UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

MARIELBA DE LOS ÁNGELES RODRÍGUEZ SALAZAR

Extração de Compostos Antioxidantes de *Cissus sicyoides* L. com Tecnologia Supercrítica e Determinação da Atividade Anti-inflamatória no Modelo de Lesão Medular

BELÉM/PA
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Tese de Doutorado VIII apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Instituto de Tecnologia, da 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
2023

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)

R696e Rodríguez Salazar, Marielba de Los Ángeles.
Extração de Compostos Antioxidantes de Cissus sicyoides L. com Tecnologia Supercrítica e Determinação da Atividade Anti-inflamatória no Modelo de Lesão Medular / Marielba de Los Ángeles Rodríguez Salazar. — 2023.
95 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, 2023.

1. Cissus sicyoides L.. 2. Extração com fluido supercrítico. 3. HPLC. 4. Atividade antioxidante. 5. Atividade anti-inflamatória. I. Título.

CDD 641.3

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Data da Avaliação: ____/____/____

Nota/ Conceito: _____

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DEDICATÓRIA

Dedico este trabalho a minhas filhas, pois são a razão de meu existir.

AGRADECIMENTOS

Agradeço a Deus e à Virgem del Valle, por me conceder sabedoria e força para concluir esta etapa de minha vida.

Aos meus pais Elbinellys del Valle Salazar e Eulalio Rafael Rodríguez, aos meus irmãos, minha família, por me apoiar, guiar e dar força em cada momento. Tornando possível a realização do meu sonho.

Ao meu esposo Glides Rafael Olivo Urbina, a pessoa com quem compartilho a vida. Obrigada pela sua capacidade de me fazer sorrir em momentos difíceis.

Ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, e a CAPES pela Bolsa de estudo.

Ao Prof. Dr. Raul Nunes de Carvalho Junior, eu agradeço a orientação e o apoio durante todo o processo de construção desta pesquisa.

À Profª. Dra. Maria Iracilda da Cunha Sampaio por seu apoio e me proporcionar a oportunidade de expansão de meus conhecimentos.

Aos membros da banca examinadora, pelas contribuições para a realização da tese.

Aos meus colegas e amigos do Laboratório de Extração (LABEX) da Universidade Federal do Pará (UFPA) que participaram da minha formação, o meu eterno agradecimento.

"*Tente uma, duas, três vezes e se possível tente a quarta, a quinta e quantas vezes for necessário. A persistência é amiga da conquista...*"

Bill Gates

RESUMO

A *Cissus sicyoides* L. ou *Cissus verticillata* L. também conhecida como insulina vegetal, é uma planta que possui uma potente atividade anti-inflamatória, devido a sua composição química, que se destaca pela presença de compostos com alta atividade antioxidante. A tecnologia supercrítica é uma alternativa apropriada para extrair compostos antioxidantes de matrizes vegetais. Neste sentido, o objetivo da tese foi realizar a extração de compostos antioxidantes presentes em *C. sicyoides* por meio da tecnologia supercrítica, utilizando CO_2 e EtOH como cossolvente, e posteriormente, no extrato supercrítico avaliar seu efeito anti-inflamatório no modelo de lesão medular. Para isto, na primeira etapa experimental da tese foi realizada a identificação de compostos fenólicos por HPLC e foi avaliado o efeito citotóxico, em glóbulos vermelhos humanos, do extrato de folhas e caules de *C. sicyoides* obtido por extração supercrítica, na condição operacional de 50 °C/ 400 bar e 10 % EtOH (ρ_{CO_2+EtOH} = 953 kg/m^3), em comparação ao extrato obtido por extração convencional (Soxhlet) e, por último, foi avaliado o efeito anti-inflamatório no modelo de lesão medular. Na análise dos extratos por HPLC, os dados espectrais UV/Visível revelaram compostos com bandas de absorção em comprimentos de onda característicos de flavonoides glicosilados. O extrato supercrítico não apresentou efeito citotóxico, pois não houve ruptura das membranas dos glóbulos vermelhos. O ensaio *in vivo* demonstrou uma aparente redução na concentração celular na área circundante à lesão nos animais tratados, sugerindo um efeito anti-inflamatório do extrato supercrítico no modelo de lesão medular. Na segunda etapa experimental da tese, se pretendeu estudar os antioxidantes presentes em cada parte da *C. sicyoides* (frutos, flores, folhas e caules). As amostras foram avaliadas quanto ao teor de CFT pelo método Folin-Ciocalteu, que variou entre 9,30 a 141,04 mg EAG/g de amostra, de FT por complexação com cloreto de alumínio de 1,03 a 44,02 mg EQ/g de amostra, sua atividade antioxidante pelo método DPPH, o valor EC_{50} variou entre 162,83 a 453,25 g de amostra/ g de DPPH e pelo método FRAP os valores foram de 199,14 e 241,26 μM sulfato ferroso/ g de amostra. Assim, as folhas e os caules antes e após SFE, são ricos em antioxidantes, o que justifica o alto teor de FT, bem como a maior atividade antioxidante pelos métodos testados. Por fim, no processo de SFE foi possível obter um extrato com potencial atividade antioxidante, que pode ser utilizado para aplicações biológicas.

Palavras-chave: *Cissus sicyoides* L.; extração com fluido supercrítico; HPLC; atividade antioxidante; atividade anti-inflamatória.

ABSTRACT

Cissus sicyoides L. or *Cissus verticillata* L., also known as vegetable insulin, is a plant that has a potent anti-inflammatory activity, due to its chemical composition, which stands out for the presence of compounds with high antioxidant activity. Supercritical technology is an appropriate alternative to extract antioxidant compounds from plant matrices. In this sense, the objective of this thesis was to perform the extraction of antioxidant compounds present in *C. sicyoides* through supercritical technology, using CO_2 and EtOH as a co-solvent, and subsequently, in the supercritical extract, to evaluate the anti-inflammatory effect in the spinal cord injury model. For this, in the first experimental stage of the thesis, the identification of phenolic compounds by HPLC and the cytotoxic effect, on human red blood cells, of the extract of leaves and stems of *C. sicyoides* obtained by supercritical extraction was evaluated, in the operating condition of 50 °C/ 400 bar and 10 % EtOH (ρ_{CO_2+EtOH} = 953 kg/m^3), compared to the extract obtained by conventional extraction (Soxhlet) and, finally, the anti-inflammatory effect in the spinal cord injury model was evaluated. In the analysis of the extracts by HPLC, the UV/Visible spectral data revealed compounds with absorption bands at wavelengths characteristic of glycosylated flavonoids. The supercritical extract did not show a cytotoxic effect, as there was no disruption of red blood cell membranes. The *in vivo* assay demonstrated an apparent reduction in cell concentration in the area surrounding the lesion in the treated animals, suggesting an anti-inflammatory effect of the supercritical extract in the spinal cord injury model. In the second experimental stage of the thesis, the aim was to study the antioxidants present in each part of *C. sicyoides* (fruits, flowers, leaves and stems). Samples were evaluated for CFT content by the Folin-Ciocalteu method, which ranged from 9.30 to 141.04 mg GAE/g of sample, of FT by complexation with aluminum chloride from 1.03 to 44.02 mg QE /g of sample, its antioxidant activity by the DPPH method, the EC_{50} value varied between 162.83 to 453.25 g of sample/g of DPPH and by the FRAP method the values were 199.14 and 241.26 μM ferrous sulfate/ g sample. Thus, leaves and stems before and after SFE are rich in antioxidants, which justifies the high FT content, as well as the higher antioxidant activity by the tested methods. Finally, in the SFE process it was possible to obtain an extract with potential antioxidant activity, which can be used for biological applications.

Keywords: *Cissus sicyoides* L.; supercritical fluid extraction; HPLC; antioxidant activity; anti-inflammatory activity.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

bs: Base seca;

CO_2: Dióxido de carbono;

CFT: Compostos fenólicos totais;

DPPH: *2,2-Diphenyl-1-picrylhydrazyl;*

EC_{50}: 50 % a concentração inicial do radical DPPH;

FT: Flavonoides totais;

FRAP: Poder antioxidante redutor férrico (*Ferric Reducing Antioxidant Power);*

HPLC: Cromatografia Líquida de Alta Eficiência (*High Performance Liquid Chromatography*);

HPTLC: Cromatografia em camada delgada de alta eficiência (*High-Performance Thin-Layer Chromatography*);

SCI: Lesão na medula espinhal (*Spinal Cord Injury*);

SC: Medula espinhal (*Spinal Cord*);

SFE: Extração com fluido supercrítico (*Supercritical Fluid Extraction*);

SPE: Extração em fase sólida (*Solid Phase Extraction*).

SUMÁRIO

TEXTO INTEGRADOR

A *Cissus sicyoides* L. ou *Cissus verticillata* L. pertence à família Vitaceae, e é conhecida popularmente como cipó-pucá ou insulina vegetal, devido a sua capacidade de redução da glicose no sangue. Na composição química da *C. sicyoides*, são encontrados compostos bioativos com alta atividade antioxidante principalmente nas folhas e nos caules pela presença de carotenoides e compostos fenólicos. A planta tem vários usos na medicina tradicional, entre eles, os mais conhecidos são para o tratamento da epilepsia, acidente vascular cerebral, abcessos, artrite e diabetes.

Devido à ausência de tratamentos eficazes para doenças relacionadas ao sistema nervoso central, várias pesquisas têm sido realizadas com o propósito de investigar os efeitos terapêuticos de plantas medicinais em lesões agudas do sistema nervoso central. No estudo realizado anteriormente pelo nosso grupo de pesquisa foi demostrado que o extrato supercrítico de *C. sicyoides*, tem consideráveis efeitos anti-inflamatórios e neuroprotetores em modelo experimental de isquemia cerebral focal. Esse estudo sustenta a premissa de que a presença de compostos fenólicos no extrato supercrítico de *C. sicyoides* pode ter influência na redução de células inflamatória. Este fato, foi a motivação para o desenvolvimento de um novo estudo para avaliar o efeito anti-inflamatório no modelo de lesão medular.

Diante disso, por se tratar de um estudo para obtenção de extrato para aplicação biológica. A extração com fluido supercrítico (SFE – *Supercritical fluid extraction*) é uma tecnologia promissora na obtenção de extratos de alta qualidade, sem efeitos citotóxicos, sendo adequada na recuperação de compostos bioativos de fontes naturais, que podem ser utilizados para testes biológicos e no desenvolvimento de produtos farmacêuticos.

Portanto, o objetivo deste estudo foi de realizar a extração de compostos antioxidantes presentes em *C. sicyoides* L. por meio da tecnologia supercrítica, utilizando CO_2 e EtOH como cossolvente, e posteriormente, avaliar o efeito anti-inflamatório no modelo de lesão medular do extrato supercrítico. O projeto de pesquisa desta tese foi dividido em 4 capítulos, descritos a seguir:

Capítulo I – Atividade antioxidante e biológica de extratos de *Cissus sicyoides* e *Rosmarinus officinalis* – apresenta a primeira parte da revisão da literatura, abordada na forma de capítulo de livro, publicado pela editora *IntechOpen*, no capítulo encontra-se a descrição da composição química, atividade antioxidante e biológica de extratos de *C. sicyoides*, bem como de *R. officinalis*, que representam uma importante fonte natural de antioxidantes.

Também é proposta uma abordagem da SFE para a obtenção de compostos bioativos de fontes naturais.

Capítulo II – Extração de antioxidantes de matrizes vegetais com solventes verdes – é apresentada a segunda parte da revisão da literatura como capítulo de livro, publicado pela editora *Elsevier*. Neste capítulo mostra-se uma revisão crítica da literatura publicada sobre algumas tecnologias utilizadas para a obtenção de antioxidantes a partir de matrizes vegetais com solvente verde e os principais métodos *in vitro* para a determinação da capacidade antioxidante desses compostos.

Os capítulos III e IV são referentes à parte experimental da tese, e que representam os resultados obtidos neste estudo.

Capítulo III – Efeito citotóxico do extrato supercrítico de cipó-pucá (*Cissus sicyoides* L.) em glóbulos vermelhos humanos e como anti-inflamatório em lesão medular em ratos adultos – retrata um artigo publicado na revista *The Journal of Supercritical Fluids*. Neste artigo são apresentados os resultados experimentais da pesquisa envolvendo a determinação do efeito citotóxico em glóbulos vermelhos humanos do extrato de *C. sicyoides* obtido por extração supercrítica, utilizando dióxido de carbono (CO_2) e etanol (EtOH) como cossolvente, em comparação ao extrato obtido por extração convencional (Soxhlet) e a avaliação do efeito anti-inflamatório na lesão da medula espinhal em ratos adultos.

Capítulo IV – Proposta para o 2º artigo – Determinação de compostos fenólicos, flavonoides e capacidade antioxidante das partes áreas e do extrato supercrítico de *Cissus sicyoides* L., a qual foi desenvolvida como continuação do projeto desta pesquisa. Neste artigo foi realizada a determinação do teor de compostos fenólicos, flavonoides e avaliação do potencial antioxidante do extrato de *C. sicyoides* obtido por extração supercrítica, utilizando CO_2 e EtOH como cossolvente, nas folhas e caules antes e após da extração, bem como nas flores e os frutos, a fim de identificar a contribuição de cada parte da planta para a obtenção de compostos antioxidantes.

Finalmente, foi elaborada uma conclusão geral e foram apresentadas as pesquisas desenvolvidas durante o período de doutoramento. A tese é mostrada nos capítulos a seguir.

OBJETIVOS

Objetivo Geral

Realizar a extração de compostos antioxidantes presentes em *Cissus sicyoides* L. por meio da tecnologia supercrítica, utilizando CO_2 e EtOH como cossolvente, e posteriormente, no extrato supercrítico avaliar o efeito anti-inflamatório no modelo de lesão medular.

Objetivos Específicos

- Descrever a atividade antioxidante e biológica de extratos de *Cissus sicyoides* e *Rosmarinus officinalis* e realizar uma revisão geral da SFE (Capítulo I);
- Apresentar uma visão geral de algumas tecnologias utilizadas para obtenção de antioxidantes a partir de matrizes vegetais e os principais métodos *in vitro* para determinação de antioxidante (Capítulo II);
- Determinar a presença de compostos fenólicos por HPLC e avaliar o efeito citotóxico em glóbulos vermelhos humanos do extrato de *C. sicyoides* obtido por extração supercrítica, em comparação com o extrato obtido por extração convencional; e avaliar seu efeito anti-inflamatório no modelo de lesão medular (Capítulo III);
- Determinar o teor de compostos fenólicos, flavonoides e o potencial de antioxidante do extrato de *C. sicyoides* obtido por extração supercrítica, nas folhas e caules antes e após da extração, bem como nas flores e os frutos da planta (Capítulo IV).

Antioxidant and Biological Activity of *Cissus sicyoides* and *Rosmarinus officinalis* Extracts

(Atividade Antioxidante e Biológica de Extratos de *Cissus sicyoides* e *Rosmarinus officinalis*)

Reprinted from:

SALAZAR, M. A. R.; URBINA, G. R. O.; BEZERRA, P. N.; CUNHA, V. M. B.; SILVA, M. P.; PIRES, F. C. S.; SILVA, A. P. S.; SOUSA, S. H. B.; CARVALHO JR, R. N. Antioxidant and Biological Activity of *Cissus sicyoides* and *Rosmarinus officinalis* Extracts. In: SHALABY, E. Antioxidants. London: IntechOpen, 2019, p. 418. Doi: 10.5772/intechopen.83733.

Chapter

Antioxidant and Biological Activity of *Cissus sicyoides and Rosmarinus officinalis* Extracts

Marielba de los Angeles Rodriguez Salazar, Glides Rafael Olivo Urbina, Priscila do Nascimento Bezerra, Vânia Maria Borges Cunha, Marcilene Paiva da Silva, Flávia Cristina Seabra Pires, Ana Paula de Souza e Silva, Sérgio Henrique Brabo de Sousa and Raul Nunes de Carvalho Jr

Abstract

This chapter will describe the antioxidant and biological activity of *Cissus sicyoides* and *Rosmarinus officinalis* leaf extracts, which represent an important natural source of antioxidants. These plants contain several bioactive compounds with high antioxidant activity, such as phenolic compounds, which are compounds that prevent or delay oxidative stress, acting as free radical scavengers (FRSs), and thus reduce the onset of cardiovascular disease, cancer, diabetes, epilepsy, stroke, among other diseases. The supercritical fluid extraction (SFE) has been studied to obtain antioxidant compounds from natural sources, without the drawbacks associated with conventional extraction processes, such as the use of organic solvents, which present toxicity and contaminate the extracts, is proposed.

Keywords: *C. sicyoides*, *R. officinalis*, antioxidant activity, biological activity, supercritical extraction

1. Introduction

The Amazonian biodiversity presents a great source of foods and medicinal plants rich in antioxidant compounds whose study and conscious exploration contribute to the region sustainable development [1, 2]. The plants have a great importance due to their medicinal and nutritional properties. About 70–90% of the world population prefers the use of medicinal plants or plant extracts to treat common diseases [3, 4]. Plants have been extensively studied in recent years for their antioxidant activity. The main classes of plant chemicals are phenolic compounds, tocopherols, carotenoids, and alkaloids. Among these compounds, phenolic compounds are the most important. They prevent or delay oxidative stress, acting as free radical scavengers (FRSs), and thus reduce the onset of different chronic diseases [5–8].

Antioxidants

Antioxidants are a set of substances that can delay or inhibit oxidation reactions and act as a defense mechanism to neutralize the harmful effects of oxidation in biological systems and foods [6, 9, 10]. Oxidative stress is considered a state of imbalance where excessive amounts of reactive oxygen and nitrogen species (ROS/RNS, for example, superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite) exceed the capacity of endogenous antioxidants (uric acid, superoxide dismutase, catalase, glutathione peroxidase), leading to the oxidation of a biomacromolecule variety such as enzymes, proteins, DNA, and lipids. Exogenous antioxidants (phenolic compounds, carotenoids, tocopherols, and ascorbates) are consumed in the diet mainly of fruits, leaves, seeds, vegetables, and cereals, they have the function of increasing or protecting the antioxidant defense in biological systems and, therefore, they are important for endogenous oxidative stability [11–13].

It is conflicting that oxygen and nitrogen, considered essential for biological processes, are also cofactors for toxic and degenerative processes. In this sense, the antioxidant compounds act through different chemical mechanisms in order to minimize or maintain redox balance in vivo [9, 14]. There are several mechanisms by which oxidation can be inhibited. In general, the mechanisms involved include FRSs, ester bond enzymatic hydrolysis, transition metal ion sequestration, and enzyme-catalyzed peroxide reduction. The last three mechanisms mentioned do not cease reactive species action, but prevent the formation of molecules capable of promoting free radical chain reactions [15].

There is a growing interest in new sources of natural antioxidant compounds due to synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) in the food industry being severely restricted, since they may show carcinogenic effects on living organisms [16–18]. In this sense, the scientific community and consumers are looking for new bioactive compounds of natural origin that can be used to develop new treatments against diseases. In addition, they may be employed in the food industry as functional food ingredients.

Cissus sicyoides L., which belongs to the Vitaceae family, is also known as vegetal insulin, anil-trepador, bejuco-caro, cipó-pucá, or puci. It is considered a plant from the Neotropical region and is usually found in the Amazon region [19, 20]. According to studies on *C. sicyoides* extract composition, the presence of bioactive compounds with high antioxidant activity as carotenoids and phenolic compounds (flavonoid, resveratrol, coumarins, and tannins) was found [21–23]. Therefore, it is a plant traditionally used by Brazilian popular medicine to treat rheumatism, epilepsy, stroke, abscesses, arthritis, and diabetes [23, 24].

Rosmarinus officinalis is an aromatic plant of the Lamiaceae family, native to the Mediterranean region. Today, it has been grown in many parts of the world and is known as rosemary [25, 26]. It has been recognized as one of the plants with great antioxidant activity. Among the most effective antioxidant constituents, cyclic diterpene diphenols, carnosic acid, rosmarinic acid, and carnosol have been identified. *R. officinalis* extracts have been used in the treatment and/or prevention of diseases such as cancer, Alzheimer's disease, urinary and gastrointestinal infections, diabetes, ischemia, and atherosclerosis [17, 25–32]. *R. officinalis* extract has been commercially exploited as a natural antioxidant [5, 16].

