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Avaliação do potencial cicatrizante de nanoemulsões a base de andiroba (*Carapa guianensis* Aublet) *in vitro*.

ISOLDA DE SOUSA MONTEIRO

Brasília-DF

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Dissertação submetida ao Programa de Pós-graduação em Ciências e Tecnologias em Saúde da Universidade de Brasília para a obtenção do Título de Mestre.

Área de concentração: Nanobiotecnologia aplicada a saúde.

Orientadora: Profa Dra Graziella Anselmo Joanitti

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Dedico este trabalho à Isolda criança que nunca desistiu
dos seus sonhos, que eu nunca me esqueça dela

.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
AO	Óleo de andiroba
AOCS	American Oil Chemists' Society
ATR-FTIR	Attenuated total reflection module
AV	Acid value
CCD	Charge Coupled Device
CG-MS	Gas chromatography-mass spectrometer
DLS	Dynamic Light Scattering
DMEM	Meio Eagle modificado por Dulbecco
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDS	Dispersive X-ray Spectroscopy
EI	Electron impact ionization
ETOH	Ethanol
FBS	Fetal bovine serum
FTIR	Infrared spectrophotometry
GAE	Gallic acid equivalent
HaCat	Human keratinocytes
HD	Hydrodynamic diarmetrer
IC50	Inhibitory concentration of 50%
MET	Microscópio eletrônico de transmissão
MTT	3,4,5-dimetilthiazol-2,5 bifenil tetrazolium bromide
NeAnd	Nanoemulsão a base de andiroba
NEs	Nanoemulsões
NPs	Nanopartículas
PBS	Phosphate Buffer Saline
PdI	Índice de polidispersão
Ph	Potencial hidrogeniônico
PV	Peroxide value
RMN	Nuclear magnetic resonance
T0	Zero time
T24	24 hours
TPC	Total phenolic content
ZP	Zeta potential

Resumo

A cicatrização é um processo complexo, envolvendo mecanismos celulares, bioquímicos e moleculares. Lesões que não cicatrizam são resultados de uma interrupção na progressão da sequência fisiológica de eventos celulares e bioquímicos responsáveis pela restauração da integridade da pele. O óleo de andiroba (AO) é extraído das sementes de *Carapa guianensis* Aublet e possui diversas propriedades terapêuticas, como efeitos anti-inflamatórios e cicatriciais. A utilização de nanoemulsões tem sido promissora na entrega e melhora da biodisponibilidade de compostos bioativos hidrofóbicos. O objetivo deste estudo foi desenvolver uma nanoemulsão tópica a base de AO e avaliar seu potencial cicatricial *in vitro*. O AO foi adquirido comercialmente e caracterizado em relação a sua acidez, índice de peróxidos, perfil de ácidos graxos, Espectroscopia de Infravermelho com Transformada de Fourier (FTIR), ressonância magnética nuclear (RMN), antioxidantes e carotenóides. A nanoemulsão a base de óleo de andiroba (NeAnd) foi preparada com uma proporção de 2:1 de surfactante e óleo (p/p) por meio de ultrassonicação. A citotoxicidade da NeAnd foi testada em queratinócitos (Hacat) pelo método de MTT. O ensaio de *scratch* foi realizado para avaliar o efeito das NeAnd na migração celular. Os dados foram analisados estatisticamente usando ANOVA e pós-teste de Tukey. O perfil lipídico mostrou a presença e proporção de ácidos graxos esperadas para o óleo de andiroba. Adicionalmente, foram encontrados compostos bioativos como 2-undecenal, oleato de etila e palmitato de etila. O FTIR exibiu bandas típicas relacionadas à cadeia acila dos lipídios e o RMN confirmou os compostos majoritários do AO. NeAnd apresentou estabilidade quando armazenada a 4°C ao longo de 120 dias, diâmetro hidrodinâmico médio de 205,7 ± 3,9 nm, índice de polidispersão (PdI) de 0,295 ± 0,05, potencial zeta de -4,16 ± 0,41 mV e pH em torno de 6,5. O ensaio de viabilidade celular mostrou que NeAnd e AO livre em diferentes concentrações (90, 180 e 360 µg/mL), em queratinócitos, apenas o grupo AO foi citotóxico na concentração de 360 µg/mL (77,29%; $p<0,05$) em 48 horas. No scratch em queratinócitos, NeAnd (360 µg/mL) mostrou um aumento significativo (88,9%; $p<0,05$) na migração de células que resultaram no fechamento do risco em comparação com o grupo controle PBS e o AO livre. Os resultados *in vitro* indicam características de biocompatibilidade e o potencial das nanoemulsões desenvolvidas em processos de cicatrização. Estudos adicionais em modelos de cicatrização são necessários para que tais formulações sejam reconhecidas como nanofitoterápicos para aplicação clínica em tratamentos de cicatrização de feridas e regeneração tecidual.

Palavras-chave: *Carapa guianensis; cicatrização; nanoemulsão, óleo*

Abstract

Healing is a complex process involving cellular, biochemical, and molecular mechanisms. Non-healing injuries result from a disruption in the progression of the physiological sequence of cellular and biochemical events responsible for restoring skin integrity. Andiroba oil (AO), extracted from Carapa guianensis Aublet seeds, possesses therapeutic properties, including anti-inflammatory and scar-healing effects. The use of nanoemulsions holds promise for delivering and improving the bioavailability of hydrophobic bioactive compounds. The aim of this study was to develop a topical nanoemulsion based on AO and evaluate its scar-healing potential in vitro. Commercially acquired AO was characterized for acidity, peroxide index, fatty acid profile, Fourier Transform Infrared Spectroscopy (FTIR), nuclear magnetic resonance (NMR), antioxidants, and carotenoids. The andiroba oil-based nanoemulsion (NeAnd) was prepared with a 2:1 ratio of surfactant to oil (w/w) through ultrasonication. The cytotoxicity of NeAnd was tested on keratinocytes (HaCat) using the MTT method. The scratch assay was conducted to assess the effect of NeAnd on cell migration. Data were statistically analyzed using ANOVA and Tukey's post-test. The lipid profile confirmed the presence and expected ratio of fatty acids in andiroba oil. Additionally, bioactive compounds such as 2-undecenal, ethyl oleate, and ethyl palmitate were identified. FTIR exhibited typical bands related to the acyl chain of lipids, and NMR confirmed the major compounds of AO. NeAnd demonstrated stability when stored at 4°C for 120 days, with an average hydrodynamic diameter of 205.7 ± 3.9 nm, polydispersity index (PdI) of 0.295 ± 0.05, zeta potential of -4.16 ± 0.41 mV, and pH around 6.5. The cell viability assay showed that NeAnd and free AO at different concentrations (90, 180, and 360 µg/mL) in keratinocytes, only the AO group was cytotoxic at 360 µg/mL (77.29%; $p<0.05$) after 48 hours. In the keratinocyte scratch assay, NeAnd (360 µg/mL) demonstrated a significant increase (88.9%; $p<0.05$) in cell migration, resulting in the closure of the scratch compared to the PBS control group and free AO. In vitro results indicate biocompatibility characteristics and the potential of the developed nanoemulsions in healing processes. Further studies in wound healing models are necessary for these formulations to be recognized as nanophytotherapeutics for clinical applications in wound healing and tissue regeneration treatments.

Keywords: *Carapa guianensis; wound healing; nanoemulsion, oil*

1. Fundamentação Teórica

1.1. Cicatrização

A cicatrização de feridas é um evento que ocorre quando um tecido é lesionado de alguma forma. Diversos processos bioquímicos são envolvidos nesse processo. É um mecanismo complexo que ocorre no corpo em resposta a lesões teciduais, como cortes, queimaduras ou feridas. Essa série de eventos coordenados tem o objetivo de fechar a ferida, restaurar a integridade da pele e minimizar o risco de infecções. O processo de cicatrização, é geralmente dividido em três fases principais: inflamação, proliferação e remodelação (1,2).

A fase de inflamação é a primeira resposta do corpo à lesão, começa imediatamente após a lesão e pode durar de 72 horas a dias. A lesão causa uma ruptura nos vasos sanguíneos, levando ao extravasamento de sangue e fluidos que criam inchaço e vermelhidão. Ocorre o aumento da permeabilidade celular e as células imunológicas, como neutrófilos e macrófagos, são atraídas para a área lesionada para combater possíveis infecções. Eles também removem detritos celulares e bactérias. A liberação de substâncias químicas, como citocinas e fatores de crescimento, ajuda a regular o processo inflamatório e a iniciar a reparação (1,3).

A fase proliferativa ou regenerativa ocorre após a fase de inflamação, quando o tecido de granulação começa a se formar ao redor da lesão. As células começam a reparar o tecido danificado. Pode durar de alguns dias a várias semanas. Ocorre migração epitelial, proliferação endotelial e as células chamadas fibroblastos entram em ação e começam a produzir colágeno, uma proteína essencial na formação de tecido conjuntivo, que auxilia no fechamento do tecido lesionado. As células epiteliais, que revestem a superfície da pele, começam a proliferar e migrar para cobrir a ferida. Isso resulta na formação de um novo epitélio (1,4).

A fase de remodelação é a etapa final da cicatrização e pode durar de meses a anos. Durante essa fase, o colágeno produzido na fase de proliferação é reorganizado para fortalecer a cicatriz. No entanto, a cicatriz geralmente não é tão resistente quanto o tecido original da pele. Com o tempo, a cicatriz se torna menos visível e mais plana à medida que a cor e a textura se aproximam do tecido circundante. A força da cicatriz aumenta, embora nunca atinja a mesma resistência da pele original (Fig. 1) (2,5).

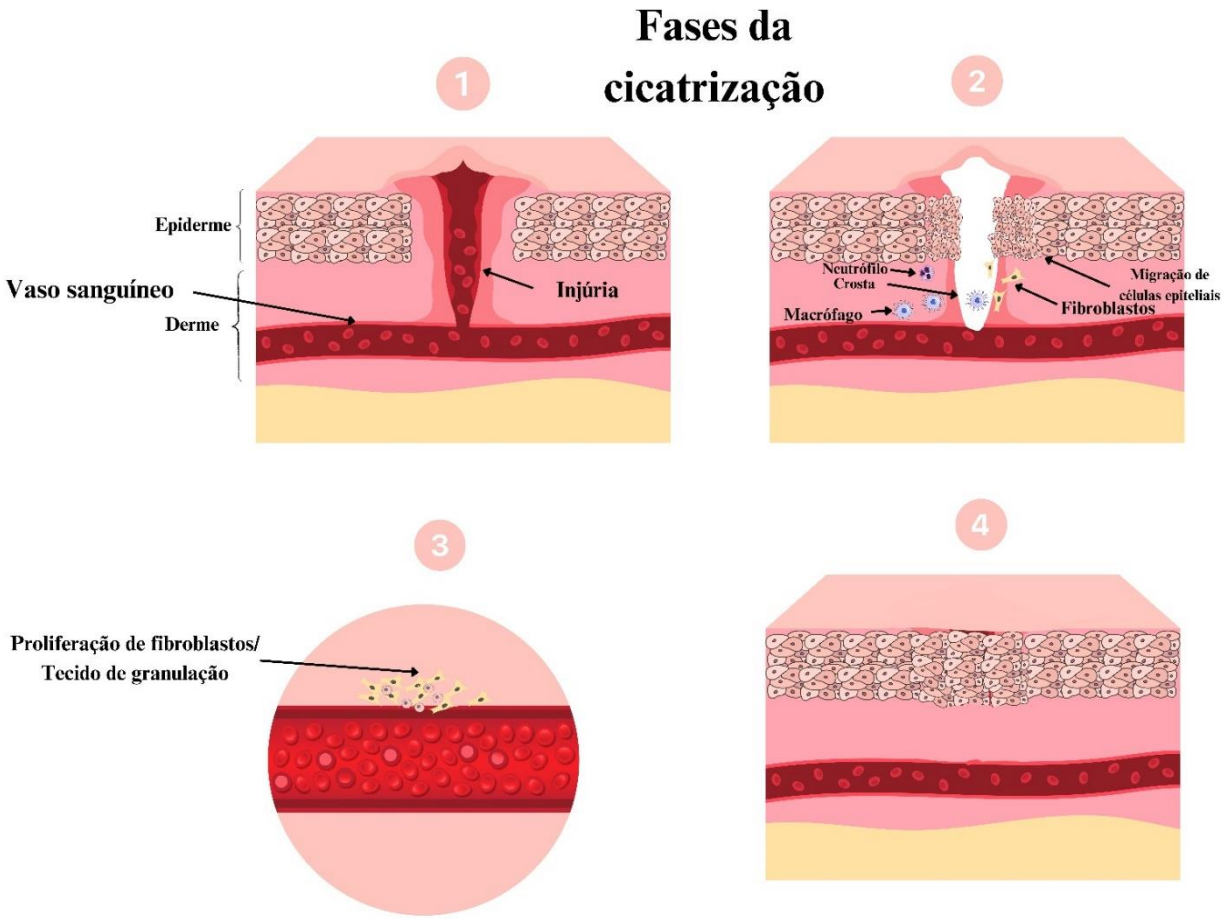

Figura 1: Imagem esquemática simulando as fases da cicatrização de feridas. Em 1- fase inflamatória, 2- fase proliferativa, 3- proliferação de fibroblastos, 4- fase final da remodelação do tecido danificado. Criada pela autora em: https://www.canva.com/pt_br/.

Feridas na pele tem um impacto negativo nos sistemas de saúde e na economia mundial. Um estudo mostrou que quase um bilhão de pessoas sofrem com feridas agudas e crônicas no mundo (2). As feridas cutâneas podem ser classificadas em agudas e crônicas de acordo com a patogênese e suas consequências (6,7). Os ferimentos agudos passam por uma série de eventos moleculares que eventualmente resultam na recuperação da integridade estrutural. Já as feridas crônicas não cicatrizam de forma adequada e são caracterizadas por processos patológicos tais como inflamação contínua, infecções persistentes e necrose (8).

Em nações desenvolvidas, entre 1% e 2% da população é afetada por feridas crônicas. Nos Estados Unidos, estima-se que cerca de 4,5 milhões de pessoas tenham esse tipo de ferida. Pesquisas mostram que a prevalência de feridas crônicas de diversas origens é de aproximadamente 2,21 casos por cada 1.000 indivíduos, enquanto a prevalência de úlceras crônicas nas pernas é estimada em cerca de 1,51 casos por 1.000 indivíduos (7,9).

