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Talyta Mayara Silva Torres

Biorefinery of ora-pro-nobis leaves for the recovery of neuroprotective, antioxidant and protein fractions

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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RESUMO

As folhas de ora-pro-nobis (*Pereskia* sp.) são amplamente utilizadas em algumas regiões do Brasil como alimento. Estudos confirmam seu potencial antioxidante e o alto valor nutricional, com teor de proteínas em torno de 20%, sugerindo possível emprego industrial da ora-pro-nobis (OPN). As tecnologias alternativas de extração assistidas por microondas (MAE), e extrações a alta pressão, como extração por fluido supercrítico (ESC), extração por meio de líquido pressurizado (ELP), extração com líquido expandido (ELE) e extração com água subcrítica (EAS), são tecnologias alternativas e ambientalmente amigáveis dependendo do solvente empregado. As técnicas de extração, empregadas de forma combinada buscam aplicar uma abordagem de biorrefinaria para melhor aproveitamento da OPN. Foram utilizadas duas espécies de OPN, *Pereskia grandifolia* e *Pereskia aculeata*, submetidas aos diferentes processos de extração para investigar o efeito das variáveis de processo. MAE realizado entre 70 e 150 °C, 5 a 15 min e água, etanol e mistura de água e etanol (50%) como solventes. A ESC foi realizada a 25 MPa, temperaturas de 40, 50 e 60 °C e CO_2 como solvente; ELP foi realizada a 10 MPa, água e etanol como solventes e temperaturas de 50, 80 e 110 °C. ELE foi realizada a 7 MPa, etanol e CO_2 como solventes (25, 45, 75 e 100% etanol) e a 40 °C, e EAS foi realizada a 10 MPa e temperaturas de 80 a 185 °C. Os processos foram integrados para a melhor recuperação dos compostos bioativos da OPN, empregando as condições otimizadas nos processos individuais. A atividade antioxidante dos extratos foi avaliada por diferentes métodos, o conteúdo de fenólicos total (CFT) foi determinado pelo método de Folin-Ciocalteu e o teor de proteína solúvel total, determinado pelos métodos de Bradford e ácido bicinconínico, foi avaliado em extratos aquosos de extratos de alta pressão isolados e combinados. Nos estudos do MAE, o perfil fenólico das amostras mostrou o ácido elágico e o ácido *p*-anísico sendo primeiramente reportados como principais fenólicos presentes em folhas de ora-pro-nobis. Para folhas de *Pereskia aculeata*, o melhor rendimento foi encontrado na ESC 40 °C, a melhor atividade antioxidante foi encontrada na ELP 110 °C com etanol e ELP 80 °C usando água como solvente. Essas condições foram utilizadas em processo integrado para avaliar a qualidade dos extratos. O uso de ESC e ELP etanol antes da ELP com água mostrou-se eficiente para melhorar a atividade antioxidante, o CFT e o teor de proteínas solúveis do extrato aquoso. Os principais compostos encontrados nas amostras de ESC foram terpenóides e nas amostras de ELP e EAS foram flavonoides. As amostras de ESC apresentaram moderada atividade anticolinérgica e atividade anti-inflamatória, ambas envolvidas no processo de Alzheimer. Um

segundo processo de integração foi sugerido para a biorrefinaria de folhas de *P. aculeata*. No geral, as melhores atividades antioxidantes foram das amostras extraídas com água subcrítica. Ácido caftárico e derivados de quercetina, kaempferol e isorhamnetin foram encontrados nas amostras de etanol e água, o que pode explicar a alta atividade antioxidante.

Palavras-chave: Ora-pro-nobis. Atividade antioxidante. Microondas. Extração supercrítica. Extração por Líquido Pressurizado. *Pereskia aculeata*. *Pereskia grandifolia*.

RESUMO EXPANDIDO

Introdução

Ora-pro-nobis é uma planta trepadeira, de folhas suculentas, conhecida por ser rica nutricionalmente e por possuir alto teor de proteína. Ela também possui potencial biológico, tendo seu potencial sido avaliado em relação à sua capacidade antioxidante, anticancerígena, diurética, entre outras. A ora-pro-nobis é a única planta do gênero Cactaceae que possui folhas, e do gênero *Pereskia*, as espécies mais comumente difundidas da ora-pro-nobis são a *Pereskia grandifolia*, *Pereskia aculeata* e *Pereskia bleo*. Dentre estas, a *Pereskia grandifolia* e *Pereskia aculeata* foram trabalhadas no presente trabalho. O uso de tecnologias verdes para a obtenção de extratos de matrizes vegetais vem ganhando atenção do mercado devido às suas vantagens em relação às técnicas de extração convencional, como o uso de menor quantidade de solvente, tempo de extração e em geral o uso de solventes GRAS (*Generally Recognized as Safe*). O conceito de biorrefinaria refere-se ao uso de matéria-prima vegetal como biomassa para a conversão de produtos, aproveitando ao máximo a matriz no processo de produção. As tecnologias que utilizam fluidos a alta pressão, como a extração por fluido supercrítico (ESC), extração com líquido pressurizado (ELP), extração com líquido expandido (GXL) e extração com água subcrítica (EAS), e tecnologias de extração não-convencional, como o micro-ondas (MAE) se inserem bem no contexto de biorrefinaria, uma vez que tem como foco minimizar os resíduos.

Objetivos

Com o intuito de aproveitar ao máximo as potencialidades da ora-pro-nobis, o objetivo do presente trabalho foi aplicar o conceito de biorrefinaria utilizando de técnicas verdes não-convencionais de extração, como extração assistida por micro-ondas, e extrações utilizando alta pressão com fluidos supercrítico e subcrítico, com combinação de processos, e caracterizar as frações quanto às suas atividades biológicas e identificar os compostos majoritários.

Metodologia

Primeiramente, a *Pereskia grandifolia* foi utilizada para entender o perfil de compostos fenólicos dos extratos de ora-pro-nobis recuperados através de extração assistida por micro-ondas, utilizando cromatografia líquida (LC-ESI-MSMS). Foi feita uma otimização por meio de design experimental do tipo Box-Behnken para determinar melhores condições de tempo, temperatura e solvente, maximizando as variáveis avaliadas. Para as próximas etapas do trabalho foi utilizada a espécie *Pereskia aculeata*. Extrações a alta pressão foram realizadas de forma isolada e integrada, em ordem crescente de polaridade, por meio das técnicas de ESC utilizando CO_2 e ELP utilizando etanol e água como solventes. Os extratos da fração apolar (ESC) foram caracterizados por meio de cromatografia gasosa (CG-QTOF-MS), enquanto que a fração mais polar foi caracterizada por meio de cromatografia líquida (UPLC-QTOF-MS^E). Aqui também foi verificado se os extratos de ESC apresentaram atividade neuroprotetiva por inibição das enzimas AChE (acetilcolinesterase, marcador da doença Alzheimer) e LOX (lipoxigenase, enzima que atua em processos inflamatórios). Os resíduos foram avaliados por meio de microscopia eletrônica de varredura (MEV) para identificar possível ruptura da superfície da matriz. Todos os extratos foram comparados com extratos de Soxhlet quanto ao rendimento, quanto à

atividade antioxidante (AA) *in vitro*, por meio das metodologias DPPH, ABTS e FRAP, e quanto ao teor de fenólicos totais. Com o objetivo de explorar uma diferente tecnologia de extração verde a alta pressão na recuperação de compostos fenólicos dos extratos de ora-pro-nobis, foi utilizada a técnica de GXL, durante um período de Doutorado Sanduíche no *Instituto de Investigación en Ciencias de la Alimentación*, em Madrid. Foi realizado uma nova integração de processos, e para aumento do rendimento da terceira etapa foi realizada EAS utilizando temperaturas maiores que no fluxograma anterior. Os extratos foram comparados com extratos convencionais por meio de rendimento de extração, atividade antioxidante por DPPH e ORAC, quanto ao teor de fenólicos e carotenoides totais, e os extratos mais polares caracterizados por meio de cromatografia líquida (HPLC-DAD-ESI-MSMS). Foi realizada uma análise estatística de componentes principais (PCA) para verificar a relação entre as atividades *in vitro* e a composição dos extratos. Aqui os extratos das etapas 1 e 2 também foram testados para as atividades de inibição das enzimas AChE e LOX. O teor de proteína também foi avaliado em todos os extratos cujo solvente foi água, por meio das técnicas de Bradford e BCA.

Resultados e Discussão

Os dados obtidos para a *Pereskia grandifolia* indicaram presença dos fenólicos: ácido cafeico, ácido elágico, ácido *p*-anisico, ácido *p*-cumárico, kaempferol e quercetina, sendo as maiores concentrações encontradas nos extratos MAE com água como solvente. Por outro lado, quando maximizadas todas as variáveis (rendimento, fenólicos totais, DPPH, ABTS e FRAP), as melhores condições foram encontradas a 150 °C, 12.5 minutos e etanol como solvente. Para os extratos de *Pereskia aculeata* a alta pressão, a ESC a 40 °C, ELP com etanol a 110 °C e ELP com água a 80 °C foram as melhores condições isoladas. A integração de processos foi positiva para aumentar o rendimento em 76% no ELP-água, quando aplicados ESC e ELP-etanol antes da terceira etapa. Houve também um incremento na recuperação de compostos fenólicos por meio da integração de processos., e as melhores atividades antioxidante foram encontradas nos extratos aquosos. Os extratos de SFE apresentaram moderada atividade anticolinérgica e atividade anti-inflamatória, e apresentaram maiores teores de terpenos que os extratos convencionais, principalmente vitamina E, γ-sitosterol e lupeol. Os extratos de ELP com água e etanol apresentaram um perfil de fenólicos similar ao da *P. grandifolia*, com quercetina sendo um dos majoritários, contudo, os ácidos cítrico e isocítrico foram encontrados apenas nos extratos aquosos, indicando que estes seriam os responsáveis pela alta AA destes. A recuperação de proteínas também aumentou com a integração de processos, sendo maior para o ELP-água como terceira etapa em 82% comparado ao ELP-água sozinho e em 38% comparado à extração convencional. Na última etapa do fluxograma, GXL foi testado como segunda etapa comparado ao ELP com etanol, contudo ELP 100% foi eleita melhor condição para a integração de processos. A extração com água subcrítica superou o rendimento do fluxograma anterior (de máx. 5%), atingindo rendimentos de 47 e 65% para 150 e 185 °C, respectivamente. Aqui também a AA foi maior nos extratos de água. O perfil de fenólicos mostrou maior conteúdo de ácido caftárico nestas amostras, e uma análise de componentes principais (PCA) mostrou que a AA está relacionada a este composto e ao composto isorhamnetin-O-pentoside-O-rutinoside. Aqui o teor de proteínas nos extratos aumentou conforme aumentou a temperatura, sendo o teor nos extratos a 185 °C 1.8 vezes maior que nos extratos convencionais.

Considerações finais

Considerando a ora-pro-nobis uma matéria-prima rica nutricionalmente e com alto potencial de atividade biológica, ela se apresenta como uma excelente matriz a ser explorada no conceito de biorrefinaria, recuperando produtos de alto valor agregado em todas as etapas do processo e utilizando de tecnologias limpas de extração. O presente trabalho apresenta uma abordagem inédita sobre a planta, como o perfil de compostos, e informações relevantes no campo de biorrefinaria para recuperação de compostos bioativos e proteína.

Palavras-chave: Ora-pro-nobis. Fenólicos. Bioprospecção. Atividade neuroprotetiva.

ABSTRACT

Ora-pro-nobis (*Pereskia* sp.) leaves are widely used in some regions of Brazil as food. Studies confirm its antioxidant potential and high nutritional value, with a protein content around 20%, suggesting a possible industrial use of ora-pro-nobis (OPN). Alternative extraction technologies such as microwave assisted extraction (MAE), and high-pressure extractions such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), gas-expanded liquids (GXL) and subcritical water extraction (SWE) are considered environmentally friendly technologies depending on the solvent used. Extraction techniques, used in combination, are inserted in a biorefinery approach to better use of OPN. Two species of OPN, *Pereskia grandifolia* and *Pereskia aculeata*, submitted to different extraction processes were used to investigate the effect of process variables. MAE was carried out at temperatures between 70 and 150°C, time from 5 to 15 min and water, ethanol and a mixture of water and ethanol (50%) as solvents. SFE was performed at 25 MPa, temperatures of 40, 50 and 60 °C and CO_2 as solvent; PLE was performed at 10 MPa, water and ethanol as solvents and temperatures of 50, 80 and 110 °C. GXL was performed at 7 MPa, ethanol and CO_2 as solvents (25, 45, 75 and 100% ethanol) and at 40 °C, and SWE was performed at 10 MPa and temperatures from 80 to 185 °C. The processes were integrated for the best recovery of OPN bioactive compounds, employing the optimized conditions in the individual processes. The antioxidant activity of the extracts was evaluated by different methods, the total phenolic content (TPC) was determined by the Folin-Ciocalteu method and the total soluble protein content, determined by the Bradford and bicinchoninic acid methods, was evaluated in aqueous extracts of isolated and combined high pressure extracts. In the MAE studies, the phenolic profile of the samples showed ellagic acid and *p*-anisic acid being first reported as the main phenolics present in OPN leaves. For *Pereskia aculeata* leaves, the best yield was found at SFE 40 °C, the best antioxidant activity was found at PLE 110 °C with ethanol and PLE 80 °C using water as solvent. These conditions were used in an integrated process to assess the quality of extracts. The use of SFE and PLE ethanol before PLE with water proved to be efficient in improving the antioxidant activity, TPC and soluble protein content of the aqueous extract. The main compounds found in SFE samples were terpenoids and in PLE and SWE samples were flavonoids. SFE samples showed moderate anticholinergic activity and anti-inflammatory activity, both involved in the Alzheimer's disease mechanism. A second integration process has been suggested for the *P. aculeata* biorefinery. Overall, the best antioxidant activities were from samples extracted with subcritical water, and

caftaric acid, quercetin, kaempferol and isorhamnetin derivatives were found in ethanol and water samples, which may explain the high antioxidant activity.

Keywords: Ora-pro-nobis. Antioxidant activity. Microwave. Supercritical Extraction. Pressurized Liquid Extraction. *Pereskia aculeata*. *Pereskia grandifolia*.

DIAGRAMA CONCEITUAL

Biorrefinaria de Ora-Pro-Nobis (*Pereskia* Sp.): Emprego de Tecnologias Verdes para Recuperação e Valorização de Proteína Vegetal

Por Quê?

- Valorização de uma planta pouco conhecida na academia, a ora-pro-nobis (OPN), que vem ganhando destaque por suas propriedades nutricionais e biológicas e por se apresentar como alternativa à proteína animal devido ao alto teor proteico de suas folhas;
- Utilizar tecnologias verdes não convencionais com o objetivo de adotar processos mais sustentáveis e obter compostos bioativos a partir dessas tecnologias;
- Aproveitamento da matéria prima em processos integrados de extração no conceito de biorrefinaria.

Quem já fez?

- Não foram encontrados trabalhos na literatura para a recuperação das proteínas de folhas de OPN utilizando tecnologias alternativas de extração;
- Existem alguns trabalhos que indicam alta atividade antioxidante de extratos de folhas de OPN e atividades biológicas associadas aos extratos obtidos com solventes orgânicos;
- Na literatura se encontram estudos que combinam processos de extração para recuperação de compostos diversos, porém nenhum relacionado à OPN.

Hipóteses

- O emprego de tecnologias verdes pode ser utilizado para recuperação de compostos bioativos a partir das folhas de OPN;
- Os extratos de folhas de OPN possuem atividade antioxidante e conteúdo de compostos fenólicos compatíveis com os descritos na literatura;
- As tecnologias verdes são eficientes na recuperação de proteínas das folhas de OPN;
- Processos combinados podem resultar em vários compostos de interesse.

Como fazer?

- Estudar os processos de extração não convencionais e comparar as respostas com as obtidas por extração convencional, a fim de otimizar as condições de operação;
- Observar se a combinação de processos resulta em maior seletividade dos extratos;
- Avaliar a qualidade dos extratos por meio de análises *in vitro* de atividade antioxidante, conteúdo de compostos fenólicos, teor de proteína solúvel total e composição química por cromatografia líquida e gasosa.

LIST OF ABBREVIATIONS

ABTS 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid

AChE Acetylcholinesterase

DPPH 2,2-difenil-1-picril-hidrazil

FRAP Ferric Reducing Ability of Plasma

GC-QTOF-MS Gas chromatography with quadrupole time-of-flight mass spectrometry

HPLC-DAD-ESI-MSMS High Performance Liquid Chromatography-Photodiode Array Detection- Electrospray Ionization-Mass Spectrometry

IC_{50} Half Maximal Inhibitory Concentration

LC-ESI-MSMS Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

LOX Lipoxygenase

MAE Microwave Assisted Extraction

OPN Ora-pro-nobis

ORAC Oxygen Radical Absorbance Capacity

PLE Pressurized Liquid Extraction

SEM Scanning Electron Microscopy

SFE Supercritical Fluid Extraction

SOX Soxhlet extraction

SWE Subcritical Water Extraction

TCC Total Carotenoid Content

TPC Total Phenolic Content

TSPC Total Soluble Protein Content

UPLC-QTOF-MS^E Liquid Chromatography-Quadrupole Time-Of-Flight Mass Spectrometry

FIGURE CAPTIONS

Fig. 1 – Ora-pro-nobis (*Pereskia* sp.) flowers and leaves. a) Leaves aspect of ora-pro-nobis plant; b) *Pereskia grandifolia* flower; c) *Pereskia aculeata* flower; d) *Pereskia bleo* flower.

Fig. 2 – Conventional refinery vs. Biorefinery.

Fig. 3 –Soxhlet apparatus.

Fig. 4 – Phase diagram of a pure substance.

MANUSCRIPT 1:

Fig. 1. Desirability function bi-plot graph maximizing the total phenolic content, effect of the factors: time (min), temperature (°C) and water (%) on microwave assisted extractions from *Pereskia grandifolia* leaves; a) Temperature x time x desirability; b) Water (%) x time x desirability and c) Water (%) x temperature x desirability.

Fig.2. Desirability function graph maximizing all the responses (Yield, DPPH, ABTS and TPC), effect of the factors: time (min), temperature (°C) and water (%) on microwave assisted extractions from *Pereskia grandifolia* leaves; a) Temperature x time x desirability; b) Water (%) x time x desirability and c) Water (%) x temperature x desirability.

MANUSCRIPT 2:

Fig.1. Scheme of SFE extraction unit. 1: CO_2 regulator valve; 2, 7, 9: Manometer; 3: Cooling bath; 4: CO_2 cylinder; 5: CO_2 pump (M111, Maximator, Niedersachen, Germany); 6: Compressed air regulator; 8: Jacketed extraction vessel (stainless steel cylinder, L = 32,9 cm height, di = 2,042 cm internal diameter; V = 107,74 mL volume); 10, 13: Heating bath; 11, 12: Regulator needle valve; 14: Extract Reservoir; 15: Rotameter.

Fig.2. Scheme of PLE extraction unit. 1: Solvent reservoir; 2: HPLC pump; 3: Electric heat exchanger; 4: Manometer; 5: Extraction vessel with electrical heating jacket; 6: Regulator valve; 7: Regulator needle valve; 8: Extract reservoir; 9: N_2 cylinder.

Fig.3. Heat-map visualization of gas chromatography-mass spectrometry (peak area $x10^6$) analysis of *Pereskia aculeata* leaves.

MANUSCRIPT 3:

Fig.1. Scanning electronic microscopy (SEM) images with 1000x magnitude from ora-pro-nobis (*P. aculeta*) leaves: (a) before extractions; (b) after SOX-hexane; (c) after SFE; (d) after PLE-ethanol; (e) after PLE-water; (f) after PLE-ethanol_PLE-water; (g) after SFE_PLE-ethanol; (h) after SFE_PLE-ethanol_PLE-water; (i) sample by Farago et al. (2004).

Fig. 2. Heat map based on the intensity of the chromatographic peaks referring to the metabolites annotated in the samples.

MANUSCRIPT 4:

Fig.1. Scheme of the downstream processing proposed for the valorization of *Pereskia aculeate* leaves using compressed fluids extraction.

Fig.2. Kinetic behavior of the extraction yield (g $100g^{-1}$) of supercritical fluid extracts from *Pereskia aculeate* leaves.

Fig.3. Kinetic behavior of the extraction yield (g $100g^{-1}$) of gas-expanded liquid extraction (GXL $_{EtOH\ 45\%}$, GXL $_{EtOH\ 75\%}$) and pressurized liquid extraction (PLE EtOH 100%) from Pereskia aculeate leaves. Conditions: 40 °C, 7 MPa and 4 mL min^{-1}.

Fig.4. Global yield (%) from subcritical water extractions (SW) at 5, 10, 15, 20 and 30 min and conventional alkaline extraction (CA) at 45 min of *Pereskia aculeate* leaves.

Fig.5. Liquid chromatography-diode-array detection chromatogram (320 nm) of a) Pressurized liquid extraction (ethanol, 40 °C, 7 MPa, 150 min), b) Conventional extraction (ethanol, room temperature and pressure, 24 h), c) Subcritical water extraction (water, 185 °C, 10,5 MPa, 15 min), d) Conventional alkaline extraction (water, room temperature and pressure). For peak identification, see **Table 2**.

Fig.6. Principal Component Analysis, a) loadings and b) scores from compressed fluids and conventional extractions from *Pereskia aculeata* leaves. For the IC_{50} value of DPPH, the inverse ($1/ IC_{50}$) was used in the data matrix to facilitate the PCA analysis

APPENDIX A

Figure A1 – Pareto chart of yield (%) response of microwave assisted extraction from *Pereskia grandifolia* leaves.

Figure A2 – Pareto chart of antioxidant activity determination by DPPH ($1/IC50$ - $\mu g.mL^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

Figure A3 – Pareto chart of antioxidant activity determination by ABTS ($\mu mol_{TEAC}.g^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

Figure A4 – Pareto chart of antioxidant activity determination by FRAP ($\mu mol_{TEAC}.g^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

Figure A5 – Pareto chart of total phenolic content ($mg_{GAE}.g^{-1}$) response of microwave assisted extraction from *Pereskia grandifolia* leaves.

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WORK STRUCTURE

This document is presented in the form of chapters, to facilitate the reading and give a logical sequence to the contents covered, this dissertation is divided as follows: **Chapter 1** consists of the general introduction. **Chapter 2** contains a brief bibliographic review in which will be presented a general approach on supercritical extraction, pressurized liquid extraction, subcritical extraction, microwave assisted extraction and ora-pro-nobis (*Pereskia* sp.) main characteristics. In **Chapter 3** an approach is taken on the *Pereskia grandifolia* species of ora-pro-nobis using microwave assisted extraction to understand the phenolic profile of the leaves extracted using green solvents. In **Chapter 4** the main focus was the extraction at higher pressures, focusing on the optimization of supercritical CO_2 ($scCO_2$), pressurized ethanol and water extractions and rehearsing a combination of processes in a biorefinery approach. The **Chapter 5** was mainly to study a better second step of the downstream process used in the chapter before using a gas-expended liquid technology with ethanol and $scCO_2$ as solvents and to test higher temperatures in a subcritical water extraction to improve yield and concentrate protein, as the last product of the productive chain. Finally, in **Chapter 6**, general conclusions of the work are presented.

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

The bioactive compounds market is growing due to the increase of consumer's concern with health, increasing the demand for products of higher nutritional quality. Most bioactive compounds are obtained from natural sources. For this purpose, conventional extraction techniques are used, which despite being well established in the market, demand large amounts of energy, time and solvent (often toxic solvents). These techniques also have difficulties in use when it comes to the quality of the extract. The use of high temperatures, in this type of extraction, for a long period of time, can degrade the compound of interest if it is thermolabile. This fact, combined with the concern for the environment, has alerted industry and academia to seek environmentally friendly technologies that preserve the quality of the final product for obtaining natural extracts. High pressure extraction techniques are inserted in this context, being techniques widely explored in the literature for the extraction of bioactive compounds from natural matrices (REVERCHON; DE MARCO, 2006), among which are supercritical fluid extraction, pressurized liquid extraction and subcritical water extraction.

The biorefinery concept refers to the use of vegetable raw material as biomass for the conversion of products, fuels and chemicals, using various processing techniques, a concept similar to that of the traditional refinery. The goal is the depletion the raw material within production of high added value products, reusing the "residue" produced within the process flowchart itself (CHEW et al., 2017).

The growing interest in the application of the biorefinery concept is due to its advantages over the traditional refinery. In addition to reducing the emission of gases derived from fossil sources, it has a range of biomass available, making it extremely interesting to use residues

from the food and agriculture industry (ZACHAROF, 2017). These residues, which include bark, leaves, seeds, among others, have a large quantity of bioactive compounds that have high added value, which can be designated for the pharmaceutical or food industry.

Ora-pro-nobis (*Pereskia* sp.) is a climbing plant, with succulent leaves, known for being very nutritionally rich, having a high protein content, and for being used in traditional medicine for the treatment of diseases like headaches or even to cancer treatment. It is known as an unconventional food plant, and is often ignored as a plant with potential for human nutrition. Finally, ora-pro-nobis (OPN) leaves has gained recognition in the natural products market, especially vegetarian foods, due to its high protein content.

The idea of combining non-conventional extraction techniques with the concept of biorefinery is interesting, since both tend to generate less waste and have a good sustainable appeal. The OPN leaves become an interesting raw material in this context, since it is a plant that is not highly valued by the food market and with high potential in the market for bioactive compounds, according to the literature.

1.2 OBJECTIVES

1.2.1 Main objective

The main objective of this study was to recover extracts from ora-pro-nobis (*Pereskia* sp.) leaves using green technologies and suggest a processing route, in a biorefinery approach, to obtain different products with high added value from OPN leaves.

1.2.2 Specific objectives

a) To obtain extracts from the leaves of OPN by means of supercritical fluid extraction using different conditions of temperature;
b) To obtain extracts from the leaves of OPN by pressurized liquid extraction evaluating the effect of temperature (50 to 110 °C) and solvent type (ethanol and water), on extraction yield and extract quality (antioxidant activity and phenolic content);
c) To obtain extracts from the leaves of OPN by means of subcritical water extraction evaluating the effect of temperature (80 to 185 °C) on extraction yield and extract quality (antioxidant activity, phenolic content and protein and carbohydrate content);
d) To obtain extracts from the leaves of OPN by means of microwave extraction assisted, using solvents of different polarities;
e) To compare the overall extraction yields obtained by the different extraction methods with the traditional extraction procedure by Soxhlet and maceration with the solvents hexane, ethanol and buffer or alkaline water;
f) To evaluate the antioxidant activity (DPPH, ABTS and FRAP methods) and phenolics content (Folin Ciocalteu) in the extracts;
g) To combine extraction methods in increasing order of polarity to assess influence on yield, antioxidant activity and phenolic recovery;
h) To evaluate the chemical composition of the extracts by means of chromatography analysis (Gas-chromatography to the non-polar extracts and Liquid chromatography to the polar extracts);

i) To improve protein recovery in the last step of the downstream process by means of optimization of extraction conditions.

CHAPTER 2: LITERATURE REVIEW

In this chapter, a brief literature review will be presented on the subjects relevant to understanding and contextualizing the work.

2.1 ORA-PRO-NOBIS

Ora-pro-nobis (*Pereskia* sp.), also called trepadeira-limão (in portuguese) and Barbados gooseberry, is a shrub climbing plant with a high content of mucilage and protein. Thus, it is widely used as an emollient and as a food source (DUARTE; HAYASHI, 2005). It is a perennial plant, with fine stems, succulent leaves, forage shape, long branches, thorns, mucilage, flowers arranged in terminal summits, and tree or shrub habits (MARTINEVSKI; OLIVEIRA; FLORES, 2013). Despite the few studies, it is known that it is a plant rich in lysine, leucine and valine (essential amino acids), and has vitamin C and carotenoids in the leaf (BARBALHO et al., 2016).

Ora-pro-nobis belongs to the Cactaceae family, and to the Pereskioideae subfamily, where the entire genus *Pereskia* is found. Species of the genus *Pereskia* are found in the Caribbean and Central and South America, in dry forest environments (EDWARDS; NYFFELER; DONOGHUE, 2005). The genus *Pereskia* includes 17 species, of which *P. grandifolia* and *P. aculeata* are known in Brazil as ora-pro-nobis (OPN), from the Latin “pray for us”, and are differentiated by the color of the flower, *P. grandifolia* has lilac flowers while *P. aculeata* has yellow flowers (**Fig. 1**). Both are used as food and emollient for healing wounds, but in many places they are used only as ornamentation, as they are climbing plants, especially *P. grandifolia* (PINTO; SCIO, 2014). Another *Pereskia* specie widely explored in the literature

is *P. bleo*, widely used in traditional medicine to treat diseases related to cancer, headache, stomach pain, gastric ulcers, hemorrhoids, dermatitis, diabetes, hypertension, rheumatism, inflammation and as a tonic to revitalize the body (SIM; NURESTRI; NORHANOM, 2010). This species is differentiated by those previously mentioned also by the color of its flower, which is orange (**Fig. 1**).

Fig. 1. Ora-pro-nobis (*Pereskia* sp.) flowers and leaves. a) Leaves aspect of ora-pro-nobis plant; b) *Pereskia grandifolia* flower; c) *Pereskia aculeata* flower; d) *Pereskia bleo* flower.

Source: a) The author. b) <http://belleideias.blogspot.com/2015/05/pereskia-grandifolia-haworth-flores-de.html>; c) <https://www.paodeacucar.com/produto/690118/cha-de-ora-pro-nobis---pereskia-aculeata---50g>; d) <https://www.belliplantas.com.br/01-mudas-de-ora-pro-nobis-laranja-pereskia-bleo-bulbos-belli>.

Due to its high protein content, ora-pro-nobis is often called “meat of the poor”, as the poorest population does not always have access to animal-based proteins, and thus vegetable proteins, especially from unconventional plant sources, are inserted as alternatives to supply

the protein needed in the diet. Unconventional vegetables or unconventional food plants (UFP's) have been the subject of a recent study because they have many nutrients and have a low market value (KINUPP; BARROS, 2008; ROCHA et al., 2008). Ora-pro-nobis (*Pereskia* sp.) has a high nutritional value, with a protein content up to 25.5 %, fat between 2.07 and 4.1 %, fibers up to 39.9 %, minerals as calcium, iron, magnesium, manganese and zinc, vitamins A, C and folic acid (PINTO; SCIO, 2014). The amino acid profile of this plant, assessed by Takeiti et al. (2009), indicates that tryptophan is the most abundant amino acid in the leaves of *P. aculeata* species. **Table 1** shows average values of proximate composition found in the literature for leaves of the species *Pereskia aculeata* Mill.

Table 1 – Chemical composition (dry base) of ora-pro-nobis (*Pereskia aculeata* Mill.) leaves (%, w/w).

Reference	Protein	Fat	Ashes	Carbohydrate	Total fiber	Moisture
Martinevski et al. (2013)	20.10	2.07	13.66	24.8	39.27	86.81 (w.b.)
Takeiti et al. (2009)	28.4	4.1	16.1	nd	9.8	89.5 (w.b.)
Souza (2014)	14.38	2.54	15.97	58.99	8.12	95.74 (w.b.)
Rocha et al. (2008)	22.93	3.64	18.07	36.18	12.64	6.53
Rodrigues (2016)	15.71	3.57	17.25	8.49	50.55	4.83
Marinelli (2016)	24.17	3.71	17.83	48.39	32.80	5.90
Guimarães (2018)	18.25	2.87	28.33	nd	16.69	87.45 (w.b.)

nd – not determined; w.b. – wet base.

Ora-pro-nobis are widely used in folk medicine for infection and inflammation treatments, and also as diuretics, and even against cancer. Due to these appreciable functions, this plant has become the object of study of several researches, in order to prove the OPN properties and the chemical compounds that promote these activities. **Table 2** shows some studies that explored the biological properties of ora-pro-nobis.

Table 2 – Biological activities detected in ora-pro-nobis extracts.

	Biological activity	**Extract**	**Reference**
Pereskia aculeata	Antioxidant and antibiotic	Ethanol 70 %	Garcia et al. (2019)
	Improved intestinal health	Methanol	Vieira et al. (2019)
	Antioxidant	Water maceration	Rodrigues (2016)
	Antinociceptive	Methanol	Pinto et al. (2015a)
	Wound healing effect (*in vitro*)	Ethanol	Carvalho et al. (2014)
	Digestibility (*in vivo*)	Food formulation (powder)	Zem et al. (2017)
	Wound healing effect (*in vivo*)	Ethanol 92.8 %	Sartor et al. (2010)
	Toxicity against *Trypanosoma cruzi*	Water 60 °C	Valente; Scheinvar (2007)
Pereskia grandifolia	Cytotoxicity against cervical cancer cells	Methanol and hexane	Karim; Sismindari (2012)
	Diuretic	Ethanol	Kazama et al. (2012)
	Cytotoxicity against human nasopharyngeal epidermoid carcinoma cells and hormone-dependent breast carcinoma cells	Methanol extraction followed by hexane and ethyl-acetate fractionation	Nurestri, Sim e Norhanom (2009a)
Pereskia bleo	Antioxidant (*in vitro*)	Supercritical extraction with ethanol as co-solvent	Sharif et al. (2015)
	Antioxidant	Ethyl-acetate	Hassanbaglou et al. (2012)
	Antibacterial	Soxhlet hexane and methanol	Wahab et al. (2009)

Biological activities ranging from antioxidant activity to cytotoxicity against cancer cells were found in ora-pro-nobis leaves. Some studies identified the metabolites from OPN with biological activities. For instance, Agostini-Costa et al. (2014) that found high content of α-carotene, β-carotene and zeaxanthin in leaves of *P. aculeata* and *P. grandifolia*, the levels of zeaxanthin were similar to those reported for green corn, a recognized source of this carotenoid.

Malek et al. (2009) identified and isolated the compounds: β-sitosterol, 2,4-di-tert-butylphenol, α-tocopherol and phytol in ethyl acetate extracts from *P. bleo* leaves, and evaluated their cytotoxicity against human nasopharyngeal epidermoid carcinoma cells. Also, these authors observed relative citotoxicity against human nasopharyngeal epidermoid carcinoma cell line and human hormone-dependent breast carcinoma cell line for the compound α-tocopherol isolated from the extract of *P. bleo*.

Phenolic compounds such as caffeic acid, catechin, epicatechin, quercetin, α and β-tocopherol, 2,4-di-tert-butylphenol, terpenes such as β-carotene and phytol, sterols such as campesterol, stigmasterol, β-sitosterol, flavonoids such as vixetin, lutein, α-carotene, among other compounds have already been identified from conventional extracts of ora-pro-nobis leaves (*Pereskia* sp.) (ABDUL-WAHAB et al., 2012; AGOSTINI-COSTA et al., 2014; GARCIA et al., 2019; HASSANBAGLOU et al., 2012; MALEK et al., 2009; MARINELLI, 2016; MARTIN et al., 2017; NURESTRI et al., 2009b; PINTO et al., 2015a; SIM; NURESTRI; NORHANOM, 2010; SOUZA, 2014).

2.2 BIOREFINERY

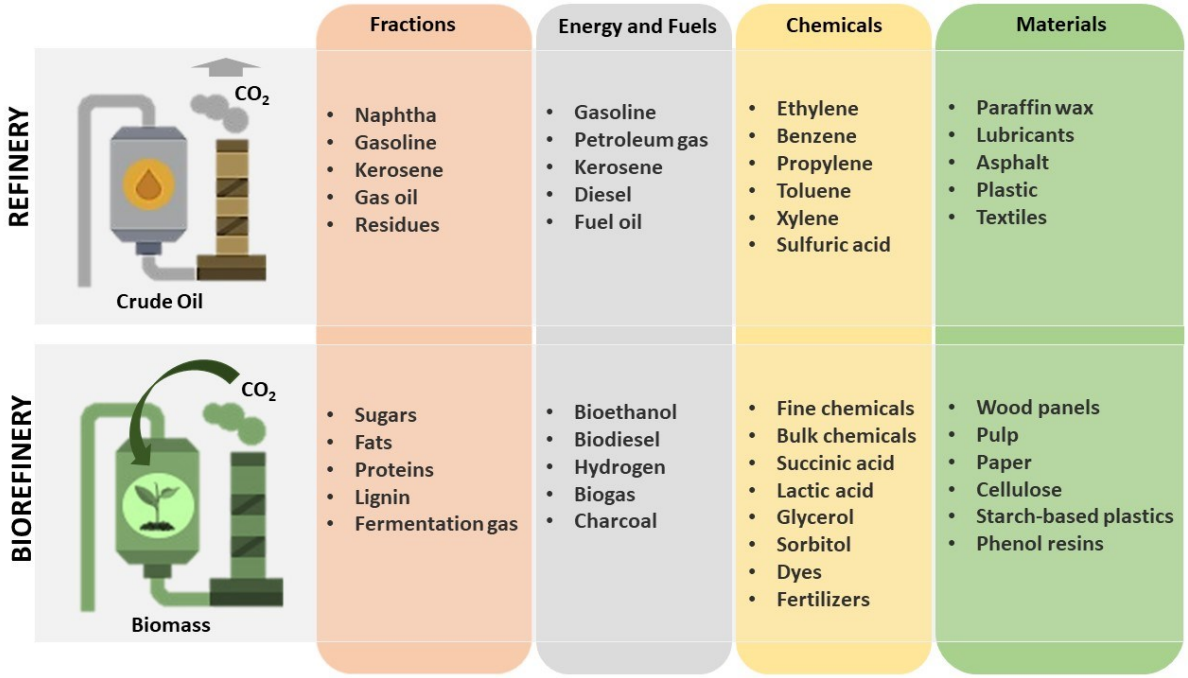
2.2.1 Biorefinery concept

The biorefinery concept is based on the use of the entire vegetable raw material, for the production of energy (biofuel, heat, etc.), molecules (fine chemicals, cosmetics, medicines, etc.), materials (plastics, biofilms, etc.), additives and food ingredients, among others (OCTAVE; THOMAS, 2009). The use of renewable raw materials as a substitute for fossil raw materials is intended to reduce the emission of gases that contribute to the greenhouse effect. The goal of the biorefinery is to separate and transform the raw material into different fractions for the use in various industrial sectors (BENEVENUTI; PEREIRA JR, 2016). An appropriate

plant matrix for the biorefinery process is one capable of generating multiple products, preferably with low competition with the food industry and the most selective composition possible (CHEW et al., 2017).

