SERVIÇO PÚBLICO FEDERAL
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INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

FERNANDA WARISS FIGUEIREDO BEZERRA

Extração de Compostos Bioativos de Folhas de *Croton matourensis* Aubl. com CO_2 Supercrítico: Determinação da Composição Química, Atividade Antioxidante e Atividade Biológica

BELÉM – PA
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Instituto de Tecnologia, Universidade Federal do Pará, como requisito necessário para obtenção do título de Doutora em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Raul Nunes de Carvalho Junior

BELÉM – PA
2022

Dados Internacionais de Catalogação na Publicação (CIP) de acordo com ISBD
Sistema de Bibliotecas da Universidade Federal do Pará
Gerada automaticamente pelo módulo Ficat, mediante os dados fornecidos pelo(a) autor(a)

Wariss Figueiredo Bezerra, Fernanda.
Extração de Compostos Bioativos de Folhas de Croton matourensis Aubl. com CO2 Supercrítico: Determinação da Composição Química, Atividade Antioxidante e Atividade Biológica / Fernanda Wariss Figueiredo Bezerra. — 2022.
103 f. : il. color.

Orientador(a): Prof. Dr. Raul Nunes de Carvalho Junior
Tese (Doutorado) - Universidade Federal do Pará, Instituto de Tecnologia, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Belém, 2022.

1. Croton matourensis. 2. CO2 supercrítico. 3. Larixol. 4. Atividade neuroprotetora. 5. SARS-CoV-2. I. Título.

CDD 664.022

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Aos seis dia do mês de maio de 2022, às 9:00 h, utilizando o recurso de videoconferência, reuniu-se a comissão julgadora integrada pelos Senhores Professores Doutores **Raul Nunes de Carvalho Júnior** (Orientador), **Rosinelson da Silva Pena** (Membro Interno), **Alessandra Santos Lopes** (Membro Interno), **Eloisa Helena de Aguiar Andrade** (Membro Externo), **Flávia Cristina Seabra Pires** (Membro Externo), para dar início à **Defesa da Tese de Doutorado** da discente FERNANDA WARISS FIGUEIREDO BEZERRA.

Título da Dissertação: "EXTRAÇÃO DE COMPOSTOS BIOATIVOS DE FOLHAS DE *Croton matourensis* Aubl. COM CO_2 SUPERCRÍTICO: DETERMINAÇÃO DA COMPOSIÇÃO QUÍMICA, ATIVIDADE ANTIOXIDANTE E ATIVIDADE BIOLÓGICA"

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Prof. Dr. Raul Nunes de Carvalho Júnior
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"Para realizar grandes conquistas, devemos não apenas agir, mas também sonhar; não apenas planejar, mas também acreditar".

Anatole France

AGRADECIMENTOS

Aos meus pais, ao meu esposo, a minha irmã, aos meus sogros e as minhas cunhadas pelo constante apoio, incentivo e inspiração e a toda minha família pela força e motivação;

Ao meu orientador pela oportunidade de aprendizado e por estar ao nosso lado para que pudéssemos evoluir e superar os desafios acadêmicos;

Aos meus companheiros de laboratório, de curso e de vida que muitas vezes estenderam a mão e me ajudaram nos momentos em que tanto precisei;

Aos professores do programa e aos demais que contribuíram na execução do trabalho direta ou indiretamente;

À professora Gracialda Ferreira por ter me apresentado e cedido o material vegetal que foi usado na tese;

Aos membros da banca examinadora pelas contribuições e sugestões científicas que auxiliaram no desenvolvimento do trabalho;

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela bolsa concedida;

Ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos, à Universidade Federal do Pará e à Hadriane Pombo por todo apoio durante o período de pós-graduação.

RESUMO

A espécie *Croton matourensis* apresenta benefícios à saúde, devido a sua composição que possuí alta concentração em compostos bioativos como compostos fenólicos, terpenos, ácidos fenólicos e fenilpropanoides. O método mais usado para obter seus extratos é a hidrodestilação, entretanto a extração com dióxido de carbono supercrítico (CO_2-SC) é um método ambientalmente amigável, com alta seletividade e capaz de obter extratos com alta concentração em larixol, um diterpeno oxigenado de alto valor econômico e com diversas atividades biológicas. Neste contexto, a presente tese objetivou usar a tecnologia supercrítica para obter extratos das folhas de *C. matourensis* com alta concentração em larixol e avaliar sua potencial atividade anti-inflamatória, neuroprotetora e antiviral contra o vírus da COVID-19. Para isso, na primeira etapa da tese foi realizada a extração das folhas de *C. matourensis* com CO_2-SC em diferentes condições de temperatura (40 e 50 °C) e pressão (100 a 400 bar), a extração supercrítica foi comparada com a hidrodestilação (HD) e a extração hexânica (EH) por meio da avaliação do rendimento mássico, da composição química e da atividade antioxidante dos extratos, posteriormente foi realizado o modelo experimental de isquemia focal no córtex motor de ratos utilizando o extrato obtido na melhor condição operacional com CO_2-SC. Os maiores rendimentos foram obtidos na EH (5,73 ± 0,26%) e na condição a 50°C/400 bar (5,60 ± 0,06%), os quais foram estatisticamente iguais ($p > 0,05$), a condição supercrítica foi mais vantajosa na obtenção do larixol (48,49%) em comparação à EH (2,93%). Os extratos obtidos pelos diferentes métodos apresentaram elevado valor de compostos fenólicos totais (51,81 ± 2,03 a 79,53 ± 1,19 mgEAG/g extrato), flavonoides totais (2,50 ± 0,20 - 6,89 ± 0,45 mgEQ/g extrato) e atividade antioxidante com percentual de inibição máximo igual a 83,26 ± 0,58% na concentração de 14 mg/mL. O estudo de isquemia cerebral focal revelou que os animais tratados com o extrato de *C. matourensis* apresentaram maior redução da área de lesão do que os tratados com a solução controle (Tween 5%), sugerindo seu potencial efeito anti-inflamatório e neuroprotetor. Na segunda etapa da tese, foi realizado o estudo *in silico* para avaliar as interações entre o ligante larixol e quatro receptores do SARS-CoV-2. Os resultados de docagem molecular mostraram que o modo de interação desempenhou papel fundamental de interação com o larixol, principalmente na interação com a Spike-protease. O estudo mostrou através da energia de afinidade que o ligante apresentou conformações estáveis, formando um complexo estável com os receptores, estando em conformidade com o sítio catalítico dos receptores. Os dados de docagem molecular, dinâmica molecular e energia livre de Gibbs apontaram que o larixol pode se ligar ao compartimento de ligação dos receptores, evidenciando sua potencial atuação como agente antiviral ou agente usado em conjunto com outras terapias que forneçam linha de defesa contra doenças associadas ao coronavírus. Desta forma, a tese mostrou que o extrato das folhas de *C. matourensis* obtido com CO_2-SC apresentou alta concentração em larixol, com potenciais atividades anti-inflamatória, neuroprotetora e antiviral, apresentando potencial para ser aplicado como insumo nas indústrias alimentícia, cosmética e farmacêutica.

Palavras-chave: *Croton matourensis*, larixol, CO_2 supercrítico, estudo *in silico*, SARS-CoV-2, atividade antioxidante, atividade anti-inflamatória, efeito neuroprotetor, atividade antiviral.

ABSTRACT

The *Croton matourensis* species has health benefits, due to its composition, which has a high concentration of bioactive compounds such as phenolic compounds, terpenes, phenolic acids and phenylpropanoids. The most used method to obtain its extracts is hydrodistillation, however the extraction with supercritical carbon dioxide (SC-CO_2) is an environmentally friendly method, with high selectivity and capable of obtaining extracts with a high concentration of larixol, an oxygenated diterpene of high economic value and with several biological activities. In this context, the present thesis aimed to use supercritical technology to obtain extracts from leaves of *C. matourensis* with a high concentration of larixol and to evaluate its potential anti-inflammatory, neuroprotective and antiviral activity against the COVID-19 virus. For this, in the first stage of the thesis, the extraction of *C. matourensis* leaves with SC-CO_2 was carried out under different conditions of temperature (40 and 50 °C) and pressure (100 to 400 bar), the supercritical extraction was compared with hydrodistillation (HD) and hexane extraction (HE) through the evaluation of the mass yield, chemical composition and antioxidant activity of the extracts, then the experimental model of focal ischemia in the motor cortex of rats was carried out using the extract obtained in the best operating condition with SC-CO_2. The highest yields were obtained in HE (5.73 ± 0.26%) and in the condition at 50°C/400 bar (5.60 ± 0.06%), which were statistically equal ($p > 0.05$) , the supercritical condition was more advantageous in obtaining larixol (48.49%) compared to HE (2.93%). The extracts obtained by the different methods showed a high value of total phenolic compounds (51.81 ± 2.03 to 79.53 ± 1.19 mgGAE/g of extract), total flavonoids (2.50 ± 0.20 - 6.89 ± 0.45 mgQE/g of extract) and antioxidant activity with maximum inhibition percentage equal to 83.26 ± 0.58% at a concentration of 14 mg/mL. The focal cerebral ischemia study revealed that the animals treated with the *C. matourensis* extract showed a greater reduction in the lesion area than those treated with the control solution (Tween 5%), suggesting its potential anti-inflammatory and neuroprotective effect. In the second stage of the thesis, an *in silico* study was carried out to evaluate the interactions between the larixol ligand and four SARS-CoV-2 receptors. The molecular docking results showed that the mode of interaction played a fundamental role in the interaction with larixol, mainly in the interaction with Spike-protease. The study showed through the affinity energy that the ligand had stable conformations, forming a stable complex with the receptors, conforming to the catalytic site of the receptors. Molecular docking, molecular dynamics and Gibbs free energy data showed that larixol can bind to the binding compartment of receptors, evidencing its potential role as an antiviral agent or agent used in conjunction with other therapies that provide a line of defense against diseases associated with the coronavirus. Thus, the thesis showed that the extract of *C. matourensis* leaves obtained with SC-CO_2 showed a high concentration of larixol, with potential anti-inflammatory, neuroprotective and antiviral activities, presenting potential to be applied as an input in the food, cosmetic and pharmaceutical industries.

Keywords: *Croton matourensis*, larixol, supercritical CO_2, *in silico* study, SARS-CoV-2, antioxidant activity, anti-inflammatory activity, neuroprotective effect, antiviral activity.

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SUMÁRIO

TEXTO INTEGRADOR

A espécie *Croton matourensis* possui grande potencialidade devido a sua composição química que apresenta elevada concentração em compostos terpênicos, compostos fenólicos e ácidos fenólicos. Quando utilizada a extração com dióxido de carbono supercrítico (CO_2-SC) é possível a obtenção de extratos com elevada concentração em larixol (>50%), composto que possui alto valor no mercado e que desperta interesse nas indústrias, pois apresenta atividades biológicas como ação antimicrobiana contra patógenos humanos, alimentares e agrícolas (*Plasmopara vitícola*, *Aspergillus flavus*, *Candida albicans*, *Penicillium funiculosum*, *P. ochrochloron*, *Trichophyton mentagrophytes*, *Microsporum gypseum* e *M. nannu*), ação contra doença renal, atividade antioxidante, ação analgésica e anti-inflamatória contra dor neuropática, melhora da função endotelial e ação no tratamento de edema de isquemia de reperfusão pulmonar.

Apesar da espécie ser nativa da Amazônia Legal, Nordeste e Centro-Oeste do Brasil, ainda são poucos os trabalhos encontrados na literatura que estudaram suas características químicas e biológicas. Desta forma, é necessária a realização de mais estudos que avaliem sua composição química, potencialidades biológicas e tecnológicas através de tecnologias mais sustentáveis que fomentem a bioeconomia, podendo trazer biodesenvolvimento para a região Amazônica e valorizando este recurso ainda pouco explorado. Neste contexto, a presente tese teve como objetivo realizar o estudo sobre a extração das folhas de *C. matourensis* com CO_2-SC, avaliando a condição operacional responsável pelo maior rendimento mássico, elevada concentração de larixol e avaliar as potencialidades do extrato como agente anti-inflamatório, neuroprotetor e antiviral. Para essa finalidade, a tese foi desenvolvida e dividida em quatro capítulos, descritos a seguir.

O Capítulo I apresenta a primeira parte da revisão da literatura, abordada na forma do artigo de revisão "Bioactive Compounds and Biological Activity of *Croton* Species (Euphorbiaceae): An Overview" publicado na revista "Current Bioactive Compounds", o artigo fez uma varredura na literatura sobre as espécies do gênero *Croton* e abordou pontos sobre suas características botânicas, taxonômicas, composição química, atividades biológicas e métodos de extração com ênfase na extração com fluido supercrítico.

O Capítulo II, referente à segunda parte da revisão da literatura, apresenta o capítulo de livro "Extraction of Bioactive Compounds" publicado pela editora "Elsevier", o capítulo aborda a obtenção de compostos bioativos por meio da tecnologia supercrítica, com ênfase no

CO_2 como solvente, apresentando algumas de suas propriedades como fluido supercrítico e algumas das principais classes de biocompostos relatadas na literatura obtidas a partir de sua aplicação.

O Capítulo III é referente ao artigo "Chemical Composition, Antioxidant Activity, Anti-inflammatory and Neuroprotective Effect of *Croton matourensis* Aubl. Leaves Extracts Obtained by Supercritical CO_2" publicado na revista "The Journal of Supercritical Fluids", o artigo apresenta o estudo experimental da obtenção de extratos das folhas de *C. matourensis* com CO_2-SC, a avaliação dos aspectos químicos e capacidade antioxidante dos extratos obtidos e a potencial atividade anti-inflamatória e neuroprotetora do extrato obtido na melhor condição operacional supercrítica.

O Capítulo IV apresenta o artigo "Potential Antiviral Activity of Larixol against Structures of SARS-CoV-2 using Computational Study" submetido na revista "Journal of Biomolecular Structure and Dynamics". O trabalho foi realizado em virtude da crise pandêmica de COVID-19 que a população passou a enfrentar desde o início do ano de 2020, desta forma, aliado aos resultados obtidos no artigo experimental apresentado no capítulo III, no qual foi obtido um extrato com elevada concentração em larixol e com potenciais atividades biológicas (anti-inflamatória e neuroprotetora), o trabalho objetivou avaliar se o larixol apresenta potencial atividade antiviral contra o SARS-CoV-2, para isto, foi realizada a interação entre o composto e quatro estruturas do vírus através de estudo de simulação computacional.

Por fim, foi elaborada uma conclusão geral e posteriormente são apresentados os trabalhos científicos desenvolvidos durante o período do doutorado. Diante do exposto, a tese é mostrada nos capítulos a seguir.

OBJETIVOS

OBJETIVOS GERAIS

Obter extratos das folhas de *Croton matourensis* Aubl. via dióxido de carbono supercrítico (CO_2-SC), determinar a melhor condição operacional em termos de rendimento mássico e obtenção de larixol, avaliar a composição química, atividade antioxidante e atividade biológica do extrato obtido na melhor condição operacional supercrítica; e, posteriormente, realizar o estudo *in silico* entre o larixol e estruturas do vírus da COVID-19.

OBJETIVOS ESPECÍFICOS

- Caracterizar físico-quimicamente as folhas de *C. matourensis*;
- Obter extratos de *C. matourensis* por extração convencional e em diferentes condições de extração com CO_2-SC;
- Determinar as isotermas de rendimento global dos extratos obtidos com CO_2-SC;
- Determinar o conteúdo de compostos fenólicos totais, flavonoides totais e atividade antioxidante dos extratos obtidos;
- Determinar e quantificar os compostos voláteis por cromatografia gasosa acoplada à espectrometria de massas (CG/EM) dos extratos;
- Realizar o estudo *in vivo* da atividade anti-inflamatória e neuroprotetora do extrato obtido na melhor condição operacional com CO_2-SC;
- Realizar o estudo de simulação computacional entre o larixol e proteínas do SARS-CoV-2.

CAPÍTULO I

Bioactive Compounds and Biological Activity of *Croton* Species (Euphorbiaceae): An Overview

(Compostos Bioativos e Atividade Biológica de Espécies de *Croton* (Euphorbiaceae): Uma Visão Geral)

Reprinted from:

BEZERRA, F.W.F.; BEZERRA, P.N.; DE OLIVEIRA, M.S.; DA COSTA, W.A.; FERREIRA, G.C.; CARVALHO JR., R.N. Bioactive Compounds and Biological Activity of *Croton* Species (Euphorbiaceae): An Overview. CURRENT BIOACTIVE COMPOUNDS, v. 16, p. 383-393, 2020. DOI: 10.2174/1573407215666181122103511

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Current Bioactive Compounds **2019**, *15*, 000-000 **1**

REVIEW ARTICLE

Bioactive Compounds and Biological Activity of *Croton* Species (Euphorbiaceae): An Overview

Fernanda W.F. Bezerra[1], Priscila do N. Bezerra[1], Mozaniel S. de Oliveira[1], Wanessa A. da Costa[2], Gracialda C. Ferreira[3] and Raul N. de Carvalho Jr[1,2,*]

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Abstract: ***Background:*** *Croton* species are widely spread around the world, and present a varied chemical composition distributed in many classes of secondary metabolites, such as terpenoides, alkaloids, phenolic compounds and phenylpropanoids. These compounds can be obtained by different extraction methods, and more recently, with supercritical fluids. The crude and isolated extracts may have applications due to their biological activities in animals and humans.

Methods: The text was written based on literature data from 1996 onwards.

Results: The research showed in a concise way the botanical and taxonomic aspects of *Croton* and the success of its application is in studies related to the biological activities of the plant parts. It was also related to the chemical composition of its extracts and isolated compounds, obtained by many methods.

Conclusion: In summary, the review feature studies reported the use of extracts and isolated *Croton* compounds due to their biological effects with antioxidant, antimicrobial, anti-inflammatory, neuroprotective, antitumor, anticancer, cytotoxic, insecticidal and allelopathic activities, with potential application in food, cosmetics, pharmaceuticals, and agrochemicals products.

ARTICLE HISTORY

Received: June 07, 2018
Revised: November 12, 2018
Accepted: November 13, 2018

DOI:
10.2174/1573407215666181122103511

Keywords: *Croton*, folk medicine, medicinal plants, bioactive extracts, phytocompounds, biological activity, supercritical fluid extraction.

1. INTRODUCTION

The use of plants in folk medicine has been an ordinary practice for thousands of years in various civilizations [1, 2]. Rational exploration can contribute to the sustainable development of local communities and stimulate global phytotherapy growth [3]. The study of these plants extracts has been carried out in order to obtain agents that allow the application in food, oleochemicals, cosmetics, and medicinal products, aiming at the prevention and treatment of diseases with few undesirable side effects and easy access to population [4-10].

Croton is a very extensive genus, belonging to the family Euphorbiaceae, and possessing diverse species such as lianas and great trees. It is reported in tropical regions of the Old and New Worlds. Several parts of its species (root, bark, stems, twigs, leaves, flowers, fruits, and seeds) are popularly used as insecticides, larvicides, and in the treatment of diseases such as inflammation, hypertension, headache, fever, gastric diseases, dysentery, and others [11-16].

Its use is associated with its vast chemical composition: diterpenes (clerodane, labdane, abietane, tigliane, ent-kaurane, and cembranolides), alkaloids, and phenolic compounds. Some species also have volatile oils with a composition rich in phenylpropanoids, mono, and sesquiterpenes like methyl eugenol, *E*-caryophyllene, 1, 8-cineole, α-pinene, and bicyclogermacrene. Such substances are of great interest as they present high biological potential with antioxidant, antimicrobial, anti-inflammatory, antitumor, neuroprotective, anticancer, and antimalarial activities [16-25].

The separation or recovery of these substances has been widely studied with the use of new technologies. Among these, the technology of supercritical fluid extraction (SFE) can be highlighted, since it is capable of obtaining natural extracts rich in bioactive compounds, offering a solution of sustainable use for the natural resources. SFE, in particular with carbon dioxide (CO_2), provides the generation of products free of toxic residues and which generally present high quality when compared to products obtained by conventional techniques, thus becoming a process of great interest for

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1573-4072/19 $58.00+.00

food, pharmaceutical, and cosmetic industries [26-29]. In this sense, the present review aims to point out *Croton* bioactive activities associated with the chemical compounds present in its extracts obtained by conventional methods and SFE.

2. BOTANICAL AND TAXONOMIC PROFILE

Euphorbiaceae belonging to the Malpighiales, is composed of about 300 genera and 8000 species grouped into 47 tribes and 7 subfamilies with the biovulate groups: Phyllanthoideae and Oldfieldioideae; and the uniovulate: Peroideae, Cheilosoideae, Acalyphoideae, Euphorbioideae, and Crotonoideae. The family is very diverse presenting from simple weeds to large woody trees, with wide distribution around the world, mainly in tropical and subtropical climates [30-32]. *Croton*, belonging to the subfamily Crotonoideae, is the second most extensive of the family with about 1300 species of trees, shrubs, and herbaceous. Only in the Brazilian biomes (Amazonia, Cerrado, Atlantic Forest, Pampa, and Caatinga) there are 311 species approximately [17, 33-35].

The genus has its name derived from the Greek word "kroton" meaning thick, for their thick and smooth seeds [11]. According to Alves *et al*. [13] the genus presents few morphological synapomorphies, being the filament apex inflexed orientation of the stamens in the bud the main characteristic. Other common characteristics presented by Berry *et al*. [36] are the conspicuous stellate or scale-like trichomes, narrow or condensed inflorescences of unisexual flowers, watery to colored sap, frequent petiolar glands, and senescent leaves that turn orange before dehiscing (Fig. **1**).

Croton species are used as a logging resource, as food, in popular medicine treating fever, pain, digestive problems, intestinal worms, and malaria. In addition to being applied as larvicides and insecticides due to the diverse composition that can be formed by terpenes, alkaloids, proanthocyanidins, flavones, and other phenolic compounds [22, 24, 37, 38].

Fig. (1). Euphorbiaceae-Crotonoideae. *Croton matourensis* Aubl. **A**) Leaves; **B**) Branch; **C**) Fruit. (Author).

3. PHYTOCHEMICAL COMPOSITION

Croton species chemical composition are well diversified. In this way, several compounds can be found, being of high or low polarity. In *Croton urucurana* the composition was predominantly acetyl aleuritolic acid, stigmasterol, β-sitosterol, campesterol, β-sitosterol-O-glucoside, sonderianin, catechin, and gallocatechin [39]. In *Croton stellulifer*, the major components were α-phellandrene, p-cymene, linalool, and α-pinene [40]. According to Pinto *et al.* [19] the essential oil chemical composition of *Croton jacobinensis* showed larvicidal effect on Aedes aegypti (*E*-caryophyllene, 1, 8-cineole, α-pinene, viridiflorene, and δ-cadinene), while in *Croton nepetaefolius* the effect may be related to the presence of the oxygenated monoterpene 1, 8-cineole, the phenylpropanoids elemicin and methyl eugenol [12].

The antibacterial effects presented by *Croton heliotropiifolius* were related to the presence of (*E*)-caryophyllene, γ-muurolene, and viridiflorene [33]. Table **1** shows the main chemical components present in Croton species and their biological activities potencies.

Table 1. Chemical composition and biological uses of *Croton* species.

