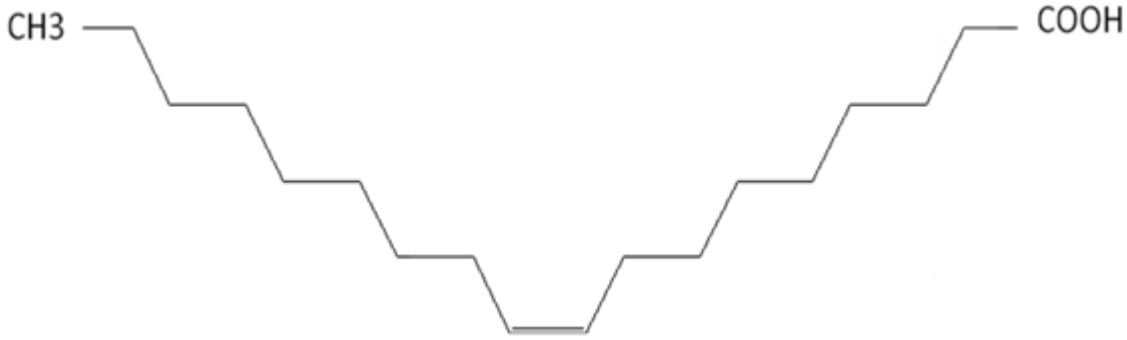
Fonte: Próprio autor

Figura 2. Cianidina 3-O-rutinosídica.



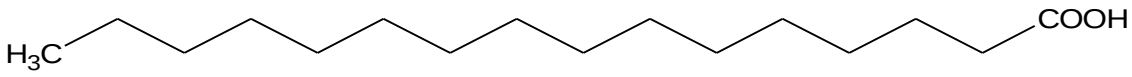
Fonte: Próprio autor

Figura 3. Ácido oléico



Fonte: Próprio autor

Figura 4. Ácido palmítico



Fonte: Próprio autor

1.2 NANOTECNOLOGIA

A primeira menção à nanotecnologia foi feita em 1959 pelo físico Richard Feymann, quando o mesmo sugeriu a manipulação de partículas diminutas. Porém, foi no ano de 1974, que o termo Nanotecnologia foi criado pelo professor Norio Taniguchi, da Universidade de Tóquio, que descreveu o termo como fabricação de

materiais em escala nanométrica (INSTITUTO INOVAÇÃO, 2005; FERNANDES; FILGUEIRAS, 2008). A partir desse momento, o mercado envolvendo a nanotecnologia tem crescido. De acordo com a Agência Brasileira de Desenvolvimento Industrial (ABDI 2013), no mercado mundial anualmente são arrecadados 11 milhões de toneladas de nanomateriais, representando aproximadamente 11 bilhões de euros, sendo que a tendência é aumentar a cada ano. No cenário da pesquisa científica mundial, o Brasil é um dos países que mais se destaca, tanto em número de pesquisadores quanto em infraestrutura, resultando em um expressivo crescimento nos números de patentes, principalmente na área de pesquisas espaciais, eletrônica, têxtil e cosméticos (FERNANDES; FILGUEIRAS, 2008; ABDI, 2011; ANNA; ALENCAR; FERREIRA, 2013).

O campo farmacêutico mostra-se como um dos mais promissores para o uso da nanotecnologia, agregando valor a seus produtos e despertando interesse da indústria (ABDI, 2010; DIMER, 2013). Diversos nanoprodutos com ampla aplicação nessa área têm sido desenvolvidos, como nanoemulsões, nanopartículas, lipossomas, entre outas (SHAH; BHALODIA; SHELAT, 2010; FATHI; MOZAFARI; MOHEBBI, 2012; SILVA; CERQUEIRA; VICENTE, 2012; SOLÈ, 2012).

1.3 NANOEMULSÕES

Na área farmacêutica, emulsões são frequentemente descritas como preparações constituídas por uma fase oleosa, uma fase aquosa e um agente emulsificante, frequentemente, um tensoativo. Uma emulsão pode ser classificada em dois tipos. Quando o óleo constitui o meio interno e a água o externo, caracterizando uma emulsão óleo em água (O/A). Caso ocorra o inverso, ela se caracteriza como uma emulsão água em óleo (A/O) (PIANOVSKI et al., 2008).

As nanoemulsões ou miniemulsões são emulsões em escala nanométrica, que possuem tamanho de gotícula situado entre 20 a 500 nm. Elas são termodinamicamente instáveis, diferindo, por exemplo, das microemulsões, que são termodinamicamente estáveis. No entanto, devido ao tamanho de partícula reduzido consegue-se alcançar uma estabilidade adequada frente a fenômenos como a cremagem, coalescência e sedimentação, levando as nanoemulsões serem

consideradas cineticamente estáveis (TADROS et al., 2004; GUTIERREZ et al., 2008; SOLÉ; SOLANS, 2012).

Para que se obtenha uma nanoemulsão estável, frequentemente são necessários os tensoativos, que são substâncias de caráter anfifílico (grupamentos hidrófilos e lipófilos) e atuam reduzindo a tensão interfacial entre os dois outros componentes da formulação. Também atuam criando um filme interfacial que impede a agregação entre as partículas da fase interna. Tensoativos mais hidrofílicos tendem a produzir nanoemulsões do tipo O/A enquando tensoativos mais lipofílicos tendem a produzir nanoemulsões do tipo A/O. (SILVA; CERQUEIRA; VICENTE, 2012; ALLEN; POPOVICH; ANSEL, 2013).

Griffin (1949) determinou uma escala numérica de 1 a 20 para classificar os tensoativos baseada no Equilíbrio Hidrófilo-Lipófilo (EHL), onde o EHL aumenta de acordo com a hidrofília da molécula. Neste contexto, o conhecimento do valor de EHL de um tensoativo é de extrema importância para se prever o sentido da formulação. Sendo assim, os tensoativos com EHL baixos geralmente formam uma formulação do tipo água/óleo, enquanto uma formulação do tipo óleo/água é formada com a utilização de tensoativos com EHL alto. Quando um tensoativo ou mistura de tensoativos resultam em um EHL que coincide com o EHL requerido da fase oleosa, ocorre a formação de uma emulsão estável (ORAFIDYA; OLADIMEJI, 2002). Essa abordagem tem sido utilizada com sucesso para obtenção de pequenos tamanhos de gotícula da fase interna e nanoemulsões, sendo demonstrado através de diversos estudos como, o desenvolvimento de nanoemulsões do óleo de babaçu (RODRIGUES et al., 2015), óleo de tucumã (SILVA et al., 2015) e nanoemulsão de *Manilkara subscericea* (FERNANDES, et al., 2014).