Supercritical fluid extraction (SFE) has already been studied to obtain bioactive compounds from natural sources. Salazar et al. and Carvalho et al. showed that the application of SFE technology is successful in obtaining extracts from *C. sicyoides* and *R. officinalis*, respectively, with high antioxidant capacity [24, 33]. SFE is based on the use of solvents with temperatures and pressures above their critical points, which have a high solvency power. One of the most commonly used solvents in SFE is carbon dioxide (CO_2) since its critical points are moderate, nontoxic,

Antioxidant and Biological Activity of Cissus sicyoides and Rosmarinus officinalis *Extracts*
DOI: http://dx.doi.org/10.5772/intechopen.83733

non-flammable, affordable, chemically inert, and apolar and has an ideal behavior for thermosensitive compound extraction. In SFE it is possible to obtain totally solvent-free extracts without compound thermal degradation due to the low operating temperatures applied [34–37].

Supercritical CO_2 (Sc-CO_2) has a limitation in dissolving polar molecules. However, this disadvantage can be solved by polar solvent addition, called modifiers or cosolvents, which modify the supercritical fluid polarity and, consequently, improve polar fraction extraction rich in bioactive substances, such as phenolic compounds related to high antioxidant activity [37, 38]. The aim of this chapter is to describe the antioxidant and biological activity of *Cissus sicyoides* and *Rosmarinus officinalis* leaf extracts that represent an important natural source of antioxidants. In addition to providing an overview of the SFE that is currently presented as a modern and environmentally safe extraction technology for antioxidant compound extraction.

2. *Cissus sicyoides*

2.1 Botanical description

Cissus sicyoides L. or *Cissus verticillata* L., which belongs to the Vitaceae family, is also known as vegetal insulin, cipó-pucá, or puci. It is considered a plant of the Neotropical region and is usually found in the Amazon region. It is a climbing plant, which can reach up to 6 m in length, and presents fleshy articulated branch, alternating leaf of ovate format, pale or yellowish-green flowers, and round fruit, with variations of color from violet to black (**Figure 1**) [19, 20].

2.2 Chemical composition

The bioactive compounds present in the leaf and stem are represented by carotenoids (α-carotene and β-carotene) [39] and phenolic compounds such as

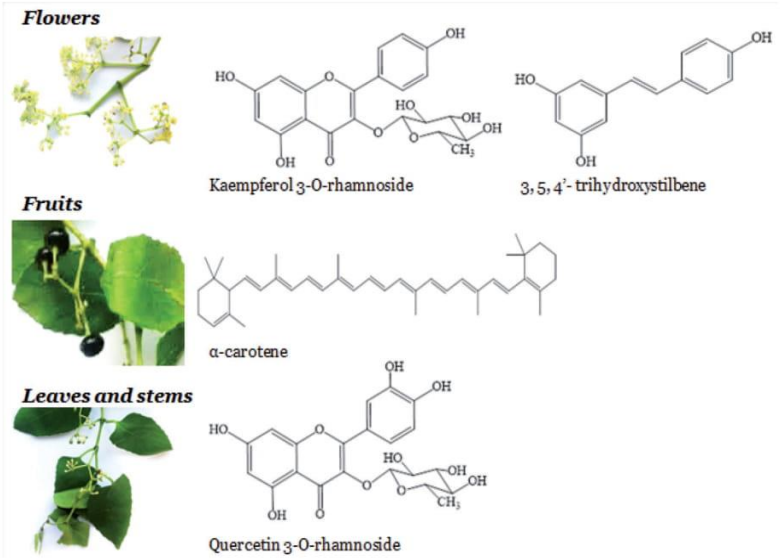

Figure 1.
Description of C. sicyoides parts and the main chemical structure of the antioxidant compounds.

flavonoids (quercetin 3-O-rhamnoside and kaempferol 3-O-rhamnoside) [21]. But also, three new flavonoid glycosides were found, denominate cissosides I, II, and III (kaempferol 3-O-α-L-(5”-O-acetyl)-arabinofuranosyl-7-O-α-L-rhamnopyranoside, quercetin 3-O-α-L-arabinofuranosyl-7-O-α-L-rhamnopyranoside, and quercetin 3-O-α-L-(5”-O-acetyl)-arabinofuranosyl-7-O-α-L-rhamnopyranoside) [22].

Recently, three different flavonoids were identified (quercetin-3-O-hexoside, quercetin-3-O-deoxyhexoside, and kaempferol-3-O-deoxyhexoside) [23]. In addition, resveratrol (3,5,4′-trihydroxystilbene) [40] and a new benzofuran-type stilbene (cissusin) [22], tannins, coumarins (glycoside 5,6,7,8-tetrahydroxycoumarin-5β-xylopyranoside and sabandin), and steroids (β-sitosterol and 3β-O-β-D-glucopyranosyl sitosterol) were found [21]. The presence of essential oils was also detected [41]. In the supercritical extracts of *C. sicyoides* phytochemical screening obtained from leaves and stems by high-performance thin-layer chromatography (HPTLC), the presence of terpenes, phenolic compounds, and flavonoids was evidenced [24]. In the fruit composition analysis, three anthocyanins were found (delphinidin-3-rutinoside, cyanidin-3-rhamnosyl-arabinoside, and delphinidin-3-rhamnoside) [42]. Therefore, the fruit of this plant may have potential use as a food coloring. **Figure 1** shows the chemical structures of the main antioxidant compounds found in *C. sicyoides*.

2.3 Antioxidant and biological activity

In *C. sicyoides* polyphenolic profile, we can find flavonoids: the quercetin and kaempferol as the majority and its various isomers. The mechanism of quercetin antioxidant action has been associated mainly with the reduction of ROS/RNS, which is a compound that prevents or retards oxidative stress, which enables the prevention of various chronic diseases [6, 43]. In the study conducted by Crespo et al., treatment with quercetin and kaempferol prevented the production of ROS such as peroxides, superoxide anion, and nitric oxide. These results confirmed the differential protective effect of these flavonoids in the diet against oxidative stress induced by pro-inflammatory stimuli in parenchymal liver cells [44]. Resveratrol plays an important antioxidant role in reducing hydroxyl radicals, superoxide, and metal-induced radicals, as well as showing antioxidant abilities in ROS-producing cells. Also, it has a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS/RNS [45]. Benzofuran is a potent radical scavenger capable of inhibiting lipid peroxidation, its FRS capacity being greater than α-tocopherol [46].

Figure 2 shows the results of the qualitative analysis of antioxidant activity by HPTLC of C. sicyoides extracts obtained by supercritical extraction (essays 1–15), hexane extract (HE), and ethanolic extract (EE) obtained with conventional extraction by Soxhlet. The plaque was derivatized with DPPH (2,2-diphenyl-1-picrylhydrazyl), and it was possible to detect the presence of yellow spots on the plaque purple bottom resulting from the reduction of the DPPH•; in the presence of antioxidant substances, 2,2-diphenyl-picryl-hydrazine is reduced, losing its purple coloration. The study results confirmed the presence of chemical constituent characteristic of this plant, with antioxidant activity. In the same study, a quantitative determination of the antioxidant activity by the DPPH method was carried out. It was demonstrated that with the extractive methodologies (SFE and Soxhlet) used it was possible to extract with low EC_{50} values, related to a high antioxidant activity; for EE, the value of EC_{50} (325.67 g of extract/g of DPPH) is similar to the value of EC_{50} (404.81 g of extract/g of DPPH) obtained with SFE [24].

In the in vitro antioxidant activity determination by the ABTS method of the *C. sicyoides* aqueous extract obtained by decoction, it was evidenced that the extract has an antioxidant activity of IC_{50} = 13.0 ± 0.2 μg/ml. These results indicate that

Antioxidant and Biological Activity of Cissus sicyoides and Rosmarinus officinalis *Extracts*
DOI: http://dx.doi.org/10.5772/intechopen.83733

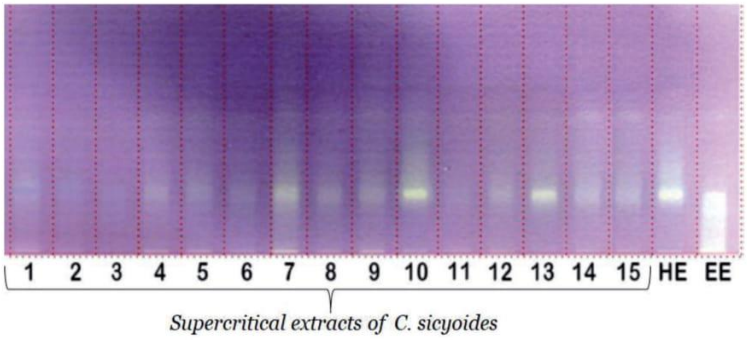

Figure 2.
Qualitative analysis of antioxidant activity by HPTLC of C. sicyoides extracts obtained by supercritical extraction and conventional extraction (Soxhlet) [29].

the extract is a potential source of natural antioxidant and may be useful in the prevention of diseases associated with oxidative stress [47]. Thus, the antioxidant activity results of *C. sicyoides* extracts are related to the extraction methods and to the solvent used.

Due to antioxidant properties, *C. sicyoides* has been used by folk medicine to treat rheumatism, epilepsy, stroke, abscesses, arthritis, and diabetes; it has also been used to treat respiratory diseases. Some biological activities are attributed to the plant as anti-inflammatory, antirheumatic, antiepileptic, antihypertensive, antimicrobial, antipyretic, antioxidant, antiallergic, anticancer, and antidiabetic activities [23, 40, 48–50].

Several studies point to the application of *C. sicyoides* in the treatment of various diseases, in order to demonstrate the cytotoxic activity of the *C. sicyoides* aqueous extract obtained by decoction against cells human epidermoid carcinoma no. 2 (HEp-2 cells), showing complete inhibition of cell division after 24 h of treatment [51]. Also, the antitumor activity of *C. sicyoides* hydroalcoholic extract obtained by maceration, in animals at doses of 300 and 600 mg/kg in weight, was investigated, being demonstrated that the extract showed an inhibition of tumor activity in sarcoma-180 of 49 and 62% and Ehrlich carcinoma of 69 and 84% [52].

Regarding the plant anti-inflammatory activity, it has been demonstrated that the oral administration of 300 and 500 mg/kg of the *C. sicyoides* stem's aqueous extract obtained by decoction in mice with induced edema has a potent anti-inflammatory activity, and administration of the extract produced an approximately 50% reduction of the induced edema [53]. Resveratrol was indicated as one of the constituents responsible for the anti-inflammatory and antiallergic properties presented by *C. sicyoides* alcoholic extract obtained by maceration [40]. However, modern ethnopharmacological use reports that the *C. sicyoides* hydroalcoholic extract by percolation has anti-inflammatory and antidiarrheal actions, due to the abundant presence of flavonol-O-glycoside derivatives of quercetin and kaempferol, which are mainly responsible for the plant pharmacological effects [23].

In addition, pharmacological effects were detected in the treatment and/or prevention of dysfunctions such as hypertension and vasoconstriction of arteries, veins, and capillaries with aqueous extract of *C. sicyoides*. These compounds act at the membrane level, increasing the calcium entry through the membrane as well as acting on the internal calcium deposits [54].

In the evaluation of the antidiabetic potential of *C. sicyoides*, the effects of leaf tea from the plant were studied; the in vivo experimental model chosen proved to be an appropriate treatment, reducing blood and urine glucose levels [48]. Later, it

was demonstrated that treatment of diabetic rats with *C. sicyoides* aqueous extract obtained by decoction, for 7 days (100 and 200 mg/kg), reduced blood glucose levels by 22 and 25%, respectively [49]. However, *C. sicyoides* leaf tea was used to investigate the plant therapeutic efficacy in volunteers who are diabetic and intolerant to glucose. A single dose of tea (1 g of dried leaf powder in 150 ml of water) was used for a period of 7 days. It was observed in people intolerant to glucose that the tea had antidiabetic activity [55].

C. sicyoides has antibacterial activity, showing inhibitory capacity against bacteria that cause food poisoning [56], which causes acute effects in the gastrointestinal tract and, in some cases, a high severity that patients come to death (*Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and *Escherichia coli*). In addition, the antifungal activity of plant leaf and stem alcoholic extracts was demonstrated, inhibiting the growth of fungi *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* [57].

Recently, Salazar et al. carried out the biological activity determination of *C. sicyoides* supercritical extract; an in vivo test using a focal cerebral ischemia model was performed, and the extract had shown to have a neuroprotective and anti-inflammatory effect, justifying the use in traditional folk medicine for central nervous system diseases. These effects were associated to the presence of phenolic compounds in the extract [24]. Therefore, the results of these studies justify the traditional use of *C. sicyoides*, pointing to the plant extract potential benefit as a possible alternative medicine in disease treatment.

3. *Rosmarinus officinalis*

3.1 Botanical description

Rosmarinus officinalis is an aromatic plant of the Lamiaceae family, native to the Mediterranean region, and is also cultivated in Central Asia, India, Southeast Asia, South Africa, Australia, the United States, and Brazil. Today, it has been grown in many parts of the world and is commonly known as rosemary. The plant is a bush that reaches from 0.50 to 1.50 m in height, with very pungent aroma leaves and blue, violet, and white flowers (**Figure 3**) [25, 26, 58].

3.2 Chemical composition

R. officinalis chemical composition varies greatly, due to some factors that directly influence the quality, amount of oil, and extract produced. However, it is possible to verify, through the literature, that its main chemical constituents are flavones, diterpenes, steroids, and triterpenes [17, 26, 27, 31]. The phenolic compounds present in *R. officinalis* were grouped into three classes: (i) phenolic acids (vanillic, caffeic, ferulic, and rosmarinic acids), (ii) diterpenes (carnosol, rosmadial, carnosic acid, methyl carbonate, rosmanol, epirosmanol, epiisorosmanol, epirosmanol methyl ether, and epiisorosmanol ethyl ether), and (iii) flavonoids (hesperetin, apigenin, genkwanin, 4′-methoxytectochrysin, cirsimaritin, scutellarein, 4″,5,7,8-tetrahydroxyflavone, homoplantaginin, and 6-hydroxyluteolin 7-glucoside) [27]. Recently, a *R. officinalis* chromatographic analysis was carried out, which revealed two large groups: oxygenated monoterpenes and hydrocarbonated monoterpenes. The main constituents of these groups were 1,8-cineole followed by camphor, borneol, and α- and β-pinene. The oxygenated and hydrocarbonated sesquiterpenes were composed of caryophyllene and caryophyllene oxide [26]. In the supercritical extracts of *R. officinalis* leaves, chemical analysis confirmed the

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DOI: http://dx.doi.org/10.5772/intechopen.83733

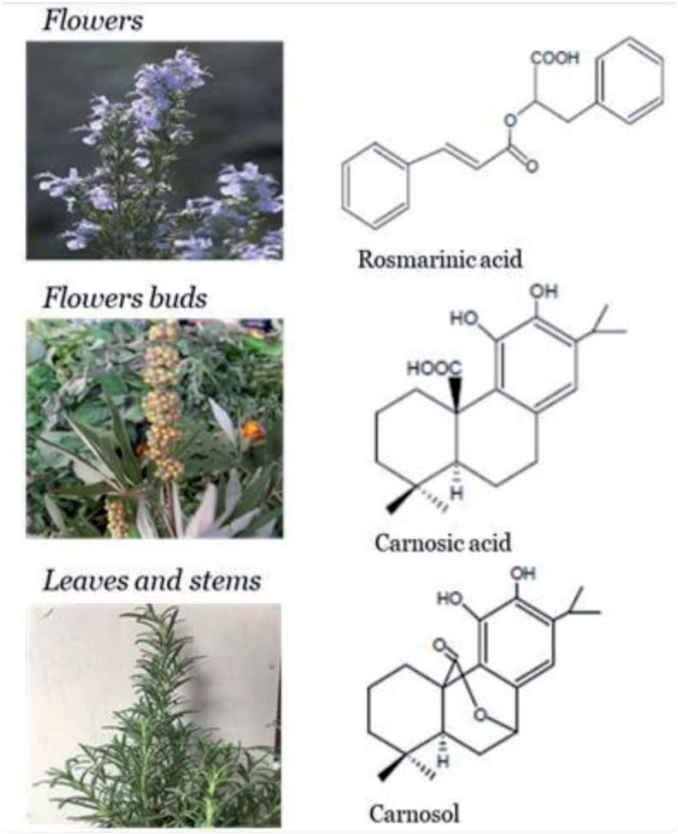

Figure 3.
Description of R. officinalis parts and the main chemical structure of the antioxidant compounds.

presence of 1,8-cineole, camphor, carnosic acid, and rosmarinic acid [17, 33, 59]. **Figure 3** shows the chemical structures of the main antioxidant compounds found in *R. officinalis*.

3.3 Antioxidant and biological activity

R. officinalis has been recognized as one of the plants with high antioxidant activity [28–30]. Its antioxidant effect is due to the phenolic compounds present in the leaves and stems [60]. Among the most effective antioxidant constituents, cyclic diterpene diphenols, carnosic acid, and carnosol were identified. In addition, its extract contains epirosmanol, rosmanol, metilcarnosato, isorosmanol, and other caffeic acid derivatives [61, 62].

The action mechanism of these compounds has been widely discussed in several studies. The carnosic acid and carnosol act as potent sequesters of peroxyl radicals and are responsible for 90% of the antioxidant properties, where both are inhibitors of lipid peroxidation in liposomal and microsomal systems, besides being good sequestrants of hydroxyl radicals. Specifically, carnosic acid removes hydrogen peroxide but may also act as a substrate for its ability to increase or maintain the superoxide dismutase and glutathione peroxidase activities. The most important elements in the *R. officinalis* structure are the diterpenes containing the aromatic ring (C_{11}–C_{12}) in the catechol group, with the conjugation of three basic rings. The

catechol group is responsible for eliminating the radical electrons formed as an oxidation result. Lactone carnosol, rosmarinic acid, and hesperetin were cited in the literature as important FRSs [31, 63–65]. Rosmarinic acid has two aromatic rings, each with two OH groups that are capable of donating hydrogen and chelating metals [66]. Caffeic acid derivatives may act as metal ion chelators (Fe^{2+}), thus reducing the formation of ROS [67].

The antioxidant of *R. officinalis* extracts obtained in SFE was confirmed. Carvalho et al. analyzed the plant antioxidant activity through a coupled reaction of β-carotene and linoleic acid; the results indicated that the extracts obtained at high pressures and low temperature (300 bar/40°C) exhibited the highest antioxidant activities, in comparison to extracts obtained in low pressures (150 bar/30°C). In any case, antioxidant activities were always above the control used (β-carotene and linolenic acid) as shown in **Figure 4**. The authors state that the antioxidant action remained approximately constant for the 3-h reaction for all extracts tested. The major compounds detected in the extracts were camphor (0.6% d.b.) and 1,8-cineol (0.043% d.b.). The extract obtained by hydrodistillation showed the highest yield of camphor (1.22% d.b.) and 1,8 cineol (0.23% d.b.) compared to other extraction methods (SFE and Soxhlet) [33].

Thus, the antioxidant capacity of *R. officinalis* leaf and stem extract obtained with supercritical CO_2 was also evaluated by the ORAC method. The extract showed a high antioxidant capacity (1.9 mol Trolox/mg) similar to that of BHT and vitamin E (2.8–3.0 mol Trolox/mg). In addition, the extract presented a high percentage of lipid oxidation inhibition (88%) of fatty acids present in an analyzed cosmetic foundation. The extract volatile fraction was characterized by compounds such as camphor, 1,8-cineol, and trans-β-caryophyllene present in relative amounts of less than 25% [59].

Due to antioxidant properties, *R. officinalis* has been used in food preservation and in disease treatment. In food preservation, components such as rosmanol and carnosol prevent oxidation and microbial contamination and also can be up to four times more effective than BHA and equal to BHT as an antioxidant [27, 68, 69]. Different studies have demonstrated the potent activity of *R. officinalis* in inhibiting the formation of hydroperoxides, reducing carotenoid color loss, and retarding lipid oxidation in corn [78] and hazelnut oils [70].