No	Species	Main Compounds	Biological Effects	Vegetable Part	Locality	References
1	*C. anisatum*	Trans-anethole and estragole	Fumigant	Bark	Madagascar	[41]
2	*C. argyrophyllus*	Bicyclogermacrene; β-pinene; and spathulenol	Antioxidant and antimicrobial	Leaves	Bahia, Brazil	[34]
3	*C. argyrophyllus*	Bicyclogermacrene; and spathulenol	Antiinflammatory	Leaves	Sergipe, Brazil	[42]
4	*C. bonplandianum*	Flavonoids	Antioxidant	Leaves	Andhra, Índia	[43]
5	*C. bonplandianum*	Glycosides	Allelopathic	Leaves	Índia	[44]
6	*C. campestris*	Gallic acid; catechin; chlorogenic acid; caffeic acid; epicatechin; rutin; quercitrin; isoquercitrin; quercetin; and kaempferol	Antiulcerogenic	Roots	Ceará, Brazil	[45]
7	*C. campestris*	Condensed tannins; flavones; flavonols; xanthones; chalcones; aurones; flavonoids; leucoanthocyanidins; catechins; and alkaloids	Antibacterial	Leaves	Ceará, Brazil	[22]
8	*C. campestris*	β-caryophyllene; 1, 8-cineol; and germacrene-D	Anti-inflammatory and anti-edematogenic	Leaves	Ceará, Brazil	[46]

Table (1) contd….

No	Species	Main Compounds	Biological Effects	Vegetable Part	Locality	References
9	*C. celtidifolius*	Proanthocyanidin	Neuroprotective	Bark	Santa Catarina, Brazil	[47]
10	*C. crassifolius*	Diterpenoids	Anti-angiogenic	Roots	Guangdong, China	[48]
11	*C. euryphyllus*	Clerodane diterpenoids	Neuroprotective	Twigs and leaves	Guangxi, China	[21]
12	*C. flavens*	Viridiflorene; germacrone; (*E*)- γ-bisabolene; β-caryophyllene	Anticancer	Leaves	Petit Canal, Guade-loupe	[49]
13	*C. gratissimus*	Cembranolides	Anticancer	Stem bark	Durban, África do Sul	[25]
14	*C. heliotropiifolius*	(*E*)-caryophyllene; γ-muurolene; viridiflo-rene	Antibacterial	Leaves and stems	Bahia, Brazil	[33]
15	*C. heliotropiifolius*	Limonene; α-pinene; and caryophyllene	Antioxidant, antimi-crobial	Leaves	Bahia, Brazil	[34]
16	*C. heliotropiifolius*	β-caryophyllene; bicyclogermacrene; germacrene-D	Larvicidal	Leaves	Sergipe, Brazil	[24]
17	*C. jacobinensis*	E-caryophyllene; 1, 8-cineole; α-pinene, viridiflorene, δ-cadinene	Larvicidal	Leaves	Ceará, Brazil	[19]
18	*C. jacobinensis*	Labdane diterpene	Antibacterial	Stems	Ceará, Brazil	[17]
19	*C. laevigatus*	Abietane-type diterpenoids	Anticancer	Twigs and leaves	Yunnan, China	[18]
20	*C. laui*	Labdane diterpenoids	Anti-inflammatory	Leaves and stems	Hainan, China	[50]
21	*C. lechleri*	Rutin and vitexin	Antitumor	Leaves	Veracruz, México	[51]
22	*C. lechleri*	Taspine	Anti-inflammatory	Resin	Haarlem, Holanda	[52]
23	*C. limae*	Cedrol; eucalyptol; α-pinene	Allelopathic	Leaves	Ceará, Brazil	[53]
24	*C. matourensis*	Fenchyl acetate; methyleugenol; isoelemi-cine; elemicine; spathulenol; valencene	Cytotoxic	Leaves	Bolívar, Venezuela	[16]
25	*C. micans*	(Flower) Fenchyl acetate; α-caryophyllene; β-cubebene; β-caryophyllene; α-cubebene; β-elemene; valencene (Leaves) Fenchyl acetate; α-caryophyllene; α-selinene; β-bourbene	Citotóxico	Flower and leaves	Bolívar, Venezuela	[16]
26	*C. nepetaefolius*	1, 8-cineole; arylpropanoids elemicin; methyl eugenol	Larvicidal	Leaves; stalks and roots	Ceará, Brazil	[12]
27	*C. penduliflorus*	Quercetin-3-O-rhamnoside; kaempferol-3-O-rhamnoside; protocatechualdehyde; p-hydroxybenzoic acid	Neuroprotective	Leaves	Ile-Ife, Nigéria	[54]
28	*C. polyandrus*	p-cymene; bornyl acetate; ascaridole	Antitumor	Leaves	Paraíba, Brazil	[35]
29	*C. pulegiodorus*	β-caryophyllene; bicyclogermacrene; germacrene-D; τ-cadinol; β-copaen-4-α-ol	Larvicidal	Leaves	Sergipe, Brazil	[24]
30	*C. stellulifer*	α-phellandrene; *p*-cymene; linalool; α-pinene	Aantimicrobial	Bark	S. Tomé island	[40]
31	*C. tiglium*	Tigliane-type diterpenoids	Antitubercular	Leaves	Sichuan, China	[20]
32	*C. tonkinensis*	ent-Kaurane diterpenoid	Anticancer	Leaves	-	[23]
33	*C. urucurana*	Gallocatechin; procyanidin B3; catechin; epicatechin; tembetarine; magnoflorine; taspine; methyl-3-oxo-12-epi-barbascoate; methyl-12-epi-barbascoate; hardwickiic acid	Anti-inflammatory and antinociceptive	Bark	Mato Grosso do Sul, Brazil	[55]
34	*C. zambesicus*	Tannin; kelakellani; saponin; flavonoid	Larvicidal	Leaves	Ondo, Nigéria	[56]
35	*C. yanhuii*	Clerodane diterpenenes	Neuroprotective	Twigs	Yunnan, China	[57]

4. OBTAINING METHODS OF BIOACTIVE EXTRACTS FROM *CROTON*

Several studies have reported extracts obtainment from *Croton* using a variety of methods. In general, the most commonly used conventional methods for collecting plant extracts are maceration, hydrodistillation, steam trapping, and solvent extraction. Other methods, called non-conventional, are also employed as microwave assisted extraction, ultrasonic assisted extraction, and supercritical fluid extraction (SFE).

Atikah [58], for example, used maceration on *C. tiglium* seeds with hexane as solvent and 1:7 molar ratio (raw material:hexane), reaching 30% yield. Bantie *et al*. [59] also used the methanol technique to obtain *Croton macrostachyus* leaf extracts, reaching 18.6% yield. Solvent extraction using Soxhlet apparatus was used in Sutthivaiyakit *et al.* [60], Santos *et al.* [61], and Kibazohi & Sangwan [14] works for extracts from *Croton joufra, Croton cajucara,* and *Croton megalocarpus*, respectively. In these studies, n-hexane, chloroform, and methanol were used to perform the extractions. Hydrodistillation was used to obtain *C. flavens* leaf [49] and *C. zehntneri* extracts [62], reaching yields of 0.18% and 0.15%, respectively.

SFE is an unconventional method that has been highlighted as being efficient in bioactive compounds extractions with a high degree of purity and standardization, besides being considered chemically green, since it does not use toxic organic solvents during the process. SFE is a unitary contact operation based on the equilibrium and physical-chemical properties of supercritical fluids (SF), which are substances that are under conditions of pressure and temperature above their critical points (Pc and Tc) [27, 63-65]. The carbon dioxide (CO_2) supercritical region is represented in the phase diagram (Fig. **2**), supercritical CO_2 densities were calculated using software developed by the National Institute of Standards and Technology, which uses the equation of state developed by Span and Wagner [66].

The SF have liquids and gases characteristics. High diffusion coefficient being close to the gases and high solubility close to the liquid, providing a fast and efficient mass transfer. SF density is higher than that of the gases, with a higher solvation power due to the high compressibility. In addition, they present low viscosity and absence of surface tension, promoting greater penetration in the solid matrix [64, 65].

CO_2 is the main substance used in SFE for being relatively inexpensive, has low critical pressure and critical temperature (73.7 bar and 31 °C), reducing the thermosensitive compounds degradation, besides being non-toxic, non-flammable, odorless, and easily separated from the extract. CO_2 is indicated for extracting nonpolar compounds, but when polar organic solvents such as water, ethyl acetate, ethanol, and methanol are added, the polarity is modified, it being possible to extract other compounds, these aggregate solvents are called cosolvents [28, 67, 68].

In Sousa *et al.* [69] study, supercritical CO_2 was used to obtain *Croton zehntner* extracts at 66.7 bar and 20 °C, its composition was formed by (*E*)-anethole, alpha-muurolene, methyl chavicol, and germacrene D, with total mass yield of 3.4%. Wang *et al.* [48] and Huang *et al.* [70] applied the SFE technique to collect *Croton crassifolius* extracts at 200 bar and 48 ºC, reaching the maximum yield of 3.45%, the composition was predominant in diterpenoids and sesquiterpenes, and the extracts presented antiangiogenic activity.

5. BIOATIVE PROPERTIES

Croton is reported in the literature due to its wide application in folk medicine to treat stomach pains, gastric and duodenal ulcers, digestive system, hematological, and respiratory disorders, wounds, inflammation, abscesses, impetigo, malaria, among others [18, 23, 45, 50]. These diseases treatment are associated with the biological activity that the genus presents due to its phytochemical, pharmaceutical, and medicinal properties [11, 18]. Studies involving the most varied species of *Croton* indicate their antioxidant, antimicrobial, anti-inflammatory, antimutagenic, neuroprotective, antidiabetic, analgesic, antidematogenic, antituberculosis, antitumor, antirheumatic, anticancer, allelopathic, and antimalarial activity [16, 20, 34, 35, 46, 53, 56, 71]. In this way, studies on these biological effects are presented below.

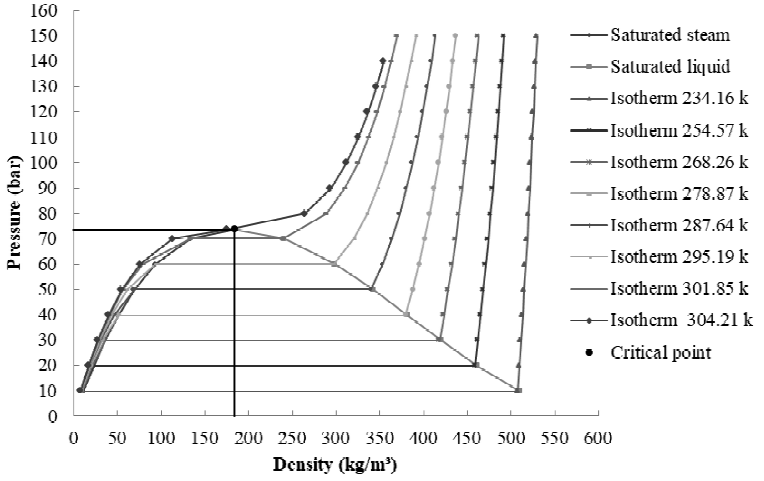

Fig. (2). CO_2 phase diagram (Author).

5.1. Antioxidant Activity

Reactive oxygen species (ROS) and nitrogen (RNS) as the superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) can be generated in the mitochondria, cytoplasm, or plasma membrane causing damage to the cellular structure such as lipids, proteins, membrane, and nucleic acids. This oxidative stress is derived from the unbalance of the antioxidant defense in the cells, which are present in the elimination of these reactive species [72-75]. In this way, natural extracts rich in antioxidants can act in the prevention and treatment of some neurological, carcinogenic, inflammatory, and tumoral diseases. In addition to being used in food products, nutraceuticals, pharmaceuticals, cosmetics, and oleochemicals [54, 76, 77].

Studies involving *Croton lechleri* species demonstrated their antioxidant activity attributed mainly to phenolic compounds and taspine alkaloid, capable of providing protection from free radicals due to the epithelial regeneration and healing, conferred to the proliferation and migration of fibroblasts and collagen production [38, 52, 77]. Cordeiro *et al.* [55] attributed the anti-inflammatory and antinociceptive activity of the *Croton urucurana* bark methanolic extract to the antioxidant action that the extract major compounds (diterpenes, alkaloids, and flavonoids) present. In Ramos *et al.* [42] study, *Croton argyrophyllus* leaves essential oil presented anti-inflammatory action in rodents, the effect can be attributed to the antioxidant action that was proven by the inhibition of nitric oxide radicals (NO) and the lipid peroxidation assays. In Brito *et al.* [34] study, leaves essential oil and leaves and stem methanolic extract from *Croton heliotropiifolius* and *Croton argyrophyllus* were evaluated for antioxidant activity by the $DPPH^{\bullet}$ and $ABTS^{\bullet}$ methods; essential oils had higher antioxidant activity than methanolic extracts; for the DPPH method, the *C. heliotropiifolius* essential oil had a higher value of EC_{50} (34.8 $mg.mL^{-1}$), whereas by the ABTS method no significant difference was found between the species *C. argyrophyllus* (EC_{50}=16.5 $mg.mL^{-1}$) and *C. heliotropiifolius* (EC_{50}=22.7 $mg.mL^{-1}$).

5.2. Antimicrobian Activity

The bioconservation of food, pharmaceutical, and cosmetic products has been used instead of synthetic preservatives such as nitrates, formaldehydes, butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), parabens, among others, as these can cause health damage such as development of bacterial resistance, allergic reactions (asthma, urticaria, abdominal pain, nausea, diarrhea, convulsions, and anaphylactic shock), inflammatory reactions, and neoplasias that can lead to death. In this way, vegetables, fungi, and bacteria have been used over the years as potential antimicrobial agents due to reports about their actions against various deteriorating and pathogenic microorganisms [48, 78-82].

In Lavor *et al.* [22] study, the *Croton* leaves ethyl acetate fraction has significantly modulated the resistance to bacteria, the synergic action between the extract and the amikacin potentiated the pharmacological action of the antibiotic against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Croton gratissimus* leaf extract presented 0.83 mg/mL of minimum inhibitory concentration (MIC) against *Candida krusei* and *Candida glabrata*. The essential oil also showed good activity against *Streptococcus mutans*, 0.42 mg/mL of MIC value; according to Akhalwaya *et al.* [83] the tree bark is popularly used to treat gums problems. Bernardino *et al.* [17] isolated the diterpene labdane from *Croton jacobinensis* stems, diterpene presented modulating effect when combined with antibiotics, synergistic effect was observed with gentamicin against *Escherichia coli*, and also with gentamicin and cephalothin against *Pseudomonas aeruginosa*. In the study of *Croton heliotropiifolius* aerial parts essential oil [33] showed inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* with 62.5 and 500 µg.mL-1 of MIC, respectively; thus demonstrating the potential application of essential oil in the food industry by controlling bacteria.

5.3. Anti-inflammatory Activity

The inflammatory reaction is triggered by endogenous and exogenous noxious organism stimuli such as fever, irritations, infections, and swelling. The reaction is an organism protective response to pathogens and tissue damage leading to the organism return to homeostasis through the migration of immune cells through endothelial cells. However, excessive inflammation can lead to chronic diseases such as arthritis, obesity, cancer, degenerative inflammatory diseases, and cardiovascular disease. The use of new drugs based on natural products has been studied over the years for the treatment of inflammation with reduction of side effects that are associated with drugs conventionally employed [84-90].

Croton urucurana bark methanolic extract reduced the response of paw edema to carrageenan in rats at 15 $mg.kg^{-1}$ dose, according to Cordeiro *et al.* [55] the effect was similar to indomethacin, it can be inferred that the extract may have reduced the release of histamine, serotonin, bradykinin, prostaglandins, and cyclooxygenase. Oliveira-Tintino *et al.* [46] studied the anti-inflammatory potential of *Croton* leaves essential oils in *in vivo* models of acute and chronic inflammation and demonstrated the action on abdominal contortions, paw edema induced by carrageenan, dextran, histamine, and arachidonic acid models, formalin test, peritonitis test, vascular permeability, and granuloma.

Zhao *et al.* [91] study evidenced *Croton crassifolius* roots ethanolic extract anti-inflammatory activity, in the study three animal models were developed: in the first model, the extract significantly inhibited the enhancement of capillary permeability induced by acetic acid in mice, evidencing that the extract may exert the inhibition of the inflammatory mediators present in the inflammation acute phase; in the second model, the extract presented response after 4 and 6 h of the carrageenan injection in the paw edema test, being effective in the second phase of inflammatory response and may involve the inhibition mechanism of the inflammatory mediators release, such as prostaglandins; and in the third model, the granuloma formation test showed that the extract presented anti-inflammatory activity in the inflammation chronic phase, since at 90 and 180 $mg.kg^{-1}$ doses there was an effective inhibition of granuloma formation of cotton pellets.

5.4. Neuroprotective Activity

Plants and plant extracts have been used for the treatment of various neurodegenerative disorders affecting the nervous

system (brain, spinal cord, and peripheral nerves). These may be related to the oxidative stress caused by the excess oxidants dysfunction or deficits in the antioxidant system, resulting in the elevation of reactive species. Antioxidant compounds present in *Croton* as glutathione peroxidase, superoxide dismutase, carotenoids, terpenoids, and polyphenols may have neuroprotective effects against this stress. The inhibitory action of such compounds on neural mediators and enzymes such as acetylcholine and acetylcholinesterase, anti-amyloidogenic effects, and inhibition of the neurotoxicity of N-methyl-D-aspartate, for example, may be favorable in the treatment of such disorders as Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, transmissible spongiform encephalopathies, myasthenia gravis, and senile d [11, 70, 92-95].

The inhibitory activity of quercetin-3-O-rhamnoside, kaempferol-3-O-rhamnoside, protocatechualdehyde, p-hydroxybenzoic acid, isolated from *Croton penduliflorus* leaves were studied by Aderogba *et al.* [54] and show high action (51.6 - 68.3 $mg.mL^{-1}$) against the enzyme acetylcholinesterase that is responsible for catalyzing the neurotransmitter acetylcholine degradation that leads to central nervous system disturbances. Moreira *et al.* [47] studied the *Croton celtidifolius* bark proanthocyanidin-rich extract, the results indicated that administration of 10 $mg.kg^{-1}$ intraperitoneally over a period of five consecutive days may confer neuroprotection against the underlying dopaminergic neurons degeneration in Parkinson's disease systemic treatment in rats and in the management of the disease non-motor symptoms such as memory problems and psychiatric symptoms that do not improve with current dopaminergic medications. Sun *et al.* [57] isolated and characterized two new diterpenes from the extract of *Croton yanhuii* branches, the constituents exhibited the property of stimulating nerve growth mediated by nerve growth factor from PC12 cells and may be potential anti-neurodegenerative agents for Alzheimer's disease and others neurological diseases. In a similar study, Pan *et al.* [21] isolated and characterized new clerodane diterpenes from *Croton euryphyllus* leaves and branches and determined that new compounds showed at 10 uM concentration an growth promoting the activity of neurites mediated by PC12 cells nerve growth factor.

5.5. Antitumor Activity, Anticancer, and Cytotoxic Effect

Although advances in diagnostic techniques and therapeutic modalities in patients have been achieved, cancer remains a major public health problem, in 2012 were over 14 million cases worldwide and for 2018 are designed more than 17 million new cases only in the United States. Cancer occurs due to uncontrolled cell growth and invasive potential in nearby tissues, which can affect most tissues in the human body. Tumor cells can metastasize, which is the migration from the primary site for installation in another distant location, or through benign tumors that display uncontrolled growth, but without invasion. Drugs obtained from natural sources may be used as supplementary agents as an alternative to existing antineoplastic therapies and can offer improved efficacy and less adverse toxicity to patients [96-99].

In Compagnone *et al.* [16] study the essential oils of *Croton matourensis* leaves and *Croton micans* flowers and leaves were tested *in vitro* by the mitochondrial metabolic activity method (MTT) and showed moderate cytotoxicity against colon carcinoma cells (LoVo and X-17), cervical cancer (HeLa), and control cells (dermal fibroblasts). The *in vivo* toxicity study of *Croton lechleri* leaf methanol extract showed LD_{50} results of 356 $mg.kg^{-1}$ i.p. (intraperitoneal route) and 500 $mg.kg^{-1}$ o.p. (oral), demonstrating that the extract has a moderate toxic effect; the extract also showed 59% inhibition of the cell lines growth derived from cervical region human tumor, indicating that *Croton* species may be an important source of antitumor compounds [51]. *Croton polyandrus* leaves essential oil showed antitumor activity *in vivo* and induced moderate gastrointestinal, hematological, and hepatic toxicity under the conditions evaluated, in contrast to the typical high toxicity of the antineoplastic drugs used [35].

5.6. Insecticidal Activity

The use of insecticides is applied due to public health risks associated with mosquitoes that may be vectors of human and veterinary pathogens and parasites such as dengue, malaria, leishmaniasis, filariasis, Chagas disease, West Nile virus, Chikungunya, and Zika [100, 101]. The use of synthetic insecticides, although effective, brings with it the disadvantages of disrupting natural control systems, the development of population resistance, and the effects on populations that are not target [102]. In this way, plants and plant extracts can be used as chemical defenses in the form of larvicides, insect growth regulators, repellents, and ovipositors. Since they are biodegradable, offer a large concentration of bioactive compounds, simple handling, and are low cost [103, 104].

Jose & Adesina [56] demonstrate that *Croton zambesicus* leaves hexanic extract showed larvicidal activity on the *Anopheles stephensi* malaria vector at 10 mL dosage with LC_{50} of 155.19 ppm and LC_{90} of 580.16 ppm, the larvae showed 91% mortality after 24 hours of exposure; the authors attributed the activity to the secondary metabolites (tannins, flavonoids, saponins, and glycosides) identified by the extract phytochemical screening. In Bagavan & Rahuman [105] study of *Croton bonplandianum* leaves ethyl acetate extract showed an effect against larvae of *Anopheles vagus* in the fourth stage (LC_{50} of 39.96 $\mu g.mL^{-1}$ and LC_{90} of 218.14 $\mu g.mL^{-1}$) with 100% mortality after exposure for 24 hours to the crude extract at 1000 $\mu g.mL^{-1}$. In Park *et al.* [106] study, *Croton anisatum* leaves essential oil presented fumigant activity against *Callosobruchus chinensis* (LC_{50} of 8.59 $mg.L^{-1}$) with 83.7% mortality after 24 hours of treatment in 11.76 $mg.L^{-1}$ concentration. Dória *et al.* [24] studied the larvicidal activity of *Croton heliotropiifolius* and *Croton pulegiodorus* essential oil against *Aedes aegypti*, the results indicated 100% mortality after 24 hours at 2000 ppm of *C. heliotropiifolius* essential oil (LC_{50} = 544 ppm) and 500 ppm for *C. pulegiodorus* (LC_{50} = 159 ppm). The study indicated the existence of a synergic effect between the essential oils constituents in a lower concentration, since when tested in isolation, the major constituent (β-caryophyllene) had a low larvicidal potential (LC50 = 1038 ppm).

5.7. Allelopathic Activity

Allelopathy is the science that studies the interaction between plants and microorganisms (viruses, fungi, and bacte-

ria) and their secondary metabolites that may influence agricultural and biological growth and development. Plant extracts present great allelopathic potential, since they can present such metabolites (alkaloids, phenolic compounds, terpenoids, and steroids) and when released into the environment can act against pathogens, affecting the development of other plants and microorganisms. In this way, there is control of agricultural pests such as weeds, reduction in the use of synthetic pesticides, reduction of environmental damages, an increase in yield of crops for food production [107-111].