A preparação de nanoemulsões possibilita uma série de vantagens, como uma atividade biológica eficaz, possibilidade de maior biodisponibilidade, melhor estabilidade e maior absorção, além de uma aparência e aceitabilidade agradável (BRUXEL et al. 2012). Além dessas vantagens, as nanoemulsões possibilitam a incorporação de fármacos ou óleos vegetais pouco solúveis (FATHI; MOZAFARI; MOHEBBI, 2012). As nanoemulsões são basicamente por dois métodos distintos, que envolvem alto ou baixo aporte de energia. O primeiro grupo de métodos utiliza equipamentos que induzem alta força de cisalhamento, sendo assim capazes de romper as gotículas da fase dispersa, como por exemplo, homogeneizadores de alta

pressão e velocidade, além de geradores de ultrassom (TADROS et al., 2004; KENTISH et al., 2008). No entanto, o método de alto aporte de energia tem uma limitação, que são os elevados custos dos equipamentos (SCHUH; BRUXEL; TEIXEIRA, 2014). O segundo grupo de métodos utiliza baixo aporte de energia, sendo que para a formação de nanoemulsões com partículas diminutas utilizam-se de fenômenos físico-químicos, como por exemplo, a emulsificação espontânea e inversão de fases (SALAGER et al., 2004; LIU et al., 2006; MARTINI et al., 2007; PREZIOSI et al., 2013).

Para verificar a estabilidade das nanoemulsões, é extremamente importante avaliar parâmetros como o potencial zeta, que estabelece a diferença de potencial elétrico no meio, sendo que o valor estabelecido como parâmetro é de +30 a – 30 mV. Outra forma de verificar a estabilidade, é medindo o tamanho de partícula e índice de polidispersão, sendo que essa medição ocorre através de equipamentos que utilizam espectroscopia de correlação de fótons, que por meio de um mecanismo de dispersão de luz fornece as medidas de tamanho de partícula e polidispersão (SILVA; CERQUEIRA; VICENTE, 2012; BRUXEL et al., 2012).

1.4 PRODUTOS NATURAIS NANOEMULSIONADOS

1.4.1 Extratos vegetais e óleos fixos nanoemulsionados

Diversos estudos se destacam por obter nanoemulsões que tem como ativos os extratos vegetais e óleos fixos. No estudo de Fernandes et al., (2014), foi possível obter uma nanoemulsão estável do tipo óleo em água com tamanho inferior a 200 nm e com uma ação inseticida a partir dos triterpenos dessa importante espécie predominante da Floresta Atlântica. Outro estudo obteve uma nanoemulsão utilizando um extrato hidroetanólico de *Vellozia squamata* a partir das suas folhas e caule, onde por meio dessa formulação foi possível desenvolver uma nanoformulação com propriedade antioxidade, principalmente em razão da presença de di e triterpenos presentes nesse extrato (QUINTÃO et al., 2013).

Quanto aos óleos fixos, os mesmos têm a vantagem de serem carreados em nanoemulsões devido em parte ao auxílio dos tensoativos. Exemplos de óleos fixos desenvolvidos em nanoemulsões são os óleos de Jojoba e Tucumã, onde em ambos

foi possível obter nanoemulsões estáveis, além da determinação do EHL (COSTA et al., 2014; SILVA et al., 2015), proporcionando à essas formulações aplicabilidade para diferentes ações farmacológicas.

1.4.2 Oleoresinas e óleos essenciais nanoemulsionados

No Brasil, há diferentes estudos inovadores utilizando matérias-primas vegetais de diferentes biomas. Uma dessas espécies é a Copaíba, onde no estudo de Rodrigues et al. (2014), desenvolveu-se uma nanoemulsão (óleo/água) com potencial larvicida, agregando valor a essa importante espécie Amazônica através do uso de uma tecnologia de baixo custo. Outra pesquisa envolvendo espécies brasileiras que possui potencial para gerar um produto nanobiotecnológico inovadore é o estudo de Oliveira et al. (2016), onde através do oleoresina de sucupira (*Pterodon emarginatus* Vogel) desenvolveu-se uma nanoelmusão com atividade larvicida contra o *Aedes aegypti*, sugerindo ainda que o possível mecanismo de ação seja por meio da inibição reversível da acetilcolinesterase.

Entre os estudos envolvendo óleos essenciais, a pesquisa desenvolvida por Duarte et al. (2015), utilizou uma espécie que não é endêmica do Brasil, mas que possui diversas ações farmacológicas que é o Alecrin (*Rosmarinus officinalis*). A partir das folhas dessa espécie foi possível obter o seu óleo essencial através do método de hidrodestilação. Portanto, utilizando esse óleo essencial desenvolveu-se uma nanoemulsão de alecrin (tamanho de partícula menor que 200 nm) estável por mais de 30 dias de armazenamento, sendo que a partir da mesma obteve-se ainda um resultado satisfatório contra as larvas de *A. aegypti*.

1.4.3 Formulações e Nanoformulações inovadoras contendo o Açaí

Há estudos na literatura que mostram o grande valor comercial e farmacológico que o açaí pode proporcionar nas mais diversas formulações farmacêuticas. Um desses estudos é o que evidencia uma promissora atividade cosmética do açaí, obtida por meio do seu extrato que possui ação antioxidante (GARBOSSA; CAMPOS, 2016). Outra pesquisa também relacionada a aplicabilidade cosmética do açaí foi desenvolvida por Ferrari; Rocha-Filho (2011), onde os mesmos

preparam uma emulsão múltipla (óleo/água/óleo) com óleo de açaí com o intuito de avaliar sua estabilidade física e o Fator de Proteção Solar (FPS), no entanto, os resultados mostraram que a partir dessa formulação não foi possível obter uma proteção solar satisfatória, mas que a mesma pode ter maior aproveitamento nessa área em pesquisas que aproveitem o seu potencial antioxidade e anti-inflamatório.

Um estudo desenvolvido por Valério et al. (2013), obteu nanopartículas de poliuretano contendo o óleo de açaí como co-surfactante no preparo das mesmas (processo de polimerização). Já em outro estudo de Monge-Fuentes et al. (2017), foi desenvolvida uma nanoemulsão com óleo de açaí que apresentou potencial na terapia de melanoma, sendo que os testes foram realizados in vivo, onde a formulação continha 9 g do tensoativo Polissorbato 80 mais 2 g de óleo de açaí.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Preparar nanoemulsões do tipo óleo em água contendo a fração lipofílica obtida dos frutos de *Euterpe oleracea* Mart.

2.2 OBJETIVOS ESPECÍFICOS

- Efetuar a caracterização química da fração lipofílica obtida de frutos de *Euterpe oleracea*;
- Preparar nanoemulsões por método com solvente orgânico;
- Determinar o Equilíbrio Hidrófilo-lipófilo requerido (EHLr) da fração lipofílica obtida de frutos de açaí;
- Preparar nanoemulsões por método com aquecimento e avaliar o processo de diluição das nanoemulsões antes do armazenamento;
- Preparar nanoemulsões por método isento de solventes / sem aquecimento;
- Caracterizar as nanoemulsões obtidas.