The *R. officinalis* extract has been successfully commercially exploited as a natural antioxidant, for its use as synthetic antioxidants such as BHA, BHT, and TBHQ, in the food industry and is severely restricted as they may have carcinogenic effects on living organisms [5, 16, 17]. In this way, *R. officinalis* extract may be useful to

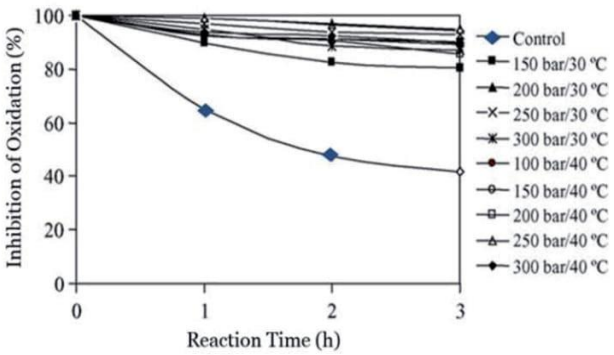

Figure 4.
Antioxidant activity of R. officinalis extracts obtained with supercritical CO_2 [38].

replace or even decrease the synthetic antioxidants in foods. As preservatives, the extracts offer several technological advantages and benefits to consumers [71].

Health problems derived from lipid oxidation have attracted consumers' and researchers' attention, since numerous diseases are linked to dietary and biological lipid oxidation products. Therefore, *R. officinalis* extracts have been related to several biological activities, such as anticancer, antidiuretic, anti-inflammatory, antibacterial, antidiabetic, antiangiogenic, antioxidant, and hepatoprotective [28, 71–74], besides allowing the use of the plant in the treatment and/or prevention of Alzheimer's disease, urinary and gastrointestinal infections, diabetes, aging, ischemia, and atherosclerosis [25, 32].

Among the most important groups of compounds isolated from the plant, phenolic diterpenes account most of their biological activity. These compounds have been indicated in recent years as inhibitors of neuronal cell-induced death by a variety of agents both in vitro and in vivo, confirming the therapeutic potential of these compounds for Alzheimer's disease, due to the compounds multifunctional nature in the neuronal protection mediated by the plant antioxidant activity [32].

Several studies show that *R. officinalis* has pharmacological activity for chemoprevention and cancer therapy. In the extract antiproliferative activity evaluation against human ovarian cancer cells, it was corroborated that the extract inhibited the proliferation of cancer cell lines, affecting the cell cycle in multiple phases. In addition, it induced apoptosis by modifying the multiple gene expression that regulates apoptosis. Thus, the extract can be considered as an adjuvant to chemotherapy [62]. Also, the antiangiogenic effect of the carnosic acid present in *R. officinalis* extract was corroborated in angiogenesis models using human umbilical vein endothelial cells in relation to the tube formation in the reconstituted basement membrane, chemotaxis, and proliferation. Carnosic acid from the extract may be useful in preventing disorders due to angiogenesis, and its antiangiogenic effect may contribute to a neuroprotective effect [72].

The actions of *R. officinalis* leaf ethanolic extract obtained with Soxhlet extraction were tested in glucose homeostasis and antioxidant defense in rabbits. Serum levels, glucose levels and insulin levels were studied in diabetic rabbits (alloxan was used to induce diabetes); it was shown that at a dose of 200 mg/kg was possible to reduce the blood glucose level and to increase the serum insulin concentration. In addition, during 1 week of animal treatment with the extract, it was demonstrated that it had an ability to inhibit lipid peroxidation and to activate the antioxidant enzymes. Due to its potent antioxidant properties, the plant extract has a remarkable antidiabetogenic effect [75]. Recently, the antidiabetic and antihypercholesterolemic action of flavonoid-rich fractions of *R. officinalis* (fractions obtained with n-butanol and diethyl ether) in diabetic mice induced by streptozotocin was evaluated. Both fractions showed a decrease in the glucose level at a dose of 400 mg/kg, especially the fraction obtained with diethyl ether; plasma glucose levels decreased up to 60.38%. The pancreas histopathological study showed that both fractions regenerated the pancreatic β cells and increased the mass of islets. *R. officinalis* fractions exhibited a potent antidiabetic effect, while the fraction obtained with n-butanol showed a high anti-hypercholesterolemic activity [76].

The anti-inflammatory activity of *R. officinalis* supercritical extracts was studied. Absorptions of extract fractions were tested on monolayers of Caco-2 cells (2–12 h of incubation). Human macrophages were treated with basolateral fractions, and TNF-α, IL-1β, IL-6, and IL-10 secretions were measured by ELISA. Fractions obtained after 8 and 12 h in absorption experiments caused a considerable reduction in the excretion of pro-inflammatory cytokines. This reduction in cytokine secretion levels was associated with the amounts of carnosol and carnosic acid. Thus, the *R. officinalis* supercritical extract can be used in formulations to inflammatory disease prevention [77].

In relation to the antibacterial activity, *R. officinalis* essential oils obtained by hydrodistillation exhibited antibacterial activity against *Escherichia coli*, *Salmonella typhi*, *S. enteritidis*, and *Shigella sonnei*; this activity was associated with the oil ability to reduce DPPH radical formation (CI_{50} = 3.82 μg/ml) [61]. However, the antibacterial and antifungal activities of *R. officinalis* leaf extracts obtained by SFE extraction were confirmed, and the extracts showed antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and antifungals against *Candida albicans*. Obtaining *R. officinalis* extracts by SFE has been shown to be a promising extraction with respect to its incorporation into various foods, cosmetics, and pharmaceuticals products that a natural aroma, color, and antioxidant/antimicrobial additive are desired. These properties are also necessary for the food industry in order to find possible alternatives to synthetic preservatives [17].

4. Supercritical fluid extraction (SFE) of antioxidant compounds from plant matrices

When a new extract from a natural source is tested, the most important aspects to take into account are the extraction method and the type of solvent used, as this will affect the antioxidant properties. Several extraction methods for the selective extraction from plant matrices such as *R. officinalis* were identified in the scientific literature [71].

Thus, the bioactive compound extraction has been considered one of the most important steps in the approach of obtaining or recovering bioactive compounds. Conventional extractions have been the most used technology for these compound recovery. It is based on the extraction power of different solvents and the application of high temperatures, promoting mass transfer. However, there are drawbacks associated with conventional extraction processes such as the use of large amounts of organic solvents, toxic to human health and the environment, extraction time, and the use of high temperatures that can degrade the thermosensitive compounds [6, 8]. They motivated the search for environmentally safe extraction techniques such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) [36, 78]. SFE has already been studied to obtain antioxidant compounds from natural sources [24, 33, 77, 79]. **Table 1** presents the antioxidant activity values of different plant extracts obtained with SFE, involving the plants under study (*C. sicyoides* and *R. officinalis*).

4.1 SFE procedure

A solvent is considered a supercritical fluid when the pressure and temperature of the system are above its critical point. This point is defined as the highest temperature and pressure at which a substance can exist in equilibrium between the liquid and vapor phases. Above its critical temperature (Tc) and critical pressure (Pc), the supercritical fluid can be considered as an expanded liquid or as a compressed gas, whose density (ρ) is relatively high and consequently has a high solvency power. This effect gives the solvent a certain degree of selectivity, in addition to allowing easy separation of the solvent from the solute, which can be achieved by a simple system depressurizing, resulting in products totally solvent-free and without thermal degradation of the compounds of interest, due to low operating temperatures [35, 38].

Plants	Extraction conditions	Solvents	Method of determination	Antioxidant capacity	Biological Activity	Refs.
C. sicyoides	40°C/400 bar	CO_2 + 10% of ethanol	DPPH	404.81 ± 2.78 EC_{50}: g of extract/g of DPPH	Neuroprotective and anti-inflammatory effect	[29]
R. officinalis	40°C/300 bar	CO_2	DPPH	12.85 ± 0.10 IC_{50}: $\mu g.ml^{-1}$	Antioxidant, antibacterial, and antifungal	[21]
	100°C/350 bar	CO_2	DPPH	0.23 ± 0.01 IC_{50}: mg/ml	Antioxidant	[84]
	50°C/300 bar	CO_2	ORAC	1.9 ± 0.10 μmol Trolox/mg extract	Antioxidant	[59]
Mangifera indica L.	55°C/100 bar	CO_2 + 20% of ethanol	DPPH	2.13 ± 0.24 EC_{50}: DPPH μg/μg dry extract	Antioxidant	[85]
Eugenia uniflora L.	60°C/400 bar	CO_2 + ethanol	DPPH	>200 EC_{50} (μg/ml)	Antioxidant	[86]
Raphanus sativus L.	35°C/400 bar	CO_2	DPPH	359 mg TE/100 g dry extract	Antioxidant and anti-inflammatory	[37]
Piper nigrum L	40°C/300 bar	CO_2	DPPH	103.28 EC_{50}: of $\mu g.ml^{-1}$	Antioxidant	[87]

Table 1.
Presentation of the antioxidant activity values of different plant extracts obtained with SFE, involving the plants under study (C. sicyoides and R. officinalis).

One of the most commonly used solvents in SFE is carbon dioxide (CO_2) because its critical points are moderate (Tc = 31.1°C, Pc = 73.8 bar, and critical density (ρc) = 0.468 g/cm^3), nontoxic, non-flammable, affordable, chemically inert, and apolar and has an ideal behavior for thermosensitive compound extraction [34, 37]. In addition to the supercritical CO_2 (Sc-CO_2), there are other substances that are also used as supercritical fluids, as shown in **Table 2**.

Due to its low polarity, Sc-CO_2 presents a limitation to dissolve polar molecules. However, this disadvantage can be solved by the addition of polar solvents, called modifiers or cosolvents, which modify the supercritical fluid polarity and, consequently, improve the extraction of polar fractions rich in bioactive substances, such as phenol compounds related to high antioxidant activity [37, 38]. Methanol is the solvent most used as a modifier for various plant matrices, but it is toxic and

Fluid	Tc (°C)	Pc (bar)	ρ_c (g/cm^3)
Nitrous oxide (N_2O)	36.5	71.0	0.457
Ethane (C_2H_6)	32.2	48.8	0.203
Propane (C_3H_8)	96.7	42.5	0.220
Propylene (C_3H_6)	91.9	46.2	0.230
Benzene (C_6H_6)	289.0	48.9	0.302
Toluene (C_7H_8)	318.6	41.1	0.290
Ammonia (NH_3)	132.5	112.8	0.240
Water (H_2O)	374.2	220.5	0.272

Table 2.
Critical properties of some substances used as solvents in supercritical extraction processes.

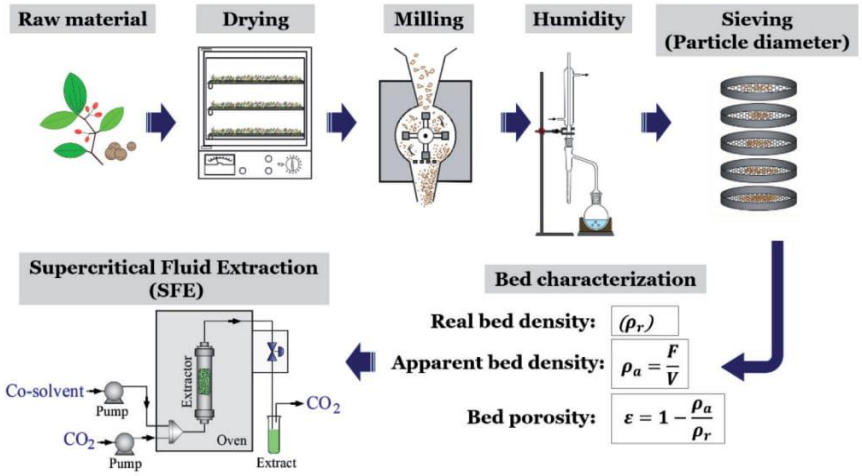

Figure 5.
Scheme of the SFE procedure of plant matrices.

different from ethanol, which is an environmentally safe solvent being a good choice for SFE processes, and can be used in the extraction of natural products [80, 81]. Water is also a very attractive cosolvent for natural product extraction due to its high polarity, which considerably increases the polarity of Sc-CO_2 [79].

For antioxidant compound extraction and recovery by SFE, several vegetable matrices were used, such as seeds, fruits, leaves, flowers, rhizomes, roots, fruit peels, and tree branches. The SFE process consists basically in the extraction of soluble compounds present in the solid matrix by a supercritical solvent and then separates these compounds from the solvent after depressurizing the system. In order to achieve an efficient and adequate extraction, several factors must be taken into account, having a careful control of the operating conditions and process step optimization [35, 36, 82].

Initially, the raw material must pass through a pretreatment stage before being fed into the fixed bed extractor; this procedure is performed to prepare the solid particles, allowing a greater efficiency to be achieved in the extraction process [83]. As shown in **Figure 5**, after the raw material is collected, one of the first stages of its pretreatment is the solid matrix moisture reduction, for example, drying leaves in an oven with air circulation. Generally, the plant matrix moisture should not exceed 14% (wet basis). Another important step is the moisture content determination by the distillation method of the Jacobs immiscible solvent, with the purpose of knowing if the quantity of water in the sample is adequate for the supercritical extraction process. The sieving stage is applied to standardize and determine the average particle size of the solid particles. The real and apparent density and bed porosity determination is also very important as they affect the particles packaging in the extraction vessel and consequently the solvent flow and the mass and heat transfer processes [35, 82].

After a suitable pretreatment, the solid matrix is placed in an extraction vessel forming a fixed bed. Depending on the compounds of interest, the supercritical solvent (Sc-CO_2) or solvent + cosolvent is fed by the solvent pump and/or cosolvent into the extraction vessel, where it continuously flows through the fixed bed and dissolves the extractable components from the solid matrix. The mixture of solutes that is removed from the solid matrix is called extract. In the separation step, the mixture formed by the solvent extraction + extract leaves the vessel and feeds the separator (collection flask) where the mixture is separated by rapid reduction of

pressure (ambient pressure). The extract precipitates in the separator, and the solvent is removed from the system [38, 83].

5. Conclusion

The identification of new natural antioxidant compounds is of great interest to the food, pharmaceutical, and cosmetic industry in order to find possible alternatives to synthetic antioxidants. In this way, plants such as *C. sicyoides* and *R. officinalis* have been extensively studied for their antioxidant activity. The *C. sicyoides* extract obtained by SFE has a neuroprotective and anti-inflammatory effect; these effects are associated with the presence of phenolic compounds and the high antioxidant activity in the extract. *R. officinalis* extract is antibacterial, antifungal, anti-inflammatory, and effective, associated with the presence of carnosic acid, carnosol, rosmarinic acids, and hesperetin. It has been corroborated that these plants contain chemical compounds that exhibit the capacity of FRSs and reduce the onset of different diseases. Finally, obtaining extracts from plant matrices using environmentally safe extraction technology such as SFE represents a great opportunity to obtain bioactive compounds.

Acknowledgements

Mar Salazar (Process Number: 1777277) thanks CAPES for the doctorate scholarship.

Conflict of interest

The authors have no conflict of interest to declare and are responsible for the content and writing of the manuscript.

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Antioxidants Extraction from Vegetable Matrices with Green Solvents

(Extração de Antioxidantes de Matrizes Vegetais com Solventes Verdes)

Reprinted from:

SALAZAR, M. A. R.; URBINA, G. R. O.; BEZERRA, P. N.; CUNHA, V. M. B.; SILVA, M. P.; PIRES, F. C. S.; SILVA, A. P. S.; FERREIRA, M. C. R.; BARBOSA, J. R.; SOUSA, S. H. B.; CARVALHO JR, R. N. Antioxidants Extraction from Vegetable Matrices with Green Solvents. In: INAMUDDIN; ALTALHI, T. Green Sustainable Process for Chemical and Environmental Engineering and Science: Green Solvents and Extraction Technology. Amsterdam: Elsevier, 2023, p. 289-308. DOI: 10.1016/B978-0-323-95156-2.00010-6.

CHAPTER 12

Antioxidants extraction from vegetable matrices with green solvents

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12.1 Introduction

Lately, 25% the drugs in the modern pharmacopoeia are plant-based and various synthetic drugs are made with compost obtained from plant matrices (Umair et al., 2017). The antioxidant compounds have become increasingly important in chemical structures. Natural antioxidants are part of various vegetable matrices (Pitaro et al., 2013; Shahidi, 2015; Xu et al., 2017). In a strong and healthy human body, the pro-oxidants production is successfully kept under control by the several levels of antioxidant defenses. It is important for the organism to maintain the balance between oxidizing compounds and antioxidant compounds to establish better physiological conditions in the body. This state of imbalance is known as oxidative stress (Birben et al., 2012; Burton-freeman, 2010; Ching et al., 2006; Devasagayam et al., 2004; Donnelly and Robinson, 1995; Kaur and Kapoor, 2001; Somogyi et al., 2007).

Several selective extraction methods are used for vegetable matrices. The extraction methods, which are replacing the conventional extractions, consist of modern extraction technologies such as supercritical technology and high pressure extraction. All of these extraction methods present great efficiency, as the physical–chemical properties of green solvents used can be modified (Gil-Chávez et al., 2013; Khaw et al., 2017; Khoddami et al., 2013; Xu et al., 2017). Solvents with high polarity are prominently being used for antioxidant compounds extraction (Figueroa et al., 2018).

In relation to the method for determine antioxidant capacity. The methods are classified depending on the reaction mechanism: the tests that involve electron transfer reaction (Bartoszek and Polak, 2016), include the Ferric reducing/antioxidant power (FRAP) (Firuzi et al., 2005), the Trolox equivalent antioxidant capacity (TEAC) test and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Aruoma, 2003; Rice-Evans et al., 1996). However, tests of hydrogen transfer reaction include the oxygen radical absorbance capacity (ORAC) (Huang et al., 2002; Miguel, 2010; Ou et al., 2001). Are indicated

Green Sustainable Process for Chemical and Environmental Engineering and Science
DOI: https://doi.org/10.1016/B978-0-323-95156-2.00010-6

the application of at least two of the above methods, but preferably the use of all is recommended to antioxidant activity quantification, evaluating the advantages and disadvantages of each test and their applicability (Sousa et al., 2018; Xu et al., 2017). This chapter pursues to deliver an overview of a few technologies used to obtain antioxidants from vegetable matrices with green solvent and the main in vitro methods for these compounds antioxidant capacity determination.

12.2 Antioxidants

Bioactive compounds can be classified through the mechanisms by which they act as control preventive compounds, "cleaning" antioxidants and/or repairers. They act in the first line of defense to avoid the development of reactive species through iron and copper metals chelation in free radical scavengers before they attack the biological molecules (Bendary et al., 2013; de Castro and Sato, 2015; Niki, 2010; Ratnam et al., 2006).

On the other hand, pro-oxidants are reactive compounds produced naturally by the organism and exert fundamental functions in biological systems. If for some reason they are produced in excess, or if the endogenous antioxidant defenses function poorly, they can cause macromolecules oxidations such as lipids, proteins, or DNA (oxidative stress) and consequent biological dysfunctions and associated diseases. But it is known that these defenses do not protect the organism completely, since they still suffer oxidative, when in contact with oxygen (Halliwell, 1995; Halliwell and Gutteridge, 1985; Losada-Barreiro and Bravo-Díaz, 2017; Lü et al., 2010).

In another way, secondary antioxidants act by blocking peroxides and hydro peroxides decomposition, converting them into inactive form by the action of antioxidants, blocking the chain reaction through the reactive intermediates capitation, such as the peroxyl and alkoxyl radicals. In this category they are natural and synthetic antioxidants. It is important for the organism to maintain the balance between oxidizing compounds and antioxidant compounds to establish better physiological conditions in the body. The state of imbalance leads to oxidative stress, which implies in the etiology of several diseases including coronary disease, and cancer (Birben et al., 2012; Burton-freeman, 2010; Ching et al., 2006; Devasagayam et al., 2004; Donnelly and Robinson, 1995; Kaur and Kapoor, 2001; Somogyi et al., 2007).

Antioxidants have become increasingly important for their chemical structure, since they delay or inhibit the oxidative stress. These compounds may be synthetic or natural antioxidants, which are part of various foods constitution, mostly vegetables, justify the beneficial activities presents in fruits, vegetables, greens, and cereals provided to the body (Pitaro et al., 2013; Shahidi, 2015; Xu et al., 2017).

12.3 Antioxidant extraction techniques with green solvents

The extraction technologies, which have been replacing the conventional extractions, consist of modern extraction technologies (Gil-Chávez et al., 2013; Khaw et al., 2017;

Khoddami et al., 2013; Xu et al., 2017). These techniques are typified by the prospect of using green solvents. Properties that an ideal green solvent should have embrace: low toxicity, biodegradable in the environment, and easily removed gives sample (Herrero and Ibañez, 2018). Table 12.1 shows the main techniques for extracting antioxidants with green solvents.

Solvents as ethanol and water are excellent to get compounds which are believed to be effective antioxidants (Alam et al., 2013). Solvents physicochemical properties in supercritical state, such as CO_2, permit the extraction of a variety of compounds (Díaz-Reinoso et al., 2006; Goyeneche et al., 2018).