À medida que a população envelhece e as doenças crônicas não transmissíveis aumentam, juntamente com a resistência microbiana e a formação de biofilmes, espera-se um aumento no número de feridas relacionadas a esses fatores. Isso acarretará custos significativos para o tratamento, o que terá um impacto financeiro nas instituições de saúde. Estima-se que os gastos com o tratamento de feridas crônicas representem de 1% a 3% dos custos totais de assistência à saúde, embora esses valores possam ser subestimados, pois não levam em conta a perda de produtividade, diminuição da qualidade de vida dos pacientes e aposentadorias precoces causadas por essas intercorrências (10,11).

Um estudo realizado nos EUA mostrou que as feridas afetaram 15% dos beneficiários do sistema de seguro de saúde Medicare, com um custo anual estimado de US$ 28 bilhões (12). No Brasil, um estudo em uma unidade de cuidados paliativos e prolongados identificou custos médios mensais de R$ 36.629,95 por paciente, projetando custos anuais de R$ 445.664,38 (13). O tratamento de feridas crônicas representa um desafio econômico para o sistema de saúde, afetando de maneira significativa a sociedade e prejudicando a qualidade de vida dos pacientes (14). Já no que diz respeito aos gastos com o tratamento de Lesões por Pressão (LPP), em um hospital em Minas Gerais, observou-se que o custo médio semestral por paciente foi de R$ 1.886,00, resultando em um custo total semestral de R$ 113.186,00. Quanto à distribuição dos custos relacionados à mão de obra dos profissionais de enfermagem, incluindo enfermeiros e técnicos de enfermagem, a maioria dos pacientes avaliados (68%) apresentou um custo máximo de até R$ 1.000,00, com alguns poucos pacientes (10%) registrando custos de mão de obra superiores a R$ 2.500,00. Os custos diretos foram atribuídos com ênfase nos materiais, coberturas/adjuvantes utilizados e no tempo de trabalho investido na execução desses procedimentos(15).

Abordagens convencionais, incluindo a administração sistêmica de agentes antimicrobianos e antibióticos, bem como aplicações locais de medicamentos, têm enfrentado obstáculos consideráveis. Problemas como a penetração limitada de medicamentos nos tecidos mais profundos da pele, o desenvolvimento de resistência bacteriana decorrente do uso prolongado de antibióticos e a ineficácia na erradicação de riscos de infecção microbiana sistêmica têm sido notáveis. Essas deficiências ressaltam a necessidade de abordagens terapêuticas inovadoras que possam mitigar obstáculos e melhorar a eficácia do tratamento de feridas crônicas. Nesse contexto, estratégias baseadas em nanotecnologia associada ao uso de compostos bioativos da biodiversidade brasileira têm surgido como alternativas promissoras,

oferecendo soluções para problemas persistentes associados aos métodos tradicionais de cicatrização de feridas (16). No presente trabalho, será abordado o uso do óleo de andiroba (*Carapa guianensis)* nanoestruturado para avaliação de seu potencial cicatrizante *in vitro*. Mais detalhes sobre o óleo de andiroba e o uso de nanocarreadores estão descritos nos tópicos a seguir.

1.2. Andiroba

A andiroba, conhecida como *Carapa guianensis* é pertencente à família Meliaceae sendo predominantemente encontrada na América Latina, na África e ao sul do Saara (17). Popularmente chamada de *crabwood* em inglês, andiroba em espanhol e *carapa blanc, ou Carapa de Guyane* em frânces (18).

Figura 2: Imagens da andirobeira (Carapa guianensis Aublet). A - a árvore inteira e outras da mesma espécie ao redor, B - folhas, C - sementes dentro do fruto e D - sementes retiradas do ouriço para extração do óleo. Imagens: Raimundo Lima.

Trata-se de uma árvore perene, capaz de alcançar aproximadamente 55 metros de altura. Possui um tronco cilíndrico e reto, bem como uma copa média densa composta por ramos verticais (19). As árvores desse gênero produzem frutos em forma de cápsulas globosas, geralmente dispersas de forma aleatória, que liberam suas sementes quando o fruto se abre após impacto no solo (Figura 1 a, b) (20). As sementes da andiroba exibem notável variação, principalmente em relação ao tamanho, peso e, em alguns casos, à forma. Dentro da mesma cápsula, é possível encontrar sementes diversas, embora elas estejam dispostas de maneira organizada, muitas vezes formando uma estrutura em espiral (19) (Figura 1 c, d).

Essa planta como um todo, desde sua casca, sementes e óleo, apresentam forte amargor característico da espécie. Mas os principais componentes utilizados dessa planta são suas sementes para extração do óleo, que é extraído e utilizado por comunidades nativas da Amazônia desde muito tempo como tradição para tratar diferentes patologias (21).

O óleo de andiroba possui atividades farmacológicas já descritas na literatura, que incluem ação anti-inflamatória, antioxidante, anticancerígena, antimicrobiana e benefícios para a saúde cardiovascular, devido à presença de ácidos graxos insaturados, como o ácido oleico e linoleico, bem como fitocompostos como limonóides, triterpenos, esteroides, cumarinas e flavonóides. Esses componentes contribuem para a redução da inflamação, proteção contra danos oxidativos, possíveis efeitos anticancerígenos, atividade antimicrobiana e angiogênica (22–26).

Os ácidos graxos insaturados, presentes no óleo de andiroba (AO), como os ácidos oleico e linoleico, desempenham um papel importante na produção de substâncias chamadas eicosanoides, que têm efeitos anti-inflamatórios, ajudam a reduzir o risco de doenças cardíacas e aumentam os níveis de colesterol HDL (27). Além disso, entre 3% e 5% do óleo consiste em substâncias como triterpenos, esteroides, cumarinas, flavonoides e, notavelmente, limonóides, que são fitoquímicos característicos da família Meliaceae (28,29). A análise físico-química do AO também revela uma quantidade substancial de polifenóis totais extraíveis, que provavelmente são responsáveis por suas propriedades antioxidantes, bem como seu potencial anticancerígeno, antimicrobiano e anti-inflamatório (27).

Alguns estudos investigaram as propriedades de cicatrização do óleo de andiroba e demonstraram sua eficácia. Por exemplo, Araújo e colaboradores em 2017, (30) observaram melhorias na regeneração da pele, proliferação de células fibroblásticas, produção de colágeno, formação de vasos sanguíneos e resposta imune em feridas tratadas com óleo de andiroba.

Outros estudos também indicaram que emulsões contendo andiroba são eficazes na aceleração da cicatrização de feridas na pele. Além disso, há evidências de que o óleo de andiroba pode ser uma opção promissora para o tratamento de queimaduras (31,32).

Apesar das propriedades cicatrizantes já documentadas na literatura do óleo de andiroba em feridas cutâneas, existem desafios na sua aplicação tópica, já que é um óleo com características hidrofóbicas, ou seja, pouco solúvel em água, o que pode influenciar na interação do mesmo com as células alvo. Diante disso, a nanotecnologia vem inovando com alternativas para a melhora da entrega desses compostos bioativos, como a nanoencapsulação de agentes bioativos e terapêuticos, que pode aumentar a sua eficiência, capacidade de interação com células e tecidos alvo, especificidade, redução das doses necessárias e diminuição da toxicidade (26,33).

1.3. Nanotecnologia

A nanotecnologia modificou a forma de funcionamento de diversas indústrias em todo o mundo. O conceito de nanotecnologia é de uso generalizado com quatro gerações de nanomateriais, sendo aplicadas em diversos campos interdisciplinares, como agricultura, alimentos, cosméticos, medicina, saúde, automóveis, petróleo, gás, química, mecânica, dentre outros. A nanotecnologia opera em uma escala nanométrica (10^{-9} m), alterando o tamanho dos materiais, suas propriedades e seus processos biológicos. Isso tem revolucionado a medicina, permitindo o tratamento de doenças anteriormente intratáveis e impactando a produção industrial (34,35).

A nanotecnologia desempenha um papel fundamental na medicina, oferecendo avanços notáveis em diagnóstico, tratamento e terapia regenerativa. Ela permite o desenvolvimento de dispositivos médicos, como nanorrobôs, sensores e ferramentas de diagnóstico em nanoescala. Além disso, a nanotecnologia é aplicada na medicina regenerativa, terapia celular e engenharia de tecidos, permitindo a melhora no reparo de celular, tecidos e órgãos. Também desempenha um papel essencial na genômica e proteômica, fornecendo informações moleculares sobre doenças. A nanotecnologia tem aplicações antimicrobianas e é usada para tratar doenças, enquanto nanosensores contribuem para a detecção de agentes tóxicos e diagnósticos mais precisos. A oncologia se beneficia com a nanotecnologia, que permite o desenvolvimento de nanoagentes e tratamentos anticancerígenos específicos, além de melhorar a eficácia dos medicamentos quimioterápicos. Esses avanços colaborativos entre médicos, pesquisadores e tecnologias vem revolucionando a medicina (36–41).

Existem diversos tipos de nanopartículas ou nanomateriais (NPs) que podem ser utilizados como nanocarreadores auxiliando no transporte de diversos fármacos e compostos bioativos. Dentre eles, pode-se citar nanopartículas metálicas, nanopartículas poliméricas, nanocarreadores baseados em lipídeos, podendo ser lipossomas e nanoemulsões, dentre outros (35). Biomateriais incorporados com essas NPs estão sendo utilizados para realizar a entrega de compostos bioativos de diferentes características, com o intuito de melhorar a aplicação, biodisponibilidade, diminuir a citotoxidade de fármacos e auxiliar na cicatrização de feridas. Essas NPs têm sido vastamente utilizadas na indústria da nanotecnologia para produção de diversos produtos (35). Estudos têm explorado o uso de nanoformulações à base de óleo de andiroba. Por exemplo, (42) investigaram os efeitos citotóxicos, genotóxicos e hematotóxicos de nanoemulsões contendo óleo de andiroba administradas por via oral e observaram que essas formulações são biocompatíveis, não apresentando efeitos genotóxicos, citotóxicos ou mutagênicos em testes realizados *in vivo*, embora tenham mostrado citotoxicidade em concentrações mais elevadas em testes *in vitro*.

No presente trabalho, foram utilizados nanocarreadores lipídicos (nanoemulsões) para investigar se esse tipo de nanoestrutura auxiliaria na melhora da biodisponibilidade dos compostos do óleo de andiroba (*Carapa guianensis* Aublet) no processo de migração de queratinócitos *in vitro*, evento importante durante o processo de cicatrização.

1.4. Nanoemulsões

As nanoemulsões (NEs) são sistemas coloidais de partículas com tamanho submícron (10 a 1.000 nm) que atuam como veículos para moléculas como fármacos e compostos bioativos. Como sistema de administração de medicamentos, as nanoemulsões melhoram a eficácia terapêutica, reduzem os efeitos adversos e reações tóxicas. Elas são aplicadas em tratamentos de infecções do sistema retículo-endotelial, terapia de reposição enzimática no fígado, tratamento do câncer, vacinação dentre outros. As NEs oferecem várias vantagens, como melhorar a biodisponibilidade de medicamentos, ter baixa citotoxidade, proporcionar maior estabilidade física, ter gotículas na escala nanométrica que aumentam a área de superfície e serem versáteis em termos de formulações e solubilização de compostos lipofílicos (35,43).

As nanoemulsões podem ter diversas configurações, mas podem ser do tipo óleo-em-água (O/A) ou água-em-óleo (A/O) (Figura 3), dependendo se o óleo está disperso como gotículas na água, ou vice-versa. Uma nanoemulsão do tipo óleo-em-água é definida como uma

dispersão coloidal termodinamicamente instável, cineticamente estável, consistindo em dois líquidos imiscíveis, sendo que um dos líquidos está disperso como pequenas gotículas esféricas no outro líquido em tamanhos nanométricos (10^{-9}m) (44). Uma nanoemulsão pode ser formada a partir de óleo e água, mas é necessário o uso de um surfactante para que o sistema seja estável quanto à coalescência das gotículas. O mesmo facilita a formação da nanoemulsão e garante sua estabilidade cinética durante o armazenamento. Às vezes, é usada uma combinação de surfactantes em vez de um único surfactante para formar e estabilizar nanoemulsões.

Uma nanoemulsão geralmente é preparada usando esses componentes: óleo, água, surfactante e possivelmente um co-surfactante. A estrutura das gotículas em uma nanoemulsão é formada por caudas apolares das moléculas de surfactante que se projetam no núcleo hidrofóbico formado pela fase oleosa, enquanto os grupos polares das moléculas de surfactante se projetam na fase aquosa circundante (Fig.3) (44).

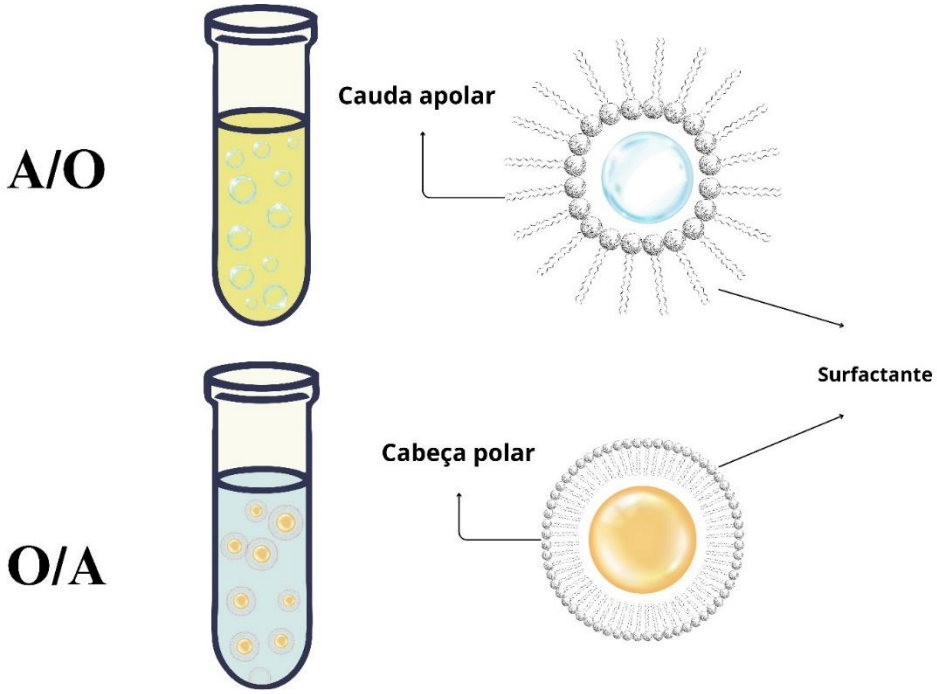

Figura 3: Imagem esquemática de nanoemulsões água em óleo (A/O) e óleo em água (O/A), em que a parte amarela representa a fase oleosa, e a parte azul clara, a fase aquosa. As caudas apolares do surfactante ficam sempre voltadas para a fase oleosa, devido às suas características lipofílicas. Fonte: Adaptada (35). *Criando pelo autor com: www.canva.com.*

Existem diversos métodos para preparação das NEs, os de alta e os baixa energia. Nos métodos de alta energia, é feita a aplicação de forças intensas para transformar gotículas

maiores em gotículas menores, criando assim emulsões com tamanho submicrométrico (45). Isso pode ser alcançado através de técnicas como a homogeneização de alta pressão, microfluidização e ultrassonicação (46). Cada método utiliza uma abordagem diferente para atingir a quebra das gotículas, e a escolha depende de vários fatores, incluindo a escala de produção, requisitos de tamanho de gotícula e composição da formulação. A presença de emulsificantes adequados é essencial para estabilizar as nanoemulsões produzidas por esses métodos (47,48).