3 ARTIGO

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Preparation of aqueous nanoemulsions with the lipophilic fraction of açaí (*Euterpe oleraceae* Mart.) fruits

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Abstract

Euterpe oleracea is a Brazilian palm species and its fruits are commonly known as açaí. The oil extracted from açaí fruits is chemically comparable to olive oil, being oleic acid considered its main constituent. Despite the great potential of açaí, to our knowledge, it remains almost unexplored concerning obtainment of nanoemulsions with its lipophilic fraction. The transesterification reaction catalyzed by lipase from *Candida antarctica* (CAL-B) was used for characterization of the fatty acids present in the sample and oleic acid was the main compound. Anthocyanins were also found in the lipophilic fraction. Nanoemulsions were prepared using different methods, using organic solvent or heating or organic solvent-free/non-heating methods. Several formulations were obtained by changing surfactant nature. Lower mean droplet size (184.7 ± 0.92 nm) and narrow distribution (polydispersity index = 0.218 ± 0.003) was observed using polyethylene glycol 400 dioleate 1% (w/w, expressed as surfactant content at former aqueous phase) in the organic solvent method. No phase separation, satisfactorily particle size distribution (mean droplet size = 313.3 ± 8.84 nm and polydispersity index = 0,400 ± 0,036) and zeta potential (-37.4 ± 0.551) were observed for the nanoemulsion prepared with polysorbate 80/sorbitan monooleate (HLB 11) in the organic solvent-free/non-heating method. The present study allowed a critical understanding of some aspects of nanoemulsification of the lipophilic fraction from *Euterpe oleracea* O/A nanoemulsion. Thus, it contributes to valorization of acai oil and development of potential phytopharmaceuticals innovative nanoproducts with this natural Amazon raw material.

Keywords: Arecaceae; colloidal system; dynamic light scattering.

1. Introduction

Euterpe oleracea is a Brazilian palm species that belongs to the family Arecaceae (Leitman et al., 2015). Its fruits are commonly known as açaí and it is recognized as an important source of phenolic compounds, including anthocyanins (Pacheco-Palencia et al., 2009; Gordon et al., 2012). Açaí fruits also have a great lipid content, being chemically comparable to olive oil (Gordon et al., 2012; Silva and Rogez, 2013). The lipophilic fraction obtained from açaí fruits is mainly constituted by monounsaturated and polyunsaturated fatty acids (Rufino, 2011), being oleic acid and palmitoleic acid the major unsaturated compounds. In general, they correspond to more than 50% of total fatty acid content (Mantovani; Fernandes; Menezes, 2003). Due to high percent of antioxidant substances, açaí lipophilic fraction was considered a potential edible oil with great potential to prevent cardiovascular diseases (Nascimento; Antoniassi; Freitas, 2008). It has anti-inflammatory and antinoceptive properties, being suggested that the mechanism of action may involve prostaglandin biosynthesis inhibition (Favacho et al., 2011). Antimicrobial activity against *Staphylococcus aureus* (Melhorança-Filho and Pereira, 2012) and antioxidant activities (Okada et al., 2011) were also observed. Moreover, its bioactive substances presented antiproliferative effects on HT-29 human colon adenocarcinoma cells (Pacheco-Palencia et al., 2008). Absence of cytotoxic and genotoxic effects in human lymphocytes suggested that this important Amazon raw material has potential application for phytomedicines (Maistro; Marques; Tsuboy, 2013).

Aqueous nanoemulsions are dispersed systems of oil and water, stabilized by one or more surfactants. They have small droplet size (Wang et al., 2009) and are considered kinetically stable, despite the fact that they are not thermodynamically stable, such as microemulsions (Bruxel et al., 2012). However, nanoemulsions

usually are formed using low surfactant concentrations, unlike microemulsions, which are formed at high surfactant concentrations and may have limited applications (Solè et al., 2012). A main concern is observed regarding definition of acceptable range of droplet size of internal phase for these nanoemulsions. The upper limit may range according to different authors, often from 200-500 nm. However, it is well established that no major influence on the properties of the nanoemulsion may occur after reaching the nanoscale, when compared to the bulk material (Solè et al., 2012). Several applications have been described for nanoemulsions, including for insecticides, cosmetics, food and medicines (Rodriguez-Rojo et al., 2012; Hadzir et al., 2013; Sugumar et al., 2014). They have many advantages that makes them potential formulations for pharmaceuticals, including improvement of water solubility for poor water soluble drugs (oil in water nanoemulsions), increased skin penetration and to enhance absorption and bioavailability of drugs (Shen; Wang; Zang, 2011; Donsi et al., 2012; Dias et al., 2014). They can be used for internal or external administration, including by oral route or topical application (Li et al., 2014; Rigo et al 2015). Moreover, oil in water (O/W) nanoemulsions may be used by parenteral route, mainly due to very small droplet size (Araujo et al., 2011; Bruxel et al., 2012). However, in this case the small droplet size should be reached, in addition to stabilization of fine droplets that must remain with low mean diameter. Several studies describing nanoemulsions as potential phytomedicines also have been related, mainly due to aforementioned advantages (Li et al., 2011; Quintão et al., 2013).

The main strategies related to nanoemulsification involves high energy or low energy methods. The high energy methods make use of devices that induce high input of energy, such high pressure homogenizers, high speed shear and ultrasound.

The low energy methods are associated to spontaneous nanoemulsification and/or phase inversions that main occur under constant temperature (Phase inversion composition – PIC) or by ranging the temperature (Phase inversion temperature - PIT) (Solè et al., 2012). Despite great potential of acai lipophilic fraction, to our knowledge, it remains almost unexplored regarding obtainment of nanoemulsions, which was focused on the phase inversion temperature method (Monge-Fuentes et al., 2017). Search for the nanoemulsification by low energy method in contrast to high energy method is especially interesting in terms of economic viability and should be encouraged. Thus, the aim of the present study was to prepare a nanoemulsion using this important Amazon raw material by low energy methods.

2. Experimental Section

2.1. Materials

Sorbitan monooleate (HLB 4.3), polyethylene glycol 400 dioleate (HLB 8.5), polyethylene glycol 600 dioleate (HLB 10.0), polyethylene glycol 400 monooleate (HLB 11.0), polyethylene glycol 600 monooleate (HLB 13.0), polysorbate 80 (HLB 15.0) were obtained from Praid® Especialidade Químicas (SP, Brazil). Polysorbate 20 was obtained from Dinâmica® Química Contemporânea (SP, Brazil). Lipase acrylic resin from *Candida antarctica* expressed in *Aspergillus niger* was purchased from Sigma Aldrich. Hexane was purchased from Labsynth Produtos para Laboratórios (SP, Brazil) and distilled water was used for nanoemulsion preparation.

2.2. Euterpe oleracea (Mart.) oil extraction

Açaí fruits were obtained from a local market on Macapá (AP, Brazil) and identified by the botanist Rosângela Sarquis. Açaí (*Euterpe oleracea*) oil was obtained according to a previous method (Silva and Rogez, 2013) with some modifications. Briefly, fresh pulp was obtained by mechanical stirring with water at 45 ºC. Further lyophilization step was performed and afforded a dry residue (1.210 kg), which was placed on a 5.0 L bottom flask and extracted with 3.5 L of hexane under reflux for 90 minutes. After this period, hexane extract was concentrated under reduced pressure using a rotary evaporator (IKA HB 05), furnishing 393.1 g of açaí fruits lipophilic fraction (AFLF).