Six specific principles were proposed to bioactive compounds extraction, most of these principles being followed to by modern extraction techniques (Chemat et al., 2012; Herrero and Ibañez, 2018):

1. Use of renewable resources;
2. Use of green solvents;
3. Lower energy expenditure;
4. Minimization of waste produced;
5. Favor safe processes and automated;
6. Obtaining extracts without degradation of its compounds and contaminants free.

12.3.1 Supercritical fluid extraction

Supercritical technology consists a unitary operation that separates compounds (solutes) from a solid matrix, placed in contact with a solvent in a supercritical state (Jesus and Meireles, 2014). A solvent supercritical form is a homogeneous phase with properties similar to liquid and gas. In the supercritical state, the solvent has densities similar to liquids and viscosity very close to the gas (Brunner, 2005; Bubalo et al., 2015; Khaw et al., 2017; Zuin and Ramin, 2018).

Most antioxidants are susceptible to degradation, and the main factors associated with it are temperature, oxygen, and light. Supercritical technology represents an alternative to obtain this class of compounds. As it provides organic solvents-free extracts, which cause some thermal degradation degree or easy oxidation, that can occur after exposure to high temperatures and oxygen, during the conventional extraction methods (Dai and Mumper, 2010; Khoddami et al., 2013).

Several solvents have been tested as supercritical solvents, but the critical properties and solubility determine which is most suitable (Bubalo et al., 2015, 2018; Pereira and Meireles, 2010). The supercritical carbon dioxide ($ScCO_2$) is the most solvent applied in the supercritical fluid extraction process, since it presents low critical conditions (31.1°C/73.8 bar); besides being nontoxic, nonflammable, accessible, chemically inert, nonpolar, and easily separated from the solute (Bubalo et al., 2018; Herrero et al., 2013; Taghvaei et al., 2016).

Table 12.1 Main techniques for extracting antioxidants with green solvents.

Sample	Vegetable part	Extraction conditions	Solvent	Method of determination	Antioxidant capacity	References
Supercritical fluid extraction						
Rosmarinus officinalis	Leave	100°C/350 bar	CO_2	DPPH	0.23 $IC_{50:}$ mg/mL	(Babovic et al., 2010)
Mentha spicata	Leave	50°C/200 bar	CO_2 + ethanol	DPPH	71% inhibition	(Mandana et al., 2011)
Raphanus sativus	Leave	40°C/400 bar	CO_2 + ethanol	DPPH	403 mg trolox/g dry extract	(Goyeneche et al., 2018)
Thymus praecox ssp.	Leave, stem, and root	40°C/300 bar	CO_2	DPPH	404.5 IC_{50} (ug/mL)	(Petrović et al., 2016)
Cinnamomum cassia	Bark	45°C/600–650 bar	CO_2	DPPH–ABTS	10.09 $IC_{50:}$ mg/mL–10.06 mmole trolox/g	(Yang et al., 2012)
Subcritical water extraction						
Geranium macrorrhizum	Leave	200°C/100 bar	Water	DPPH	197 trolox/g dry extract	(Nastić et al., 2018)
Panax ginseng CA meyer	Stem and root	160°C/100 bar	-	DPPH	39.97% inhibition	(Lee et al., 2018)
Vitis sp.	Fruit	140°C/150 bar	-	DPPH	13.85 µg of extract DPPH/µL	(Aliakbarian et al., 2012)
Matricaria chamomilla	Flower	200°C/1.6 bar	-	DPPH	IC_{50}: 0.03 mg/mL	(Cvetanovíc et al., 2014)
Coriandrum sativum	Seed	200°C/30 bar	-	DPPH	IC_{50}: 0.01 mg/mL	(Zeković et al., 2014)
Pressurized liquid extraction						
Phyllostachys nigra	Leave	200°C/103 bar	Ethanol/water	DPPH	SC_{50}:140 µg/mL	(Shang et al., 2014)
Camellia sinensis	Leave	75°C/50–200 bar	Ethanol	DPPH	74.40% inhibition	(Jaiswal and Naik, 2017)

Haematococcus pluvialis	Algae	200°C/103.4 bar	Ethanol	ABTS	0.287 mmol trolox/g extract	(Jaime et al., 2010)
Physalis angulata L	Fruit	25°C/100 bar	Ethanol	DPPH	76.33% inhibition	(Corazza et al., 2018)
Malus pumila	Fruit	102°C/103.4 bar	Ethanol/water	DPPH	1091 mg trolox/ 100 g dry material	(Wijngaard and Brunton, 2009)
Microwave-assisted extraction						
Satureja macrostema	leave	500 W of microwave power	Ethanol	ABTS	IC_{50}: 10.85 µg total s.s./mL	(Alonso-carrillo et al., 2017)
Achillea millefolium	Leave and stem	170 W of microwave power	Water/ethanol	DPPH	IC_{50}: 7.89 µg/mL	(Milutinovic et al., 2015)
Urtica dioica	Aerial part	407 W of microwave power	Water	DPPH	4.48 mg DPPH/g dry material	(Ince et al., 2012)
Prunus amygdalus	Almond skin	100 W of microwave power	Ethanol/water	DPPH	46.3% inhibition	(Valdés et al., 2015)
Vaccaria segetalis	Seed	400 W of microwave power	Methanol/ water	DPPH	IC_{50}: 0.48 mg/mL	(Yuan et al., 2014)
Ultrasound-assisted extraction						
Cassia auriculata	Leave	pH 6.2 and power 50 W	Methanol/ water	FRAP - DPPH	96.2 mM Fe^{2+}/g–90.5% inhibition	(Sharmila et al., 2016)
Prunella vulgaris L	Fruit	79°C/40 kHz frequency	Ethanol/water	DPPH	91.62% inhibition	(Zhang et al., 2011)
Lycium ruthenicum Murr.	Fruits	power 100 W	Ethanol/water	FRAP	8.04 mM ferrous sulfate/100 g	(Luo et al., 2018)
Benincasa hispida	Seed	65% power level/52°C	Ethanol	DPPH–ABTS	35.48–43.10% inhibition	(Bimakr et al., 2012)
Camellia oleifera l	Seed	88°C/45 kHz frequency	Ethanol/water	DPPH	82.6% inhibition	(Feng et al., 2014)

Table 12.2 The critical properties of CO_2 and the substances most commonly used as cosolvents (ethanol and water).

Substances	*Tc* (°C)	*Pc* (bar)	ρ_c (g/cm³)
Ethanol (C_2H_5OH)	243.6	63.3	0.280
Dióxido de carbon (CO_2)	31.1	73.8	0.468
Water (H_2O)	374.2	220.5	0.272

Supercritical CO_2 can dissolve polar compounds. It is required to alter the fluid polarity through a modifier or a cosolvent addition (de Melo et al., 2014; Goyeneche et al., 2018). Table 12.2 shows the critical properties of CO_2, ethanol, and water.

The cosolvents most repeatedly used are ethanol, water, isopropanol, methanol, and mixtures of these substances. The solvent + cosolvent mixture composition has to be controlled within narrow limits. The most suitable cosolvents for applications related to the food and pharmaceutical industry are ethanol and water as they can be effortlessly separated from the final product, have low toxicity, and miscibility in supercritical CO_2 (Easmin et al., 2014; Gil-Chávez et al., 2013; Jiao and Pour, 2018; Rosa et al., 2009).

As shown in Fig. 12.1, a simple supercritical extraction system consists of three main steps (Cunha et al., 2018; Espinosa-Pardo et al., 2014):

1. Solvent feed system (CO_2) and/or solvent (CO_2) + cosolvent (ethanol and water) (compressor, solvent/cosolvent pumps, cooling bath, and mixer);

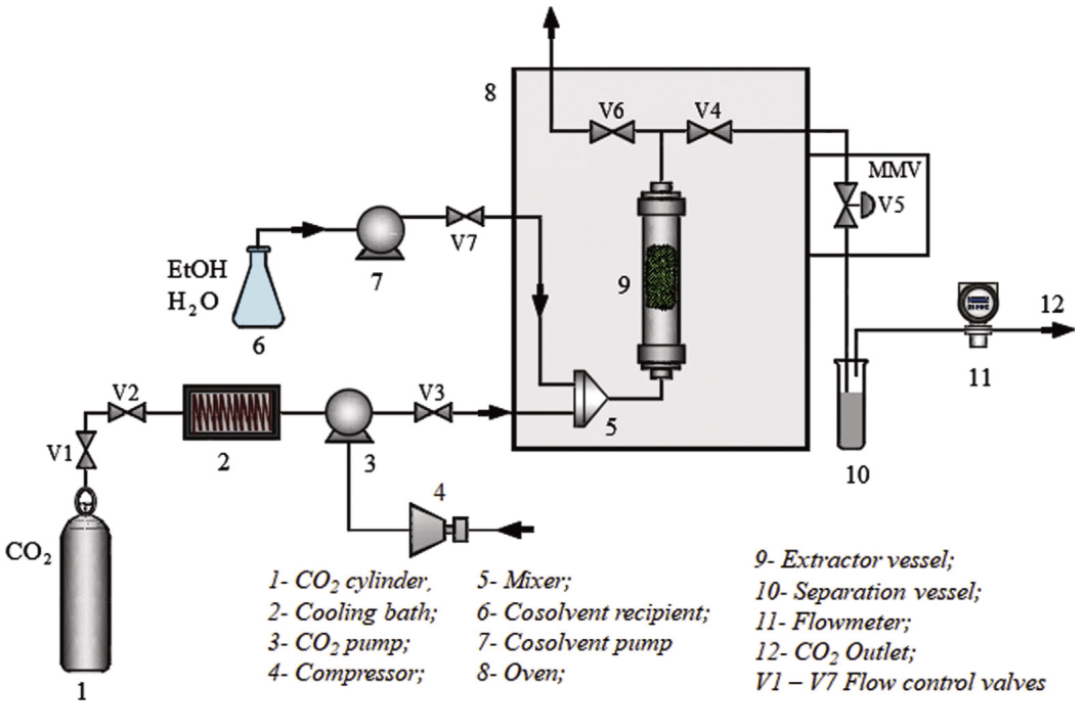

Fig. 12.1 ***Schematic diagram of a supercritical fluid extraction system.*** *Source: Author.*

2. Extraction with the supercritical solvent of the soluble substances present in the solid matrix (oven and extraction vessel);
3. Separation of compounds of interest from the solvent (control valve for extraction pressure reduction, separation, and extract collection container).

12.3.2 Subcritical Water Extraction

Is a type extraction that uses of water a temperature of 100–374°C and pressures of 1–221 bar (Castro-Puyana et al., 2013; Saldaña and Valdivieso-Ramírez, 2015). The subcritical water extraction benefits of some physical properties of water. These properties are density, surface tension, and viscosity (Missopolinou et al., 2012).

Subcritical water extraction is most effective when performed at high temperatures. However, very high temperatures may cause components of interest degradation. This last effect is considered the most important, in relation to the increase in water temperature (Rodríguez-Meizoso et al., 2006; Vitas et al., 2018).

Among advantages using subcritical water extraction are the possibility of working with a wide temperature range, its polarity decreasing as its temperature increases, which makes the water dielectric constant with characteristics alike to ethanol and methanol (Herrero et al., 2013). Demonstrates the possibility of selectively extracting several of compounds, for compounds with high polarity milder temperatures are used, whereas for less polar compounds higher temperatures are used (Rahmanian et al., 2015; Rodríguez-Meizoso et al., 2006). Thus, use subcritical water extraction can be alternative to obtain compounds antioxidant (Aliakbarian et al., 2012).

Water being used as solvent in extractions techniques is interesting, because water is fire-resistant, harmless, and eco-friendly (Lachos-Pereza et al., 2018). However, subcritical water extraction has been used in the extraction of polycyclic aromatic hydrocarbons, herbicides, and pesticides (Saldaña and Valdivieso-Ramírez, 2015).

Antioxidants extraction is usually accomplished by conventional processes and the use of these techniques often requires a secondary process of extracts purification (Luo et al., 2018). In this context, subcritical water extraction shows up as an efficient technology and meets the requirements of clean technologies as well as being low price, efficient, and safe (Zeković et al., 2014). Some publications present studies related to this type of extraction to attain antioxidant compounds from plant matrices (Lee et al., 2018; Nastić et al., 2018). The subcritical water extraction, the raw material preparation must first be taken into account. Some papers use *in natura* samples, others use previously dehydrated samples. The extraction procedure is composed by few steps as displayed in Fig. 12.2:

1. Starting with the solvent feed, a pressurizing and heating system;
2. The process of extracting the solute present in the matrix under study;
3. The extract collection. This last step is generally coupled to a refrigeration system, since the extract is obtained at an elevated temperature.

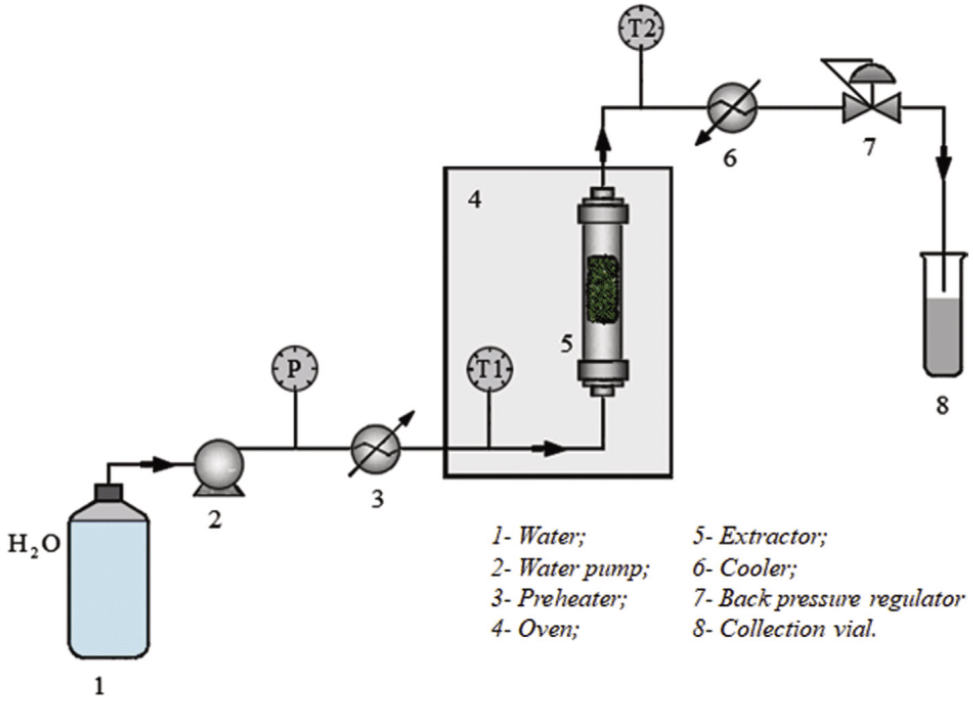

Fig.12.2 ***Schematic diagram of subcritical water extraction system.*** *Source: Author*

12.3.3 Pressurized liquid e xtraction

This extraction combines temperature and pressure with liquid solvents to obtain compounds of interest (Mello et al., 2019; Rahmanian et al., 2015). The extraction can be carried out in employing operating conditions from 40°C to 200°C, 50–200 bar, and a suitable solvent (water and ethanol) (Etxabide et al., 2018). In these conditions, it is possible to keep the solvent in a liquid state, which facilitates cell permeability, intermolecular physical interactions, and increases mass transfer, reducing the amount of solvents (Mello et al., 2019; Picó, 2017; Strati et al., 2014). This extraction also called pressurized solvent extraction or pressurized fluid extraction (Wu, 2017). Due to the modification of the water polarity and change of properties, it becomes suitable to extract compounds, moderately polar or nonpolar, modifying the process temperatures (Hassas-roudsari et al., 2009; Zaibunnisa et al., 2009).

Pressurized liquid extraction to obtain antioxidant compounds depends on solvent types, number of cycles and/or solvent washing, pressure, temperature, extraction time, and also should be optimized for substances of interest extractions (Moret et al., 2014). The use of toxic solvents, such as halogen compounds, provides good extraction yields, however, induce the need for an additional extracts purification stage, besides being harmful to the environment. Some alternative solvents used in extractions, such as more ecological and friendly solvents with the environment, have been extensively studied

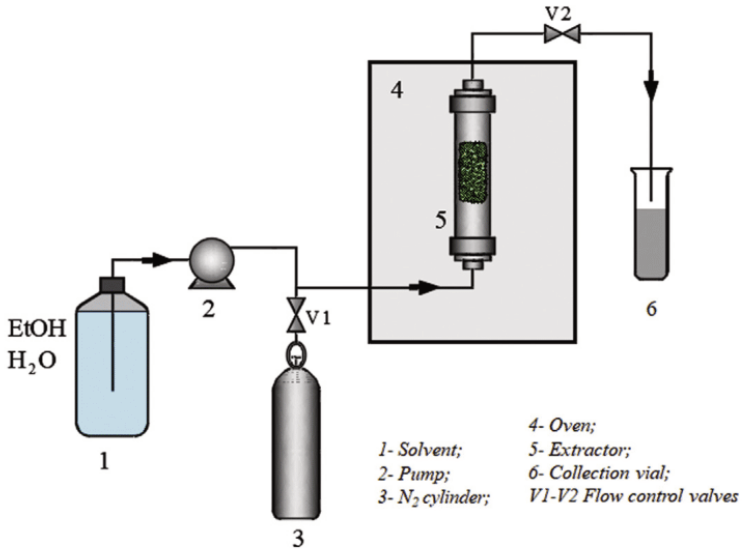

Fig. 12.3 ***Schematic diagram of the pressurized liquid extraction system.*** *Source: Author.*

to obtain extracts rich in antioxidant compounds (Herrero et al., 2013; Saravana et al., 2018). Some studies demonstrated an extraction advanced efficiency with green solvents in relation with pressurized liquid extraction (Etxabide et al., 2018; Jaime et al., 2010). As shown in Fig. 12.3, the procedure has the following steps (Herrero et al., 2013; Picó, 2017):

1. The dry, milled, and sieved sample is loaded onto the extraction cells;
2. The solvent is fed into the cell with a certain pressure;
3. The cells are heated and static extraction takes place (closed system) and subsequently initiates the dynamic extraction (open system);
4. The solvent inside the cell is removed with inert gas (nitrogen);
5. Depressurizing and finally the solvent present in the extracts are evaporated.

Pressurized liquid extraction employing temperatures from 100°C to 200°C is adequate to obtain antioxidant compounds from vegetable matrices. Some studies have used a mixture of water with organic solvents and found that there was an increase in bioactive compounds recovery (Palma et al., 2002; Piñeiro et al., 2004). Comparing the pressurized liquid extraction with conventional extraction, minimization of solvent consumption and extraction times, and the possibility of using green solvents with good extraction efficiencies are some advantages that can be listed. In addition, the technique allows the expansion for small industrial productions (Castejón et al., 2017; Moret et al., 2014; Señorans and Luna, 2012).

12.3.4 Microwave-assisted extraction

This extraction represents an excellent different option to conventional techniques for extracting with reduced extraction time, less or no solvent consumption, good

selectivity, increased yield, greater heat transfer, reduction of processing steps, lower energy consumption, and consequently, lower costs. In addition to the prospect of be given products labeled as "green" as stated by the environmental standards, with high added value (Périno et al., 2016).

Microwave-assisted extraction is established on the dielectric heating system thanks to the energy absorption by polar molecules, directly using the energy supplied by the microwaves to heat them inside the matrix structure, contributing to obtaining the analytes of interest in the matrix. Polar molecules align with the continuously variable electric field generated by microwaves, leading to accelerated chemical, biological, and physical processes (Ince et al., 2012; Li et al., 2013). Unlike conventional methods with external heating, such as solid–liquid extraction, heat and mass gradients work from within biological matrix out where the whole sample is heated while causing the desorption of the target compound, and in the solid matrices heating occurs more rapidly (Ciriminna et al., 2016).

As nonpolar solvents do not absorb microwave energy, at least some polarity must be ensured in the system and for this purpose polar and nonpolar solvents mixtures or nonpolar solvents have been proposed to obtain organic compounds. The solvent polarity is a significant factor in bioactive compounds selective extraction. The solvent choice for the microwave-assisted extraction depends on the interaction between solvent and matrix, on the dissolution factor, and on the absorption solvent properties (dielectric constant, dielectric loss, and dissipation factor) which indicate the microwave energy absorption capacity, the energy conversion efficiency in heat, the solvents capacity for energy absorption, and its transformation into heat transferred to the molecules. Therefore, the precise solvent choice is very important to reach an ideal extraction (Alonso-carrillo et al., 2017; Handa et al., 2016; Ruth Alara et al., 2018; Setyaningsih et al., 2015).