A ultrassonicação é uma técnica onde são utilizados ultrassonicadores que emitem ondas ultrassônicas para promover forças de cavitação necessária para a quebra das gotículas. Quando se varia o tempo da energia ultrassônica, pode-se atingir o tamanho desejado das partículas (49–52). O processo de preparação de uma nanoemulsão óleo-em-água (O/A) usando o método de ultrassonicação se inicia com o primeiro passo envolvendo a preparação de uma macroemulsão O/A, que é feito misturando óleo, água e surfactante em um sistema de agitação por um período adequado. Em seguida, a macroemulsão é transformada em nanoemulsão por meio de um ultrassonicador, ondas de choque de alta energia criam turbulência, devido à cavitação, que rompe as gotículas (53).

A lecitina é um agente emulsificante natural amplamente utilizado na indústria de alimentos, farmácia, cosméticos e biotecnologia. Consiste em uma mistura de fosfolipídios com glicolipídios, sendo a fosfatidilcolina o componente mais comum. A característica anfipática das moléculas de lecitina as torna eficazes como agentes emulsificantes, permitindo interações tanto aquosas quanto lipofílicas. Embora em NEs as lecitinas naturais frequentemente sejam combinadas com surfactantes sintéticos, elas permanecem a escolha preferencial devido à sua biocompatibilidade e baixa ocorrência de reações alérgicas. Essas propriedades fazem com que as lecitinas naturais sejam seguras para uso em nanoemulsões lipídicas, mesmo em aplicações sensíveis (54–57).

NEs vem sendo investigadas a respeito da entrega de compostos derivados de plantas medicinais para o tratamento de ferida. Nanoemulsões à base de lecitina mostraram ser eficientes e de baixa toxicidade para tratamentos tópicos. Essas NEs contendo extrato de casca de bétula enriquecido com betulina (BET) ou tampão fosfato salino (PSB), nas concentrações 1:200 e 1:400, demonstraram maior viabilidade celular e atividade proliferativa, indicando potencial para promover a cicatrização de feridas. NEs com PSB e BET também mostraram maior eficácia no fechamento de feridas *in vitro* em comparação com controles, sugerindo seu

valor como candidatos para o tratamento de feridas (58). Outro estudo com hidrogel carregado com extrato de *A. satureioides* demonstrou promover a cicatrização de feridas, reduzir inflamação e dano oxidativo, e estimular a formação de novos vasos sanguíneos em modelos de pele suína, indicando seu potencial terapêutico (59).

Devido à sua versatilidade, nanoemulsões podem ser formuladas em várias formas farmacêuticas, como líquidos, cremes, sprays, géis, aerossóis, e espumas, e administradas por diversas vias, incluindo tópica, oral, intravenosa, intranasal, pulmonar e ocular. Estas nanoemulsões apresentam maior capacidade de dispersão do que formulações micelares simples, bem como estabilidade cinética superior em relação a microemulsões. Além de seu uso estabelecido na indústria cosmética e de pesticidas como base aquosa para produtos orgânicos, as nanoemulsões oferecem benefícios significativos na administração de medicamentos. A estabilidade física a longo prazo é atribuída ao pequeno tamanho das gotas, mitigando fenômenos convencionais de desestabilização. Na forma parenteral, as nanoemulsões protegem medicamentos contrafatores ambientais agressivos, alcançam órgãos específicos e podem evitar o sistema retículo-endotelial. Por via oral, as nanoemulsões melhoram drasticamente a dissolução e a biodisponibilidade de medicamentos hidrofóbicos (60).

Quando pensamos na interseção da biodiversidade e bioeconomia, a nanotecnologia e sua aplicação vêm desempenhando papéis cruciais na agregação de valor aos recursos biológicos. A capacidade de explorar a diversidade de plantas, animais e microorganismos em um nível molecular oferece oportunidades para o desenvolvimento de produtos, como medicamentos, cosméticos e produtos agrícolas. Abre-se um caminho para a descoberta de novas substâncias naturais com aplicações em setores como medicina, energia e alimentos. A nanotecnologia, integra diretamente a biodiversidade em sua escala mais fundamental, cria um ambiente propício para a promoção da bioeconomia e o uso responsável dos recursos biológicos em benefício da sociedade e do meio ambiente (61–64).

Diante do exposto, não foram encontradas, até o momento, publicações que abordem o desenvolvimento e uso de nanoemulsões contendo óleo de andiroba para avaliar em processos envolvidos na cicatrização *in vitro*. Portanto, este trabalho teve como objetivo desenvolver e caracterizar nanoemulsões à base de óleo de andiroba como base para um produto fitoterápico e avaliar seus efeitos na citotoxicidade e migração de queratinócitos *in vitro*, que são células envolvidas no processo de cicatrização de feridas.

2. Objetivos

2.1. Geral: O objetivo desta pesquisa foi desenvolver formulações de nanoemulsões a base do óleo de andiroba (*Carapa guianensis* Aublet) e avaliar seus efeitos na citotoxicidade e migração de queratinócitos *in vitro.*

2.2. Específicos

- Obter e caracterizar o óleo de andiroba (*Carapa guianensis* Aublet), quanto aos seus compostos bioativos, índice de acidez e índice de peróxidos;

- Formular, caracterizar e analisar a estabilidade da nanomeulsão a base de óleo de andiroba (NeAnd) ao longo do tempo;

- Avaliar a citotoxicidade das NeAnd e do óleo de andiroba livre em linhagem de queratinócitos *in vitro*;

- Avaliar o potencial cicatrizante das NeAnd e do óleo de andiroba livre (*Carapa guianensis* Aublet) em ensaio de migração celular *in vitro* (*Scratch assay*);

3. Capítulo I

Este capítulo apresenta um artigo científico resultante desta dissertação, submetido a revista Journal of Drug Delivery Science and Technology.

Development and characterization of an Andiroba oil-based nanoemulsion (*Carapa guianensis*) and its potential healing effects *in vitro.*

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Abstract

Andiroba oil, extracted from *Carapa guianensis* seeds, possesses therapeutic properties including anti-inflammatory and wound healing effects. This study aimed to develop a nanoemulsion formulation containing andiroba oil and assess its wound healing potential *in vitro*. The andiroba oil underwent evaluation for acidity, antioxidant properties, and fatty acid profiling. Nanoemulsions (NeAnd) were prepared with a 2:1 ratio of surfactant and oil (w/w) through ultrasonication. Characterization was conducted using FTIR, Raman spectroscopy, dynamic light scattering, and transmission electron microscopy. Cytotoxicity assessments were performed on HaCat cells (human keratinocytes) while a scratch assay was employed to evaluate their impact on cell migration. Data were statistically analyzed using ANOVA. The oil exhibited expected acidity and fatty acid profiles consistent with andiroba oils, with notable antioxidant activity. NeAnd displayed a spherical shape and stable properties, with an average hydrodynamic diameter (DH) of 205.7 ± 3.9 nm, a polydispersity index (PdI) of 0.295 ± 0.05, a negative zeta potential of –4.16 ± 0.414 mV, and pH around 6.5. These nanodroplets remained stable for 120 days when stored at 4°C and maintained their DH and PdI, even when subjected to pH variations. FTIR and Raman analyses confirmed the functional groups and the arrangement of the fatty acid chains in NeAnd. Cell viability assay showed that NeAnd and free andiroba oil were not cytotoxic to keratinocytes at various concentrations (90, 180, and 360 µg/mL) after 24 and 48 h *in vitro*. In the scratch wound healing assay, NeAnd showed a

significant improvement in wound closure compared to the PBS control and free andiroba oil in keratinocyte cells ($p<0.05$). These promising findings indicate NeAnd as a potential nanophytomedicine for future clinical applications in wound healing and tissue regeneration treatments.

Keywords: *Carapa guianensis*; Wound healing; nanoemulsion; oil.

1. Introduction

Healing is a complex process involving various cellular, biochemical, and molecular mechanisms, encompassing different phases. In the inflammatory phase, the body responds with blood clotting, cellular infiltration, and the release of antimicrobial agents and cytokines, which initiates the proliferative response for wound repair. In the proliferative phase, epithelial cells cover the wound surface, and granulation tissue grows to fill the space. This tissue formation includes the proliferation of fibroblasts, deposition of collagen, development of new blood vessels, and other extracellular components. Once the new tissue is formed, the remodeling phase begins, restoring the structural and functional integrity of the tissue [1,2]. Non-healing wounds are caused by disruptions in the physiological healing process, influenced by factors like comorbidities (diabetes, peripheral vascular disease, immunosuppression), complications (infections), and an intensified inflammatory state**.** Poor wound healing has a negative impact on both the global economy and public health [3,4].

The andiroba tree (*Carapa guianensis*) plays a crucial role across various domains, particularly in indigenous, bioeconomic, pharmaceutical, and wound tissue regeneration contexts. The importance of andiroba underscores its holistic value, integrating cultural, economic, medicinal, and therapeutic aspects in traditional medicine, its oil is employed to address a variety of skin conditions, inflammations, and as an insect repellent. Within the pharmaceutical field, studies emphasize its anti-inflammatory, antioxidant, and antimicrobial properties. Recently, the andiroba has gained prominence due to its wound tissue regeneration capabilities. Compounds present in andiroba oil have demonstrated promising efficacy in accelerating the healing process, suggesting its potential application in promoting skin health and managing chronic wounds [5].

Nanotechnology refers to the study, creation, manipulation, and exploration of a wide range of areas, such as biology, physics, and chemistry at the nanoscale [6]. There are different types of nanostructures that can be used as carriers for bioactive compounds, such as liposomes, nanoemulsions, and polymeric nanoparticles, among others [7]. Nanoemulsions are heterogeneous systems in which a liquid (dispersed phase) is dispersed in another liquid (continuous phase) as nanosized droplets. The size of these droplets varies between 20-500 nm, and the types of nanoemulsions vary according to the class of dispersed and continuous phases [8]. The use of nanoemulsions has shown promise in the delivery and improvement of the bioavailability of hydrophobic bioactive compounds, as medications based on these compounds can have solubility and bioavailability issues in biological fluids [9,10]. Additionally, nanosystems enhance the efficacy, stability, and safety in delivering natural bioactive compounds [11]. Nanotherapies have been significantly growing in the field of wound healing, with various studies and techniques analyzing how nano-scale strategies can influence different phases of the wound repair process [12,13].

The encapsulation of vegetable oils has become a promising strategy to facilitate the application of natural products and enhance their actions [14]. Studies that evaluated AO in *in vivo* models to assess its wound healing potential showed a reduction in healing time in wounds

treated with AO [15–18]. The oil from *Carapa guianensis* appears to decrease the density of collagen type I fiber networks, forming them in a reticular pattern and, notably, increasing the presence of type III fibers. Additionally, there is an increase in fibroblast proliferation and greater angiogenesis in the injury region, which is essential for tissue regeneration [16,19] Nevertheless, to the best of our knowledge, no study has investigated if a nanostructured AO improves the oil wound healing effect.

Therefore, considering the advantages of nanotechnology and the wound healing effects of AO, in this study, we employed nanotechnology techniques to develop and characterize a nanoemulsion formulation based on andiroba oil (NeAnd). We also assessed its cytotoxicity and wound healing potential *in vitro*. Our findings showed that it was possible to obtain and successfully characterize a nanoemulsion based on andiroba oil (NeAnd). It is noteworthy to highlight that NeAnd did not induce cytotoxicity in any of the times and concentrations evaluated. In addition, the present nanotechnological strategy improved andiroba oil's effect on wound healing *in vitro* by stimulating keratinocytes migration and accelerating wound closure.

2. Material and methods

2.1. Materials

The andiroba oil was commercially acquired by the company FERQUIMA LTDA (SP, Brazil). The oil was extracted through cold pressing. Egg lecithin (E-80) was purchased from Lipoid (Ludwigshafen, Germany). Hexane (P.A.) was purchased from Dinâmica Química Contemporânea LTDA (SP, Brazil). MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), methanol, dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, and trypsin were all purchased from Thermo (Gibco, USA).

2.2. Andiroba oil Characterization

The lipid profile was characterized according to the methodology described in our previous study [20]. Briefly, approximately 15 mg (±0.1 mg) of oil was weighed in glass tubes with caps and 1.5 mL of a 0.5 M KOH solution in methanol was added. Full esterification of fatty acids was obtained using 14% BF_3 in methanol. Extraction of fatty acid methyl esters was performed by adding 2.5 mL of saturated NaCl solution and 1 mL of hexane, followed by vortex agitation for 1 min and centrifugation at 241× g for 5 min. Samples were then analyzed by gas chromatography (Shimadzu GCMS-QP2010 Plus with an AOC-5000 injection system, Japan; and a J&W Scientific DB-23 column (60 m × 0.25 mm ID × 0.25 µm), Folsom, CA, USA). Quantification was achieved with a calibration curve of certified reference material (F.A.M.E Mix C8–C24).