Croton hirtus leaves showed 75.91% inhibition of the radicle growth of lettuce seedlings by the Sandwich method at 50 mg concentration, and 40.82% inhibition of growth on lettuce radicle at 44.14 mm of volatilization distance by the dish pack method in Yusoff *et al.* [112] study. *Croton limae* leaves essential oil presented allelopathic activity on tomato seeds (*Lycopersicum esculentum* Mill.) germination at 2.5% concentration and affected stem and radicular growth at 2.5, 3.75, and 5% concentration. Leite *et al.* [53] attributed the synergistic effect activity to the essential oil chemical composition (α-pinene, β-pinene, eucalyptol, and cedrol). Sisodia & Siddiqui [113] demonstrate that *Croton bonplandianum* root, stem, and leaves aqueous extracts investigated in different concentrations (0.5, 1.0, 2.0, and 4.0%) for their allelopathic effects on seed germination, seedling growth of cultivated plants (*Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., and *Brassica rapa* L.), and weeds (*Melilotus alba* Medik., *Vicia sativa,* and *Medicago hispida*); had the inhibitory effect directly proportional to the extracts concentration; while the length of the radicle, the length of the plumule, and the dry weight of the seedlings were significantly reduced in response to all extracts.

CONCLUSION

Based on an extensive literature review, it was possible to conclude that *Croton* has been used in several countries essentially as a medicinal plant due to its high chemical potential and biological value. In addition, its crude and purified extracts obtained from conventional and non-conventional methods such as SFE have potential applications as antimicrobial, antioxidant, anti-inflammatory, antinoceptive, neuroprotective, anticancer, and bioherbicide, being able to be applied in several types of products in the food, cosmetic, and pharmaceutical industries.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Fernanda Wariss Figueiredo Bezerra thanks to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, for the scholarship.

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CAPÍTULO II

Extraction of Bioactive Compounds

(Extração de Compostos Bioativos)

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BEZERRA, F.W.F.; DE OLIVEIRA, M.S.; BEZERRA, P.N.; CUNHA, V.M.B.; SILVA, M.P.; DA COSTA, W.A.; PINTO, R.H.H.; CORDEIRO, R.M.; DA CRUZ, J.N.; CHAVES NETO, A.M.J.; CARVALHO JR., R.N. Extraction of bioactive compounds. In: INAMUDDIN; ABDULLAH M. ASIRI; ARUN M. ISLOOR. (Org.). Green Sustainable Process for Chemical and Environmental Engineering and Science. 1ed. Amsterdam: Elsevier, 2020, v. 1, p. 149-167. DOI: 10.1016/B978-0-12-817388-6.00008-8

CHAPTER 8

Extraction of bioactive compounds

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Contents

1. Introduction

Secondary metabolites, such as antioxidants, vitamins, and minerals, are bioactive compounds produced by different plants species, and are also used as medicines and flavorings. Their antioxidant properties and possible uses in food industry have been investigated in the recent years [1].

Among the various methods of obtaining natural substances, such as maceration, solvent extraction, ultrasound-assisted extraction, and microwave-assisted extraction, the supercritical fluid extraction (SFE) has become an important alternative for food, pharmaceutical, and cosmetic industries. The preparation of solvent-free products is one of the most important advantages of this process. Carbon dioxide (CO_2) is the most used solvent in SFE due to its inertness, nontoxicity, high solubility, and perfect conditions for thermosensitive compounds extraction [1, 2].

Green Sustainable Process for Chemical and Environmental Engineering and Science
https://doi.org/10.1016/B978-0-12-817388-6.00008-8

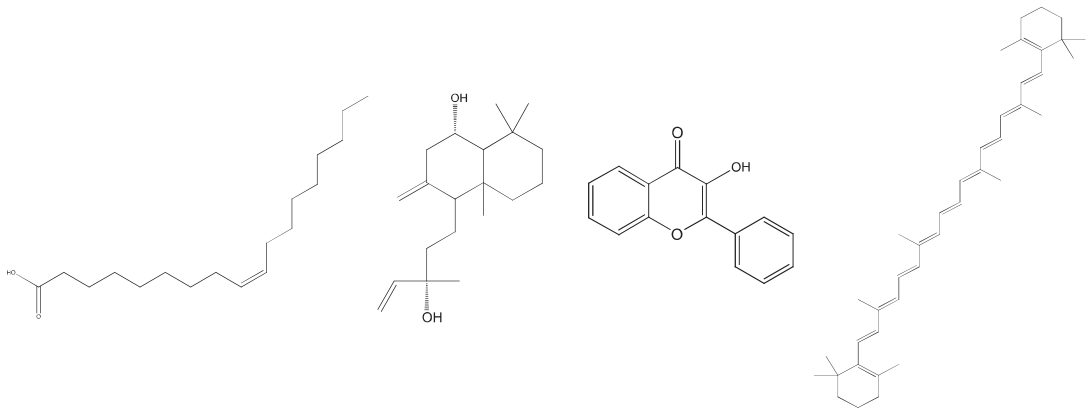

Fig. 1 Representation of bioactive compounds of the class of fatty acids (oleic acid), essential oils (larixol), phenolic compounds (flavonol), and carotenoids (β-carotene).

A fluid is said to be supercritical when it reaches a certain point above its critical temperature and pressure (T_c and P_c). Upon reaching the supercritical state, some properties of the fluid such as viscosity, density, and diffusivity present changes with intermediate values to the gaseous and liquid states [3, 4]. Such characteristics are important to extract high added-value compounds with biological activities, such as fatty acids, essential oils, phenolic compounds, and carotenoids, etc. (Fig. 1).

2. CO_2 as supercritical fluid

Over the years, several extraction methods have been developed with the purpose of obtaining bioactive compounds from natural raw materials. However, such methods (maceration, hydrodistillation, solvent extraction, etc.) are associated with some disadvantages such as the need for high extraction time, use of toxic solvents which are harmful to health and the environment, and they need of high temperature, which can promote the degradation of thermosensitive compounds [5].

Some solvents used in industries bring impacts on economic, ecological, and health issues, in this way, the concept of "green solvents" involves the reduction of environmental impacts that are caused by organic solvents. Thus, some characteristics are important for a given solvent to be considered "green," such as being biodegradable, having low volatility, low potential of ozone layer degradation, being produced with renewable resources, and having a low impact on human health and in the environment [6–9].

The extraction with supercritical fluids (SFs), in particular with CO_2, provides products free of toxic waste, having a higher quality when compared to the products obtained by conventional methods, becoming a process of great interest for the food, pharmaceutical, and cosmetic industries [10, 11]. The CO_2 offers several additional advantages such as:

residual toxicity elimination of organic solvents, low critical temperature (31.1°C), and critical pressure (73.7 bar), low thermosensitive compounds deterioration, selectivity for desired compounds, and sensory properties preservation of the material in food and pharmaceutical applications [12, 13]. Fig. 2 shows the pressure *versus* temperature (*P-T*) CO_2 phase diagram, marking its different aggregation regions (solid, liquid, and gaseous), phase change lines (sublimation, melting, and boiling, or vapor pressure). It is also possible to observe the triple point that occurs in 5.2 bar pressure and −56.6°C temperature, at this point it starts the vapor pressure, which ends at the critical point at 73.7 bar and 31.1°C and from this point it starts the supercritical region.

CO_2 is considered to extract apolar and moderately polar compounds. However, when smaller volumes of more polar solvents (modifiers or cosolvents) such as water, ethyl acetate, ethanol, or methanol are added the polarity is modified and more polar compounds can be extracted due to the CO_2 increased solvency and the polar compounds solubility. According to the type of sample and the analyte-sample affinity, the cosolvents may influence the extraction of polar compounds in three ways: (i) the cosolvent may produce an analyte-cosolvent interaction, increasing the analytes solubility during extraction; (ii) the polar cosolvents can interact with the sample facilitating the analytes desorption; and (iii) the sample swelling that occurs in the cosolvent presence may favor SF penetration, increasing the polar compounds extraction [4, 5, 13, 14].

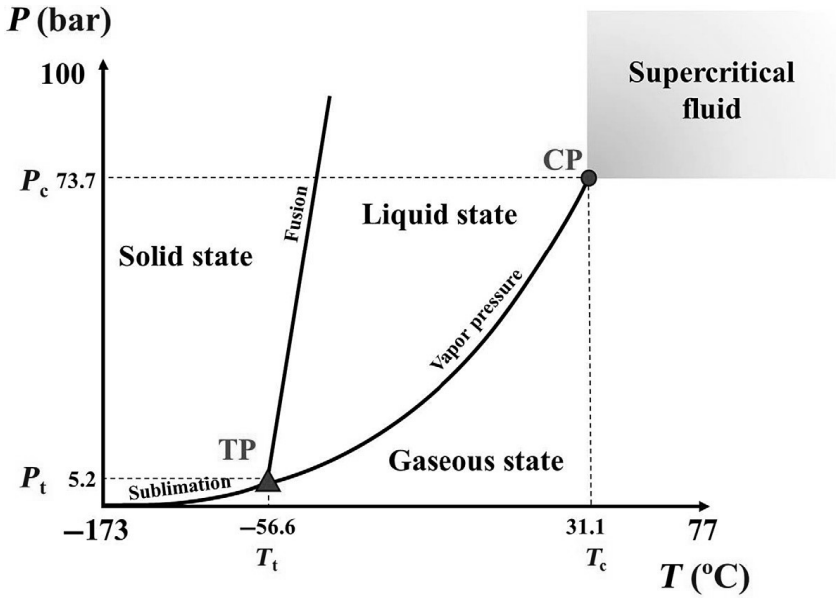

Fig. 2 *P-T* phase diagram for CO_2. P = pressure, P_c = critical pressure, *CP* = critical point, P_t = triple point pressure, T (°C) = temperature (°Celsius), T_P = triple point, T_t = triple point temperature, and T_c = critical temperature.

3. Supercritical carbon dioxide properties

3.1 Thermodynamics proprieties

3.1.1 Density

Density is defined as the inverse of the specific volume ($\rho = 1/V$) and can be obtained from thermodynamic tables or calculated by volumetric properties using an equation of state (EoS). When there is addition of cosolvents in the extraction process, the calculation can be performed by applying a cubic EoS such as Peng-Robinson or other EoS combined with mixing rules with appropriate interaction parameters [15, 16].

This thermodynamic property is extremely important in the SFE process. The supercritical carbon dioxide (SC-CO_2) density is highly dependent on temperature and pressure variation. The increase in pressure favors the increase in density at constant temperature, while the increase in temperature decreases the density at constant pressure, this behavior is due to the state of molecular approximation that occurs when the pressure increases, in this case the SC-CO_2 density is similar to a liquid, presenting high intermolecular interaction of its respective electron clouds (hydrogen bonds). The increase of density causes increase in its power solvation [15, 17, 18].

3.1.2 Solubility

Solubility is defined as the amount of a solute (mass fraction or mole fraction) that is dissolved by the supercritical solvent in thermodynamic equilibrium [19]. In this way, this equilibrium determines the mass transfer limit between the system phases, providing information about the phase compositions, the distribution of the individual components, and their quantities in equilibrium, as well as their variations with temperature and pressure, and the various components concentration. The parameters that influence solubility are temperature, pressure, and solute properties (molecular mass, polarity, and vapor pressure). Among these, the temperature deserves more attention due to its detrimental effect on solubility of SF [20, 21].

3.2 Transport properties

CO_2 is well established for use as a processing solvent in applications in the food and pharmaceutical industries and is a good alternative to replace organic solvents in conventional processes [22, 23]. Studies have reported that SC-CO_2 offers many advantages, for example in improving the selectivity of the compounds of interest present in a plant matrix in a binary system where mass and heat transfer occur between different phases equilibrium involved in separation processes [24]. Beyond temperature and pressure, other factors must be considered, such as the physicochemical properties of transport that depend on these two variables which are responsible for their changes. Although there are several studies on phase equilibrium thermodynamics, experimental data on transport properties (viscosity and diffusivity) are scarce in the literature [25].

In the supercritical state, CO_2 has diffusion coefficient close to that of a gas, which facilitates the penetration of the fluid in the solid matrix, being of great interest not only to study the structure and the thermophysical behavior of the fluid, but also to perform practical engineering applications and, depending on the process conditions, viscosity reduction is especially important as it determines not only the mass transfer but also the convective heat transfer [26, 27].

4. SC-CO_2 extraction

SFE is a unitary contact operation based on the equilibrium and the physicochemical properties of the SF, influenced by operational conditions as: temperature, pressure, solvent flow, material morphology, bed porosity, and particle size [20]. The SFE process can be performed through two stages of operation: extraction and separation. The extraction step involves the relation of the solvent solubility capacity by manipulating the thermodynamic properties (pressure and temperature) and/or modifying the chemical nature of the fluid upon addition of a cosolvent. Separation is achieved by depressurization, heating, or gradually cooling the extract, thereby providing a controlled fractionation. The separation can also be obtained by combining of the extraction process to purification/enrichment methods of extracts, for example the adsorption [28].

Fig. 3 shows an SC-CO_2 extraction system formed by a solvent feed system CO_2, a high-pressure pump, an extraction cell and a separation system. The extraction process is relatively simple, in which the preprocessed solid sample (dried and comminuted) is inserted into the extractor vessel which is heated. The system is subsequently pressurized to predetermined values by the action of a high-pressure pump, which allows the CO_2 to flow through the system and favor the solubilization of the compounds of interest present in the sample. Finally, the SC-CO_2 and extract obtained pass through an expansion valve and the CO_2 has its pressure reduced until the ambient pressure. The extract then precipitates in the collection flask and the CO_2 in the gas phase passes through a flow meter for the control and recording of its flow and subsequent release to the environment or recycle for a new process.

Pressure is the preferred variable for adjusting density values in SC-CO_2 extraction behavior, since it offers higher manipulation margins compared to other properties [15]. The temperature effect on the extraction can be observed through inflection points (intersection) between isotherms. The increase of the temperature causes a greater intermolecular distance, and consequently, the reduction of the CO_2 solubility power, by decreasing the density. The presence of inflection points will depend on the interaction between SF and the extract to be obtained [29]. From the date listed in Table 1, it is possible to observe the influence of temperature, pressure, flow rate, and density of the supercritical CO_2 in the bioactive compounds extraction process from vegetable raw materials.

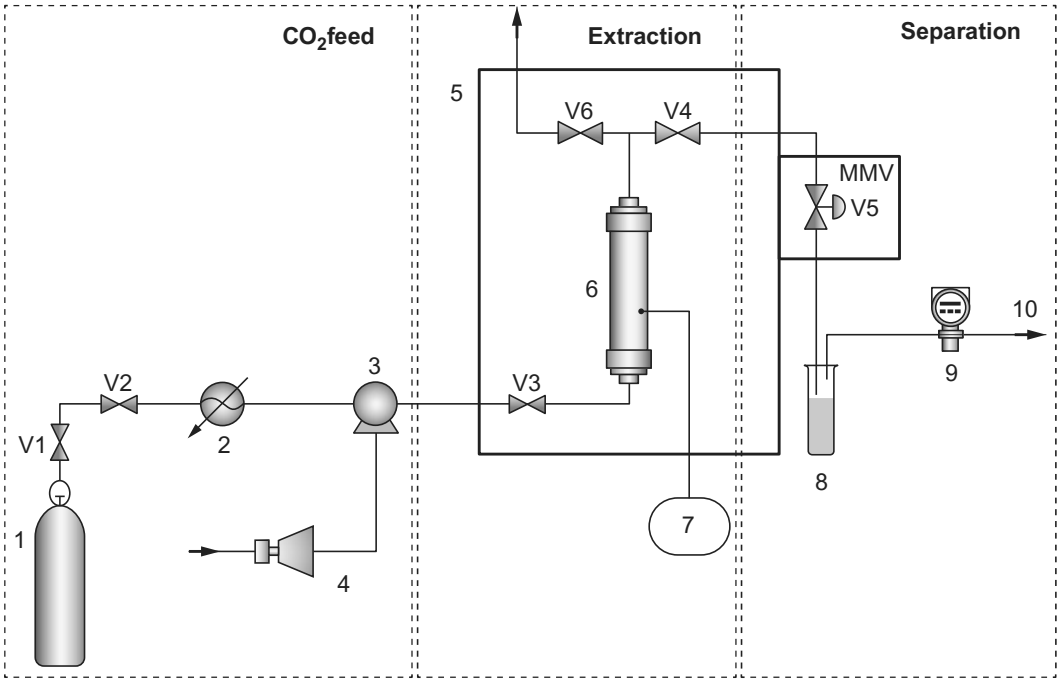

Fig. 3 Flow diagram of SC-CO_2 extraction. (1) CO_2 cylinder; (2) cooling bath; (3) pump; (4) compressor; (5) oven; (6) extractor vessel; (7) monitor; (8) collecting bottle; (9) flowmeter; (10) CO_2 outlet; V1–V6 flow control valves. *(Adapted from F.W.F. Bezerra, W.A. da Costa, M.S. de Oliveira, E.H. de Aguiar Andrade, R.N. de Carvalho Jr., Transesterification of palm pressed-fibers (Elaeis guineensis Jacq.) oil by supercritical fluid carbon dioxide with entrainer ethanol, J. Supercrit. Fluids 136 (2018) 136–143. doi:10.1016/j.supflu.2018.02.020.)*

5. Bioactive compounds extraction by SC-CO_2

5.1 Fatty acids

Fatty acids are a class of lipid compounds that have from 2 to 80 carbon atoms in their chain, and may or may not have double bonds and as a radical function may have hydroxyl groups, epoxy, and halogen atoms. Fatty acids maintain the energy balance of human organism, and when ingested properly can reduce the risk of some diseases like diabetes, hypertension, and inflammation [18]. These compounds can be obtained by some methods like solvent accelerated extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and Bligh and Dyer [39–41].

However, some conventional techniques may imply losses of compounds by thermal degradation, which may lead to fixed oil hydrolytic rancidity [40, 42, 43]. Ranjith Kumar et al. [44] have reported that solvent free methods may be promising to enable large-scale commercialization of high-quality oils. With this, the supercritical technology as an alternative has presented good results for lipid compounds extraction from several vegetal matrices, as evident from Table 2.

Table 1 Extraction parameters of bioactive compounds from vegetable raw materials

Raw material	Compounds	*T* (°C)	*P* (bar)	CO_2 density ($kg\,m^{-3}$)	Flow rate CO_2 ($kg\,s^{-1}$)	Yield (%)	References
Euterpe oleracea	Unsaturated fatty acids (C18:1 ω-9 and C18:2 ω-6)	50–70	150–490	700–900	8.85×10^{-5}	9.07–45.40	[30]
Oenocarpus bacaba	Unsaturated fatty acids (C18:1 ω-9, C18:2 ω-6, C18:3 ω-3).	40–60	120–420	700–900	8.85×10^{-5}	4.32–60.39	[31]
Oenocarpus distichus Mart.	Unsaturated fatty acids (C18:1 ω-9, C16:0, C18:2 ω-6)	50–60	150–350	~700–~900	8.85×10^{-5}	17.51–45.90	[32]
Rosmarinus officinalis	Camphor and 1,8-cineole	30–40	100–300	~650–~950	8.33×10^{-5}	1.00–5.00	[33]
Sesamum indicum L.	Unsaturated fatty acids and phytosterols	40–60	200–400	~700–~1000	5.67×10^{-5}	~32.5–~55.0	[34]
Spilanthes acmella	Spilanthol	50–70	150–490	700–900	7.40×10^{-5}	6.89–8.94	[35]
Syzygium aromaticum	Essential oil (Eugenol, *E*-caryophyllene, eugenol acetate)	40–50	100–300	~400–~900	8.85×10^{-5}	12.70–13.14	[36]
Cissus sicyoides L.	Terpenes, phenolic compounds.	40–60	200–400	~760–953	1.34×10^{-4}	1.27–3.21	[37]
Virola surinamensis	Saturated fatty acids (C14:0, C12:0)	40–80	350	~790–~936	7.90×10^{-5}	59.21–64.39	[38]
Piper divaricatum	Essential oil (methyl eugenol, eugenol, β-elemene)	35–55	100–500	325–1005	8.85×10^{-5}	4.68–7.40	[39]

Table 2 Fatty acids obtained by SC-CO_2 from vegetable raw materials

Raw material	Condition	Yield (%)	Major compounds	References
Watermelon seeds	$T=60°C$; $P=400$ bar	51.83	Linoleic acid	[45]
Scenedesmus sp.	$T=53°C$; $P=500$ bar	7.41	Trans-9-elaidic methyl ester	[46]
Tetraselmis sp.	$T=40°C$; $P=150$ bar	10.88	Palmitic acid	[47]
Scenedesmus obliquus	$T=60°C$;	8.00	Palmitic acid	[48]
Chlorella protothecoides	$P=300$ bar;	30.40		
Nannochloropsis salina	5% ethanol	13.00		
Italian Walnuts	$T=50°C$; $P=270$ bar	62.30	Linoleic acid and sitosterol	[49]
Euterpe oleracea	$T=70°C$; $P=490$ bar;	45.40	Oleic acid	[30]
Nannochloropsis sp.	$T=50°C$; $P=400$ bar	6.90	Oleic acid	[50]
Elaeis guineensis Jacq.	$T=40°C$; $P=450$ bar	6.09	Hexadecanoic acid	[29]

5.2 Essential oils

Essential oils are secondary metabolites produced by plants for protection against diseases and herbivorous insects. Several industrial segments use essential oils for the most varied applications due to their beneficial biological properties for the human health maintenance [50, 51].

The isolation of essential oils from aromatic plants usually occurs by conventional methods, the most widespread being hydrodistillation [51, 52]. However, this technique has limitations, as losses of compounds that may occur during extraction due to hydrolysis [52]. In this sense, it is recommended to use techniques that can be selective and do not degrade their bioactive compounds. The essential oils extraction with SC-CO_2 can be a viable alternative, mainly because it is a green technology suitable for lipophilic molecules extraction and capable of providing high-quality extracts and totally organic solvents free [35, 43, 53, 54]. Table 3 lists work reported during the last years using SC-CO_2 as extractant to obtain essential oils.

5.3 Phenolic compounds

Phenolic compounds are a secondary metabolites group characterized by having in their structure an aromatic ring attached to one or more hydroxyl substituents and can be

Table 3 Major compounds of essential oils obtained by SC-CO_2 from vegetable raw materials

Raw material	Condition	Yield (%)	Major compounds	References
Eryngium billardieri	$T=35°C$; $P=300$ bar	0.85	γ-cadinene	[53]
Juniperus communis L.	$T=55°C$; $P=300$ bar	6.55	1-octadecene	[55]
Helichrysum italicum	$T=52°C$; $P=200$ bar	4.09	1-[2-(2-Methyl-2,3-dihydroxypropyl)-2,3-dihydro-1-benzofuran-5-yl] ethanon	[56]
Echinophora platyloba	$T=55°C$; $P=240$ bar	1.18	Linalool	[57]
Cleome coluteoides	$T=35°C$; $P=220$ bar	0.65	α-cadinol	[58]
Mentha spicata	$T=48°C$; $P=151$ bar	1.40	Carvone	[59]
Rosmarinus officinalis	$T=50°C$; $P=103$ bar	1.41	Camphor	[60]
Launaea acanthodes B.	$T=55°C$; $P=240$ bar	1.02	n-dodecanal	[61]
Croton zehntneri	$T=15°C$; $P=66.7$ bar	3.40	*E*-anethole	[62]

classified as individual phenols or polyphenols according to the molecule phenol units number [15]. Their antioxidant properties are important in determining their role as protective agents against processes of free-radical-mediated diseases [62]. When added to foods, antioxidants control the rancidity development, delay the formation of toxic oxidation products, maintain nutritional quality, and extend product shelf life [63].