Biorefinery is compared to the conventional refinery process, while the main difference is associated with the raw material and the products obtained. Conventional refineries use petroleum, a raw material of fossil origin, exhaustible, for the generation of fuels, chemicals and materials. For the fossil processing, large quantities of greenhouse gases are generated, mainly carbon dioxide (RAMASWAMY, 2013). Biorefineries use raw materials of renewable sources, also generating fuels, chemicals and materials. Although some processes, including microbiological ones, also generate CO_2, this by-product is reabsorbed in the process (BENEVENUTI; PEREIRA JR, 2016). **Fig. 2** shows a scheme that summarizes the differences between the conventional refinery and the biorefinery.

Fig. 2 – Conventional refinery vs. Biorefinery.



Source: Adapted from Cherubini (2010); Hülsey (2018) and Katakojwala; Mohan (2020).

This concept is relatively new, having started in the 1980s, due to the increase in energy demand caused by the increase in the world population, requiring an alternative to the use of non-renewable fuels (natural gas, oil, among others) (ZACHAROF, 2017). The concern with the future scarcity of energy from fossil sources, coupled with the growing environmental concern due to climate change, made biomass an interesting alternative for obtaining energy in a renewable way. The discovery of new sources of oil (such as the discovery of the hydrocarbon resources in the pre-salt layer of the Brazil Southeastern coast) has reduced the concern about the scarcity of this source, however, the environmental concern has driven the search for sustainable alternatives for energy generation. One of the alternatives is the use of agroindustry residues, biomasses, to production of high added-value products.

Octave and Thomas (2009) classified the types of refineries into: sugar biorefinery, Ligno cellulosic biorefinery and lipid biorefinery. The first has the sugar as the main product from the vegetable biomass, which is used in the food industry. The second uses the lignocellulosic fraction of the plant, which represents about 70% out of total, and is used in the paper, construction and textile industry. The third one basically uses oilseeds and the extracted oil is used for the production of biofuels and high added value fatty acids, also generating by-products that are widely used, such as lubricants and detergents. The second and third types have in common the fact that they do not compete with the food industry. Despite the advantages of using plant biomass as a raw material in the biorefinery, this process has some difficulties, since the matrix is too complex and requires several pre-treatments to be readily available to microorganisms in fermentation processes (ZACHAROF, 2017).

This classification is one of the many proposed in literature (KHOSHNEVISAN; ANGELIDAKI, 2018). The biorefinery can also be classified according to the type of raw material as follows: first generation (1G), which are edible crops such as soybean, rice, wheat;

second generation (2G), where the non-edible materials are inserted, like residues from agricultural and forest, wood; third generation (3G), mainly algae (MONCADA B; ARISTIZÁBAL M; CARDONA A, 2016). The lignocellulosic feedstock biorefinery or second generation biorefinery involves the use of agricultural and forests residues that contains a high lignocellulosic content. The ora-pro-nobis leaves are inserted in the first or second generation biorefinery classification, since it is a material rich in lignocellulose and the main product of the leaves are protein, which are edible crops.

2.2.2 Separation processes to be applied in the biorefinery

There are several separation processes that can be applied in the biorefinery process. They can be classified into or based on the type of separation in: the ones based on balance (absorption, distillation, liquid-liquid extraction, supercritical fluid extraction, pressurized liquid extraction and subcritical water extraction), affinity (chromatography), membranes (microfiltration, ultrafiltration and reverse osmosis), solid-liquid separation (conventional filtration, solid-liquid extraction, precipitation and crystallization), among others (RAMASWAMY, 2013).

Conventional extraction techniques have been used for decades (maceration, Soxhlet, hydro distillation), however, despite being well established, these techniques require large amounts of energy, time and solvents (AMEER; SHAHBAZ; KWON, 2017). As a result of this, and with the growing concern with product quality and environmental damage, alternative extraction methods have been developed and studied over the years (REVERCHON; DE MARCO, 2006). The most widespread green extraction methods are: supercritical fluid

extraction, pressurized fluid extraction, subcritical water extraction, ultrasound-assisted extraction and microwave assisted extraction (AMEER; SHAHBAZ; KWON, 2017).

In this study, green extraction techniques such as microwaves, supercritical fluid extraction, pressurized liquid extraction, gas-expended liquid extraction and subcritical water extraction were used to obtain bioactive compounds from a very interesting plant matrix to be used in a biorefinery concept.

2.2.3 Ora-pro-nobis applied to the concept of biorefinery

There are some studies that present downstream processes based on the concept of biorefinery using plant matrices (DÁVILA et al., 2017; MACKĖLA; ANDRIEKUS; VENSKUTONIS, 2017; PATHAK; MANDAVGANE; KULKARNI, 2017), most suggesting the use of waste from agriculture industry or algae. The biorefinery associated with green technologies is proven to be a topic of great interest in the academia, as we can see well discussed by some author's when evaluating the biorefinery approach impact on peanut by-products (SORITA; LEIMANN; FERREIRA, 2020), jabuticaba fruit by-product (BENVENUTTI; ZIELINSKI; FERREIRA, 2021), tamarindo fruit by-product (MARTINS et al., 2020) and *Kappaphycus alvarezii* macroalgae (RUDKE et al., 2019), for instance. The authors advocate that the use of alternative extraction processes such as supercritical fluid extraction, pressurized liquid extraction, ultrasound assisted extraction, microwave assisted extraction and subcritical water extraction, allied with green solvents such as ethanol, water, CO_2 and Natural Deep Eutectic Solvent (NADES) in the biorefinery approach enhance the sustainability appeal of these processes. **Table 3** shows a brief list of some raw materials already studied in the concept of biorefinery and the separation processes used. No study was found about ora-pro-nobis associated to the concept of biorefinery.

Ora-pro-nobis is an unconventional food plant, with a high protein content, up to 28.59% (TAKEITI et al., 2009). Queiroz (2012), when evaluating the cultivation of ora-pro-nobis (*Pereskia aculeata* mill.) under intermittent water deficit in the soil, classified ora-pro-nobis as a food rich in protein, since it corresponds to more than 20 % of the daily protein requirement for an adult, according to Brazilian Health Regulatory Agency ANVISA (n° 27/1998). The plant is widely used as food in the region of Minas Gerais, Brazil, and is known as "meet of the poor" for its high protein content. There are few digestibility studies of this plant, and the digestibility value found by Takeiti et al. (2009) (76 %, *in vitro*) meets FAO requirements for children aged 2-5 years.

In folk medicine, ora-pro-nobis leaves are used to treat anemia, and also as a source of anti-inflammatory and emollient molecules. Studies prove the nutritional richness of ora-pro-nobis, containing large amounts of dietary fiber, minerals such as calcium, iron, zinc, manganese and magnesium, vitamins, C, B9 and E, and low levels of lipids such as palmitate, methyl, methyl oleate and methyl stearate (PINTO; SCIO, 2014). Reported levels of protein and essential amino acids are substantially greater than the minimum amount recommended by the Food and Agriculture Organization of the United Nations (FAO) as needed for human consumption (SIERAKOWSKI et al., 1987). The ora-pro-nobis is considered a plant that is easy to propagate by seeds and by stem cuttings, with fast and vigorous growth, with low incidence of pests and diseases and adaptability to different soils and climates, thus being a good option for cultivation (QUEIROZ, 2012). As it is considered an unconventional food plant, the cultivation is quite limited to domestic use (KINUPP; BARROS, 2008), but the scenario is changing due to the demand for plant-based protein and government incentives.

Among the functional properties of ora-pro-nobis, the following stand out: antioxidant activity, antimicrobial activity, cytotoxicity activity against cancer cells, diuretic activity,

antinociceptive activity and anti-inflammatory activity. Antioxidant activity is the most cited functional property of the ora-pro-nobis plant, of all species. Authors such as Garcia et al., 2019; Hassanbaglou et al., 2012; Rodrigues, 2016; Sharif et al., 2015; Souza et al., 2014; Turra, 2007 observed high antioxidant capacity of ora-pro-nobis (*Pereskia* sp.) extracts. Garcia et al. (2019) observed antimicrobial activity of *Pereskia aculeata* extracts against Gram-positive and Gram-negative bacteria. Valente and Scheinvar (2007) observed toxicity of *P. aculeata* extract against the protozoan *Trypanosoma cruzi*. Wahab et al. (2009) also observed antibacterial action in ora-pro-nobis (*Pereskia bleo*) extracts. Some studies focused on the cytotoxicity of extracts against cancer cells, such as Karim and Sismindari (2012) who observed cytotoxicity of extracts against cervical cancer and Nurestri et al. (2009b) who observed cytotoxicity against nasopharyngeal squamous cells carcinoma and hormone-dependent breast carcinoma cell line. Kazama et al. (2012) observed diuretic activity in extracts of *Pereskia grandifolia*. Abdul-wahab et al. (2012) and Guilhon et al. (2015) observed *in vivo* antinociceptive activity in wistar rats of *Pereskia bleo* extracts. Pinto et al. (2015a) also observed potential analgesic activity *in vivo* in wistar rats and promising anti-inflammatory activity for acute and chronic inflammation in *Pereskia aculeata* leaves extracts.

As shown in **Table 2**, ora-pro-nobis is a plant that has gained interest from researchers for its properties and beneficial health effects, like the uses for preventing diseases such as varicose veins, colon cancer, hemorrhoids, intestinal tumors and diabetes, as well as the reduction bad cholesterol level and treating boils and syphilis, requiring further studies to prove these effects (MARTIN et al., 2017). As it is a plant that has been little explored in the literature, the idea was to apply the concept of biorefinery in the extraction of bioactive compounds from this matrix, using green extraction techniques, such as supercritical extraction and extraction by pressurized liquids.

Table 3 – Biomass studied in the biorefinery approach and separation processes used.

Biomass	**Separation process**	**Reference**
Avocado	Drying Size reduction Thermo-mechanical oil extraction Supercritical CO_2 extraction (ethanol as co-solvent) Fermentation Enzyme assisted extraction	(DÁVILA et al., 2017)
Buckwheat	Supercritical CO_2 extraction Pressurized fluid extraction Enzyme assisted extraction	(MACKĖLA; ANDRIEKUS; VENSKUTONIS, 2017)
Pomegranate Peel	Size reduction Drying Pressurized water extraction Fermentation Pyrolysis	(PATHAK; MANDAVGANE; KULKARNI, 2017)
Microalgae (*Nannochloropsis* sp.)	Drying Size reduction Soxhlet Supercritical fluid extraction Fermentation	(FERREIRA et al., 2013)

Microalgae (*Scenedesmus obliquuus*)	Supercritical CO_2 extraction Pressurized fluid extraction Pressurized CO_2 and ethanol extraction	(GILBERT-LÓPEZ et al., 2017)
Microalgae (*Nannochloropsis* sp.)	Drying Size reduction Cold extraction (ethyl-acetate and acetone) Supercritical fluid extraction Fermentation	(NOBRE et al., 2013)
Mango peel	Blanching Pectin extraction	(BANERJEE et al., 2018)
Olive pomace	Aqueous extraction Filtration Liquid Hot Water Organosolv pretreatment Enzymatic hydrolysis Lignin precipitation and purification	(GÓMEZ-CRUZ et al., 2021)
Spent coffee grounds	Subcritical water extraction Protein hydrolysis Hydrothermal carbonization	(MASSAYA et al., 2021)

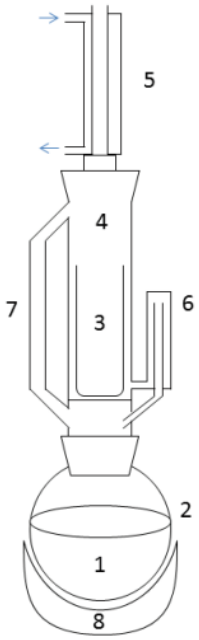
2.3 EXTRACTION METHODS

2.3.1 Soxhlet extraction

The Soxhlet extractor was developed in 1879 by Franz Ritter von Soxhlet, and has long been the most widely used solid-liquid extraction method. It has become a standard technique to which new technologies are compared to validate the results. The Soxhlet apparatus is shown in **Fig. 3** and the parts are explained as follows: the sample is placed in a filter (3), which is gradually filled with the extractor solvent, which is evaporated in the flask (1) and condensed (5) and once the solvent level reaches the siphon (6), it returns for the balloon and the cycle starts again. (JENSEN, 2007; REDFERN et al., 2014). Soxhlet is often described as a continuous extractor, since the solvent is recirculated, but it can be better described as an automated batch process, since the extract is not collected continuously (JENSEN, 2007).

Despite being a highly established method, it has some disadvantages such as long extraction times (1 to 72 hours), exposure to flammable and hazardous organic solvents, the probability of thermal decomposition of the extracted compounds because the extract is recovered in the flask that it is at the boiling temperature of the solvent and the possible reaction of the extract compounds with the solvent (AZWANIDA, 2015). As an advantage, the method promotes a continuous cyclical treatment of a multicomponent substance with fresh solvent, uses less solvent than maceration, there is no need for filtration to separate the raw material from the solvent and several extractions can be carried out simultaneously, due to at the low cost of the extractor (AZWANIDA, 2015; LUQUE DE CASTRO; PRIEGO-CAPOTE, 2010).

Fig. 3 –Soxhlet apparatus.



Source: Redfern et al. (2014)

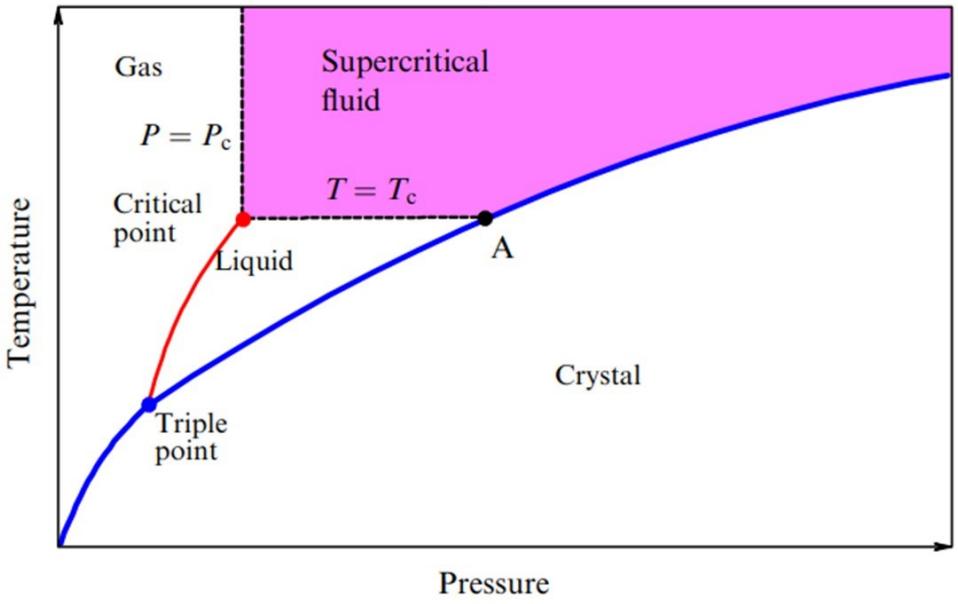
2.3.2 Supercritical fluid extraction

The extraction of natural products is one of the most used applications of supercritical fluid, due to its advantages of use compared to conventional extraction processes, such as the flexibility of the process, the possibility of using GRAS (Generally Recognized As Safe) solvents instead of organic solvents and the rapid elimination of the solvent from the matrix, eliminating the post-processing steps to remove the solvent (REVERCHON; DE MARCO, 2006).

Supercritical fluid extraction occurs when the solvent used is at temperature and pressure conditions above the critical point in a phase diagram (**Fig. 4**). The critical point represents the highest temperature and pressure at which the substance exists in liquid-vapor equilibrium (KERTON; MARRIOTT, 2013). The fluid, under these conditions, has

very peculiar characteristics of intermediate properties between liquids and gases, with lower density and viscosity and greater diffusivity than that of the liquid state. Above the critical point the thermal conductivity is increased, as well as other physical properties, increasing the heat and mass transfer in this region (MARTINEZ, 2007).

Fig. 4 – Phase diagram of a pure substance.



Source: Brazhkin et al. (2012).

Various compounds can be used as solvents in supercritical extraction, such as hexane, pentane and butane, nitrous oxide, sulfur hexafluoride and fluorinated hydrocarbons. However, carbon dioxide (CO_2) is the most widely used solvent because it is safe, readily available and not expensive. In addition, it has interesting critical properties for the extraction process of natural compounds, since it uses lower temperatures than other solvents, being interesting for maintaining the quality of extracts with thermolabile compounds. **Table 4** shows critical properties of some solvents used in supercritical extraction (DA SILVA; ROCHA-SANTOS; DUARTE, 2016; REVERCHON; DE MARCO, 2006).

Table 4 – Critical conditions of solvents used in supercritical fluid extraction.

Solvent	Critical condition	
	Temperature (°C)	Pressure (bar)
Carbon dioxide	31.2	73.9
Ethane	32.4	48.8
Ethene	10.1	51.2
Methanol	-34.4	80.9
Nitrous oxide	36.7	72.7
n-Butene	-139.9	36.5
n-Pentane	-76.5	33.7
Water	101.1	220.5

Source: Adapted by Silva, Rocha-Santos, Duarte (2016).

Carbon dioxide, CO_2, as solvent has high affinity for nonpolar compounds, being one of the limitations of supercritical CO_2 extraction. To expand the range of products for this type of extraction, co-solvents are often used with different polarities than CO_2, modifying the solvent's properties, changing its polarity in the supercritical medium (KERTON; MARRIOTT, 2013). Also, when supercritical CO_2 extraction is integrated with other processes as the first step, it helps to break down the extraction cell and improve recovery of other matrix fractions. An example of that was the study conducted by (FERRO et al., 2019), when applying supercritical CO_2 before pressurized liquid extraction (PLE) on the recovery of antioxidant compounds from *Sida rhombifolia* leaves, observed not only higher selectivity of supercritical CO_2 extraction towards non-polar compounds compared to Soxhlet with hexane, but the depressurization process in this step helped to improve PLE efficiency due to the rupture of the raw material cell wall.

One of the advantages of using supercritical extraction as an extraction method is the flexibility of the process, which can change the density of the solvent by modifying the pressure/temperature binomial, as well as having several solvents in one, controlling only two physical properties of the process. In addition, the process has shorter extraction times compared to conventional techniques (DA SILVA; ROCHA-SANTOS; DUARTE, 2016). A disadvantage of the process is the high investment cost involved, in addition to requiring skilled workers, and high security demanded in the process, for making use of high pressures (REVERCHON; DE MARCO, 2006).

Silva, Rocha-Santos and Duarte (2016) presented a very complete review of bioactive compounds extracted by means of supercritical fluid extraction from several matrices. They observed that most extracts have antioxidant activity (41%), followed by extracts with antitumor activity (18%) and antibacterial activity (10%), and extracts with antiviral, anti-inflammatory activity, among others (total of 5%). These results show the importance of the study of supercritical extraction applied to these purposes, as they are compounds with high added value and extensive application in several areas, such as medical, pharmaceutical, food additives and food in general, among others.

2.3.3 Pressurized liquid extraction

Pressurized liquid extraction (PLE) is a technique that involves extraction using liquid solvents at elevated temperatures and pressures, with increased extraction rates when compared to techniques performed at room temperature and atmospheric pressure. PLE was first introduced in 1995 by Dionex Corporation and presented as Solvent Accelerated Extraction Technology. It also has other names as pressurized solvent extraction, accelerated solvent extraction, heated solvent extraction, pressurized fluid

extraction, high pressure solvent extraction, high pressure and high temperature solvent extraction and subcritical solvent extraction. When water is used as the pressurized fluid, the technique is named pressurized hot water extraction, and will be treated in more detail in this review (CARABIAS-MARTÍNEZ et al., 2005; MUSTAFA; TURNER, 2011).

Pressurized liquid extraction can be carried out in a static mode, dynamic mode or a combination of the two. In the static mode, the solvent and the matrix are kept in contact for a period of time at constant pressure and temperature, in the dynamic the solvent passes through the matrix continuously, dragging the extract. A combination of the two can also be applied to try to increase reaction rates (CARABIAS-MARTÍNEZ et al., 2005). The use of high temperatures in the extraction improves the solvent extraction capacity, increasing the mass transfer and the penetration power in the sample (HUIE, 2002). High pressure is required to keep the solvent liquid during extraction.

The advantages of using this method are: less time and greater extraction efficiency when compared to conventional techniques. They are more complete extractions, but less selective, which is a disadvantage of the process, as well as the high costs of equipment and the need for safety and qualified labor, for working at high pressures and temperature; the disadvantages of PLE extractions are associated with the use of high-pressures, that requires expensive equipment and also the process needs a sequential step to remove the solvent, different from supercritical fluid extraction (MUSTAFA; TURNER, 2011). There are several PLE studies for obtaining bioactive compounds from plant matrices, making this technology interesting for application in a biorefinery process.

2.3.4 Subcritical water extraction

Extraction using subcritical water as a solvent is a type of pressurized liquid extraction, being called various names such as heated water extraction, pressurized hot water extraction, superheated water extraction, high temperature water extraction or liquid water extraction heated (CARABIAS-MARTÍNEZ et al., 2005). The use of temperatures above the boiling temperature of water is possible in pressurized systems. The increase in process temperatures increases the effectiveness of the process, as it changes the dielectric constant and viscosity of the water, causing a change in the affinity of this solvent. The greater affinity of water for less polar compounds makes it an interesting substitute for organic solvents in extraction processes (LACHOS-PEREZ et al., 2017).

The water is in the subcritical state when the temperature used is between 100 and 374 °C, it is supercritical when the working temperature is greater than 374 °C. As it is a very high temperature, that of supercritical water, lower temperatures are generally used, so the process is extraction with subcritical water, because the process conditions are below the critical condition (HERRERO; CIFUENTES; IBAÑEZ, 2006).

The advantages of using this method compared to conventional methods are associated with the choice of the solvent, which is a green solvent, and the shorter operating time. The disadvantages are due to the use of high temperatures, which can degrade some compounds and the residual water in the extract, requiring post-processing operations to purify the extract (BORISOVA et al., 2017). This process, as well as supercritical fluid and pressurized liquid extractions, has great potential for application in a biorefinery process to obtain bioactive compounds.

2.3.5 Microwave assisted extraction

The use of microwaves dates from the second world war, being developed at that time, and later used on a commercial scale in domestic ovens. The use of microwaves as a heat source in laboratories started in the 1970s with acid digestions, for extraction, the first work published with this objective was in 1986 (KAUFMANN; CHRISTEN, 2002). Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz to 300 GHz. Thus, microwaves wavelength is between X-rays and infrared rays in the electromagnetic spectrum. The action of magnetic waves in the material causes heating, which occurs in a closed system, without loss of heat to the environment (MANDAL; MOHAN; HEMALATHA, 2007).

The electric field causes heating through two phenomena: ionic conduction and dipole rotation (MANDAL; MOHAN; HEMALATHA, 2007). Dipole rotation occurs by aligning the field with molecules with dipole moment, either of the solvent or of the sample. This alignment causes collisions with the surrounding molecules, thereby releasing thermal energy into the medium. In this way, the heat is released simultaneously by the entire sample, one of the advantages of this method. The dielectric properties of the sample and solvent significantly influence the efficiency of the applied electromagnetic waves (KAUFMANN; CHRISTEN, 2002; ROUTRAY; ORSAT, 2012). **Table 5** shows some dielectric constants for solvents at 2,450 MHz and room temperature. The migration of dissolved ions increases the penetration of the solvent into the matrix, thus facilitating the solvation of the analyte. The electric field also generates ionic currents in the solution, the resistance to these currents causes heat that is released in the environment by the Joule effect (KAUFMANN; CHRISTEN, 2002).

Table 5 – Dielectric constant of some solvents at 2,450 MHz and room temperature.

Solvent	Dielectric constant
Water	80.4
Ethylene glycol	37.0
Methanol	32.6
Ethanol	24.3
Chloroform	4.8
Hexane	1.9

Source: Chemat (2012).

Microwave Assisted Extraction (MAE) systems can be classified in two ways, 'closed system' and 'open system', with the closed system generally being operated at pressures above atmospheric pressure, and the open system operates under atmospheric pressure. The closed system or multi-mode system allows the random dispersion of microwave radiation in the cavity by an agitator, while the open system or focused system (mono-mode) allows microwave radiation focused on a restricted zone of the cavity (CHAN et al., 2011). The use of microwaves for extraction means a significant reduction in the extraction time (<30 min) (MANDAL; MOHAN; HEMALATHA, 2007).

The microwave extraction mechanism differs from traditional solid-liquid extraction mechanisms because the extraction occurs due to changes in the cell matrix caused by electromagnetic waves. The high yields and speed of the process in microwave assisted extraction can be explained by the combination of the gradients of heat and mass transfer in the same direction (CHEMAT, 2012).

2.3.6 Gas-expanded liquid extraction

Gas-expanded liquids (GXL) were defined by Jessop and Subramaniam (2007) as mixed solvents formed by a compressible gas and an organic solvent. This definition is broad, because it can embrace the supercritical fluid extraction with use of co-solvents, however to narrow down the definition, GXL was defined as any mixture of compressible gas and an organic solvent at conditions below the mixture critical point (ECKERT et al., 2004). To avoid confusion, and to set a more specific definition, Akien and Poliakoff (2009) define GXLs as "a mixture of a condensable gas with other components such that there are at least 2 fluid phases or, a single phase above the bubble point curve but below the critical composition, where the properties of the liquid phase(s) are substantially different from those at atmospheric pressure", excluding all mixtures which are adjacent to the dew point curve, but including mixtures where the components are solid at atmospheric pressure.

The most used gas in GXL is CO_2 because of the same advantages of supercritical technology, been the mixture called CO_2-expanded liquids (CXL) (AKIEN; POLIAKOFF, 2009). The liquids used in GXL can be classified according to their ability to dissolve CO_2 in three classes: Class I liquids are the liquids that have a low solubility in the gas (such as water and CO_2) and do not expand much, Class II liquids are the ones that are highly soluble in the gas and have a high expansion (such as methanol and CO_2) and Class III liquids are the ones with moderate gas solubility but the small expansion (such as ionic liquids and CO_2) (JESSOP; SUBRAMANIAM, 2007). The CO_2 presents a high solubility at moderate pressures (3 to 8 MPa) in many organic solvents such as alcohols, ketones, ethers, and esters, providing change on the polarity, dielectric constant, and solubility of CO_2 neat and increasing tunability of the solvent compared to supercritical CO_2 ($scCO_2$) (ECKERT et al., 2004; HALLETT et al., 2006).

CXLs and $scCO_2$ share some advantages regarding the use of CO_2 as solvent, nonetheless there are some limitations in using CO_2 neat due to the very non-polar nature. The use of co-solvents in $scCO_2$ tend to minimize this disadvantage, however, the use of higher amounts of organic solvents can enhance the solubility of the mixture, hence favoring the use of CXLs (usually, 10-50% of organic solvent) instead of co-solvents in $scCO_2$ (usually, up to 10% of organic solvent) (HALLETT et al., 2006). The GXLs combine the properties of liquid solvents and supercritical fluids and have some advantages such as: ease of removal of the gas (usually CO_2), enhanced solubility and miscibility of reagent gases (compared to liquid solvents at ambient conditions) and milder process pressures (tens of bars) compared to $scCO_2$ (hundreds of bars) (HALLETT et al., 2006; JESSOP; SUBRAMANIAM, 2007).

The GXL extractions have some advantages compared to conventional extractions due to the nature of the solvents (when green solvents are used, as CO_2 and ethanol), providing more environmentally friendly alternatives, with faster solvent removal (JESSOP; SUBRAMANIAM, 2007). The applications of GXLs, specially using CO_2 as the compressible gas goes from processes of separation, precipitation of fine particles, polymer processing, and as reaction media for catalytic reactions. The technology has an enormous potential to be applied in a biorefinery approach when using green solvents.

2.4 CONSIDERATIONS OF THE STATE-OF-THE-ART

The bioactive ingredient market exceeded USD 27 billion in the year 2015 and is expected to reach USD 51.71 billion by 2024, according to a recent report by (GRAND VIEW RESEARCH, 2016). Consumers are increasingly interested in healthy products,

including diet foods and compounds that contain nutrients and are capable of preventing disease. This growing concern has driven the market for bioactive compounds, prompting the industry to supply these products and meet the demand. The academy is already aware of this trend, as shown by the increase in research in this area. Using the Scopus database with the keywords: “Bioactive AND compounds AND extraction AND plant”, it was possible to find a total of 2,324 publications in the last 5 years, with a growing trend.

The vegetable protein ingredients market is also growing, been valued at USD 38.5 billion in 2020 and is expected to expand at a compound annual growth rate (CAGR) of 10.5% from 2021 to 2028 (GRAND VIEW RESEARCH, 2021). According to the same report, the search for plant-based proteins is been rising alongside with animal-based protein prices. The main plant sources that move the market are wheat, soy, pea, canola, rice, potato, hemp, oat, and almond. Soy and wheat emerged as the dominant product sources in 2020. In fact, soy is the most popular, been the responsible for 75% of demand for vegetable proteins in the European Union (JERZAK; ŚMIGLAK-KRAJEWSKA, 2020). Different protein sources, especially native protein crop species have been economically neglected while not using their potential.

Ora-pro-nobis leaves (OPN) are classified as unconventional food plants (UFP), which means that they are used locally as feedstock, but are not commercialized as frequently as other traditional plants (KINUPP; BARROS, 2008). The OPN are known for their high protein content, up to 28% DW, compared with some usual vegetable protein sources like rice bran (up to 15% DW), red beans (up to 24% DW), oat (up to 18% DW), among others (SANT’ANA et al., 2011; WATCHARARUJI et al., 2008). The OPN production is only local, in cooperatives to be sell in local fairs or small companies, which makes difficult to creating a supply chain. However, from the cultivation point the plants advantage relies on the fact that it is hardy and perennial, compared to other green

leaves, been a production up to 6.4 t ha^{-1} $year^{-1}$ already reported for *Pereskia aculeata* (SOUZA et al., 2020).

OPN leaves essential and non-essential amino acid content are higher than the values recommended by the FAO for the human diet (TAKEITI et al., 2009). Some authors presented digestibility values ranging from 68 to 83% for the OPN leaves, been the average true digestibility around 77% (SILVEIRA et al., 2020; TAKEITI et al., 2009; ZEM et al., 2017), which is comparable to soy, the most consumed vegetable protein source (digestibility up to 78%) (MENDES et al., 2007). Its most abundant amino acids are tryptophan and lysine (MERCÊ, 2001; TAKEITI et al., 2009), which are generally found in animal meat, essential in animal nutrition and deficient in cereals. The L-lysine content can reach 2-23 times the content found in various vegetables (MERCÊ, 2001). The OPN leaves are not only nutritionally enriched, but also possess several biological activities, as shown previously in **Table 2**.

The search for more sustainable ways of chemicals and food production is been a hot topic in the research area as well as industry. In this sense, the biorefinery concept have been studied, as well as green technologies to be used in the biorefinery. According to Scopus, a total of 282 studies were found related to the "Biorefinery AND green AND extraction" key-words and in the last 5 years a total of 121,881 studies were written related to the "plant protein" theme. When searching "Biorefinery plant protein" a total of 180 studies were found between 2016 and 2021. However, for the key-words "Biorefinery AND protein AND subcritical AND extraction", only a total of 12 studies were found at all times searched, proven that besides the need of such data, research studies are still scarce. When searching the key-words: biorefinery, *Pereskia*, ora-pro-nobis, protein and plant protein, at different combinations, none results were found, proven the novelty of the present work.

CHAPTER 3: VALORIZATION OF ORA-PRO-NOBIS LEAVES BY MEANS OF MICROWAVE ASSISTED EXTRACTION

In this chapter, the study of extraction extracts rich in phenolic compounds from ora-pro-nobis leaves from the species *Pereskia grandifolia* produced in the Pytotechnics' department of the Federal University of Santa Catarina in a specially cultivated environment, by means of microwave-assisted extraction will be shown. The study of this matrix will enlighten the extracts profile of ora-pro-nobis leaves using green extracts, which will be used in the next chapters. The manuscript is presented in form of submitted article, to be presented to a journal of high impact factor from the area of study.

MANUSCRIPT 1: Phenolic compounds recovered from ora-pro-nobis leaves by microwave assisted extraction

Abstract

Ora-pro-nobis is an ornamental plant, often used as food in some regions of Brazil, and the *Pereskia grandifolia* is one of the less explored specie of this nutritional plant. To understand the antioxidant potential and the phenolic profile of ora-pro-nobis leaves, a micro-wave assisted extraction (MAE) was held using the green solvents ethanol and water. An optimization was made using Box-Behnken experimental design (3^2), with process time, temperature and solvent (% of water/ethanol) as variables. The evaluated responses were the process yield, combined with antioxidant capacity (DPPH, ABTS and FRAP methods) and total phenolic content (TPC) of the recovered extracts. Soxhlet method with ethanol was performed for comparison purpose. The phenolic profile of extract samples was assessed by LC-ESI-MS/MS analysis. High yield values were provided by 50% ethanol as solvent at 150 °C. Best antioxidant potential from DPPH and FRAP methods were provided by ethanolic extracts at 110 °C, while water extracts at 150 °C provided best ABTS results. Best TPC recovery was found in 50% ethanol samples at 70 °C. Overall, 24 phenolic compounds were identified, within caffeic acid, ellagic acid, *p*-anisic acid, p-coumaric acid kaempferol and quercetin as the main components. Ellagic acid and p-anisic acid were firstly reported associated to ora-pro-nobis leaves. The optimization of the results indicates that 150 °C, 12.5 minutes of MEA and ethanol as solvent provided the best combined responses. The use of MAE for ora-pro-nobis is a novelty that must be followed to explore green methods to value natural products.

Keywords: *Pereskia grandifolia*, Microwave assisted extraction, ellagic acid, *p*-anisic acid, phenolics

3.1. Introduction

The demand for minimally processed foods of processed by environmentally sustainable methods has been constantly increasing. Products that attend these characteristics contribute to the growing "natural" market. Green extraction technologies are inserted in this new context, representing an alternative to conventional techniques that often use toxic organic solvents, with high energy demand. Microwave assisted extraction (MAE) is an alternative technology that is used to overcome some disadvantages of the traditional Soxhlet and maceration methods, such as long extraction time and high amount of solvent and energy use. The main advantages are the safe use of the technology, easy applicability and a broad range of raw materials and solvents that can be applied, with little limitation (MANDAL; MOHAN; HEMALATHA, 2007).

Ora-pro-nobis is a plant of the genus *Pereskia*, the only genus in the family *Cactaceae* that has a green leaf (SHARIF *et al.*, 2013). This genus includes about 17 species, and the most widespread ones are *Pereskia grandifolia*, *Pereskia aculeata* and *Pereskia bleo* (PINTO ; SCIO, 2014). Their differences are mainly related to leaf size, thorn content and color of the flowers. Because *Pereskia grandifolia* is less cultivated and little used as food supplement in Brazil, there are less scientific studies related to its characteristics or properties, compared to other species.

The production of leafy vegetables with high nutritional value is very important to ensure food security for a sustainable human development (SOUZA *et al.*, 2020). Ora-pro-nobis leaves, due to the nutritionally rich matrix and biological properties associated, are an interesting source to be explored and to diversify family farming (MADEIRA *et al.*, 2016). Therefore, the present work aims to explore the antioxidant capacity of the underestimated *Pereskia grandifolia* leaves and determine the main compounds present in its extracts with good performance in terms of antioxidant potential.