2.3. Chemical characterization

2.3.1. Esterification reaction of the oleic acid by CAL-B

The standard ethyl oleate (EO) was prepared by classic esterification using the modified protocol of Rosset et al. (2013) and used in the analytical curve for quantify the oleic acid present in the sample of AFLF. Briefly, 1.0 mL (0.895 g) of oleic acid, 1.2 mL of ethanol (99.5%) and 50 mg of *Candida antarctica* lipase (CAL-B ≥ 5000 U/g) were added in a flask (5.0 mL). The reaction was maintained at 30 °C (± 2 °C) under magnetic stirring (300 rpm) for 24 h. At the end of the reaction, anhydrous sodium sulfate was added and the mixture was filtered and washed with hexane (3 x 1.0 mL). Excess solvent was evaporated under reduced pressure and the product purified by flash silica gel column chromatography eluted with hexane

(Rosset et al., 2013). The isolated yield of the ethyl oleate product was 65.2% (0.641 g). The mixtures of stock solution of EO (20 mg/mL) in chloroform and a stock solution naphtol as internal standard (4 mg/mL) were prepared and analyzed by GC-MS. The quantification of the EO and internal standard were plotted with the concentration of the OA in the AFLF.

2.3.2. Transesterification reaction of AFLF by enzymatic catalysts

The AFLF was transesterified prior to gas-chromatography analysis. The reaction followed the method described by Rosset (2011) with some modifications. In a vial (5 mL), it was added the AFLF (1.0 mL, 0,813 g), 5% (50 mg) of the lipase from *C. antarctica* B and 3.0 mL of ethanol (99.5%) on magnetic stirring (300 rpm, 30 ± 2 ºC) by 24 h. After this period, the enzyme was filtered and washed with hexane (3 x 1.0 mL). The filtrate was evaporated under reduced pressure and purified by column chromatography on silica gel using hexane:ethyl acetate (9:1) as eluent. The product, fatty acid ethyl esters (*FAEE*), obtained were analyzed by Gas-Chromatography-mass spectrometry and Infrared - Fourier transform.

2.3.3. Gas-Chromatography-mass spectrometry (*GC-MS*)

The FAEE was pooled together and used for the analysis on a GCMS-QP 2010 gas-chromatograph coupled to mass spectrometer (Shimadzu MS2010 Plus) using electron ionization (70 eV). It was solubilized in dichloromethane (1 µg/mL) and 1.0 µL of the solution was subjected to following experimental conditions: injector temperature, 210 °C; detector temperature, 250 °C; carrier gas, Helium; flow rate 1

mL/min; split injection with split ratio 1:30. The oven temperature was programmed from 120 °C, with an increase of 5 °C/min to 260 °C, ending with a 4 min isothermal at this temperature, the total analysis time was 36 min. RTX-5MS column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 µm). Mass spectrometry (MS) conditions were ionization voltage, 70 eV and scan rate, 1 scan/s. Mass range was *m/z* from 50 to 500. Identification of the fatty esters was by comparison literature data and CG-MS library (MS database, NIST 5.0) of the fragmentation spectrum.

2.3.4. Fourier transform infrared analysis

The AFLF and FAEE were analyzed by a Shimadzu IRAffinity spectrometer (FTIR), the samples were prepared on films of KBr disks in the 4000–400 cm^{-1} region with resolution of 4 cm^{-1} and 64 scans.

2.3.5. Determination of total anthocyanins contente

The total anthocyanins content on AFLF was determined by the pH differential method (Inácio et al., 2013) with some modifications. Two portions of AFLF were individually suspended in KCl buffer (pH 1.0) or AcONa buffer (pH 4.5) and vigorously homogenized using a vortex. Then, the systems were centrifuged. The final volume of the supernatants were adjusted to final concentration of approximately 1.0 mg/mL and absorbance of solutions at pH 1.0 and 4.5 was measured at 520 and 700 nm using a UV-Mini spectrophotometer (Shimadzu). The total anthocyanins content (TAC) was calculated as follows:

$$TAC\ (\%, w/w) = \frac{A}{e\ x\ l}\ x\ MW\ x\ DF\ x \frac{V}{W}\ x\ 100\%$$

Where, A = (A520nm - A700nm) pH1.0 (A520nm - A700nm) pH4.5; MW (molecular weight) = 449.2 g.mol1 for cyanidin-3-glucoside (cyd- 3-glu); DF = dilution factor; W = sample weight (mg); l.0 = path length in cm; *e* = 26.900 M extinction coefficient in L mol^{-1} cm^{-1} for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

2.4. Nanoemulsions

2.4.1. Emulsification-evaporation method

Nanoemulsions were prepared according to previous method (Leong et al., 2011), with some modifications. Several formulations were prepared as function of surfactant type and concentration. Organic phase was constituted by AFLF hexane solution (1 %, w/v) and surfactant aqueous solution constituted aqueous phase (1% or 3% w/w). Aqueous phase was heated (60 ± 2 ºC) and emulsification process involved addition of organic phase drop wise on aqueous phase (1:9) under constant mechanical stirring (500 rpm). The system was continuously stirred for 10 min. Final homogenization was performed using an Ultra-Turrax T-25 digital (IKA-Werke, Germany) at 8000 rpm for 3 min. Organic solvent was removed under reduced pressure using a rotary evaporator. Distiller water was added in order to obtain 100 mL of each nanoemulsion. They were stored protected from light under room temperature (25 ± 2 ºC).

2.4.2. Heating method

Several stock solutions of surfactant (s) and AFLF were prepared at a wide range of hydrophile-lipophile balance values. Each stock solution presented a surfactant (s) to AFLF ratio of 1:1. The resulting HLB values were calculated as follows:

$$HLB = \frac{HLBa\ x\ ma + HLBb\ x\ mb}{ma + mb}$$

Where:

HLB_a is the HLB value of the most hydrophobic surfactant

HLB_b is the HLB value of the most hydrophilic surfactant

ma is the mass of most hydrophobic surfactant

mb is the mass of most hydrophilic surfactant

Two sets of formulations were prepared using polysorbate 20/sorbitan monooleate (HLB 4.3 – 16.7) or polysorbate80/sorbitan monooleate (HLB 4.3 – 15). Each formulation presented a final mass of 50 g as follows: 2.5 % (w/w) of surfactant (s); 2.5% (w/w) of AFLF and 95 % (w/w) of water. The stock solutions constituted the oily phase, which was kept under magnetic stirring and controlled temperature (80 ± 5º C) for 30 min. Then, deionized water (80 ± 5º C) was added using a syringe under constant flow and the system was stirred for 1 h. After this period, final mass was restored to 50 g using deionized water and the system was stirred for additional 10 min.