Different techniques have been developed as traditional variations of microwave extraction method, such as hydrodiffusion, solvent, and solvent-free extraction, microwave-assisted hydrodistillation, among others. These methods allow the obtainment of high value-added compounds using only the raw materials supplied or with the aid of solvents to bioactive compounds extract (Boukroufa et al., 2015; Périno et al., 2016; Rombaut et al., 2014; Yahya et al., 2018). These are considered "green" techniques from the perspective of energy expenditure, due to the lower waste production and energy demand, in a fast and efficient way (Golmakani and Moayyedi, 2015; Jeyaratnam et al., 2016; Kusuma et al., 2018).

Microwave-assisted extraction process takes place in a microwave oven adapted according to the needs. The sample or the sample and solvent (when necessary to use) are accommodated in a vessel which is placed in the middle of an rotary table, and the operational data are determined in order to generate volumetric heating by microwaves irradiation causing cellular structure perturbation, facilitating the target compounds separation from the matrix (Swamy and Muthukumarappan, 2017).

Studies point out the advantages of microwave-assisted extraction from plant matrices applying the various methodologies, confirming a higher yield of antioxidant compounds in relation to other extraction types, besides maintaining the bioactive compounds desirable properties. The molecules activation and the heat generated in the process break the cell walls, easily releasing the matrix compounds from the material to the solvents. Its efficiency depends the sample characteristics, volume, concentration, and the solvent used property, the components to be obtained, and their dielectric constants, equipment power, temperature, extraction time, pressure, pH, particle size, among others. These parameters are essential for an efficient extraction process to ensure minimum degradation and thus obtain higher antioxidant compounds yield. However, these methods are applicable to obtain temperature stable compounds. Currently, the microwave-assisted extraction has been used on an industrial scale as it increases the processes viability and ensures bioproduct of higher quality (Angoy et al., 2018; Périno et al., 2016; Ruth Alara et al., 2018).

12.3.5 Ultrasound-assisted extraction

This technique is well known for being a green technology in terms of solvent and energy consumption. This extraction method is considered fast, efficient, easy to handle, and increase the extracts yield, since the mass transfer is intensified using ultrasonic vibration, causing cellular rupture, which facilitates the solvent or reagent solution penetration, thereby increasing the extraction rate (Chemat et al., 2016; Tiwari, 2015; Vinatoru, 2015).

Physical parameters such as equipment power and frequency, waves amplitude, intensity and length, pulsating bubble acoustic pressure and mean diameter, reactors temperature, shape, and size influence the extraction efficiency (Pingret and Chemat, 2013; Suslick et al., 2011). The most usual ultrasonic devices used for bioactive compounds extraction are bath type and probe type system.

In the case of ultrasonic bath devices with transducers located at the ultrasonic bath base, which convert energy into ultrasound where the sample or mixture of sample and solvent is placed in a water bath under a specific ultrasonic power and time. While the equipment that uses the probe, the sample or mixture is placed in a vessel and the probe is introduced into the sample, and then the ultrasound is applied (Albuquerque et al., 2016; Priego-Capote and Luque de Castro, 2004).

Ultrasound-assisted extraction of natural products is performed in an ultrasonic bath having transducers (Kulkarni and Rathod, 2014). However, the probe-type systems increases the effects and allows a high possibility of process parameters in relation to the ultrasonic baths (Ahmad-Qasem et al., 2013; Bimakr et al., 2012; Chen et al., 2018).

The ultrasound-assisted extraction process is adjustable and polar and nonpolar solvents can be used at various temperatures. The solvent selection and its polarity is based on the target metabolites solubility knowledge to obtain maximum extraction efficiency and selectivity required (Alonso-carrillo et al., 2017). Ultrasound extraction is widely

used to obtain antioxidants and their biological activities depend process parameters. Extraction with solvents judged "green," such as water and ethanol, become more suitable for this type of process. The ultrasound application to optimize the phenolic compounds yield with high antioxidant activity contributes to industrial applications in a positive way (Luo et al., 2018).

12.4 Main methods for in vitro antioxidant activity quantification

12.4.1 TEAC method

TEAC test is established on antioxidants potential to scavenge the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical cation, which is characterized by having blue–green coloration. By adding antioxidant compounds to this solution, the ABTS•+ radical reverts to its original stable ABTS form, which is colorless. The discoloration indicates the radical species decomposition by their reduction after the antioxidant addition (Rice-Evans et al., 1996; Tafulo et al., 2010). Decomposition produces a decrease in absorbance, which corresponds quantitatively antioxidants capacity. Absorbance readings of the radical and antioxidant mixture at different times are plotted along with the blank readings (Pérez-Jiménez and Saura-calixto, 2006). ABTS•+ radical cation has wavelength absorption spectrum at 660–820 nm (Somogyi et al., 2007). Allows hydrophilic and lipophilic compounds antioxidant capacity determination, representing an advantage over other methods (Alam et al., 2013; Durmaz, 2012).

12.4.2 FRAP method

One of the mechanisms for determining the antioxidant capacity, widely applied to evaluate plant extracts functional potential, is the ferric reducing antioxidant power test, the FRAP, have its origin in the production of Fe^{2+} from Fe^{3+} ion reduction present in the 2,4,6-tripyridyl-s-triazine complex (TPTZ). When the reaction occurs, there is an adaptation in the reaction mixture hue from light purple to intense purple whose absorbance can be taken at 595 nm wavelength. The greater the color absorbance or intensity, higher the antioxidant potential. FRAP antioxidant assay is water and buffered solutions soluble (Antolovich et al., 2002; Benzie and Strain, 1999; de Castro and Sato, 2015; Firuzi et al., 2005). The test was developed to determine iron reduction in biological fluids and aqueous solutions. Later, this method was adapted by other authors. However, the commonly used protocols high reagents and extracts volumes, which can make the method impracticable for samples or extracts that are difficult to obtain or have low yield (Pulido et al., 2000; Xu et al., 2017).

12.4.3 DPPH method

The in vitro test using DPPH• radical is one of the most used, since it is a practical and stable method. DPPH• radical reduction is monitored at a typical wavelength until

the reaction reaches a stable state. DPPH• radical absorbs a 515 nm and exhibits violet-intense coloration (Aruoma, 2003; Brand-Williams et al., 1995). Test involve DPPH• radical reduction by the antioxidant action of some plant extract compounds, through the donation of an electron to the radical (SET—single electron transfer), stabilizing it and changing the color of the reaction medium (Musa et al., 2016).

A test on the antioxidant nature influences in the different kinetics reaction evolution. Therefore, depending on the compound, the DPPH• reaction may occur slowly or rapidly. The free radical produced can be subjected to other reactions that control the DPPH molecules reduced number by a reductant molecule. The advantage of this test is the availability of the DPPH radical commercially, facilitating its use, since there is no need to generate the radical (Alam et al., 2013; Brand-Williams et al., 1995). The DPPH method is widely used because it is a method that has a stable and simple reaction system (Ghosh et al., 2014).

12.4.4 ORAC method

ORAC test is established on the capture a specific radical (peroxyl), generated from 2,2-azino-bis (2-methylpropionamidine dihydrochloride) organic molecule (AAPH). The radicals attack the oxidized fluorescein molecule that no longer emits fluorescence, thus producing a decrease in it. In antioxidants presence, peroxyl radical reaction with fluorescein causes it to maintain the same fluorescence emission. The ORAC method has been widely used in plants, fruits, and vegetables recent studies (Alvarez-suarez et al., 2009; Huang et al., 2002; Krishnaiah et al., 2010; Ou et al., 2001).

ORAC mechanism originally established by Cao et al. (Cao et al., 1993) is relatively simple and sensitive, measures the antioxidant ability to scavenge peroxyl radicals. However, due to some limitations in the use of this probe (photosensitivity, high variability, among others), Ou et al. (Ou et al., 2001) decided to use fluorescein (FL) instead of B-PE, producing the assay cheaper, reproductive, and robust. The decrease in FL fluorescence indicates the damage extent caused by its reaction with the generated radicals. The test result is calculated by utilizing the area under the fluorescence curve of the sample and a "control" sample, without antioxidant (Amorati and Valgimigli, 2018; Huang et al., 2002; Ou et al., 2001).

12.5 Considerations

Environmentally safe extraction technologies are those that comply with the main principles in relation to obtaining of bioactive compounds. However, most antioxidant compounds are unstable and susceptible to degradation and the main factor associated with its degradation is the temperature, therefore, among the extraction methods studied, the supercritical extraction method, where mild conditions are used, providing low processing temperatures, avoiding thermosensitive compounds degradation. In addition,

the solvent can be effortlessly removed from the solute, presenting minor energy expenditure when compared to other methods and allowing a higher extraction rate. In relation to the methods with in vitro antioxidant activity quantification, DPPH and TEAC methods are mostly used for their simplicity and their reaction system.

Acknowledgments

The authors Marielba A.R. Salazar, Vânia M.B. Cunha, Marcilene P. Silva, Flávia C.S. Pires, Ana P.S. Silva, Maria C.R. Ferreira, Sérgio H.B. Sousa thank CAPES and CNPq - Brazil for the doctorate's and master's scholarship.

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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

(Efeito Citotóxico do Extrato Supercrítico de Cipó-pucá (*Cissus sicyoides* L.) em Glóbulos Vermelhos humanos e como Anti-inflamatório em Lesão Medular em Ratos Adultos)

Reprinted from:

SALAZAR, M. A. R.; URBINA, G. R. O.; CUNHA, V. M. B.; BEZERRA, F. W. F.; DIAS, M. N. C.; SANTOS, I. R.; TEIXEIRA, B. J. B.; COSTA, W. A.; GOMES-LEAL, W.; SILVA E SOUZA, J. N.; DA SILVA, S. H. M.; CARVALHO JUNIOR, R. N. 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. **The Journal of Supercritical Fluids**, v.169, p. 105105, 2021. DOI: 10.1016/j.supflu.2020.105105.

J. of Supercritical Fluids 169 (2021) 105105

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

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

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

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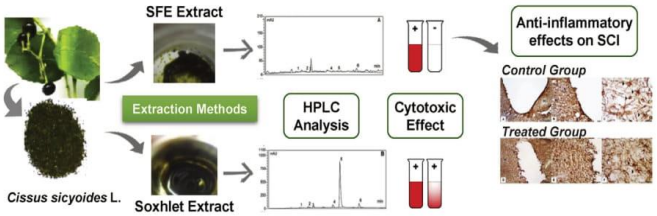
HIGHLIGHTS

- The global yield of *C. sicyoides* supercritical extract was 3.03 ± 0.10 %.
- In *C. sicyoides* extracts, glycosylated flavonoids were identified by HPLC.
- The supercritical extract did not present rupture of red blood cell membranes.
- The supercritical extract showed anti-inflammatory effects in the SCI model.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 March 2020
Received in revised form
22 September 2020
Accepted 4 November 2020
Available online 12 November 2020

Keywords:
Cissus sicyoides L.
Supercritical technology
HPLC
Cytotoxicity
Anti-inflammatory effect

ABSTRACT

Cissus sicyoides L. is a plant that has great anti-inflammatory activity, due to the presence of phenolic compounds. The objective of this work was to identify such substances by HPLC and to evaluate the cytotoxic effect on human red blood cells, of the *C. sicyoides* extract obtained by supercritical extraction, using CO_2 and EtOH as co-solvent, in comparison to the extract obtained by conventional extraction (Soxhlet), as well as, to evaluate its anti-inflammatory effect on spinal cord injury (SCI) in adult rats. In HPLC analysis, the UV/Visible spectral data revealed compounds with absorption bands in wavelengths characteristic of glycosylated flavonoids. The supercritical extract did not show cytotoxic effect, as there was no rupture of red blood cell membranes. The *in vivo* test demonstrated reduction in cell concentration in the lesion surrounding area in treated animals, suggesting an anti-inflammatory effect of the supercritical extract on the SCI model.

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https://doi.org/10.1016/j.supflu.2020.105105
0896-8446/

1. Introduction

Spinal cord injury (SCI) lead to neurological complications and, eventually, to paraplegia or quadriplegia. These extremely debilitating conditions are the main contributors to morbidity. SCI consists of two phases: the initial impact that causes primary injury, followed by a prolonged secondary injury, which can last for years. Vascular disorder is also a notable feature of SCI [1]. It usually results from a sudden and traumatic impact on the spine that fractures or displaces the vertebrae, and occurs more often in adults. Spinal cord injuries are mainly caused by traffic accidents, falls or violence. Therefore, it is essential to unravel the cellular and molecular mechanisms of SCI and develop new treatments for this devastating condition [2]. Due to the absence of an effective treatment for SCI, several studies have been carried out with the objective of investigating the therapeutic effects of medicinal plants on lesions of the central nervous system.

Various extraction methods are used to obtain plant extracts. However, the inconveniences associated with conventional extraction processes, such as the use of organic solvents, toxic to human health and environment, and the use of high temperatures, that can degrade thermosensitive compounds, have motivated the search for environmentally safe extraction techniques [3,4]. Supercritical fluid extraction (SFE) is an alternative to conventional methods for extraction of bioactive compounds from plant matrices, such as phenolic compounds, which are substances of great interest due to their high potential in different applications, since they have the ability to eliminate free radicals, and can play an important role in the treatment and/or prevention of certain diseases [5,6]. Supercritical carbon dioxide ($scCO_2$) is the most applied solvent in SFE, and according to the solubility and selectivity of the target compounds, cosolvents can be used in the extraction with $scCO_2$, such as EtOH, which may be suitable for food and pharmaceutical applications [7,8]

In previous studies, the anti-inflammatory and neuroprotective effects of plant extracts, obtained by supercritical extraction, were evaluated using an experimental model of ischemia in adult rats, such as cipó-pucá (*Cissus sicyovides* L.) [9], black sesame (*Sesamum indicum* L.) [10], and copaíba (*Copaifera* sp.) [11] and maravuvuia (*Croton matourensis* Aubl.) extracts [49]. They promoted better tissue preservation and decreased pathological recruitment of inflammatory cells. *C. sicyoides* is a plant that belongs to the Vitaceae family, and is normally found in the Amazon region, with great occurrence in the Northeast of Pará state (Brazil) [12,13]. Its therapeutic and anti-inflammatory actions are associated with the presence of phenolic compounds, which are the main responsible for the pharmacological effects of this plant [14–17].

The objective of this work was to identify the phenolic compounds by HPLC and to evaluate the cytotoxic effect, on human red blood cells, of *C. sicyoides* extract obtained by supercritical extraction, using CO_2 as solvent and EtOH as co-solvent, in comparison to the extract obtained by conventional extraction (Soxhlet) and, finally, to evaluate its anti-inflammatory effect on SCI in adult rats.

2. Material and methods

2.1. C. sicyoides *extract*

The leaves and stems of *C. sicyoides* were collected in the medicinal plant garden, located in the city of Salinópolis (06º20′58.8 ″S – 473º32′30.6 W, Pará, Brazil). Then, the raw material was pretreated and stored.*C. sicyoides* leaf and stem extracts were obtained by SFE based on the work developed by Salazar et al. [9]. The process conditions were: 50 °C, 400 bar, and 10 % EtOH. These conditions were selected so that a high content of phenolic compounds could be obtained as a result of the high density of CO_2 + EtOH mixture used (953 kg/m^3). The extractions were performed in a Spe-ed^{TM} SFE (Applied Separations, model 7071, USA), previously described by Oliveira et al. [18], equipped with a co-solvent pump (LabAlliance, model 1500, USA). The volume of the extractor vessel used was 100 mL, and the solvents were CO_2 (99.9 %, White Martins®, Brazil) and EtOH (99.8 %, Neon, Brazil). The mass of raw material used was 0.010 kg, with static extraction time of 0.5 h, 3 h of dynamic extraction, and mass flow rates of CO_2 and EtOH of 4.52 g/min and 0.53 g/min, respectively. Ten extraction assays were performed. Conventional extraction, to obtain the ethanolic extract, was carried out according to the method described by the Brazilian Pharmacopoeia [19], in which EtOH (99.5 %, Dinamica®, Brazil) at 1:10 (m/v) ratio (*C. sicyoides*:solvent), was refluxed for 3 h in a 500 mL Soxhlet apparatus. Three extraction assays were performed. The supercritical and ethanolic extracts of *C. sicyoides* were subjected to evaporation under vacuum at 40 °C using a CentriVap (Labconco®, USA) for EtOH removal and, finally, were weighed using an analytical balance (Quimis®, model AS 210, Brazil). The extraction yields were expressed as percentage (%), and calculated from the mathematical ratio between the extract mass and the mass of raw material used, on dry basis (db). The extracts were stored at 5 °C, in a bottle, protected from light and oxygen.

2.2. Solid phase extraction (SPE)

Solid phase extraction (SPE) of the supercritical and ethanolic extracts of *C. sicyoides* was carried out according to the procedure described by Irakli et al. [20], using C18 cartridges (Strata, 55 μm, 70 A, 200 mg/3 mL, Phenomenex, USA). They were packed with 3 mL of methanol (99.9 %, Tedia®, USA) and 3 mL of ultrapure water, acidified with 1% formic acid (85 %, ISOFAR, Brazil). Then, 10 mg of each extract was diluted in methanol (99.9 %, Tedia®, USA) at 33 %, placed in the C18 cartridge, and eluted. After that, 3 mL of acidified ultrapure water was poured on the cartridge. The analytes adsorbed were eluted with 70 % acidified methanol (99.9 %, Tedia®, USA). The final fraction was dried under vacuum at 40 °C, using a CentriVap (Labconco®, USA). Later, they were analyzed by HPLC-DAD.

2.3. Identification of phenolic compounds by high performance liquid chromatography (HPLC)

The identification of phenolic compounds of *C. sicyoides* supercritical and ethanolic extracts was carried out according to the methodology proposed by Beserra et al. [16], using a high performance liquid chromatography system (HPLC) (Thermo Fisher Scientific, series UltiMate^{TM} 3000, Germany), consisting of a diode array detector (DAD – 3000), equipped with a quaternary pump (LPG – 3400RS) and automatic sample injector. Chromatographic separation was performed using a C18 reverse phase column (250 × 4.6 mm, 4 μm particle size, Phenomenex, USA). The extracts were dissolved in 80 % methanol solution (99.9 %, Tedia®, USA), at concentration of 1 mg/mL, and filtered with syringe filters (0.45 μm pore size, Filtrilo®, Brazil). The injection volume was 20 μL of samples and the separations were made with flow rate of 0.6 mL/min, at temperature of 25 °C. The mobile phases used were ultrapure water (solvent A) and methanol (99.9 %, Tedia®, USA) (solvent B), both acidified with 0.1 % formic acid (85 %, Isofar, Brazil), with proportion of 5–100% B in A, for 70 min. Chromatograms were recorded at 254 nm. The identification of compounds was based on retention times and spectra, compared to external standards (quercetin 3 – rhamnoside and quercetin) (Sigma-Aldrich®, Brazil).