3.2 Material and methods

3.2.1 Sample preparation and proximate composition

Pereskia grandifolia leaves used in this study were planted and collected at coordinates (27 ° 34'56"S; 48 ° 29'58"W), as part of a project for the valorization of local plants from Professor Ilyas Siddique from Agricultural Sciences of the Federal University of Santa Catarina, Brazil. The leaves of ora-pro-nobis were harvested in batches between August 2017 and March 2018, dehydrated with circulated air for 48 h at 40 °C, reaching a moisture content of 86.50 ± 0.13 (%) on wet basis. The dried ora-pro-nobis leaves were ground in a Willey knife mill, packaged and stored in a domestic freezer at -18 ºC. The moisture content analysis was determined according to method 925.09 (AOAC, 2005a), the total sugar content was determined by the dinitrosalicylic acid (DNS) method described by (MILLER, 1959) which uses 3,5-dinitrosalicylic acid as an oxidizing agent and the total lipid content by the method 920.39C (AOAC, 2005b). The total protein content was evaluated using the Kjeldahl procedure (method 928.08, AOAC, 2000), with a nitrogen-protein matching factor of 6.25.

3.2.2 Soxhlet extraction

Soxhlet (SOX) extraction was carried out according to method 920.39C (AOAC, 2005b), using ethanol P.A. and hexane P.A. as extraction solvents. The extraction procedure consists in placing 5g of the sample in the Soxhlet extractor. 150 mL of solvent is added in the system for circulating into the extractor at a constant rate of 4 to 5 drops per second. The total extraction time was 6 h. The experiments were held in triplicate and extraction yield was expressed as means ± standard deviation.

3.2.3 Microwave-assisted extraction

The extractions were carried out in a microwave reactor (Monowave 300 from Anton Paar GmbH). The mass ratio of dry sample to solvent was 1:20 (1g of sample and

20 mL of solvent), placed in the reactor in a vial, model G30 (maximum capacity of 30 mL) along with a magnetic stirrer, for extraction yield evaluation. The maximum power assessed was 850 W, with constant agitation at 1,000 rpm. After extraction, the solvent was removed from the samples using a rotary evaporator (Fitason Model 802, São Paulo / SP - Brazil) or freeze dryer (Liotop®, model L101, São Carlos, Brazil). Then the extracts were packed in amber flask and kept in a freezer (Freezer Consul, Joinville / SC - Brazil) at -18 ° C until further analysis.

3.2.4 Extraction yield (%)

The overall extraction yield (X_0) was calculated as the percentage of the ratio between the extract mass (m_e) and the dry sample mass (m_s) (**Eq. 1**). The tests were performed in duplicate and the results expressed as mean ± standard deviation (SD).

$$Xo = \frac{me}{ms} . 100 \quad \textbf{(1)}$$

3.2.5 Antioxidant capacity

DPPH method: For the evaluation of antioxidant activity, the method of capturing the free radical DPPH (2,2-diphenyl-1-picrilhidrazil), based on the methodology by (MENSOR *et al.*, 2001) was used. The samples were diluted in the extraction solvent at different concentrations. Then, the DPPH radical solution was added. After homogenization, the samples were incubated in the absence of light and at room temperature for 45 min. The samples were read in a spectrophotometer at a wavelength of 517 nm. The experiments were carried out in triplicate, the results expressed as mean ± standard deviation and the effective concentration to reduce by 50% the antioxidant activity of the radical (IC_{50}) was calculated by linear regression of antioxidant activity (AA) vs. concentration curve.

ABTS method: The extracts were analyzed for their antioxidant capacity according to the methodology described by (RE *et al.*, 1999). The $ABTS^{*+}$ radical was produced by

reacting 2.45 mM potassium persulfate and 7 mM ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) in the dark at room temperature, 16h before application of the method. The ABTS radical formed was then diluted in PBS buffer (phosphate buffered saline) 5mM pH 7.4 to an absorbance of 0.7 (± 0.2) at 734 nm. A volume of 10µL of sample, with at least 5 different concentrations, was added to 990µL of the ABTS radical solution. The sample was read at 734 nm after 45 minutes of incubation at room temperature and absence of light. The Trolox was used as a standard, so that the results were expressed in Trolox equivalents (TEAC value), according to a calibration curve (125-2,500 $\mu mol.L^{-1}$), with a coefficient of determination of (R^2) 0.9529. All analyzes were performed in triplicate and expressed as mean ± standard deviation.

FRAP method: The FRAP method determines the antioxidant activity by means of the iron reduction capacity. In this study, the method described by (BENZIE; STRAIN, 1996) was used. The FRAP reagent is prepared using a 0.3 M sodium acetate buffer solution (pH 3.6), a 20 mM ferric chloride solution ($FeCl_3.6H_2O$) and a diluted TPTZ solution (2,4,6-tripidylstriazine) in a 40 mM hydrochloric acid solution. The reagent is prepared using 60 ml of the sodium acetate buffer solution with 6 ml of the TPTZ solution and 6 ml of the ferric chloride solution. Samples previously diluted in the appropriate solvent are added to the reagent and the reaction takes place in the dark for 30 minutes and after that is read at a wavelength of 593 nm. The Trolox is used as standard, with a calibration curve being made with at least 5 different dilutions (50-500 $\mu mol.L^{-1}$), with a coefficient of determination of (R^2) 0.9935. The results are expressed in $mmol_{TEAC}.g^{-1}$ (TEAC - Trolox equivalent).

3.2.6 Total Phenolic Content (TPC)

The methodology used to analyze the total phenolic content was described by (KOŞAR; DORMAN; HILTUNEN, 2005). A standard curve of gallic acid was prepared with a stock solution of 2 mg mL^{-1} of gallic acid (98% pure, Sigma Aldrich) diluted in distilled water in different concentrations, with the obtained curve with a coefficient of determination of (R^2) 0.9965. Samples were diluted at concentrations up to 10 mg mL^{-1}, with the reaction mixture containing 10 µL of extract or solvent, 50 µL of reagent Folin-Ciocateau (Sigma Aldrich), 150 µL of 20% sodium carbonate (Lafan) and 800 µL of distilled water. Then, the samples were incubated at room temperature, in the absence of light. After 2 hours of incubation, the absorbance of the samples was read on a UV-vis spectrophotometer (Femto 800 XI, São Paulo / SP - Brazil) at a wavelength of 760 nm. The experiments were carried out in triplicate and the results expressed in milligrams of gallic acid equivalent per gram of dry matter ($mg_{GAE}\ g_{extract}^{-1}$).

3.2.7 Determination of phenolic compounds using LC-ESI-MS/MS

Sample preparation: The samples for analysis were prepared according to procedure describe by Schulz et al. (2015), with modifications. Defatted extracts (1.0 mL) were mixed with HCl 6 mol L^{-1} (5 mL), methanol (5 mL) and maintained at 85 °C for 30 min. After hydrolysis, the pH was adjusted to 2 with NaOH 6 mol L^{-1}. Then, samples were partitioned with diethyl ether (10 mL), shaken and centrifuged at 4000 rpm for 10 min, performed three times for each sample. The supernatants were dried using a rotary evaporator at 40 °C for solvent removal. The dried sample was resuspended with methanol (1 mL) and diluted ten times using methanol:water (30:70, v/v) before injection.

LC-ESI-MS/MS analysis: The evaluation of the chemical composition of the extracts of ora-pro-nobis (*P. aculeata*) was carried out according to the methodology described by (SCHULZ *et al.*, 2015) and performed by (LIMA, RENAN DA SILVA *et*

al., 2019). The equipment used was the 1200 Series (Agilent Technologies, Waldbronn-BW, Germany), A Synergi column (4.0 μm, 2.0 × 150 mm dia.; Phenomenex, Torrance-CA, USA) was used for the separations under gradient elution with mobile phase composed of methanol:water (95:5, v/v) and 0.1% (v/v) aqueous formic acid solution. The liquid chromatography system coupled to a mass spectrometry system composed of a hybrid tri-quadrupole/trap mass linear spectrometer (Q Trap 3200 Applied Biosystems/MDS Sciex, Concord-ON, Canada) was used in the analysis. The mass spectrometer was operated in negative electrospray ionization mode (TurboIonSpray Applied Biosystems/MDS Sciex, Concord-ON, Canada) and the MS/MS parameters were capillary needle kept at -4500 V; curtain gas at 10 psi; the temperature at 400 ° C; gas 1 and gas 2 at 45 psi; and CAD gas, medium. The chromatographic separation conditions and the mass spectrometer parameters for each phenolic compound were the same as described by (SCHULZ *et al.*, 2015). System control and data analysis were performed using the Analyst software (1.5.1).

3.2.8 Statistical analysis

The effect of process variables, time (X_1), temperature (X_2) and solvent (X_3), on the responses, yield (Y_1), antioxidant activity (DPPH (Y_2), ABTS (Y_3), FRAP (Y_4)), and total phenolic content (Y_5), for the microwave assisted extraction of ora-pro-nobis leaves, were evaluated with a Box-Behnken experimental design (**Table 2**), with 3 levels (−1, 0, +1) and second order. Then, 13 experiments and 1 central point (in triplicate) were conducted. The desirability function, as described by Derringer & Suich, (1980), was used to calculate the optimal conditions of the studied variables. The results were submitted to statistical analysis using the Statistica 7.1 program (StatSoft, Tulsa, OK, USA), being expressed as mean ± standard deviation. For quantitative data, one-way ANOVA analysis

of variance was performed, followed by the Tukey post-hoc test, with a significance level of 5%.

The influence of time, temperature, and solvent on the responses was assessed according to **Eq. 2**.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^{\,2} + \sum_{i=1}^{j-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \textbf{(2)}$$

Where Y represents the responses, β_0 is the expected average value of the response (constant), βj is the linear coefficient, βjj is the quadratic coefficient, βij is the interaction coefficient and Xi and Xj are the independent variables.

The statistical analyses used real values of the analyzed variables, except for the solvent: 0 represented 100% ethanol, 1 represented 100% water and 0.5 represented the mixture of 50% ethanol.

3.3 Results and discussion

3.3.1 Proximate composition

The proximate composition of *Pereskia grandifolia* leaves are presented in **Table 1**. The results corroborate with data from the literature for the proximate composition of ora-pro-nobis of various species (*Pereskia* sp.). Martinevski et al. (2013) found similar values for moisture (86.81% in b.u.), total proteins (20.10%) and carbohydrates (24.80%) for *Pereskia aculeata* leaves. It is not common to find data of centesimal composition for *P. grandifolia* in the literature. Almeida et al. (2014) made the proximate composition of the flours of *P. aculeata* and *P. grandifolia*. The authors found higher protein content for the species *P. grandifolia*, of 32.02%, compared to Takeiti et al. (2009), who found protein content of 28.4% for leaves of *Pereskia aculeata* Miller.

Table 1 – Proximate compositions of *Pereskia grandifolia* leaves.

	Quantity (%)
Moisture (wet base)	86.50 ± 0.13
Protein	14.64 ± 0.87
Fat	2.97 ± 0.86
Carbohydrate	24.41 ± 0.35

Source: The authors.

Most studies provided a protein content of ora-pro-nobis leaves around 20%, showing that this plant is a good source of protein. The total protein content found in a variety of most consumed vegetable flours, ora-pro-nobis (*Pereskia grandifolia*) has a protein content higher than coffee powder and rice bran, that is of 15.75 and 12.25%, respectively (MORO; ROSA; HOELZEL, 2004; SILVA; ASCHERI; PEREIRA, R. G. F. A., 2007).

The lipid content from ora-pro-nobis (**Table 1**) was relatively low compared to that found by Takeiti et al. (2009), of 4.1% for *Pereskia aculeata* Miller. Considering that the material is a leaf, the lipid content should not be very high, which can be an advantage because, according to Rocha et al. (2008), ora-pro-nobis can be used in low-calorie diets (low in carbohydrates) and with lipid restriction. The lipid content found by Almeida et al. (2014) for *P. grandifolia* was also higher, reaching 6.72%. The proximate composition varies according to each species and growing conditions.

The carbohydrate content (24.41%) corroborates with that from literature, such as the values found by Martinevski et al. (2013) for *P. aculeata* (24.8%) and by Almeida et al. (2014) for *P. grandifolia* (29.86%).

3.3.2 Effect of process variables on extraction yield

The yield values for the microwave assisted extraction (MAE), conducted at various process conditions, compared with Soxhlet method with ethanol as solvent, are shown in **Table 2**. Within the MAE assays, the lowest yield values were provided by ethanol as solvent (assays 5, 6 and 9), while the highest yields were obtained using ethanol/water mixture (50 %) at 150 ° C (assays 3 and 4). The Soxhlet method with ethanol showed yield of 18.91%, higher than found by MAE with ethanol, probably due to the longer extraction time, higher solvent amount (6h, 1:30 ratio of sample to solvent) and recycling, for Soxhlet, compared to MAE (up to 15 min; 1:20 ratio). This behavior is an indication of the presence of polar compounds at the ora-pro-nobis leaves.

A Pareto chart was made to evaluate the influences of process variables on yield (**Appendix A**, **Figure A1**). Temperature and solvent type influenced significantly the yield, with linear and quadratic functions, respectively, with solvent type providing the highest influence. The ethanol/water mixture increased significantly the yield, compared to other solvents; however, it is probably less selective due to the increase in the number of solubilized components (MUSTAFA; TURNER, 2011).

The microwave energy effect is extremely dependent on the nature of the solvent and the irradiated sample. The solvents have different dielectric constants and dissipation factors. The higher the solvent dissipation factor, the lower the penetration of microwave energy into the sample at a given frequency. Water, with lower dissipation factor than ethanol, presents higher penetration capacity in the matrix than ethanol, explaining the water higher yields (TEO; CHONG; HO, 2013). The temperature effect was also significant, i.e., higher temperatures elevated the yield due to the increase in the mass transfer (diffusion rate), increasing the solute solubility and reducing the solvent viscosity and increasing its penetration into the matrix (TRIPODO *et al.*, 2018).

The highest yield values were obtained at the highest temperature (150 °C), for 5 and 15 min submitted to microwave, with no significant difference in yield at 95% confidence (Tukey's test), however the Pareto chart shows a significant and positive influence of the time. Although the MAE process time (5 to 15 min) is much lower than the conventional Soxhlet method (6 h), higher yield values were quickly obtained.

The influence of process variables on yield was provided by applying **Eq. 2**, using the second order model to evaluate the quadratic effects of process parameters. The regression coefficients are presented in **Table 3** and the resulting model that represent the MAE yield is given by **Eq. 3**, with a good fit to experimental data (0.9680 and 0.9597 for R^2 and R^2-adjusted, respectively).

$$Y = 8.616 + 0{,}726.X_1 - 0.262.X_2 + 68.000.X_3 + 0.017X_1^2 + 0.02.X_2^2 - 48.143.X_3^2 - 0.008.X_1.X_2 + 0.070.X_1X_3 - 0.054.X_2.X_3 \quad \textbf{(3)}$$

Table 2 - Process variables, for the MAE of ora-pro-nobis: time, (t), temperature (T) and solvent (S), and the responses of yield, DPPH, ABTS, FRAP and TPC.

Assay	Codified variables			Real variables*			Responses				
	t (min)	T (°C)	S (%)	t (min)	T (°C)	S (%)	Y_1	Y_2	Y_3	Y_4	Y_5
							Yield (%)	DPPH IC_{50} ($\mu g.mL^{-1}$)	ABTS ($\mu mol.g^{-1}$)	FRAP ($\mu mol.g^{-1}$)	TPC ($mg_{GAE}.g^{-1}_{extract}$)
1	-1	-1	0	5	70	50% EtOH/water	21.72	226.04	1153.53	263.82	129.11
2	1	-1	0	15	70	50% EtOH/water	28.39	213.81	816.36	258.67	78.25
3	-1	1	0	5	150	50% EtOH/water	34.76	214.10	696.40	257.39	79.68
4	1	1	0	15	150	50% EtOH/water	35.17	185.66	119.17	135.45	100.18
5	-1	0	-1	5	110	ethanol	6.96	147.03	869.66	344.82	111.48
6	1	0	-1	15	110	ethanol	7.95	273.58	991.07	317.07	102.80
7	-1	0	1	5	110	water	20.63	2209.57	1045.71	214.57	112.33
8	1	0	1	15	110	water	22.32	2538.18	1002.61	267.78	106.93
9	0	-1	-1	10	70	ethanol	4.55	163.00	862.64	308.01	108.78
10	0	1	-1	10	150	ethanol	15.27	179.04	1520.74	334.37	100.10
11	0	-1	1	10	70	water	21.97	2565.01	1517.53	257.44	106.51
12	0	1	1	10	150	water	28.39	2014.21	1968.78	141.31	90.40

13	0	0	0	10	110	50% EtOH/water	27.04	203.88	997.19	172.86	97.82
14	0	0	0	10	110	50% EtOH/water	22.84	228.01	900.91	194.34	101.53
15	0	0	0	10	110	50% EtOH/water	28.32	215.96	965.71	194.73	101.02
Sox-EtOH	-	-	-	-	-	-	18.91 ± 2.32	151.69 ± 2.27	887.66 ± 3.23	-	103.98 ± 1.17
BHT	-	-	-	-	-	-	-	628.54 ± 11.95	2743.79 ± 81.45	-	-

232

233 Table 3 – Regression coefficients for responses to microwave assisted extraction of ora-pro-nobis (*P. grandifolia*) expressed in real variables[#].

Variables	Regression coefficients									
	Yield (%)		IC_{50} ($\mu g.mL^{-1}$)		ABTS ($\mu mol_{TEAC}.g^{-1}$)		FRAP ($\mu mol_{TEAC}.g^{-1}$)		TPC ($mg_{EAG}.g^{-1}$)	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
β_0	8.616	0.153	-168.296	0.634	2,341.821	0.000*	382.190	0.000*	199.652	0.000*
β_1	0.726	0.198	-19.804	0.550	107.190	0.001*	-17.451	0.000*	-12.535	0.000*
β_2	-0.262	0.003*	6.612	0.190	-38.354	0.000*	0.583	0.204	-0.304	0.039*
β_3	68.001	0.000*	-1,474.670	0.000*	-315.901	0.192	-251.210	0.000*	-20.629	0.009*
β_{11}	0.017	0.447	1.115	0.408	-9.968	0.000*	1.346	0.000*	0.072	0.066
β_{22}	0.002	0.000*	-0.021	0.315	0.151	0.000*	0.005	0.014*	-0.003	0.000*
β_{33}	-48.143	0.000*	4,193.105	0.000*	1,087.450	0.000*	260.399	0.000*	25.799	0.000*
β_{12}	-0.008	0.006*	-0.00002	0.890	0.950	0.000*	-0.146	0.000*	0.089	0.000*
β_{13}	0.070	0.747	20.205	0.124	-16.451	0.169	0.8.095	0.000*	0.328	0.377
β_{23}	-0.054	0.054	-7.085	0.000*	-2.586	0.087	-1.781	0.000*	-0.093	0.051
R^2	0.9680		0.9860		0.75056		0.9256		0.9243	
$R^2_{adjusted}$	0.9597		0.9822		0.68642		0.9065		0.9049	

234 [#]For calculation purpose the value of 0 represented 100% EtOH, 1 represented 100% water and 0.5 represented mixture 50% water/EtOH. *Significative ($p<0.05$).

3.3.3 Effect of the process variables on antioxidant activity

3.3.3.1 DPPH assay

The extracts were evaluated for antioxidant activity by DPPH radical scavenging method. The principle of this method is to observe the sample's ability to eliminate or neutralize the DPPH radical by means of a UV/visible spectrophotometer (OLIVEIRA, 2015). The results, expressed in IC_{50}, represents the sample concentration that reduces 50% of the DPPH radical, then lower IC_{50} represents better antioxidant capacity. The IC_{50} values from **Table 2** varied from 151.69 μg mL^{-1}, for Soxhlet with ethanol, to 2565.01 μg mL^{-1} for MAE with water at 70°C. The antioxidant values were compared with a commercially used antioxidant, the butylated hydroxytoluene (BHT), and the IC_{50} values for MAE and Soxhlet extracts, except the water extracts, were lower than the standard, indicating good antioxidant activity potential.

Few studies have observed the antioxidant potential from different ora-pro-nobis species. The DPPH results from **Table 2** corroborate with that from Sim et al. (2010) for *P. grandifolia* extract recovered by hexane and ethyl acetate (285 and 140 μg.mL^{-1}, respectively), while aqueous extracts showed the lowest antioxidant activity (> 5 mg.mL^{-1}), similar from the present study for water samples (> 2 mg.mL^{-1}). According to Hassanbaglou et al. (2012), *P. bleo* extracts by hexane and methanol showed IC_{50} values of 244 and 277 μg mL^{-1}, respectively, similar that from ethanol/water 50% samples (**Table 2**).

Soxhlet is a very well-established conventional technique, however it demands high extraction time. The antioxidant activity evaluated by DPPH showed statistically equal values of IC_{50} for samples recovered by MAE ethanol/water 50% and Soxhlet ethanol extracts, showing better MAE efficiency because similar results were provided in

short time and with less solvent used. The IC_{50} values for MAE with ethanol/water 50% were lower than the BHT standard, showing high antioxidant capacity.

MAE is widely used to recover extracts rich in antioxidant compounds, with several studies carried out to identify optimal extraction conditions for maximum recovery of bioactive compounds (BALLARD *et al.*, 2010; LI, H. *et al.*, 2012; PAN *et al.*, 2008; PÉRINO-ISSARTIER; ABERT-VIAN; CHEMAT, 2011; SINGH, A. *et al.*, 2011). The high recovery of antioxidant compounds by MAE can be explained by the microwave action in plant cells. When microwave energy is applied, the moisture from the samples is converted into heat by agitating and aligning the molecules to the electromagnetic field, whereupon the water begins to evaporate. The steam generates pressure inside the cell wall that eventually leads to cell disruption, facilitating the leaching of the active constituents into the surrounding solvent and improving the extraction yield (BALLARD *et al.*, 2010; MANDAL; MOHAN; HEMALATHA, 2007).

A second order model was used to describe the IC_{50} values, considering first-order interactions (**Eq. 4**). The model presented a good fit with an R^2 of 0.9860 and an adjusted R^2 of 0.9822.

$$Y = -0.170 - 0.020.X_1 + 0.007.X_2 - 1.475.X_3 + 0.001.X_1^2 - 0.00002.X_2^2 + 4.193.X_3^2 - 0.00002.X_1.X_2 + 0.020.X_1.X_3 - 0.007.X_2.X_3 \quad \textbf{(4)}$$

The Pareto chart describes the influence of process variables on IC_{50} values (**Appendix A**, **Figure A2**), represented by the inverse values ($1/IC_{50}$), since lower IC_{50} is better activity. The highest effects were the solvent type (negative), process time and solvent-time interaction (negative), with the best IC_{50} values found for samples recovered by ethanol at lower time. MAE using ethanol/water 50% also provided good DPPH performance. According to literature, the same solvents, ethanol and ethanol/water mixtures, were effective for the antioxidant components recovery by MAE from different matrices (DAHMOUNE *et al.*, 2015; LI, H. *et al.*, 2012). Inglett et al. (2010) also found

285 higher antioxidant activity from samples recovered by MAE with ethanol from
286 buckwheat, compared with water or ethanol/water 50% as solvents. According to the
287 authors, at high temperatures, compounds with antioxidant activity are more stable in the
288 presence of ethanol than in aqueous media, and more degradation may occur when water
289 is present.

290 3.3.3.2 ABTS assay

291 The results for antioxidant activity by ABTS method, in Trolox equivalents, are
292 presented in **Table 2**. The Pareto chart (**Appendix A**, **Figure A3**) shows the significant
293 effects for ABTS values. The most significant effects were the quadratic of the solvent
294 type, the time and the temperature. The model for calculating the ABTS value for ora-
295 pro-nobis extracts is presented by **Eq.5**.

296 $$Y = 2.342 + 0.107.X_1 - 0.038.X_2 - 0.316.X_3 - 0.01.X_1^2 + 0.0002.X_2^2 +$$

297 $$1.087.X_3^2 + 0.001.X_1.X_2 - 0.016.X_1.X_3 - 0.003.X_2.X_3 \quad \textbf{(5)}$$

298 The ABTS highest values were found from MAE water samples, followed by
299 ethanol (150 °C, 10 min). Here we have a similar logic as DPPH method as to the capacity
300 of neutralizing the radical generated. Yet, the ABTS has a more polar characteristic than
301 DPPH because it is basically water soluble. Therefore, the compounds acting more
302 directly in the mechanism are different from the other method, which explains the results
303 variation, which is why more than one method is necessary to understand the antioxidant
304 capacity of a sample. Rodrigues (2016) evaluated the antioxidant activity of conventional
305 extracts with water and ethanol 70% in water from ora-pro-nobis (*P. aculeata*) leaves by
306 ABTS method and found lower values (up to 5.20 $\mu mol_{TEAC}.g^{-1}$) than those from the
307 present study.

308

309 3.3.3.3 FRAP

310 The FRAP values for MAE samples from ora-pro-nobis leaves are also shown in
311 **Table 2**. The FRAP values are higher than those found by Hassanbaglou et al. (2012) for
312 *P. bleo* ethanolic extracts (of 40.45 μmol.g^{-1}). The model for FRAP values is presented
313 by **Eq. 6**, considering the second order model and first order interactions. The model
314 presented a good fit, of 0.9256 and 0.9065 (R^2 and R^2-adjusted, respectively). The MAE
315 ethanol extracts presented the highest FRAP values, corroborating with DPPH results.

316 $$Y = 0.382 - 0.017.X_1 + 0.0006.X_2 - 0.251.X_3 + 0.0013.X_1^2 + 0.000005.X_2^2 +$$

317 $$0.260.X_3^2 - 0.00015.X_1.X_2 + 0.008.X_1.X_3 - 0.002.X_2.X_3 \quad \textbf{(6)}$$

318 3.3.4 Effect of process variables on total phenolic content

319 The values of total phenolic content (TPC) for the *Pereskia grandifolia* leaves
320 extracts are shown in **Table 2**. The TPC was expressed in gallic acid equivalents
321 ($mg_{GAE}.g^{-1}$), with values ranging from 78.25 to 129.11 $mg_{GAE}.g^{-1}$ (**Table 2**), and the
322 highest value obtained by MAE at 5 min, 70°C and ethanol/water 50%. The TPC results
323 from **Table 2** were higher than found by Sim et al. (2010) for *P. grandifolia* leaf extracts
324 (19.08 to 45.99 $mg_{GAE}.g^{-1}$), and similar to that for *P. bleo* from ethanolic conventional
325 extract (109.43 $mg_{GAE}.g^{-1}$) (HASSANBAGLOU *et al.*, 2012) and for *P. aculeata* (108.2
326 to 139.4 $mg_{GAE}.g^{-1}$) from sample recovered by maceration with 95% ethanol
327 (CARVALHO *et al.*, 2014).

328 From the Pareto chart (**Appendix A**, **Figure A5**), the combined effect of time and
329 temperature was the most significant for TPC, followed by temperature, time and solvent
330 type. The solvent ethanol/water 50% was selective for phenolic compounds at shorter
331 time and lower temperature. Phenolic compounds, mostly polar compounds, have affinity
332 to polar solvents such as ethanol and water. Also, high temperatures may have degraded
333 the most sensitive compounds, reducing TPC values at higher temperatures. Liazid et al.

(2007) evaluated the stability of 22 phenolic compounds recovered by microwave, with temperatures ranging from 50 to 175 °C. They observed that increasing temperature, the degradation of these compounds also increased, mainly catechin and resveratrol, which are easily degradable. Otherwise, Casazza et al. (2012) observed higher TPC from methanolic extract at 150 °C in a range of temperature from 30 to 150 °C, for grape skins. Singh & Saldaña (2011) also observed an increase in TPC with temperature in subcritical water extraction from potato skins, up to 180°C, with degradation of these compounds above that temperature.

The Pareto chart **(Appendix A, Figure A5**) also shown the significant negative effect of time also on the TPC for MAE of ora-pro-nobis leaves. The MAE results suggest a relation between power and time for the recovery of phenolic compounds. High microwave power requires short time for the phenolics recovery, and vice-versa (MANDAL; MOHAN; HEMALATHA, 2007; SONG *et al.*, 2011). In the present study, the MAE power varied according to the assay temperature, with the maximum power of 400W used. During the extraction, a power peak occurs at the beginning of the extraction until reaching the desired temperature, and then is constant during extraction. It was noticed that the maximum power used in this study was relatively higher suggested by Jokić et al. (2012) for the extraction of phenolic compounds from broccoli (the optimal conditions for extraction were 71.51°C, 159.33W and methanol concentration of 72.06% and 16.9 min), with differences due to the equipment characteristics.

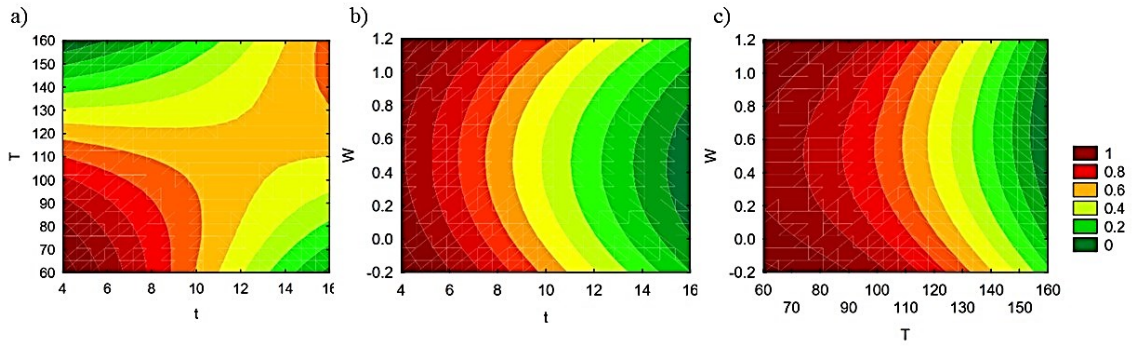
The TPC model for ora-pro-nobis is presented by **Eq. 7**, considering first and second order interactions. The equation presented adjustment values of 0.9243 and 0.9049 for R^2 and R^2-adjusted, respectively.

$$Y = 199.652 - 12.535.X_1 - 0.304.X_2 - 20.629.X_3 + 0.072.X_1^2 - 0.003.X_2^2 + 25.799X_3^2 + 0.089.X_1.X_2 + 0.328.X_1.X_3 - 0.093.X_2.X_3 \quad \textbf{(7)}$$

3.3.5 Phenolic profile of *Pereskia aculeate* leaves extracts

The identification and quantification of the phenolic compounds present in the MAE extracts of *Pereskia grandifolia* leaves were carried out by means of high-performance liquid chromatography (LC-ESI-MS/MS). The identification and quantification were based on standards and all the chromatographic data was treated with Analyst software. The parameters used were retention time, parent ion, quantitative ion and limits of identification (LOD) and quantification (LOQ). The results for TPC (**Section 3.3.4**) calculated by the Folin-Ciocalteu method indicated that the best set of phenolics recovery was achieved using water as solvent and this trend is confirmed by the desirability data for TPC presented in **Fig. 1**, where it shows the tendency of the optimum TPC recovery at water as solvent and lowest temperature and time. Therefore, the phenolics profile analysis was carried out for the samples obtained by water, the assays number 7, 8, 11 and 12, and compared with the central point (average of assays 13, 14 and 15) and Soxhlet with ethanol.

Fig. 1. Desirability function bi-plot graph maximizing the total phenolic content, effect of the factors: time (min), temperature (°C) and water (%) on microwave assisted extractions from *Pereskia grandifolia* leaves; a) Temperature x time x desirability; b) Water (%) x time x desirability and c) Water (%) x temperature x desirability.



A total of 44 phenolic compounds were tested as standards and 24 compounds were identified and quantified from the samples, as shown in **Table 4**. The phenolics identified were predominantly phenolic acids and flavonoids. The ones with higher concentration in the samples were Caffeic acid, Ellagic acid, *p*-Anisic acid, *p*-Coumaric acid, Kaempferol and Quercetin. To our best knowledge, Ellagic acid and p-Anisic were never identified as main compounds from *Pereskia* sp. leaves. Ellagic acid and *p*-Anisic acid are phenolic acid reported in the literature as having a very high antioxidative capacity, having effect against oxidation-linked chronic diseases such as cancer and cardio-vascular diseases (LOSSO *et al.*, 2004; MAKSYMIAK *et al.*, 2016; TOŠOVIĆ; BREN, 2020; VATTEM; SHETTY, 2005), which may explain the antioxidant activity of the MAE extracts.

Flavonoids are most abundant phenolic compounds found in *Pereskia* sp. extracts, so far presented in the literature, and Kaempferol and Quercetin are the most cited ones (GARCIA *et al.*, 2019; HASSANBAGLOU *et al.*, 2012). In fact, the MAE sample from *P. grandifolia* leaves (by ethanol/water 50%) presented Quercetin and Kaempferol (2.111 and 0.918 $mg.g^{-1}$, respectively) concentrations comparable with found by Garcia et al. (2019) for these compound's derivatives (2.11; 0.738; 3.56 $mg.g^{-1}$ and 0.810 $mg.g^{-1}$, respectively) from hydroethanolic extracts from *P. aculeata*.

In general, MAE extracts were more efficient for the recovery of phenolic compounds, mainly phenolic acids and other phenolics than Soxhlet conventional extraction, as shown in **Table 4**, corroborating with TPC *in vitro* results (**Section 3.4)**. Among the phenolic acids, the MAE extracts presented higher recovery values. For instance, the caffeic acid and *p*-coumaric acid recovered in the MAE extract at 110 °C, 5 min and water as solvent were 17 and 14 times the values of the compounds found in the Soxhlet ethanol extract, respectively. The flavonoids recovery was a bit higher in the

Soxhlet samples, but with an increment of only 13 % of the better condition, that was the assay 7 (5 min, 110 °C, Water). The values from **Table 4** indicate the order of phenolic recovery as: assay 7 (5 min, 110 °C, Water) > assay 8 (15 min, 110 °C, Water) > assay 11 (10 min, 70 °C, Water) > assay 12 (10 min, 150 °C, Water) > assays 13-15 (10 min, 110 °C, 50 % EtOH). Therefore, the combination time-temperature was the most significant variable affecting the TPC recovery demonstrated by *in vitro* analysis, and it is also demonstrated by chromatography results.