2.4.3 Influence of dilution prior to storage

The most stable formulations prepared during rHLB evaluation were diluted (1:2) with distilled water or two diferente aqueous surfactant dispersions (1%, w/w), with polyethylene glycol 400 dioleate or polyethylene glycol 600 dioleate. After dilution, the systems were stirred for 10 min using a magnetic stirrer and stored protected from light under room temperature (25 ± 2 ºC). The optimal dispersed

system was used as template for the nanoemulsion prepared in the solvent-free/non-heating method.

2.4.4. Solvent-free/non-heating method

The effect of enhancement of surfactant to oil ratio on the optimal dispersed system was evaluated. Five stock solutions were prepared ranging the SOR (90:10, 80:20, 70:30, 40:60, 50:50). Each formulation had 1.0 g of stock solution and final mass was 4 g. The water was added drop wise to the stock solution placed on a screw-top vial under vigorous agitation using a vortex stirrer. The formulations were stored protect from light under controlled temperature (25 ± 2 ºC).

2.4.5. *Characterization of nanoemulsions*

Macroscopical analysis was performed in order to verify presence of creaming, phase separation and determine emulsion type (W/O or O/W). Nanoemulsions were characterized on Day 0 and Day 7 using a Zeta Sizer Nano-ZS (Malvern, UK). Droplet size and polydispersity index were obtained after dilution of each nanoemulsion with distilled water for injection (1:25). All measurements were performed in triplicate and results expressed as mean and standard deviation.

3. Results and Discussion

Extraction of açaí fruits yielded 32.5% (w/w) of a green oily lipophilic fraction (AFLF). The transterification of AFLF revealed the presence of unsaturated and saturated fatty acids in the chromatogram (Figure 1). The utilization of CAL-B enzyme as a biocatazylser allows several advantages, such high efficiency, complete chemical conversion and purity of the product. It causes hydrolysis of the

tryacilglycerol and reaction with ethanol (Mehrasbi et al., 2017; Ferreira et al., 2016). Moreover, the lipase from *C. antarctica* B is used in several studies, being considered very efficient (Rosset et al., 2011; Stamatis; Seretis; Kolisis, 2001).

The analysis by GC-MS showed that oleic acid (**3**) is the main compound, with 56% of fatty acid content in the AFLA. It was also possible to identify the palmitic (**1**), linoleic (**2**) and stearic acids (**4**) in the AFLA sample. Rufino et al. (2011), and Mantovani, Fernandes and Menezes (2003) also found oleic acid as major compound of the lipophilic fraction of açaí, corresponding to more than 50% of fatty acid content.

The oleic acid is very interesting in terms of pharmacological action, being useful to revert the resistance to insulin and also in inflammatory processes (Marinho et al., 2015; Carrillo et al., 2017), reduction of LDL levels and thus acting in hyperlipidemias and hypertrigleceremies (Reaven; Grasse; Tribbl, 1994). It also prevent cardiovascular diseases, acting specifically on aterogenesis inhibition and monocytes recruitment (Carluccio et al., 1999). Debbabi et al., (2016) showed that this fatty acid prevent neurodegenerative disorders. A pre-clinical study indicated that the high oleic acid content olive oil diminished the free radical production and augmented the level of cellular markers that protect the organism against cytokines and organic dysfunctions (Albuquerque et al., 2016).

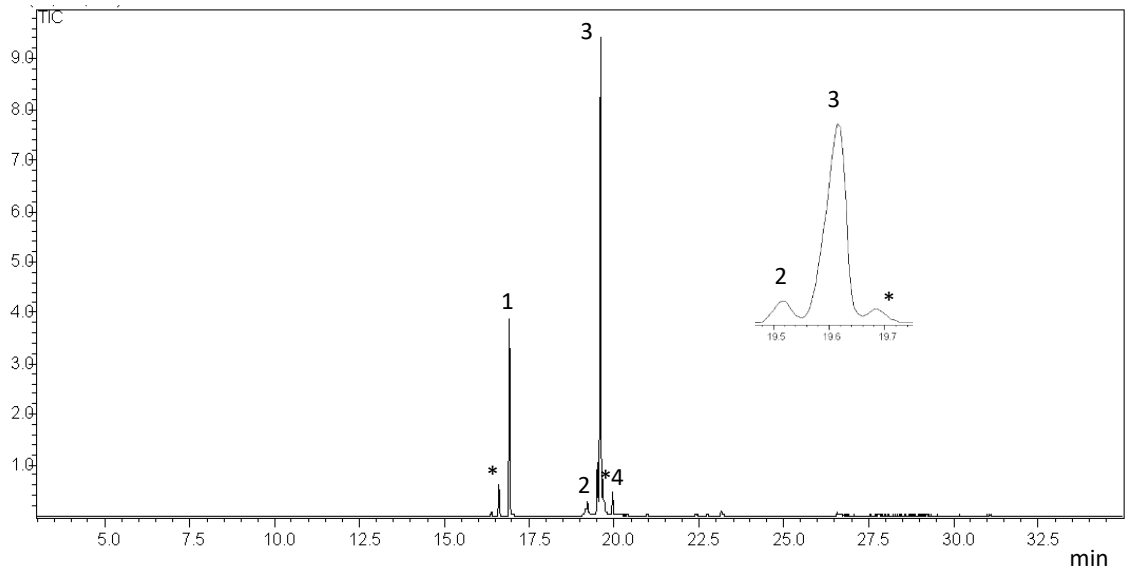


Figure 1. Chromatogram of FAEE from *Euterpe oleracea* Mart. Fatty acids: 1 = palmitic acid, 2 = linoleic acid, 3 = oleic acid, 4 = stearic acid, * = non identified.

The infrared spectra of AFLF and FAEE (Figure 2) shows an absorption band in the range of 2922-2854 cm^{-1} which is attributable to axial vibration deformation of C-H methyl ($-CH_3$) and methylene bonds ($-CH_2$). The medium intensity peaks at 1500 cm^{-1} are also associated to C-H bond, specifically to angular deformation vibration. Around 1300 cm^{-1}, the bands are attributable to C-O bond, suggesting sobreposition of signals with diferente intensities. The peaks around 717 cm^{-1} are associated to assimetric angular deformation vibrations of C-H bond (Purkayastha et al., 2012; Inácio et al., 2013). The intense absorption band at 1745 cm^{-1} and 1738 cm^{-1} are characteristic of axial deformation of C=O bond of aliphatic esters. The slight shift of the signal C=O is related to lower necessary energy for deformation, when compared to the triglyceride (Pavia et al., 2010).