2.4. Cytotoxicity of the plant extract

To evaluate the cytotoxicity effect of *C. sicyoides* extracts on human red blood cells, the hemolytic activity was developed according to the methodology described by Sharma & Sharma [21] and Khan et al. [22]. 2 mL of human red blood cells were centrifuged 3 times (Kubota®, model 2100, Japan), at 2500 rpm/10 min in phosphate buffered saline (PBS) (Sigma-Aldrich®, USA), and then, a suspension of red blood cells was obtained at 10 % concentration. 2.5 mL aliquots of red blood cell suspension were prepared and added to 500 μL of the extract at concentrations of 250, 500, 1000, and 2000 mg/L. Three 1 mL aliquots were generated in test tubes, which were placed in a water bath (Fanem®, model 102/6-R, Brazil) and incubated at 37 °C. Then, 40 μLl of 2.5 % glutaraldehyde (25 %, Vetec®, Brazil) was added at 0, 20, and 40 min intervals to end the reaction. After these intervals, the tubes were centrifuged at 2500 rpm for 15 min, the supernatant was removed, and the released hemoglobin was measured by absorbance spectrophotometry (ABS), using a spectrophotometer (Thermoplate, model TP-Reader, USA), at 492 nm. As a positive control, 0.2 % Triton X-100 (Sigma-Aldrich®, USA), diluted in PBS (100 % hemolysis) was used, and as a negative control, dimethyl sulfoxide (99.5 %, Nuclear, Brazil) (extract diluent) (diluted in PBS 0% hemolysis). The analysis was performed in triplicate, and the means and standard deviations (SD) were calculated. The percentages of hemolysis were calculated using equation Eq. (1).

$$\%\,\text{hemolysis} = \frac{ABS_{extract} - ABS_{\text{PBS}}}{ABS_{\text{Triton}} - ABS_{\text{PBS}}} \times 100 \quad (1)$$

2.5. Anti-inflammatory effect of the extract obtained by SFE on spinal cord injury

2.5.1. Animals and experimental groups

Seven adult male Wistar rats, weighing between 250 and 390 g, were used. They were housed in standard cages with food and water provided *ad libitum*. The animals were divided into two experimental groups: control group (n = 3) and treated group (n = 4), with survival time of 7 days. After the surgical procedure to induce spinal cord injury, the control group consisted of animals that received only a 5% tween solution intraperitoneally (i.p.). The animals of the treated group received a solution containing supercritical extract of *C. sicyoides* at concentration of 50 mg/kg [23]. The experimental procedures were carried out in accordance with the guidelines established by the Research Ethics Committee of Federal University of Pará (CEPAE-UFPA 137-13), in compliance with the international standards suggested by the National Institute of Health (NIH, USA) and Society for Neuroscience.

2.5.2. Surgical procedures and treatment of experimental groups

The animals were anesthetized with ketamine hydrochloride (Vetanarcol®, Köning, 72 mg/kg) and xylazine hydrochloride (Kensol®, Köning, 9 mg/kg) through intraperitoneal injection. Then, they were positioned on a simple vertebral stabilizer. A 2 cm incision was made in the dorsal region to expose the spine. The laminectomy was performed at T3-T8 level with the aid of a dental drill, and the hemisection of the spinal cord was performed using a scalpel blade (N-12). The dorsal incision was closed using suture (black monofilament - TECHNOFIO - Nylon 4–0). During surgery, the body temperature was maintained between 36 and 37 °C due to exposure to an incandescent lamp. After the procedure, the animals were accommodated in standard cages with food and water supplied *ad libitum*, and controlled temperature of 24 °C. During the 7 days related to the survival time, a daily dose of the respective treatment was applied - supercritical extract of *C. sicyoides* (treated) or 5% tween solution (control) - through intraperitoneal injection using an insulin syringe (BD Ultra Fine Mini, 1.0 mL, and 6 mm needle).

2.5.3. Perfusion and histological processing

The animals were sacrificed by intraperitoneal injection of ketamine hydrochloride (Vetanarcol®, Köning, 90 mg/kg) and xylazine hydrochloride (Kensol®, Köning, 10 mg/kg). They were perfused with 400 mL of 0.9 % heparinized saline solution, and 400 mL of 4% paraformaldehyde in phosphate buffer, which were applied through the left ventricle. The right atrium, at the same time, was cut to remove the injected fluid during 40–60 min. The injured spinal cords of each animal were extracted, maintained in formalin for 24 h and transferred to cryoprotection solutions with concentrations of 10–30% sucrose. Then, 5 μm-thick cross and longitudinal sections were obtained with the aid of a cryostat (Leica CM 1850).

2.5.4. Histopathological analysis

The analysis of the sections of each experimental group was performed using an optical microscope (binocular Eclipse Model - E200MVR) with cresyl violet stain. The images of the stained areas were obtained with a digital camera (Moticam-5500-5 M pixels) coupled to an optical microscope (Nikon, elipse 50i).

2.5.5. Immunohistochemical analysis

The immunohistochemical analysis aimed to identify activated and non-activated microglia by staining with anti-Iba1 antibody (Wako, 1:1000) [24], and also identify astrocytes by staining with anti-glial fibrillary acidic protein (GFAP) antibody (Dato®, Z0334, 1:1000) [25]. The sections analysis of each experimental group was performed using an optical microscope (Binocular Eclipse Model - E200MVR). The images of the marked areas were obtained with a digital camera (Moticam-5500-5 M pixels) coupled to an optical microscope (Nikon, elipse 50i).

3. Results and discussion

3.1. Extraction yield

The overall yield of SFE at 50 °C/400 bar and 10 % EtOH, and density of 953 kg/m^3 was 3.03 ± 0.10 % (db), with about 0.302 ± 0.01 g of *C. sicyoides* extract obtained in each procedure, (total of 3.02 g). Soxhlet extractions were performed as a reference extraction, being the yield of ethanolic extract 10.93 ± 0.09 % (db), with 0.768 ± 0.01 g collected in each extraction (total of 2.30 g), with sufficient quantities to perform the analyses described in this work. These results are in accordance with yield values previously found in SFE (3.21 %) and Soxhlet extraction (11.02 %) of *C. sicyoides* [9]. However, in studies on the leaves of the plant using other conventional methods of extraction and solvents, yield values lower than those obtained in this study were reported. Xu et al. [27] found a yield of 4.40 % in the methanolic extraction and Garcia et al. [43] achieved a yield of 8.82 % in the aqueous extraction. According to other studies, yield values similar to those found in this work were achieved for other plants. For burdock leaves (*Arctium lappa*), extraction yields of 4.84 % and 11.40 % were obtained with $scCO_2$ and EtOH and by Soxhlet extraction using EtOH, respectively [44]. Also, working with eucalyptus leaves (*Eucalyptus globulus*), Rodrigues et al. [45] found yields of 3.16 % and 25.90 %, obtained with $scCO_2$ and 5% EtOH and by Soxhlet extraction with EtOH, respectively. These results confirm that, in SFE, the use of small amounts of EtOH as a co-solvent has a positive effect on the extraction yield [46,47]. In general, the yield obtained by Soxhlet was higher than that obtained by SFE, however, Soxhlet extraction has certain disadvantages, such as low selectivity and obtaining extracts with various undesirable substances.

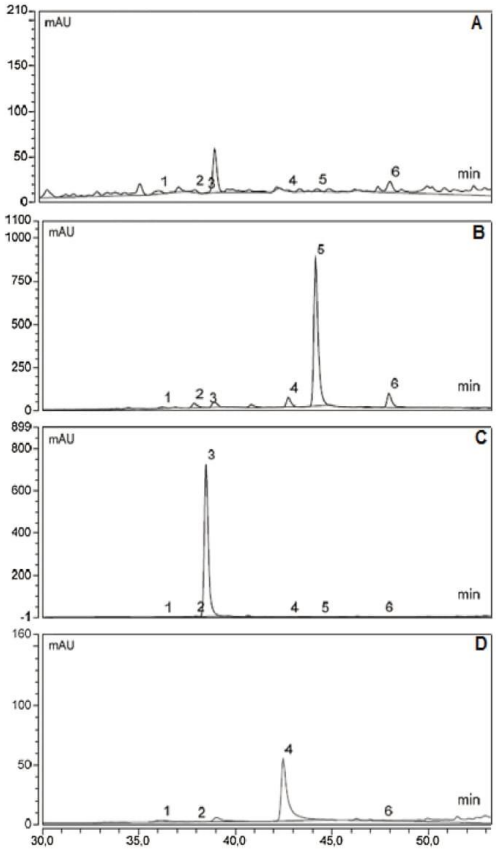

Fig. 1. HPLC of *C. sicyoides* extract: (A) obtained by SFE; (B) obtained by Soxhlet; (C) quercetin 3 – rhamnoside standard; (D) quercetin standard; mobile phase: ultrapure water and metanol, acidified with formic acid; the chromatograms were recorded at 254 nm; peaks have been numbered according to the elution order.

3.2. Identification of phenolic compounds by high performance liquid chromatography (HPLC)

The *C. sicyoides* extracts obtained by SFE and the ethanolic extract obtained by Soxhlet showed, in qualitative terms, a dark green color indicating the presence of chlorophyll. Therefore, during sample preparation, SPE was performed with the purpose of removing interfering compounds, such as chlorophyll and, thus, continuing with the development of chromatographic analysis and allowing the identification of target compounds.

In Fig. 1, the chromatograms of the extracts are presented. The identified peaks are numbered from 1 to 6, and the peaks heights show the relative concentrations of the compounds present in the extracts. It is observed that the substances showed the same retention times as the available standards (quercetin 3-rhamnoside and quercetin), however, they showed absorption spectra similar to the external standards used. The absorption spectra are fingerprints of specific classes of compounds, which allow their identification. The UV/Visible spectral data revealed, for peak 2, two absorption bands of wavelengths (λ) around 254 and 346 nm; for peak 3, λ of 269 and 338 nm; for peak 4, λ of 268 and 335 nm; and for peak 5, λ of 255 and 350 nm, which correspond to wavelengths characteristic of glycosylated flavonoids (see the Supplementary material S1). This is in accordance with the studies reported by Beltrame et al. [26], Xu et al. [27], and Beserra et al. [16], who found different glycosylated flavonoids derived from quercetin in extracts from the aerial parts of *C. sicyoides*, obtained by conventional extraction. It should be noted that the compounds identified showed the same retention time as the standards. This is a parameter based on the molecule polarity, influenced by the degree of glycosylation and the nature of sugars present in molecules [28,29].

Table 1
Hemolytic property of *C. sicyoides* extract.

	Percentage of red blood cells (%)			
	Concentrations of compounds (mg/L)			
	250	500	1000	2000
SFE extract	0.74 ± 0.04	1.39 ± 0.09	3.04 ± 0.05	5.02 ± 0.12
Soxhlet extract	2.84 ± 0.02	8.51 ± 0.03	18.36 ± 0.08	42.07 ± 0.54

All the experiments were performed in triplicate and data is presented as mean ± SD.

The results obtained confirm that $scCO_2$ can extract compounds with large molecular weight, or polar ones, with increasing pressure and solvent density. In addition, low polarity can be improved with the use of polar co-solvents, which increase its solvation power [30–32]. Comparing SFE results with the Soxhlet ones, in relation to phenolic compounds, the ethanolic extract, obtained by Soxhlet, presented a better identification of such substances. Salazar et al. [9] identified, in ethanolic extract of *C. sicyoides*, a content of phenolic compounds (150.19 mg GAE/g de extract) higher than that obtained by $scCO_2$ + EtOH (65.20 mg GAE/g de extract), measured by the spectrophotometric method with Folin-Ciocalteu. The authors suggested that the differences are due to high polarity, quantity, recycling, and/or reflux of EtOH, which may improve the extraction of these compounds.

In the polyphenolic profile of *C. sicyoides*, quercetin, kaempferol, and its various isomers are found among the flavonoids [27,33]. The biological activities attributed to*C. sicyoides*, such as anti-inflammatory, anti-rheumatic, anti-epileptic, anti-hypertensive, antibacterial, anti-flu, antipyretic, antioxidant, anti-allergic, and anti-diabetic properties are mainly associated with the presence of quercetin and quercetin 3-rhamnoside in the plant [12,15,40]. The action mechanism of quercetin has been associated with a decrease in reactive oxygen and nitrogen species (ROS/RNS). These compounds prevent or delay oxidative stress, which enable the prevention and/or treatment of several chronic diseases [34,35]. Oxidative stress is considered a state of imbalance, in which excessive amounts of ROS/RNS exceed the capacity of endogenous antioxidants, leading to oxidation of a variety of biomacromolecules. Exogenous antioxidants, such as phenolic compounds, have the function of increasing or protecting the antioxidant defense in biological systems [4].

3.3. Effect of cytotoxicity of the plant extract

Hemolysis represents the rupture of integrity of the red blood cell membrane, causing hemoglobin release. Therefore, hemolysis is manifested by the presence of free hemoglobin in the erythrocyte suspension, such as plasma or additive solutions [36,37]. Table 1 shows the hemolytic property of *C. sicyoides* extracts, in which it was possible to note that the supercritical extract did not show hemolysis. Whereas in the ethanolic extract, obtained by Soxhlet, hemolysis of 42.07 % was evidenced in concentration of 2000 mg/L. Data are expressed as a percentage of hemoglobin release compared to 100 % hemolysis of the same number of cells using Triton 100-X (positive control). The mechanical stability of the erythrocytic membrane is an indicator of the effect imposed by var-

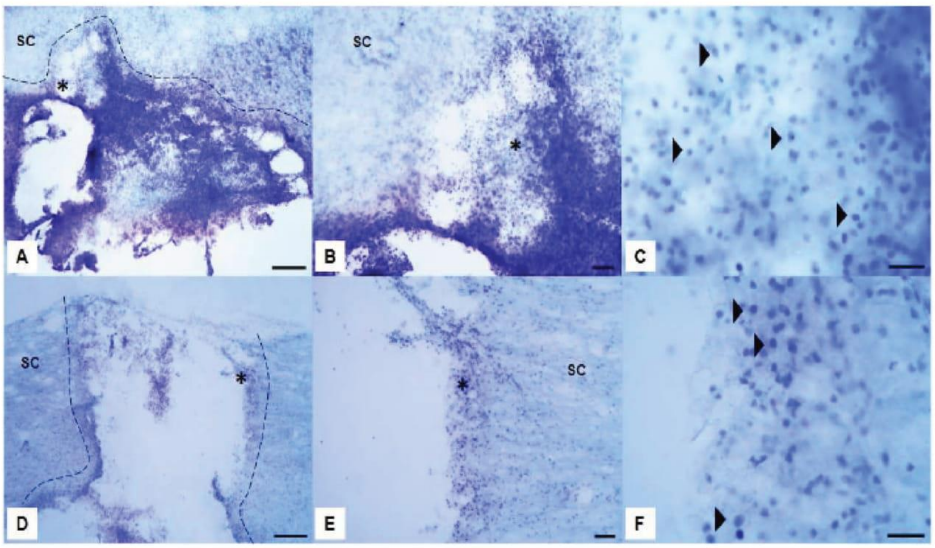

Fig. 2. Histopathological analysis of SCI areas by cresyl violet staining: (A—C) control group; (DF—) treated group; (→) arrows point to inflammatory cells (C, F); (*) asterisks indicate the expanded area of the image; (SC) spinal cord; the dashed area delimits the injury area and the cell infiltrate present on the lesion area; with increase: A, D x 40; B, E x 100; C, F x 400.

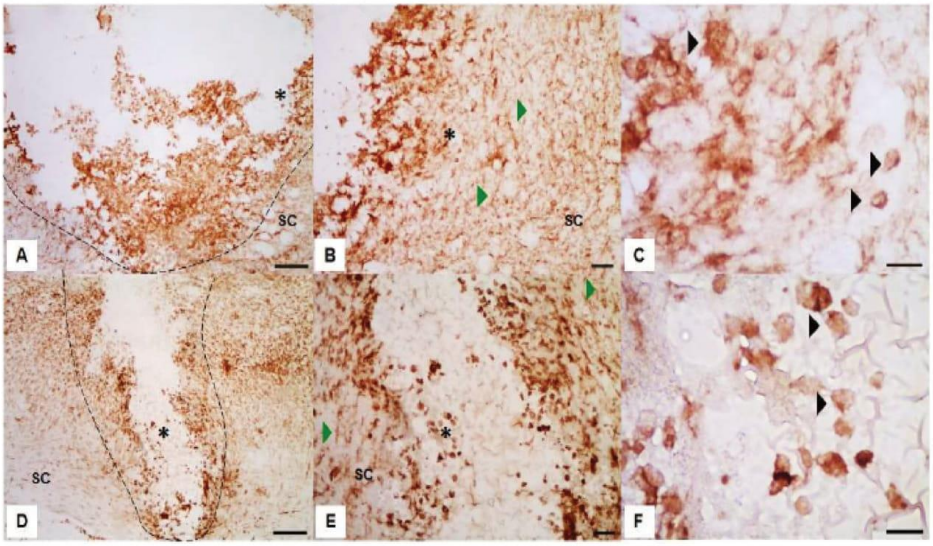

Fig. 3. Immunohistochemical analysis of the injured area by immunolabeling of activated and nonactivated microglia: (A—C) control group; (DF—) treated group; (→) green arrows point to non-activated microglia (B, E); (→) black arrows point to activated microglia (C, F); (*) asterisks indicate the expanded area of the image; (SC) spinal cord; the dashed area delimits the injury area and concentration of activated microglia present in the site; with increase: A, D x 40; B, E x 100; C, F x 400.

ious compounds for cytotoxicity determination, and depends on its physical and structural properties [21].

As this is a study to obtain extract for biological application, the use of ethanolic extract is not considered adequate, according to the cytotoxicity results, based on hemolytic activity. The cytotoxic effect of *C. sicyoides* ethanolic extract can be comparable to that of amphotericin B, which is a relatively toxic drug, and even though it was used in low concentrations (0.25 to 8.0 mg/L), an 8.64 to 46.11 % hemolysis has been reported [22]. Therefore, these facts were the motivation for the development of biological tests only in the group of treated animals, using a solution containing the supercritical extract of *C. sicyoides*. Supercritical technology is promising in obtaining high quality extracts, without cytotoxic effects, being suitable for the recovery of bioactive compounds from natural sources, which can be used for biological tests and in the development of pharmaceutical products [50,51].

In *in vivo* toxicological and pharmacological studies with *C. sicyoides* extracts, Vasconcelos et al. [39] reported the presence of liver

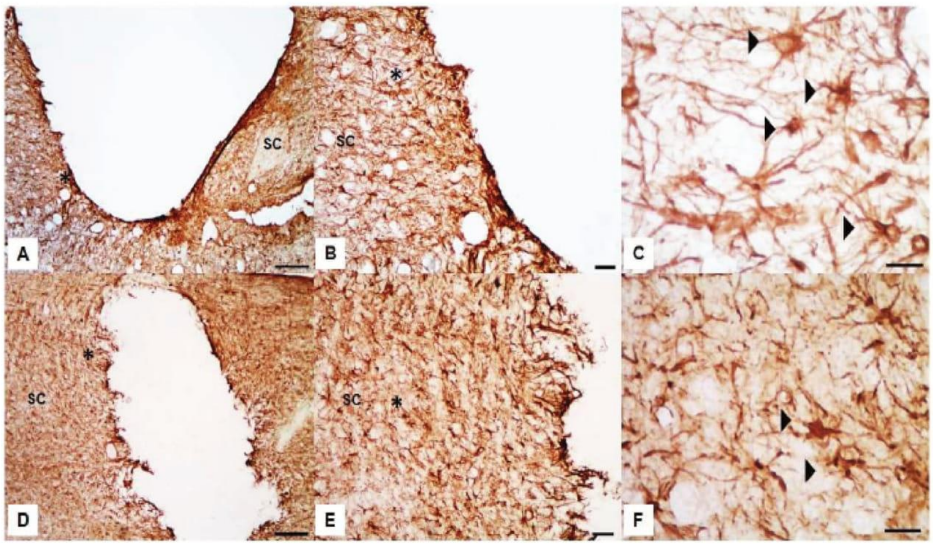

Fig. 4. Immunohistochemical analysis of the injured area by astrocyte immunolabeling: (A—C) control group; (DF—) treated group; (→) arrows point to astrocytes visualized near the lesioned area (C, F); (*) asterisks indicate the expanded area of the image; (SC) spinal cord; with increase: A, D x 40; B, E x 100; C, F x 400.

changes in Wistar rats, after being given doses of 5.0 g/kg orally, and 2.0 g/kg *via* intraperitoneal, of extract obtained with 35 % EtOH, demonstrating the toxicity of this extract. However, Ferreira et al. [40] reported that a single oral administration of 5000 mg/kg of extract obtained with methanol by maceration did not produce any signs or symptoms of toxicity. In addition, Beserra et al. [16] indicated that oral administration of a single dose of 5 g/kg of extract obtained with 70 % EtOH, by percolation, did not produce visible symptoms or signs of toxicity.

3.4. Anti-inflammatory effects of the extract obtained by SFE on spinal cord injury

The experimental model used in the study was the hemisection of spinal cord (SC) in the thoracic segment of adult rats. Histopathological analysis of SC tissues used in the study was performed by cresyl violet staining. It was observed that the hemisection promoted formation of progressive cavitation, and increased inflammatory infiltrate at the site, a phenomenon that was identified in all experimental groups, as shown in Fig. 2. The treatment with *C. sicyoides* supercritical extract (50 mg/kg), for seven days, demonstrated an apparent reduction in cell concentration at the lesion surrounding area (Fig. 2D – F), when compared to the control group treated with 5% tween solution (Fig. 2A – C). The technique used for SCI was effective in promoting cavitation formation and intense inflammatory infiltrate in the place where the lesion was induced; therefore, it is a useful experimental model for the purpose of this study. According to Gomes-Leal et al. [41], SCI induce intense local inflammatory response, which results in activation of macrophages and microglia.