411 Table 4 – Phenolic profile of ora-pro-nobis (*Pereskia grandifolia*) leaves extracts (μg
412 g^{-1} of extract).

Phenolic compounds	**SOX**	**MAE**				
	Ethanol	**7 assay** 110 °C water 5 min	**8 assay** 110 °C water 15 min	**11 assay** 70 °C water 10 min	**12 assay** 150 °C water 10 min	**13-15 assay** 110 °C 50% ethanol 10 min
Phenolic acids						
1 4-Aminobenzoic acid	3.14	1.91	9.69	15.78	1.90	4.69
2 4-Hydroxymethylbenzoic acid	1.81	4.35	2.79	2.75	9.12	1.81
3 Caffeic acid[I, II]	12.46	210.32	54.02	39.25	32.75	13.76
4 Chlorogenic acid[II]	< LOQ	< LOQ	0.76	2.43	0.85	0.95
5 Cinnamic acid	< LOQ	20.50	6.24	5.53	2.75	6.38
6 Ellagic acid	1453.99	1702.20	1307.63	1101.89	1003.51	899.42
7 Ferulic acid[II]	nd	21.93	89.34	100.15	< LOQ	76.29
8 Gallic acid	< LOQ	1.82	1.81	1.59	1.45	1.48
9 Mandelic acid	5.93	11.45	5.71	3.33	5.38	3.84
10 p-Anisic acid	170.18	188.70	190.17	208.08	80.09	51.62
11 p-Coumaric acid[II]	60.37	845.58	291.83	269.32	230.66	135.21
12 Protocatechuic acid	23.19	30.80	22.40	27.30	14.14	33.93
13 Salicylic acid	10.83	15.76	15.28	15.06	11.11	7.79
14 Sinapic acid	29.91	43.07	20.55	22.14	13.74	15.62
15 Syringic acid	10.62	4.57	3.74	5.30	3.65	6.65
16 Vanillic acid	11.77	21.94	15.08	18.23	12.76	11.07
Flavonoids						
17 Epicatechin[III]	4.79	8.54	4.94	3.94	1.34	4.42
18 Kaempferol[I]	1594.15	86.04	55.39	79.74	824.14	918.84
19 Myricetin[III]	2.61	2.55	2.38	2.51	1.83	2.64
20 Quercetin[I, III]	3132.27	4076.57	3757.37	3745.63	2378.95	2111.18
21 Rutin	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	4.49
Phenolic aldehydes						
22 Syringaldehyde	< LOQ	2.91	< LOQ	1.94	6.56	< LOQ
23 Vanillin	1.35	5.87	4.43	2.26	10.53	3.37
Coumarins						
24 Umbelliferone	3.34	16.14	13.27	17.53	10.82	7.89
Total phenolic acids	1794.20	3124.89	2037.05	1838.13	1423.88	1270.51
Total flavonoids	4733.81	4173.69	3820.08	3831.82	3206.26	3041.56
Other phenolics	4.69	24.92	17.69	21.73	27.91	11.26
Total phenolic compounds	6532.70	7323.50	5874.83	5691.68	4658.05	4323.34

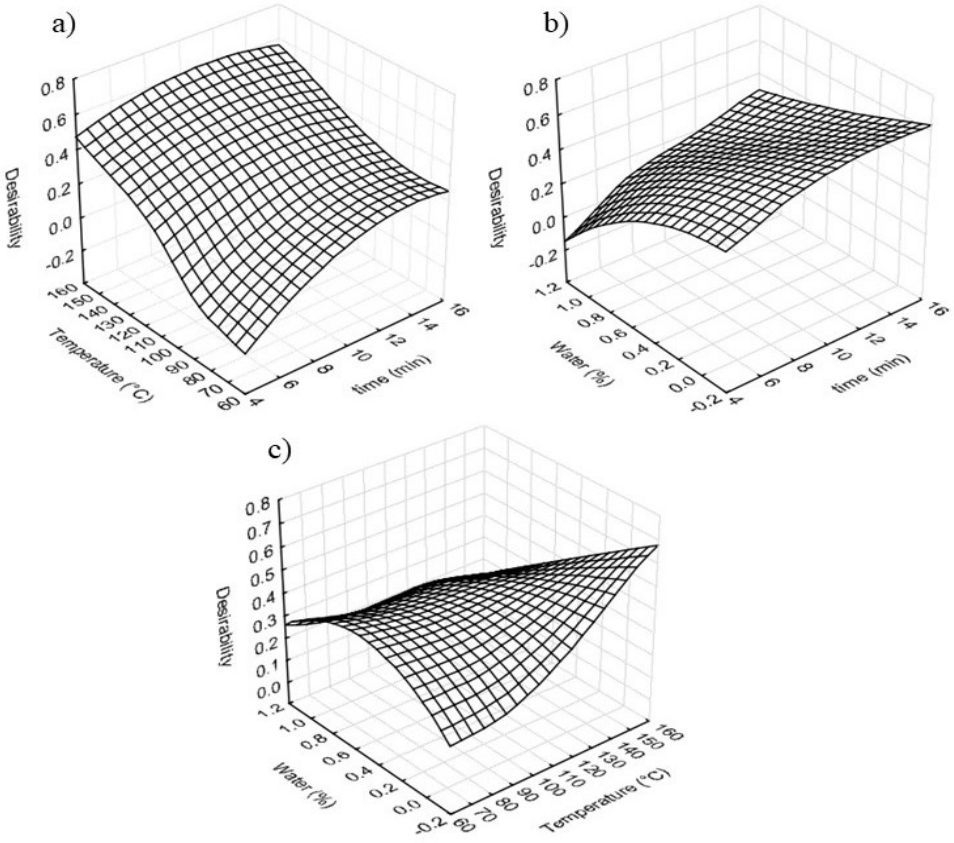
413 nd - not detected. < LOQ – not quantifiable. [I] Also determined by (Garcia et al., 2019). [II] Also
414 determined by (Souza, 2014). [III] Also determined by (Hassanbaglou et al., 2012).

415

3.3.6 Optimization

The desirability function was applied to determine the global optimum extraction conditions for the responses Yield, DPPH ($1/IC_{50}$ µg mL^{-1}), ABTS (µmol g^{-1}), FRAP (µmol g^{-1}) and TPC (mg g^{-1}). Acceptable desirability (D) responses range from 0 to 1, with more sensitive responses at higher D value (closer to 1), representing the optimized condition of the system. The overall desirability value, maximizing all responses, was determined as 0.561, indicating the optimum conditions at 12.5 minutes, 150 °C and ethanol as solvent (**Fig. 2**).

Fig.2. Desirability function graph maximizing all the responses (Yield, DPPH, ABTS and TPC), effect of the factors: time (min), temperature (°C) and water (%) on microwave assisted extractions from *Pereskia grandifolia* leaves; a) Temperature x time x desirability; b) Water (%) x time x desirability and c) Water (%) x temperature x desirability.



Other simulations were held to evaluate desirability value: first maximizing only TPC, DPPH, ABTS and FRAP with desirability value going to 0.68 (t = 5 min, T = 70 °C, S = ethanol), then maximizing only antioxidant activity DPPH, ABTS and FRAP with D value of 0.71 (t = 10 min, T = 150 °C, S = ethanol) and the maximum D value was obtained when maximizing only TPC, DPPH and FRAP, 0.95 (t = 5 min, T = 70 °C, S = ethanol). However, only the optimization maximizing all responses was tested experimentally, as follows.

The desirability values for the individual responses were also calculated and are presented in **Table 5**, alongside with the results found for the optimum conditions, done experimentally in triplicate to evaluate the model predicted values. The results corroborate with the model for prediction of optimum conditions for the responses Yield (%), TPC, DPPH and ABTS, as seen in **Table 5**, which means that the model has a good fit. As presented in the table, the relative standard deviation between the predicted and the observed values were less than 10% for the cited variables, except for FRAP analysis, which in this case the observed values were 1.7 times the predicted value.

445 Table 5 - Individual and global optimum responses for Yield, TPC and antioxidant activity (DPPH, ABTS and PLE).

	Desirability value (DV)	**Time (min)**	**Temperature (°C)**	**Water (%)**	**Predicted**	**Observed**	**RSD (%)**
Individual							
Yield (%)	0.96	5	150	0.75	36.50	-	
TPC ($\mu g_{GAE}.g^{-1}$)	1.00	5	70	1	133.35	-	
DPPH ($\mu g.mL^{-1}$)	0.75	5	70/150	0	143.06	-	
ABTS ($\mu mol_{TEAC}.g^{-1}$)	0.76	12.5	150	1	1,718.8	-	
FRAP ($\mu mol_{TEAC}.g^{-1}$)	1.00	7.5	150	0	360.85	-	
Global	0.56	12.5	150	0			
Yield (%)					15.77	16.19 ± 1.90	1.9
TPC ($\mu g_{GAE}.g^{-1}$)					103.84	115.51 ± 0.01	7.5
DPPH ($\mu g.mL^{-1}$)					194.93	192.87 ± 2.44	0.8
ABTS ($\mu mol_{TEAC}.g^{-1}$)					1540.7	1446.34 ± 51.51	4.5
FRAP ($\mu mol_{TEAC}.g^{-1}$)					298.61	498.15 ± 5.97	34.2

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3.4 Conclusions

MAE applied for ora-pro-nobis laves provided extract samples with high antioxidant capacity and also high content of phenolic compounds. These results corroborate with the literature, showing the high potential of this non-conventional food plant for application in cosmetic, pharmaceutical and food industries. The best yield performance was provided by water and 50% ethanol as solvents, at 150 ° C. For the TPC, the best response was found at 70 °C, 5 min and a 50% EtOH. For DPPH, the greatest antioxidant activity was found in extracts with ethanol at the highest temperatures evaluated, and the time was not significant. In the ABTS method, the best responses were found with water and mixture of the solvents (50% EtOH) without significant influence of time and optimal condition tending to higher temperatures. FRAP results were similar to DPPH for these samples. Around 24 compounds were identified in the samples, the main compounds being Caffeic acid, Ellagic acid, *p*-Anisic acid, *p*-Coumaric acid Kaempferol and Quercetin. Ellagic acid and *p*-Anisic acid were firstly reported in the present study for the ora-pro-nobis leaves. The optimal conditions of global extraction evaluated by MAE were 150 °C, 12.5 minutes and ethanol as solvent. Compared with the conventional extraction technique (Soxhlet) the MAE proved efficient in obtaining extracts rich in antioxidant compounds and with high phenolic content, known for their bioactive activities, using a considerably lower time of extraction for the recovery of compounds.

3.5 Acknowledgments

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CHAPTER 4: APPLICATION OF HIGH-PRESSURE TECHNOLOGIES TO RECOVERY OF BIOACTIVE COMPOUNDS FROM ORA-PRO-NOBIS (*Pereskia aculeata*) LEAVES USING THE BIOREFINERY CONCEPT

This chapter presents the study related to the extraction of bioactive compounds from ora-pro-nobis leaves from *Pereskia aculeata* species by means of high-pressure techniques, supercritical fluid extraction (SFE) and pressurized liquids extraction (PLE). Besides, the influence of process combination (SFE and PLE), on the studied responses was also evaluated. The species *Pereskia aculeata* is a more abundant variety, compared to *Pereskia grandifolia* (studied in previous sections), which proximate this study to the most available raw materials, and contributes to detect variations within species of ora-pro-nobis. The data from the previous chapter help the solvents definition for the following studies. The results from this chapter are divided in two parts: (1) optimization of the high-pressure extraction methods to recover valuable compounds from ora-pro-nobis; (2) application of the biorefinery approach by combining extraction methods. Each study was written in the form of a scientific article to be submitted to international journals with a high impact factor.

MANUSCRIPT 2: Neuroprotective potential of extracts from leaves of ora-pro-nobis (*Pereskia aculeata*) recovered by clean compressed fluids

Abstract

Ora-pro-nobis (*Pereskia aculeata*), an emerging unconventional food plant belonging to the Cactaceae family, is known as a source of protein with high nutritional value, traditionally used in folk medicine. In this study, different compressed fluid technologies were used to obtain antioxidant rich extracts from *P. aculeata* leaves. The recovered extracts from ora-pro-nobis leaves showed, in general, high antioxidant potential in DPPH and FRAP *in vitro* assays. Complementary, supercritical fluid extracts presented acetylcholinesterase inhibition and anti-inflammatory activity. Overall, 11 compounds were identified from the non-polar extracts belonging to terpenoids and phenolic compounds. Metabolites like 2,4-Ditert-butylphenol, β-sitosterol, campesterol, phytol, Vitamin E were already identified in other *Pereskia* sp. extracts, but compounds like fucosterol and lupeol were also tentatively identified in the present study. Terpenes and phenolic compounds are known for its biological activities, which shows the potentiality of the *P. aculeata* leaves extracts recovery by compressed fluids technologies.

Keywords: Supercritical fluid extraction; pressurized liquid extraction; antioxidant; acetylcholinesterase; anti-inflammatory.

4.1 Introduction

Bioactive compounds from natural sources have been of global interest due to some characteristics like their low or absent toxicity, complete biodegradability and availability from renewable sources (TRINGALI, 2000). However, trials are needed to proof the natural extracts efficiency and several researches are growing related to extraction methods with sustainable appeal. Green techniques can enhance the economical relevance of the natural products (ROMBAUT et al., 2014). High-pressure extraction techniques like supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are within the green technology concept, when using green solvents like CO_2, ethanol and water, for example. *Pereskia aculeata*, commonly known as ora-pro-nobis, is a plant from *Pereskia* genus. They are classified as Unconventional Food Plants (UFPs), usually not valued as food materials, but are popularly used and have high nutritional value and several medicinal properties (KINUPP; BARROS, 2008). Then, environmentally friendly methods, allied with very rich plant matrices such as ora-pro-nobis, may create a product of high industrial interest.

Neurodegenerative diseases are defined by the disfunction and loss of neuronal cells in the nervous system and are associated with protein aggregates (FU; HARDY; DUFF, 2018). Alzheimer's disease (AD) is a multifactorial progressive neurodegenerative disorder. Free radical oxidative stress, neuroinflammation, head trauma and diabetes are considered risk factors of AD (BENZI; MORETTI, 1995). Various chemical classes of compounds can act as antioxidants, such as phenolic compounds (BARBA et al., 2016), terpenes and isoprenoids (TETALI, 2019), among others. Therefore, it is natural to associate bioactive compounds to an effectiveness in Alzheimer's disease. AD is also associated with the depletion of the neurotransmitter acetylcholine (ACh) (ANAND; SINGH, 2013). Acetylcholinesterase (AChE) enzyme is

considered a therapeutic target of Alzheimer's disease. *In vitro* assessment of inflammatory process applied to plants are often related to pro-inflammatory enzymes mechanism, such as Lipoxygenases (LOXs) that can act in the biosynthesis of inflammatory lipid mediators, such as prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) and hydroxyeicosatetraenoic acids (HETE) (JIMÉNEZ-ASPEE et al., 2015).

In the present study, a careful optimization of the extraction conditions (e.g., temperature, pressure and solvent) was carried out to obtain high recoveries of compounds with bioactivity potential as neuroprotective agents. With that in mind, acetylcholinesterase, lipoxygenase activity and antioxidant activity assays of ora-pro-nobis extracts were assessed to understand their enzyme inhibition capacity in cholinergic, inflammatory and oxidation processes, all of them involved in Alzheimer's disease progression.

4.2 Materials and methods

4.2.1 Raw material and sample preparation

The ora-pro-nobis (*Pereskia aculeata*) leaves were purchased by the Sítio Flora Bioativas company (Tijucas, SC, Brazil). The raw material drying and grinding was held before acquisition and followed the steps: the harvest was done in the morning, then the leaves were washed in stainless-steel vats very quickly to avoid losing the mucilage of the leaves, the leaves were then spread through the drying trays and subjected to drying at 45 °C in an air-circulating dehydrator (MS Metalúrgica e Comércio, Canoinhas, SC, Brazil) for 18 to 24 h, the rotation of the trays made every 2 h. After drying the leaves were grinded in a home-made stainless-steel hammer mill and packet at room

temperature. The samples were then acquired and stocked at -18 °C, to extend shelf life of the product, until further extractions.

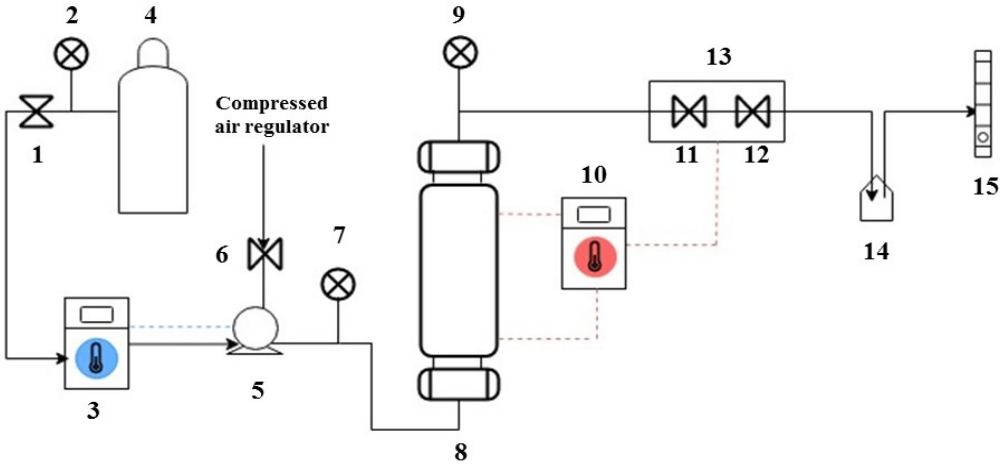
4.2.2 Extraction procedures

4.2.2.1 Supercritical Fluid Extraction (SFE)

Supercritical fluid extractions of ora-pro-nobis (*Pereskia aculeata*) leaves were performed in an extraction unit developed by Zetzl et al. (ZETZL; LOZANO; BRUNNER, 2007), and adapted by Michielin et al. (MICHIELIN et al., 2005) and Mazzutti et al. (MAZZUTTI et al., 2018). A schematic design of the extraction unit is shown in **Fig. 1**. The solvent used was CO_2 (99.9%, pure) delivered at pressure up to 6 MPa (White Martins Ltda., Joinville-SC, Brazil). The extraction procedure was described by Michielin et al. (MICHIELIN et al., 2005) and the time of extraction was determined by a kinetic study. The extraction conditions were chosen according to previous studies of the group (ANDRADE et al., 2012; MAZZUTTI et al., 2018), and on the basis of previous results from a SFE study with ora-pro-nobis (*Pereskia bleo*) leaves (SHARIF et al., 2015). The SFE conditions evaluated were 40, 50 and 60 °C; solvent flow rate and pressure were kept constant at $3.33x10^{-4}$ $kg.s^{-1}$ and 25 MPa, respectively. A previous kinetics evaluation of the overall extraction curve (OEC) was performed to establish the extraction time. The kinetics was performed at 50 °C, 25 MPa and CO_2 flow rate of $3.33x10^{-4}$ $kg.s^{-1}$ and samples were collected at pre-established time intervals. Based on the OEC, the extraction time was fixed at 120 min for the SFE assays, representing the diffusional period of extraction (CAMPOS et al., 2005). Briefly, the extraction procedure consisted of placing 20 g of dried and milled sample inside a stainless-steel extraction vessel (329 mm length, 20.42 mm internal diameter), completed with glass beads. The extracts were collected in amber glass flasks previously weighted, and stored at -18 °C

(in a domestic freezer). The experiments were performed in duplicate and the extraction yield (X_0), calculated as the ratio between the extract mass and the dried sample mass represented as percentage (%, w/w), was expressed as mean values ± standard deviation (SD).

Fig.1. Scheme of SFE extraction unit. 1: CO_2 regulator valve; 2, 7, 9: Manometer; 3: Cooling bath; 4: CO_2 cylinder; 5: CO_2 pump (M111, Maximator, Niedersachen, Germany); 6: Compressed air regulator; 8: Jacketed extraction vessel (stainless steel cylinder, L = 32,9 cm height, di = 2,042 cm internal diameter; V = 107,74 mL volume); 10, 13: Heating bath; 11, 12: Regulator needle valve; 14: Extract Reservoir; 15: Rotameter.

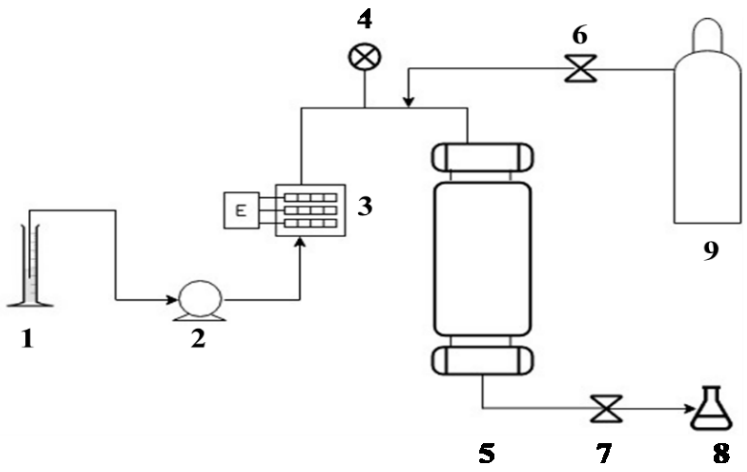


4.2.2.2 Pressurized Liquid Extraction (PLE)

A self-assembled apparatus was used to perform the pressurized liquid extractions using ethanol and distilled water as solvents. The equipment and the procedure were described by Gonçalves Rodrigues et al. (GONÇALVES RODRIGUES et al., 2019). The scheme of the extraction unit is shown in **Fig. 2**. The extraction conditions were chosen based in previous studies from the research group (MAZZUTTI et al., 2018;

GONÇALVES RODRIGUES et al., 2019). The yield assays were conducted at three different temperatures (50, 80 and 110 °C), 10 MPa, 4 mL.min^{-1} of solvent and at fixed extraction time, defined according to the kinetics study. The kinetic study was conducted at 80 °C, 10 MPa and 4 mL.min^{-1} solvent flow rate for 36 min, where aliquots of extract were collected at pre-established time intervals to design the OEC, for each solvent (water and ethanol) (GONÇALVES RODRIGUES et al., 2019). The extraction time was set at 15 min, determined when approximately 90% of the extract was obtained. Briefly, 8 g of dried sample and 60 g of glass beads form the fixed extraction bed. The pressure was set by pumping the solvent into the extraction cell using an HPLC pump (Waters, model 515, USA). After the extraction, the recovered samples were dried to solvent withdrawal by rotary evaporator (Fisatom, model 801, São Paulo, Brazil) or freeze dryer (Liotop®, model L101, São Carlos, Brazil) and stored at -18 °C with absence of light. The experiments were carried out in duplicate and the extraction yield (X_0) expressed as mean values ± SD.

Fig.2. Scheme of PLE extraction unit. 1: Solvent reservoir; 2: HPLC pump; 3: Electric heat exchanger; 4: Manometer; 5: Extraction vessel with electrical heating jacket; 6: Regulator valve; 7: Regulator needle valve; 8: Extract reservoir; 9: N_2 cylinder.



4.2.2.3 Soxhlet extraction (SOX)

Soxhlet extraction was performed according to 920.39 method of AOAC (2005) (AOAC, 2005b), using hexane P.A. (Synth, São Paulo, Brasil) or ethanol P.A. (Neon, São Paulo, Brazil) as solvents, for separate extractions. Briefly, the experimental procedure consists in adding 150 mL of solvent to the Soxhlet extractor with 5 g of dried sample for 6 h. The extracts recovered were dried to solvent withdrawal and stored at -18 °C with absence of light. The extraction was performed in duplicate and the extraction yield (X_0) was represented as mean values ± SD.

4.2.3 Total phenolics content (TPC)

The total phenolics content was determined according to Singleton, Orthofer, & Lamuela-Ravento´s (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1999). A stock solution of 2 mg mL^{-1} of gallic acid (98% purity, Sigma Aldrich, St. Louis, MO, USA) was prepared and diluted in distilled water to assessment of a calibration curve. The standard curve was obtained with a determination coefficient (R^2) of 0.9952. The samples were prepared by dilution in ethanol P.A. (Neon, São Paulo, Brazil) up to a concentration of 10 mg mL^{-1}. The reaction mixture with 10 µL of extract or solvent of extraction (blank), 50 µL of reagent Folin-Ciocateau (Sigma Aldrich, St. Louis, MO, USA), 150 µL of 20% sodium carbonate (Lafan, São Paulo, Brazil) and 800 µL of distilled water. Then, the samples were incubated at room temperature, in the absence of light for 2 h and read at 760 nm (Femto 800 XI, São Paulo / SP - Brazil). The experiments were carried out in triplicate (mean±SD) and the results expressed in milligrams of gallic acid equivalent (GAE) per gram of dry extract ($mg_{GAE}\ g_{extract}^{-1}$).

4.2.4 DPPH free radical scavenging assay

The method of DPPH was described by Brand-Williams et al. (BRAND-WILLIAMS; CUVELIER; BERSET, 1995) and adapted by Staško et al. (STAŠKO et al., 2007). The extracts were diluted in different concentrations (5 to 8 dilutions for each extract). Then, 20 µL of the samples were added in a microplate followed by 130 µL of DPPH 0.1 mM (Sigma Aldrich, St. Louis, MO, USA) diluted in ethanol, and 130 µL of monobasic phosphate buffer pH 6.0. The samples were incubated in the absence of light and at room temperature for 30 min. The absorbance values were read at 525 nm (TECAN, multi-plate reader, Infinity M200). The results were expressed as IC_{50} (the effective concentration to reduce 50% the antioxidant activity of the radical DPPH, compared to a blank solution) as the average of triplicate assays (mean±SD). The results are compared with BHT (butylated hydroxytoluene), a standard synthetic antioxidant used as food additive.

4.2.5 FRAP assay

The FRAP method was described by Benzie & Strain (BENZIE; STRAIN, 1996). The FRAP reagent was prepared using a 0.3 M sodium acetate buffer solution (pH 3.6), a 20 mM ferric chloride solution ($FeCl_3x6H_2O$) and a diluted TPTZ solution (≥ 98% purity, Sigma Aldrich, St. Louis, MO, USA) diluted in 40 mM hydrochloric acid (HCl, 37% P.A.-A.C.S., Synth, São Paulo, Brazil), in a 10:1:1 proportion. Samples previously diluted were added to the reagent and the reaction took place in the absence of light for 30 min. After incubation time, the samples were analyzed on spectrophotometer at 593 nm (TECAN, multi-plate reader, Infinity M200). The Trolox (97% purity, Sigma Aldrich, St. Louis, MO, USA) was used as standard in a calibration curve. The analysis was

performed in triplicate (mean±SD) and the results were expressed as Trolox equivalent (TE) ($mmol_{TE}.g^{-1}$).

4.2.6 Acetylcholinesterase (AChE) activity assay

Acetylcholinesterase (AChE) activity was determined by Ellman's method with modifications (ELLMAN et al., 1961). The velocity of consumption of the substrate is verified with the help of a fluorescent reagent, 4-fluoro-7-sulfamoylbenzofurazan (ABD-F, Sigma Aldrich, St. Louis, MO, USA), which directly links to the sulfur atom of thiols (SÁNCHEZ-MARTÍNEZ et al., 2021). Initially, the K_M (Michaelis–Menten constant) of the enzyme is calculated to determine the substrate concentration of the reaction rate is half of maximum velocity rate. Reactions mixtures in the wells contained the following reagents: 100 µL of substrate acetylthiocholine iodide (ACth) (Sigma Aldrich, St. Louis, MO, USA) was added at different concentrations (0.4 - 4 mM) in H_2O; 50 µL of pure EtOH; 100 µL of buffer (150 mM Tris-HCl pH=8); 25 µL of ABD-F (125 µM) in buffer; and 25 µL of 0.8 U/mL AChE (Sigma Aldrich, St. Louis, MO, USA). To determine the inhibition capacity of the extracts against the acetylcholinesterase enzyme, each well was filled with 100 µL of extract sample (diluted in EtOH/H_2O [1:1, v/v]) at different concentrations; 100 µL of buffer; 25 µL of 0.8 U/mL AChE in buffer and 25 µL of ABD-F (125 µM) in buffer. The mixture was incubated for 10 minutes**.** Reaction started by adding 50 µL of ACth at concentration of K_M in H_2O. The V_{mean} of the substrate consumption is recorded each 10 s (V_{mean} corresponds to enzymatic mean velocity during kinetic) in fluorescence mode (λ_{ex} = 389 nm and λ_{em} = 513 nm) during 10 min in a Synergy HT 96-well microplate reader (Biotek, Winooski, VT, USA). The velocity of the reaction is compared to a control (assay without extract sample). Galantamine was used as a positive control. Tests were carried out in triplicate. Results were expressed as IC_{50}, which

is the sample concentration when 50% inhibition is reached and was obtained plotting the inhibition percentage against sample concentrations.

4.2.7 Lipoxygenase (LOX) activity assay

Lipoxygenase (LOX) activity was determined by fluorescence assay based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxydes, which can degrade the fluorescence of fluorescein (NUÑEZ; FOGLIA; PIAZZA, 1995; SÁNCHEZ-MARTÍNEZ et al., 2021). The assay consists in first determining K_M of the enzyme with linoleic acid (substrate) at different concentrations (0.7 – 7 mM). Then, the inhibition assay mixture containing the extract (diluted in EtOH/H_2O [1:1, v/v]) at different concentrations, 1 µM of fluorescein diluted in buffer (150 mM Tris-HCl pH=9), soybean lipoxygenase (948 U/µL) (Sigma Aldrich, St. Louis, MO, USA) (diluted in buffer), and linoleic acid at concentration of K_M in EtOH/H_2O [1:1, v/v], was added to a 96-well microplate and fluorescence is recorded every 10 s during 15 min using a Synergy HT 96-well microplate reader (Biotek, Winooski, VT, USA) in fluorescence mode (λ_{ex} = 485 nm and λ_{em} = 530 nm). Negative control without extract sample were also included. The enzymatic activity was graphically determined from the slope of the linear portion of the curve and the V_{mean} of the reaction is compared to a control. Quercetin was used as reference compounds. LOX-1 from soybean is routinely used since it resembles human LOXs in its substrate specificity and inhibition characteristics (JIMÉNEZ-ASPEE et al., 2015). Tests were carried out in triplicate, with results expressed as IC_{50}, which is the sample concentration providing 50% inhibition of the LOX enzyme and was obtained plotting the inhibition percentage against sample concentrations.

4.2.8 Tentative identification of chemical composition by (GC-q-TOF-MS)

The composition of non-polar extracts of *Pereskia aculeata* leaves was studied by GC-q-TOF-MS. Samples were analyzed in an Agilent 7890B gas chromatograph coupled to an Agilent 7200 quadrupole time-of-flight (q-TOF) mass spectrometer, equipped with an electronic ionization (EI) interface. An Agilent Zorbax DB5- MS^{+} 10 m Duragard Capillary Column (30 m × 250 μm, 0.25 μm) was used for chromatographic separation. Sample injection volume was 1 μL. The injector was operated in split mode (ratio of 10:1 and a split flow of 8.4 mL/min) at 250 °C. Helium was used as carrier gas at a constant flow (0.8 mL/min). The oven temperature was programmed to start at 60 °C, heated to 325 °C at 10 °C/min and held at this temperature for 10 min, totalizing injection time at 36.5 min. MS parameters were the following: electron impact ionization at 70 eV, filament source temperature of 250 °C, quadrupole temperature of 150 °C, m/z scan range 50–600 amu at a rate of 5 spectra per second. Systematic mass spectra deconvolution of chromatographic signals and tentative identification of unknowns was performed using the Agilent Mass Hunter Unknown Analysis tool and mass spectral databases (i.e. NIST MS Search v.2.0 and Fiehn Lib).

4.2.9 Statistical analysis

All extraction procedures were carried out in duplicate and all *in vitro* analysis were carried out in triplicate. The results were expressed as the mean ± standard deviation (SD). Antioxidant activity, total phenolics content and identified compounds were analyzed by the Pearson test correlation coefficients with 95 % confidence. The one-way ANOVA followed by post hoc Tukey's HSD test at the $p < 0.05$ level were applied to all the results, using software Statistica version 7.1 (Stat-Soft Inc., Tulsa, OK, USA).

4.3 Results and discussion

4.3.1 Extraction yield (X_0)

The extraction time was defined by the kinetics procedure for SFE and PLE, performed at the intermediary temperature condition. For the SFE, the extraction time was set as 120 min; while for PLE the time was set as 15 min, both representing the time where the overall extraction curve reached the diffusional period (WEINHOLD et al., 2008). The values obtained for global extraction yield for SFE, PLE and Soxhlet extraction (SOX) are presented in **Table 1**. The highest yields were obtained by SOX and PLE using ethanol as solvent, with significant difference from other values. Lower yield values were obtained by SFE, SOX hexane and PLE water, with no significant differences (Tukey's test, $p<0.05$).

Table 1 – Extraction yield, total phenolic content and antioxidant activity (DPPH and FRAP) of ora-pro-nobis (*Pereskia aculeata*) leaves extracts by different extraction methods (SOX, SFE and PLE).

T	Solvent	Yield (%)	TPC (mg_{GAE} $g^{-1}_{extract}$)	DPPH IC_{50} (mg mL^{-1})	FRAP ($mmol_{TE}$ g^{-1})
Soxhlet extraction – atmospheric pressure, 6 h/360 min					
	Hexane	4.66 ± 0.40^{ab}	4.96 ± 0.29^{a}	7.83 ± 0.13^{j}	0.09 ± 0.01^{c}
	Ethanol	15.08 ± 2.13^{c}	38.18 ± 6.78^{cde}	1.00 ± 0.03^{d}	0.16 ± 0.01^{e}
Supercritical Fluid Extraction (SFE) – 25 MPa, 1.2 kg h-1, 120 min					
40 °C	CO_2	1.78 ± 0.08^{a}	3.71 ± 0.12^{a}	3.09 ± 0.17^{g}	0.02 ± 0.01^{a}
50 °C	CO_2	1.69 ± 0.01^{a}	4.19 ± 0.01^{a}	6.35 ± 0.22^{i}	0.03 ± 0.01^{ab}
60 °C	CO_2	1.68 ± 0.11^{a}	4.90 ± 0.01^{a}	5.05 ± 0.22^{h}	0.05 ± 0.01^{b}
Pressurized Liquid Extraction (PLE) – 10 MPa, 4 mL min-1, 15 min					
50 °C	Ethanol	6.87 ± 0.12^{ab}	37.24 ± 1.96^{cde}	2.88 ± 0.08^{g}	0.12 ± 0.01^{d}
80 °C	Ethanol	10.06 ± 0.15^{bc}	43.02 ± 2.26^{e}	2.54 ± 0.03^{f}	0.11 ± 0.01^{cd}
110 °C	Ethanol	13.09 ± 0.09^{c}	60.09 ± 1.77^{f}	1.64 ± 0.04^{e}	0.17 ± 0.01^{e}
50 °C	Water	2.29 ± 0.27^{a}	30.58 ± 2.02^{bc}	1.38 ± 0.18^{e}	0.10 ± 0.01^{cd}
80 °C	Water	3.07 ± 0.02^{a}	39.52 ± 2.27^{cde}	0.31 ± 0.02^{a}	0.25 ± 0.01^{g}
110 °C	Water	4.65 ± 0.38^{ab}	33.01 ± 0.80^{cd}	0.72 ± 0.07^{c}	0.21 ± 0.02^{f}
BHT		-	266.4*	0.17 ± 0.01	1.54 ± 0.01**

*Cruz (2016).

**Cui, Gu e Kang (2016).

Values with same letters in the same columns are not significantly different, according to ANOVA test (Tukey, $p < 0.05$).

In general, extraction yield values were different between high-pressure and conventional methods, when comparing solvents with similar polarities. For instance, SFE presented lower yield than SOX hexane (both non-polar solvents), probably due to hexane and CO_2 characteristics at extraction conditions. Thus, SOX hexane may have recovered cuticular waxes from the ora-pro-nobis leaves, mostly high molecular weight non-polar compounds, not particularly easily recovered by carbon dioxide (DE LUCAS et al., 2002). Besides, carbon dioxide is more selective than hexane to lipid components. Therefore, SOX hexane provide higher yield than SFE probably due to solvent recycling, prolonged extraction time and higher amount of solvent used (DE LUCAS et al., 2002; GUEDES et al., 2020). Additionally, Sharif et al. (2015) performed SFE at 25 MPa and 40 °C for ora-pro-nobis leaves from the *P. bleo* specie, and obtained lower yield performance (0.85 %) compared to the present study for *P. aculeata* leaves, at the same conditions of pressure and temperature (1.78 %) (**Table 1**). This difference was probably due to the species variations.

On the other hand, the yield values of PLE-ethanol at 110 °C and Soxhlet ethanol (13.09 and 15.08%, respectively) presented no significant difference. Even though, PLE and Soxhlet were held at different temperatures, with Soxhlet been held at temperature close to the solvent boiling point (78 °C). Comparing PLE at similar temperature (80°C), lower yield was reached (67% of Soxhlet-ethanol yield), probably because the extraction time and solvent volume were 24 and 2.5 times lower, respectively, for PLE related to Soxhlet method. The same behavior was observed by Rebelatto et al. (REBELATTO et al., 2020) for pink pepper extraction, where the yield of PLE with ethanol was 75% of the Soxhlet yield with ethanol. Nevertheless, for PLE, the yield increased with temperature.

PLE-water presented the lowest yield, although it is worth mention that pressure variation was detected during extraction because of changes in the flow of the

280 solute/solvent stream. This was probably caused by formation of a colloidal phase due to
281 the high content of mucilage (heteropolysaccharide) from ora-pro-nobis plant. The
282 mucilage retained water, affecting the flux and reducing extraction yield. The same
283 behavior was observed by Kanmaz and Ova (KANMAZ; OVA, 2013), during subcritical
284 water extraction from flaxseed (*Linum usitatissimum* L.), with a mucilage formation
285 which made the extraction difficult.

286 The use of ethanol for PLE and SOX provided intermediate values of yield,
287 compared to other solvents (water, CO_2 and hexane), which can be associated to the
288 intermediate polarity of ethanol. The increase in temperature on PLE provided higher
289 yield values for both studied solvents (ethanol and water). Higher temperatures increase
290 the solute-solvent interactions, reducing solvent surface tension, enhancing penetration
291 into solid matrix, improving extraction rates (MUSTAFA; TURNER, 2011). However,
292 this increase in temperature is also associated with thermal degradation of compounds of
293 biological interest, such as flavonoids (OKIYAMA et al., 2018), which might be
294 evaluated by investigating the properties and composition of the extracts, as follows.

295

296 4.3.2 Total phenolics content (TPC)

297 Total phenolics content values ranged from 3.71 to 60.09 $mg_{GAE}.g^{-1}$ (**Table 1**).
298 The highest TPC values were found at PLE-ethanol and PLE-water extracts. Also, these
299 TPC results were higher than obtained by Garcia et al. (GARCIA et al., 2019) for the
300 hydro methanolic extract from *Pereskia aculeata* leaves (23.75 $mg.g^{-1}$) and similar to that
301 reported by Cruz et al. (CRUZ et al., 2021), also for *P. aculeata* leaves, with values
302 ranging between 26 and 66 mg_{GAE}/g.