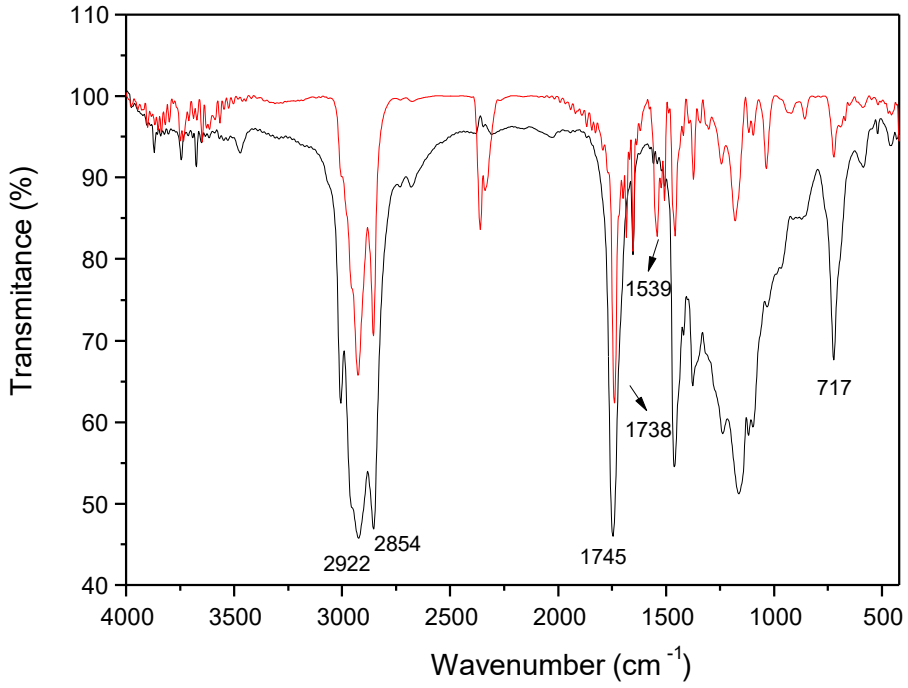
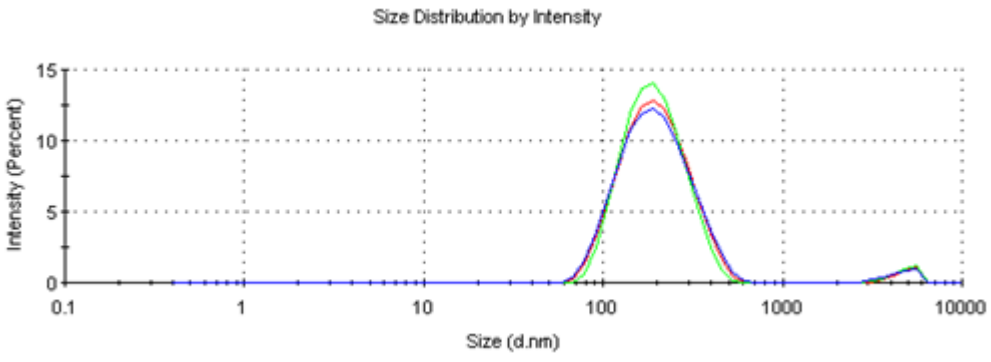


Figure 2. FTIR spectra of AFLF (—) and *FAEE (—)*.

The anthocyanins are major compounds of açaí fruits. Despite the lipophilic fraction has a great content of oleic acid, some level of anthocyanins was found on this sample (0.0718 %, w/w), expressed and cyanidin-3-glucoside. Despite the anthocyanins are present on AFLF, they occur on lower percentage, when compared to the content obtained direct from the fruits pulp, such 0.33 % (w/w) (Inácio et al., 2013) detected and 0.36 % (w/w) (Junior et al., 2016). Pacheco-Palencia, Mertens-Talcott & Talcott (2008), indicated the presence of phenolic acids on the lipophilic fraction of açaí, such vanilic acid (1.616 mg/kg) and syringic acid (1.073 mg/kg). Moreover, the anthocyanins cyanidin, cyanidin 3-glucoside and cyanidin-3-rutinoside were also found on the lipophilic fraction obtained by extraction of the fresh pulp with petroleum ether (Silva and Rogez, 2013).

Set of different formulations were prepared using hexane solution of AFLF (organic phase) and different aqueous surfactant solutions (1%, w/w) (aqueous phase). It was observed phase separation in the formulations prepared with polysorbate 80, polysorbate 20, polyethylene glycol 400 monooleate and polyethylene glycol 600 monooleate. Formulations prepared with polyethylene glycol 400 dioleate and polyethylene glycol 600 dioleate presented homogeneous appearance. Droplet analysis revealed that polyethylene glycol 400 dioleate and polyethylene glycol 600 dioleate formulations also presented low mean droplet diameter and low polydispersity index (polyethylene glycol 400 dioleate: size: 184.7 ± 0.9 nm; pdi: 0.218 ± 0.003 and polyethylene glycol 600 dioleate: size: 215.2 ± 1.82 nm; pdi: 0.299 ± 0.014) (Figure 3). However, after 7 days of preparation it was also observed phase separation. Spontaneous emulsification method using volatile organic solvent (hexane) followed by an evaporation step have been considered very promising due to its ability to generate small droplets (Kelmann et al., 2007). However, despite nanoemulsions were formed, desired stability of the systems were not achieved. This instability may be attributed to insufficient amount of surfactant. This component allow stabilization of nanoemulsions and must form a film around all droplets, allowing steric stabilization against coalescence (Wang et al., 2009).



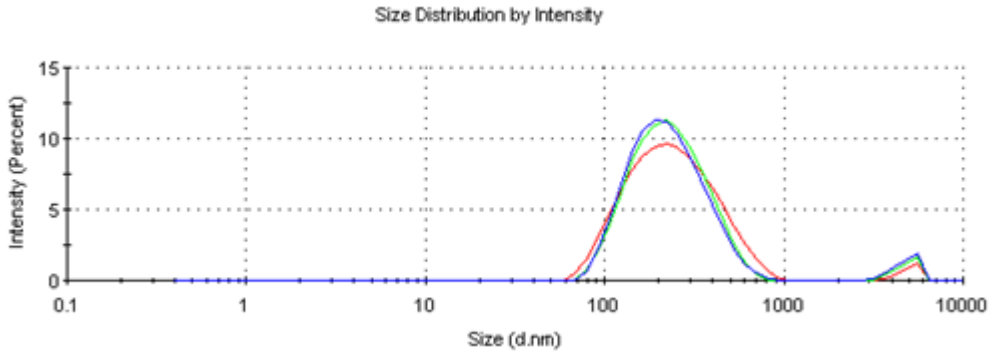


Figure 3. Characterization of nanoemulsion prepared using hexane solution of AFLF (organic phase) and different aqueous surfactant solutions (1%, w/w) (aqueous phase). **Upper figure** (polyethylene glycol 400 dioleate) **-** Day 0: droplet size: 184.7 ± 0.9 nm; polydispersity index: 0.218 ± 0.003. **Lower figure** (polyethylene glycol 600 dioleate) **-** Day 0: droplet size: 215.2 ± 1.82 nm; polydispersity index: 0.299 ± 0.014.