To investigate the presence of other bioactive compounds in the oil, analyzes by gas chromatography-mass spectrometer (GC-MS) were performed in Agilent equipment, model 5975C masses coupled to a 7890 A gas chromatograph. Sample insertion was performed using a 10 µL syringe in a model 7693A automatic injector. A capillary column (HP5 ms, 30 m × 250 µm × 0.25 µm of (5 % phenyl) -methylpolysiloxane film) was used with helium as a carrier gas at a constant flow of 1.0 mL/ min. The injected volume was 1 µL in split mode 1:2. Temperature program was at 50 ◦C for 1 min, rising to 180 ◦C (2 ◦C/min), subsequently rising to 250 ◦C (10 ◦C/min) for 10 min and finally rising to 280 ◦C. Mass detector analysis was performed, applying the following parameters: interface, ionization source and quadrupole

analyzer temperature at 280, 230 and 150 ◦C, respectively, and electron impact ionization (EI) mode at 70 eV. The results were identified by comparison with the equipment's NIST library database, observing similarity index and visual inspection of the mass spectra obtained versus the equipment's library.

2.2.1. Nuclear magnetic resonance (NMR) analysis

The chemical characterization of andiroba oil was performed by nuclear magnetic resonance (NMR) analysis. For this, 1D NMR data were acquired at 298 K in CDCl3 on a Bruker AVANCE III 400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 9.4 T, observing 1H and 13C at 400 and 100 MHz, respectively. The NMR spectrometer was equipped with a 5 mm direct detection probe with a z-gradient. All 1H and 13C NMR chemical shifts (δ) were given in ppm related to the TMS signal at 0.00 as an internal reference. The spectra were processed in the TOPSPIN software.

2.2.2. Peroxide and acidity index

Acidity level was measured following the AOCS (American Oil Chemists' Society) method Ca 5a-40 (2004) and expressed as a percentage of oleic acid; Peroxide value was determined following the AOCS method Cd 8b-90 (2000) and expressed in meq/kg.

2.2.3. Methods for determining antioxidant activity.

Two methods were used to determine the antioxidant activity: The first method used was the Phosphomolybdenum Complex described by Prieto et al. (1999), with results expressed in milligrams of ascorbic acid in 100 g of sample. The second method is based on the scavenging of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•), described by Rufino et al. (2010). The antioxidant activity of this method was expressed as an IC_{50}, therefore it is the concentration of the sample in mg ml-1 that inhibits the absorption of DPPH• by 50%. In this assay, the percentage of inhibition is obtained from the absorption difference between the absorbance of DPPH• and the absorbance of the sample measured by a spectrophotometer. Reading was performed using a microplate reader (EZ Read 2000, Biochrom®) at 695 nm for phosphomolybdenum complex, and 515 nm for DPPH•. Values were expressed as mean ± standard deviation. The analyzes were performed in three repetitions.

2.2.4. Total phenolic content and total carotenoids

The total phenolic content (TPC) was determined by the Fast Blue method described by Medina (2011). The results were expressed in milligrams of gallic acid equivalent (GAE) in 100 g of sample. Total carotenoids were determined using the spectrophotometric method described by Rodriguez-Amaya (2001), with results expressed in μg 100 g-1. Reading was performed using a microplate reader (EZ Read 2000, Biochrom®) at 420 nm to TPC, and different absorbances for total carotenoids: 444 nm (α-carotene); 450 nm (β-carotene); 456 nm (δ-carotene); 462 nm (γ-carotene) and 470 nm (lycopene). Values were expressed as mean ± standard deviation. The analyzes were performed in three repetitions.

2.3. Development of Andiroba Oil-Based Nanoemulsion (NeAnd)

The development of the Andiroba oil-based nanoemulsion (NeAnd) was an adaptation of a method previously described in Ombredane et al, 2020 [20]. Briefly, the method consisted of two steps: First, a coarse emulsion was prepared by adding egg lecithin and andiroba oil in PBS in concentration of 32,4 mg AO and 68,4 mg egg lecithin in 15mL of PBS. Each formulation was produced in a 2:1 ratio for surfactant (egg lecithin) and oil (w/w). Sonication at 20 kHz, under an ice bath for 3 min was used in both steps. The developed formulations were stored at 4 °C in the dark until further analysis. A blank formulation (without the oil) was prepared in a similar manner as described above.

2.4. Physicochemical Characterization of NeAnd

2.4.1. Dynamic light scattering (DLS)

The average diameter, polydispersity index, and zeta potential [21] were determined by means of ZetaSizer® Nano ZS90 (Malvern, UK) at room temperature and with detection of light scattering in an angle of 90°. Before the analysis, samples were diluted (1:10, v/v) in PBS to be optically clear and avoid the attenuation of the laser beam by the particles along with the reduction of the scattered light that can be detected [22]. Three readings of each sample were obtained to calculate the Z average. The pH of the samples was measured with a pH indicator stripe before each zeta potential analysis.

2.4.2. Stability of NeAnd under different storage and pH conditions

Exposure of NeAnd to stress conditions (temperature, time of storage, and pH variation) was performed to determine their stability. NeAnd was stored at 4°C, 37 °C protected from light. Their physicochemical parameters were analyzed after 1, 7, 15, 60, 90, and 120 days as described in 2.4.1 section. For the pH analysis, the pH change was performed with the addition of 2 M NaOH and 2.5 M HCl to achieve pH 3, 5, 7, 9, and 11. Then, the physicochemical parameters of each sample were analyzed as described in section 2.4.1.

2.4.3 Morphology of NeAnd by transmission electron microscopy (TEM)

A drop of approximately 20 microliters of the sample was placed onto a piece of Parafilm, and a grid was placed over the drop for 10 minutes. After this time, the excess sample was removed with filter paper. Subsequently, a drop of 2% uranyl acetate solution (contrast) was added to the Parafilm, and the grid was placed over this drop for 1 minute. Following that, excess uranyl acetate was removed with filter paper. The samples were air-dried at room temperature. After drying, the samples were analyzed using the Transmission Electron Microscope (TEM), JEM-2100, Jeol, Tokyo, Japan, equipped with Energy Dispersive X-ray Spectroscopy (EDS), Thermo Scientific, operating at 200 kV.

2.4.4 Infrared spectrophotometry (FTIR) analysis of NeAnd

The FTIR measurements were conducted using a Bruker Fourier transform infrared spectrometer, model Vertex 70. The analysis was performed using the attenuated total reflection module (ATR-FTIR). An average of 96 scans were collected for each sample, with a resolution of 4 cm^{-1}, in the range of 400 to 4000 cm^{-1}, with the same background sampling before each measurement. It is important to mention that the instrument's wavenumber precision is better than 0.01 cm^{-1} at 2000 cm^{-1} in the ATR-FTIR measurement (Bruker Vertex 70). Therefore, wavenumber shifts of approximately 1 cm^{-1} are considered significant.

2.4.5. Raman analysis of NeAnd

The RAMAN measurements were performed using the LabRAM HR Evolution spectrometer, manufactured by Horiba. The Raman spectrometer is equipped with a confocal microscope, a CCD (Charge Coupled Device) detector, and an 1800 lines/mm grating. The measurements of each sample were conducted using a He-Ne laser tuned to the 633 nm line (1 mW) and focused on the sample using a 50x objective lens.

2.5. Cell culture

HaCat cells (human keratinocytes) was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % (v/v) fetal bovine serum (v/v) and 1 % of antibiotic solution (100 IU/mL Penicillin – 100 µg/mL Streptomycin – v/v) at 37 ◦C and 5 % CO_2.

2.5.1 Cytotoxicity assay

Cells were seeded into 96-well culture plate at a density of 5 x 10^3 HaCat cells per well in culture medium overnight at 37°C, 5 % CO_2 in a humid atmosphere. Then, the medium was changed and various concentrations of NeAnd, blank nanoemulsion (without andiroba oil), and free andiroba oil were added (90, 180 and 360 µg/mL, considering andiroba oil concentration). The free andiroba oil was previously diluted in ethanol with final concentration of ethanol lower than 1 % per well, which is nontoxic for cells. The plates were incubated for 24 and 48 h at 37° C, 5% CO_2 in a humid atmosphere. The cell viability assay was performed using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The MTT assay is based on the reduction of tetrazolium derivatives in living cells by mitochondrial dehydrogenases, allowing the estimation of the metabolic activity of cells. This assay enables the assessment of cell viability and proliferation as parameters of cell survival and growth [23]. After 24 and 48 h of incubation, the treatments were removed and 150 µL of the MTT solution (0.5 mg/mL in DMEM) was added to each well. The plates were incubated for 2 h at 37° C and 5 % CO_2 in humid atmosphere. The culture medium was discarded and 150 µL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was monitored using a spectrophotometer with a microplate reader at 595 nm The control group was considered as 100% cell viability. The results were obtained from two independent experiments (duplicate) for both the 24- and 48-h time points. In addition, each concentration was performed in triplicate. The mean cell viability values were calculated relative to the control mean (PBS and EtOH).

2.5.2. Scratch Assay

After trypsinization, 3 x 10^4 HaCat cells/well were seeded in 24-well plates to grow as a monolayer for 48 hours in DMEM medium supplemented with 1 % (v/v) fetal bovine serum (v/v), incubated overnight at 37°C, 5 % CO_2 in a humid atmosphere. After that, the scratch assay was performed based on the protocol by [24]. For this, a straight-line scratch was made in the central region of each well using the tip of a 200 µL pipette, simulating a wound. After scratch, the cells were exposed to NeAnd, blank, oil, and controls for a period of 24 hours. The chosen concentration for NeAnd and its respective blanks was 360 µg/mL diluted in PBS. For the treatment with free oil, a solution in EtOH was prepared, and then a concentration of 360 µg/mL was prepared in PBS. PBS was used as the control for NeAnd and blank; ethanol was used as the control for the oil. The scratch was recorded under an optical microscope with an attached camera before applying the treatment (T0), and after 24 hours (T24). The migration records of HaCat were evaluated using ImageJ software and the plugin described by Suarez-Arnedo et al [25].

2.6. Statistical analyses

Statistical differences between experimental groups were evaluated by the analysis of variance (ANOVA) and Tukey post-hoc test at a significance level of 0.05 using Graph Pad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA). All values were expressed as means ± standard error of the mean (SEM and SD) and a value of $p < 0.05$ was considered statistically significant, to determine if there was a statistical difference between the groups, and the normality and lognormality tests (Shapiro-Wilk test). All assays were performed in triplicates in two independent experiments.

3. Results

3.1. Chemical characterization of andiroba oil.

Before starting the preparation of NeAnd, the quality and properties of andiroba oil were evaluated. Analyzes of fatty acid profile of andiroba oil demonstrated the presence of saturated and unsaturated fatty acids, mainly oleic acid (47.62 % ± 0.426) and palmitic acid (28.6%± 0.343), and lower content of linoleic (9.65% ± 0.145) and stearic acid (8.83% ± 0.089) (Table 1). Additionally, other bioactive compounds were also detected in andiroba oil such as 2-undecenal, ethyl oleate, and methyl palmitate (Fig.1).

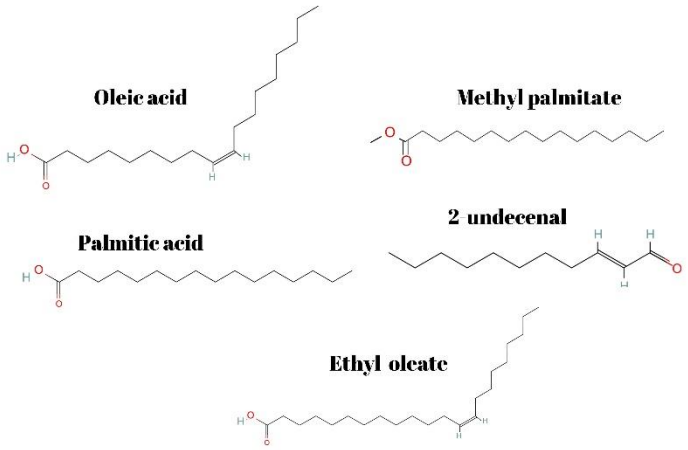


Figure 1. Bioactive compounds of andiroba oil (*Carapa guianensis*).

Table 1. Andiroba oil (*Carapa guianensis*) lipid profile.

Fatty acid Content	(%)
Oleic acid	**47.62 ± 0.426**
Palmitic acid	**28.60 ± 0.343**
Linoleic acid	**9.65 ± 0.145**
Stearic acid	**8.83 ± 0.089**

The andiroba oil was also evaluated in terms of its acidity index, acidity level, and peroxide content to assess the oil's quality. Results showed an acid value of 7.62 ± 0.01 (mg $KOH \cdot g^{-1}$), peroxide value of 1.44±0.76 MEQ·KG−1), and degree of acidity of 3.83±0.01 (%).

3.1.1 Nuclear magnetic resonance (NMR) analysis of andiroba oil.

Nuclear magnetic resonance is a spectroscopic technique that provides the identification of primary and secondary metabolites in complex mixtures. The chemical profile obtained by ^{1}H (Fig. 2) and ^{13}C (Fig. 3) NMR was characterized mainly by the presence of fatty acids esterified to the glycerol moiety due to the existence of signals in the region between

4.10 to 5.50 and the signals at 62.09 and 68.98 ppm, respectively [26]. The presence of the signals at 172.66 and 173.08 can be attributed to the presence of carbonyl group in a fatty acid chain.

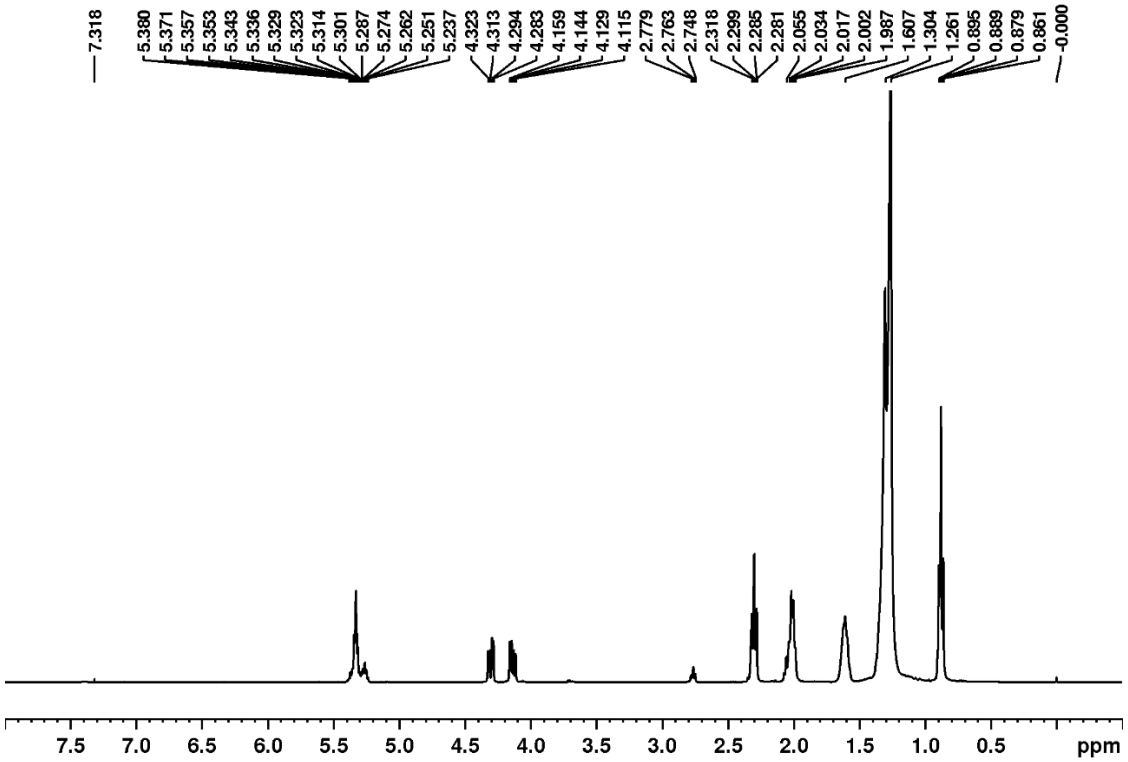


Figure 2. ^{1}H NMR spectrum of andiroba oil (*Carapa guianensis*) in $CDCl_3$ at 400 MHz.