303 The TPC values were higher in the PLE-ethanol extracts than PLE-water extracts,
304 recovery increasing with temperature with ethanol and best TPC found at 80 °C using

305 water as solvent. The TPC increase with temperature in PLE-ethanol is probably due to
306 the increase in mass transfer rates due to reduction in solvent viscosity and surface
307 tension, increasing solute-matrix disruption (MUSTAFA; TURNER, 2011). Besides, in
308 the present study, PLE-ethanol was more efficient to recover phenolic compounds than
309 Soxhlet extraction. The technics at higher pressure presented better TPC recovery
310 compared to the conventional extraction at lower pressure, even though the extraction
311 time was 24 times lower.
312
313 4.3.3 Antioxidant activity
314 The antioxidant activity of extracts from ora-pro-nobis leaves was determined by
315 DPPH and FRAP methods, as shown in **Table 1**. The sample recovered by PLE-water
316 (80 °C) exhibited the highest antioxidant activities, whereas the lowest values were found
317 for SOX hexane and SFE samples (non-polar solvents). Although the influence of
318 temperature on PLE depends on the solvent applied, for PLE-ethanol samples, best
319 antioxidant potential was obtained at 110 °C. In general, the water-based extracts
320 provided the best antioxidant activities from the evaluated methods. Few authors
321 evaluated antioxidant activity of water-based extracts from *Pereskia* sp. leaves, although
322 some works are reported in this regard using conventional extraction methods (SIM;
323 NURESTRI; NORHANOM, 2010; SOUZA et al., 2016).
324 Polar solvents were more efficient in recovering compounds with antioxidant
325 activity from ora-pro-nobis (*Pereskia aculeata*) leaves, in agreement with Solana et al.
326 (SOLANA et al., 2015), who state that water and ethanol are essential to obtain extracts
327 with high antioxidant capacity. Besides, the increase in extraction temperature enhances
328 the recovery of antioxidant components and also contributes to the formation of new
329 antioxidant compounds by Maillard reaction. Plaza et al. (PLAZA et al., 2010) suggested

that compounds formed by Maillard reactions, caramelization and thermo-oxidation contribute to antioxidant activity of extracts obtained by subcritical water. However, high temperatures can affect the stability of some compounds with antioxidant activity, causing degradation, molecular structural changes or the formation of pro-oxidant compounds; as observed in PLE-water extracts.

A Pearson's correlation was held to evaluate the relationship between the antioxidant activity assays with the total phenolic content values, at 95% significance confidence. A significative and negative correlation was found between IC_{50} (DPPH method) and TPC (r = -0.75), negative because low IC_{50} values represent better antioxidant performance. Otherwise, the FRAP results had a significant and positive correlation with TPC (r = 0.67). Schober et al. (SCHOBER; BOER; SCHWARTE, 2018) classified the correlations magnitude based on the obtained results as: negligible (0.00 – 0.10), weak (0.10 – 0.39), moderate (0.40 – 0.69), strong (0.70 – 0.89) and very strong (0.90 – 1.00). Following this classification, correlation between TPC and DPPH is a strong correlation while between TPC and FRAP is a moderate correlation. This indicates that antioxidant activity is related to the presence of phenolic compounds from extracts of ora-pro-nobis leaves, recovered by SFE, PLE and Soxhlet.

4.3.4 Anti-cholinergic and anti-inflammatory activity of *P. aculeata* leaves extracts

Initially, to observe the anti-inflammatory potential from ora-pro-nobis extracts, polar and non-polar SOX extracts were compared. As expected, the non-polar extract was more efficient as lipoxygenase inhibitor, since non-polar compounds are more related to acetylcholinesterase (AChE) and lipoxygenase (LOX) activity. AChE inhibitory activity is more related to alkaloids, terpenes, flavonoids and phenolic compounds, while

lipoxygenase inhibitors are mainly flavonoids and others classes of compounds found in essential oils (ANAND; SINGH, 2013; BAYLAC; RACINE, 2003; KIM et al., 2004; SANTOS et al., 2018). Hence, enzymatic inhibitory activity assays were conducted for AChE inhibitory activity and anti-inflammatory activity (LOX) using the non-polar extracts. The results from *P. aculeata* extracts, expressed as IC_{50}, are presented in **Table 2** for SOX-hexane and SFE extracts obtained at different temperatures. The strongest AChE inhibitor was the sample recovered by SFE at 50 °C, while the most effective LOX inhibitor was the sample by SFE at 40 °C.

Table 2 – IC_{50} values from LOX and AChE enzymatic inhibition assays of *Pereskia aculeata* leaves extracts.

Samples	AChE IC_{50} $\mu g.mL^{-1}$	LOX IC_{50} $\mu g.mL^{-1}$
SOX hexane	197.20 ± 25.33^{a}	103.95 ± 9.58^{a}
SFE 40 °C	295.79 ± 47.96^{b}	90.60 ± 8.71^{a}
SFE 50 °C	174.59 ± 52.82^{a}	163.92 ± 6.46^{b}
SFE 60 °C	207.11 ± 52.29^{ab}	141.29 ± 11.62^{c}

Values with same letter in the same column are not significantly different, according to ANOVA test (Tukey, $p < 0.05$).

Santos et al. (2018) classified AChE inhibition potency as: high potency, $IC_{50} < 20$ $\mu g.mL^{-1}$; moderate potency, $20 < IC_{50} < 200$ $\mu g.mL^{-1}$; and low potency, $200 < IC_{50} < 1{,}000$ $\mu g.mL^{-1}$. According to this classification, SOX and SFE at 50 °C presented moderate potency, while SFE at 40 and 60 °C present low potency. Such classification is not found for anti-inflammatory assay using LOX enzyme. However, comparing the IC_{50} values obtained in the present study with values found for other plants, it is possible to see the high potential of ora-pro-nobis extracts as an anti-inflammatory agent. The IC_{50}

was equivalent to found for bellyache bush leaves (*Jatropha gossypifolia* L.) and superior to many other plants well described by Santos et al. (SANTOS et al., 2018). The anti-inflammatory capacity of non-polar extracts of *Pereskia* sp. plants was also evaluated by *in vivo* assays, in agreement with our results. Pinto et al. (PINTO et al., 2015b) observed strong *in vivo* anti-inflammatory activity from methanolic extracts from *P. aculeata* in mice.

4.3.5 Tentative compounds identification of ora-pro-nobis non-polar extracts

The chemical composition of non-polar extracts with demonstrated bioactive potential was determined by GC-MS analysis. GC-MS analysis of SFE and SOX hexane extracts with observed biological activity was carried out. **Table 3** shows the retention time (min) and a tentative identification of compounds from *P. aculeata* leaves extracts recovered by SFE (at different temperatures) and SOX by hexane. Eleven compounds were tentatively identified, including 10 terpenoids and one di-tert-butylphenol isomer, previously reported in ora-pro-nobis leaves (NURESTRI et al., 2009b; NURESTRI; SIM; WAHAB, 2009; PINTO et al., 2015a). 2,4-Di-tert-butylphenol is a phenolic compound with several bioactive properties, as shown by Varsha et al. (2015), which presented activity against *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum*, and cytotoxicity against HeLa cancer cells. Terpenoids and steroids were the major compounds found in the samples. Isocamphane was the only monoterpene identified, only in SOX sample. Monoterpenes are the most volatiles terpenoids and, therefore, the most difficult to recover by SFE due to loses during the depressurization step.

399 Table 3 – Tentatively identified compounds in non-polar extracts of *Pereskia aculeata* leaves by GC-QTOF-MS.

#	Rt (min)	Molecular formula	Tentative identification	Monoisotopic mass	MSQMF	MS/MS product ions (m/z)	Samples (Peak Area x10^6)				Also determined by
							SOX hexane	SFE 40 °C	SFE 50 °C	SFE 60 °C	
1	12,75	$C_{14}H_{22}O$	2,4-Di-tert-butylphenol	206.1671	92.3	191.0022, 192.0016, 57.0691, 206.0498, 162.9693	1.62	2.60	2.78	2.26	[48–50]
2	16,69	$C_{10}H_{18}$	Isocamphane	138.1409	80.4	67.0536, 95.0842, 81,0690, 79.0535, 55.0534	0.10	0.00	0.00	0.00	-
3	19,64	$C_{20}H_{40}O$	Phytol	296.3079	75.0	73.0461, 143.0860, 75.0261, 81.0690, 67.0536	0.60	0.78	0.86	0.77	[16,40,47,48,50,52]
4	24,80	$C_{30}H_{50}$	Squalene	410.3913	88.6	81.0690, 41.0387, 69.0689, 95.0842, 67.0536	1.48	2.92	4.54	5.27	-
5	26,47	$C_{28}H_{48}O_2$	γ-Tocopherol	416.3654	74.4	151.0303, 416.0974, 191.0022, 417,1006, 152.0593	0.17	0.00	0.53	0.61	-
6	26,99	$C_{29}H_{50}O_2$	Vitamin E	430.3811	93.6	164.9819, 430.0884, 164.0827, 431.0924, 205.0361	4.37	0.82	12.78	16.08	[48]
7	27,03	$C_{29}H_{50}O_3$	α-Tocopherolquinone	446.3760	54.6	150.1031, 221.0836, 177.9826, 203.0007, 179.0011	0.00	3.63	1.41	1.79	

8	27,75	$C_{28}H_{48}O$	Campesterol	400.3705	80.8	91.0533, 315.3047, 145.1005, 79.0531, 213.1629	1.00	2.86	3.57	4.09	[47]
9	28,35	$C_{29}H_{50}O$	γ-Sitosterol	414.3862	82.1	105.0675, 91.0532, 145.0972, 329.0277, 79.0535	6.98	16.84	24.06	25.85	[47,48,50,53]
10	28,46	$C_{29}H_{48}O$	Fucosterol	412.3705	60.5	281.0513, 314.2597, 91.0533, 105.0688, 79.0631	0.48	0.76	1.39	1.27	-
11	28,62	$C_{30}H_{50}O$	Lupeol	426.3862	72.6	189.1622, 105.0688, 119.0830, 204.1861, 107.0842	3.09	6.96	8.22	9.92	-

400 MSQMF - mass spectrum quality match factor;

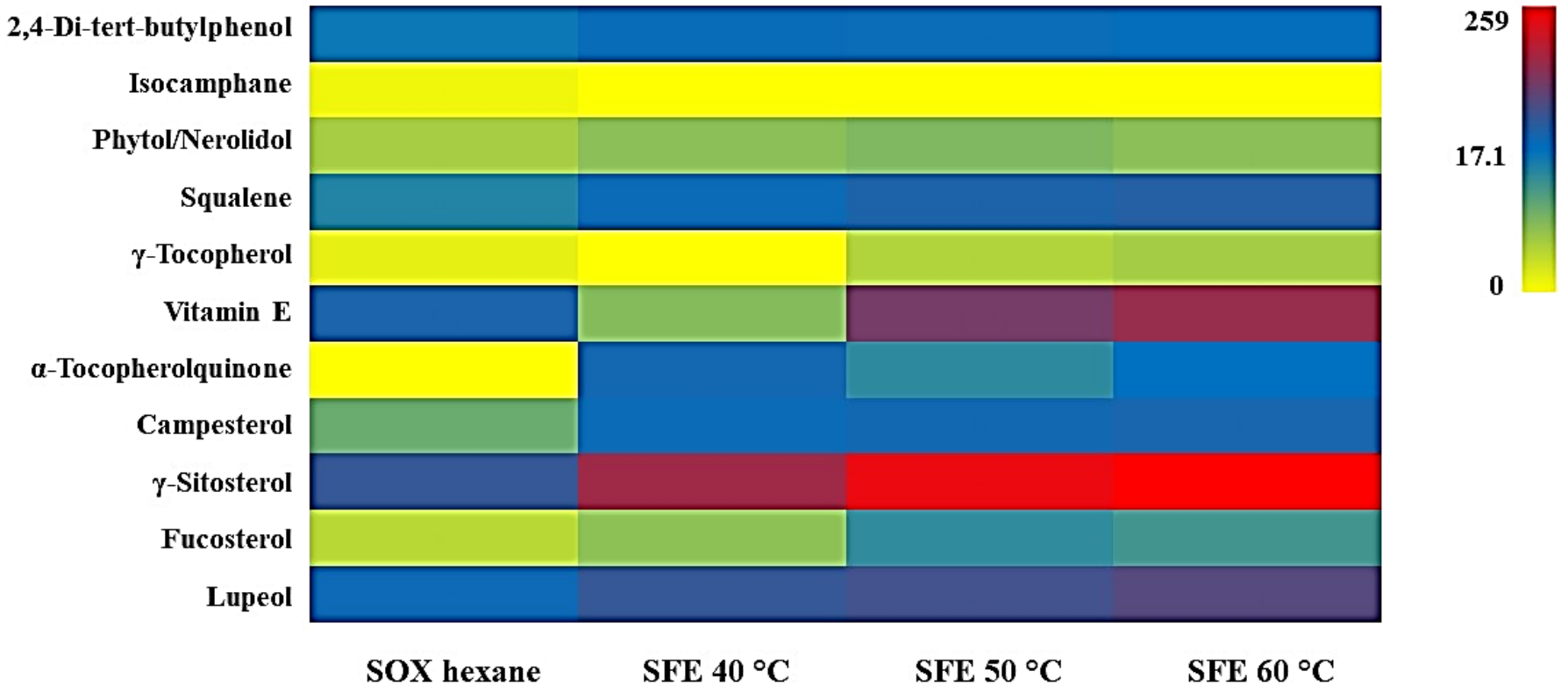
401 *Tentative identification using NIST v17 (National Institute of Standards and Technology – USA) mass spectral library.

The abundances (peak area x 10^6) of the identified terpenoids and phenolic compounds from SFE samples obtained at different temperatures are compared in **Table 3**. In general, the abundance of the compounds increases with temperature for most metabolites, except α-tocopherolquinone, which is higher for SFE at 40 °C. Diterpenes like phytol and vitamin E, triterpenes like squalene and lupeol, sterols like campesterol and fucosterol were also identified. Vitamin E (α-tocopherol) and γ-tocopherol peaks were very low in the SFE extracts at 40 °C. This may be explained by the differences in solubility with temperature increase. From 40 to 60 °C, at constant pressure (25MPa), the solvent power decreases due to CO_2 density reduction, although the recovery of α and γ-tocopherol presented opposite performance, with temperature increase. This behavior was also noticed by Bashipour and Ghoreishi (2014), for SFE from almond leaves up to 26 MPa, where the recovery of vitamin E (α-tocopherol) increased when varying temperature from 40 to 60 °C. This behavior was attributed to the increase in α-tocopherol vapor pressure with temperature, contributing to solute solubility, and which was dominant over the decrease in solvent density.

A heat-map analysis was conducted to detect the effect of the extraction process (SOX-hexane and SFE at 40, 50 and 60 °C) on the compounds identified from ora-pro-nobis extract samples. The result is presented in (**Fig. 1**) by displaying quantitative values of the identified compounds from the extract samples. The intensity of each component from the evaluated samples (represented by the chromatogram peak area x10^6) is identified by colour-coded range, placed in two dimensions, with red representing the higher level, blue the intermediate level and white the lower level of component incidence (KIRK, 2021). The results were distributed in percentile, with medium value represented by the median, and show that α-tocopherolquinone was exclusively found in SFE extracts, with higher abundance in sample by SFE at 40 °C (**Fig. 1**). Also, the supercritical samples

427 provided higher amount of vitamin E (SFE at 50 and 60 °C) and γ-sitosterol (SFE at 40,
428 50 and 60 °C), compared to Soxhlet sample. These composition differences were
429 probably the responsible for the better performance of the SFE samples, where the extract
430 recovered at 50 °C was the strongest AChE inhibitor, and the 40 °C extract was most
431 effective LOX inhibitor, compared to Soxhlet-hexane sample (**Table 2**). In summary, the
432 SFE from ora-pro-nobis leaves provide extracts with various bioactive compounds, in
433 agreement with data reported in literature for conventional extractions of this plant.
434 Supercritical CO_2 extracts presented *in vitro* anti-inflammatory activity and a moderate
435 AChE activity, demonstrating the potential of *Pereskia aculeata* leave extracts obtained
436 by the proposed green extraction technology.

437 Fig.3. Heat-map visualization of gas chromatography-mass spectrometry (peak area x10^6) analysis of *Pereskia aculeata* leaves.



438

4.4 Conclusions

Considering the two classes of solvents used (polar and non-polar) and the different extraction methods, the PLE provided the polar extracts with better antioxidant capacity. SFE-CO_2 and SOX hexane samples showed anti-inflammatory and anti-cholinergic activity, with better performance from sample recovered by SFE at 50 °C. Eleven compounds, mostly terpenoids, were identified by GC-MS from the non-polar extracts (SFE and SOX-hexane). In general, the samples obtained by supercritical fluid presented better results in terms of antioxidant, AChE and LOX activities than SOX extracts.

The results suggest that different fraction from ora-pro-nobis leaves, i.e., the polar and the non-polar fractions, presented characteristics of interest, with antioxidant, anti-inflammatory or anti-cholinergic potentials. Thus, the combination of green extraction methods may provide a better use of this underestimated raw material, preserving the characteristics of the plant and fractionating the target compounds. Then, PLE would provide the compounds with antioxidant activity, while SFE enables the recovery of inflammatory or anti-cholinergic components. With that in mind, a subsequent work is undergoing to combine the best conditions of SFE and PLE, in a biorefinery/circular economy concept, to provide the best from ora-pro-nobis leaves.

4.5 Acknowledgments

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MANUSCRIPT 3: High-pressure biorefining of ora-pro-nobis (*Pereskia aculeata*)

Abstract

Ora-pro-nobis (OPN) is an unconventional food plant used in some Brazilian regions in traditional culinary. A combination of high-pressure extraction processes was used, following the biorefinery concept, to value this underestimated raw material. A sequence of extractions was performed, with solvents of increasing polarity: supercritical fluid extraction (SFE-CO_2), pressurized liquid extraction using ethanol (PLE-ethanol) and water (PLE-water). Soxhlet and buffer maceration were used for comparison. Extraction yield, antioxidant capacity, phenolics and soluble protein contents were evaluated. Samples recovered by water had better antioxidant capacity, while ethanol samples presented higher phenolic content. The process combination improved yield, antioxidant potential, phenolics and soluble protein recovery, especially combining SFE + PLE-ethanol + PLE-water. Overall, 13 phenolics were identified, mainly flavonoids and organic acids, known for its antioxidant and biological activities, confirming the extracts high value-added. Thus, downstream process combination for OPN leaves enabled the recovery of high value-added products following the biorefinery concept.

Keywords: Ora-pro-nobis; supercritical fluid extraction; pressurized liquid extraction; phenolic compounds; biorefinery.

4.6 Introduction

Ora-pro-nobis (OPN), as usually named in Brazil, belongs to Cactaceae family and is used as feedstock, due to high nutritional content, and in traditional medicine, associated to fine bioactivities good to human health. *Pereskia aculeata* is the most common OPN specie found in Brazil, widely used by vegan and vegetarian consumers due to high protein content.

The vegetable protein market is represented mostly by soybean, in fact, over 75% of vegetable proteins demand from European Union is attended by soya bean meal and soya beans (JERZAK; ŚMIGLAK-KRAJEWSKA, 2020). Ora-pro-nobis are classified as unconventional food plants, i.e., usually non domesticated plants with very high nutritional potential (KINUPP; BARROS, 2008). Their leaves are known to have very high protein content for a vegetable, up to 28 % DW, higher than usual vegetable protein sources like rice bran (up to 15% DW), red beans (up to 24% DW) and oat (up to 18% DW), but lower than soybean, the most spread vegetable protein source (up to 44% DW) (SANT'ANA et al., 2011; WATCHARARUJI et al., 2008).

Some works suggest biorefinery flowcharts for vegetable raw materials, such as residues from the food industry (pomegranate, mango peel and peanut by-products) or micro and macroalgae (BANERJEE et al., 2018; GILBERT-LÓPEZ et al., 2017; PATHAK; MANDAVGANE; KULKARNI, 2017; RUDKE; DE ANDRADE; FERREIRA, 2020; SORITA; LEIMANN; FERREIRA, 2020). Most works focus on the recovery of bioactive compounds from these matrices, while only one work was found in the literature for protein recovery by conventional extractions, from *Panicum virgatum*, a typical North American grass (BALS et al., 2007),.

Pereskia aculeata leaves are suitable matrix for biorefinery application since it contains various high-valuable products. The biorefinery concept defends maximum use

of the raw material by combining extraction procedures to make the most potential of the processed matrix (BENEVENUTI; PEREIRA JR, 2016) This concept fits the current market scenario associated to sustainable and environmentally friendly products.

Therefore, the present work presents a combination of green extraction methods as an alternative to improve recovery of bioactive compounds from a very rich matrix as ora-pro-nobis in a biorefinery concept, following our previous study (TORRES et al., 2021) about characterization of non-polar fraction from ora-pro-nobis and evaluating its neuroprotective potential. Then, this work focusses on the polar antioxidant fraction from OPN, and the potential of process combination to improve the recovery of valuable compounds.

4.7 Material and methods

4.7.1 Sample preparation

The ora-pro-nobis (*Pereskia aculeata*) leaves were purchased dried and milled from *Sítio Flora Bioativas*, a family farm company (Tijucas, SC, Brazil). The process started morning harvest of the leaves, followed by quick wash to avoid mucilage lost, and then drying in trays at 45°C in an air-circulating dehydrator (MS Metalúrgica e Comércio, Canoinhas, SC, Brazil) for 18 to 24h, with tray rotation every 2h. Dryed leaves were grinded in stainless-steel hammer mill, packet and stocked at -18 °C until use.

4.7.2 PBS buffer maceration

A maceration with 0.2 M pH 6.7 PBS (phosphate-buffered saline) buffer was carried out, according to Lima, Piza and Brasil (2003), to obtain a protein-rich extract. The maceration consists in adding 5 mg of sample in 5 mL of phosphate buffer, agitate for few minutes and centrifuge at 10,000 rpm for 5 min at 5 °C (Eppendorf 5804; Hamburg, Germany). The supernatant was used to determine total soluble protein content.

4.7.3 Soxhlet extraction

Soxhlet extraction was performed according to 920.39C method of AOAC (AOAC, 2005b), using ethanol P.A. (Neon, São Paulo, Brazil), hexane P.A. (Synth, São Paulo, Brasil) and distilled water as solvents. Briefly, the procedure consists in mixing 150 mL of solvent with 5 g of dried sample, with extraction was carried out for 6 h. The recovered extracts were dried in rotary evaporator (Fisatom, model 801, São Paulo, Brazil) or freeze dryer (Liotop®, model L101, São Carlos, Brazil) and stored at -18 °C with absence of light for further analysis.

4.7.4 Supercritical Fluid Extraction (SFE)

Supercritical fluid extractions were performed in an extraction unit described and adapted by Michielin et al. (MICHIELIN et al., 2005) and Mazzutti et al. (MAZZUTTI et al., 2018). The solvent was CO_2 (99.9%, pure) delivered at pressure up to 6 MPa (White Martins Ltda., Joinville-SC, Brazil). The procedure consisted of placing 20 g of dried and milled sample inside a stainless-steel extraction vessel (32.9 cm length, 2.042 cm internal diameter), completed with glass beads. Extract samples were collected in amber glass flasks previously weighted, and stored at -18 °C (in a domestic freezer). The extraction conditions, chosen according to previous study (TORRES et al., 2021), were set at 40 °C, 25 MPa and 3.33 x 10^{-4} $kg.s^{-1}$ of CO_2 for 2 hours and solvent/biomass mass ratio of 136 (v/m).

4.7.5 Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction was performed in a self-assembled apparatus described by Gonçalves Rodrigues et al. (2019), using ethanol and distilled water as solvents. Briefly, 8 g of dried powdered sample and 60 g of glass beads were placed in the stainless-steel extraction vessel of 90 mL (internal diameter of 25 mm and a height of 180 mm), where the solvent is pumped into the extraction cell using an HPLC pump

(Waters, model 515, USA), where the pressure was controlled by a needle valve (model 20-11LF4, NFA, HIP, USA), performed in a continuous mode. The recovered samples were dried to solvent withdrawal by rotary evaporator (Fisatom, model 801, São Paulo, Brazil) or freeze dryer (Liotop®, model L101, São Carlos, Brazil) and stored at -18 °C with absence of light. Extraction conditions were set at 110 °C, 10MPa and 4 $mL.min^{-1}$ solvent flow rate for 15 min and solvent/biomass mass ratio of 7.5 (v/m), the optimized condition provided by a previous study (TORRES et al., 2021).

4.7.6 Sequential extraction processes

Different extraction techniques were sequentially combined, using increasing solvent polarity, to provide maximum recovery from ora-pro-nobis leaves. To evaluate the biorefinery performance of the extraction procedures and solvents, five treatments (extraction routes), were conducted, as presented in **Table 1**. The conditions selected, for each route, were the optimum conditions defined by Torres et al. (2021). Each route uses one initial raw material, with the individual steps recovering different samples. The extracts recovered are identified in Table 1, representing the extractions steps.

Table 1 – Combined extraction processes from ora-pro-nobis leaves (*P. aculeata*) (sequential treatments).

Code	Treatment	Steps conditions	Sample
T1	SOX_PLEwater 	Step 1: Soxhlet hexane; Step 2: PLE water, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}.	1 2
T2	SFE_PLEwater 	Step 1: SFE CO_2, 40 °C, 120 min, 1.2 kg.h^{-1}, 25 MPa; Step 2: PLE water, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}.	3 4
T3	SFE_PLEethanol 	Step 1: SFE CO_2, 40 °C, 120 min, 1.2 kg.h^{-1}, 25 MPa; Step 2: PLE ethanol, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}.	3 5
T4	PLE-ethanol_PLE-water 	Step 1: PLE ethanol, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}; Step 2: PLE water, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}.	6 7
T5	SFE_PLE-ethanol_PLEwater 	Step 1: SFE CO_2, 40 °C, 120 min, 1.2 kg.h^{-1}, 25 MPa; Step 2: PLE ethanol, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}; Step 3: PLE water, 80 °C, 10 MPa, 15 min, 4 mL.min^{-1}.	3 5 8

93 4.7.7 Extraction yield (%)

94 The overall extraction yield (X_0) was calculated as the percentage of the ratio
95 between the extract mass (m_e) and the dry sample mass (m_s) used to perform the
96 extractions (**Eq. 1**). The tests were performed in duplicate and the results expressed as
97 mean ± standard deviation.

98 $$Xo = \frac{me}{ms} . 100 \quad \textbf{(1)}$$

99 4.7.8 Total phenolic content (TPC)

100 The total phenolic content was determined according to Koşar, Dorman, and
101 Hiltunen (2005). A stock solution of 2 mg mL^{-1} of gallic acid (98% purity, Sigma Aldrich,
102 St. Louis, MO, USA) was diluted in distilled water to different concentrations to obtain
103 the standard curve of gallic acid with R^2 coefficient of 0.9952. The samples were diluted
104 in ethanol P.A. (Neon, São Paulo, Brazil) (concentration up to 10 mg mL^{-1}. The reaction
105 mixture contained 10 µL of extract, 50 µL of reagent Folin-Ciocateau (Sigma Aldrich,
106 St. Louis, MO, USA), 150 µL of 20% sodium carbonate (Lafan, São Paulo, Brazil) and
107 800 µL of distilled water. Then, samples were incubated at room temperature, in absence
108 of light. After 2 h of incubation, the absorbance was measured on UV-vis
109 spectrophotometer (Femto 800 XI, São Paulo / SP - Brazil) at 760 nm. The experiments
110 were carried out in triplicate and the results expressed in milligrams of gallic acid
111 equivalent per gram of dry matter ($mg_{GAE}\ g_{extract}^{-1}$) ± SD.
112

113 4.7.9 DPPH free radical scavenging assay

114 The method of capture of DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) was
115 used to evaluate the antioxidant activity of the extracts from OPN leaves. The method
116 was described by Brand-Williams et al. (1995) and adapted by Stasko et al. (2007).

Extracts were diluted in different concentrations (5 to 8 dilutions), added in a microplate followed by the solution of 0.1 mM DPPH radical (Sigma Aldrich, St. Louis, MO, USA) diluted in ethanol P.A. (Neon, São Paulo, Brazil). Monobasic phosphate buffer pH 6.0 was added before incubation in absence of light and at room temperature for 30 min. The samples read on spectrophotometer TECAN, multi-plate reader (Infinity M200) at 525 nm. Blank sample was the solvent used to dilute the extracts and control was the sample without DPPH radical, to remove color interference. The antioxidant activity was calculated according to **Eq. 2**. The experiments were carried out in triplicate, the results expressed as mean ± SD and the effective concentration to reduce by 50 % the antioxidant activity of the radical DPPH (IC_{50}) was calculated by linear regression.

$$AA\ (\%) = \left[1 - \frac{A_{525\ (Sample)} - A_{525\ (Control)}}{A_{525\ (Blank)}}\right] \quad \textbf{(2)}$$

4.7.10 ABTS assay

The antioxidant capacity of the extracts was evaluated according to the methodology by Re et al. (1999). The $ABTS^{*+}$ radical was produced by the reaction of 2.45 mM potassium persulfate and 7 mM of ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (≥ 98% purity, Sigma Aldrich, St. Louis, MO, USA), protected from light and at room temperature for 16 h before use. The ABTS radical was diluted in PBS buffer 5mM pH 7.4 to an absorbance of 0.7 (± 0.2) at 734 nm. A volume of 10 µL of the sample, diluted in at least 5 different concentrations were added to 990 µL of ABTS radical solution. The sample inhibition capacity was evaluated by absorbance measured on spectrophotometer at 734 nm after 45 min incubation at room temperature and absence of light. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97 % purity, Sigma Aldrich, St. Louis, MO, USA) was used as a standard, with results were

expressed in Trolox equivalent (TEAC), according to calibration curve. The calculation, first considering percentage of ABTS inhibition by Trolox standards, and then for the samples discounting the blank value of each sample. The control is made by replacing the sample with diluted solvent. **Eq. 3** is used to calculate ABTS inhibition. Equivalent calculation is made for 1 mM Trolox, finding sample concentration equivalent to % inhibition in 1 mM Trolox. All analyses were performed in triplicate and expressed as mean ± SD.

$$In\ (\%) = \frac{A_{734\ (Control)} - (A_{734(Sample)} - A_{734(Blank)})}{A_{734\ (Control)}} \quad \textbf{(3)}$$

4.7.11 FRAP assay

The FRAP (ferric reducing antioxidant power) method was used to evaluate the the antioxidant capacity of the extracts through The method described by Benzie and Strain (1996). The FRAP reagent is prepared using 0.3 M sodium acetate buffer solution (pH 3.6), 20 mM ferric chloride solution ($FeCl_3.6H_2O$) and diluted TPTZ solution (2,4,6-tripidylstriazine, ≥ 98% purity, Sigma Aldrich, St. Louis, MO, USA) in 40 mM hydrochloric acid (HCl, 37% P.A.-A.C.S. (1190g), Synth, São Paulo, Brasil) solution. The reagent is prepared using 60 mL of sodium acetate buffer solution with 6 mL of TPTZ solution and 6 mL of ferric chloride solution. Samples previously diluted in proper solvent were added to reagent, reacting in the absence of light for 30 min. After incubation time, the samples were analyzed on spectrophotometer TECAN, multi-plate reader, Infinity M200. The samples were read at 593 nm. The blank was made with the solvent. The Trolox was used as standard, with a calibration curve made with at least 5 different dilutions. The analysis was performed in triplicate and the results were expressed in $mmol_{TEAC}.g^{-1}$ (TEAC - Trolox equivalent) as mean ± SD.

4.7.12 Bradford assay

Total soluble protein content was determined by the method described by Bradford (1996). The protein content is quantified by a protein complex with Coomassie Brilliant Blue G-250. The Bradford method is based on the interaction of high molecular weight proteins with the Coomassie Brilliant Blue G-250 dye, shifting the dye balance to the anionic form, being absorbed at 595 nm (ZAIA; ZAIA; LICHTIG, 1998). Initially, Bradford's reagent is prepared by dissolving 0.01 g of the dye in 100 mL ethanol, then adding another 100 mL of 85% orthophosphoric acid, then completing with water to final volume of 1 L. The reaction mixture is formed by 2.5 mL of the reagent and 50 µL of the sample. After 5 min incubated in absence of light, the samples were read at 595 nm on spectrophotometer. The total soluble protein content is calculated using a standard bovine albumin serum curve (BSA, Sigma Aldrich, St. Louis, MO, USA). The analysis was performed in triplicate and the results expressed as mean ± standard deviation, in mg of protein per gram of extract ($mg_{protein}.g_{extract}^{-1}$) as mean ± SD.

4.7.13 Scanning electron microscopy (SEM) analysis

The scanning electron microscopy analysis was performed to analyze the surface of the dried samples before and after the extraction processes. The analysis was carried out at microscope JEOL JSM 6390 LV (Musashino, Akishima, Japan). The samples were visualized under a voltage of 5 kV with magnifications of 100 to 400 times. The sample was prepared by fixing on metallic support with double-sided carbon adhesive tape, covered with a thin gold layer, under vacuum.

4.7.14 Tentative identification of chemical profile by (UPLC-QTOF-MS^E)

Ultra-performance liquid chromatography (UPLC) analysis was used to identify the compounds from OPN leaves. The analysis was performed in an Acquity UPLC system (Waters), coupled to a Quadrupole/Time of Flight system (QTOF, Waters). The chromatographic runs were carried out on Waters Acquity UPLC BEH column (150 x 2.1 mm, 1.7 μm), at 40 °C, mobile phases containing water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), gradient ranging from 2% to 95% B (15 min), flow of 0.4 mL min^{-1} and injection volume of 5 μL. The chromatograms were analyzed at ESI^- mode, acquired at 110-1180 Da, acquired between 110-1180 Da, source temperature of 120 °C, desolvation temperature of 350 °C, desolvation gas flow of 500 L h^{-1} and capillary voltage of 3.2 kV. Leucine enkephalin was used as a lock mass. The acquisition mode was MSE. The data were analyzed by Masslynx 4.1 software (Waters Corporation).

Imported data on Mass Spectrometry – Data Independent AnaLysis software (MS-DIAL 3.82) were prerequisite for compound identification (LAI et al., 2018; TSUGAWA et al., 2015, 2019). The unknown metabolites were identified by their elemental formulas and *in silico* mass spectral fragmentation with MS-FINDER 3.22 (LAI et al., 2018; TSUGAWA et al., 2015). Structural elucidation and metabolite identification were based on molecular formula and MS/MS fragmentation with activated heuristic rules (KIND; FIEHN, 2007; SUMNER et al., 2007). Putative compounds identification was performed comparing with database such as KNApSAcK Core System database, PubChem and ChemSpider. Following the parameters of the metabolic standards initiative (MSI) level 2.1 (SUMNER et al., 2007), presenting a putative identification, including molecular formula and MS^E fragments, and based on the chemotaxonomy (family, genus, and species).

4.7.15 Statistical analysis

Antioxidant activity, total phenolic content and identified compounds were analyzed by the Pearson correlation test, coefficients with 95 % confidence. The one-way ANOVA followed by post hoc Tukey's HSD (honestly significant difference) test at $p < 0.05$ level were applied to all results using software Statistica version 7.1 (Stat-Soft Inc., Tulsa, OK, USA).

4.8 Results and discussion

4.8.1 Process integration

The biorefinery approach, applied for the recovery of valuable fractions from OPN leaves, combines extraction processes carried out sequentially with different solvents and methods, following the optimization procedures presented by Torres et al. (2021). The extraction conditions selected are justified as follows:

1) SFE with CO_2, conducted at 40 °C and 25 MPa, provided high extraction yield of 1.78 %, with extract quality represented by and *in vitro* anti-inflammatory potential and moderate acetylcholinesterase inhibition, activities involved in Alzheimer's disease mechanism, according to Torres et al. (2021);
2) PLE-ethanol, conducted at 10 MPa and 110 °C, provided samples with the high antioxidant performance (IC_{50} of 1.64 $mg.mL^{-1}$ by DPPH and 0.17 $mmol_{TE}.g^{-1}$ by FRAP), and high total phenolic content (TPC) of 60.09 $mg_{GAE}.g_{extract}^{-1}$, within the conditions studied for the recovery of the ethanolic fraction from OPN leaves (TORRES et al., 2021);
3) PLE-water, conducted at 10 MPa and 80 °C, provided the protein-rich fraction, with high antioxidant capacity (IC_{50} of 0.31 $mg.mL^{-1}$ by DPPH and 0.25 $mmol_{TE}.g^{-1}$ by FRAP).

237 The results from the sequential treatments (extraction routes from **Table 1**) are
238 presented in **Table 2** in terms of process yield and quality of the extracts (samples
239 numbers provided by **Table 1**). The results quality is provided by TPC values and
240 antioxidant performance by DPPH (as IC_{50} values), ABTS and FRAP. Also, the
241 extraction yield listed at **Table 2** represent only the yield from the last step of the
242 treatment.