The evaluation of activated and non-activated microglia distribution in the injured area was done by immunolabeling of microglial cells with anti-Iba1 antibody. The presence of activated microglia (ameboid form) in the lesion was detected in all experimental groups, with inactive microglia (ramified form) being observed in the peripheral areas (Fig. 3). However, the distribution of activated microglia was limited to the lesion center in the treated group (Fig. 3D–F), while in the control group, the activation of these cells was identified both in the center and in the periphery of the lesion (Fig. 3A–C). Microglial cells have a branched form when they are at rest, and assume an amoeboid form when activated. They can have a negative or positive action on the injured spinal tissue by releasing free radicals, growth factors, and pro and anti-inflammatory cytokines, after SCI has occurred [25].

The concentration of astrocytes (star-shaped cells) around the injured region was visualized by anti-GFAP antibody immunolabeling. It is possible to observe an apparent decrease of cells in SC tissue of the treated group (Fig. 4D–F), in comparison with the control group (Fig. 4A–C), which demonstrates a probable influence of the treatment with *C. sicyoides* supercritical extract on the proliferation of astrocytes after SCI. In particular, an important component of SCI is ischemia-reperfusion injury (IRI), which triggers increasing inflammatory cascades, resulting from the activation of immune cells such as astrocytes, which participate in axonal and neuronal deficit [1].

The results demonstrate that the treatment with *C. sicyoides* supercritical extract was able to reduce the inflammatory response, demonstrated by the reduction of the number of inflammatory cells. These results suggest that the supercritical extract presented anti-inflammatory activity after SCI. In the study previously carried out by Salazar et al. [9], *C. sicyoides* supercritical extract obtained at 50 °C/400 bar with $scCO_2$ + 10 % EtOH was considerate to have anti-inflammatory and neuroprotective effects on an experimental model of focal cerebral ischemia. The action mechanisms involved are unknown. However, in their study, modulation of microglia activation was suggested to be involved. In the work developed by Taylor et al. [52], who aimed to identify the microglial topography in the brain after injury and its influence on chronic pain, the anti-Iba1 antibody was also used for the labeling of activated microglia, but with a longer treatment period using minocycline, which resulted in a better distinction between activated and non-activated cell distribution patterns. Therefore, for microglial activation analysis, labeling with Ed-1 and groups with longer survival times can optimize the results and clarify the effects of the treatment conducted in this work and in future studies.The analysis of the chemical composition of *C. sicyoides*

showed that the plant contains glycosylated flavonoids derived from quercetin. According to Kim et al. [53], flavonoids may present anti-inflammatory activity through antioxidant action, modulation of macrophages, lymphocytes, and neutrophils activation. Crespo et al. [54] reported that treatment with quercetin prevented the production of ROS. These results confirmed the differential protective effect of this flavonoid against the oxidative stress induced by pro-inflammatory stimuli in liver parenchymal cells. Also, Kumar et al. [55] demonstrated that the treatment with *Cissus quadrangularis* extract, a plant that also belongs to Vitaceae family, with an abundant presence of quercetin in its composition, reduced the serum level of TNF-α, and decreased oxidative stress and synovial expression of inflammatory and angiogenesis markers. These studies support the premise that the presence of phenolic compounds in the *C. sicyoides* supercritical extract may have influenced the reduction of cellular infiltrates and activated microglia in the region near the SCI.

4. Conclusion

In the analysis of *C. sicyoides* extracts by HPLC, the UV/Visible spectral data revealed compounds with absorption bands in wavelengths characteristic of glycosylated flavonoids. As this is a study to obtain extract for biological application, the use of ethanolic extract is not considered adequate, as it had cytotoxic effect. Therefore, the supercritical extract of *C. sicyoides* was suitable, because there was no rupture of red blood cell membranes. The *in vivo* test demonstrated reduction in inflammatory cells concentration in the lesion surrounding area, in treated animals, suggesting an anti-inflammatory effect on the SCI model.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

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

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.supflu.2020.105105.

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CAPÍTULO IV – PROPOSTA PARA O 2º ARTIGO

DETERMINAÇÃO DE COMPOSTOS FENÓLICOS, FLAVONOIDES E CAPACIDADE ANTIOXIDANTE DAS PARTES ÁREAS E DO EXTRATO SUPERCRÍTICO DE *Cissus sicyoides* L.

1 INTRODUÇÃO

Os antioxidantes são compostos que protegem o sistema biológico contra o efeito nocivo de processos ou reações que podem causar o estresse oxidativo. O consumo de antioxidantes na dieta pode produzir uma ação protetora contra esses processos oxidativos que ocorrem no organismo (XU *et al.*, 2017). Portanto, é importante para o organismo manter o equilíbrio entre os compostos oxidantes e os antioxidantes para estabelecer melhores condições fisiológicas no corpo. O estado de desequilíbrio leva ao estresse oxidativo, que desencadeia doenças crônicas, como as doenças cardiovasculares, diabetes, câncer e doenças neurodegenerativas (LOBO *et al.*, 2010; MARTEMUCCI *et al.*, 2022).

O uso de plantas medicinais com propriedades antioxidantes tem sido explorado por sua capacidade de tratar ou prevenir as doenças nas quais o estresse oxidativo parece ser uma das causas (KASOTE *et al.*, 2015; SHARIFI-RAD *et al.*, 2020). A *Cissus sicyoides* L. pertencente à família Vitaceae, é uma planta popularmente conhecida como cipó-pucá, insulina vegetal e uva brava, é utilizada no tratamento de diversas doenças, por apresentar atividade antidiabética, anti-inflamatória, antimicrobiana e antidiarreica (DROBNIK; DE OLIVEIRA, 2015; FEITOSA *et al.*, 2021). As diferentes partes da planta apresentam composição distinta. Os compostos presentes na folha e no caule são representados por carotenoides (α-caroteno e o β-caroteno) (DA SILVA *et al.*, 1996) e compostos fenólicos como os flavonoides (kaempferol e quercetina) (BESERRA *et al.*, 2016). Apenas em um estudo foi encontrada a avaliação da composição química dos frutos, os autores identificaram três antocianinas (delfinidina-3- rutinosídeo, cianidina-3-ramnosil-arabinosídeo e delfinidina-3-raminosídeo) (TOLEDO *et al.*, 1983). A descrição das partes da planta e a estrutura química dos principais componentes é mostrada na Figura 1 do Capítulo I.

Em relação à atividade antioxidante e anti-inflamatória atribuída à planta, Salazar *et al.* (2018) realizaram uma análise qualitativa da atividade antioxidante dos extratos supercríticos de folhas e caules de *C. sicyoides*. Os resultados do estudo confirmaram a presença de

constituintes químicos característicos com atividade antioxidante. Na determinação da atividade biológica do extrato foi realizado um ensaio *in vivo* utilizando um modelo de isquemia cerebral focal, onde foi demonstrado que o extrato tem um efeito neuroprotetor e anti-inflamatório. Salazar *et al.* (2021) no extrato supercrítico de *C. sicyoides* identificaram flavonoides glicosilados. Também, foi demostrado que o extrato não apresenta efeito citotóxico, e no ensaio *in vivo* realizado houve uma redução da concentração de células inflamatórias na lesão medular.

O processo de extração com fluido supercrítico (SFE – *Supercritical Fluid Extraction*) é um método seguro e ecologicamente correto, que pode ser eficiente tanto na obtenção de óleos e extratos a partir de matrizes vegetais, quanto na obtenção de um material residual desengordurado e/ou subproduto gerados da extração, ricos em compostos antioxidantes (CUNHA *et al.*, 2019; SILVA *et al.*, 2019).

Diante disso, o objetivo deste artigo é determinar o teor de compostos fenólicos, flavonoides e avaliar o potencial antioxidante do extrato de *C. sicyoides* obtido por extração supercrítica, utilizando CO_2 e EtOH como cossolvente, nas folhas e caules antes e após da extração, bem como nas flores e nos frutos, a fim de identificar a contribuição de cada parte da planta para a obtenção de compostos antioxidantes.

2 MATERIAIS E MÉTODOS

2.1 Matéria-prima

As partes aéreas (folhas, caules, flores e os frutos) de *C. sicyoides* foram coletadas no município de Belém, PA, Brasil (1°20'40.8"S 48°26'32.6"W, PA, Brasil). Uma exsicata encontra-se depositada no herbário do Museu Paraense Emílio Goeldi (MG 241.373). Após a coleta, a matéria-prima foi colocada em embalagens plásticas e transportadas até o Laboratório de Tecnologia Supercrítica (LABTECS) para a realização do presente estudo.

2.2 Pré-tratamento da Matéria-prima

As folhas, caules e as flores de *C. sicyoides* foram pré-tratadas, realizando a secagem a 45 ºC por 72 h em estufa de circulação de ar (Solab, Brasil) e moagem com um moinho de facas (Marconi, modelo ma 048, China). Os frutos verdes e maduros da planta, após a colheita foram selecionados e lavados em água corrente. Seguidamente, foram triturados em liquidificador e congelados. Posteriormente, foram liofilizados a -57 °C por 48 h em

liofilizador de bancada (Liotop, modelo L101, Brasil), logo foram embaladas a vácuo em embalagens de polipropileno e armazenadas a temperatura inferior a 5 ºC, até a análise química.

Na matéria-prima de folhas e caules utilizadas nas extrações, foi determinado o teor de umidade segundo o método de destilação do solvente imiscível de Jacobs (JACOBS, 1973). Posteriormente, para a análise da distribuição do tamanho de partícula foram utilizadas peneiras da série Tyler (Bertel, Brasil) de 20 a 60 mesh e o diâmetro médio geométrico foi determinado de acordo com o método recomendado pela ASAE Standart (ASAE, 1993). A densidade aparente (ρa) do leito foi calculada usando o volume do leito e a massa de amostra acomodada dentro dele, assim, a porosidade do leito (ε) foi calculada pela relação matemática da densidade real e aparente.

2.3 Extração com Fluido Supercrítico (SFE)

As extrações foram realizadas no Spe-ed™ SFE (Applied Separations, modelo 7071, USA). O Procedimento operacional foi realizado segundo o manual de operações da Applied Separations (2004), utilizando uma célula de extração com volume de 1 x 10^{-4} m^3 (altura de 0,1254 m e diâmetro interno de 0,0320 m), os solventes usados foram CO_2 (99,9 %, Veloxgas, Brasil) e EtOH (99,5 %, Dinamica®, Brasil) e a massa da matéria-prima utilizada foi de 10 g. Nos ensaios, a pressão, temperatura e percentual de cossolvente foram mantidos constantes a 50 °C/ 400 bar, e 10 % de EtOH (ρ_{CO_2+EtOH} = 953 kg/m^3) tendo em vista dados relatados por Salazar *et al.* (2018). Os tempos de extração estática e dinâmica foram de 30 min e 2 h, respectivamente e a vazão de CO_2 de 3 l/min. 5 extrações foram realizadas para coleta do extrato.

Os extratos foram submetidos a evaporação para remoção do EtOH. Em seguida, foram armazenados em frascos protegidos da luz, em temperatura inferior a 5 ºC. Os rendimentos das extrações foram calculados de acordo com a Equação 1.

$$X_{0\ B.S}(\%) = \frac{m_{extrato}}{m_{amostra} \times \left(1 - \frac{U_{amostra}}{100}\right)} \times 100 \qquad (1)$$

2.4 Extração Convencional por Soxhlet

As extrações foram realizadas de acordo com o método descrito na Farmacopeia Brasileira (2010), a partir de uma relação soluto-solvente (*C. sicyoides*:solvente) de 1:10 (m/v), utilizando-se 15 g de amostra para 150 ml de EtOH (99,5%, Dinamica®, Brasil) em aparelho Soxhlet de 500 ml. O período de extração foi de 3 h, os solventes foram removidos por evaporação. 3 extrações foram realizadas para coleta do extrato etanólico e, por último, o rendimento foi calculado em base seca (b.s.) pela Equação 1.

2.5 Determinação dos Compostos Antioxidantes

2.5.1 Preparo das Amostras

Para a preparo das amostras utilizadas nas análises, foi utilizado a procedimento descrito por CUNHA *et al*. (2019) os extratos e as amostras de folhas e de caules antes e após da SFE e das flores e dos frutos, foram dissolvidas em 25 ml de solução de EtOH (96 %, ACS Cientifica, Brasil) a 70 % (v/v). Estas últimas amostras foram homogeneizadas e centrifugadas por 20 min a 3000 rpm (Termo Multifige Scientific, modelo XR1, USA). Após a centrifugação, o sobrenadante foi filtrado em papel filtro e armazenado protegido da luz em temperatura inferior a 5 ºC para utilização nas análises seguintes.

2.5.2 Compostos Fenólicos Totais (CFT)

A determinação de CFT foi realizada utilizando o método de Folin-Ciocalteu segundo a metodologia descrita por Singleton; Orthofer e Lamuela-Raventós (1999) e Georgé *et al.* (2005) as amostras, foram dissolvidas em EtOH (96 %, ACS Cientifica, Brasil) a 7 % (v/v), (extratos de *C. sicyoides*, na concentração de 140 mg/l, para folhas e caules antes e após SFE, as flores e os frutos na concentração de 4000 mg/l). Em seguida, 0,5 ml destas soluções, foram submetidas à reação com 2,5 ml de Folin-Ciocalteu (Sigma-aldrich, Brasil) a 10 % (v/v) e 2 ml de solução de carbonato de sódio (99,5 %, Vetec®, Brasil) a 7,5 % (m/v). Após 60 min de incubação à temperatura ambiente e no escuro, as medidas das absorbâncias foram realizadas a 760 nm em um espectrofotômetro UV-VIS (BEL Engineering®, modelo UV-M51, Itália). O teor de CFT foi expresso em miligrama equivalente de ácido gálico (EAG) (98 %, Dinamica®, Brasil) por grama de amostra em b. s. (mg EAG/g de amostra) a partir da equação da curva de calibração ($y=0,0103x+0,0229$; com $R^2=0,9963$).

2.5.3 Flavonoides Totais (FT)

A determinação de FT foi realizada com o procedimento descrito por Dowd (1959) e Meda *et al.* (2005) onde 0,5 ml de solução etanólica de cloreto de alumínio ($AlCl_3$) (pureza 99,0 %, Fluka, Alemanha) a 2 %, foi misturada com o mesmo volume de amostra (extratos de *C. sicyoides*, dissolvidos em EtOH na concentração de 140 mg/l, para folhas e caules antes e após a SFE, as flores e os frutos na concentração de 4000 mg/l). Após 10 min, à temperatura ambiente e no escuro, a absorbância foi lida à 425 nm em espectrofotômetro UV-VIS (BEL Engineering®, modelo UV-M51, Itália). Para a quantificação foi utilizado como padrão a quercetina (pureza ≥ 95,0 %, Sigma-Aldrich, Brasil) para a curva analítica. O cálculo do teor de FT, foi expresso como mg de equivalentes de quercetina (EQ) por grama de amostra em b. s. (mg EQ/g de amostra) a partir da equação da reta ($y=0,0312x+0,0005$; com $R^2=0,9918$).

2.6 Avaliação da Capacidade Antioxidante

2.6.1 Método DPPH

A determinação da atividade antioxidante das amostras pelo método baseado na captura do radical DPPH• (DPPH - *2,2-Diphenyl-1-picrylhydrazyl*) foi realizada de acordo com o procedimento proposto por Brand-Williams; Cuvelier e Berset (1995) e Sánchez-Moreno; Larrauri e Saura-Calixto (1998). Para isso, foi preparada uma solução de DPPH (Sigma-Aldrich, Brasil) a 60 μM em EtOH (96 %, ACS Cientifica, Brasil). Em seguida, 3,9 ml desta solução foi adicionada em 0,1 ml de amostra em diferentes concentrações (extratos de *C. sicyoides*, dissolvidos em EtOH nas concentrações de 10, 7 e 6 mg/l, para folhas e caules antes e após SFE, flor e os frutos nas concentrações de 4, 3, 2 e 1 mg/l). Essas soluções foram mantidas à temperatura ambiente e no escuro. Após estabilização do t_{EC50} (tempo necessário para reduzir em 50 % a quantidade inicial do radical DPPH•) a absorbância foi medida a 515 nm utilizando um espectrofotômetro UV-VIS (BEL Engineering®, modelo UV-M51, Itália). Foi realizado o cálculo do EC_{50} (quantidade de antioxidante necessária para reduzir em 50 % a quantidade inicial do radical DPPH•) e a atividade antioxidante foi expressa como grama de amostra por grama de DPPH em b. s. (EC_{50} expresso em g de amostra/g de DPPH).

2.6.2 Método FRAP

A determinação da atividade antioxidante por meio da redução do ferro (FRAP – *Ferric Reducing Antioxidant Power*) foi realizada de acordo com o procedimento proposto por

Pulido; Bravo e Saura-Calixto (2000) e Firuzi *et al.* (2005). A solução FRAP foi preparada por adição de 25 ml de tampão acetato 3 M, com 2,5 ml de cloreto férrico hexa-hidratado 20 mM e 2,5 ml de uma solução de TPTZ (2,4,6-Tris (2-piridil) -s-triazina) a 10,0 mM. A partir da amostra foram realizadas diluições diferentes (4, 3 e 2 mg/l), em seguida uma alíquota de 90 µl de cada diluição da amostra foi misturada com 270 µl de água destilada e com 2,7 ml do reagente FRAP. Após 30 min de incubação a 37 °C, a absorbância foi medida a 595 nm em espectrofotômetro (BEL Engineering®, modelo UV-M51, Itália). Os valores foram expressos em µM sulfato ferroso/g de amostra (b.s.).

2.7 Análise Estatística

Todas as determinações foram realizadas em duplicata e os resultados foram expressos como a média ± desvio padrão. Para verificar a existência de diferença significativa nos valores de teor de CFT, FT e nos métodos *in vitro* para a determinação da capacidade antioxidante nas amostras, as médias dos resultados foram submetidas à análise de variância (ANOVA) e, quando significativas, comparadas pelo teste de Tukey a 5% de probabilidade, com auxílio do programa STATISTICA® versão 7.0 (Statsof. Inc. Tulsa, EUA).

3 RESULTADOS E DISCUÇÃO

3.1 Rendimentos dos Extratos Obtidos por SFE e Soxhlet

A matéria-prima constituída de folhas e caules de *C. sicyoides* apresentou 7,30±0,05 % de umidade, valores de diâmetro das partículas, densidade aparente, e porosidade adequados para SFE. O rendimento do extrato de *C. sicyoides* obtido por SFE foi de 3,30±0,10 % (b.s.). O rendimento do extrato etanólico, obtido por Soxhlet foi de 9,10±0.10 % (b.s). Os resultados encontrados neste trabalho estão em conformidade com os obtidos previamente por Salazar *et al.* (2018), onde os rendimentos dos extratos foram de 3.21 % na SFE e 11.02 % na extração por Soxhlet, e Salazar *et al.* (2021), reportaram que os rendimentos dos extratos foram de 3.03 % na SFE e 10.93 % na extração por Soxhlet da mesma planta. Estes valores similares de rendimento confirmam que, na SFE, a utilização de condições operacionais otimizadas, pode ser garantia de um processo reprodutível e sem variações.

Resultados semelhantes aos deste trabalho já foram relatados na literatura para SFE de outras plantas do bioma amazônico, no trabalho de Dias *et al.* (2012) para folha de jambú (*Spilanthes oleracea*), o rendimento foi de 1,59 a 5,82 % utilizando CO_2 e CO_2 + 50% de

EtOH. Nas folhas de copaíba (*Copaifera langsdorffii*), Costa *et al.* (2015) encontraram um rendimento entre 0,31 a 7,01% utilizando CO_2 e CO_2 + 25% de EtOH. No entanto, na SFE utilizando CO_2 foram encontrados valores inferiores aos citados pelos autores acima. Barbosa *et al.* (2017) para as flores, folhas e caules de jambú (*Spilanthes oleracea*), o rendimento variou de 1,41 a 1,61 %. Almeida *et al.* (2021) nas folhas de murici (*Byrsonima crassifolia*) o rendimento foi de 0,45 a 1,24 %.