Depending on the plant species, SC-CO_2 can be used to obtain extracts rich in various phenolic groups such as coumarins, cinnamic acids, quinones, flavonoids, and lignans [15]. However, to modify the CO_2 apolar nature, some cosolvents need to be added, to increase the phenolic compounds extraction yield by the increase of solvation power. Table 4 lists some recent researches performed on phenolic compounds extraction using SC-CO_2 and cosolvents.

5.4 Carotenoids

The term "Carotenoids" is used to denote most of the pigments found naturally in animal and plant kingdoms. This group of fat soluble pigments has biological properties and contains more than 700 compounds responsible for red, orange, and yellow colors. Most carotenoids are hydrocarbons containing 40 carbon atoms and two terminal rings. These

Table 4 Phenolic compounds obtained by SC-CO_2 from vegetable raw materials

Raw material	Condition	Cosolvent	Major compounds	References
Paullinia cupana	$t=40$ min; $T=40$°C; $P=300$ bar	Ethanol, Methanol	Caffeine, catechin epicatechin	[64]
Vaccinium myrtillus L.	$T=40$°C; $P=200$ bar	Ethanol, Water	Anthocyanins	[65]
Syzygium cumini	$T=50$°C; $P=162$ bar	Ethanol	Anthocyanin, phenolic compounds	[66]
Citrus sinensis	$T=40$°C; $P=350$ bar	Ethanol	Phenolic compounds	[67]
Feijoa sellowiana	$T=50$°C; $P=250$ bar	Ethanol, Methanol	Gallic acid, catechin, rutin, ferulic acid, apigenin, quercetin	[68]
Opuntia stricta	$T=40$°C; $P=350$ bar	–	Polyphenols	[69]

pigments have been used mainly in the food, pharmaceutical, and cosmetic industries due to the potential for health benefits [69]. Nowadays, industrial production is performed through chemical synthesis, but natural alternatives of carotenoid production/obtaining are under development, mainly through the extraction of plants or algae [70].

The carotenoids extraction usually occurs with organic solvents, but the supercritical technology is an alternative technique for these compounds recovery, presenting many advantages when compared to the conventional processes. As a solvent for supercritical extraction, CO_2 has several advantages that make it the ideal solvent for thermosensitive molecules extraction such as carotenoids [71, 72]. Table 5 presents information from recent studies on SC-CO_2 extraction of carotenoids from plant materials.

5.5 Applications of bioactive compounds obtained from SC-CO_2

The production of new drugs from natural products and their analogous molecules obtained from the SC-CO_2 use has gained great importance. In the literature, the pharmacological activities of natural products against pathologies caused by bacteria, fungi, protozoa, viruses, besides cytoprotective activity, anticancer, among others have been documented [80–83]. Table 6 shows some biological activities that have already been related to extracts obtained through SC-CO_2.

SC-CO_2 has also been applied as a solvent and antisolvent in other industrial uses such as obtaining micro and nanoparticles for encapsulation [94, 95]. Particularly in the nanocomposite synthesis process, the use of SC-CO_2 is advantageous due to its high permeability of the structure and can be used in various compounds impregnation [96, 97].

Table 5 Carotenoids obtained by SC-CO_2 from vegetable raw materials

Raw material	Condition	Major compounds	References
Cucurbita sp.	$T=47.75°C$; $P=300$ bar	β-carotene and α-tocopherol	[73]
Solanum lycopersicum L.	$T=50°C$; $P=400$ bar	Lycopene	[74]
Dunaliella salina	$T=55°C$; $P=400$ bar	Total carotenoids	[75]
Lycopersicum esculentum L.	$T=80°C$; $P=400$ bar	Lycopene and β-carotene	[76]
Diospyros kaki L.	$T=60°C$; $P=300$ bar	Xanthophylls, β-carotene	[77]
Spinacia oleracea	$T=56°C$; $P=390$ bar	Lutein	[78]
Bactris gasipaes	$T=40°C$; $P=300$ bar	Total carotenoids	[79]
Mangifera indica L.	$T=40°C$; $P=300$ bar	Total carotenoids, β-carotene	[80]

Table 6 Biological activity related to the plant and its chemical compounds

Plant	Major Compounds	Activity	References
Cinnamomum zeylanicum	Cinnamaldehyde (77.10%), β-caryophyllene (6.00%), and α-terpineo (4.40%)	Antityrosinase	[84]
Piper nigrum L.	B-caryophyllene (25.38%), limonene (15.64%), and sabinene (13.63%)	Antioxidant	[43]
Stellera chamaejasme	Squalene (37.24%), 9,12-octadecadienoic acid *Z,Z*-(26.61%), and n-hexadecanoic acid (8.72%)	Acaricidal	[85]
Origanum vulgare	Carvacrol (39.89%), trans-sabinene hydrate (22.07%), and cis-piperitol (7.77%)	Antimicrobial	[86]
Lavandula angustifolia L.	Linalool (42.82%), linalyl acetate (23.40%), and camphor (8.05%)	Antioxidant and Antimicrobial	[87]
Rosmarinus eriocalyx	β-Amyrin (17.70%), olean-12-ene (8.2%), and epi-α-bisabolol (6.10%)	Antioxidant	[88]
Melaleuca cajuputi	β-caryophyllene (31.50%), Humulene (14.60%), and eugenin (13.60%)	Allelopathic	[89]
Apium nodiflorum	Dillapiol (57.5%), Phytol (12.7%), and *Z*-Falcarinol (6.8%)	Antifungal	[54]
Syzygium aromaticum	Eugenol (62.88%), Eugenol acetate (18.67%), and *E*-caryophyllene (15.60%)	Phytotoxic	[36]

Continued

Table 6 Biological activity related to the plant and its chemical compounds—cont'd

Plant	Major Compounds	Activity	References
Myristica fragrans	Myristicin (32.8%), Sabinene (16.1%), and α-Pinene (9.8%)	Cytotoxic	[90]
Zingiber officinale R.	A-Zingiberene (18.18%), β-Sesquiphellandrene (11%), and α-curcumene	Antibacterial	[91]
Tanacetum parthenium	Camphor (75.54%), trans-chrysanthenyl acetate (28.01%), and *Z*-Spiroether (16.78%)	Insecticidal	[92]
Pelargonium graveolens	Citronellol (14.44%), Citronellylformate (7.74%), and geraniol (7.73%)	Pesticidal and Antifungal	[93]
Chrysanthemum indicum	D-Camphor (8.58%), Caryophyllene oxide (8.46%), and α-curcumene (5.93%)	Antiinflammatory	[94]

The encapsulation technique has been successfully applied for stabilization and controlled release of nutrients such as vitamins and antioxidants [98–100]. The process has been widely used by the food, cosmetic, and pharmaceutical industries in order to stabilize certain compounds which under normal conditions would be unstable so that they can be used in different media [101–103]. Karim et al. [104] used hydroxypropyl methyl cellulose (HPMC) as a carrier material for the fish oil microencapsulation using the supercritical antisolvent process and obtained 81.75% efficiency in the process. Thus, micronized fish oil can be introduced into a variety of food matrices since it is a source rich in omega-3, omega-6, eicosapentaenoic acid, and docosahexaenoic acid that reduce the risks of cardiovascular disease, hypertension, and inflammation.

Another application involving the supercritical technology that can also be cited is the supercritical solvent impregnation (SSI), which is defined as a saturation process of a given material by compression and mixing with the pressurized CO_2 which may be in the sub or supercritical state [105]. The use of this technique in active packaging production brings an innovative alternative for several products conservation, because it maintains its nutritional and sensorial quality, prolonging its shelf life due to the action of an active agent added to the packaging material that can confer antimicrobial and/or antioxidant activity [106–108].

6. Considerations

The SC-CO_2 extraction is an important technique for obtaining biocompounds, since the obtained extracts are of high quality and strongly concentrated. The advantages of

this process include the rapid separation of CO_2 from the extracts (due to the simple depressurizing) and the not requirement of the use of organic solvents. In addition, bioactive compounds can be used in other technologies that employ SC-CO_2, obtaining materials of high added value for application in several sectors in an environmentally safe way.

Acknowledgment

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001.

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CAPÍTULO III

Chemical Composition, Antioxidant Activity, Anti-Inflammatory and Neuroprotective Effect of *Croton matourensis* Aubl. Leaves Extracts Obtained by Supercritical CO_2

(Composição Química, Atividade Antioxidante, Efeito Anti-inflamatório e Neuroprotetor de Extratos das Folhas de *Croton matourensis* Aubl. Obtidos por CO_2 supercrítico)

Reprinted from:

BEZERRA, F.W.F.; SALAZAR, M.A.R.; FREITAS, L.C.; DE OLIVEIRA, M.S.; DOS SANTOS, I.R.C.; DIAS, M.N.C.; GOMES-LEAL, W; ANDRADE, E.H.A.; FERREIRA, G.C.; CARVALHO JR., R.N. Chemical Composition, Antioxidant Activity, Anti-inflammatory and Neuroprotective Effect of *Croton matourensis* Aubl. Leaves Extracts Obtained by Supercritical CO_2. JOURNAL OF SUPERCRITICAL FLUIDS, v. 165, p. 104992, 2020. DOI: 10.1016/j.supflu.2020.104992

J. of Supercritical Fluids 165 (2020) 104992

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

Chemical composition, antioxidant activity, anti-inflammatory and neuroprotective effect of *Croton matourensis* Aubl. Leaves extracts obtained by supercritical CO_2

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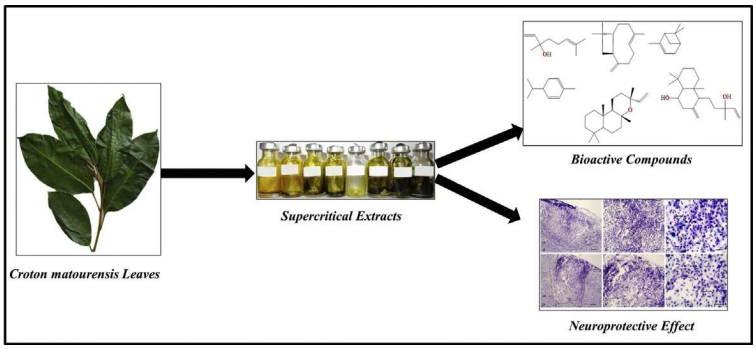
HIGHLIGHTS

- Larixol was found as major compound in SC–CO_2 extracts (24.88–50.57 %).
- The highest extraction yield with SC–CO_2 was at 50 °C/400 bar (5.60 ± 0.06 %).
- The extracts showed high TPC (51.81 ± 2.03–79.53 ± 1.19 mg GAE/g extract).
- All *Croton matourensis* leaves extracts exhibited antioxidant activity.
- SC–CO_2 extract presents anti-inflammatory and neuroprotective effect.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 February 2020
Received in revised form 24 June 2020
Accepted 15 July 2020
Available online 21 July 2020

Keywords:
Croton matourensis Aubl.
Supercritical extraction
Larixol
Antioxidant activity
Neuroprotection
Anti-inflammatory effect

ABSTRACT

Croton matourensis leaves extract was studied for its anti-inflammatory and neuroprotective effects after experimental stroke. The study investigated the extracts obtained with supercritical CO_2 (SC–CO_2) under different operational conditions of temperature (40 and 50 °C) and pressure (100–400 bar), assessing their global yield isotherms, chemical composition and antioxidant activity, in comparison to extracts obtained by hydrodistillation (HD) and *n*-hexane (HE). The highest yields were obtained with SC–CO_2 at 50 °C and 400 bar (5.60 ± 0.06 %) and in the HE (5.73 ± 0.26 %). The major compound found in HD and HE was linalool (35.26 and 69.98 %, respectively), whereas in SC–CO_2 extraction was larixol (24.88–50.57 %). The extracts showed high total phenolic contents, total flavonoids and antioxidant activity with maximum percent inhibition of DPPH radical of 83.26 ± 0.58 %. The treatment suggests a potential anti-inflammatory and neuroprotective effect, showing reduction in the injury of the animals treated with the SC–CO_2 extract.

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https://doi.org/10.1016/j.supflu.2020.104992
0896-8446/

1. Introduction

Stroke is among the top 10 causes of death worldwide, generating life expectancy reduction in men and women. The damage is caused by the momentary rupture or obstruction of blood flow (ischemia) supplied to the brain, which may cause temporary or irreversible impairment of motor, cognitive and sensory abilities. In the ischemic stroke, the post-injury phase is a neuroinflammation, in which there is an infiltration of inflammatory blood cells followed by the activation of resident cells such as microglia/macrophages releasing pro-inflammatory mediators in the final stages, contributing to secondary damage [1–4]. The search for new neuroprotective agents in anti-inflammatory therapies from medicinal plants has been studied in recent years as an alternative to traditionally used drugs [3,5–7].

Croton matourensis Aubl. (synonym *Croton lanjouwensis* Jabl.), popularly known as "maravuvuia", "orelha de burro", "dima", or "sangra-d'água", has phytogeographic domain in the Brazilian Amazon and in some areas of Central America. In the folk medicine its use is reported in the treatment of infections, fractures and colds [8–11]. Although the genus *Croton* is widely studied, the *C. matourensis* species still has few manuscripts reporting its chemical composition and biological activities [9–13].

Compagnone et al. [9] reported as major compounds fenchol acetate, methyleneugenol, isoelemicine, elemicine, spatulenol and valencene in the essential oil of *C. matourensis* leaves collected in Venezuela. This essential oil presented a potential cytotoxic effect against colon carcinoma (LoVo) and cervical cancer (HeLa). In the essential oil from *C. matourensis* leaves collected in Manaus (Brazil) were reported as major compounds β-caryophyllene, thunbergol, cembrene, *p*-cymene, and β-elemene. This essential oil presented *in vitro* cytotoxicity and *in vivo* antitumoral effect in C.B-17 severe combined immunodeficient mice with HepG2 cell xenografts [10]. Gottlieb et al. [12] determined α-pinene, elemicin, p-cymene and felandrene as the major compounds of the essential oil from the tree bark collected in Pará (Brazil), whereas in the study conducted by Schneider et al. [13] a new seco-labdane diterpene called maravuic acid was isolated from the bark of the *C. matourensis* tree collected in Pará (Brazil) ((12*E*)-3,4-seco-labda-4(18),8(20),12,14-tetraen-3-oic acid) obtained by supercritical carbon dioxide ($SC-CO_2$) and then purified by high performance liquid chromatography (HPLC).

The use of supercritical technology has been reported in obtaining bioactive compounds as a promising technique due to its advantages over conventional methods, making it possible to obtain extracts free of toxic agents, which facilitate its applications in biologically active products. The most used supercritical fluid is CO_2 as it is environmentally friendly, inert, non-toxic, non-flammable, has low critical pressure and temperature points (73.7 bar and 31.1 °C) that avoid the degradation of thermosensitive compounds. Moreover, this technology enables an easy separation of the obtained product, reducing post-processing costs [14–16].

Thus, the aim of this study was to evaluate the process variables (temperature and pressure) in the *C. matourensis* leaves extraction with $SC-CO_2$, as well as to analyze the effects on the chemical characteristics of the extracts compared to the ones obtained by conventional methods (hydrodistillation and *n*-hexane extraction). Subsequently, it was evaluated the neuroprotective and anti-inflammatory effect of the extract obtained by the best operational condition with $SC-CO_2$.

2. Material and methods

2.1. Plant material

The *Croton matourensis* Aubl. leaves were collected in a natural regeneration area of the Degraded Areas Recovery Program (PRAD) of the mining company Paragominas S.A, located in the State of Pará (Brazil), on the Miltônia 3 plateau (02° 02′21.5″S – 020° 22′40 .3″W). Part of the sample was herborized to obtain the exsiccatae that had the HCUFRA6136 voucher as identification.

2.2. Preparation and characterization of the raw material

Leaves were dried in an air recirculation oven (model SL - 102, Solab, Brazil) at 50 °C for 48 h and were comminuted using a knife mill (model MA048, Marconi, Brazil). Then, the moisture content was determined by distillation with xylene (Ecibra, PA-ACS, Brazil) [17]. The granulometric determination was performed on standard Tyler series sieves ranging from 24–48 mesh, coupled to a sieve shaker (Bertel, Brazil). The average particle diameter was determined according to the method from American Society of Agricultural Engineers [18]. The real density of the particles (ρ_r) was determined at the Analytical Center of the Chemistry Institute of the University of Campinas (UNICAMP), by helium pycnometer (model Ultrapyc 1200e, Quantachrome, USA) in triplicate. The bed porosity (ε) was calculated based on the mathematical relation between the real and apparent densities.

2.3. Extraction methods

2.3.1. Hydrodistillation

Hydrodistillation extraction was performed in a Clevenger extractor. In this assay, 100 g of sample and 500 mL of distilled water were deposited in a 1000 mL round-bottomed flask for a period of 10,800 s. The flask was connected to the extraction system with cooling coming from a thermostatic bath (model Q215u2, Quimis, Brazil), and then it was placed in contact with a heating blanket (model Q321A29, Quimis, Brazil). After the extraction period, the sample was centrifuged (model Q-222T18, Quimis, Brazil) for 5 min at 2500 rpm, and then for more 300 s with anhydrous sodium sulfate (PA ACS Anidro, Alphatec, Brazil). The yield was determined on a dry basis using Eq. 1.

$$X_{0_{d.b}}(\%) = \left(\frac{m_e}{m_{sample} \times \left(1 - \frac{U_a}{100}\right)} \right) \times 100 \quad (1)$$

Where: $X_{0_{d.b}}$ is the overall yield of the extract on a dry basis (%), m_e is the extract mass in grams (g), m_{sample} is the sample mass used in grams (g), e U_a is the moisture content of the sample (%).

2.3.2. n-Hexane extraction

The extraction using *n*-hexane (95 %, Vetec, Brazil) was carried out according to the methodology described in the Brazilian Pharmacopoeia [19]. The solute/solvent mixture (1:10, w:w) was kept under reflux at the boiling temperature of the solvent in a Soxhlet apparatus for 10,800 s. After the extraction period, the solvent was evaporated using a rotary vacuum evaporator (Laborota 4000, Heidolph, Germany). The yield of the extract obtained on a dry basis was calculated according to Eq. 1.

2.3.3. Supercritical CO_2 extraction

Extractions using supercritical carbon dioxide ($SC-CO_2$) were carried out in the system Spe-ed™SFE (model 7071, Applied Separations, USA). The volume capacity of extractor vessel used was of 5.74×10^{-5} m^3 (height of 0.3248 m and inside diameter of 0.015 m). Isotherms were determined using 7 g of sample, two temperature values (40 and 50 °C) and four pressure values (100, 200, 300 and 400 bar). Extractions were performed in two periods: static (1800 s) and dynamic (7200 s). In the second period, the outflow of CO_2 was kept constant at 1.40×10^{-4} kg/s. The density of the $SC-CO_2$ was calculated using software developed by the National Institute of

Standards and Technology, which uses the state equation developed by Span and Wagner [20]. The calculation of the overall yield on dry basis was carried out according to Eq. 1.

2.4. Characterization of the extracts

2.4.1. Volatile compounds

The chemical composition of the extracts was evaluated according to the methodology reported by Gurgel et al. [21], using gas chromatography/mass spectrometry (QP-2010 plus system, Shimadzu, Japan) under the following conditions: silica capillary column Rtx-5MS (30 m ×0.25 mm, film thickness =0.25 μm), program temperature of 60–240 °C (3 °C/min), injector temperature of 250 °C, helium as drag gas (linear velocity of 32 cm/s, measured at 100 °C), and splitless injection (1 μL of a 2:1000 *n*-hexane solution). Ionization was obtained by the electronic impact technique at 70 eV; the temperature of the ion source and other parts was 200 °C. The compounds were quantified by gas chromatography using a flame ionization detector (FID) (Shimadzu, QP 2010 system) under the same conditions as GC/MS, except that nitrogen was used as the drag gas. The retention index was calculated for all the volatile constituents using a homologous series of *n*-alkanes (C_8–C_{20}). They were identified by comparison of their mass spectra and retention indices to those reported in the literature [22,23].

2.4.2. Total phenolic contents (TPC)

The total phenolic contents was determined according to the methodology described by Singleton et al. [24] and Georgé et al. [25]. For the analysis, it was used 500 μL of the extract diluted in ethanol (95 %, Vetec, Brazil) at 7% (v:v), 2500 μL of Folin-Ciocalteau (Tedia, Brazil) at 10 % (v:v) and 2000 μL of sodium carbonate solution (99.5 %, Vetec, Brazil) at 7.5 % (w:v). After 60 min reaction at room temperature in dark conditions, absorbance was measured at 760 nm using an ultraviolet-visible spectrophotometer (model Evolution 60, Thermo Scientific, USA). Quantification was performed using gallic acid (98.0 %, Vetec, Brazil) as standard for construction of the calibration curve at concentrations of 1, 5, 10, 20, 40, 60, 80 and 100 mg/L. From the line equation, the total phenolic contents were calculated, and the result was expressed in mg of gallic acid equivalent per gram of extract on a dry basis (mg GAE/g extract). The analysis was performed in triplicate.

2.4.3. Total flavonoids (TF)

The analysis of total flavonoids was performed according to the methodology described by Dowd [26] and Meda et al. [27]. For the analysis, 1000 μL of extract diluted in ethanol (95 %, Vetec, Brazil) was added in 1000 μL of ethanolic solution with 2% (w:v) of aluminum chloride (99.0 %, Fluka, Germany). The reading was performed after 10 min reaction at 425 nm on an ultraviolet-visible spectrophotometer (model Evolution 60, Thermo Scientific, USA). Quantification was performed using quercetin (purity ≥ 95.0 %, Sigma-Aldrich, Brazil) to construct the analytical curve at concentrations of 0.5, 1, 5, 10, 15, 20, 25, 30 and 35 mg/L. From the line equation, the calculation was performed, and the result was expressed in milligrams of quercetin equivalent per gram of extract on a dry basis (mg QE/g extract).

2.4.4. Antioxidant activity (AA)

The antioxidant activity was determined by capturing the free radical 2,2-diphenyl-1-picrylhydrazi ($DPPH^{\bullet}$), according to the methodology described by Brand-Williams et al. [28] and modified by Sánchez-Moreno et al. [29]. The samples were diluted in ethanol (99 %, Vetec, Brazil) at concentrations of 8, 10, 12 and 14 mg/mL. Then, 0.1 mL solution of each concentration was added in 3.9 mL of ethanolic solution containing the $DPPH^{\bullet}$ radical (60 μM). The solutions were kept at room temperature and in dark conditions until the stabilization period t_{IC50}, necessary for the sample to reduce 50 % from the initial quantity of $DPPH^{\bullet}$ radical. After this reaction period, the samples were read at 515 nm on an ultraviolet-visible spectrophotometer (model Evolution 60S, Thermo Scientific). The construction of the analytical curve was performed at concentrations of 10–60 μM of the $DPPH^{\bullet}$ radical. Assays were performed in triplicate and calculation of the inhibitory concentration of the capture of free radicals by $DPPH^{\bullet}$ (IC_{50}) was expressed in gram of extract per gram of $DPPH^{\bullet}$ in dry basis (g extract/g DPPH). The result was also expressed by calculating the scavenging activity in terms of percent inhibition using Eq. 2.

$$\text{Inhibition } (\%) = \left(\frac{A_{DPPH} - A_S}{A_{DPPH}}\right) \times 100 \quad (2)$$

Where: A_{DPPH} was the absorbance of control and A_S was the absorbance of test sample.