Table 2 – Yield, TPC and antioxidant activity (DPPH, ABTS and FRAP methods) for the extract samples recovered by isolated (assays 1-4) and by combinate extraction processes (assays 5-9) from ora-pro-nobis leaves (*Pereskia aculeata*).

Sample	Treatments	Extraction steps	Accumulated yield (%, w/w)	Yield (%, w/w)	TPC ($mg_{EAG}.g^{-1}$)	IC_{50} ($mg.mL^{-1}$)	ABTS ($mmol_{TEAC}.g^{-1}$)	FRAP ($mmol_{TEAC}.g^{-1}$)
1	SOX	1	-	4.66 ± 0.40^{ab}	4.96 ± 0.29^{a}	7.83 ± 0.13^{j}	-	0.09 ± 0.01^{b}
2	PLEwater from T1	2	6.8	2.14 ± 0.02^{a}	10.49 ± 0.34^{a}	1.65 ± 0.05^{gh}	0.37 ± 0.02^{cd}	0.10 ± 0.01^{b}
3	SFE	1	-	1.78 ± 0.08^{a}	3.71 ± 0.12^{a}	3.09 ± 0.17^{i}	-	0.02 ± 0.01^{a}
4	PLEwater from T2	2	4.23	2.45 ± 0.04^{a}	26.73 ± 0.31^{bc}	0.29 ± 0.02^{a}	0.42 ± 0.03^{acd}	0.13 ± 0.01^{cd}
5	PLEethanol from T3	2	13.41	11.63 ± 0.67^{cd}	81.71 ± 1.85^{g}	1.74 ± 0.05^{h}	1.32 ± 0.05^{e}	0.35 ± 0.03^{h}
6	PLE-ethanol	1	-	13.09 ± 0.09^{d}	61.89 ± 3.36^{f}	1.64 ± 0.04^{gh}	0.44 ± 0.03^{ad}	0.17 ± 0.01^{e}
7	PLEwater from T4	2	15.96	2.87 ± 0.54^{a}	30.85 ± 1.93^{bcde}	0.69 ± 0.01^{de}	0.53 ± 0.01^{b}	0.24 ± 0.01^{f}
8	PLEwater from T5	3	18.82	5.41 ± 0.34^{abc}	55.42 ± 2.08^{f}	0.44 ± 0.01^{ab}	0.36 ± 0.01^{c}	0.35 ± 0.01^{h}
9*	PLE-water	1	-	3.07 ± 0.02^{a}	41.71 ± 4.12^{e}	0.31 ± 0.02^{a}	0.54 ± 0.03^{b}	0.25 ± 0.01^{f}

Column with the same letter are not significantly different from Tukey's HSD test ($\alpha = 0.05$). * Sample 9 is not presented at **Table 1** because it is not part of a combined process.

For one step processes, the non-polar solvents (CO_2 or hexane), used respectively by SFE and SOX methods, provided the lowest yield values. PLE with water, although a polar solvent, also presented low yield performance. The best yield was provided by PLE with ethanol (13.09%). The lower PLE-water yield was mainly due to the formation of a colloidal phase on the extraction cell that difficulted the solvent flux, lowering the extraction yield. To overcome this difficulty the defatting of the sample prior to PLE-water extraction aimed to increase yield removing the oiled fractions, increasing solute/matrix interaction. Otherwise, water provide the water-soluble components like protein and the ethanol as solvent recover mainly the phenolic fraction.

Process combination is an alternative to fractionate valuable compounds from ora-pro-nobis, and the efficiency of process integration must combine performance in terms of process yield and product quality. Then, the best yield value was obtained by treatment 5, combining SFE + PLE-ethanol + PLE-water, with combined yield of 18.82%. This increase, compared to the isolated extractions (5% increase) may be a result from the depressurization between extractions, which causes cell wall disruption of the solid material, increasing the solute/solvent contact area, affecting the yield. Similar behavior was observed by Ferro et al. (2019) for the recovery of antioxidant compounds from guanxuma leaves (*Sida rhombifolia*) by process integration. The authors combined SFE and PLE (ethanol/water 70% mixture), with an increase in yield due to fast depressurization between SFE and PLE steps.

The influence of process combination on samples quality, regarding TPC and antioxidant capacity, is also shown on **Table 2**. PLE-ethanol provided the highest TPC values, of 61.89 and 81.71 $mg_{EAG}.g^{-1}$, respectively obtained by one step extraction, and by treatment 3 (SFE + PLE-ethanol). The increase in TPC value when PLE-ethanol was downstream from SFE shows that the previous recovery of the oily fraction from the raw

material (by SFE) followed by depressurization after SFE, facilitates the recovery of phenolics by PLE- ethanol. Besides, although PLE-water could be appropriate to recover the protein fraction (high polar compounds), compared to PLE-ethanol, treatment 5 also enable phenolics recovery at the third step PLE-water (sample 8).

Regarding the antioxidant capacity, the best IC_{50} values (lower values) were provided by PLE-water as first, second or third steps (0.29, 0.31 and 0.44 $mg.mL^{-1}$, respectively). For ABTS method, the best performance was detected by second step PLE-ethanol (sample 5: 1.32 $mmol_{TEAC}.g^{-1}$), probably because SFE, with oily fraction recovery, followed by depressurization, increased the extraction of antioxidant components like phenolics (see TPC results). The FRAP results also the best performance for sample 5, with no significant difference to sample 8 (recovered by PLE-water as 3rd step).

A study by Sharma et al. (2015) evaluated temperature influence on TPC and antioxidant activity of six onion varieties (conventional extraction). An increase in TPC and antioxidant activity was observed with increasing temperature up to 120 °C, and then reduced up to 150 °C for all varieties. The authors associated this behavior to quercetin (and isomers) content, the main flavonoids from onion, which content in onions extracts presented the same behavior of TPC and antioxidant activity with temperature changes. Besides, quercetin has also been identified from ora-pro-nobis samples, from *P. bleo* specie (HASSANBAGLOU et al., 2012), and from *P. aculeata* (GARCIA et al., 2019). In order to corroborate the literature data, a tentative identification of compounds from samples of OPN leaves, recovered by SFE and PLE is presented in **Section 3.4**. Several phenolic compounds derived from flavonoids were identified in the extracts, including quercetin 3-O-pentosylrutinoside, quercetin rhamnosyl hexoside and quercetin 3-O-rutinoside.

The Pearson correlation applied for **Table 2** results shows a significant negative correlation between the FRAP and DPPH methods (-0.58) and a significant positive correlation between TPC and FRAP (0.74). This behavior indicates the important role of phenolic components, which enhanced at PLE-ethanol after SFE, on the antioxidant activity of the extracts.

4.8.2 Influence of combined processes on total protein content

Pressurized water extraction has been used for the recovery of proteins and amino acids from rice (SEREEWATTHANAWUT et al., 2008), soy (NDLELA et al., 2012), flaxseed (HO; CACACE; MAZZA, 2007), and to obtain bioactive peptides from tuna skin (AHMED; CHUN, 2018) and apple pomace pectin (WANG; CHEN; LÜ, 2014). Therefore, extract samples recovered from OPN using water as solvent were evaluated in terms of protein content by Bradford method [21], and the results, expressed in milligrams of protein per gram of extract are presented in **Table 3**. The highest protein values were provided by samples 4 and 8 (35.77 and 38.74 $mg_{protein}.g_{extract}^{-1}$ respectively), with no significant difference, provided by treatments 2 and 5. These values, higher than conventional maceration of the integral sample, show that previous SFE , followed by depressurization, aid the protein recovery.

Table 3 – Total Soluble Protein Content (TSPC) from ora-pro-nobis (*Pereskia aculeata*) leaves extracts by Bradford (1976) method.

Treatments	Sample	TSPC ($mg_{protein}.g_{extract}^{-1}$)
Maceration (standard procedure)	**control**	28.03 ± 2.91[a]
PLEwater from T1	**2**	25.88 ± 1.91[a]
PLEwater from T2	**4**	35.77 ± 2.42[b]
PLEwater from T4	**7**	22.30 ± 2.53[a]
PLEwater from T5	**8**	38.74 ± 3.14[b]
PLE-water	**9**	21.31 ± 2.38[a]

* Means with the same letter are not significantly different based on Tukey's HSD test ($\alpha = 0.05$).

The total soluble protein content (TSPC) from PLE-water process, comparing samples 9 (one step) with samples 2 and 7 (two steps processes) were similar to conventional buffer maceration extraction (standard procedure), around 28%. A significant increase in protein content was observed for the samples 8 (PLE-water from T5) and 4 (PLE-water from T2), increasing up to 82% and 68% compared to PLE-water without pretreatment (sample 9) and been 38% and 28% higher than standard extraction. This shows that extraction combination, in a biorefinery approach, is effective for the fractionation of a complex raw material and also can increase the recovery of target compounds.

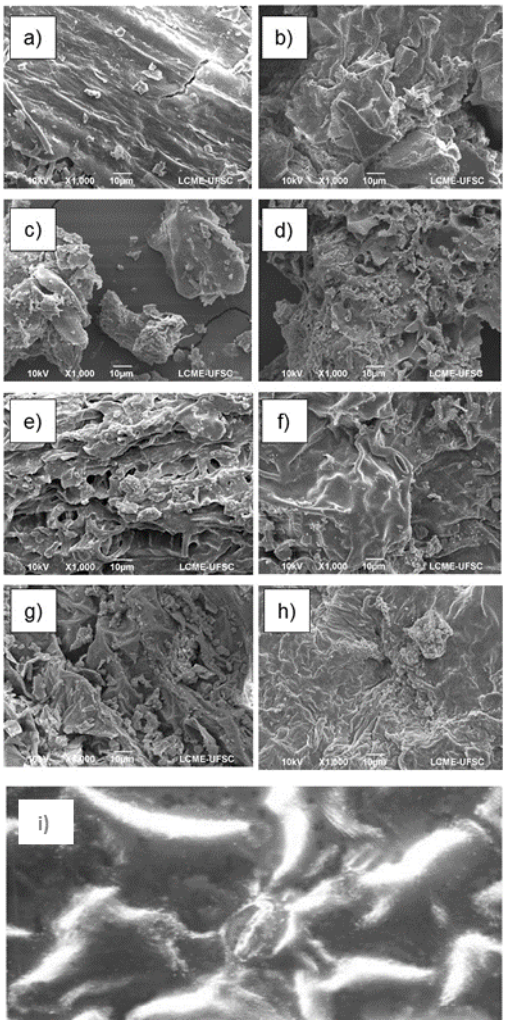
Regarding treatment 5 it is relevant to observe the different characteristics of the three recovered products: an oily fraction by SFE (sample 3), with *in vitro* anti-inflammatory and moderate anti-cholinergic activities, as presented by Torres et al. (2021); followed by phenolic fraction by PLE-ethanol (sample 5), shown at **Table 2,** and the protein fraction by PLE-water (sample 8), with the best protein content, 38% higher than the conventional extraction method.

4.8.3 Scanning electronic microscopy (SEM) analysis

Ora-pro-nobis (dry and grind) was submitted to SEM analysis to observe the effect extraction treatments on solid samples: (a) before extractions; (b) after SOX-hexane; (c) after SFE; (d) after PLE-ethanol; (e) after PLE-water; (f) after PLE-ethanol +PLE-water; (g) after SFE+PLE-ethanol; (h) after SFE+PLE-ethanol+PLE-water; (i) sample by (FARAGO; TAKEDA; BUDEL, 2004). The results as compared at **Fig. 1**.

Sample before extraction (**Fig. 1-a**) shows a compact surface compared to other samples submitted to extraction (**Fig. 1-b to h**), and similar to **Fig. 1-i** (FARAGO; TAKEDA; BUDEL, 2004), SEM analysis held for a different ora-pro-nobis specie (*Pereskia grandifolia*) without processing. Comparing samples after one extraction (**Fig. 1-b, 1-c, 1-d, 1-e**) with samples after two (**Fig. 1-f, 1-g**) or three (**Fig. 1-h**) extractions it shows an increase in cracks on surface with processing, which agrees with higher yields, higher TPC, antioxidant potential and TSPC found for samples by combined processes.

Fig.1. Scanning electronic microscopy (SEM) images with 1000x magnitude from ora-pro-nobis (*P. aculeta*) leaves: (a) before extractions; (b) after SOX-hexane; (c) after SFE; (d) after PLE-ethanol; (e) after PLE-water; (f) after PLE-ethanol_PLE-water; (g) after SFE_PLE-ethanol; (h) after SFE_PLE-ethanol_PLE-water; (i) sample by Farago et al. (2004).



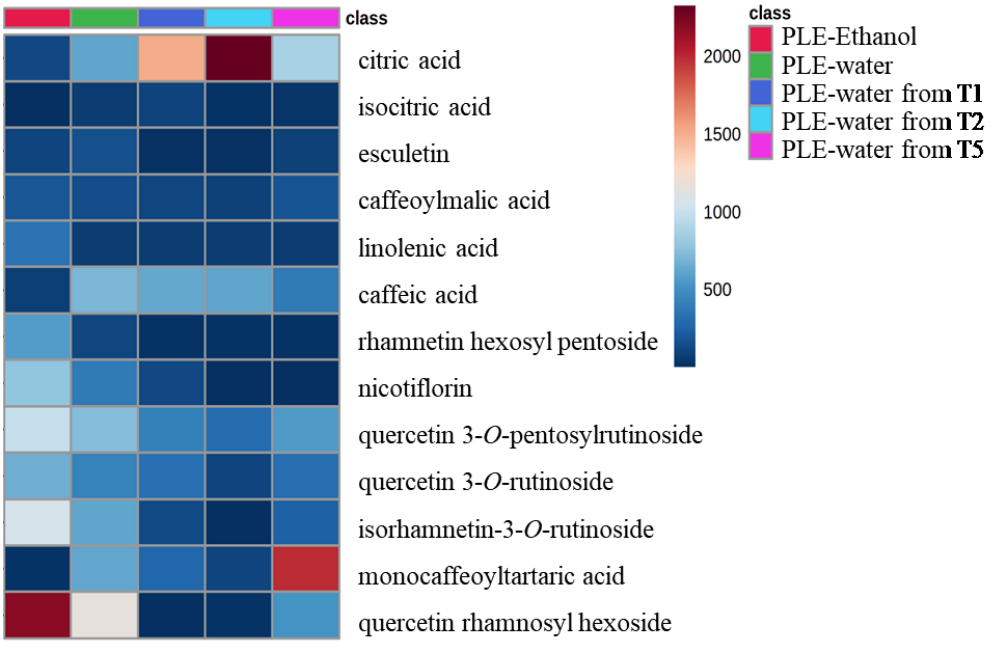
4.8.4 Chemical characterization of the ora-pro-nobis extracts

Extracts from isolated and integrated processes (samples 2, 4, 6, 8 and 9) were analyzed by UPLC-QTOF-MS^E, where 13 metabolites were tentatively identified. **Table 4** shows the retention time (t_R), error, molecular formula, deprotonated ions ([M-H]$^-$) from MS mass spectra associated with fragment ions from MS/MS mass spectra.

Various phenolic compounds were tentatively identified, and mostly defined as derived from flavonoids and organic acids. The phenolic profile from the extracts is similar to that presented by Garcia et al. (2019) from hydroethanolic extract from *P. aculeata* leaves. The authors identified, using the same UPLC–MS/MS methods, quercetin derivatives, caffeic and cartaric acid, kaempferol 3-O-rutinoside, and Isorhamnetin derivatives, same as compounds 3, 4, 7, 8, 9, 11 and 12 from **Table 4**, found in the present study. Pinto et al. (2015a), on the other hand, analyzing methanolic leaf extract of *P. aculeata* by UPLC–MS/MS in the positive ion mode, found different compounds, only quercetin was the same as the present study.

The heat map was designed to provide an overview of the distribution of metabolites noted in the samples (**Fig. 2**). In the graph, each column represents an analyzed sample and each row an annotated metabolite, where red boxes indicate higher concentration levels while blue boxes indicate lower levels, the intensity of colors indicating the degree of presence of the metabolites.

Fig. 2. Heat map based on the intensity of the chromatographic peaks referring to the metabolites annotated in the samples.



Citric and isocitric acids were recovered by water (samples 2, 4, 8 and 9), and those compounds were firstly identified from *Pereskia aculeata* extracts. Although these acids are present in many fruits and vegetables, including other plants from Cactaceae family. For instance, citric acid was identified in crude ethanolic extract from *Corryocactus brevistylus* fruits by UHPLC-ESI-HR-MS/MS analysis (ARECHE et al., 2020) and in *Coryphantha macromeris* (Engelm.) fruits methanolic extracts by UHPLC-PDA-HESI-Orbitrap-MS/MS. Caftaric acid (monocaffeoyltaric acid) was also identified only in the water samples, especially PLE-water from T5 (three steps), which indicates that the combination of process improved recovery of this phenolic acid. On the other hand, quercetin and isorhamnetin isomers were recovered better by ethanol as solvent in the PLE extraction. Even though some differences were found on the intensity of the chromatographic peaks, in general the profile of the ethanolic and water samples was very similar and showed that the compressed fluids were efficient to provide extracts rich in bioactive compounds.

The ethanolic extract (samples 6) presented linoleic acid, a fatty acid that have more affinity with ethanol and CO_2 as solvents. Linoleic acid was also identified by Souza et al. (2014) in an essential oil from *P. aculeata* obtained by hydrodistillation. Soxhlet with ethanol was also evaluated by UPLC-QTOF-MS^E, presenting the same profile as PLE-ethanol, however the compound esculetin appeared only in the PLE extract, what can possibly show a higher selectivity from compressed fluid extraction methods. The PLE-ethanol from T3 sample was not analyzed.

411 Table 4 – Chemical compounds identified using the negative ionization mode (ESI^-) from *Pereskia aculeata*.

Peak	t_R (min)	Negative ion mode (ESI^-)			Error (ppm)	Molecular formula	Tentative identification	Also determined by
		$[M-H]^-$ observed	$[M-H]^-$ calculated	MS/MS				
1	0.96	191.0184	191.0192	85.0328; 87.0098; 111.0127	-4.2	$C_6H_8O_7$	isocitric acid	(AL KADHI et al., 2017; ZHUANG et al., 2018)
2	1.23	191.0184	191.0192	85.0312; 87.0091; 111.0091	-4.2	$C_6H_8O_7$	citric acid	(AL KADHI et al., 2017; ZHUANG et al., 2018)
3	3.00	179.0342	179.0344	133.0266; 135.0440	-1.1	$C_9H_8O_4$	caffeic acid	(CARVALHO et al., 2019)
4	3.03	311.0398	311.0403	149.0079; 179.0334	-1.6	$C_{13}H_{12}O_9$	monocaffeoyltartaric acid (caftaric acid)	(FARAG et al., 2016)
5	3.04	177.0188	177.0188	105.0325; 133.0281; 149.0078	0.0	$C_9H_6O_4$	esculetin	(LIN et al., 2018)
6	3.67	295.0454	295.0454	115.0044; 133.0165; 179.0335	0.0	$C_{13}H_{12}O_8$	caffeoylmalic acid	(ABU-REIDAH et al., 2013)
7	3.79	741.1868	741.1878	300.0274; 301.0310; 591.1494; 609.1663	-1.3	$C_{32}H_{38}O_{20}$	quercetin 3-*O*-pentosylrutinoside	(ENGELS et al., 2012)
8	4.11	609.1451	609.1456	300.0220; 301.0326; 343.0720	-0.8	$C_{27}H_{30}O_{16}$	quercetin rhamnosyl hexoside	(ENGELS et al., 2012)
9	4.17	609.1444	609.1456	300.0257; 301.0364	-2.0	$C_{27}H_{30}O_{16}$	quercetin 3-*O*-rutinoside	(ENGELS et al., 2012)
10	4.20	609.1437	609.1456	300.0240; 301.0317	-3.1	$C_{27}H_{30}O_{16}$	rhamnetin hexosyl pentoside	(ENGELS et al., 2012)
11	4.46	593.1506	593.1506	255.0264; 284.0305; 285.0382	0.0	$C_{27}H_{30}O_{15}$	nicotiflorin (kaempferol 3-*O*-rutinoside)	(ENGELS et al., 2012)
12	4.54	623.1614	623.1612	300.0369; 315.0528	0.3	$C_{28}H_{32}O_{16}$	isorhamnetin-3-*O*-rutinoside	(SONG et al., 2019)

13	11.38	277.2159	277.2166	277.2168	-0.7	$C_{18}H_{30}O_2$	linolenic acid	(FARAG et al., 2014a, 2014b)

412

4.9 Conclusion

The present study suggested a downstream processing for the valorization of ora-pro-nobis (*Pereskia aculeata*) leaves using green extraction processes as supercritical CO_2 extraction and pressurized ethanol extraction and pressurized water extraction. Different flowcharts were compared with the ones using conventional Soxhlet extraction, using two and three steps and it was observed that the use of subsequent extraction processes using the residue of the previous steps as raw material for the followings, was not only more selective than conventional extraction (higher antioxidant and phenolic recovery), but increased yield of the last step (PLE-water) when applied the sequential extractions supercritical CO_2 + pressurized ethanol (around 76% of increment). At all steps we recover a very interesting product, with supercritical CO_2 the non-polar extracts rich in terpenoids and carotenoids, with pressurized ethanol we recovered extracts rich in phenolics and with subcritical water we could recover protein (at an increment up to 82% protein yield when compared to the process isolated). The combination of processes was efficient to enhance yield and phenolic recover in the second and third steps of the biorefinery downstream process suggested. To best of our knowledge, this is the first downstream process to be suggested for the recovery of bioactive compounds and protein from ora-pro-nobis leaves.

4.10 Acknowledgments

The authors are grateful to CNPq, project number 404347/2016-9, CAPES/PROEX-1624/2018, CAPES-PRINT, project number 88887.310560/2018-00, the Laboratório Central de Microscopia Eletrônica (LCME/UFSC) and Laboratório Multiusuário de Estudos Biológicos (LAMEB/UFSC) from Universidade Federal de

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438 Embrapa Agroindústria Tropical.

CHAPTER 5: EXTRACTION OF ORA-PRO-NOBIS (*PERESKIA ACULEATA*) LEAVES BIOACTIVE COMPOUNDS IN SEQUENTIAL PROCESSES USING GAS EXPANDED EXTRACTION AND EXTRACT CHARACTERIZATION.

This chapter refers to a stage of the doctorate that was carried out in the sandwich doctoral stage at the institution *Consejo Superior de Investigaciones Científicas* (CSIC), at the *Instituto de Investigación en Ciencias de la Alimentación* (CIAL) in Madrid, Spain, in the period from November 2019 to October 2020. The following results were also compiled in the form of a scientific article and submitted to an international journal with a high impact factor, in conjunction with the Spanish research group. The main goal with this chapter was to test a different extraction method based on compressed fluids, and trying to enhance protein yield on the third step of the downstream process suggested in Chapter 4. The experience as an exchange student contributed to my relationship with my research and also helped bringing knowledge into this specific area to the research group in Brazil, also increasing international partnership between the graduation program and an international institution.

MANUSCRIPT 4: Protein valorization from ora-pro-nobis leaves by compressed fluids biorefinery extractions

Abstract

Ora-pro-nobis (*Pereskia aculeata*) leaves were selected as raw material for biorefinery application based on compressed fluid extractions with green solvents. The fractionation followed the sequential processes: (1) Supercritical fluid extraction (SFE) with CO_2, (2) Gas expanded liquid extraction (GXL), using CO_2:Ethanol, and pressurized liquid extraction (PLE) with ethanol, and (3) Subcritical water extraction (SWE). *In vitro* analysis of total phenolic and total carotenoid contents and antioxidant activity (DPPH and ORAC) were assessed, defining PLE as the second step in a sequential process. Total protein and carbohydrate contents were evaluated from the SWE samples. Phenolics profile from PLE and SWE samples was assessed by LC-DAD-ESI-MS/MS, identifying 8 phenolic compounds, with quercetin, isorhamnetin and kaempferol as the main detected flavonoids. SWE at 150 and 185 °C provided the highest protein recovery, as the third step in a sequential process.

Keywords

Biorefinery, Protein, Ora-pro-nobis, Compressed fluid extraction, Flavonoids, Bioprospecting.

5.1 Introduction

The biorefinery concept involves the depletion of renewable raw materials, many represented of by-products from industrial processing chain, providing high-added value products (BENEVENUTI; PEREIRA JR, 2016; CHEW *et al.*, 2017). Combining green extraction techniques with the biorefinery concept contributes to reduce waste or residues generation. The most studied green extractions, in a biorefinery downstream processing, are: supercritical fluid extraction, pressurized liquid extraction, pressurized hot water extraction, ultrasound-assisted extraction and microwave-assisted extraction (AMEER; SHAHBAZ; KWON, 2017).

High-pressure extraction techniques are well inserted in the biorefinery context, as they are well stablished methods for the recovery of bioactive compounds from natural matrices (REVERCHON; MARCO, DE, 2006). Natural products extraction is the most common application of the supercritical fluid extraction (SFE) due to its advantages over conventional methods, such as process flexibility, use of GRAS (Generally Recognized As Safe) solvents, compared to traditional organic solvents, and fast solvent removal from the solid matrix, avoiding post-processing steps (REVERCHON; MARCO, DE, 2006). Carbon dioxide (CO_2) is the most used supercritical solvent because it is of low cost, safe and easily available. Also, it has low critical point, meaning moderate processing conditions (SILVA, R. P. F. F. DA; ROCHA-SANTOS; DUARTE, 2016). Pressurized Liquid Extraction (PLE) involves extraction at elevated temperatures, and pressure adequate to maintain the solvent in its liquid state, which increase extraction rates compared to methods at room temperature and atmospheric pressure. When water is used as the pressurized fluid, the technique is called pressurized hot water extraction (PHWE) or subcritical water extraction (SWE) (CARABIAS-MARTÍNEZ *et al.*, 2005; MUSTAFA; TURNER, 2011). Extraction with gas expanded liquids (GXL) combines

the advantages of using compressed gas (low viscosity) and traditional organic liquids (polarity and solubility) as solvents; the pressures are normally lower than at SFE, with the advantage of lower volume of organic solvents compared to low-pressure liquid extractions. Again, CO_2 is the most common gas on GXL (AKIEN; POLIAKOFF, 2009; HALLETT *et al.*, 2006).

An appropriate plant matrix for biorefinery is one capable of generating multiple products, preferably with low competition with the food industry and containing a wide composition range (CHEW *et al.*, 2017). Plants of the genus *Pereskia*, known as Ora-pro-nobis, are classified as UFPs (Non-Conventional Food Plants), which are generally classified as weeds with popular use as food product with known nutritional and/or medicinal properties (KINUPP; BARROS, I. B. I. DE, 2008). This is the only genus of the *Cactaceae* family that has green leaves, being used as food in some regions of Brazil (EDWARDS; DONOGHUE, 2006). Its high protein content has attracted interest to be used as substitute for animal protein; moreover, is a plant rich in lysine, leucine, and valine (essential amino acids), and has vitamin C and carotenoids in the leaf (MARINELLI, 2016). Despite it is an interesting raw material to exploit its properties, there are still very few works in the literature that investigate the profile of compounds present in the plants of the genus *Pereskia*, especially *Pereskia aculeata*, being interesting to explore the quality of these compounds, confirming their properties through specific analysis. In this sense, ora-pro-nobis is a very interesting raw material to be exploited in the concept of biorefinery, because it can generate different products that can be applied in food and pharmaceutical industry.

With that in mind, integrated sequential extractions were proposed, using green compressed fluids in increasing order of polarity, for the fractionation of bioactive compounds from *Pereskia aculeata* leaves for bioprospection purposes. The biorefinery

processes include: SFE (CO_2), GXL (EtOH:CO_2), PLE (EtOH) and SWE (H_2O), where the recovered extracts were characterized by *in vitro* and chromatographic analysis.

5.2 Material and methods

5.2.1 Sample preparation

Ora-pro-nobis (*Pereskia aculeata*) leaves were purchased from *Sítio Flora Bioativas* company (Tijucas, SC, Brazil). The raw material collection, preparation, drying and grinding were performed as follows: the leaves were harvest in the morning, then quickly washed in stainless-steel vats to avoid losing the mucilage; in sequence, the leaves were spread in trays for drying at 45°C in air-circulating dehydrator (MS Metalúrgica e Comércio, Canoinhas, SC, Brazil) for 18 to 24h, the trays were rotated every 2h. After drying, the leaves were grinded in a home-made stainless-steel hammer mill and packet. The samples were stored at -18 °C in domestic freezer until further use.

5.2.2 Conventional extraction method

Conventional maceration extractions were performed (in triplicate), to compare with high-pressure extraction, from *P. aculeata* leaves using the method of Reyes et al., (2014) with ethanol and hexane as solvents. Briefly, 0.2 g of dried sample were mixed with 20 mL solvent containing 0.1% (w/v) BHT in a 50 mL Falcon tube/glass container and the mixture was shaken for 24 h in an orbital shaker (DOS-20L, Elmi Ltd, Riga, Latvia) at 250 rpm in the dark. Following the extraction, the exhausted substrate was precipitated out in a refrigerated centrifuge (Sorvall Evolution RC, Thermo Electron, Asherville, NC, USA) operating at 11.952g at 4°C for 10 min. The supernatant was collected, and the solvent was removed using a stream of N_2. Dry extracts were weighed to yield assessment and stored at −20°C protected from light.

5.2.3 High pressure extraction methods

In order to apply the biorefinery concept, a sequence of extractions was performed using solvents with increasing polarity. The process design proposed is presented in **Fig. 1**, with each step detailed as follows:

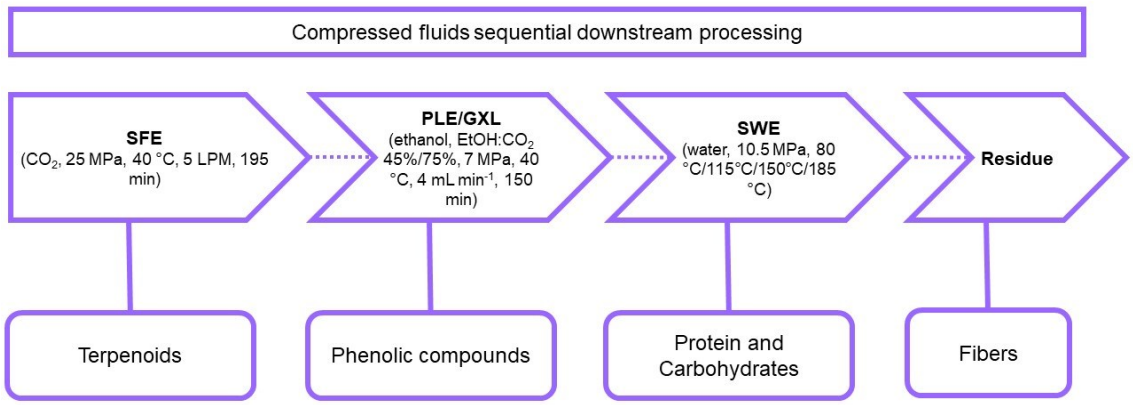
Step 1: The first step was SFE, carried out in a Spe-ed Helix unit from Applied Separations (Allentown, PA, USA). Briefly, 80 g of dried sample, together with glass spheres were placed in a 300 mL basket and inserted at stainless-steel (SST) extraction cell. The conditions were 25 MPa and 40 °C, according to previous study (TORRES *et al.*, 2021). A kinetics study was performed to establish the extraction time. In this step, fractions were collected every 10 min until one hour, and then every 15 min until 210 min and finally every 30 min for a total extraction of 270 min. Flow rate was kept at 5 $L.min^{-1}$ of CO_2, measured by a CO_2 gas flow meter placed at the exit of the extraction cell and corrected to standard conditions (SLPM). The extracts were stored at -20 °C in the absence of light, until further analysis.

Step 2: SFE residue (from Step 1) was used as raw material in sequential extractions by PLE or by GXL using ethanol and CO_2:ethanol, respectively. The extraction was carried out in a home-made SFE unit. CO_2 was provided from a pressurized cylinder and subcooled in a heat exchanger placed within the high-pressure pump (model PU-2080 CO_2 Plus from Jasco, Tokyo, Japan). Ethanol was pumped by a co-solvent pump (model PU-2080 Plus from Jasco, Tokyo, Japan). The total flow was set as 4 $mL.min^{-1}$, been the sum of supercritical CO_2 and EtOH. The temperature was set as 40 °C and pressure at 7 MPa, to minimize operational costs (GILBERT-LÓPEZ *et al.*, 2015). Three different percentages of EtOH were tested, 45, 75 and 100 % (where 100% represented the PLE condition). The kinetics was performed for all ethanol concentrations to determine optimal extraction time. Briefly, 3 g of sample was mixed with glass spheres and glass

wool, placed at both ends of the SST cell to avoid loses. The cell was placed in the oven set at 40 °C. The pressure was kept at 7 MPa by manual metering valve (Swagelok, Ohio, USA). The residual ethanol from the extract samples was evaporated under N_2 stream and stored at -20 °C in the absence of light.

Step 3: The residue from Step 2 was the raw material for SWE (water as solvent). The extractions were carried out in an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA, USA). The solvent was degassed by an ultrasound bath, to prevent oxidation during the extraction, before placed in the solvent controller. Extractions were carried out in 11 mL stainless steel cell at 10.5 MPa containing 0.5 g of sample from step 2 (mixed with glass spheres), and glass wool and filter paper were packed at both ends of the cell. The extraction starts by heating-up the cell, up to operational temperature. The temperatures evaluated were 80, 115, 150 and 185 °C. The residual water from the recovered extract samples was removed by freeze-drying (Lyobeta, Telstar, Terrassa, Spain) and the samples were stored at -20 °C in the absence of light, to prevent from degradation until analysis.

Fig.1. Scheme of the downstream processing proposed for the valorization of *Pereskia aculeate* leaves using compressed fluids extraction.



5.2.4 Conventional alkaline extraction method

The conventional maceration alkaline extraction of proteins from ora-pro-nobis leaves was performed according to the adaptation of the method described by Sereewatthanawut et al., 2008. The dry sample was suspended in distilled water to a suspended solids content of 20% (1 g in 5 g of water). Then, 0.2 M NaOH solution was added until pH 11, for 45 min in a thermomixer (Thermomixer comfort; Eppendorf, Hamburg, Germany) at 400 rpm at 30 °C. After the reaction time, the pH was adjusted with 0.2 M HCl solution to pH 7.0. The final mixture was centrifuged at 3500 rpm for 20 min (Eppendorf 5804; Hamburg, Germany). The soluble portion is the extracted protein fraction. The process was carried out in triplicate and the soluble portion was lyophilized, weighed to yield assessment and stored at −20°C to be used in the determination of total protein content.

5.2.5 Total phenols content (TPC) determination

The total phenolics content was determined by the method described by Koşar et al. (2005), using Folin-Ciocalteau reagent. A mixture with 10µL sample and 600µL of distilled water was added with 50µL of Folin-Ciocalteau reagent. After one minute, an aliquot of 150µL of sodium carbonate (Na_2CO_3) 20% (w/v) was added and water set the final reaction volume of 1mL. After 2 hours at room temperature and absence of light, 300µL aliquot were transferred to 96-well plate and read at 760nm on a spectrophotometer. A calibration curve of gallic acid (0.025 - 2 mg / ml) is made, and the total phenolic content is expressed as milligrams of gallic acid equivalent per gram of extract (mgGAE/gextract). All analyzes were performed in triplicate.

5.2.6 Total carotenoids content (TCC) determination

A spectrophotometric method described by Gallego et al., (2019) was used to determine the total carotenoids and total chlorophylls concentration, based on their

145 characteristic absorbance. Extracts were dissolved in methanol at concentrations ranging
146 0.05–1 mg.mL^{-1}. Absorbance of these solutions was recorded at 470 nm, for. External
147 standard calibration curve of lutein (0.5–10 μg.mL^{-1}) was used to calculate the total
148 carotenoid content. Total carotenoids were expressed as mg lutein g^{-1} extract, by
149 interpolating the absorbance of the extract at 470 nm in the calibration curve of lutein.
150 All the analyses were performed in triplicate and results expressed as mean ± SD.

151 5.2.7 DPPH radical scavenging assay

152 The antioxidant activity was evaluated by the method of neutralizing DPPH
153 radicals (2,2-diphenyl-1-picrilhidrazyl), described by Brand-Williams et al. (1995) and
154 adapted by Mensor et al. (2001). In this method, 100μL of each sample (diluted in ethanol
155 at different concentrations) reacts with 150μL of DPPH solution prepared by diluting 23.5
156 mg of DPPH in 100mL of methanol and after this solution, a further 1:10 dilution in
157 methanol is made and then used for analysis. After a period of 30 min at room temperature
158 and absence of light, and a 516nm reading in a spectrophotometer. The inhibition
159 percentage of DPPH radical was plotted against the concentration value of the extracts,
160 and the concentration required to inhibit 50% of the initial DPPH concentration,
161 expressed in IC_{50}, was calculated. The lower the IC_{50} value is, the higher is the antioxidant
162 activity. The tests were performed in triplicate.