De modo geral, no extrato obtido por Soxhlet o rendimento foi superior ao obtido por SFE. No entanto, esse método de extração apresenta certas desvantagens, entres elas se destacam a obtenção de extratos com diversas substâncias indesejáveis e com certo efeito citotóxico, que impossibilitam sua aplicação biológica. As extrações por Soxhlet foram realizadas apenas como extração de referência, mas sem realizar comparações dos resultados de rendimento e a composição química entre os extratos.

3.2 Compostos Fenólicos Totais (CFT)

Na Tabela 1 são apresentados os teores médios de CFT das amostras analisadas de *C. sicyoides*, os resultados obtidos apresentaram diferenças significativas entre si ($p \leq 0,05$), com a exceção da amostra de flores (9,30 mg EAG/g de amostra), de folhas e caule antes (10,52 mg EAG/g de amostra) e após da SFE (9,67 mg EAG/g de amostra) que não apresentaram diferenças entre si ($p > 0,05$), a análise de variância (ANOVA) é apresentada na Tabela 2.

Entres as amostras analisadas a maior concentração de CFT foi observada para o extrato de *C. sicyoides* obtido por Soxhlet (141,04 mg EAG/g), seguido do extrato obtido por SFE (64,23 mg EAG/g de amostra). De tal modo, pode-se inferir que as diferenças nos teores de CFT podem estar associadas ao solvente usado, método de extração e a matriz vegetal. Geralmente, são extraídos usando solventes mais polares como o etanol, pelo fato de que os compostos fenólicos têm caráter polar.

Comparando estes resultados com os estudos realizados empregando plantas do mesmo gênero e diferente espécie, observou-se que Chipiti *et al.* (2015) em extrato etanólico de *Cissus cornifolia* reportaram um teor de compostos fenólicos de 136,1 mg EAG/g de extrato, sendo este valor semelhante ao do extrato etanólico de *C. sicyoides*. Assim, Akomolafe *et al.* (2013) em extrato aquoso de caule de *Cissus populnea* reportaram um teor de 17,33 mg EAG/g de extrato, sendo que este valor é similar aos obtidos nos frutos da planta. É importante destacar que na literatura não existe nenhum estudo sobre as flores da *C. sicyoides*, e em relação à composição dos frutos, foi encontrado só um estudo, onde foram identificadas

três antocianinas. Os autores sugeriram, que o fruto desta planta pode ter uso potencial como corante alimentício (TOLEDO *et al.*, 1983).

O resultado de CFT do extrato obtido por SFE segue a tendência apresentada por Salazar *et al.* (2018) em extratos de folhas e caules de *C. sicyoides* encontraram um valor de 65,20 mg EAG/g de extrato a 50 °C/ 400 bar utilizando CO_2 + EtOH. Bezerra *et al.* (2020) em extratos de folhas de maravuvuia (*Croton matourensis*) encontraram um valor de 67,66 mg EAG/g de extrato obtido a 50 °C/ 400 bar utilizando CO_2. Almeida *et al.* (2021) em extratos de folhas de murici (*Byrsonima crassifolia*) um valor de 68,85 mg EAG/g de extrato obtido a 40 °C/ 300 bar com CO_2. Estes resultados confirmam que CO_2 supercrítico é capaz de extrair compostos polares com o emprego de altas pressões e com o uso de cossolventes polares.

3.3 Flavonoides Totais (FT)

Em relação ao conteúdo de FT, os resultados obtidos permitem constatar que os teores médios de FT em base seca de *C. sicyoides*, apresentam diferenças significativas entre si ($p \leq 0,05$) (Tabela 1), exceto as amostras de folhas e caule antes (6,59 mg EQ/g de amostra) e após da SFE (7,36 mg EQ/g de amostra) assim como, as amostras dos frutos da planta, que não apresentam diferenças entre si ($p > 0,05$). A maior concentração de flavonoides foi observada para o extrato obtido por Soxhlet (44,02 EQ/g de amostra), seguido do extrato obtido por SFE (14,26 EQ/g de amostra), a menor concentração foi apresentada na amostra de flores (1,03±0,19 EQ/g de amostra).

Normalmente, os valores de FT são inferiores aos de CPT, pois os flavonoides são uma classe especifica destes compostos. Conforme ao observado na Tabela 1, os valores de FT das diferentes amostras de *C. sicyodes*, encontrados neste estudo, são inferiores ao obtidos para CFT, e os valores estão dentro dos padrões.

O resultado de FT do extrato obtido por Soxhlet, apresentou teor superior ao reportado por Texeira e Guimarães (2013) onde a partir de extratos etanólicos de *C. sicyoides* encontraram um valor de 10,59 mg EQ/g de extrato. Em relação ao resultado de FT do extrato obtido por SFE, este apresentou valor similar ao obtido por Salazar *et al.* (2018), em extratos de folhas e caule de *C. sicyoides* a 40 °C/ 300 bar, utilizando CO_2 + EtOH (13,44 mg EQ/g de extrato). No entanto, valores inferiores foram reportados por Costa *et al.* (2015) em extratos de folhas de copaíba (*Copaifera langsdorffii*) a 40 °C/ 250 bar utilizando CO_2 (8,32 mg EQ/g de extrato). Bezerra *et al.* (2020) em extratos de folhas de maravuvuia (*Croton matourensis*) obtidos a 50 °C/ 400 bar, utilizando CO_2 (4,09 mg EQ/g de extrato).

Segundo Salazar *et al.* (2021) a planta possui diversos fitoconstituintes, no qual os flavonoides foram identificados no extrato supercrítico e etanólico desta espécie. As atividades biológicas atribuídas à *C. sicyoides*, como as atividades anti-inflamatória, antirreumáticas, antiepilépticas, anti-hipertensivas, antibacterianas, antigripal, antitérmica, antioxidante, antialérgica e antidiabéticas, são associadas, principalmente, à presença desta classe de compostos na planta. Assim, a análise do FT é uma estimativa do teor de todos os subgrupos dessa classe de compostos, e podem fornecer uma estimativa da capacidade antioxidante.

3.4 Avaliação da Capacidade Antioxidante

3.4.1 Método DPPH

Os resultados obtidos na análise da atividade antioxidante pelo método DPPH (Tabela1), permitiram verificar que os valores de EC_{50} apresentaram diferenças significativas entre si ($p \leq 0,05$). No entanto, as amostras de folhas e caule após a SFE (162,83 g de amostra/ g de DPPH) e de frutos verdes (164,42 g de amostra/ g de DPPH), não diferiram estatisticamente, ou seja, apresentaram médias estatisticamente iguais ($p > 0,05$), da mesma maneira que, como as amostras de folhas e caule antes da SFE (175,79 g de amostra/ g de DPPH) e os frutos maduros (177,78 g de amostra/ g de DPPH). Estes valores de EC_{50} foram os menores valores observado. Além disso, o extrato SFE (453,25 g de amostra/ g de DPPH) e o extrato Soxhlet (389,70 g de amostra/ g de DPPH) também apresentaram capacidade antioxidante, sendo suas médias estatisticamente diferentes das demais ($p \leq 0,05$). O potencial antioxidante é inversamente proporcional ao valor do EC_{50}, ou seja, quanto menor o valor de EC_{50} apresentado pela amostra, menor quantidade é necessária para reduzir em 50 % a concentração inicial do radical DPPH• e dessa forma, maior sua atividade antioxidante.

Comparando os resultados da atividade antioxidante dos extratos com outros estudos, Texeira e Guimarães (2013) a partir de extratos etanólicos de *C. sicyoides* encontraram um valor de EC_{50} (248,32 g de extrato/g de DPPH) inferior aos reportados. Resultados semelhantes os obtidos para os extratos já foram relatados por Salazar *et al.* (2018) que encontraram valores de EC_{50} no extrato obtido por Soxhlet de 325,67 g de extrato/g de DPPH e o extrato obtido com SFE de 460,81g de extrato/g de DPPH.

De acordo como os resultados, verificou-se que dentre as amostras de *C. sicyoides*, os frutos verdes e a amostra de folhas e caule após a SFE apresentaram maior atividade antioxidantes. A tecnologia supercrítica já foi aplicada na obtenção do óleo de bacaba-de-

leque (*Oenocarpus distichus* Mart.) (CUNHA *et al.*, 2019) e do óleo de açaí (*Euterpe oleracea* Mart.) (SILVA *et al.*, 2019) para avaliar os compostos antioxidantes presentes na polpa antes e depois da extração. Os resultados dos estudos demostraram que houve melhor identificação destes compostos na polpa após a extração. Neste contexto, o aproveitamento de folhas e caule *C. sicyoides* após a SFE traria benefícios para a obtenção de novos extratos ou insumo, bem como os frutos e os extratos da planta, visto que possuem potencial antioxidante.

De acordo com a Associação Brasileira da Indústria de Insumos Farmacêuticos (ABIQUIFI), o principal insumo farmacêutico comprado no exterior pelo Brasil é a insulina, utilizada no tratamento de diabetes. Nesse tipo de doença crônica, o controle glicêmico é muitas vezes o mais recomendado, assim como o uso de drogas antidiabéticas e terapia com insulina (LIM *et al.*, 2021). Por conseguinte, a *C. sicyoides* é uma planta medicinal utilizada popularmente no Brasil para o tratamento de diabetes. Apontando então para o potencial benéfico do extrato supercrítico da planta como um possível medicamento alternativo no tratamento de diabetes.

3.4.2 Método FRAP

Em relação à análise da atividade antioxidante pelo método FRAP, os resultados obtidos apresentaram diferenças significativas entre si ($p \leq 0,05$), com exceção da amostra de frutos verdes (208,80 μM sulfato ferroso/g de amostra) e do extrato obtido por SFE (211,59 μM sulfato ferroso/g de amostra) que não apresentaram diferença entre si ($p > 0,05$), a mesma tendência foi observada na amostra de flores (199,14 μM sulfato ferroso/g de amostra), de folhas e caules antes da SFE (201,48 μM sulfato ferroso/g de amostra). A atividade antioxidante medida pelo método FRAP foi observada nas amostras analisadas.

Em geral, a capacidade antioxidante das plantas está relacionada à quantidade de compostos fenólicos. Sendo assim, os resultados da atividade antioxidante das amostras apresentados na Tabela 1 estão relacionados aos resultados de CFT, pois os extratos obtidos por SFE e Soxhlet com maior teor de CFT (64,23 e 141,04 mg EAG/g de extrato) foram aqueles com maior atividade antioxidante (211,59 e 241,26 μM sulfato ferroso/g de amostra). Assim como a amostra de frutos verdes com maior teor de CFT (17,89 e mg EAG/g de extrato) foi aquela com maior atividade antioxidante (164,42 g de extrato/ g de DPPH e 208,80 μM sulfato ferroso/g de amostra).

Os valores observados com este método foram similares aos relatados em outros estudos, Silva *et al.* (2018) encontraram em extratos de folhas de *Myrcia sylvatic* que a

capacidade antioxidante pelo método FRAP apresentou valor de 193,47 ± 2,63 de µM de sulfato ferroso/g de amostra. Chipiti *et al.* (2015) em extrato etanólico de *Cissus* cornifolia, afirmaram que os extratos apresentaram poder redutor (Fe^{3+} - Fe^{2+}) e capacidade de sequestro de radicais DPPH.

4 CONCLUSÕES

O presente trabalho, pretendeu estudar os antioxidantes presentes em cada parte da *C. sicyoides* (frutos, flores, folhas e caules). Diante de tais resultados foi possível concluir que as folhas e os caules antes e após a SFE, são ricos em antioxidantes, o que justifica o alto teor de FT, bem como a maior atividade antioxidante pelos métodos testados (DPPH e FRAP). Também, o emprego dos frutos verdes, poderia ser uma boa opção para obtenção de antioxidantes a partir da planta. Por fim, no processo de SFE foi possível obter um extrato com alto teor de compostos fenólicos e com potencial antioxidante, que pode ser utilizado para novas aplicações biológicas como na determinação de sua atividade antidiabética.

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Tabela 1. Valores médios de compostos fenólicos totais (CFT), flavonoides totais (FT) e atividade antioxidante, referentes à planta em estudo.

Amostra	CFT (mg EAG/g de amostra)	FT (mg EQ/g de amostra)	DPPH (EC_{50} g de amostra/ g de DPPH)	FRAP (μM sulfato ferroso /g de amostra
Flores	9,30±0,05^{f}	1,03±0,19^{f}	171,79±0,40^{d}	199,14±0,23^{d}
Frutos verdes	17,89±0,05^{c}	3,22±0,02^{d}	164,42±0,42^{f}	208,80±0,30^{b}
Frutos maduros	15,88±0,14^{d}	3,01±0,66^{d}	177,78±0,43^{c}	205,74±0,74^{c}
Folhas e caules	10,52±0,08^{f}	6,59±0,08^{c}	175,79±0,79^{c}	201,48±0,48^{d}
Folhas e caules após SFE	9,67±0,08^{f}	7,36±0,03^{c}	162,83±0,40^{f}	205,48±0,74^{c}
Extrato SFE	64,23±0,25^{b}	14,26±0,01^{b}	453,25±1,25^{a}	211,59±0,59^{b}
Extrato Soxhlet	141,04±0,47^{a}	44,02±0,01^{a}	389,70±0,30^{b}	241,26±0,26^{a}

Dados representam a média ± desvio-padrão (base seca). Médias seguidas da mesma letra na coluna não diferem estatisticamente entre si, a 5 % de probabilidade, pelo Teste de Tukey.

Tabela 2. Análise de variância (ANOVA) para a determinação de compostos fenólicos totais (CFT), flavonoides totais (FT) e atividade antioxidante, referentes à planta em estudo.

	GL	SQ	QM	F	P
Análise de CFT					
Tratamento	6	29158,04	4859,67	43133,00	0,00*
Resíduo	7	0,79	0,11		
Total	13	29158,83			
Análise de FT					
Tratamento	6	2713,30	452,22	3304,53	0,00*
Resíduo	7	0,96	0,14		
Total	13	2714,26			
Método DPPH					
Tratamento	6	184329,70	30721,60	36303,20	0,00*
Resíduo	7	5,90	0,80		
Total	13	184335,60			
Método FRAP					
Tratamento	6	2412,20	402,00	770	0,00*
Resíduo	7	3,70	0,50		
Total	13	2415,80			

GL= Graus de liberdade; SQ= Soma de quadrados; QM=Quadrado médios; F= estadística de Fischer; (*)= $p \leq 0,05$.

CONCLUSÃO GERAL

Conforme mostrado no capítulo I, o extrato supercrítico de *C. sicyoides*, tem potencial efeito neuroprotetor e anti-inflamatório; esses efeitos podem estar associados à presença de CFT. De acordo com o que foi apresentado no Capítulo II, os antioxidantes são suscetíveis à degradação, e dentre os métodos de extração estudados, destaca-se o método de SFE como uma tecnologia que atendem aos princípios fundamentais em relação à obtenção de compostos. Entre aos métodos de quantificação da atividade antioxidante *in vitro*, o método DPPH é um dos mais utilizados por sua simplicidade e seu sistema de reação.

De acordo com o estudo apresentado no capítulo III, na análise dos extratos de *C. sicyoides* por HPLC, os dados espectrais UV/Visível revelaram a presença de flavonoides glicosilados. Por se tratar de um estudo para obtenção de extrato para aplicação biológica, a utilização do extrato etanólico não é considerada adequada, pois apresentou efeito citotóxico, ao contrário do extrato supercrítico que não apresentou tal efeito e pode ser utilizado. O ensaio *in vivo* demonstrou uma redução na concentração celular na área circundante à lesão, sugerindo um efeito anti-inflamatório do extrato supercrítico.

No estudo dos antioxidantes em cada parte da *C. sicyoides*, apresentado no Capítulo IV, pode-se concluir que as folhas e os caules antes e após a SFE, são ricos em antioxidantes, o que justifica o maior teor de FT, bem como a maior atividade antioxidante pelos métodos testados. No processo de SFE foi possível obter um extrato com alto teor de CFT, com potencial atividade antioxidante.

Por fim, a tese mostrou que o extrato supercrítico das folhas e caules de *C. sicyoides* L. apresentou boa concentração de CFT, com alta atividade antioxidante, e com potenciais atividades anti-inflamatórias, representando um excelente insumo para ser aplicado na indústria farmacêutica, na produção de medicamentos para o tratamento de doenças do sistema nervoso. Também, sugere-se a realização de novos estudos para que possam determinar outros potenciais biológicos, como o potencial antidiabético, e assim, contribuir para a literatura e oferecer baseamentos para os novos estudos sobre a *C. sicyoides*.

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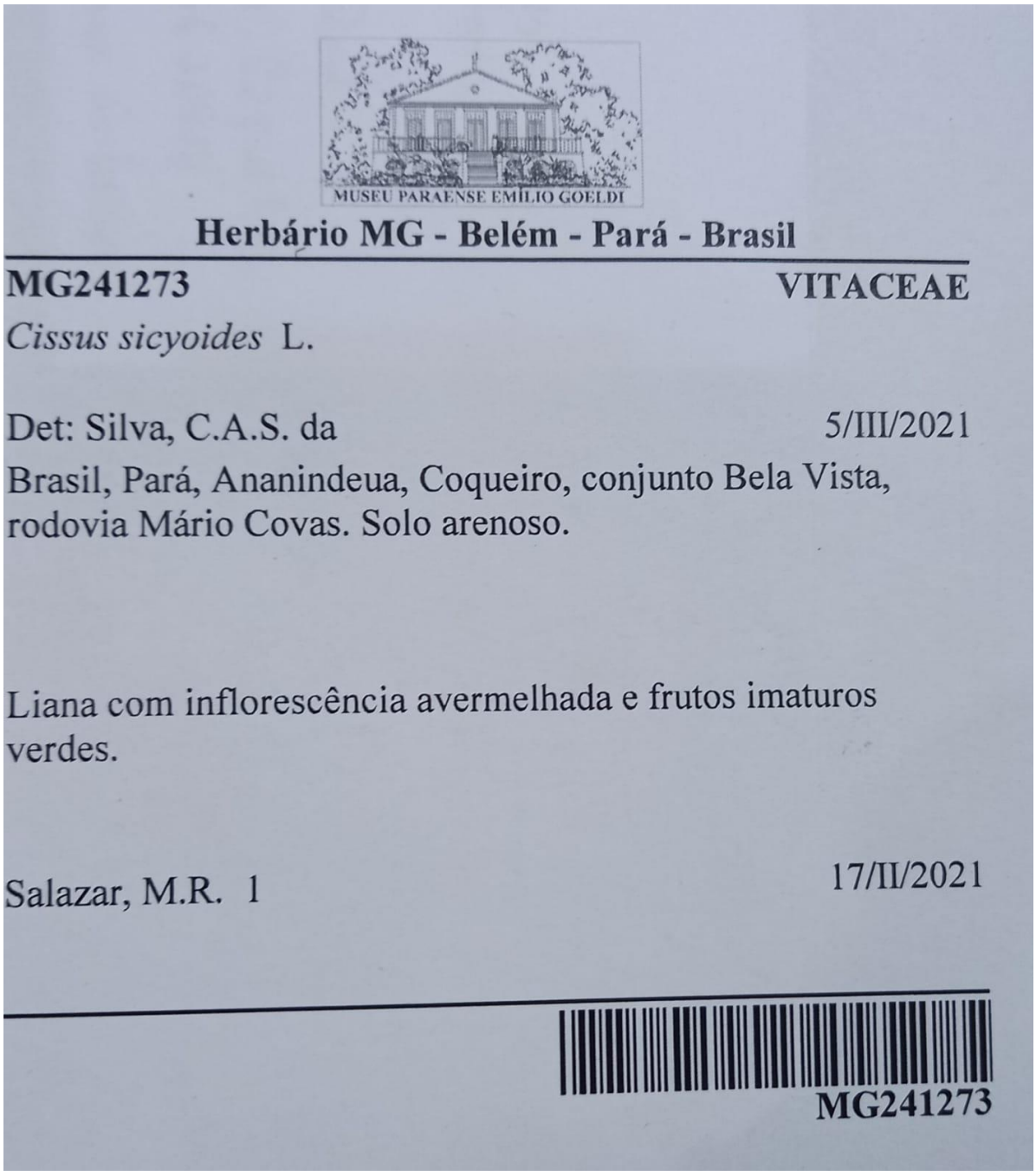
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Det: Silva, C.A.S. da 5/III/2021

Brasil, Pará, Ananindeua, Coqueiro, conjunto Bela Vista, rodovia Mário Covas. Solo arenoso.

Liana com inflorescência avermelhada e frutos imaturos verdes.

Salazar, M.R. 1 17/II/2021

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