Some authors consider nanoemulsions only when mean droplet size in the range 20-200 nm. Despite they are thermodynamically instable, these systems have great kinetic stability, which is intrinsically associated to small droplet size (Gutierrez et al., 2008; Solè et al., 2012). It presents fine aspect and bluish reflect, which are characteristic for these systems (Tadros et al., 2004). In the case of aqueous (O/W) nanoemulsions, they have a major advantage to increase water release of low water-soluble substances, including natural compounds (Dias et al., 2012). Using this definition, only the system prepared with polyethylene glycol 400 dioleate would be considered an aqueous nanoemulsion. However, several other acceptable ranges are presented in the literature, for example, 30-300 nm (Zhang et al., 2011). On this context, the nanoemulsion prepared with polyethylene glycol 600 dioleate could also be considered a nanoemulsion. A critical review about this colloidal systems highlights that no major drastic changes in the physico-chemical properties may occur when nanosize is achieved. Moreover, it says that due to the fact that the

classification often consider the intended applications, this concern may remain open (Solè et al., 2012).

Utilization of a heating method showed that most of formulations at various HLB values presented unstable behavior (data not shown). Low creaming index were observed using polysorbate 20/sorbitan monooleate (HLB 9 and 10) and polysorbate 80/sorbitan monooleate (HLB 10, 11 and 12) (Table 1). No major macroscopical increase of stability was found using water or surfactant dispersion prior to storage in the formulations prepared with sorbitan monooleate/polysorbate 20 (HLB 9, 10 and 11) and sorbitan monooleate/polysorbate 80 (HLB 10). Lower creaming index was observed after dilution with surfactant dispersions when compared to water, in the formulation prepared with sorbitan monooleate/polysorbate 80 (HLB 12). The lowest creaming index and better macroscopical appearance was observed on the formulation prepared with sorbitan monooleate/polysorbate 80 at HLB 11 and diluted with water.

Stable emulsions and therefore with smallest mean droplet size, including nanoemulsions, are achieved when hydrophile-lipophile balance (HLB) of surfactant (s) coincide with required HLB value of oil phase (Fernandes et al., 2013). To our knowledge, no previous HLB determination of acai oil and/or lipophilic fraction was performed. Considering HLB value of best surfactant used in the present study, we may suggest that required HLB value of acai oil is around 11.0. Moreover, better indicatives of stability and homogeneity (absence of phase separation and lower creaming) suggest that polysorbate 80/sorbitan monooleate was the best pair. Therefore, the formulation constituted by water, polysorbate 80 at HLB 11 and AFLF was used as template for solvent-free/non heating method.

Table 1. Creaming index of dispersed systems prepared with AFLF and non-ionic surfactants by heating method.

	Day 0			Day 1		
HLB	H_2O	PEG400DO	PEG600DO	H_2O	PEG400DO	PEG600DO
9[a]	2.8	2.8	2.8	PS	PS	PS
10[a]	2.5	2.5	2.5	PS	PS	PS
10[b]	2.1	2.1	2.1	2.4	2.4	2.4
11[b]	1.1	1.3	1.4	1.7	2.1	2.3
12[b]	2.5	1.6	1.8	2.5	2.6	2.6

[a]HLB value achieved with sorbitan monooleate polysorbate 20

[b]HLB value achieved with sorbitan monooleate/polysorbate 80

Formulations diluted with water (H_2O), aqueous polyethylene glycol 400 dioleate dispersions (1%, w/w) (PEG400DO) or aqueous polyethylene glycol 600 dioleate dispersions (1%, w/w) (PEG600DO)

PS = phase separation

On the solvent-free/non-heating method, only the formulation prepared at the highest SOR (90:10) did not present creaming. DLS characterization showed that mean droplet size and polydispersity index were around 313.3 nm and 0.400, respectively, on the day of preparation. It presented almost no change regarding zeta potential and a major alteration on particle size distribution (lower mean droplet size and narrower droplet size population (Figure 4). Spontaneous decrease of mean droplet size may occur if volatile organic solvents are used during emulsification process, being induced by evaporation of residual solvent fraction from internal phase to external phase (Silva et al., 2011). However, in this case no organic solvent was used and the change for a more homogeneous particle size distribution may be due to the high content of unusual hydrophilic compounds in AFLF. The presence of

compounds that constitute the internal phase with some finite solubility in the water is associated to Ostwald ripening, the main breakdown process that is associated to loss of stability of aqueous nanoemulsions (Tadros et al., 2004). However, in the case of açaí oil, the presence of phenolics with great water solubility than usual components of vegetable oils, may be the responsible this phenomena. This may be due to the fact that, once released to the continuous phase, they will not re-aggregate and keep on solution. Further physic-chemical studies to validate this hypothesis should be carried out.

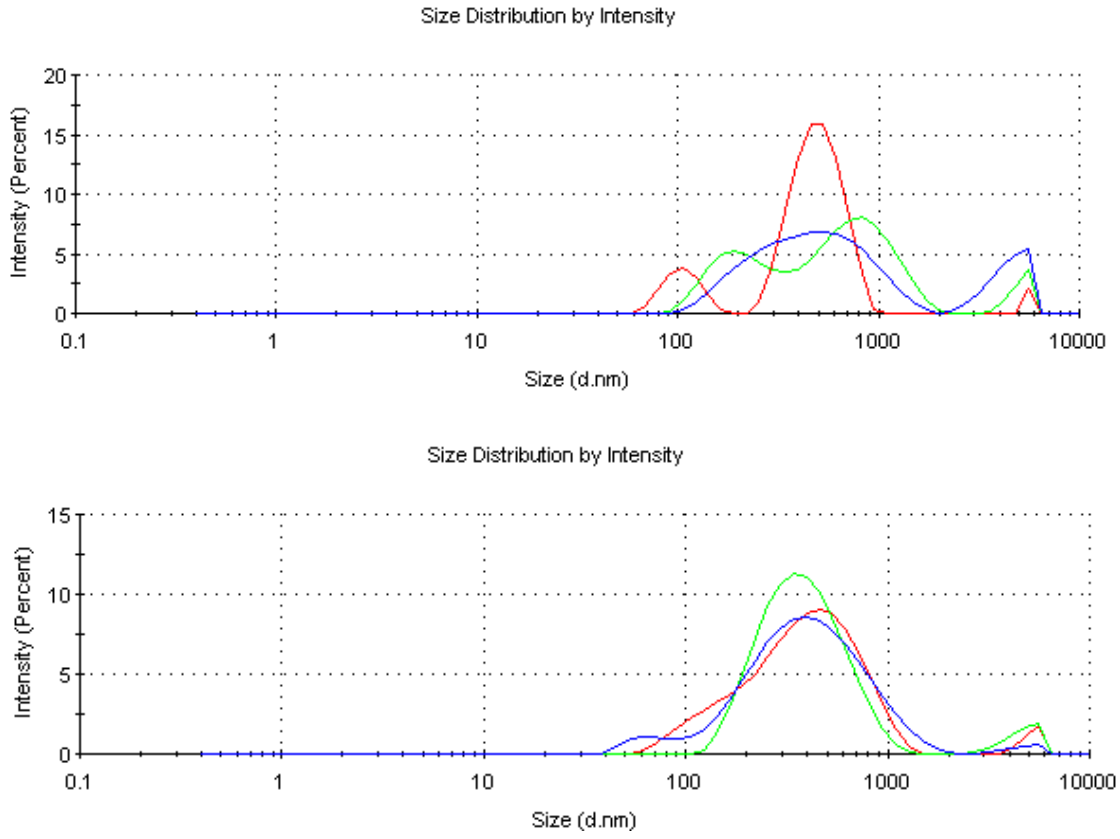


Figure 4. Characterization of nanoemulsion prepared with sorbitan monooleate/polysorbate 80 (HLB 11) at SOR 90:10, AFLF and water by solvent-free/non-heating method. **Upper figure -** Day 0: droplet size: 470.7 ± 39.41; polydispersity index: 0.685 ± 0.044; zeta potential: -38.2 ± 0.850. **Lower figure -** Day

7: droplet size: 313.3 ± 8.84; polydispersity index: 0.400 ± 0.036; zeta potential: -37.4 ± 0.551.