163 5.2.8 ORAC assay

164 The antioxidant activity of ora-pro-nobis extracts were also assessed by oxygen
165 radical absorbance capacity assay, according to Ou et al. (2013) method, with some
166 modifications. The analysis was carried out as follows: 100 μL of extract sample at
167 different concentrations (5 μg–50 μg mL^{-1}) in EtOH/H_2O (1:9, v/v) were added in the
168 wells along with 100 μL of AAPH (590 mM) in 30 mM phosphate-buffered saline (PBS)
169 at pH = 7.5, 25 μL of fluorescein (10 μM) in PBS buffer and 100 μL of PBS buffer.

Fluorescence was measured ($\lambda_{excitation}$ = 485 nm; $\lambda_{emission}$ = 530 nm) every 5 minutes at 37 °C for 1 hour. Ascorbic acid was used as the reference standard. The capacity of each extract for scavenging peroxyl radicals was calculated through the inhibition percentage of the difference between the area under the curve (AUC) of fluorescence decay in the presence (AUCsample) or absence (AUCcontrol) of the sample (**eqn (1)**).

$$\text{Inhibition } \% = \frac{\text{AUCcontrol} - \text{AUCsample}}{AUCcontrol} \quad (1)$$

The AUC is calculated using following **eqn (2)**:

$$AUC = 0.5 + \sum \frac{f_i}{f_0} \quad (2)$$

where f_0 is the fluorescence at time 0 and f_i is fluorescence every 5 min. The results may be expressed as IC50 values by plotting concentration vs. inhibition percentage, or by Trolox equivalent. A standard curve of Trolox was made (from 0 to 50 µM) and the results were expressed as milligrams of Trolox equivalent per gram of extract.

5.2.8.1 Acetylcholinesterase (AChE) activity inhibition assay

Acetylcholinesterase (AChE) activity was determined by Ellman's method (ELLMAN *et al*. 1961) with modifications. The rate of consumption of the substrate is verified with the help of a fluorescent reagent, 4-Fluoro-7-sulfamoylbenzofurazan (ABD-F, Sigma Aldrich, St. Louis, MO, USA), which directly links to the sulfur atom of thiols (SÁNCHEZ-MARTÍNEZ, José David *et al.*, 2021). Initially, the K_M (Michaelis–Menten constant) of the enzyme is calculated to determine the substrate concentration of the reaction rate is half of maximum velocity rate. Reactions mixtures in the wells contained the following reagents: 100 µL of substrate acetylthiocholine iodide (ACth) (Sigma Aldrich, St. Louis, MO, USA) was added at different concentrations (0.4 - 4 mM) in H2O; 50 µL of pure EtOH; 100 µL of buffer (150mM Tris-HCl pH=8); 25 µL of ABD-F (125 µM) in buffer; and 25 µL of 0.8 U/mL AChE (Sigma Aldrich, St. Louis, MO, USA). To

194 determine the inhibition capacity of the extracts against the acetylcholinesterase enzyme,
195 each well was filled with 100 µL of extract sample (diluted in EtOH/H2O [1:1, v/v]) at
196 different concentrations; 100 µL of buffer; 25 µL of 0.8 U/mL AChE in buffer and 25 µL
197 of ABD-F (125 µM) in buffer. The mixture was incubated for 10 minutes. Reaction started
198 by adding 50 µL of ACth at concentration of KM in H_2O. The V_{mean} of the substrate
199 consumption is recorded each 10 s (V_{mean} corresponds to enzymatic mean velocity during
200 kinetic) in fluorescence mode (λ_{ex} = 389 nm and λ_{em} = 513 nm) during 10 min in a Synergy
201 HT 96-well microplate reader (Biotek, Winooski, VT, USA). The velocity of the reaction
202 is compared to a control (assay without extract sample). Galantamine was used as a
203 positive control. Tests were carried out in triplicate. Results were expressed as IC50,
204 which is the sample concentration when 50% inhibition is reached and was obtained
205 plotting the inhibition percentage against sample concentrations.
206

207 5.2.9 Lipoxygenase (LOX) activity assay

208 Lipoxygenase (LOX) activity was determined by fluorescence assay based on the
209 enzymatic oxidation of linoleic acid to the corresponding hydroperoxydes, which can
210 degrade the fluorescence of fluorescein (NUÑEZ; FOGLIA; PIAZZA, 1995; SÁNCHEZ-
211 MARTÍNEZ, *et al.*, 2021). The assay consists in first determining KM of the enzyme
212 with linoleic acid (substrate) at different concentrations (0.7 - 7mM). Then, the inhibition
213 assay mixture containing the extract (diluted in EtOH/H_2O [1:1, v/v]) at different
214 concentrations, 1 µM of fluorescein diluted in buffer (150mM Tris-HCl pH=9), soybean
215 lipoxygenase (948 U/µL) (Sigma Aldrich, St. Louis, MO, USA) (diluted in buffer), and
216 linoleic acid at concentration of KM in EtOH/H_2O [1:1, v/v], was added to a 96-well
217 microplate and fluorescence is recorded every 10 s during 15 min using a Synergy HT
218 96-well microplate reader (Biotek, Winooski, VT, USA) in fluorescence mode (λ_{ex} = 485

nm and λ_{em} = 530 nm). Negative control without extract sample were also included. The enzymatic activity was graphically determined from the slope of the linear portion of the curve and the V_{mean} of the reaction is compared to a control. Quercetin was used as reference compounds. LOX-1 from soybean is routinely used since it resembles human LOX in its substrate specificity and inhibition characteristics (JIMÉNEZ-ASPEE *et al.*, 2015). Tests were carried out in triplicate, with results expressed as IC_{50}, which is the sample concentration providing 50% inhibition of the LOX enzyme and was obtained plotting the inhibition percentage against sample concentrations.

5.2.10 Proximate composition

The moisture content of the raw material was determined gravimetrically in a vacuum oven at 70°C and 3,333 Pa until reach constant weight (method 926.12, AOAC (1996)). The total protein content from raw material was determined by Kjeldhal method, (method 928.08, AOAC (2000), Nx6.25), lipids (Soxhlet method 920.39, AOAC, 2005b). The enzymatic-gravimetric method (991.43 of AOAC, 2000), proposed by Lee et al. (1992) and Prosky et al. (1984), was used to determine total dietary fiber (insoluble and soluble). The total dietary fiber was calculated as the sum of soluble dietary fiber and insoluble dietary fiber after correcting for ash and undigested protein. Dietary fiber was expressed as grams per 100 g sample on a wet weight basis. All analyses were conducted in triplicate. Total carbohydrates were determined by the phenol-sulfuric acid colorimetric assay method (DUBOIS *et al.*, 1956). The absorbance was read at 490 nm. Standard curves were prepared and the results were calibrated against glucose standard solution up to 1 g/mL.

5.2.11 Bicinchoninic acid (BCA) protein assay

The total protein concentration was determined using the Pierce Bicinchoninic (BCA) assay kit (Thermo Fisher Scientific, Scoresby, Australia) following

manufacturer's protocol. Bovine serum albumin (BSA) with known concentration was used as standard. Extracts were diluted in water (0.5 $mg.mL^{-1}$) and after reaction samples were read at 562 nm. The tests were performed in triplicate and expressed as mg protein (BSA equivalent) per gram extract.

5.2.12 HPLC-DAD-ESI-MS/MS analysis

Pereskia aculeata PLE, GXL and SWE extracts (concentrations 5 $mg.mL^{-1}$ ethanol) were analyzed by high-performance liquid chromatography using an Agilent 1100 liquid chromatograph coupled to a diode array detector (DAD) (Agilent, Palo Alto, CA) and autosampler, directly coupled to an ion trap mass spectrometer (Esquire 2000, Bruker Daltonics, Bremen, Germany) via an electrospray interface. Extract solutions were filtered through 0.45 μm diameter nylon syringe filters (Sartorius, Göttingen, Germany) before injection. Separation of phenolic compounds was performed on Poroshell 120 EC-C18 column (100 mm x 4.6 mm i.d. 2.7 μm particle size) (Agilent Technologies, Palo Alto, CA, USA) at 35 °C. The mobile phase consisted of solvent (A): 0.1% formic acid in water and solvent (B): 0.1% of formic acid in acetonitrile, following method described in Garcia et al. (Garcia et al., 2019). Using a linear gradient 0 min, 5% B; 5 min, 10% B; 10 min, 15% B; 20 min, 20% B; 30 min, 25% B; 40 min, 35% B and 45 min, 50% B. This latter kept for 5 min, before re-equilibration to starting condition at 50 min. The injection volume was 10 μL and the flow rate was 0.3 mL/min. The diode array detector acquisition was run at 280, 330 and 370 nm. On the other hand, the MS was operated under ESI negative ionization mode using the following parameters: dry temperature set at 350 °C, dry gas flow (N2) 9.0 L/min, capillary at 3500 v, 51 nA, nebulizer pressure at 40 psi, and the mass spectra were recorded between 50 and 2200 m/z.

5.2.13 Statistical analysis

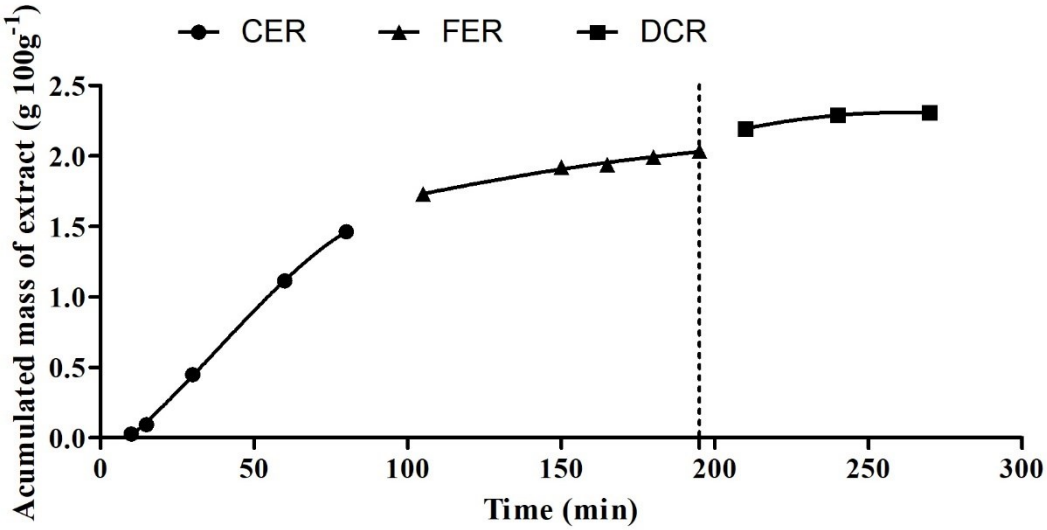
All extraction procedures were carried out in duplicate and all in vitro analysis were carried out in triplicate. The results were expressed as mean ± standard deviation (SD). Data sets were evaluated by one-way ANOVA followed by post hoc Tukey's HSD test at ($p < 0.05$) level. The principal component analysis (PCA) was held for the data from Step2 and Step3 (GXL or PLE, and SWE), using software Statistica version 7.1 (Stat-Soft Inc., Tulsa, OK, USA). Graphs were determined using GraphPad PRISM software (GraphPad Software, Inc.).

5.3 Results and discussion

5.3.1 Extraction yield (%)

The sequential extraction processes were performed considering the biorefinery concept, where the "residue" from Step1 was used for Step2, which "residue" was then submitted to Step3. The purpose was maximizing the use of the raw material, producing a series of high added-value products. To determine extraction time for each Step, an overall Extraction Curve (OEC), constructed with accumulated mass of extract vs. time, was obtained for SFE, GXL and PLE operations (**Fig. 2** and **3**, respectively). The extraction time was determined considering that 90% of possible extract was recovered. The SFE kinetics was performed at 40 °C, 25 MPa and 5 SLPM CO_2 flow rate (measured as gas). The OEC for GXL and PLE were determined at 40 °C, 7 MPa and 4 mL min^{-1} (v/v, measured in liquid state at pump head). Extraction time was 195 min for SFE, and 150 min for GXL and PLE, regardless of the ethanol concentration. SWE assays were performed in batch mode due to equipment structure. Then, OEC was simulated by yield determination at different extraction time and at 80°C and 10.5 MPa, as presented at **Fig.**

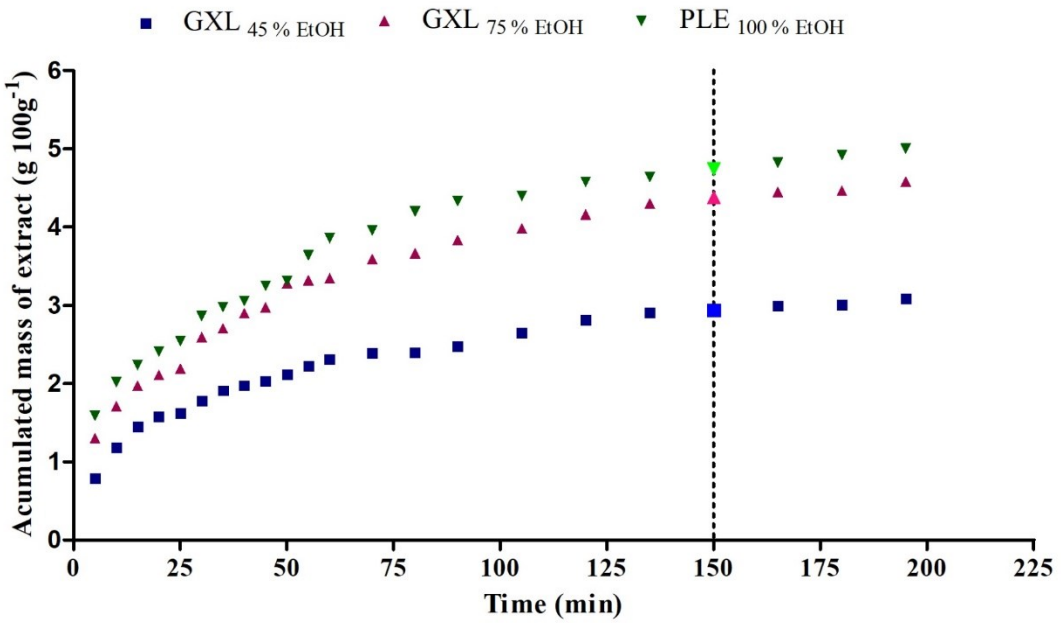
292 **4**. Extraction time SWE was set at 15 min, since increasing yield was obtained at longer
293 times.
294
295 Fig.2. Kinetic behavior of the extraction yield (g $100g^{-1}$) of supercritical fluid extracts
296 from *Pereskia aculeate* leaves.



297
298

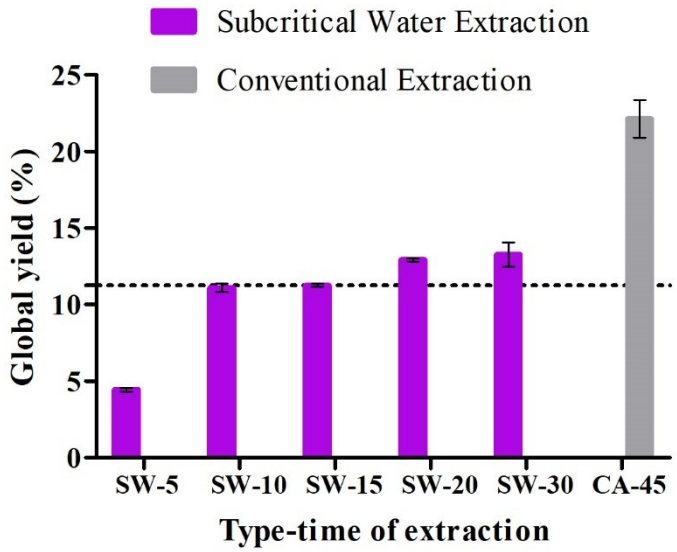
299 Fig.3. Kinetic behavior of the extraction yield (g $100g^{-1}$) of gas-expanded liquid extraction ($GXL_{EtOH\ 45\%}$, $GXL_{EtOH\ 75\%}$) and pressurized liquid

300 extraction ($PLE_{EtOH\ 100\%}$) from *Pereskia aculeate* leaves. Conditions: 40 °C, 7 MPa and 4 mL min^{-1}.



301

Fig.4. Global yield (%) from subcritical water extractions (SW) at 5, 10, 15, 20 and 30 min and conventional alkaline extraction (CA) at 45 min of *Pereskia aculeate* leaves.



Extraction yield results for SFE, GXL, PLE are compared with conventional maceration extraction at **Table 1**. SFE presented the lowest yields compared to other methods, even maceration with hexane, suggesting SFE as a selective method towards the oily fraction from *Pereskia aculeata*., enriching in carotenoids (section 3.2). This behavior was also detected by Torres et al., (2021). Then, SFE was used as Step1 in the biorefinery process to increase yield and selectivity in the following steps since this initial CO_2 defatting helps the action of more polar solvents in the following steps.

To improve the phenolics recovery, a second step using ethanol was performed. A combination of ethanol and pressurized CO_2 in GXL method, and pure ethanol in PLE method, under the same pressure and temperature conditions, were tested. Extraction yield of GXL 75% EtOH and PLE 100% EtOH were significantly different than GXL 45% EtOH. PLE provided the highest yield and therefore was selected as Step2 in a biorefinery route, compared with GXL, because high sample recovery, from Step2, aids the following extraction because the recovered compounds do not interfere in Step3.

319 Although maceration with EtOH presents higher yield than PLE (**Table 1**), it was
320 conducted at much longer process, and also lower selectivity. Then, the advantage of the
321 high-pressure method is shown on the extract quality, by the analysis of recovered
322 compounds, discussed in the sequence.

323 The last step was SWE, with yield higher than the previous steps, partly because
324 two fractions were already recovered, leaving mostly polar compounds for water
325 extraction. The yield values at the higher temperatures (150 and 185 °C) were very higher,
326 which can be justified by the increase in mass transfer at high temperatures (MUSTAFA;
327 TURNER, 2011). The maceration alkaline extraction provided higher yields compared to
328 maceration with ethanol and hexane, showing the higher affinity of the compounds from
329 *Pereskia aculeata* leaves for polar solvents. The non-polar fraction recovered by SFE
330 (Step1), and the phenolics fraction, recovered by PLE (Step2), left carbohydrates and
331 proteins at the solid material for water recovery, as discussed by the quality of the
332 fractions.

Table 1 – Yield, TPC, TCC and antioxidant activity (DPPH, ORAC methods) of sequential extraction processes from ora-pro-nobis leaves (*Pereskia aculeata*).

Treatments	Solvents / Conditions	Yield (%)	DPPH (IC_{50}ug/mL)	ORAC ($mg_{TE}/g_{extract}$)	TPC $mg_{GAE}/g_{extract}$	TCC ($mg_{LUTEIN}/g_{extract}$)
Maceration	Alkaline	22.13 ± 1.22^{d}	79.17 ± 0.97^{abd}	67.07 ± 2.42^{b}	41.94 ± 2.26^{a}	-
	Ethanol	10.87 ± 0.72^{b}	12.97 ± 0.54^{c}	76.74 ± 2.33^{b}	251.40 ± 7.13^{e}	38.90 ± 0.54^{d}
	Hexane	11.04 ± 0.49^{b}	10.36 ± 0.24^{c}	85.17 ± 2.28^{bc}	20.15 ± 2.16^{c}	17.62 ± 1.27^{c}
SFE	CO_2	1.87 ± 0.07^{a}	330.32 ± 32.80^{e}	88.75 ± 0.01^{bc}	7.79 ± 0.01^{b}	66.88 ± 0.01^{e}
GXL	45 % EtOH + 55 % $scCO_2$	2.66 ± 0.25^{a}	94.93 ± 3.54^{d}	126.63 ± 2.11^{ad}	35.17 ± 2.18^{a}	62.05 ± 1.27^{b}
	75 % EtOH + 25 % $scCO_2$	3.85 ± 0.22^{a}	80.43 ± 2.93^{bd}	129.27 ± 1.59^{ad}	37.79 ± 0.98^{a}	56.69 ± 0.16^{a}
PLE	EtOH	3.88 ± 0.16^{a}	68.50 ± 1.68^{abc}	147.88 ± 4.09^{a}	38.63 ± 0.95^{a}	58.73 ± 1.17^{ab}
SWE	80 °C	8.01 ± 0.41^{c}	48.23 ± 5.47^{abc}	146.53 ± 12.37^{a}	35.92 ± 3.33^{a}	-
	115 °C	8.38 ± 0.21^{bc}	72.17 ± 4.12^{abd}	145.57 ± 7.03^{a}	35.18 ± 3.70^{a}	-
	150 °C	45.21 ± 1.05^{e}	39.25 ± 0.40^{ac}	106.54 ± 10.98^{cd}	43.13 ± 0.47^{a}	-
	185 °C	65.28 ± 1.20^{f}	41.43 ± 2.69^{abc}	136.97 ± 8.35^{a}	54.13 ± 1.52^{d}	-
BHT	-	7.09 ± 0.01	-	-	-	-

Values are expressed as mean ± standard deviation (n = 3). The levels of significant differences between the mean values were determined using the Tukey's HSD test (α = 0.05); different letters in the same column show significant statistical differences ($p < 0.05$).

5.3.2 Total phenolics and carotenoids content

Total phenolics content from SFE, GXL and PLE samples are also shown at **Table 1**. The highest values were provided by ethanol maceration, SWE at 185 °C, EtOH and SWE 150 °C (with no significant difference). Then, followed by alkaline maceration, PLE, GXL 75% EtOH and SWE 80 °C, also with no significant differences, and last by hexane maceration and by SFE.

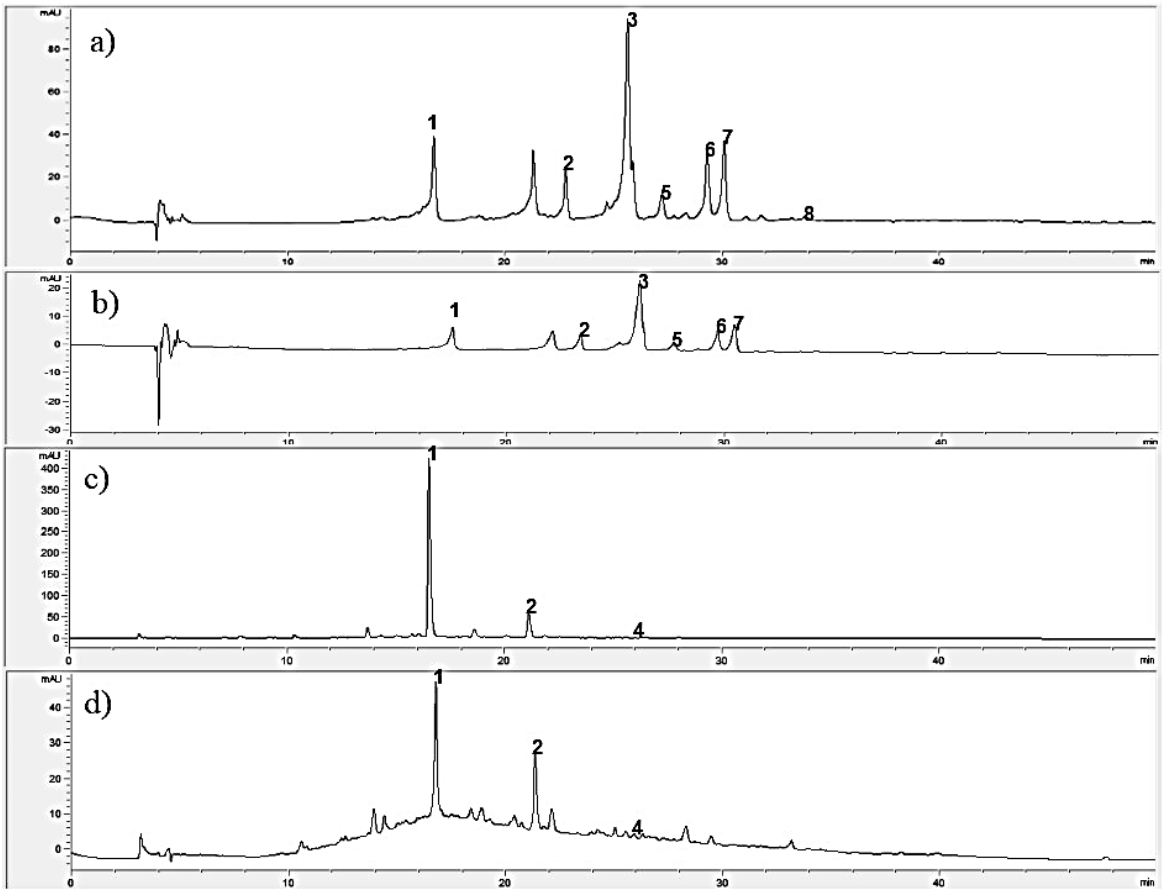
The method by Garcia et al., (2019) was used to characterize the phenolic compounds from the extract samples. The phenolics profile was tentatively identified by LC-DAD-ESI-MS/MS and the identified compounds are presented at **Table 2**, with 8 tentatively identified compounds: one phenolic acid (caftaric acid isomers) and 7 flavonoids (quercetin, isorhamnetin and kaempferol glycoside derivatives). All compounds were identified by comparing the UV-vis spectrum and the MS fragmentation pattern with literature. Caftaric acid ($[M-H]^-$ at *m/z* 311) presented the same MS^2 fragmentation pattern as the methanolic extracts from *P. aculeata* described by Garcia et al. (2019). Flavonoids derivatives were also identified, as follows: quercetin-O-pentoside-O-rutinoside ($[M-H]^-$ at *m/z* 741) and quercetin-3-O-rutinoside ($[M-H]^-$ at *m/z* 609), both presenting MS^2 fragment at *m/z* 301 and λ_{max} around 350 nm; isorhamnetin-O-pentoside-O-rutinoside ($[M-H]^-$ at *m/z* 755) and isorhamnetin-3-O-rutinoside($[M-H]^-$ at *m/z* 623) presenting MS^2 fragment at *m/z* 315 and λ_{max} around 350 nm; and Kaempferol-3-O-rutinoside ($[M-H]^-$ at *m/z* 593), with MS^2 fragment at *m/z* 285 and λ_{max} around 350 nm. The MS^2 fragment that these derivative compounds had in common was the *m/z* of the compound without the glycoside residues pentosyl (*m/z* 132), rutinose (deoxyhexosyl-hexose) (*m/z* 308), and hexosyl (*m/z* 162). Following this logic, the glucosyl residues (*m/z* 162) were also present and the quercetin-glucoside and isorhamnetin-3-O-glucoside compounds were also identified (λ_{max} around 350 nm).

Table 2 – Tentative identification of phenolic and flavonoids compounds from *Pereskia aculeate* leaves PLE and SWE extracts.

#	Rt (min)	Tentative identification	λmax (nm)	$[M-H]^-$	Main fragments	Also determined by
1	16.7	Caftaric acid isomers	328	311	179(100), 149(90)	(GARCIA *et al.*, 2019)
2	22.8	Quercetin-O-pentoside-O-rutinoside	255, 355	741	609(100), 301(84)	(GARCIA *et al.*, 2019)
3	25.7	Quercetin-3-O-rutinoside	255, 355	609	301(100)	(GARCIA *et al.*, 2019)
4	25.9	Isorhamnetin-O-pentoside-O-rutinoside	254, 354	755	623(24), 605(84), 315(100)	(GARCIA *et al.*, 2019)
5	27.3	Quercetin-glucoside	255, 355	463	301(100)	(JUSTESEN, 2000)
6	29.4	Kaempferol-3-O-rutinoside	265, 350	593	285(100)	(GARCIA *et al.*, 2019)
7	30.2	Isorhamnetin-3-O-rutinoside	254, 354	623	315(100)	(GARCIA *et al.*, 2019)
8	32.0	Isorhamnetin-3-O-glucoside	342	477	314(100)	NIST(Pubchem) (BRITO *et al.*, 2014)

The TPC results corroborate the data obtained for *Pereskia* Sp. by conventional maceration; for instance, Rodrigues, (2016), studying *P. aculeate* and using conventional water extraction (95 °C for 1h) found similar TPC results as provided by SWE at 185 °C, from the present study; Sim et al., (2010) also found similar TPC values for *P. bleo* extracts. On the other hand, TPC values found in the present study were higher than those found by Johari & Khong, (2019) and by Souza et al., (2016). It is expected high phenolic extraction performance by ethanol as solvent due to their affinity with ethanol, however water fractions also recovered phenolic components, although the chemical profile was more selective, according to *in vitro* and chromatography analysis (**Table 1**, **Table 2**, **Fig.**

374 **5**). The chromatographic results for the samples PLE, Ethanol maceration, SWE 185 °C,
375 and Alkaline maceration are compared in Figure 2, with the components peaks identified
376 as presented at Table 2. In fact, the peak area of caftaric acid isomers (Compound 1, **Fig.**
377 **5c**) from SWE samples is much superior than PLE sample (Compound 1, **Fig. 5a**), which
378 are also superior than the conventional macerations (Compound 1, **Fig. 5b** and **Fig. 5d**).
379
380 Fig.5. Liquid chromatography-diode-array detection chromatogram (320 nm) of a)
381 Pressurized liquid extraction (ethanol, 40 °C, 7 MPa, 150 min), b) Conventional
382 extraction (ethanol, room temperature and pressure, 24 h), c) Subcritical water extraction
383 (water, 185 °C, 10,5 MPa, 15 min), d) Conventional alkaline extraction (water, room
384 temperature and pressure). For peak identification, see Table 2.



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Carotenoids are natural pigments with considerable high antioxidant capacity that can act as photo-protectants and modulating gene activity, resulting in protection from experimentally-induced inflammatory damage and neoplastic transformation (HIX; LOCKWOOD; BERTRAM, 2004). Then, the total carotenoids content (TCC) was determined for SFE, GXL and PLE samples, considering the solvent (ethanol) affinity with these compounds. The TCC values are also presented in **Table 1**. Compressed fluids extracts, except SWE, presented lutein recovery values higher than conventional extractions, showing the selectivity towards the carotenoids. Due to the hydrophobic nature of carotenoids, they are conventionally extracted using organic solvents, usually, non-polar solvents. Supercritical CO_2 extraction is proven to be an effective method to extract carotenoids since they are thermolabile compounds and temperatures used at SFE are low compared to some conventional methods (SAINI; KEUM, 2018). In fact, many studies use SFE to recover carotenoids from plant matrices. For instance, (ANDRADE LIMA, DE *et al.*, 2019) applied optimized SFE condition for 15 different vegetable waste matrices, verifying a total carotenoid recovery higher than 90% (m/m) for most samples.

Agostini-Costa et al. (2014) presented a profile of carotenoids from *Pereskia* leaves and observed that lutein was the predominant carotenoid detected in this type of plant, therefore, the TCC from the present study was expressed as mg lutein per g of extract. The authors evaluated two species of ora-pro-nobis, *P. aculeata* and *P. grandifolia*, in terms of carotenoids profile, detecting lutein values ranging from 53.6 to 102 $\mu g.g^{-1}$, lower than observed in the present work, with TCC varying from 17.6 to 100.3 $mg.g^{-1}$, the differences may be most related to the extraction procedures applied to the solid samples. The data by Agostini-Costa et al. (2014) provided carotenoids content from sample recovered by saponification, a conventional extraction, while data from Table 1 show TCC from fractions recovered by pressurized CO_2 and ethanol, which proved to be

more selective towards these compounds. The high significance of the carotenoid fraction recovered by pressurized solvents from ora-pro-nobis, most particularly by supercritical CO_2, suggests TCC as a relevant fraction to be obtained from this raw material, and concentrated in one step of the biorefinery from ora-pro-nobis leaves.

5.3.3 Antioxidant activity

Antioxidant capacity of the extracts from *P. aculeate* leaves were evaluated by DPPH (expressed as IC_{50}), an electron-transfer assay, and by ORAC (expressed as Trolox equivalent), a hydrogen atom transfer assay. The antioxidant activity from ora-pro-nobis extracts is presented at **Table 1**, with the best values from SWE and PLE samples, compared to SFE, GXL. Conventional ethanol and hexane macerations provided the lowest IC_{50} (12.97 ± 0.54 and 10.36 ± 0.24, respectively), which can be related to the highest TPC. According to Rai et al. (2017), extracts can be categorized, according to IC_{50} values, in highly active ($IC_{50}<50\mu g.mL^{-1}$), moderately active ($50<IC_{50}<100\mu g.mL^{-1}$), weakly active ($100<IC_{50}<200\mu g.mL^{-1}$) or inactive ($IC_{50}>200\mu g.mL^{-1}$). The samples listed at Table 1 can be classified as highly active in decreasing order of antioxidant activity: Conventional Hexane > Conventional EtOH > SWE 150 °C > SWE 185 °C > SWE 80; other extracts were moderately active, obtained by conventional alkaline extraction, SWE 115 °C, GXL 45 and 75% EtOH and PLE-EtOH. The SFE sample was inactive.

A multivariate data analysis based on principal components analysis (PCA) was carried out to correlate the chemical composition with bioactivities of the extracts from *Pereskia aculeata* leaves. The data used were: abundance of UV-vis spectra at 330nm and IC_{50} values from DPPH, and also ORAC and TPC values, providing **Fig. 6**, where PC1 and PC2 can explain 98% of the total variance. The response differences among the

samples are presented at **Fig. 6a** (PC1), while the differences between the responses from compressed fluid extracts and conventional extracts are detected from **Fig. 6b** (PC2). From **Fig. 6a** we observe correlation between DPPH and TPC values, and that ORAC results are correlated with compounds 1 and 4. Also, **Fig. 6b** shows higher concentration of compounds from compressed fluids extracts, compared with conventional samples.

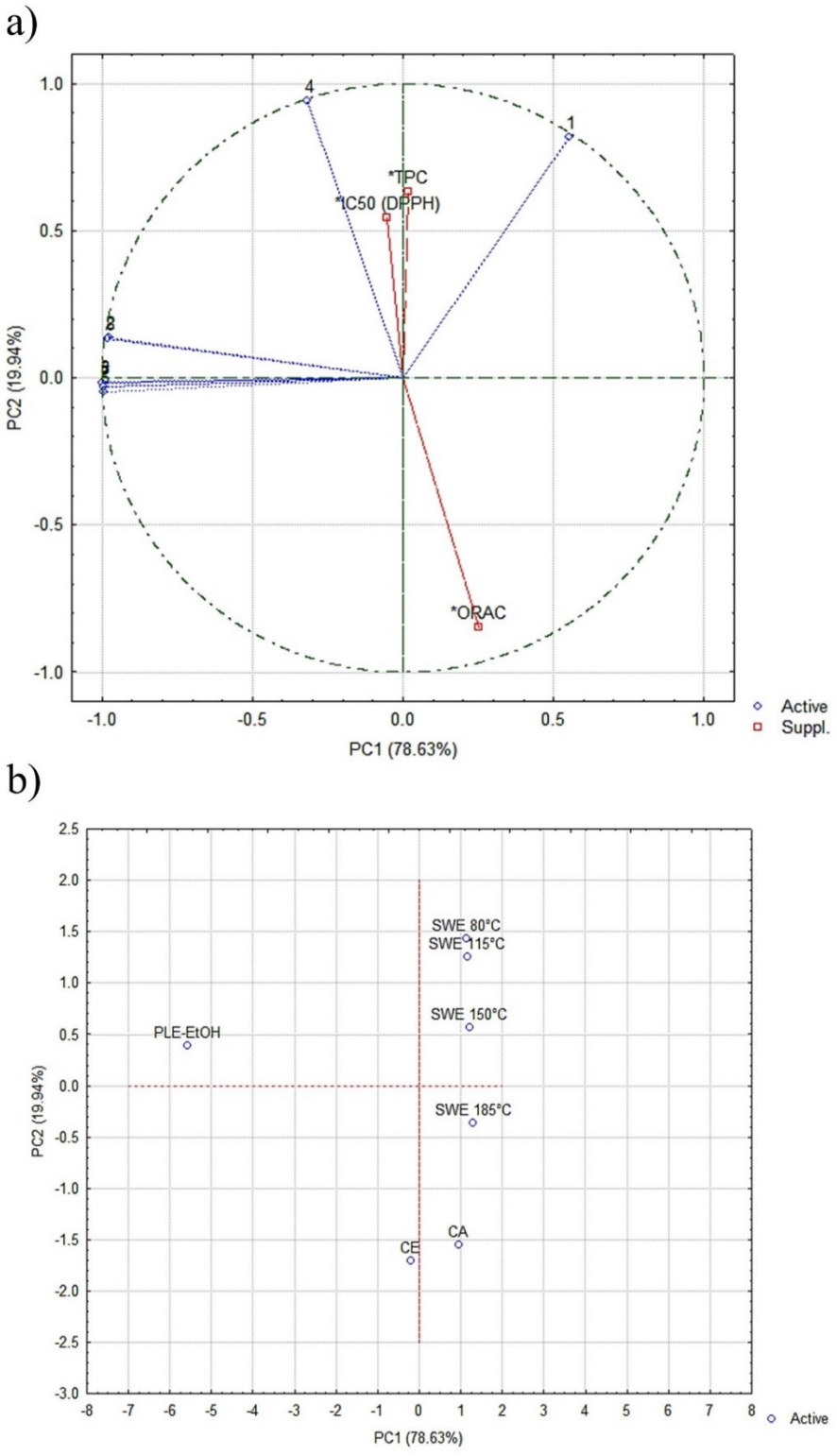
The PCA also provides the values of data correlation, as presented at **Table 3**, where the significant correlations, with 95% confidence, are highlighted (bold). Higher correlations were found between DPPH (1/ IC_{50}) and TPC ($r = 0.98$), and also ORAC and compounds 1 and 4 ($r = 0.60$ and $r = 0.84$, respectively), confirming that antioxidant activity by DPPH are due to phenolic content, while ORAC activity was provided by caftaric acid and Isorhamnetin-O-pentoside-O-rutinoside. In fact, Caftaric acid is a phenolic acid that, together with other phenolic acids, present high antioxidant activity, while Isorhamnetin is a flavonoid that also presents high antioxidant activity due to the presence of a hydroxyl group (PENGFEI *et al.*, 2009; SHAHIDI; CHANDRASEKARA, 2010).