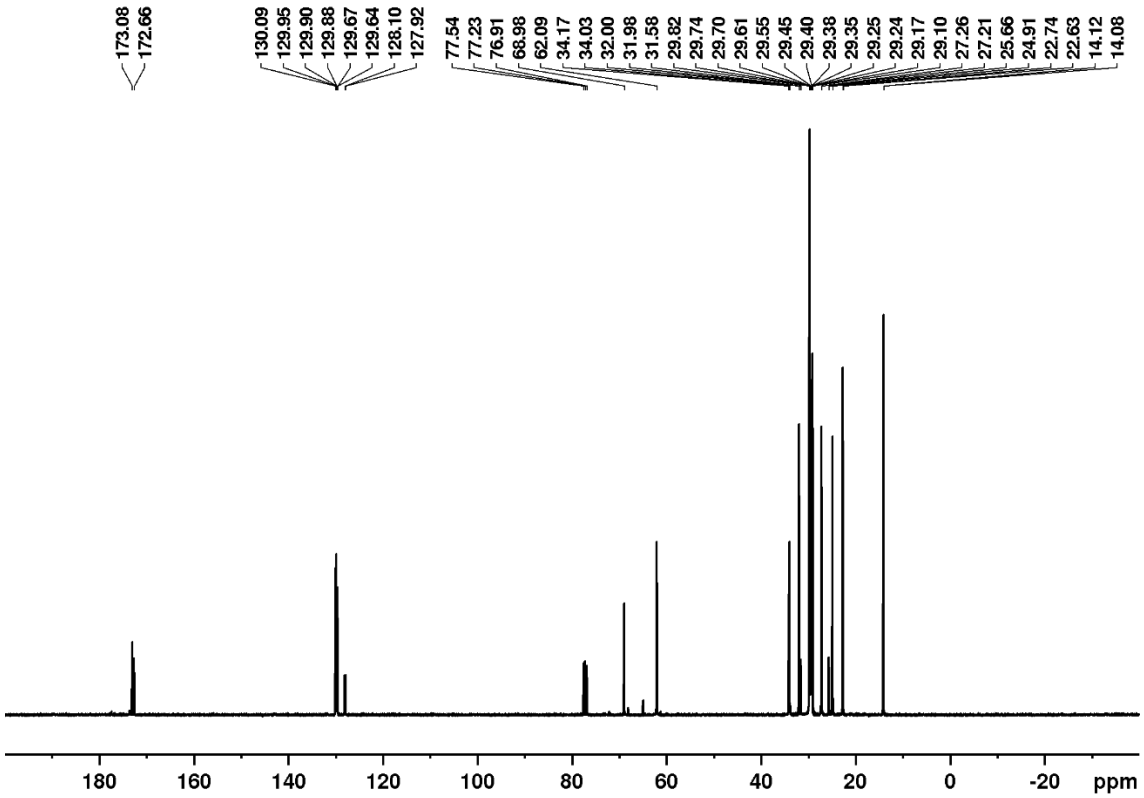


Figure 3. $^{13}C\{^{1}H\}$ NMR spectrum of andiroba oil (*Carapa guianensis*) in $CDCl_3$ at 100 MHz.

3.1.2. Total phenolic content and total carotenoid of andiroba oil.

Table **2** presents the antioxidant activity by two different methods, total phenolic compounds, and five carotenoids detected in andiroba oil (AO). In our study, we found a value of 1014.21 mg AA 100 g^{-1} for phosphomolybdenum complex content. For DPPH analysis, an IC_{50} of 7.44 mg mL^{-1} was detected for AO.

As for total phenolic compounds, our content was found to be 5164.77 mg GAE 100 g^{-1}. Finally, the total carotenoid content found in *C. guianensis* was 7.99 μg 100 g^{-1}, with emphasis on α-carotene and β-carotene, with 2.02 and 1.89 μg 100 g^{-1}, respectively.

Table 2. Antioxidant activity, total phenolic compounds, and carotenoids detected in *C. guianensis* oil.

Parameter	Value obtained	
Phosphomolybdenum complex	1014.21 ± 25.66	mg of AA. 100 g-1
DPPH•	7.44 ± 0.37	mg.mL-1
TPC	5164.77 ± 209.43	mg GAE. 100 g-1
α-Carotene	2.02 ± 0.06	μg.100 g-1
β-Carotene	1.89 ± 0.16	μg.100 g-1
δ-Carotene	1.42 ± 0.11	μg.100 g-1
γ-Carotene	1.39 ± 0.12	μg.100 g-1
Lycopene	1.27 ± 0.07	μg.100 g-1

3.2. Hydrodynamic diameter, polydispersity index, and zeta potential of NeAnd

NeAnd presented an average hydrodynamic diameter of 205.7 ± 3.9 nm, polydispersity index (PdI) of 0.295 ± 0.05, negative zeta potential of –4.16 ± 0.414 and kept the pH around 6.5 for 120 days at 4°C storage (Fig. 4).

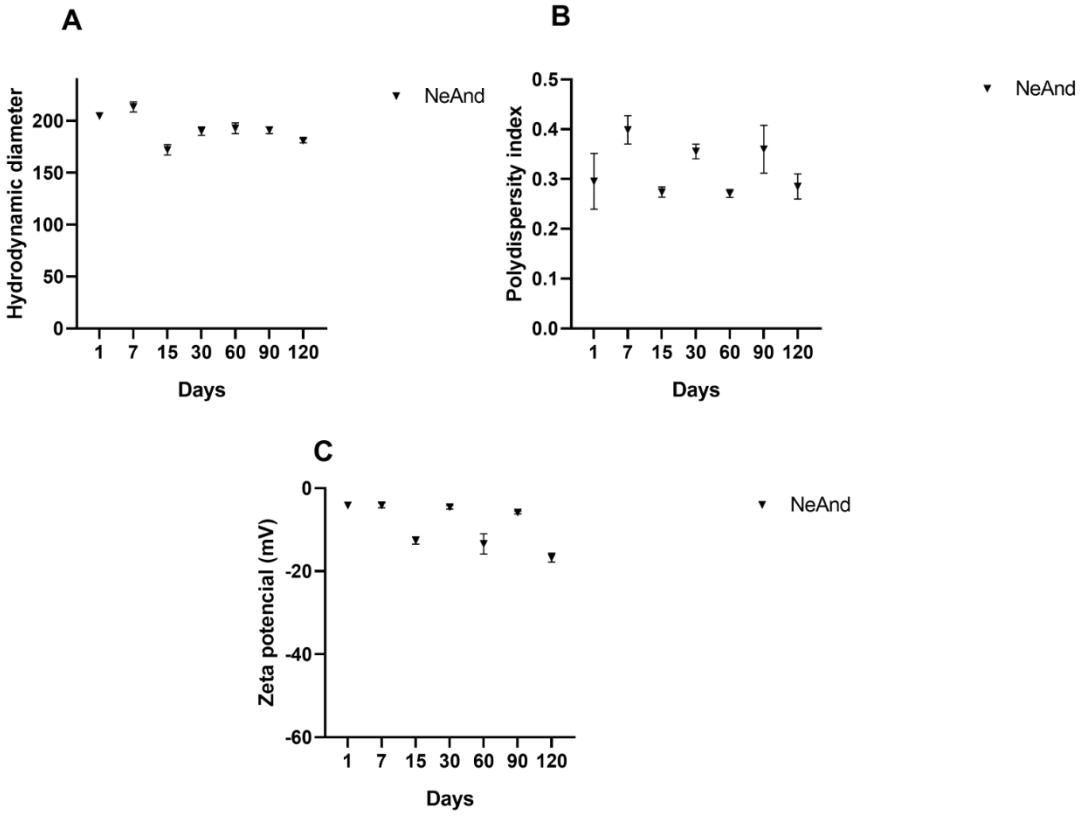


Figure 4. Evaluation of the stability of andiroba oil-based nanoemulsion (NeAnd) stored at 4 °C for 120 days, considering hydrodynamic diameter (A), polydispersity index (B), and zeta potential (C).

3.2.1. Stability evaluation of NeAnd under pH stress

Variations in pH values lead to changes in NeAnd physico-chemical characteristics related to DH and zeta potential parameters (Fig. 5). When compared to nanoemulsion at pH 7 (144.2 nm DH), we observed slight variations of approximately 34 nm in the DH ($p < 0.0001$) at acidic pH and no significant differences at basic pH (Fig. 5A). PdI values remained slight variations ($p < 0.0001$) of approximately 0.0836 at pH 3, and 0.573 at pH 5 (Fig. 5B). As for zeta potential, values increased 6.7 mV at acidic pH and decreased approximately –15.88 mV at basic pH ($p < 0.0001$) (Fig. 5C).

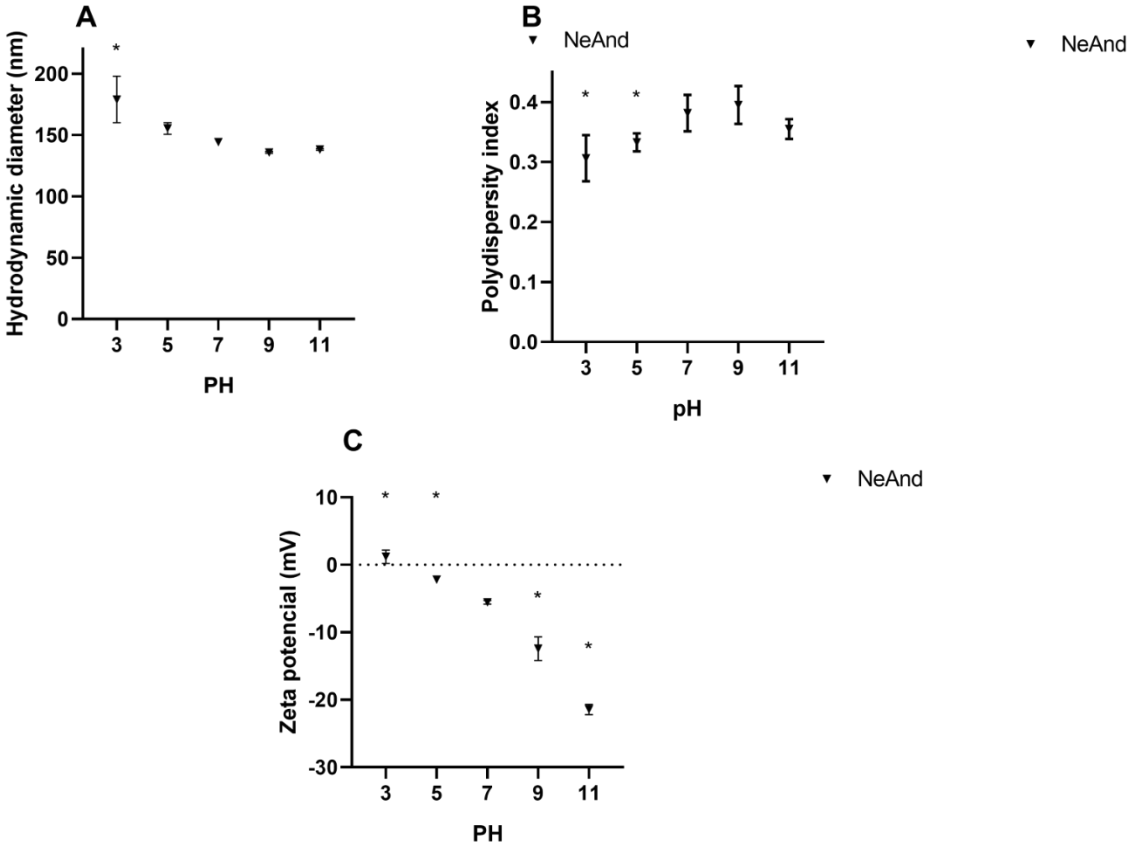


Figure 5. Evaluation of stability of andiroba oil based nanoemulsion (NeAnd) at different pH values. Hydrodynamic diameter (A), polydispersity index (B), and zeta potential (C). The values are expressed as mean ± SD. 2-way ANOVA: significant difference pH 7 versus other groups $p < 0.05$ (Sidak test). Asterisks indicate statistically significant differences between groups.

3.2.2. Transmission electron microscopy (TEM) of NeAnd

Figure 6 shows TEM images of NeAnd produced by sonication, where it can be observed that the spherical-shaped nanodroplets appear as white against a dark background. From the obtained images, it is worth noting that most of the droplets have diameters in the range of 100-150 nm.