2.5. Statistical analysis

The essays were performed in triplicate. Thus, the experimental results of mass extraction yield, total phenolic compounds, total flavonoids and antioxidant activity, obtained in the different extraction techniques, had their means and standard deviations calculated. Then, the data were statistically evaluated through analysis of variance with Tukey's post-hoc test with 95 % confidence level, using the STATISTIC 7.0 software (StatSoft, Inc., Oklahoma, USA).

2.6. Experimental model of focal ischemia using Croton matourensis extract obtained by supercritical CO_2

2.6.1. Animals and surgical procedures

Six adult male Wistar rats weighing approximately 0.25 kg were used. Three control animals and three treated animals got focal ischemic injury in the motor cortex. The animals were accommodated in cages containing three animals, each one being properly supplied with water and food. The experimental procedures were performed in accordance with the guidelines established by the Ethics Committee for Research on Animal Experimentation of the Federal University of Pará (CEPAE-UFPA 137−13).

The animals were anesthetized with ketamine hydrochloride (Vetanarcol®, Köning 72 mg/kg) and xylazine hydrochloride (Kensol®, Köning 9 mg/kg) through intraperitoneal injection. After confirming the loss of reflexes, the rats were placed in a stereotactic device. An incision of approximately 1.5 cm was made in the skin of the upper region of the animal's head to expose the skull. Then, the coordinates were removed: 1.2 antero-posterior, 2.5 mid-lateral, 0.5 dorsoventral, and it was injected 80 pmol of endothelin-1 (ET-1). The cranial opening was done with a surgical drill and the incision was closed with non-absorbable cotton suture [30–32].

After surgery, the animals were accommodated in cages supplied with water and food, and then they were kept in a room with controlled temperature at 24 °C during the seven days related to the survival time. After this period, the animals were again anesthetized by intraperitoneal injection and perfused with 0.9 % heparinized saline solution followed by 4 % paraformaldehyde.

2.6.2. Microtomy and immunohistochemical analysis

The brains of the control animals and the brains of the animals treated were removed and cryoprotected in different concentrations of sucrose solution, and then sections with 30 μm thickness were obtained with the aid of a cryostat (CM 1850, Leica). The injury area and the infiltration of inflammatory cells were identified by the violet cresyl technique [2,3].

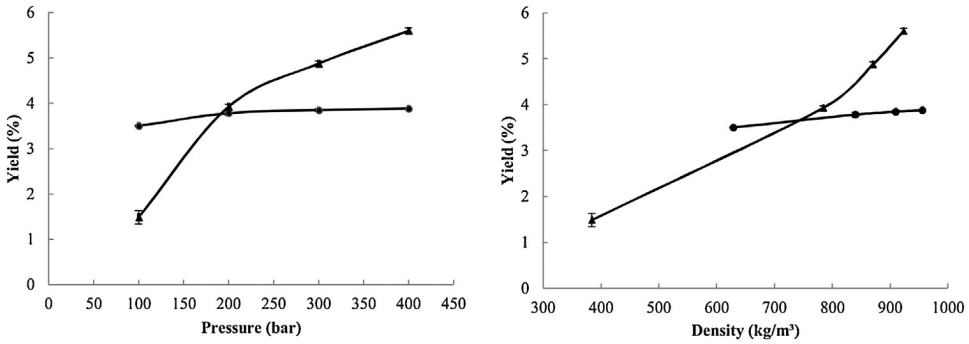

Fig. 1. Overall yield isotherms of *Croton matourensis* leaves, (●) 40 °C and (▲) 50 °C.

2.6.3. Treatment with C. matourensis *extract*

During the seven days related to the survival time, a daily dose of 100 mg/kg of the extract of *C. matourensis* leaves obtained at 50 °C and 400 bar with SC–CO_2 was administered intraperitoneally in the treated group immediately after ET–1 injection. The control group consisted of injured animals that were treated with saline solution with 5 % tween. The extract obtained with supercritical CO_2 was selected to this study because it showed a significant extraction yield, in addition to a composition with a high content of oxygenated diterpenoids (larixol and manool oxide)

3. Results and discussion

3.1. Raw material characterization

The *Croton matourensis* Aubl. leaves presented a moisture content of 10.80 ± 0.13 %, particle size of 3.17×10^{-4} m, particle real density of 1250 ± 10 kg/m^3, apparent density of 2882 kg/m^3 and bed porosity equal to 0.7694.

3.2. Extraction yields by hydrodistillation, n-hexane and supercritical CO_2

Hydrodistillation extraction showed the lowest extraction yield (1.01 ± 0.01 %), while extraction with *n*-hexane (5.73 ± 0.26 %) and extraction with SC–CO_2 at 50 °C and 400 bar (5.60 ± 0.06 %) showed higher yields and were statistically equal. Compagnone et al. [9] carried out the hydrodistillation of *Croton matourensis* leaves and *C. micans* flowers and leaves where it was obtained yields of 1.0 %, 0.13 % and 0.25 %, respectively. Same yield of essential oil from *C. matourensis* leaves was obtained in this study. In the work of Sousa et al. [33] it was done extractions with SC–CO_2, steam distillation and ethanol from *C. zehntneri* leaves, the authors obtained yields of 3.80, 3.06 and 2.68 %, respectively, lower than those obtained in this study by the method of extraction with SC–CO_2 and *n*-hexane. In general, hydrodistillation can present some disadvantages, such as low yield due to the boiling point of the mixture being lower than the boiling point of water and essential oil, occurrence of chemical changes and loss of the most volatile compounds due to the unsaturation thermal degradation or hydrolysis, in addition to a long extraction period [34–36]. Despite the extraction with organic solvent, such as *n*-hexane, often present high yields, it also has some disadvantages such as carrying compounds of greater polarity as chlorophyll, long extraction periods, contamination of the extract by toxic waste of the organic solvent resulting in a less pure product than that obtained by green technologies, such as supercritical extraction [7,15,16].

Fig. 1 shows the overall yield isotherms for *C. matourensis* leaves extracted with SC–CO_2. The yield ranged from 1.49 ± 0.15 %–5.60 ± 0.06 %. The operational condition that showed the lowest yield was 50 °C and 100 bar with a density of 384.33 kg/m^3, while the condition that obtained the highest yield was the one that operated at 50 °C and 400 bar with a density of 923.32 kg/m^3. It can be observed that near to the pressure of 200 bar, the isotherms showed an inflection point, occurring the phenomenon known as retrograde condensation in which the solute vapor pressure prevails with a strong influence of the temperature and pressure on density. Thus, in an isobaric system, below the inflection point, the increase in temperature causes a reduction in the solvation power of the fluid due to the decrease in density. However, with pressure above the inflection point, the increase in temperature can lead to an increase in the efficiency of the extraction even with the reduction of the fluid density, since the vapor pressure of the solute is increased [5,34,37].

3.3. Volatile compounds

Table 1 shows the chemical composition of *Croton matourensis* leaves extracts obtained by the methods of hydrodistillation, *n*-hexane extraction and SC–CO_2 extraction under different operational conditions. Fifty-eight constituents were identified in hydrodistillation, five in *n*-hexane extraction and twenty-eight in SC–CO_2 extraction. The compounds were distributed in the following classes: monoterpenes hydrocarbons, oxygenated monoterpenes, benzenoid, phenylpropanoids, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenoid hydrocarbons, oxygenated diterpenoid, fatty acid esters, androgens and derivatives, acyclic isoprenoid, and n-alkane.

The extract obtained by hydrodistillation presented in its composition 37.52 % of oxygenated monoterpenes, 27.88 % of monoterpenes hydrocarbons, 27.46 % of sesquiterpene hydrocarbons, 6.23 % of oxygenated sesquiterpenes, 0.45 % of phenylpropanoids, 0.19 % of oxygenated diterpenoid and 0.12 % of benzenoid. The major constituents were linalool (35.26 %), *E*-caryophyllene (9.94 %), α-pinene (8.82 %) and α-phellandrene (6.20 %). Other minority constituents have also been identified as α-humulene (3.56 %), terpinolene (3.09 %), limonene (2.94 %), γ-terpinene (2.80 %), germacrene D (2.60 %) and *p*-cymene (1.98 %). Nine compounds showed concentrations below 0.10 %. The extract showed a chemical composition slightly different from that reported by other authors who also worked with the extract obtained by hydrodistillation of *C. matourensis* leaves. In the study of De Lima et al. [10] were identified as major compounds: β-caryophyllene (12.41 ± 1.02 %), thunbergol (11.74 ± 1.11 %), cembrene (7.12 ± 0.55 %), *p*-cymene (5.05 ± 0.49 %), and β-elemene (4.94 ± 0.35 %); Compagnone et al. [9] obtained as major compounds fenchyl acetate (19.5 %), methyleugenol (14.2 %), isoelemicine (11.3 %), elemicine (7.6 %), spathulenol (6.9 %) and valencene (5.8 %); and Leão et al. [11] identified α-pinene (26.6 %) and α-phellandrene (8.5 %). The differences in chemical composition found in the present work in

Table 1
Comparative chemical composition of *Croton matourensis* leaves extracts obtained by hydrodistillation (HD), *n*-hexane extraction (HE) and different conditions of supercritical carbon dioxide extraction ($SC-CO_2$).

RI[a]	Compounds			Concentrations (% Area)[2]							
		HD	HE	$SC-CO_2$							
				40 °C				50 °C			
				100 bar	200 bar	300 bar	400 bar	100 bar	200 bar	300 bar	400 bar
	Monoterpenes hydrocarbons										
926	α-Thujene	1.08									
933	α-Pinene	8.82									
1006	α-Phellandrene	6.20									
1017	α-Terpinene	0.97									
1024	*p*-Cymene	1.98									
1029	Limonene	2.94									
1058	γ-Terpinene	2.80									
1089	Terpinolene	3.09									
	Oxygenated monoterpenes										
1099	Linalool	35.26	69.98	0.45		0.25				3.07	8.49
1178	Terpinen-4-ol	0.67									
1183	Naphthalene	0.23									
1192	α-Terpineol	1.03									
	Benzenoid										
1174	1,3-Diisopropylbenzene		6.70								
1185	*m*-Cymen-8-ol	0.12									
1235	Thymol methyl ether										0.51
	Phenylpropanoids										
1288	Safrole										1.11
1358	Eugenol	0.38									
1522	Myristicin			0.98			0.46			0.78	
1603	methoxy-Eugenol			1.97	1.37	0.77	1.14	1.67	1.47	2.08	2.01
	Sesquiterpene hydrocarbons										
1340	δ-Elemene	0.36									
1352	α-Cubebene	0.14									
1379	α-Copaene	1.12									
1388	β-Bourbonene	0.18									
1395	β-Elemene	1.34									
1423	*E*-Caryophyllene	9.94	6.53	1.80	0.36	0.63			1.18	3.55	6.58
1433	β-Copaene	0.27									
1437	γ-Elemene	0.32									
1453	Spirolepechinene	0.13									
1457	α-Humulene	3.56		0.21						0.70	1.32
1479	γ-Muurolene	1.16								0.24	
1485	Germacrene D	2.60		0.40						0.61	1.54
1492	β-Selinene	0.88									
1496	γ-Amorphene	0.11									
1500	α-Selinene	1.51									
1504	α-Muurolene	0.32									
1512	β-Bisabolene	0.77									0.50
1519	γ-Cadinene	0.70									
1526	δ-Cadinene	1.70		0.49		0.11				0.65	1.02
1545	*E*-α-Bisabolene	0.15									
	Oxygenated sesquiterpenes										
1552	Elemol	0.57		0.64	0.41	0.19		0.72	0.38	0.54	0.38
1581	Spathulenol	0.53		0.65	0.39	0.22			0.30	0.71	0.90
1587	Caryophyllene oxide	1.25		1.45	0.88	0.61	0.77		0.94	1.67	2.41
1597	Viridiflorol	0.47									
1609	Ledol	0.14									
1615	Humulene epoxide II	0.23									
1620	110-di-epi-Cubenol	0.21									
1634	Eremoligenol	0.37								0.18	
1637	γ-Eudesmol	0.32									
1646	α-Muurolol	0.25									
1651	*epi*-α-Muurolol	0.12									
1655	β-Eudesmol	0.60		1.20	0.75	0.38	0.57	1.05	0.73	1.10	1.39
1658	α-Eudesmol	1.06		2.11	1.36	0.70	1.19	1.85	1.34	1.88	2.39
1676	Germacra-4(15),510(14)-trien-1-α-ol	0.11									
1818	Cryptomeridiol							0.68			
	Diterpenoid hydrocarbons										
1926	Cembrene			0.47		11.22	13.95			0.63	
	Oxygenated diterpenoid										
2024	Manool oxide	0.19		19.60	17.87	10.25	17.31	26.72	16.79	16.44	17.49
2114	Phytol			2.72	2.76	1.57	2.05	7.47	2.29	2.64	2.43
2277	Larixol		2.93	50.57	43.06	24.88	33.81	49.96	42.91	39.51	48.49
2301	3-α-hydroxy-Manool			1.75	7.09	0.45	1.12	1.47	0.94	1.13	
	Fatty acid ester										
2095	Methyl linoleate						4.50	3.11			
	Androgens and derivatives										

Table 1 (*Continued*)

RI[a]	Compounds	HD	HE	Concentrations (% Area)[2]							
				SC-CO_2							
				40 °C				50 °C			
				100 bar	200 bar	300 bar	400 bar	100 bar	200 bar	300 bar	400 bar
2114	3-α,5-β,17-β-Androstane-317-diol			3.75		0.87	3.74	1.83		1.92	1.04
	Acyclic isoprenoid										
2327	491,317-Tetramethyl-481,216-octadecatetraenal					22.99			14.22	12.38	
	n-Alkane										
2400	Tetracosane		8.84	7.36	18.35	18.81	15.26	2.26	9.08	6.81	
	No identified	0.15	5.02	1.43	5.35	5.10	4.13	1.21	7.43	0.78	
	Other Compounds	0.60									
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[a] Retention index (DB-5 ms column); [2] Concentrações expressas em percentuais relativo área (%Area).

relation to other literatures may be related to extrinsic and intrinsic factors that may include edaphoclimatic factors, geographic location of the material collection, altitude, period of collection, part of the plant studied, preparation of raw material, extraction method used, chemotypes, genetic factors, among others [9,38–43].

The extract obtained with *n*-hexane presented linalool as the major constituent (69.98 %), in addition to other constituents such as tetracosane (8.84 %), 1,3-diisopropylbenzene (6.70 %), *E*-caryophyllene (6.53 %) and larixol (2.93 %). The chemical composition identified in this study was different from other studies that evaluated the hexanic extraction of *Croton* species. Motta et al. [44], in their study of *C. macrobothrys* leaves, determined steroid β-sitosterol (15.4 %) and the triterpenoid β-amyrin (11.0 %) as the major constituents of the extract. In the study of Dey et al. [45] the hexane semi-purified extract of *C. caudatus* leaf showed a positive result for the presence of terpenoids through the Salkowski's assay. Palmeira Jr. et al. [46] studied neutral fractions from hexane extract obtained from the leaves, stems and roots of *C. sellowii*, where they identified as major compounds of the leaves caryophyllene oxide (46.8 %) and *trans*-caryophyllene (40.8 %), in the stems caryophyllene oxide (26.5 %) and cubenol (16.7 %), and in the roots mesitylene (15.2 %), and the sesquiterpenes γ-(7.6 %), α-(6.9 %) and β-eudesmol (5.5 %).

The main constituents identified in the extraction with SC−CO_2 were larixol (24.88–50.57%), manool oxide (10.25–26.72%), 491,317-tetramethyl-481,216-octadecatetraenal (12.38–22.99 %), tetracosane (2.26–18.81 %) and cembrene (0.47–13.95%). Other minority compounds have also been identified, such as linalool under conditions of 40 °C/100 bar (0.45 %), 40 °C/300 bar (0.25 %), 50 °C/300 bar (3.07 %) and 50 °C/400 bar (8.49 %); methyl linoleate identified under conditions of 40 °C/400 bar (4.50 %) and 50 °C/100 bar (3.11 %); phytol was identified in all extraction conditions with concentration ranging from 1.57 to 7.47 %; *E*-caryophyllene (0.36–6.58%); caryophyllene oxide (0.61–2.41%); α-eudesmol (0.70–2.39%); 3-α,5-β,17-β-Androstane-317-diol (0.87–3.75%); and methoxy-eugenol (0.77–2.08 %).

The extracts obtained with SC−CO_2 showed a predominant composition in oxygenated diterpenoids (37.15–85.62%). Schneider et al. [13] also reported in their study the identification of a diterpenoid called maravuic acid ((12*E*)-3,4-seco-labda-4(18),8(20),1214-tetraen-3-oic acid) from extraction with SC−CO_2 and subsequent isolation by HPLC of *C. matourensis* bark. Larixol and manool oxide (second most concentrated compound identified) belong to the class of labdane diterpenes that have industrial, medicinal, pharmacological, and nutraceutical applications [47–51].

It can be seen that the conventional extraction methods through hydrodistillation and extraction with solvent *n*-hexane were more selective for obtaining linalool, which is an oxygenated monoterpene, while SC−CO_2 was more selective for oxygenated diterpenoids such as manool oxide and larixol. This can be associated with the used solvent and the process parameters such as temperature and pressure. Fig. 2 shows the main compounds identified in the different extraction methods.

3.4. Total phenolic contents (TPC)

The TPC results of the extracts from *Croton matourensis* leaves obtained by different methods are presented in Table 2. The extracts showed high TPC content, ranging from 51.81 ± 2.03–79.53 ± 1.19 mg GAE/g extract. The extract obtained with *n*-hexane showed the lowest content (51.81 ± 03.02 mg GAE/g extract), while the extract obtained from the hydrodistillation showed the highest content (79.53 ± 01.19 mg GAE/g extract). Thus, it can be inferred that for the extraction of TPC the solvent polarity index influenced the achievement of a higher content. This result can be explained by the fact that phenolic compounds have, in general, a polar character. However, due to the wide polarity range that these compounds present, other solvents such as *n*-hexane and SC−CO_2 can also be used to solubilize other groups [52,53].

In SC−CO_2 extraction, the extract obtained at 50 °C, 100 bar and density of 384.33 kg/m^3 was the one with the lowest TPC value (58.68 ± 1.99 mg GAE/g extract), while in the conditions of 40 °C, 400 bar and density of 956.07 kg/m^3 and 50 °C, 400 bar and 923.32 kg/m^3 showed higher contents (69.29 ± 0.76 and 67.66 ± 1.65 mg GAE/g extract, respectively) with no statistically significant difference between these two conditions ($p > 0.05$). The results obtained in this work were slightly similar to those obtained by Dutta et al. [54] who studied the hexane extract of *C. bonplandianus* stem and obtained a TPC value of 67.37 ± 0.46 mg GAE/g extract. Dutta et al. [55] who studied the hexane extract of *C. bonplandianus* leaves obtained a value equal to 75.29 ± 3.19 mg GAE/g extract, which are values much higher than the hydroalcoholic extracts (80 % methanol) from leaves of nine Argentinian species of *Croton* (*C. andinus*, *C. argentinus*, *C. catamarcensis*, *C. cordobensis*, *C. curiosus*, *C. lachnostachyus*, *C. lanatus*, *C. saltensis*, and *C. serratifolius*) that ranged from 1.71 to 4.85 mg EAG/g extract [56].

3.5. Total flavonoids (TF)

The TF concentration of *Croton matourensis* leaves extracts obtained by different extraction methods is shown in Table 2. The concentration ranged from 2.50 ± 0.20–6.89 ± 0.45 mg QE/g extract, where the highest values were obtained by the methods of hydrodistillation (6.89 ± 0.45 mg QE/g extract) and extraction with SC−CO_2 (6.53 ± 0.39 mg QE/g extract), which showed no statistically significant difference between them ($p > 0.05$). The results obtained in this study were superior to the extract of *C. bonplandianum* leaves (4.36 ± 0.48 mg/g) [57], to the extract of *C. bonplandianus* stem (3.86 ± 0.12 mg/g) [54], and to the

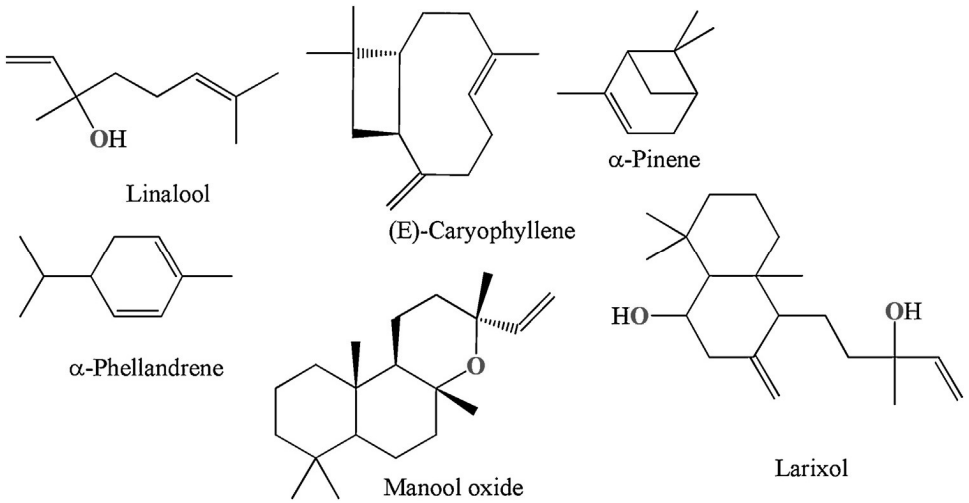

Fig. 2. Chemical structures of the main compounds identified in *Croton matourensis* leaves extracts obtained by hydrodistillation, *n*-hexane extraction and supercritical carbon dioxide extraction.

Table 2
Total phenolic contents (TPC), total flavonoids (TF) and antioxidant activity (AA) of *Croton matourensis* leaves extracts obtained by hydrodistillation (HD), *n*-hexane extraction (HE) and different conditions of supercritical carbon dioxide extraction (SC−CO_2).