Several studies were carried out with some herbal oils aiming to generate nanoemulsions by different methods. Small droplet size around 60 nm was achieved using citronella oil and polysorbate 80/sorbitan monooleate. However, high input of energy was required, using an ultrasound generator (Agrawall et al., 2017). Another study revealed the ability of low energy method with heating step to induce formation of nanoemulsions with droplet size around 150 nm. The bioactives oleoresin that constituted the internal phase was obtained from the seeds of *P. emarginatus* and the chemical composition of this oil is very complex, including some diterpenes (Oliveira et al., 2016).

Some phytopharmaceuticals dispersed systems have been prepared with açaí fruits derived materials. O/W emulsion containing açaí glycolic extract (Daher et al., 2014) and O/W/O multiple emulsion containing açaí oil (Ferrari and Rocha-Filho, 2011) were prepared for photoprotective purposes. High storage stability and antioxidant activities of açaí oil make this Amazon raw material very potential for novel nutraceuticals, cosmetics and phytopharmaceuticals, instead of traditional vegetable oil-based products (Pacheco-Palencia, 2008). Valério et al., (2013) used açaí oil as co-surfactant on polymerization of polyurethane nanoparticles. A recent study also prepared açaí-oil based nanoemulsions. Small droplet size was achieved at surfactant to oil ratio of 9:2. It was estimated around 117 nm (pdi around 1.144) by DLS, while TEM analysis suggested mean diameter around 74 nm (Monge-Fuentes et al., 2017).

The anthocyanins that are remarkable phytochemicals of açaí fruits, being even present in the lipophilic fraction are susceptible to thermal degradation. Monge-Fuentes et al. (2017) used a phase inversion temperature method in which the coarse emulsion have to be heated to 85 ºC prior to addition of cold water. Thus, considering the specificity of this raw material and needs for ecofriendly methods (eg. solvent-free), we believe that our data opens perspectives for preparation of this nanoemulsions by non-heating and solvent-free methods.

Despite some studies in the literature indicate the formation of nanoemulsions by energy methods, they call them erroneously as phase inversion methods. Most of methodologies use classical steps that successfully allowed a phase inversion, however, it does not mean that always a phase inversion is involved. Solè et al. (2012) detailed the main mechanism in low energy methods. In addition to phase inversion methods, the self-emulsification (also spontaneous emulsification) can allow achievement of nanoemulsion without any phase transition. The nanodroplets can be formed during a dilution process with water and diffusion of water-miscible components, even at constant temperature, in which the dilution of oil in water microemulsions or cubic liquid crystals occur without a change in the surfactant spontaneous curvature occurs.

Regarding the phase inversion methods for preparation of oil in water nanoemulsions, a change in the spontaneous curvature of the surfactant from negative to positive must occur (Solé et al., 2012). Thus, to ensure that a phase inversion exist, the investigation of variation in any property that occurs during the phase inversion change (e.g. conductivity) either on the phase inversion composition (Calderó et al., 2011) or on the phase inversion temperature (Izquierdo et al., 2012).

The complexity of the lipophilic fraction of açaí fruits, with potential water-soluble secondary metabolites and absence of makes necessary further studies to better investigate the occurrence of spontaneous emulsification of phase inversion. Moreover, thermal degradability should be performed to ensure that methods related to PIT (or any heating method) can be used with no major degradation of some compounds (eg. anthocyanins). Pseudo ternary phase diagrams, which as still scarce for studies with more complex herbal oils (secondary metabolites-rich oils) are also a good approach to achieve small droplets and stable systems (Rodrigues et al., 2014), being also interesting for further studies aiming to generate açaí lipophilic fraction-based nanoemulsions.

4. Conclusion

Natural products are recognized as potential bioactive products. However, many of them have poor water solubility. Therefore, nanoformulations appear as an important alternative to solve this main problem. The present study allowed achievement of *Euterpe oleracea* O/A nanoemulsions by different methods. The fact that a solvent-free/non-heating method was also capable to form nanodroplets opens perspectives for ecofriendly utilization of this herbal derivative, which can be produced through a sustainable use of biodiversity. Thus, the present study contributes to valorization of acai oil and development of potential phytopharmaceuticals innovative nanoproducts with this natural Amazon raw material.

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Authors' Contributions

D.C.S.H. (Mastering student) contributed running the laboratory work, analysis of the data and drafted the paper. B.M.S.H. and J.C.T.C contributed to the extraction of plant material. R.A.S.C, F.B.A. and I.M.F contributed to chemical analysis. J.L.D. contributed to nanoemulsion preparation and characterization. A.E.M.F.M.O. contributed to critical reading of the manuscript. CPF designed the study, supervised the laboratory work and contributed to draft and revision of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A partir da realização desse estudo, foi possível obter nanoelmulsões estáveis por três diferentes métodos, utilizando alto aporte de energia e solvente orgânico, um método com aquecimento, mas sem solvente orgânico que delimitou a faixa de EHL requerido da fração lipofílica de açaí como sendo o de número 11, logo, esse passo foi primordial para a obtenção da nanoemulsão por um método sem solvente / sem aquecimento, em que houve redução do tamanho de partícula e polidispersão após o período de sete dias de armazenamento. Nossos dados indicam ainda que o par de tensoativo Polissorbato 80 e Span 80 foi eficiente para o preparo das nanoemulsões de açaí utilizando-se uma metodologia de baixo aporte de energia.

Estudos adicionais a fim de investigar a estabilidade dessa nanoformulação por um maior período é necessária, além de outros testes que servem de parâmetro para o controle de qualidade, como a rampa de aquecimento e termogravimetria. O desenvolvimento dessa nanoemulsão abre novas possibilidades para utilização da fração lipofílica de açaí, que é um sub-produto da polpa, tendo em vista suas diversas atividades biológicas, como nanofitoproduto promissor. Adicionalmente, este trabalho contribui para a valorização dessa matéria-prima amazônica, permitindo a geração de produtos de alto valor agregado.

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Anexo 3 – Normas de publicação da Revista Brasileira de Farmacognosia

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The manuscript should be arranged in the following order: Graphical abstract, Title, Abstract, Keywords, Introduction, Material and Methods, Results, Discussion, Acknowledgements, Authorship, References, Figures with Legends, Tables, Structural Formulae and Supplemental files (if applicable).

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Results should be clear and concise.

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This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

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