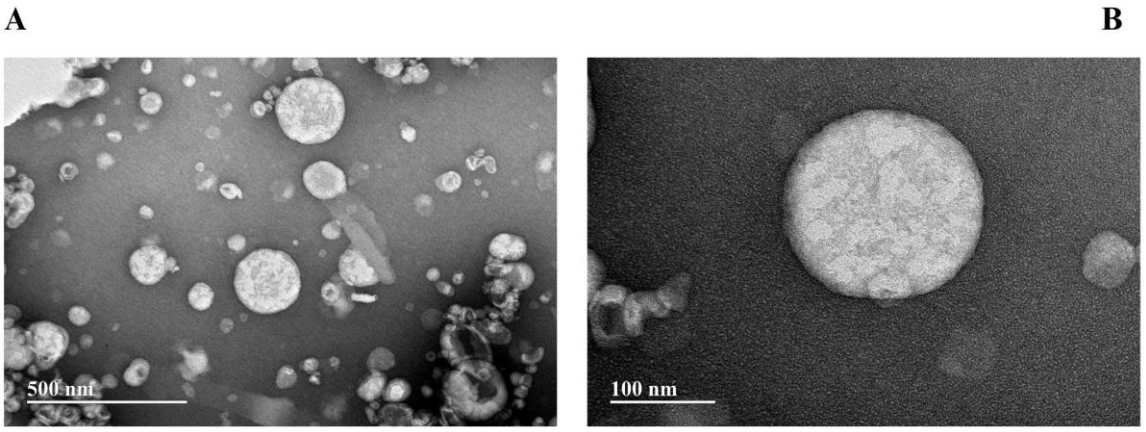


Figure 6. Transmission electron microscopy micrographs of andiroba oil-based nanoemulsion (NeAnd).

3.2.3. Infrared spectra (FTIR) of NeAnd

Figure 7 shows the FTIR spectra of andiroba oil (i), andiroba oil-based nanoemulsion (NeAnd) (ii), and the blank formulation (without the oil). The FTIR spectrum of the andiroba oil (Fig. 1 (i)) shows typical bands related to the acyl chain of lipids, such as the stretching (=CH) of the cis aliphatic doublet bond at 3010 cm^{-1} and the vibrational modes of symmetric and antisymmetric stretching of (CH_2) and (CH_3) at 2850, 2920, 2870 and 2960 cm^{-1}, respectively [27].

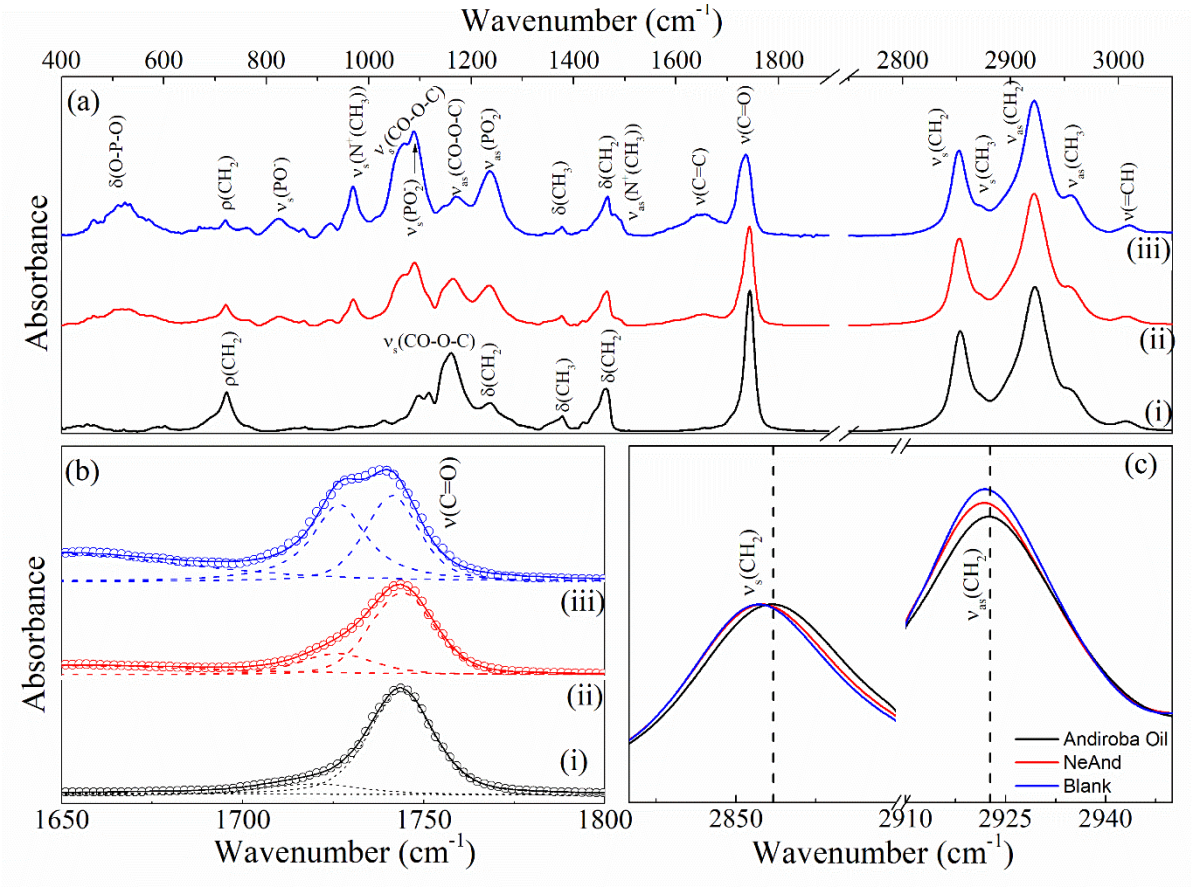


Figure 7. FTIR spectra of the (a) free andiroba oil (i), blank formulation (ii) and andiroba oil-based nanoemulsion (NeAnd) (iii). Zoom of the FTIR spectra in the range 1650 – 1800 cm^{-1} (b) and 2840 – 2950 cm^{-1} (c). The dashed lines show the Gaussian + Gaussian + Lorentzian fit of the data.

3.2.4. Raman of NeAnd

Figure 8 shows the Raman profile of AO, blank formulation, and NeAnd. To determine the ratios between the intensities of trans/gauche, trans/trans, and trans/trans, the Raman spectra were fitted using a Gaussian + Lorentzian function. The values obtained for these ratios are listed in Table 4. Note from Table 4 that the trans/gauche ratio is higher for the blank formulation and NeAnd when compared to AO. This behavior indicates that the lipid chains of NeAnd and the blank formulation are more ordered than those of free AO, which is consistent with the FTIR data.

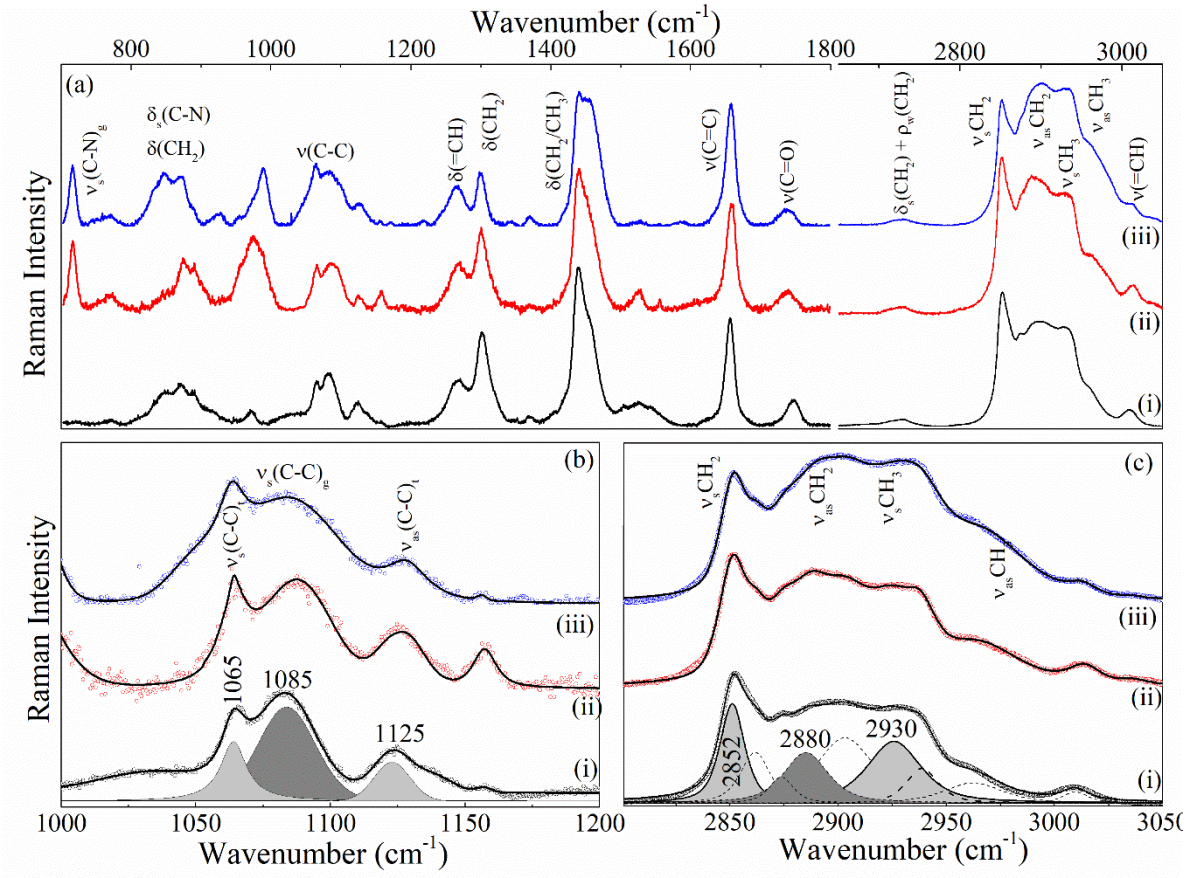


Figure 8. Raman spectra of the (a) free andiroba oil (i), blank formulation (ii) and andiroba oil-based nanoemulsion (NeAnd) (iii). Zoom of the FTIR spectra in the range 1000 – 1200 cm^{-1} (b) and 2800 – 3050 cm^{-1}(c). The dashed lines show the Gaussian + Gaussian + Lorentzian fit of the data.

Table 4. Calculated It/Ig ratio (from Raman spectra data) for free andiroba oil, blank formulation, and andiroba oil-based nanoemulsion (NeAnd).

Samples	I1065/I1085	I1125/I1085	I2880/I2850	I2880/I2930
Andiroba oil	0.63	0.40	0.38	0.81
Blank	1.12	0.60	0.50	1.06
NeAnd	1.02	0.59	0.52	1.02

3.3. Cytotoxicity of NeAnd in human keratinocytes *in vitro*

The study was conducted with keratinocytes (HaCat), treated with free (AO) and nanostructured andiroba oil (NeAnd) at three concentrations (90, 180, and 360 µg/mL) for 24 and 48 hours. For comparison, groups treated with nanoparticles without oil (blank), ethanol, and PBS were used. The results are represented in Fig. 9.

Cell viability analyses of human keratinocytes showed no cytotoxicity of the tested treatments and controls at 24 and 48 hours, except for the treatment of free AO at a

concentration of 360 µg/mL at the 48-hour time point, which showed an average cell viability percentage of 77.29% ($p<0.05$) (Figs. 9, A and B).

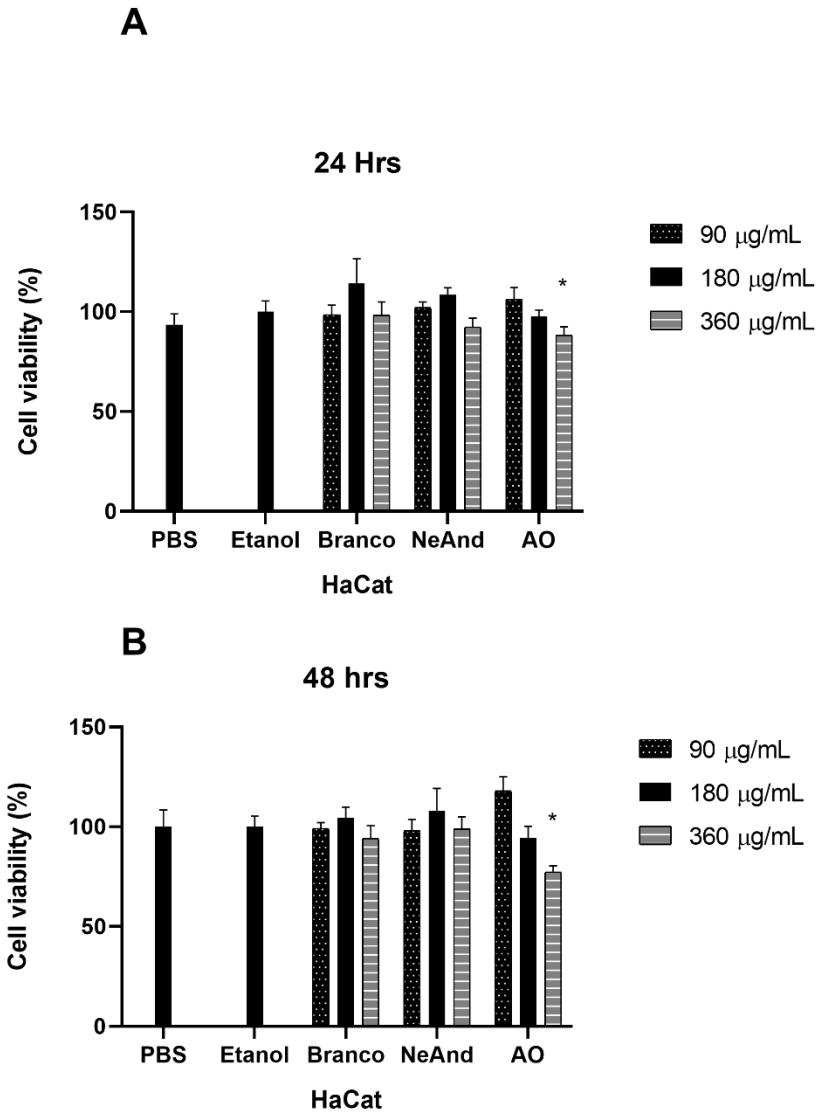


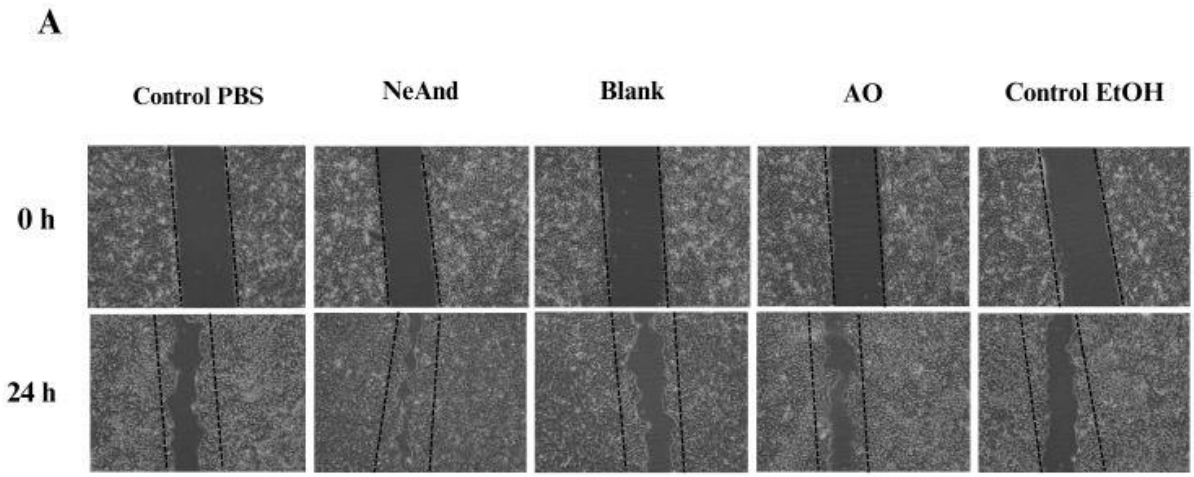
Figure 9. Cytotoxicity of andiroba oil-based nanoemulsion (NeAnd), free andiroba oil (AO), and blank formulation (without oil) on the viability of HaCat. The cells were exposed to NeAnd and free AO at 90 – 180 and 360 µg/mL for 24 h (A) and 48 h (B). Two-way ANOVA: significant difference between groups control vs treatments $p<0.05$ (Tukey post hoc test). * indicate statistically significant differences among groups.