Extraction method	Solvent polarity index[a] / CO_2 density[2] (kg/m^3)	TPC (mg GAE/g extract)	TF (mg QE/g extract)	AA		
				Concentration (mg/mL)	**Inhibition %**	**IC_{50} (g extract/g DPPH)**
HD	9	79.53 ± 1.19^{e}	6.89 ± 0.45^{f}	8	25.76 ± 1.28	1531.34 ± 146.02^{c}
				10	30.41 ± 0.99	
				12	34.76 ± 0.13	
				14	38.90 ± 0.95	
HE	0	51.81 ± 2.03^{a}	5.11 ± 0.57^{c}	8	15.05 ± 0.06	2680.11 ± 109.14^{d}
				10	16.78 ± 1.67	
				12	21.92 ± 0.93	
				14	22.42 ± 0.19	
SC-CO_2						
40 °C/100 bar	628.61	66.51 ± 0.76^{cd}	6.53 ± 0.39^{ef}	8	52.20 ± 0.68	614.73 ± 25.75^{a}
				10	63.04 ± 1.22	
				12	72.20 ± 1.20	
				14	83.26 ± 0.58	
40 °C/200 bar	839.10	66.27 ± 0.80^{cd}	2.50 ± 0.20^{a}	8	42.82 ± 0.09	763.91 ± 60.39^{a,b}
				10	54.24 ± 2.63	
				12	62.66 ± 3.71	
				14	72.97 ± 2.71	
40 °C/300 bar	909.89	65.66 ± 1.75^{cd}	5.70 ± 0.11^{ce}	8	39.19 ± 2.06	836.06 ± 12.39^{a,b}
				10	49.72 ± 2.35	
				12	57.37 ± 2.81	
				14	63.83 ± 0.39	
40 °C/400 bar	956.07	69.29 ± 0.76^{d}	3.18 ± 0.13^{ab}	8	35.04 ± 4.11	1002.46 ± 49.23^{a,b}
				10	42.73 ± 4.60	
				12	49.18 ± 5.57	
				14	53.74 ± 0.19	
50 °C/100 bar	384.33	58.68 ± 1.99^{b}	2.94 ± 0.29^{ab}	8	49.45 ± 6.83	629.83 ± 143.30^{a}
				10	62.20 ± 13.90	
				12	70.66 ± 10.65	
				14	80.13 ± 5.99	
50 °C/200 bar	784.29	65.01 ± 0.76^{c}	5.22 ± 0.22^{c}	8	39.70 ± 0.14	825.49 ± 28.57^{a,b}
				10	50.29 ± 1.11	
				12	58.23 ± 0.25	
				14	68.86 ± 0.35	
50 °C/300 bar	870.43	60.22 ± 1.18^{b}	3.68 ± 0.06^{bd}	8	39.28 ± 1.75	840.80 ± 4.29^{b}
				10	48.11 ± 2.23	
				12	57.03 ± 3.04	
				14	66.66 ± 3.46	
50 °C/400 bar	923.32	67.66 ± 1.65^{cd}	4.09 ± 0.22^{d}	8	31.77 ± 0.25	1065.55 ± 4.32^{b}
				10	38.16 ± 0.91	
				12	47.12 ± 1.68	
				14	52.99 ± 2.71	

Mean ± standard deviation, different letters indicate significant difference between values ($p < 0.05$) by Tukey test.
[a] According to [53]; [2] As described in topic *2.3.3*.

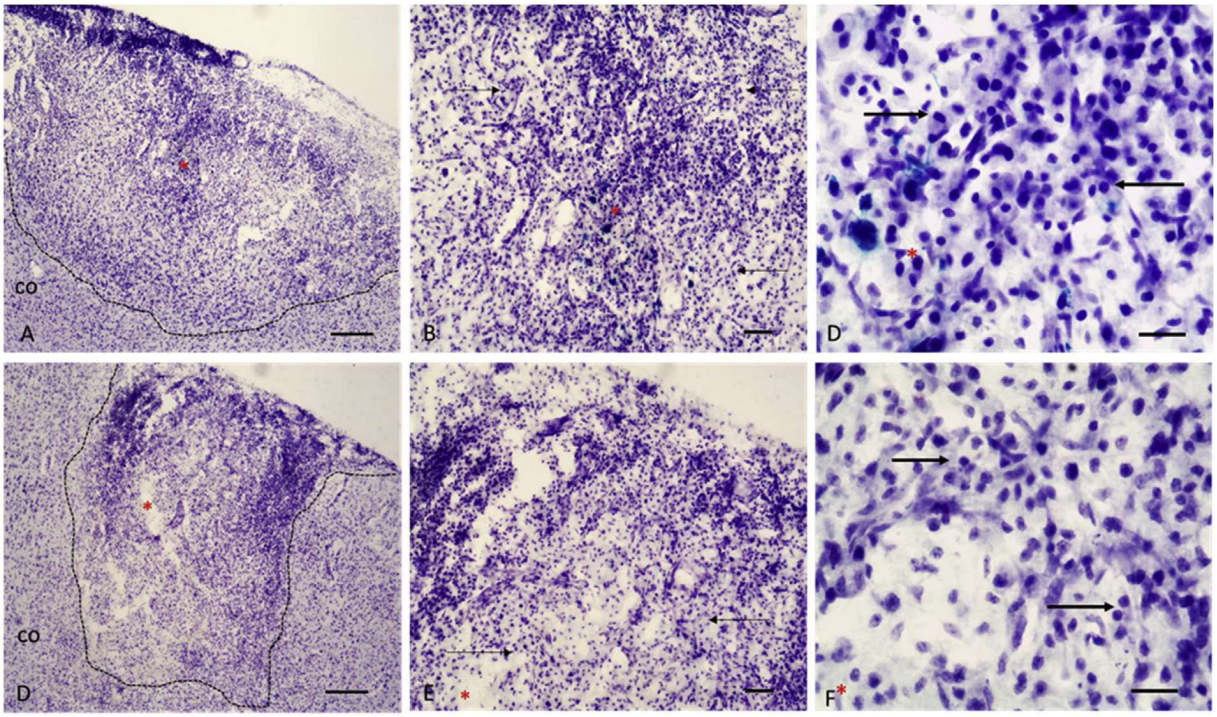

Fig. 3. Histopathological analysis of the ischemic focal lesion area. Coloring: Cresyl violet; **Upper line:** male ischemic control rats after 7 days of survival; **Bottom line:** male ischemic rats treated after 7 days of survival. **Injury area:** dotted line; **Asterisk**: ET-1 injection site; **Arrows:** point to polymorphonuclear cells - neutrophils and / or activated macrophages. **Scale bar:** 40 μm (A, D); 100 μm (B, E); 400 μm (C, F); cortex (CO).

extract obtained with petroleum ether from *C. roxburghii* leaves (4.25 ± 0.35 mg QE/g dry material) [58].

Flavonoids are an important group that is inserted in the class of polyphenols, having more than 600 compounds. TF analysis provides information about the content of its subgroups, such as chalcones, flavones, flavonoids, flavanones, isoflavones, flavan-3-ols (catechins), and anthocyanins, besides providing an estimate of the material's antioxidant activity [7,54].

3.6. Antioxidant activity (AA)

Table 2 shows the free radical scavenging effect of *Croton matourensis* leaves extracts obtained by different techniques. The IC_{50} value represents the amount of sample needed to reduce the initial concentration of the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 50 %. Thus, the lower the sample required, the greater its antioxidant potential [7]. In this sense, the hexane extract showed a higher IC_{50} value (2680.11 ± 109.14 g extract/g DPPH) and lower inhibition percentage (22.42 ± 0.19 %) at a concentration of 14 mg/mL, followed by the extract obtained by hydrodistillation, which presented an IC_{50} equal to 1531.34 ± 146.02 g extract/g DPPH and a inhibition percentage of 38.90 ± 0.95 % at a concentration of 14 mg/mL.

The extracts that showed lower IC_{50} values were those obtained with SC−CO_2 under conditions of 40 °C/100 bar (614.73 ± 25.75 g extract/g DPPH) and 50 °C/100 bar (629.83 ± 143.30 g extract/g DPPH), being statistically equal ($p > 0.05$), and with percent inhibition in the concentration of 14 mg/mL equal to 83.26 ± 0.58 % and 80.13 ± 5.99 %, respectively. Thus, it can be inferred that the extracts which showed greater antioxidant activities were those obtained at lower temperature and pressure, indicating the sensitivity to more abrupt operational conditions. This similar behavior was reported by Almeida et al. [59] and Cunha et al. [60]. The antioxidant activity may be related to the presence of phenolic compounds due to the hydroxyl groups and the double bond between the carbon atoms causing radicals elimination, as well as the synergistic effect of other compounds present in the extracts [7,16,61].

Anti-inflammatory and neuroprotective effect of C. matourensis leaves extract obtained by SC−CO_2 on experimental cerebral ischemia in rats

The cresyl violet staining method revealed the histopathology of ischemic brain tissue after endothelin-1 (ET-1) microinjections internally into the rats' cortex. Fig. 3. A, B and C show the density of cells infiltrated in the lesion area of the control animals that received treatment with 5 % tween solution after the seven-day period, whereas Fig. 3. D, E and F show the animals that received treatment with *Croton matourensis* leaves extract obtained with SC−CO_2 in the same period.

The lesion was characterized by areas with pallor, tissue loss, loss of cells, remarkable infarct area (Fig. 3. A) and infiltrates of inflammatory polymorphonuclear cells at high densities in and around the nucleus of the lesion (Fig. 3. B and C). After the treatment of ischemic animals for a period of seven days with *C. matourensis* leaves extract there was a reduction in the remarkable infarct area (Fig. 3. D). Moreover, there was also a decrease in tissue injury area, the lower cell density, the smaller amount of infiltrates of polymorphonuclear cells (Fig. 3. E and F) and consequent reduction in the area of inflammatory response, compared to control animals that received treatment with tween at 5 % (Fig. 3. A, B and C).

The focal cortical ischemia induced by ET-1 in rats has been established from neurochemical and morphological evidence due to its prolonged and marked vasoconstrictor action that induce precise and reproducible focal ischemic lesions, thus, focal microinjection of ET-1 has been used in the development of a model for focal cortical ischemia to be used for testing anti-stroke drugs [30,31]. Previous studies [3,5–7] report the anti-inflammatory and neuroprotective effects in experimental models of excitotoxicity and stroke from extracts from Amazonian plants obtained with SC−CO_2. The action mechanism of these extracts, as well as those from the *C. matourensis* leaves on the anti-inflammatory and neu-

roprotective response have not yet been elucidated. However, it may be associated with the modulatory effect of extracts on the release of pro-inflammatory substances, neutrophil recruitment and modulation of microglia, macroglia and macrophage activation, leading to reduction in tissue damage due to releasing oxygen radicals, proteases, and cytokines by inflammatory cells [3,7]. Furthermore, biological activity of *C. matourensis* leaves extract obtained by SC−CO_2 can be related to the presence of oxygenated diterpenoids, especially to the major compounds (larixol and manool oxide), which have biological effects reported as anti-malarial, antileishmanial, laxative, antimicrobial, anticonvulsive, cytotoxic, anti-inflammatory, neuroprotective, antiparasitic and analgesic activity [47–51].

In addition, studies involving *Croton* species demonstrate the biological activity effects present in this genus, such as an antitumor effect, effect against colon carcinoma and cervical cancer in essential oils of *C. matourensis* leaves and in flowers and leaves of *C. micans* [9,10]; acetylcholinesterase inhibitory activity by *C. gratissimus* ethyl acetate and butanol fractions [62]; neurodegenerative agents for Alzheimer's disease and other neurological disorders due to nerve growth factor-potentiating activities from the twigs of *C. yanhuii* [63]; antiulcer activity from *C. campestris* root extract [64] and cardioprotective effect of proanthocyanidin-rich fraction obtained from *C. celtidifolius* barks [65].

The authors suggest that in future researches the preservation and quantification of neurons, as well as the identification and quantification of infiltrated cells (microglia/macrophages), are performed in the center and periphery of the lesion after immunostaining.

4. Conclusions

The extraction with supercritical CO_2 (SC−CO_2) is presented as an efficient method for obtaining the extract from *Croton matourensis* leaves. The method showed good selectivity in comparison to conventional methods for obtaining oxygenated diterpenoids (37.15–70.78%), especially larixol (24.88–50.57%), with higher mass yield in the experimental condition of 50 °C/400 bar. The extracts presented high values of total phenolic contents, total flavonoids and antioxidant activity. In the histopathological analysis of ischemic injury in the rats' motor cortex, the extract obtained with SC−CO_2 showed an influence on tissue reconstruction and on the reduction of cell density, suggesting potential neuroprotective effect and anti-inflammatory activity. In this way, *C. matourensis* leaves extract proved to be a source of bioactive compounds with antioxidant, anti-inflammatory and neuroprotective activity, presenting potential to be applied as a supplement in the pharmaceutical and food industries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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CAPÍTULO IV

Potential Antiviral Activity of Larixol against Structures of SARS-CoV-2 using Computational Study

(Potencial Atividade Antiviral do Larixol contra Estruturas do SARS-CoV-2 usando Estudo Computacional)

Artigo submetido à revista *Journal of Biomolecular Structure and Dynamics* (Online ISSN - 1538-0254 and Print ISSN - 0739-1102) em 11/04/2022.

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Potential Antiviral Activity of Larixol against Structures of SARS-CoV-2 using Computational Study

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Abstract

The search for drugs to treat SARS-CoV-2 has been a priority in the world to reduce the severity and deaths caused by the disease. In this study, the combination of docking and molecular dynamics was used in order to obtain reliable results on the pharmacological potential of larixol against SARS-CoV-2 structures. The analysis of the interaction between larixol and the receptors showed that it can covalently bind to its active sites, suggesting that the covalent bond is preceded by a variation in the conformational structure of the receptors. From the analysis of interactions and affinity energy, it can be observed that the mode of interaction predicted by the compounds poses plays a crucial role, presenting conformation and formation of stable complexes, especially with the Spike-protease, which has a catalytic agent with hydrophobic characteristics, suggesting that larixol may be a potential candidate for inhibitor of this protease. In addition, the results of MD simulation and free energy calculations showed greater accuracy of molecular interaction energies and atomic forces in the continuous parameter sets commonly used as Poisson-Boltzmann calculations. Results support the hypothesis that larixol has the potential to act as an antiviral agent alone or in combination with other therapies, bringing a potential line of defense against the coronavirus.

Keywords: COVID-19; Larixol; *Croton matourensis*; *In silico* study; Antiviral effect.

1. Introduction

In March 2020, the World Health Organization (WHO) declared coronavirus disease 2019 (COVID-19) as a worldwide pandemic, a highly infectious disease, responsible for the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Formisano et al., 2021). The clinical picture can range from an asymptomatic upper respiratory infection to severe pneumonia that can result in multi-organ failure (Hu et al., 2021). Infecting occurs due to SARS-CoV-2 virus uptake by the type 2 transmembrane serine protease (TMPRSS2) of the host cell, that cleaves angiotensin-converting enzyme 2 (ACE2) cellular receptor, which binds Spike-protein expressed by SARS-CoV-2, invading the body through infection of the pulmonary capillary endothelial cells, inducing an inflammatory reaction (endotheliitis) that triggers thrombotic events in various blood vessels, causing failure of organs such as heart, kidneys, liver, and central nervous system, which can lead to death (Das, 2021; J. Xu, Gao, Liang, & Chen, 2021).

Antiviral medicines, immunomodulators, immunosuppressants, among others (remdesivir (Beigel et al., 2020), lopinavir/ritonavir (Cvetkovic & Goa, 2003; Oldfield & Plosker, 2006), camostat mesilate/nafamostat (Erku et al., 2021; Yin, Noda, & Yotsuyanagi, 2006), favipiravir (Baranovich et al., 2013), ribavirin (Baranovich et al., 2013), chloroquine (Touret & de Lamballerie, 2020), hydroxychloroquine (Graef et al., 2020), azithromycin (Cavalcanti et al., 2020), tocilizumab (Stone et al., 2020), ivermectin (Schmith, Zhou, & Lohmer, 2020), ibuprofen (Sodhi & Etminan, 2020), nitric oxide (Kobayashi & Murata, 2020; Mir & Maurya, 2021)) have been used in alternative treatment due to empirical basis with pharmacological justification, however, there is still no specific drug for the disease with an effective success rate and no risk of toxicity. Although new vaccines are available against COVID-19, truly safe and effective drugs must be also developed to block the entry and replication of the virus and improve the immune and inflammatory state that leads to the progression and worsening of the disease (Alrasheid, Babiker, & Awad, 2021; Erku et al., 2021; Meeran et al., 2021).

Natural products are the basis of folk medicine being sources of compounds used in the development of new therapeutic agents, the list of drugs approved by the Food and Drug Administration (FDA) between 1981 and 2010 revealed that 34% of these drugs are based on molecules of natural products or their direct derivatives (Harvey, Edrada-Ebel, & Quinn, 2015; Sepay, Sekar, Halder, Alarifi, & Afzal, 2021). The genus *Croton* presents reports of popular use associated with the treatment of inflammation, hypertension, headache, fever,

gastric diseases, dysentery, and others (Fernanda W.F. Bezerra et al., 2020). The species *Croton matourensis* has demonstrated in recent studies (Fernanda Wariss Figueiredo Bezerra et al., 2020; Compagnone et al., 2010; De Lima et al., 2018) neuroprotective, anti-inflammatory, antioxidant, antitumor and anticancer activities. In the study by Bezerra et al. (Fernanda Wariss Figueiredo Bezerra et al., 2020), the extract of the leaves of *C. matourensis* obtained with supercritical CO_2 showed high antioxidant activity (*in vitro*) and potential anti-inflammatory and neuroprotective activities (*in vivo*) that may be associated with the major compound larixol (LAX), which corresponds to 48% of the extract composition.

The *in silico* study of the therapeutic target compound present in a plant extract is a tool of economic and rapid investigation through techniques such as molecular docking (DOC) and molecular dynamics (MD) that allow estimating the affinity between molecules such as protein-ligand and protein-protein and the side and cytotoxic effects of the compounds studied. Thus, the study of computational simulation and ethnopharmacological knowledge combined with experimental trials can help in the discovery and design of new drugs (Alrasheid et al., 2021; Arouche et al., 2020; Sepay et al., 2021).

In the development of this research, the intermolecular interactions of LAX, treated as a ligand, with SARS-CoV-2 proteins, treated as receptors, were studied by DOC and MD simulations. In the DOC, data were obtained regarding the electrostatic potential, molecule coupling system, binding energy, bonding types and hydrophobicity, in addition to the validation of the adjustment of the binding positions in the active site of the receptors. Subsequently, deepening the studies, the MD was used, which allowed more complex calculations such as root-mean-square deviations (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), hydrogen bonds (HBs) and solvent-accessible surface area (SASA), with analysis of molecular interactions and possible structural mutations to analyze whether there are significant changes or deformations in the sequence of receptors and whether these changes may be related to the use of LAX as a defense agent against the coronavirus.

2. Materials and methods

2.1.Background about the receptors and ligand

The main protease (M-pro) or 3CLpro (Fig 1a) catalyzes most maturing cleavage events after protein activation. Thus, M-pro is an essential enzyme for viral replication and is

one of the most well-characterized drug targets among coronaviruses (Jo, Kim, Shin, & Kim, 2020). The crystalline structure of this SARS-CoV-2 protein is highly similar to that of other coronaviruses (Needle, Lountos, & Waugh, 2015). The active site of the protein is located in a crack between a histidine catalytic dyad/cysteine. In M-pro, Cys 145 acts as a nucleophile during the first step of the His 41-assisted hydrolysis reaction, which acts as a basic catalyst (Nukoolkarn, Lee, Malaisree, Aruksakulwong, & Hannongbua, 2008).

The Spike-protein (S-pro) or peak glycoprotein (S) (Fig 1b) is a homotrimer that protrudes from the outside of the virus surface and serves as the main driving force for recognition and entry into the host cell and is among the four structural proteins encoded by viral RNA (Lan et al., 2020; X. Xu et al., 2020). The primary entry of the virus is premeditated by ACE2 receptors forming complexes with S-pro, which then regulates invasive processes important for viral replication during cellular stress (Plana, Nadra, Estrin, Luque, & Capece, 2019).

The RNA polymerase structure dependent on RNA of SARS-CoV-2 (R-pol) (Fig 1c), that has an active site highly conserved and contains aspartate residues that protrude at the junction of its beta domains b15 and b16 (Hage-Melim et al., 2020). With a vital role in viral genome replication, R-pol active site residues are mostly accessible on the surface and can bind to free nucleotides such as ATP, GTP, CTP and UTP (Habibzadeh & Stoneman, 2020). Similar to viral proteases, R-pol is considered a molecular target for the development of new drugs against SARS-CoV-2 (Rabaan et al., 2020).

The endoribonuclease activity of SARS-CoV Nsp15 (sNsp15), Fig 1d, occurs by mutations at the active site of Nsp15 which exclude scanning the activity of endoribonuclease *in vitro*, reducing viral infectivity (Rodriguez-Torres et al., 2014). However, some mutations outside the active site are reported to have a greater effect on the viability of the virus, suggesting that Nsp15 plays a role in coronavirus infection in addition to its function as endoribonuclease (Beaulieu, 2010). Also, SARS-CoV Nsp15 has been identified in a screening of viral proteins that can suppress apoptosis, demonstrating that can affect host cell processes (Beaulieu, 2010; Kandula, Khanlou, & Farthing, 2005).

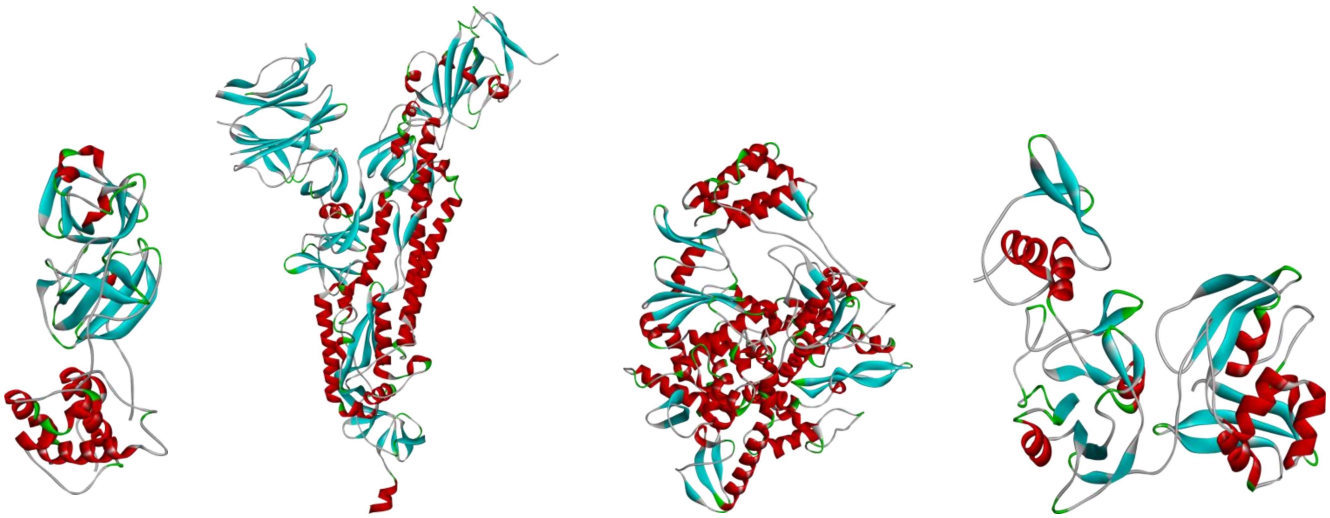

Fig 1. Molecular structure of: a) M-pro, b) R-pol, c) S-pro and d) Nsp15.

Larixol (LAX), shown in Fig 2, was first identified in an extract of *Croton matourensis* in the work of Schneider et al. (Schneider et al., 1995) and later by Bezerra et al. (Fernanda Wariss Figueiredo Bezerra et al., 2020), in both works the supercritical CO_2 extraction technique was used, until then the compound had not yet been identified in the species through the hydrodistillation technique, the most commonly used method (Compagnone et al., 2010; De Lima et al., 2018; Gottlieb et al., 1981; Leão et al., 1998). The compound, a seco-labdane diterpene, presents interesting biological activities, but with few natural sources available (Herlem & Khuong-Huu, 1997; Thuerig et al., 2018). The study by Bezerra et al. (Fernanda Wariss Figueiredo Bezerra et al., 2020) performed the extraction on the leaves of *C. matourensis* and found as the best operational condition that operated at 50 °C and 400 bar and obtained higher extract yield, consisting of 48.49% of the compound, besides, the authors reported that the extract has high antioxidant activity and potential anti-inflammatory and neuroprotective activities. Thus, to better understand the role of LAX in viral infection of Sars-CoV-2, we performed computer simulations to verify its action on this mechanism.

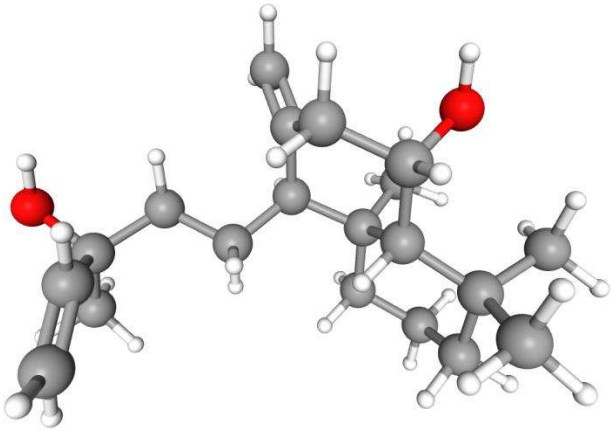

Fig 2. LAX 3D structure used in the *in silico* study.

2.2.Preparation of receptors

The crystalline structures of Main protease (M-pro), RNA polymerase (R-pol), Spike protease (S-pro) and Nsp15 Endoribonuclease (sNsp15) (PDB ID: 6M2N, 7BW4, 6VXX, and 6VWW) were retrieved from the Protein Data Bank (PDB) (https://www.rcsb.org) repository (Berman, Henrick, & Nakamura, 2003). The structures were prepared using the software GaussView 6 (Dennington, Keith, & Millam, 2009) and subsequently minimized using Assisted Model Building with Energy Refinement (AMBER) ff14SB force field (Maier et al., 2015). After minimizing the structures were submitted to a virtual screening process by molecular docking (DOC) and molecular dynamics (MD).

2.3.Preparation of the ligand

LAX molecule was subjected to calculations in the Gaussian 09W software (Frisch et al., 2009) using the Density Functional Theory (DFT) (Neto, Marques, Amador, Ferreira, & Neto, 2019) with restrained electrostatic potential (RESP) method (Bayly, Cieplak, Cornell, & Kollman, 1993). Then, the structure was submitted to calculations for geometric optimization to decrease the energy of the ligand to produce a more stable conformation (Machado, Camiletti, Neto, Jorge, & Jorge, 2009). The ligand (LAX) was optimized using GaussView 6 software (Dennington et al., 2009), following the Protocol Single Optimized Gaussian Basis Sets (SOGBS) (Bofill, 1995). For this procedure, B3LYP (Andersson & Uvdal, 2005) and 6-311G* (Kruse, Goerigk, & Grimme, 2012) functionals were used.

2.4.Molecular docking (DOC)

After the processes of minimization of the receptors and optimization of the ligand, the three-dimensional structures of proteins have been added to the DOC platform as PDB formats. The molecular coupling was performed using the software package AutoDock Vina 4.2.6 (ADV) and AutoDock Tools (ADT) (http://autodock.scripps.edu/) (Trott & Olson, 2012), the procedure was performed using a flexible ligand in a flexible active site protocol, where the ligand was allowed to be flexible, with twist inside a grid box that encompasses the binder connection cavity of each receptor predicted using algorithms. After each run, the ADV provided a set of snap poses for the ligand with calculated binding affinities in which the fit pose with the best affinity was chosen to represent the set and submitted to post-snap analysis. Visualization and analysis of the conformation of the ligand-receptor complex were performed using BIOVIA Discovery Studio (version 4.1) (BIOVIA, 2020).

All torsion angles in the ligand were relaxed, allowing the flexible anchorage to be performed, while the receivers remained rigid throughout the process. With polar hydrogens added to the protein, the model of partial charges of the united Kollman atoms (Bhattacharjee, Devi, & Mishra, 2015) was assigned to the ligand and receptors. The Gridbox (Cornell et al., 1995), that defines the potential of Lennard-Jones with electrostatic factors and provides values for all receptor atoms, was used for the simulations and had the following dimensions: 62 Å × 58 Å × 48 Å for M-pro, 42 Å × 56 Å × 45 Å for R-pol, 40 Å × 30 Å × 20 Å for sNsp15 and 60 Å × 60 Å × 60 Å for S-pro, for directions x, y and z respectively at the binding site of each of the macromolecules.

The empirical function of free energy and the Lamarckian genetic algorithm (LGA) were used for anchorage simulations according to the recommended parameters (Fuhrmann, Rurainski, & Neumann., 2010; Mlynsky et al., 2015; Trott & Olson, 2012). Based on the results obtained in the DOC, 40 main conformations were selected through the software for each ligand-receptor system. For each molecular system, we chose a conformation with lower affinity energy (AE) and with chemically reasonable ligations, that is, with suitable lengths for the chemical bonds. In this step, we use the parameters of the active sites specified in the input file for the tuning method, which is acceptable and able to retrieve the structure and interactions of a known complex (Harrach & Drossel, 2014). Thus, this computational process involved the original position of the ligand in the first simulation of the complexes in an induced manner, to evaluate whether the positions and adjustment parameters obtained by the

various programs were capable of recovering the structure and interactions of a known complex and its active site.

2.5.Molecular dynamics

After the DOC process, were used the poses as initial conformations in the Molecular Dynamics (MD) process simulations, helping to understand the prediction of the position in which could be the active site of macromolecules and the essential interactions to predict the stability of the ligand-receptor complex. For this, four octahedral boxes were created with a spacing of 10 Å between the solute and each octahedral face, with approximately 15,163 water molecules using the TIP3P model for the solvation of the systems (Harrach & Drossel, 2014; Kolafa & Perram, 1992). Then, Na^+ ions were added to neutralize the amino acid terminals to ensure the neutral liquid charge of the systems.

Periodic boundary conditions were performed using the Particle Mesh Ewald (PME) method (Golub & Ye, 1999; Kolafa & Perram, 1992) to ensure that the solute (ligand-receptor) was immersed in the solvent during not only minimization but also in MD. The systems were kept fixed with positional constraints of 50 kcal/mol.$Å^2$, with a cut of 10 Å to truncate the long-range interactions of van der Waals (VDWAALS), with constant volume by NVT (canonical ensemble - moles (N), volume (V) and temperature (T) conserved) (Noguti & Go, 1983), thus ensuring periodic boundary conditions optimized in 20,000 cycles, where 10,000 cycles were through the steepest descent algorithm (Kim & Fessler, 2016) and 10,000 cycles by the conjugate gradient algorithm (Machireddy, Kalra, Jonnalagadda, Ramanujachary, & Wu, 2017). The system went through the heating step for 10 ns with an integration time of 2 fs using the SHAKE algorithm (Dupradeau et al., 2010), restricting and fixing the bonding lengths of hydrogen atoms. To understand the dynamic behavior of all the highest scoring complexes, MD simulation was employed using GROMACS 2021.1 software (Abraham et al., 2015).

The main results with the best pharmacokinetic properties were selected and further analyzed by molecular dynamics simulations using GROMACS 2021.1 (Abraham et al., 2015). Energy minimization of the systems was accomplished using the 5,000-step teepest descent minimization (maximum number of minimization steps to be performed) to remove the weekly van der Waals contacts. Energy minimization was stopped when the maximum force was less than 1.0 kJ/mol. The system was further balanced to 50 ps at constant volume

and a temperature of 300 K. Molecular dynamics simulations were run for 20,000 ps for each protein-ligand complex, where coordinates were saved at every 2 ps interval. The van der Waals interactions were defined at 1.6 nm. The Linear Constraint Solver (LINCS) algorithm (Hess, Bekker, Berendsen, & Fraaije, 1997) was applied to constrain covalent bonds including H-atom heavy bonds during molecular dynamics (MD) simulations. The gmx rms, rmsf, gyrate, gmx hbond, gmx SASA were used to calculate the root-mean-square deviations (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), hydrogen bonds (HBs) and solvent-accessible surface area (SASA), respectively. Finally, the trajectories were saved for further analysis using Xmgrace.

2.6.Root-mean-square deviation and root-mean-square fluctuation (RMSF)

To perform the RMSD and RMSF calculations was used the CPPTRAJ tool, included in the GROMACS package (Abraham et al., 2015). The necessary informations were extracted through the program module for the creation of graphs of RMSD values as a function of time (100 ns). The RMSD values indicate the deviations of the structures generated during the MD simulation in relation to the initial structure, that is, the stability and equilibrium of the system over time, corresponding to the conformations of a given set of points already obtained, providing a measure of the difference in the starting position of these same sets, therefore, the higher the RMSD value, the greater the difference between the structures (Damm & Carlson, 2006). The RMSF calculation refers to the displacement of a specific atom or group of atoms in relation to the reference structure, contributing to the understanding of the region of the drug-protein complex that is being fluctuated during the simulation, in this way, the result of calculating the flexibility of each residue obtains an insight into the extent to which the binding of the ligand affects the flexibility of the protein (Islam et al., 2021; Martínez, 2015).

2.7.Hydrogen bonds, Radius of gyration and Solvent-accessible surface area

Hydrogen bonds (HBs), radius of gyration (Rg) and solvent-accessible surface area (SASA) were calculated from the MD trajectories for the four systems to measure the interaction between protein-ligand complexes and solvents using GROMACS software (Abraham et al., 2015). The quantification of the number of HBs was simulated in order to explore the molecular basis of the binding between the complex and the selected molecules,

providing information on the percentage of time occupation, the angle and the maximum number of HBs formed during the simulation time, thus, the strong network of HBs, hydrophobic interactions and van der Waals interactions inhibit receptors which act on virus entry and replication (Kumar et al., 2021; Maroli, Bhasuran, Natarajan, & Kolandaivel, 2020). The Rg measures the strength of the macromolecular system, through the root mean square distance between the axis of rotation and the center of mass obtained in the MD simulation, the parameter indicates the changes over time in the protein structure, evaluating the compaction and stability of the system (Nayeem, Sohail, Ridhima, & Reddy, 2021; Vishvakarma, Singh, Jain, Kumari, & Singh, 2021). The calculated surface area is the canonical SASA surface area and includes the hydrophobic, hydrophilic and total accessible surface area to the solvent of the protein molecule, the extent to which the amino acids interact with the solvent and the protein core being proportional to the surface area exposed to the solvent. Polar and non-polar surface areas are often defined using partial atomic charges taken from the molecular potential used, these partial charges differ significantly between force fields, the *g_SAS* module residue option of the GROMACS package.

2.8.Gibbs free energy

To automatically perform all necessary steps to estimate the free binding energy of the complex we use the MM/PBGBSA (MM: Molecular Mechanics; PB: Poisson–Boltzmann; GB: Generalized Born; SA: Surface Area) scripts (Genheden & Ryde, 2015; Netz & Orland, 2000; Qi, Botello-Smith, & Luo, 2017), included in GROMACS (Abraham et al., 2015). However, it is generally expected that no significant conformational change occurs after connection, so snapshots of the three species can be taken from a single trajectory. The free binding energies of complexed complexes with the most promising successful compounds were calculated using the MM-GBSA (Molecular Mechanics Poisson–Boltzmann Surface Area) method (Case et al., 2005).

3. Results and discussions

3.1.Molecular docking (DOC) analysis

LAX was considered flexible and the M-pro, sNsp15, S-pro and R-pol receptors were treated as rigid bodies, in this way the search space became limited, considering only six

degrees of freedom of translation and three degrees of rotation for the ligand. In this case, the flexibility of the binder can be approached using a calculated set of ligand conformations or allowing a degree of atom-atom overlap between the protein and the ligand. The affinity energies (AEs) obtained for the interactions between these molecules demonstrated the formation of a favorable complex since the AEs showed relatively low values.

Fig 3a shows the interaction between LAX and Main protease (M-pro), chosen based on the final pose with the most favorable AE value (-6.8 kcal/mol) among the 40 conformations obtained. In the interaction there were four interactions of the alkyl type (MET165, MET49, CYS145, HID163), in pink. Analyzing the interactions from the DOC results, it is observed that the mode of interaction predicted by the DOC poses of the selected compounds in visual inspection was similar to the poses of the crystallographic structures at the M-pro binding site. Another important aspect to note is that the catalytic site of M-pro has hydrophobic characteristics and this type of interaction is present in the analyzed compound.

The interaction of LAX with Nsp15 Endoribonuclease (sNsp15) (Fig 3b) obtained AE of -5.8 kcal/mol, of which, three bonds are of the conventional carbon-hydrogen type (LAX-GLN198, PHE196 and GLU193), in green, and three of the alkyl type (LAX-TYR195, LYS160 and VAL157), in pink. Thus, taking into account the distances of such connections, the DOC results revealed a total of seven interactions with the so-called active site of NSP15, where there may be a greater occurrence of reaction. Hydrogen bonding can induce changes in NSPRO.

Fig 3c shows the interaction between LAX and Spike protease (S-pro). The interaction obtained the lowest AE value (-7.4 kcal/mol) among the analyzed structures, indicating that this interaction can be more easily disaggregated. Four bonds are of the Pi-alkyl type (LYS330, TYR364, TYR334 and LYS166), in pink, and two bonds were of the conventional carbon-hydrogen type (PRO375 and ARG330), in green. Another important aspect to emphasize is that the S-pro catalytic site has hydrophobic characteristics because of the amino acid residues.

The DOC between LAX and RNA polymerase (R-pol) (Fig 3d) obtained AE value equal to -6.6 kcal/mol, the results showed that the alkyl bonds involving PRO225, TYR248, PHE250, PRO3663, ARG251 and VAL577 were maintained and were more close after linking with LAX due to VDWAALS forces. LAX also showed Pi-Sigma binding (PHE288), in blue, being able to bind to the active pockets of the protein interface.

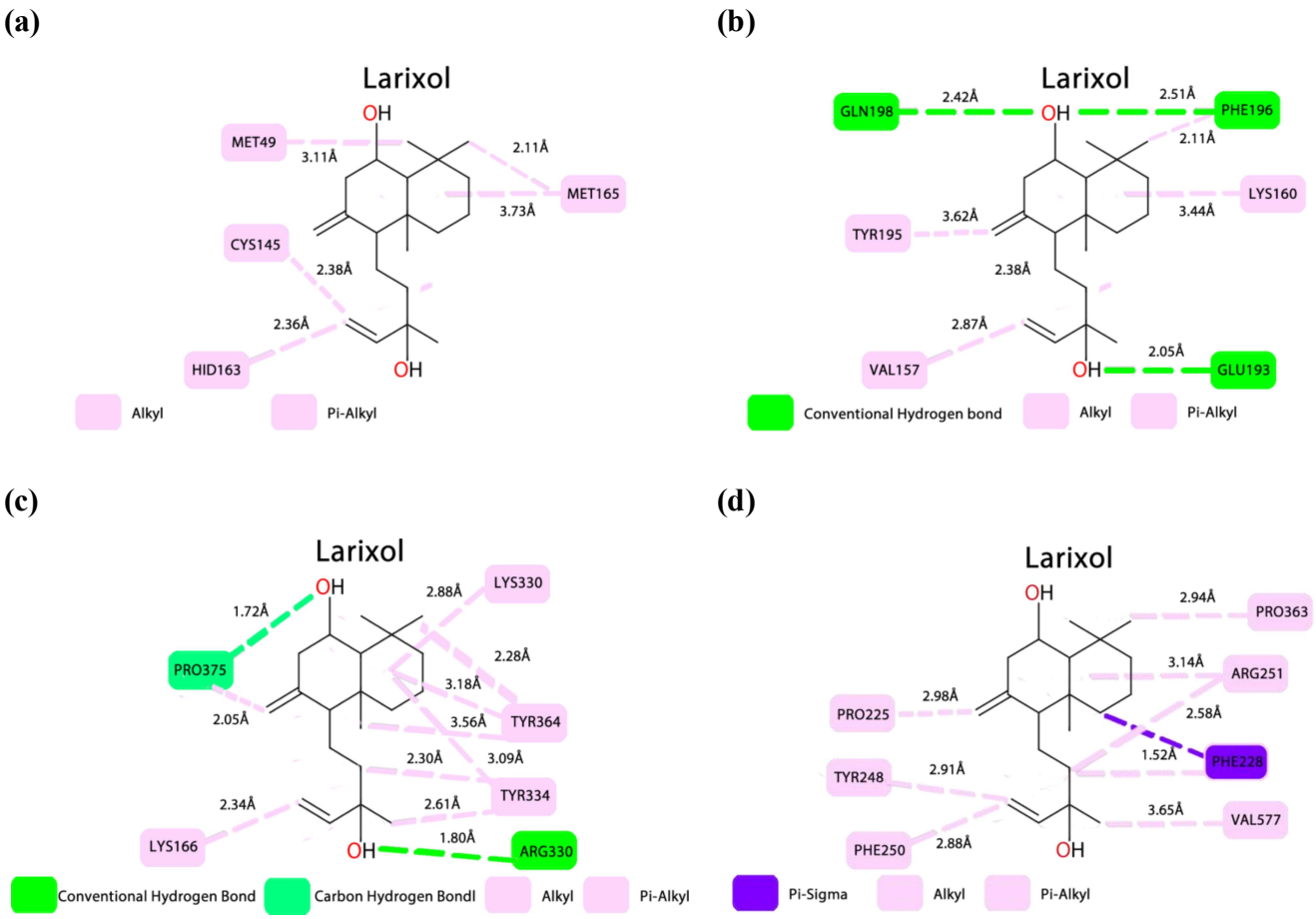

Fig 3. LAX interaction with: a) M-pro, b) sNsp15, c) S-pro and d) R-pol.

The LAX molecule has certain degree of freedom of translation, vibration and rotation, so the DOC process was positioned at the active site of the receptors. The interactions were able to produce changes in the charge distribution of the ligand, which favored the appearance of electrical dipoles at its ends. DOC research data suggest that the ligand has the potential for intercalation to form a complex with the four receptors, due to the interaction with the binding sites of each. The process was able to identify a promising conformation that could represent future solutions in critical areas of human health.

3.2.Molecular dynamics (MD) and Root-mean-square deviations (RMSD) analysis

The RMSD of the atoms of the main structure of the SARS-CoV-2 receptors were studied concerning the initial structures of the complexes to make the conformational analysis and check the stability of the complexes. Overall, the RMSD of the four complexes gradually increased at the start of the MD simulation, this behavior was expected, because, in general, the interaction of the inhibitor with the protein decreases the overall flexibility of the protein.

On the other hand, a decrease in flexibility was observed in the substrate binding region, which revealed the influence of inhibitor interactions mainly in the simulation with the residues located in the binding pocket of S-pro and R-pol.

The MD simulations described allow the atoms in the system to reach speeds compatible with thermal equilibrium. Electrostatic interaction plays an important role in the dynamic stability of the ligand-protein complex. Thus, variations in the RMSD of the structures are used to illustrate the conformation fluctuation around the mean, being an important tool to study protein stability in MD simulation. The MD simulations were completed when the values of α-carbon atoms of the main protein chains and RMSD of the ligand atoms, except the hydrogen atoms, obtained their described trajectories during the calculated simulation time.

The RMSD graphs as a function of the MD time of the positions of the ligand-receptor complexes of the four simulations are shown in Fig 4. In general, the ligand was positioned for most of the simulation time in the active site of the receptors, presenting varied behavior when the RMSD values are compared. There were no changes in the LAX positions in any of the receptors, but there was a growth trend with some deviations for M-pro, S-pro and R-pol, the interaction with Nsp15 was the one that remained more stable over time. We can relate this behavior to the large amount of hydrogen and hydrophobic interactions that occurred between the ligand and the amino acids of the proteases. In the LAX-Mpro conformation, the ligand remained relatively stable in the trajectory of the beginning of the simulation with an RMSD, from the initial structure, from 0.5 to 1.0 Å, and showed distance at the end of the simulation and later approach to the end of 100 ns, there were hydrogen bonds and this is reflected in a larger RMSD in the region of 70 in 80 ns, where the main source of instability between the simulations occurred. The LAX-Spro complex showed a lot of variation but remained stable in the final stages, resulting in a final RMSD of 2.0 to 2.5 Å during the simulation. The complex with the R-pol showed greater instability, which maintained the largest deviation until the end of the simulation. The complex with Nsp15 showed the greatest fluctuation between 20 and 40 ns, but it was the one that obtained the greatest stability until the end of the simulation.

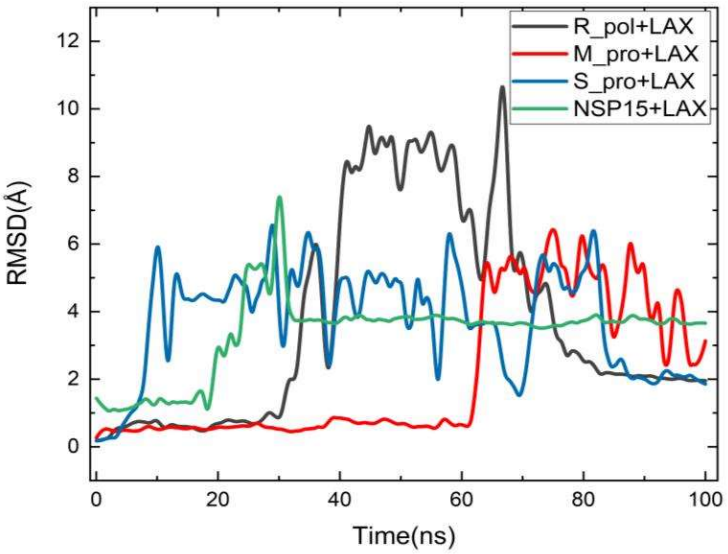

Fig 4. RMSD plot during MD simulations.

3.3.Root-mean-square fluctuation (RMSF)

RMSF values (Fig 5) measure the average deviation of a particle (eg a protein residue) over time from a reference position (typically the time averaged position of the particle). Thus, RMSF analyzes the portions of the structure that are floating from its average structure. In analogy, we assume that stochastic fluctuations of macrostructures can facilitate the entry of LAX in the active site deeply buried within proteins. In order to obtain the first clues about such a predicted opening in the s-Yju3p conformation, four 100 ns MD simulations of the explicit water-solvated protein from the closed conformation were performed. To understand the fluctuation of LAX between viral structures (Fig 5a), the RMSF values are represented graphically, we can observe a pattern of RMSF behavior, clearly showing the dynamic movement that occurs in the regions of the catalytic site. In all four simulations in Fig 5b, the M-pro and R-pol regions have more significant openings demonstrating significantly greater flexibility than the other two macrostructures. During the simulations, the NSP15 and the S-pro remained in a much closed state. The MD simulation results for the complex and for the receptors are analyzed on the 100 ns timescale to understand dynamic behavior and stability.