Considering that NeAnd showed no significant cytotoxicity in any of the concentrations evaluated above, the highest concentration (360 µg/mL) was chosen for the scratch assays.

3.4. Scratch Assay in keratinocytes

Scratch wound healing assay was carried out to observe the effects of NeAnd and AO (360 µg/mL) on cell migration of keratinocytes treated with free and nanostructured andiroba oil. The results are presented in Fig.10.

Figure 10-A shows images depicting the closure of a simulated wound in a keratinocyte monolayer before (T0) and after 24 hours of intervention (T24) with NeAnd or free AO. Figure 10-B represents the data regarding the percentage of remaining area after treatments, and there was a significant statistical difference between two groups, represented by letters. The NeAnd group induced a higher proportion of wound closure when compared to the PBS control ($p < 0.0005$) and when compared to free AO ($p = 0.0323$).



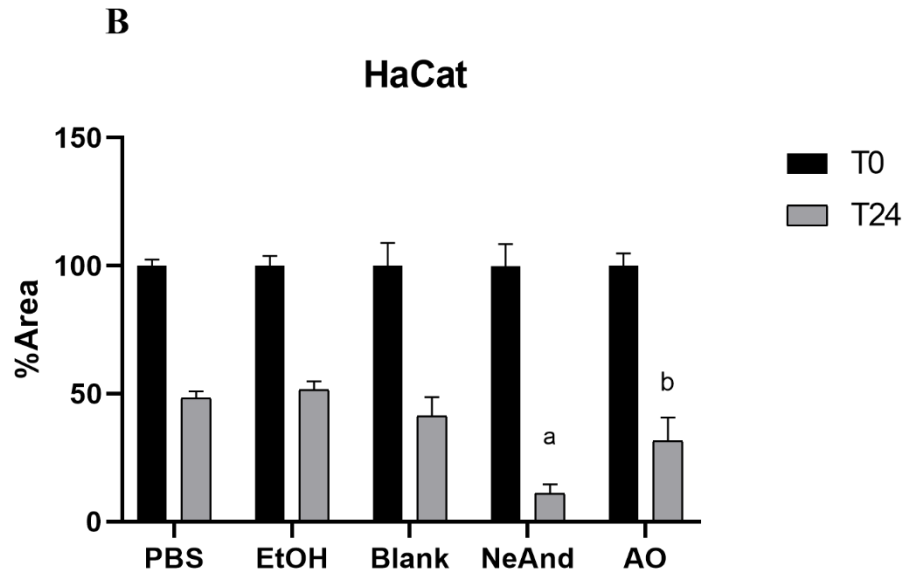


Figure 10. Scratch Assay of keratinocytes *in vitro.* A, images of the simulated wound in human keratinocytes, which were recorded under an optical microscope with an attached camera. Images were obtained before (T0) and after cells were incubated with PBS, NeAnd (360 µg/mL), blank (egg lecithin without oil), free andiroba oil (AO - 360 µg/mL), or control EtOH for 24 h (T24). In B, the values representing the injury area at time 0 and the remaining area after 24 hours with the treatments are shown. One-way ANOVA, Tukey post hoc test). Letters indicate significant difference among groups ($p<0.05$).

4. Discussion

The aim of the present study was to synthesize and characterize a nanoemulsion based on andiroba oil (NeAnd) and, in addition, observe whether NeAnd and AO would be cytotoxic at different concentrations, as well as whether it would influence the migration of two human cell lines when compared to AO in *in vitro* simulated wound (scratch assay).

Initially, the characteristics and compounds of andiroba oil were analyzed. The lipid profile of unsaturated and saturated fatty acids present in the oil was confirmed and presented oleic, palmitic, linoleic, and stearic acids (Table 1) in proportions like the ones found in the literature [28,29]. The quantitative analysis carried out by gas chromatography coupled to mass spectrometry (Table 1) revealed oleic and palmitic acid as the major compounds of andiroba oil. The results obtained from NMR spectra are in accordance with the GC-MS analysis (Figs. 2 and 3).

The acidity level, acid value (AV), and peroxide value (PV) were also investigated. It was verified that the oil presented values below those already found in the literature [30]. However, there are still no specific parameters for the analysis of andiroba oil regarding quality characteristics. It is emphasized the need for further studies with the characterization of andiroba oil.

The antioxidant properties and bioactive compound content in various plant species have been positively associated with the enhancement of the immune system and the ability to prevent, protect against, and even reverse the harmful effects induced by oxidative stress and various diseases [31]. Due to the diverse mechanisms of action that confer antioxidant capacity and the effects of bioactive compounds [32], different evaluation methods are employed to determine these parameters.

In the phosphomolybdenum complex method, the total antioxidant activity is determined by reducing Mo (VI) to Mo(V) in the presence of electron-donating compounds from the sample, followed by the formation of the phosphomolybdenum complex. This process occurs under acidic pH conditions and involves the application of high temperature for a relatively extended period [33]. Therefore, based on this test, we conclude that andiroba oil exhibits robust antioxidant activity (1014.21 ± 25.66 mg of AA. 100 g^{-1}) when compared to other types of oils (Table 3). In essential oils from leaves of different species, values of 76 mg 100 g^{-1} and 43.2 mg 100 g^{-1} were reported for *Ocimum basilicum* and *Thymus algeriensis*, respectively [34] and 185 mg 100 g^{-1} in *Trichopus zeylanicus* ssp. *travancoricus* [35].

In the DPPH• method, the IC_{50} value represents the concentration of the test compound required to reduce free radicals by 50%. A low IC_{50} value indicates a higher free radical scavenging activity [36]. We propose that the andiroba oil examined in this study

demonstrates strong antioxidant activity using this method (IC_{50} result was 7.44 mg mL^{-1}) (Table 3). In fact, it even approaches the value of the synthetic antioxidant BHT (IC_{50} of 6.80 mg mL^{-1} [41]. In a study with essential oils from different types of basil, IC_{50} values of 11.23 mg mL^{-1}, 17.52 mg mL^{-1} and 55.15 mg mL^{-1} were found [37].

Phenolic compounds constitute the most abundant class of secondary metabolites among natural antioxidants derived from the plant kingdom. They are characterized by a common aromatic ring linked to a hydroxyl group, which can be substituted by other groups [38,39]. In oils from other plant species, the TPC of the faveleira seed oil was 108.11 mg GAE 100 g^{-1} (Ribeiro et al., 2021a) [40] and 820 mg GAE 100 g^{-1} in *Carapa procera* [41]. In a study also using *Carapa guianensis* oils, Araujo-Lima et al., 2018, found TPC of 900 to 1034 mg 100 g^{-1}. Our results (5164.77 ± 209.43 mg GAE. 100 g^{-1}) surpass those reported in the literature, even in comparison to oils from the same species.

Carotenoids, lipophilic pigments synthesized by various organisms, including plants, offer numerous health benefits [42]. β-carotene and α-carotene serve as precursors of vitamin A, capable of being converted into retinol through dioxygenase enzyme activity [43] While present at a concentration of 1.27 μg per 100 g, lower than other carotenoids, lycopene stands out as a potent and effective inhibitor of reactive oxygen species due to its conjugated double bonds and elongated structure. This bioactive compound safeguards DNA and suppresses mutations that can lead to chronic diseases [43]. Nevertheless, based on the classification of carotenoid content in dietary sources, when a plant-based product contains less than 100 μg per 100 g, it is considered a low-carotenoid source [42]. Therefore, andiroba oil in this study does not qualify as a rich source of these bioactive compounds, as similarly reported by [4].

After analyzing oil properties, the preparation and characterization of nanoemulsion based on andiroba oil was carried out. NeAnd formulation showed hydrodynamic diameter (HD; or size of the nanodroplet) in the range expected for nanoemulsions produced with phosphatidylcholines – described to be approximately 150–300 nm [44]. Corroborating with our result of HD, findings in literature with similar nanoemulsion fabrication conditions are close of those obtained in our study[45,46]. Studies have shown that the zeta potential of nanoemulsions can be estimated based on their composition and pH [46] found that nanoemulsions formed using emulsifiers with anionic groups, such as sulfate, carboxyl, or phosphate groups, tend to have negatively charged oil droplets. The presence of egg lecithin in nanoemulsions, which is mainly composed of phosphatidylcholine, can significantly impact the zeta potential inducing a negative zeta potential, [47]. In our study, PdI values showed an irregular pattern throughout 120 days of storage at 4ºC. A similar pattern was observed in ZP values which showed a discrete variation of –7 mV throughout the 120 days of storage (Fig. 4).

Regarding pH analysis, the results show slight variations in DH and PdI, specially in pH 3 and 5 (Fig. 5). In this sense, stability in particle size despite pH variation can translate bioavailability in different biological systems, such as in the GIT and topically, and was therefore considered as efficient encapsulation of AO [48–50]. Furthermore, pH displayed an expressive influence in ZP of NeAnd, with statistically significant changes in surface nanoparticle charge at pH levels different from pH 7. It may be explained by the presence of H^+, cationic or anionic ion binding to nanoparticle, what turns ZP more positive or negative, respectively [51,52].

Vibrational spectroscopy techniques like FTIR and Raman spectroscopy are valuable for investigating chemical interactions among bioactive molecules in lipid nanoemulsions [53]. Spectral changes correlate with conformational and structural changes in membrane-forming molecules due to their interaction processes. FTIR analysis allows for the examination of

carbonyl group stretching (ν(C=O)) bands, along with methyl and methylene group vibrations indicative of hydrocarbon chain conformations. The ν(C=O) band is sensitive to changes in its local polarity and influenced by hydrogen bonds and other interactions with neighboring ligands [54]. When crystallized in the β-phase, triglyceride ν(C=O) bands split into two around 1743 and 1725 cm-1, assigned to free and hydrogen-bonded ester carbonyl groups [55]. In a less ordered phase, these bands collapse into a broad, asymmetric band around 1730 cm^{-1}. Symmetric and asymmetric stretching vibrations of C-C and C=O bonds are sensitive to acyl chain conformation and indicate changes in the *trans/gauche* rotamer ratio of fatty acid acyl chains in natural membrane systems. Transition from a more ordered phase to a less ordered one slightly increases the vibrational energy of the C=O stretching band [20]. To investigate interactions between andiroba oil and egg lecithin surfactant, Raman and FTIR spectra of andiroba oil-based nanoemulsion (Neand) were analyzed. Comparisons were made with a blank formulation (without oil) and free andiroba oil.

Figure 7 shows the FTIR spectra of andiroba oil (i), Andiroba oil-based nanoemulsion (AndNE) (ii) and blank formulation (without the oil). Andiroba oil (*Carapa guianensis Aubl*) are constituted basically of fatty triglyceride (TG) esters with different substitution patterns, lengths and degrees of saturation of the chains and of other minor components. Its absorption spectra have characteristics common to most vegetable oil spectra, as previously reported [56,57]. The FTIR spectrum of AO shows typical bands related to the acyl chain of lipids, such as the stretching (=CH) of the *cis* aliphatic doublet bond at 3010 cm^{-1} and the vibrational modes of symmetric and antisymmetric stretching of (CH_2) and (CH_3) at 2850, 2920, 2870 and 2960 cm^{-1}, respectively [56]. Other vibrations associated with (CH_2) and (CH_3) bonds also are found at 1460 cm^{-1} (CH_2 scissorian), 1376 cm^{-1} (CH_3 symmetric deformation (umbrela)), 1234 cm^{-1} (CH_2 twisting) and 720 cm^{-1} (CH_2)). The strong band at 1160 cm-1 can be attributed to stretching vibrations of the C-CO-C) bonds of the carbonyl or ketone groups [55]. A band associated with the ν(C=O) stretching steres and a weak band associated with the $\nu(C=C)_{cis}$ vibrations of the unsaturated bonds are found at 1745 and 1650 cm^{-1}, respectively [56].

Comparisons between the FTIR spectra of the nanoemulsion, blank formulation and free AO show that the NeAnd spectrum is similar to the IR absorption spectrum of the blank formulation, with absorption bands characteristic of the polar head of lecithin. This feature is more evident in the spectral region of 800 – 1350 cm^{-1}, where are observed the vibrational modes associated with symmetric (~1090 cm^{-1}) and antisymmetric (~1235 cm^{-1}) stretching modes of the groups, partially overlapping with the symmetric stretching modes of the CO-O-C bond (~1065 cm^{-1}). Finally, the symmetric and antisymmetric stretching bands of bonds are found at 970 and 1480 cm cm^{-1}, respectively.

Note from Fig. 7 (b) that the spectral profile of the ν(C=O) band can be deconvolved into two components using Gaussian + Lorentzian function. As previously reported, these bands can be attributed to populations of 'free' (~1743 cm^{-1}) and 'bound' (~1725 cm^{-1}) carbonyl ester groups via hydrogen bonds. Note that the intensity of the component $\nu(C=O)_{livre}$ decreases with the introduced andiroba oil (see Figs. 7 (b) (iii) and (ii)). This result suggests that the lipid chains that make up the membrane of the blank formulation are more ordered than the lipid chains of the NeAnd, which in turn is more ordered than that of free andiroba oil. This hypothesis is strengthened by the behavior of the vibrational energies of the modes and, which decrease by ~ 1,5 cm^{-1}, in the spectra of the structured samples when compared to the free andiroba oil.