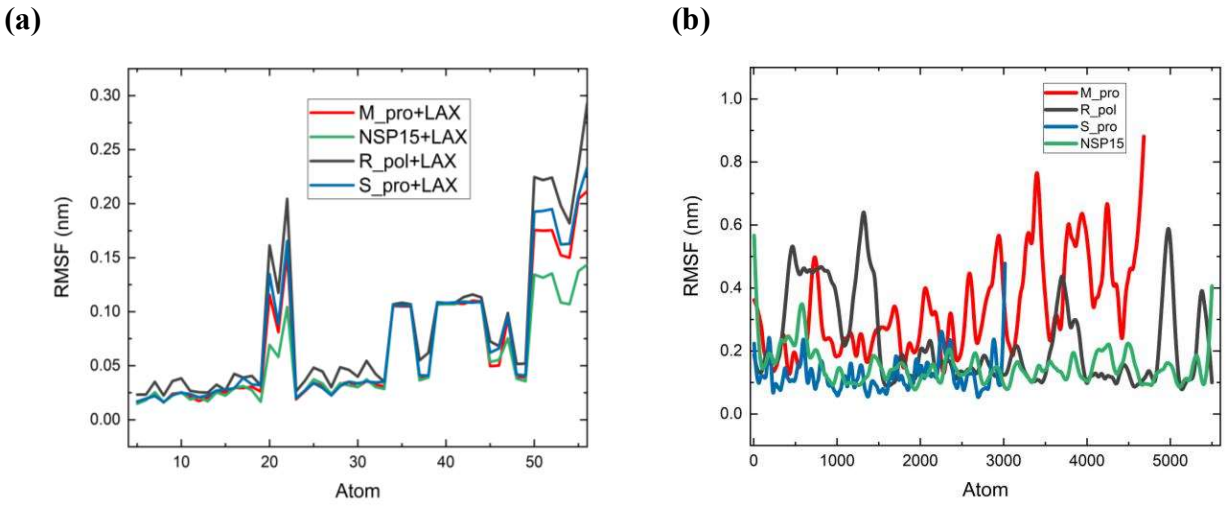

Fig 5. RMSF of complexes (a) and receptors (b).

3.4.Radius of gyration (Rg)

The Rg calculations are used for the study of folding of peptides and proteins and the result is related to the displacement of the center of mass of the protein in relation to the axis in a certain period of time. Rg is used to describe the compaction of protein-ligand complexes during MD, generally, the compact protein or globular protein shows less variation in spin value, while the expanded form of the structure has higher Rg value. The bulkier the protein, the greater its Rg. In this case, it can be said that when the protein is denaturing, its Rg increases. In this study, the variations that occurred in the values of Rg were plotted against time for all complexes as shown in Fig 6. The Rg remained standard for R-pol, but showed unstable behavior for M-pro, NSP15 and S-pro during the MD simulation, which indicates that the binding of both ligands leads to the unpacking of these macrostructures.

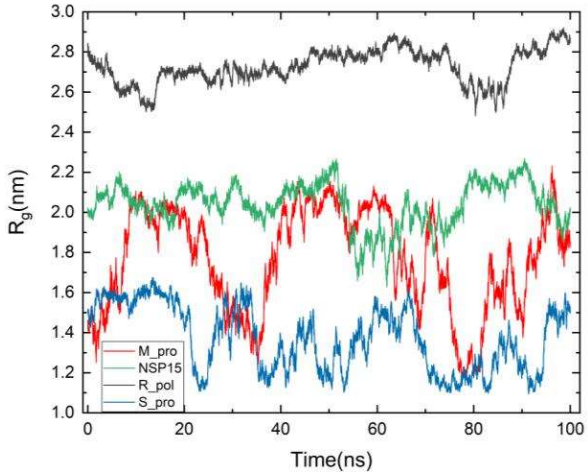

Fig 6. LAX radius of gyration with receptors.

3.5.Hydrogen bonds (HBs)

Time-dependent interactions were also evaluated by quantifying the number of hydrogen bonds established both between segments of the macrostructure itself (intramolecular) and between it and LAX. In the analysis of the HBs averages of the interaction (Fig 7) it was observed that the NSP15 made more connections with the LAX in relation to the other structures with the passage of the simulation time. From the analysis of the interaction of LAX with NSP15, Fig 7a, and the graph in Fig 4, it was observed that the amount of hydrogen bonds is interconnected with the RMSD values, considering that NSP15 showed greater stability of RMSD and more hydrogen bonds. In Fig 7b, between 40 and 80 ns the R-pol did not make hydrogen bonds, returning to do so in the period from 80 to 100 ns, the same behavior was observed in the interaction with S-pro (Fig 7c). The interaction with M-pro (Fig 7d) had a drop in its HBs with LAX at the end of the simulation. Thus, the exposed results suggest that NSP15 may be modifying the structure at some level more than the others, which may have consequences on the structure's function.

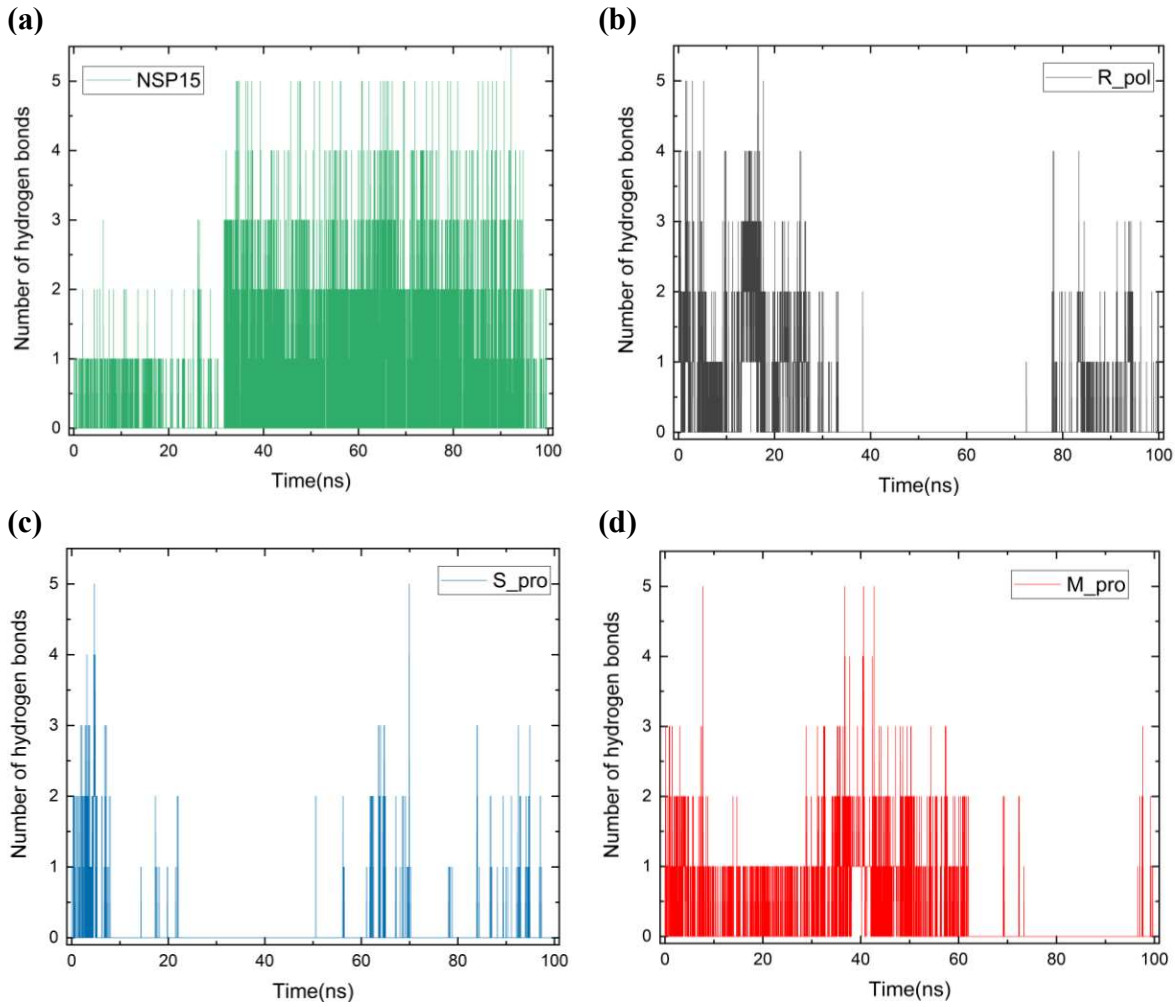

Fig 7. Hydrogen bonds *versus* time.

3.6.Solvent-accessible surface area (SASA)

Modeled conformational changes over the simulation period were estimated using SASA calculations. The average SASA values calculated for the simulations with M-pro, R-pol and S-pro, presented significant variations in some periods as seen in Fig 8 (a, c and d). For NSP15, during the 100 ns simulation the SASA value was relatively stable, showing that there were no significant changes in the structure (Fig 8b). The solvent-accessible surfaces of terminal amino acids are much larger and do not depend on force fields, indicating that they are extensively exposed to solvent. The central residues that present the smallest mean square fluctuation are also the residues that present the smallest area exposed to the solvent, as they are in the most central part of the receptor structure and, therefore, protected from the solvent. The free energy of the nonpolar solvation of each atom in a molecule is proportional to the SASA. The term nonpolar is responsible for the rearrangement of solvent molecules around the solute and for the VDWAALS interaction between the solute and the solvent molecules.

The results confirmed that the macrostructure residues were well exposed and accessible to the solvent. In Fig 8, SASA shows the surface area of receptors that are accessible to a solvent. The SASA range of this protein structure for M-pro and LAX interaction between 60 and 80 ns shows a larger solvent accessible area where the receptor surface becomes more accessible. We can see the smallest SASA value in Fig 8b which remains stable even with the presence of LAX. In Fig 8d the presence of LAX decreases SASA. We can see from all the graphs in Fig 8 that increasing the value of SASA showed a decrease in the amount of protein, indicating a decrease in the surface area accessible to the solvent, which increases the stability of the protein.

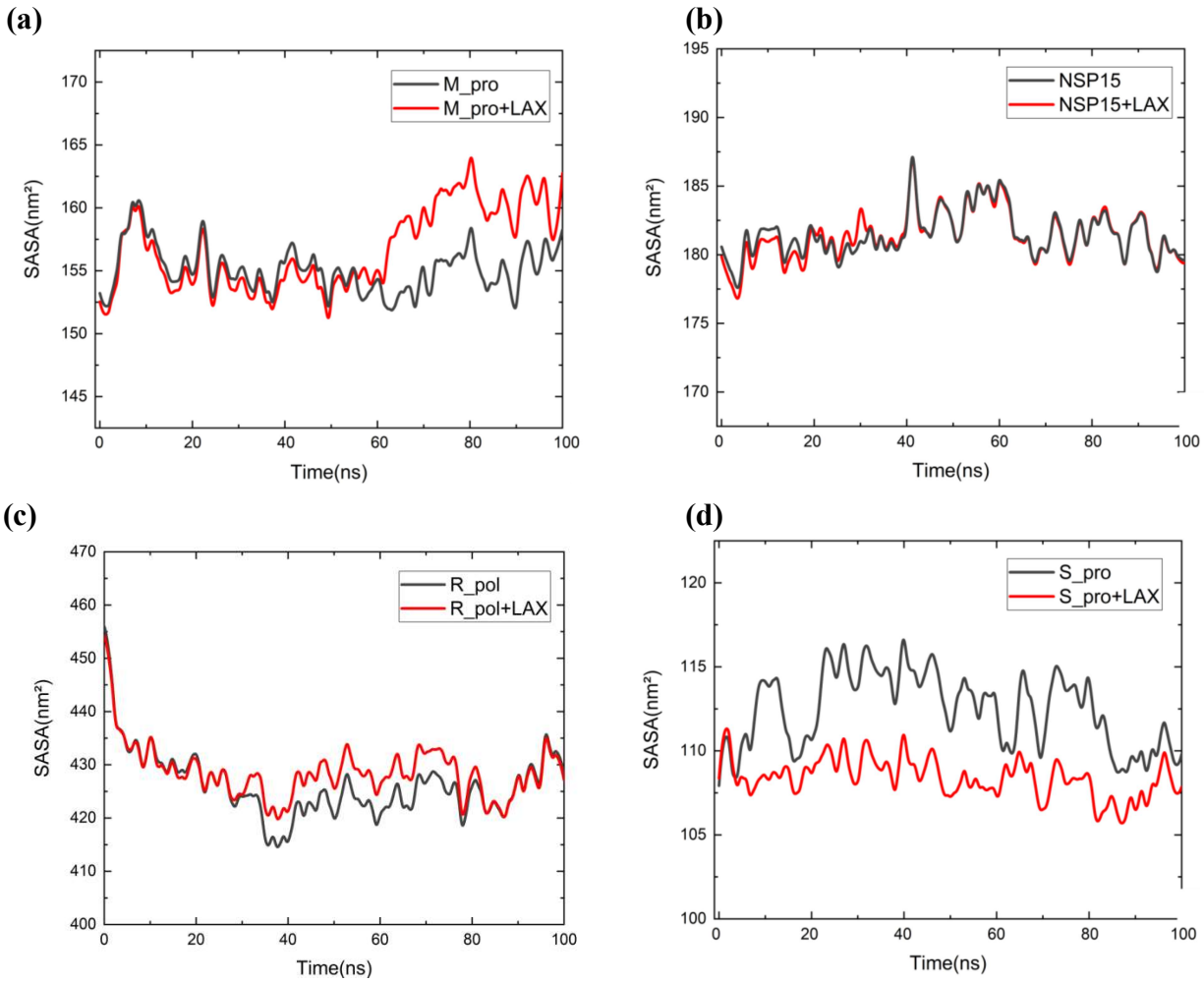

Fig. 8. SASA in relation to: M-Pro (a); NSP15 (b); R-Pol (c) and (d) S-pro.

3.7.Gibbs free energy analysis

The free binding energy and relative standard deviations in percentage of single-system simulations and system simulations above 100 ns were calculated for system simulations assuming that the ligand-protein complexes were moving separately, however, single-system simulations were still stable. From the total negative binding free energy, the ΔGBinding values indicated a higher occurrence of interactions. Furthermore, the total binding free energy of the last 20 ns period revealed a favorable ΔGBinding for interactions with NSP15. The interaction with the receptors is largely controlled by the adsorption that occurs on them, since the interaction occurs directly with the adsorbed ligand. Due to this adsorption, there is a change in the ΔG of the system, which decreases during the interaction process. Electrostatic interactions between charged peptide residues presented by the surface of receptors with LAX, have an important contribution to the negative ΔG.

Table 1. Power components for all simulations.

Receptor	VDWAALS (kJ/mol)	Electrostatic (kJ/mol)	Polar solvation (kJ/mol)	Apolar solvation (kJ/mol)	ΔGBinding (kJ/mol)
NSP15+LAX	-101.71 ± 1.01	-1.13 ± 0.64	50.93 ± 3.03	-15.31 ± 1.03	-96.24 ± 3.86
M_pro+LAX	-92.92 ± 1.89	-0.20 ± 0.02	43.10 ± 3.45	-11.08 ± 1.96	-89.09 ± 3.92
S_pro+LAX	-83.92 ± 1.48	-0.27 ± 0.04	37.11 ± 4.45	-9.08 ± 0.96	-64.08 ± 4.96
R_pol +LAX	-77.29 ± 3.57	-0.69 ± 3.19	34.30 ± 3.12	-10.06 ± 1.52	-42.73 ± 3.02

Mean ± relative standard deviation (%)

The results of the energy analysis of the complexes provided in Table 1 demonstrate that the ΔGBinding were -96,239 kJ/mol (NSP15), -89,091 kJ/mol (M-pro) and -64,084 kJ/mol (S-pro) and -42,730 kJ/mol (R-pol). The results show that the contributions to the ligand coupling were the polar solvation terms, SASA and van der Waals. These identified inhibitors do not represent any major changes in their free binding energies. Details of the MM-PBSA calculation of the complexes are summarized in Table 1. The VDWAALS potential energy varies mainly by two factors, specifically, surface area (molecule geometry) and electronic polarizability (molecular size), between the different calculated energy types. Large molecules are generally associated with greater polarizability. Electrostatic potential energy is influenced by the polarity of the interacting molecules which can be expressed by a dipole moment. The greater the difference in the electronegativity values of the bonded atoms, the greater the dipole moment. The existence of central heteroatoms plays a significant role in increasing the polarity of the molecule. Among the interactions, those with NSP15 stand out, the values of ΔGBinding have a strong affinity value, demonstrating the effectiveness of the interaction with glycoprotein residues. M-pro also showed a high affinity value, due to the ligands not interacting significantly with the binding site residues and also having a high instability, which translates into a high standard deviation value, the interaction with S-pro remains with an intermediate value, even so, it demonstrates stability when compared to R-pol, this last receptor is not so suitable when it comes to interaction for SARS-CoV-2 inhibition, but the interaction within the binding site persists throughout of the simulation.

The RMSD results showed that the system simulation method can be effective in a short period of time, but it is not suitable for long-term simulations due to the large conformational variation during MD simulations. In this way, single system simulation was used for further analysis. For this phenomenon, we decompose the binding free energy into van der Waals energy (VDWAALS), the sum of electrostatic energy and polar solvation energy and apolar solvation energy based on the main residuals, finding the sum of electrostatic energy and polar solvation energy that played a vital role in free energy binding values, as in Table 1.

4. Conclusions

This study reports through an integrated computational approach to reposition Larixol (LAX) as a potential inhibitory agent against the main surface structures of SARS-CoV-2 (M-pro, R-pol, S-pro and NSP15). After the fit tests, a divergent tie posture was generated the posture with the optimal fit score and interactions was considered the best posture for further processing and analysis. The DOC of the compounds for the surface structures was visualized in terms of interactions in the protein substrate recognition pockets and the dynamic stability of the receptor-ligand contacts was evaluated using MD simulations of each macrostructure. Analyzing the obtained conformations, S-pro obtained the best affinity energy, the behavior may be related to the hydrophobic interactions and hydrogen bonds that occurred between the active site of the receptor and the hydroxyls present in the ligand, evidencing the more stable behavior of the ligand in this complex, with several twists in the structure around its primary structure and a greater tendency towards stability. As shown through this integrated approach, computational prediction for the inhibition of key external structures of SARS-CoV-2 has yielded some promising leads for further experimental validation. Overall, our computational repositioning strategy for LAX envisions it as a promising drug candidate that, if confirmed through experimental and clinical approaches, could contribute to solving the global crisis of the COVID-19 pandemic.

Acknowledgments - The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES).

Funding details - This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Disclosure statement - The authors declare no conflict of interest.

Data availability statement - All relevant data is provided in the manuscript.

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CONCLUSÃO GERAL

Conforme abordado nos capítulos I e II foi possível inferir que o gênero *Croton* apresenta diversas aplicações biológicas que podem ser atribuídas a sua extensa e variada composição química. Dentre os métodos usados na obtenção de seus compostos bioativos, a extração com CO_2-SC apresenta-se como uma alternativa aos métodos tradicionalmente usados, devido às propriedades do fluido que favorecem a extração seletiva dos compostos desejados e a manutenção de suas propriedades químicas.

De acordo com o estudo apresentado no capítulo III, pode-se inferir que a extração com CO_2-SC apresentou-se como um método eficaz na obtenção do extrato de *C. matourensis*, com rendimento mássico máximo igual a 5,60 ± 0,06% na condição operacional a 50 °C e 400 bar, a qual foi estatisticamente igual à extração com hexano (5,73 ± 0,26%). A condição supercrítica foi responsável pela concentração de 48,49% de larixol, elevado valor de compostos fenólicos totais (67,66 ± 1,65 mg EAG/g extrato) e flavonoides totais (4,09 ± 0,22 mg EQ/g extrato) e alto percentual de inibição ao radical DPPH. Na análise histopatológica da lesão isquêmica no córtex motor de ratos o extrato influenciou na reconstrução tecidual e na redução da densidade celular, indicando potencial atividade anti-inflamatória e efeito neuroprotetor. Sugere-se que em trabalhos futuros sejam realizados estudos sobre a citotoxicidade do extrato, além de sua aplicação em outros estudos biológicos para verificar sua possível atividade anticâncer, antimalárica, alelopática, entre outras.

A partir da aplicação do estudo de simulação computacional, apresentado no capítulo IV, foi possível concluir que o larixol apresenta-se como um ligante que pode agir como inibidor de estruturas do SARS-CoV-2, especialmente a Spike-protease que atua como principal força motriz de reconhecimento do vírus na célula hospedeira e posterior replicação viral, neste sentido, o larixol apresenta potencialidade para atuar como agente antiviral e atenuar as consequências dessa doença. Ressalta-se a importância de estudos futuros *in vivo*, testes clínicos e sobre a dosagem necessária para validar o efeito antiviral em humanos do extrato ou composto isolado.

Em síntese, a presente tese buscou ressaltar a importância da espécie *C. matourensis* que ainda é pouco conhecida e estudada, mas que possui potencial aplicação como insumo na elaboração de produtos com fins alimentícios, nutracêuticos, cosméticos, farmacêuticos, biomédicos e agroquímicos, podendo trazer biodesenvolvimento tanto para a região Amazônica quanto para as indústrias.

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ANEXO II- PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO

Artigos completos publicados em periódicos

1. PINTO, R. H. H., **BEZERRA, F. W. F.**, DO NASCIMENTO BEZERRA, P. D. N., & DE CARVALHO JUNIOR, R. N. (2021). Phytochemical Composition and Functional Potential of Uxi (*Endopleura Uchi*): An Overview. Food Reviews International, v. 37, p. 1-15. DOI: 10.1080/87559129.2021.1926479.

2. SALAZAR, MARIELBA DE LOS ÁNGELES RODRÍGUEZ; URBINA, GLIDES RAFAEL OLIVO; CUNHA, VÂNIA MARIA BORGES; **BEZERRA, FERNANDA WARISS FIGUEIREDO**; DIAS, MICHELLE NERISSA COELHO; SANTOS, IJAIR ROGÉRIO; TEIXEIRA, BRUNO JOSÉ BRITO; COSTA, WANESSA ALMEIDA; GOMES-LEAL, WALACE; SILVA E SOUZA, JESUS NAZARENO; SILVA, SILVIA HELENA MARQUES; CARVALHO JUNIOR, RAUL NUNES. Cytotoxic effect of cipó-pucá (*Cissus sicyoides* L.) supercritical extract on human red blood cells and as anti-inflammatory in spinal cord injury in adult rats. JOURNAL OF SUPERCRITICAL FLUIDS, v. 169, p. 105105, 2021. DOI: 10.1016/j.supflu.2020.105105

3. FREITAS, LUCAS CANTÃO; BARBOSA, JHONATAS RODRIGUES; DA COSTA, ANA LAURA CALDAS; **BEZERRA, FERNANDA WARISS FIGUEIREDO**; PINTO, RAFAEL HENRIQUE HOLANDA; CARVALHO JUNIOR, RAUL NUNES DE. From waste to sustainable industry: How can agro-industrial wastes help in the development of new products?. RESOURCES CONSERVATION AND RECYCLING, v. 169, p. 105466, 2021. DOI: 10.1016/j.resconrec.2021.105466

4. **BEZERRA, FERNANDA W.F.**; DO N. BEZERRA, PRISCILA; DE OLIVEIRA, MOZANIEL S.; DA COSTA, WANESSA A.; FERREIRA, GRACIALDA C.; DE CARVALHO, RAUL N.. Bioactive Compounds and Biological Activity of *Croton* Species (Euphorbiaceae): An Overview. CURRENT BIOACTIVE COMPOUNDS, v. 16, p. 383-393, 2020. DOI: 10.2174/1573407215666181122103511

5. **BEZERRA, FERNANDA WARISS FIGUEIREDO**; SALAZAR, MARIELBA DE LOS ANGELES RODRIGUEZ; FREITAS, LUCAS CANTÃO; DE OLIVEIRA, MOZANIEL SANTANA; DOS SANTOS, IJAIR ROGÉRIO COSTA; DIAS, MICHELLE NERISSA COELHO; GOMES-LEAL, WALACE; Andrade, Eloisa Helena de Aguiar; FERREIRA,

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