Figure 8 shows the Raman spectra of the free andiroba oil (i), NeAnd (ii) and blank formulations (iii). With exception of the peak at 720 cm^{-1} (ν(CN)) the Raman spectra of the NeAnd and bland formulations are very similar to the Raman spectrum of the free andiroba oil. Raman spectra of phospholipids presents various peaks which can be classified into three

regions: (i) the hydrophobic chain consisting of C-H stretching modes (symmetric and asymmetric stretching of (2852 and 2880 cm^{-1}) and (2930 and 2960 cm^{-1}), deformation (~1450) and twisting (1300 cm^{-1}) and C-C stretching (1000 – 1200 cm^{-1}) modes, (ii) the interfacial regions containing C=O stretching, and (iii) polar headgroup regions comprising of a band of C-N stretching at 720 cm^{-1} [58].

The relative intensities of Raman active modes arising from the hydrophobic chains of hydrocarbons have been used to identify TG polymorphs and to probe the conformation, environment and dynamics of hydrocarbon chains in TGs. The band s(CH3) at 2930 cm^{-1} as well the band ν(C-C) at 1085 cm^{-1} can be used as a general measure of *gouche* content [59]. On the other hand, the bands s(CH3) (2930 cm^{-1}), asC-C (1065 cm-1) and sC-C (1125 cm^{-1}) are associated with ordered all-trans conformation. Therefore, the *trans/gauche* (It/Ig) ratio ((I1065/I1085, I1125/I1085 and I2880/I2930) can be used to determine the relative content of gauche rotomers, being a measure for intra- and intermolecular disorder. In addition, the ratio between I2880/I2850 measures the strength of the lateral chain-chain interaction within the lipid layer [60].

In order to determine the ratios between the intensities I1065/I1085, I1125/I1085, I2880/I2850 and I2880/I2930 the Raman spectra were fitted using Gaussian + Lorentzian function. The values found for these ratios are listed in Table 4. Note from the Table 4 that the It/Ig ratio is greater for blank formulation and NeAnd when compared to andiroba oil. This behavior indicates that the lipid chains of the NeAnd and blank formulation are more ordered than those of free andiroba oil, which is in agreement with the FTIR data.

Hence, it was concluded that the synthesized nanoemulsions for this study exhibited satisfactory characteristics. The subsequent step involved evaluating the biocompatibility of the formulation in cells involved in the wound healing process, such as keratinocytes.

The results show that a 24- and 48-hour incubation of NeAnd and AO in the cell line keratinocytes did not exhibit significant cytotoxicity in concentrations ranging from 90 to 360 ug/mL, except at a concentration of 360ug/mL and a 48-hour exposure, where AO showed toxicity of 77.29%. This data indicates a potential biocompatibility of NeAnd with the cells involved in the wound healing process (keratinocytes). Several studies have examined the use of andiroba oil and its cytotoxicity in various ways. For instance,[61] investigated an andiroba-based nanoemulsion and arrived at conclusions akin to those obtained in this study. They emphasized that Andiroba Oil (AO) is more toxic than nanoformulations, observing a direct correlation between the concentration of oil and its cytotoxicity. A more recent study by [62] once again affirms, in their research, that higher concentrations of oil and longer exposure times to this free oil led to increased cytotoxicity.

The *in vitro* scratch assay was performed on a monolayer of keratinocytes to evaluate the ability of NeAnd and AO to promote wound closure by enhancing cell migration. As mentioned earlier, the concentration chosen for the scratch assay was determined based on cytotoxicity analysis, as the highest concentration of NeAnd (360ug/mL) was not toxic to HaCat. In HaCat cells, within 24 hours, the percentage of closure for untreated cells (PBS and ETOH) was approximately 38%. When treated with NeAnd and AO, it significantly improved 88,9% and 68,6% respectively (Fig.10). It is already known in the literature that the oil extracted from andiroba seeds has various medicinal properties, including anti-inflammatory and wound healing effects. When topically applied to wounds, andiroba oil can help reduce inflammation, alleviate pain, and promote tissue regeneration [5, 16, 18, 63,64]. However, to the best of our knowledge, there are no published data on nanoformulations that have tested the wound healing effect of nanostructured andiroba oil *in vitro*. There are studies available on

formulations that analyze parasitic infections, cytotoxicity, and genotoxicity [61,65]. These findings indicate that *C. guianensis* oil holds promising properties in wound healing, reducing healing time, enhancing tissue strength, and diminishing inflammation. However, they also underscore the significance of monitoring concentration and administration duration to prevent cytotoxicity in specific contexts.

These therapeutic effects are associated with fatty acids present in this oil, such as linoleic acid and oleic acid, which have demonstrated a positive role in wound healing, thanks to their ability to modulate inflammation and enhance the *in vivo* reparative response [66]. These fatty acids have also proven effective in increasing the presence of neutrophils in the wound and reducing the thickness of necrotic tissue [67]. These compounds were confirmed in the andiroba oil used in this study, confirming that the nanostructured version enhanced cell migration in keratinocytes compared to the free oil. In a study conducted by [68], it was observed that the nanostructured version showed better results in combating intracellular parasitic forms, suggesting that such formulations may hold greater promise for therapeutic applications.

The composition of andiroba oil nanoemulsion, including long-chain fatty acids, may have different effects on HaCat cells. This can be attributed to various factors such as differences in cell membranes, anti-inflammatory activity, cell viability, and cellular signaling. For instance, fatty acids can interact differently with the cell membranes of these cell types, affecting membrane permeability and cellular response [69–71].

5. Conclusion

In the present work, it was possible to obtain and successfully characterize a nanoemulsion based on andiroba oil (NeAnd). It important to highlight that the steps involved in the production of NeAnd involved since the identification of the main compounds and aspects of the natural product used (andiroba oil) to the physico-chemical, stability, and morphology of the nanodroplets obtained. In addition, a nanotechnological approach improved andiroba oil's effect on wound healing *in vitro* by stimulating keratinocytes migration and accelerating wound closure. It is noteworthy to highlight that NeAnd was biocompatible without inducing cytotoxicity in any of the times and concentrations evaluated. This promising data points out NeAnd as a potential as nanophytomedicine for clinical application in wound healing and tissue regeneration treatments in the future.

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3. Discussão geral

Este estudo teve como objetivo principal a formulação e caracterização de uma nanoemulsão à base de óleo de andiroba (NeAnd), bem como a investigação de sua citotoxicidade em diferentes concentrações e seu impacto na migração de uma linhagem celular humana em comparação com o óleo de andiroba livre em um ensaio de cicatrização *in vitro*. Inicialmente, foram analisadas as características e compostos do óleo de andiroba, com destaque, a presença de ácidos graxos insaturados,saturados e carotenoides, além de sua alta atividade antioxidante, o que o torna promissor para aplicações terapêuticas (65).

Esses resultados, mostram primeiramente que o óleo de interesse continha os compostos bioativos já vistos na literatura (ácido oleico, ácido palmítico, ácido linoleico), e continha os ácidos graxos característicos do óleo de andiroba. Quanto às características de qualidade do óleo, como índice de peróxidos e acidez, foi verificado que o óleo apresentava os valores abaixo dos já encontrados na literatura (66). Porém, ainda não existem parâmetros específicos para a análise do óleo de andiroba em relação às características de qualidade, ressalta-se a necessidade de mais estudos com a caracterização do óleo de andiroba (67–69).

A caracterização da NeAnd mostrou um tamanho de partícula na faixa esperada 150 – 300 nm (70) para nanoemulsões e apresentou um potencial zeta negativo. A estabilidade da nanoemulsão teve maiores alterações em relação ao potencial zeta na análise de variação pH, o que já era esperado devido à mudança de pH do meio. Além disso, a análise das propriedades químicas da nanoemulsão utilizando espectroscopia vibracional (FTIR e Raman) indica que os ácidos graxos do óleo de andiroba na NeAnd se organizam de forma mais estável, em

comparação ao óleo livre, sugerindo uma ordenação maior das cadeias lipídicas na nanoemulsão.

Em relação à citotoxicidade, as análises de MTT mostraram que a NeAnd não apresentou valores abaixo de 80% de viabilidade celular em células de queratinócitos nas três concentrações testadas, porém o óleo em sua forma livre diminuiu significativamente a viabilidade celular na concentração de 360 µg/mL em 24 e 48 horas, enfatizando que o óleo em concentrações mais elevadas pode ser citotóxico. Já a NeAnd indica uma possível melhora no óleo quando está em uma nanoemulsão, já que aumentou a migração celular de queratinócitos e não foi tóxica em nenhuma concentração o que pode indicar potencial para aplicações. A NeAnd e o óleo de andiroba livre promoveram uma maior migração celular de células HaCat em monocultura, por ensaio de *Scratch assay*. Células tratadas com a nanoemulsão migraram significativamente mais rápido que as células tratadas com controles e com o óleo livre.

Os resultados sugerem que a NeAnd e o óleo de andiroba possuem compostos interessantes para aplicação na cicatrização de feridas devido a compostos bioativos, como ácidos graxos, que modulam a inflamação e aceleram a reparação tecidual (26). No entanto, a concentração e a duração da administração devem ser monitoradas para evitar citotoxicidade em contextos específicos.

4. Conclusões

O presente estudo teve como objetivo principal a produção e caracterização de uma nanoemulsão à base de óleo de andiroba (NeAnd) e a avaliação de sua citotoxicidade em comparação com o óleo de andiroba (AO) em queratinócitos, bem como sua influência na migração celular em um ensaio de ferimento *in vitro* simulado (*scratch assay*).

Seguem as principais conclusões obtidas no presente trabalho:

- O óleo de andiroba exibiu os ácidos graxos já descritos na literatura, como ácido oleico (47.62 % ± 0.426), ácido palmítico (28.6%± 0.343), ácido linoleico (9.65% ± 0.145) e ácido esteárico (8.83% ± 0.089). Foram observados também os compostos bioativos presentes no óleo de andiroba, como 2-undecenal, oleato de etila e palmitato de metila. Nas análises de RMN foi confirmado a presença dos ácidos graxos de interesse, confirmado os dados obtidos por CG-MS. O índice de acidez foi de 7,62 ± 0.01 (mg KOH·G−1) e o índice de peróxidos 1.44 ± 0.76 (MEQ·Kg−1).

- Nas ensaio de atividade antioxidante, foram encontrados um valor de 1014,21 mg de equivalente de ácido ascórbico (AA) por 100 g de óleo para o conteúdo de complexo de fosfomolibdênio. Para a análise do DPPH, foi detectado um valor de IC_{50} de 7,44 mg por mL de OA. No que diz respeito aos compostos fenólicos totais, o teor foi de 5164,77 mg de equivalente de ácido gálico (GAE) por 100 g de óleo. Por fim, o teor total de carotenoides encontrado em *C. guianensis* foi de 7,99 μg por 100 g, com destaque para o α-caroteno e o β-caroteno, com 2,02 e 1,89 μg por 100 g, respectivamente.

- NeAnd apresentou um diâmetro hidrodinâmico médio de 205,7 ± 3,9 nm, índice de polidispersão (PdI) de 0,295 ± 0,05, potencial zeta negativo de -4,16 ± 0,414 mV e pH em torno de 6,5 por 120 dias de armazenamento a 4°C. Quando alterado o pH das soluções e comparando à nanoemulsão com pH 7, observou-se mudanças discretas de aproximadamente 34 nm no DH ($p < 0,0001$) em pH ácido e nenhuma diferença significativa em pH básico. Os valores de PdI apresentaram pequenas variações ($p < 0,0001$) de aproximadamente 0,0836 em pH 3 e 0,573 em pH 5. Quanto ao potencial zeta, os valores aumentaram 6,7 mV em pH ácido e diminuíram aproximadamente -15,88 mV em pH básico ($p < 0,0001$), sendo apta para as análises biológicas.

- Na análise por FTIR do óleo de andiroba, da NeAnd e da formulação em branco (sem o óleo), o espectro de FTIR do óleo de andiroba apresentou bandas características relacionadas à estrutura da cadeia de lipídios, como carbonilas e metilas. Além disso, ao comparar os espectros de FTIR da NeAnd, da formulação em branco e do óleo de andiroba livre, observa-se que o espectro da NeAnd é semelhante ao espectro de absorção no infravermelho da formulação em branco. Isso ocorre devido à presença de bandas de absorção características da porção polar da lecitina na NeAnd.

- O perfil Raman do OA, da formulação branco e da NeAnd foram determinados. Notou-se que a razão trans/gauche é maior na formulação branco e na NeAnd quando comparada ao óleo de andiroba livre. Esse padrão indica que as cadeias lipídicas da NeAnd e da formulação branco estão mais organizadas do que as do óleo livre, e esse resultado está de acordo com os dados obtidos na análise de FTIR.
- A citotoxicidade da NeAnd, óleo de andiroba livre, e controles PBS e ETOH em linhagens de HaCat *in vitro* foi avaliada. Foramobservados que nas três concentrações testadas (90, 180 e 360 µg/mL), o óleo de andiroba e a NeAnd em HaCat não foram citotóxicos em 24 e 48 horas, exceto o óleo de andiroba livre na concentração de 360 µg/mL em 24 e 48 horas.
- Foi avaliado também o potencial cicatrizante das NeAnd e do óleo de andiroba livre (*Carapa guianensis* Aublet) e controles PBS e ETOH em ensaio de migração celular *in vitro* (*Scratch assay*) em queratinócitos na concentração de 360 µg/mL. NeAnd aumentou a migração celular de HaCat significativamente em relação ao controle PBS ($p < 0.0005$) e ao óleo livre OA ($p = 0.0323$).

Este estudo contribuiu para a caracterização do óleo de andiroba e para formulação e caracterização de uma nanoemulsão à base deste óleo andiroba, destacando que nanoemulsões podem diminuir a citotoxidade de óleos, como o óleo de andiroba e aumentar a migração celular de queratinócitos *in vitro*, enfatizando uma melhor resposta celular quando tratadas com o óleo em nanoemulsão (NeAnd). São necessárias investigações adicionais para a otimização das formulações e sua aplicação terapêutica em contextos clínicos. Essas descobertas podem ter implicações importantes no desenvolvimento de possíveis fitoterápicos para a cicatrização de feridas e outras aplicações na área da saúde.

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