Table 3 – Pearson correlations for the variables: 1:Caftaric acid, 2: Quercetin-O-pentoside-O-rutinoside, 3: Quercetin-3-O-rutinoside, 4: Isorhamnetin-O-pentoside-O-rutinoside, 5: Quercetin-glucoside, 6: Kaempferol-3-O-rutinoside, 7: Isorhamnetin-3-O-rutinoside, 8: Isorhamnetin-3-O-glucoside, acting as active variables and $1/IC_{50}$ (DPPH), ORAC and TPC, acting as supplementary variables.

	1	2	3	4	5	6	7	8	$1/IC_{50}$ (DPPH)*	ORAC*	TPC*
1	1.00	-0.43	-0.56	0.58	-0.56	-0.58	-0.57	-0.43	-0.39	0.60#	-0.50
2	-0.43	1.00	0.96	0.44	0.96	0.95	0.96	0.99	-0.24	0.35	-0.17
3	-0.56	0.96	1.00	0.30	1.00	1.00	1.00	0.97	0.00	0.25	0.07
4	0.58	0.44	0.30	1.00	0.30	0.27	0.28	0.43	-0.51	0.84#	-0.57
5	-0.56	0.96	1.00	0.30	1.00	1.00	1.00	0.97	0.01	0.24	0.07
6	-0.58	0.95	1.00	0.27	1.00	1.00	1.00	0.95	0.05	0.22	0.12
7	-0.57	0.96	1.00	0.28	1.00	1.00	1.00	0.96	0.03	0.23	0.09
8	-0.43	0.99	0.97	0.43	0.97	0.95	0.96	1.00	-0.24	0.38	-0.18
$1/IC_{50}$ (DPPH)*	-0.39	-0.24	0.00	-0.51	0.01	0.05	0.03	-0.24	1.00	-0.48	0.98#
ORAC*	0.60#	0.35	0.25	0.84#	0.24	0.22	0.23	0.38	-0.48	1.00	-0.54
TPC*	-0.50	-0.17	0.07	-0.57	0.07	0.12	0.09	-0.18	0.98#	-0.54	1.00

*Supplementary variables; #Significative ($p<0.05$).

464 Fig.6. Principal component analysis, a) loadings and b) scores from compressed fluids
465 and conventional extractions from Pereskia aculeata leaves. For the IC50 value of DPPH,
466 the inverse (1/ IC50) was used in the data matrix to facilitate the PCA analysis



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5.3.4 Total protein and carbohydrate content and proximate composition

Total protein and carbohydrate contents were assessed from the SWE and conventional alkaline samples. The SWE samples were recovered from Step3 biorefinery process from *Pereskia aculeate* leaves, which the main goal was the recovery of protein fraction. Ora-pro-nobis leaves are known for their high protein content, been recognized as "meat of the poor" because it is used in many regions of Brazil as substitute of animal protein. The proximate composition of *Pereskia aculeata* leaves, from this study, is presented at **Table 4**, showing protein content from raw material of 13%, lower than literature values (around 20%) (MARTINEVSKI; OLIVEIRA; FLORES, 2013; TAKEITI *et al.*, 2009). The total soluble protein content, measured by bicinchoninic acid method, and total carbohydrate content, from the recovered extracts, are presented at **Table 5**. The results show that SWE provided higher protein recovery. Mostly, the increase in temperature, from 150 to 185 °C, effectively increased the protein recovery from the samples, with concentration higher than obtained by conventional alkaline extraction.

Table 4 - Proximate composition of *Pereskia aculeata* dried raw leaves

	Content (g/100g)[a]
Moisture	6.92 ± 0.04
Total Protein	13.68 ± 0.06[b]
Total Carbohydrate	16.11 ± 1.82
Soluble dietary fiber	9.73 ± 0.01
Insoluble dietary fiber	38.16 ± 0.01
Total dietary fiber	47,89 ± 0.01

[a]Values are means in triplicate determinations. Values expressed on a dry basis, except for moisture.
[b]6.25xN g/100 g.

Table 5 – Total protein and carbohydrate content of SWE extracts of Ora-pro-nobis leaves.

Treatments	Protein (mgBSA/$g_{extract}$)	Carbohydrates (mg/$g_{extract}$)
Conventional alkaline maceration	292.07 ± 9.63[a]	269.84 ± 8.48[c]
SWE 80 °C	362.70 ± 2.83[ab]	157.54 ± 10.47[a]
SWE 115 °C	377.11 ± 30.01[ab]	161.39 ± 3.07[a]
SWE 150 °C	338.25 ± 17.99[ab]	456.43 ± 45.13[b]
SWE 185 °C	409.95 ± 38.94[b]	478.47 ± 0.91[b]

Values are expressed as mean ± standard deviation (n = 3). The levels of significant difference between the mean values were determined using the Tukey's HSD test (α = 0.05).

5.3.5 Neurodegenerative activities

Neurodegenerative potential from samples of Step 1 and Step 2 were evaluated in terms of inhibitory activity against acetylcholinesterase (AChE), the Alzheimer's disease marker, and against lipoxidase (LOX), an anti-inflammatory marker. The results, presented at **Table 6**, show comparable inhibitory behavior obtained by conventional samples and by compressed fluid samples. The extracts did not present high AChE inhibition activity, with IC_{50} values higher than that presented by galantamine, a positive control used at Alzheimer's disease treatment. However, for the anti-inflammatory activity, the results from ora-pro-nobis extracts were compared with that from the flavonoid quercetin, known as a potent LOX inhibitor (BORBULEVYCH *et al.*, 2004). All extract samples provided good LOX inhibition, especially Step 2 sample (PLE 100% EtOH) and the conventional hexane samples, with IC_{50} values lower than the standard. LOX inhibition was significantly higher from Step 2 samples ($p < 0.05$), which presents quercetin in its composition, according to **Table 2**.

Table 6 - Enzymatic inhibition of *Pereskia aculeate* leaves extracts in the sequential steps

	Treatments						
	Step 1[A]	Step 2	Step 3[B]	Conventional EtOH	Conventional Hexane	Galantamine [C]	Quercetin [C]
IC_{50} (AChE)	472.77 ± 0.01[a] (295.79 ± 47.96)	422.59 ± 25.42[a]	nd	584.76 ± 15.38[b]	418.89 ± 35.50[a]	0.4 ± 0.0	-
IC_{50} (LOX)	197.13 ± 3.35[a] (90.60 ± 8.71)	78.38 ± 1.47[b]	nd	141.95 ± 8.07[c]	97.12 ± 0.01[d]	-	125 ± 20

Values are expressed as mean ± standard deviation (n = 3). The levels of significant difference between the mean values were determined using the Tukey's HSD test; different letters in the same row show significant statistical differences ($p < 0.05$). Values in parenthesis are referred to (TORRES *et al.*, 2021). [A] Step 1: SFE (CO_2, 40 C, 25 MPa); Step 2: PLE (ethanol, 40 C, 7 MPa); Step 3: SWE extracts. [B]AChE and LOX inhibition were not able to be determined in SWE extracts. nd – not determined. [C] Medicine used as positive control, with values obtained by Sánchez-Martínez et al., (2021) using the same methodology as the present study.

5.4 Conclusion

In the present work a biorefinery method, based on green compressed fluids, for integral use of *Pereskia aculeate* leaves has been proposed. The extracts recovered by different extraction methods were chemically characterized by HPLC-DAD-ESI-MS/MS. Besides, *in vitro* analysis for antioxidant, anti-inflammatory and anti-neurodegenerative activities were carried out for the samples. The sequential extractions consisted of using SFE (CO_2), GXL (EtOH:CO_2), PLE (EtOH) and SWE (H_2O). Each extraction step, in a biorefinery concept, provide samples with different bioactivities and composition, useful for raw material fractionation, in a relevant novel approach for total valorization of this underestimated and very promising ora-pro-nobis.

5.5 Aknowledgments

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CHAPTER 6: FINAL CONSIDERATIONS

This chapter's goal is to summarize the results of the previous chapters, explaining the logic of the dissertation.

The dissertation's title "Ora-Pro-Nobis Biorefinery (*Pereskia* Sp.): Use of Green Technologies for Recovery and Valorization of Vegetable Protein" proposes a study focused on a biorefinery approach using green technologies. Therefore, we are introduced in the first chapters to the ora-pro-nobis plant, and how it became an interesting matrix to be explored in a biorefinery concept, along with some important definitions regarding the green technologies and the biorefinery concept used throughout the work.

Then, in **Chapter 3** we are invited to a study of *Pereskia grandifolia* valorization using microwave assisted extraction. The leaves used in this chapter were from a collaboration project with professor Ilyas Siddique, whom produced a variety of *Pereskia grandifolia* under specific conditions. The goal was to understand the plant's profile using green solvents that were latter used in the compressed fluid extractions. Although the species are different and have been submitted to different cultivation process, it is possible to see that the main profile is similar. The results in chapter 3, using water, ethanol and the 50% mixture of these two solvents showed that the antioxidant activity, measured by DPPH, ABTS and FRAP was not directly related with phenolic compounds and were increased if using ethanol as a solvent. On the other hand, total phenolics recovering was increased when using water as solvent. The mixture of solvents only improved significantly the yield, and a better yield does not mean a better selectivity, but quite the

opposite, as proven by the phenolics profile (**Chapter 3, Manuscript 1, Section 3.3.5**). The phenolic profile was held based on optimization maximizing total phenolic recovery, therefore the water extracts were analyzed by chromatography means. The main phenolics were Caffeic acid, Ellagic acid, *p*-Anisic acid, *p*-Coumaric acid, Kaempferol and Quercetin, all of them present antioxidant and biological properties interesting in the pharmaceutical, cosmetic and food industry. That was interesting because the water solvent is the one used to recover proteins, the main product of the proposed biorefinery chart, showing that these extracts are not only nutritionally enriched, but have antioxidant potential thanks to the phenolic acids and flavonoids recovered. We can conclude from that the water and ethanol solvents, alone, were good solvents to recover bioactive compound with high added value from *Pereskia* sp. leaves.

With that in mind, the work advances to **Chapter 4**, this time using compressed fluids, the main laboratory expertise, and here the dissertation proposition starts to get in shape. A downstream processing of ora-pro-nobis leaves is proposed, but the chapter is divided in two parts, first the optimization of the separated process. The extraction methods used were supercritical CO_2 extraction and pressurized liquid extraction (PLE) using ethanol and water as solvents. As the main product was the protein recovered using water as solvent, prior to the use of (PLE) with water, to better use of the raw material it was proposed a downstream processing in crescent order of polarity, with the non-polar fraction withdraw of the plant in a degrease process to improve ethanol and water solvent interaction with the matrix. On the first part (**Chapter 4, Manuscript 2**), we see that the $scCO_2$ yield extraction was not very high, compared to Soxhlet conventional extraction, but was way more selective and presented better *in vitro* activities such as moderate acetylcholinesterase (AChE) inhibition and anti-inflammatory by means of lipoxygenase (LOX) inhibition. The gas chromatography characterization showed the presence of

terpenoids in the extracts, been a n interesting product for the first step of the process. Here we also observed that ethanol was more efficient on the total phenolic recovery than water, however the water extracts presented better antioxidant activity. The second part (**Chapter 4, Manuscript 3**) focused on the polar extracts, and confirming data found in **Chapter 3**, phenolics and flavonoids were found in the ora-pro-nobis (*Pereskia aculeata*) extracts, and direct correlation between total phenolic content and antioxidant activity. which may explain the antioxidant activity on the extracts. Here, we also observed that the use of $scCO_2$ and pressurized ethanol before PLE with water significantly influenced the yield and phenolic recovering when comparing with conventional extraction. Another *in vitro* assessment held was the total soluble protein from the water extracts. For PLE water extracts, three temperatures were tested in a dynamic extraction process. The yield was inferior to the ones found in MAE extracts, but the application of the prior steps did increased yield and also influenced on protein recovering. The lower yield on this step was attributed to the compaction of the extraction bed due to mucilage production. The extraction matrix absorbed the water, and combined with the temperature, produced a gel that hindered the interaction of the solvent with the material.

To overcome this, a second downstream process was proposed to increase protein recovering (**Chapter 5**), also recovering high added value products in the previous steps of the biorefinery process. For starters, a $scCO_2$ was held, at the optimal condition chosen in the previous chapters, but a new kinetic was held in a different equipment, to a higher scale, and the extraction was stopped when reaching about 90% of the extracts yield. A different type of compressed fluid was tested as the second step, also using ethanol, the gas-expended liquid extraction, where the $scCO_2$ is used to expend the ethanol, increasing extraction rate. Total carotenoids assessment was held for the samples, and it was observed that it is another class of compound extracted by $scCO_2$ from ora-pro-nobis

leaves. In fact, there is one study that shows carotenoid recovery from ora-pro-nobis leaves extracts. Ethanol concentrations of 45, 75 and 100% ethanol were tested. To select the best conditions, *in vitro* total phenolic, total flavonoids and total carotenoids content and antioxidant activity were held. The best results were found at PLE 100% EtOH extracts. To overcome the lower yield of pressurized water extracts, the extractions were held in a batch reactor, causing the solvent to interact with the matrix and then recovering the extracts. This, allied with the use of higher temperatures, allowed the extraction rate to increase and the yields recovered were higher than in the previous study, achieving around 45 and 65% at 150 and 185 °C, respectively. The samples were tested for antioxidant activity and phenolic recovery, and again, water extracts presented good antioxidant activity, corroborating with previous observations. The chromatography profile of the ethanol and water samples showed the same as the precious chapter, that the main phenolics of the extracts were flavonoids, mainly quercetin derivatives. The main phenolic compound present in the water samples, was Caftaric acid, showing that the phenolic acid may be responsible for the antioxidant activity.

To summarize, the biorefinery downstream process proposed by this dissertation shows an interesting use of ora-pro-nobis leaves using only green extraction methods. The sustainability approach was successfully applied with the production of very high added value products at each step of the fractionation process. The supercritical CO_2 extraction used in the first step recovered an extract rich in terpenoids and carotenoids compounds, and allowed the increase of general yield of the entire process. The pressurized liquid extracts with ethanol allowed the recovery of an extracts rich in phenolic compounds. And finally, the subcritical water extracts concentrated the proteins of the raw material and promoted an antioxidant effect due to some phenolic acids and flavonoids yet recovered in this step. The final residue, rich in fibers, could be used as

crops fertilizer, for example, allowing the entire raw material to be used, in a circular economy process.

1

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APPENDIX A – Pareto charts and Global desirability

Figure A1 – Pareto chart of yield (%) response of microwave assisted extraction from *Pereskia grandifolia* leaves.

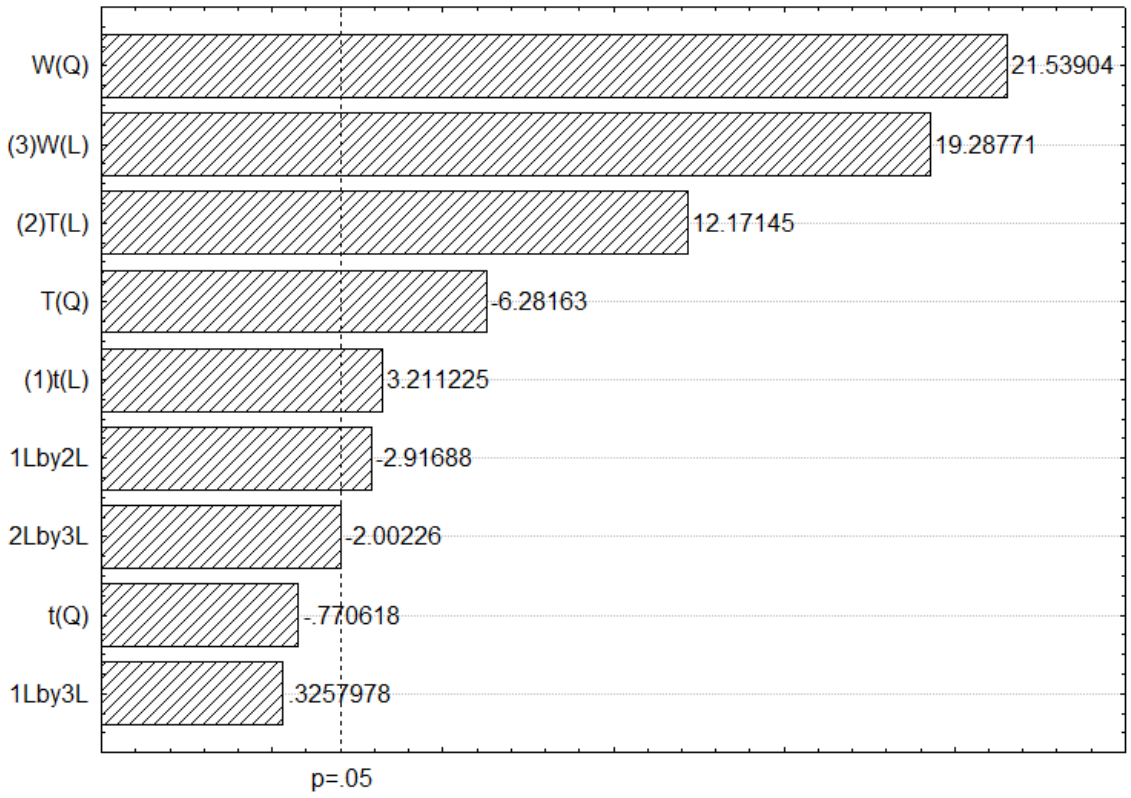


Figure A2 – Pareto chart of antioxidant activity determination by DPPH (1/IC50 - $\mu g.mL^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

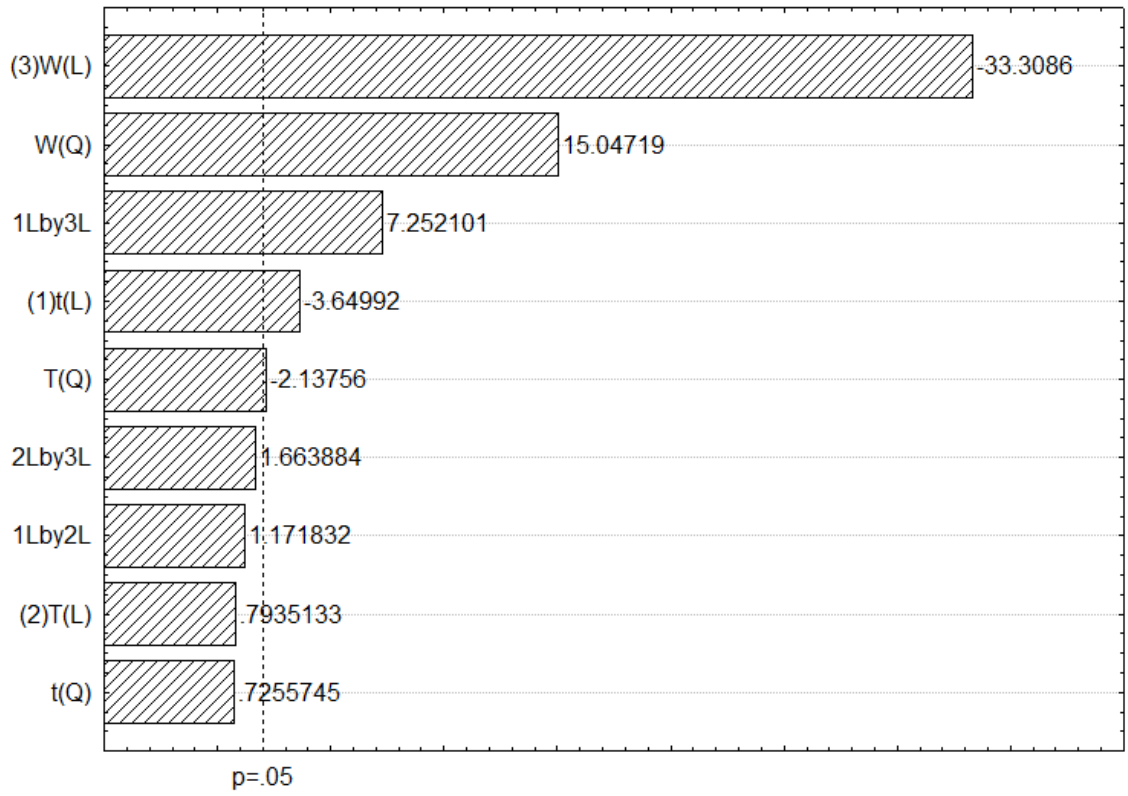


Figure A3 – Pareto chart of antioxidant activity determination by ABTS ($\mu mol_{TEAC}.g^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

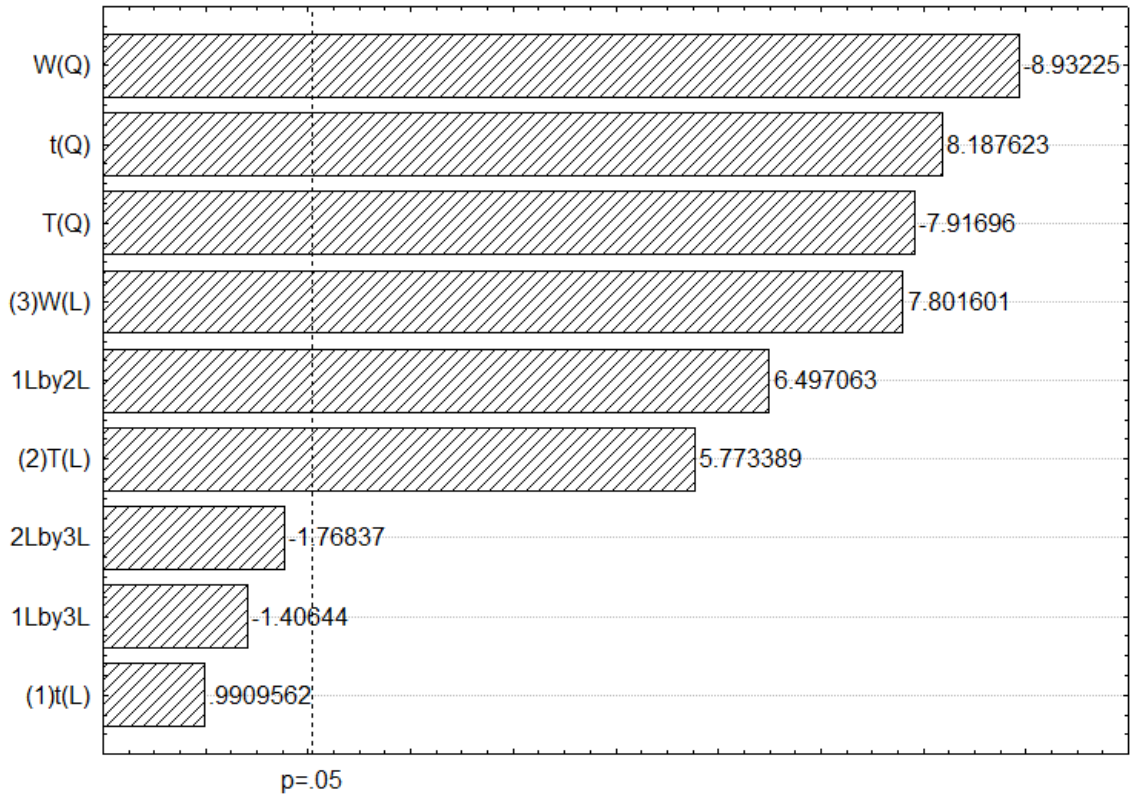


Figure A4 – Pareto chart of antioxidant activity determination by FRAP ($\mu mol_{TEAC}.g^{-1}$) of microwave assisted extraction from *Pereskia grandifolia* leaves.

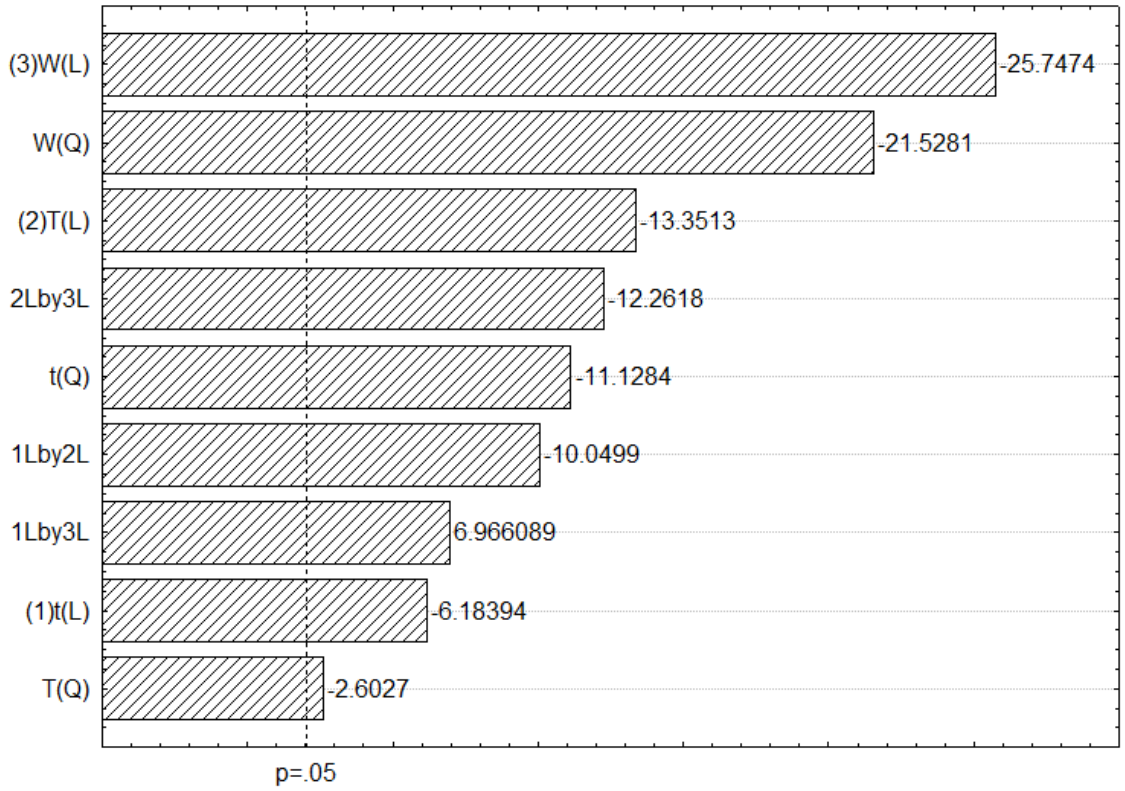


Figure A5 – Pareto chart of total phenolic content ($mg_{GAE}.g^{-1}$) response of microwave assisted extraction from *Pereskia grandifolia* leaves.

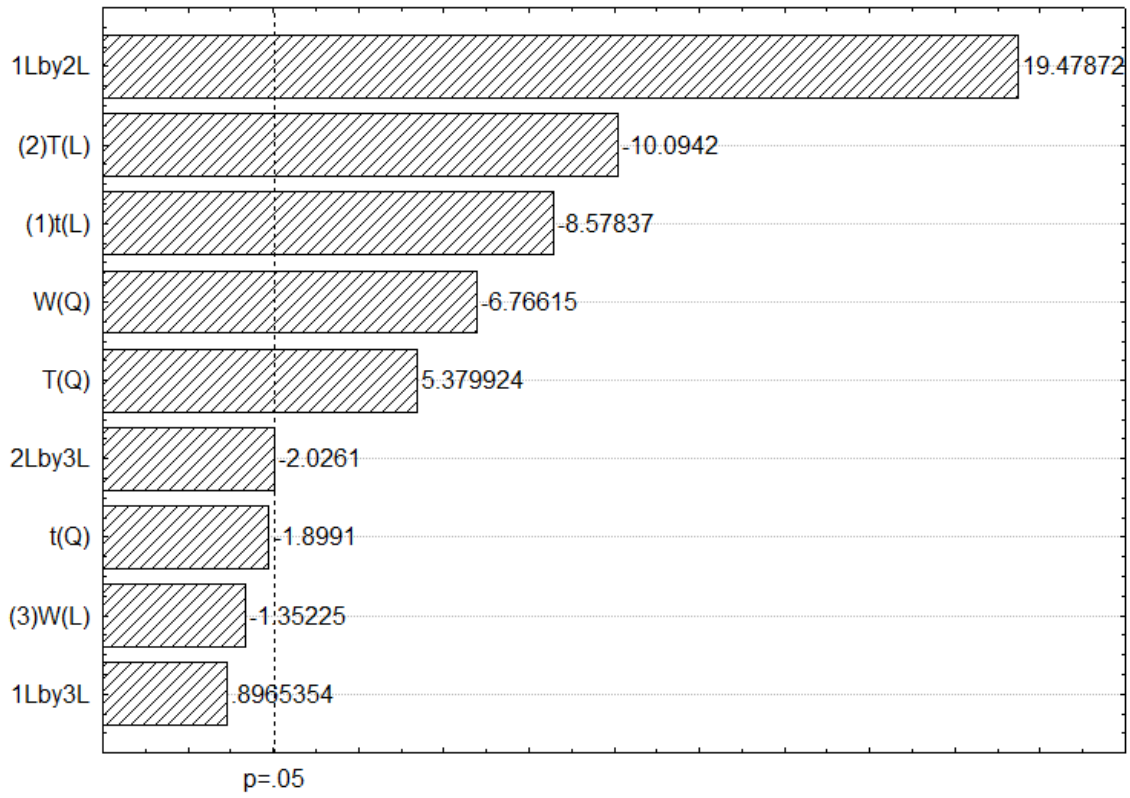
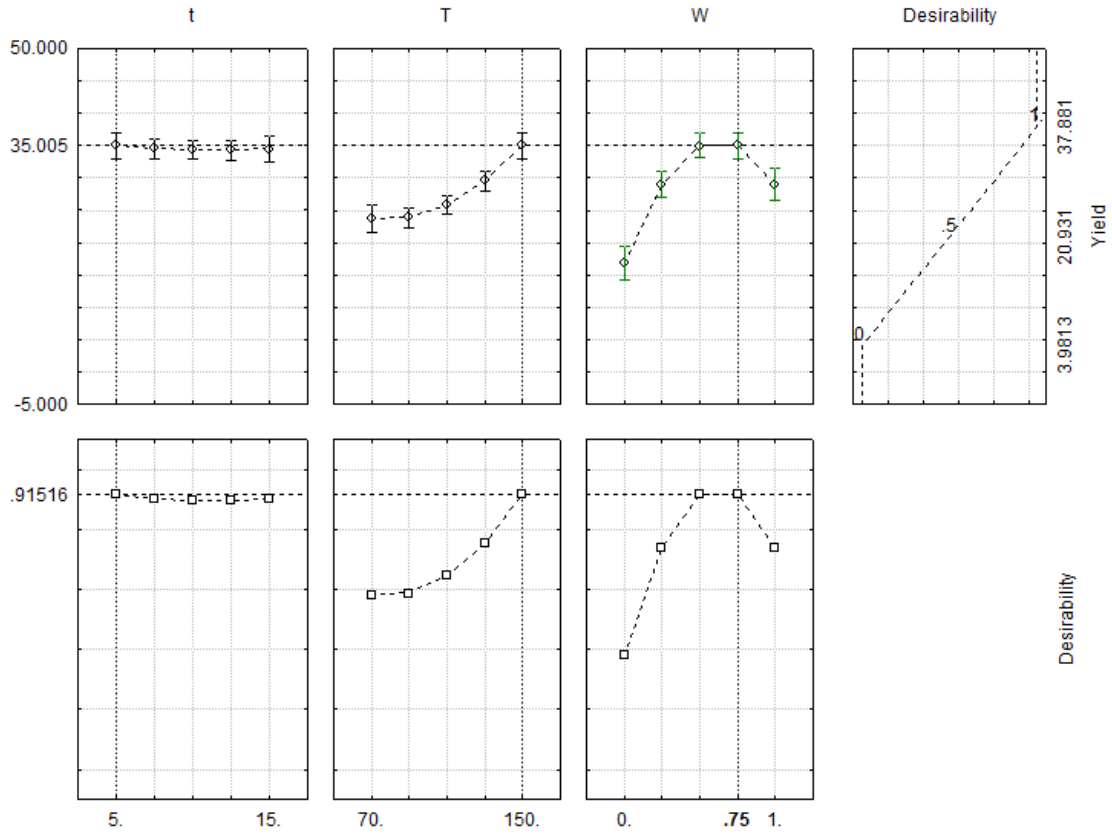


Figure A6 – Desirability function graph, profiles for predicted value and desirability of Yield (%), effect of the factors: time (min), temperature (°C) and water (%) on microwave assisted extractions from *Pereskia grandifolia* leaves.



APPENDIX B – Calibration curves

Figure B1 – Gallic acid calibration curve used in the total phenolic content determinations.

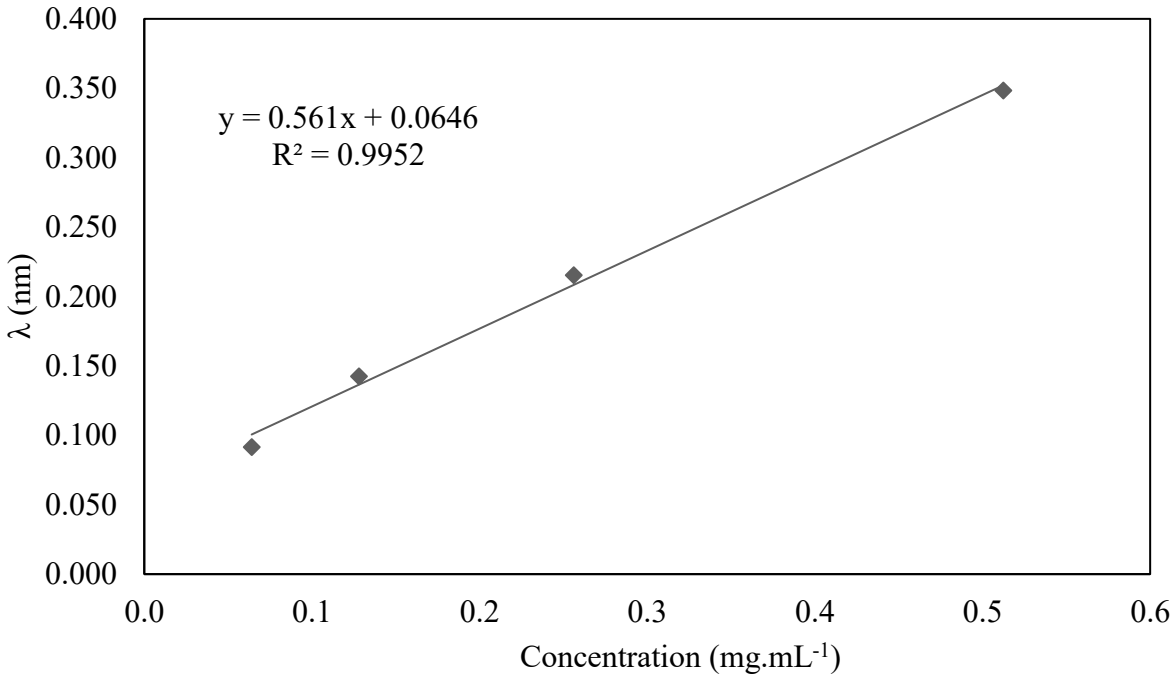


Figure B2 – Trolox calibration curve used in the antioxidant activity determinations (FRAP).

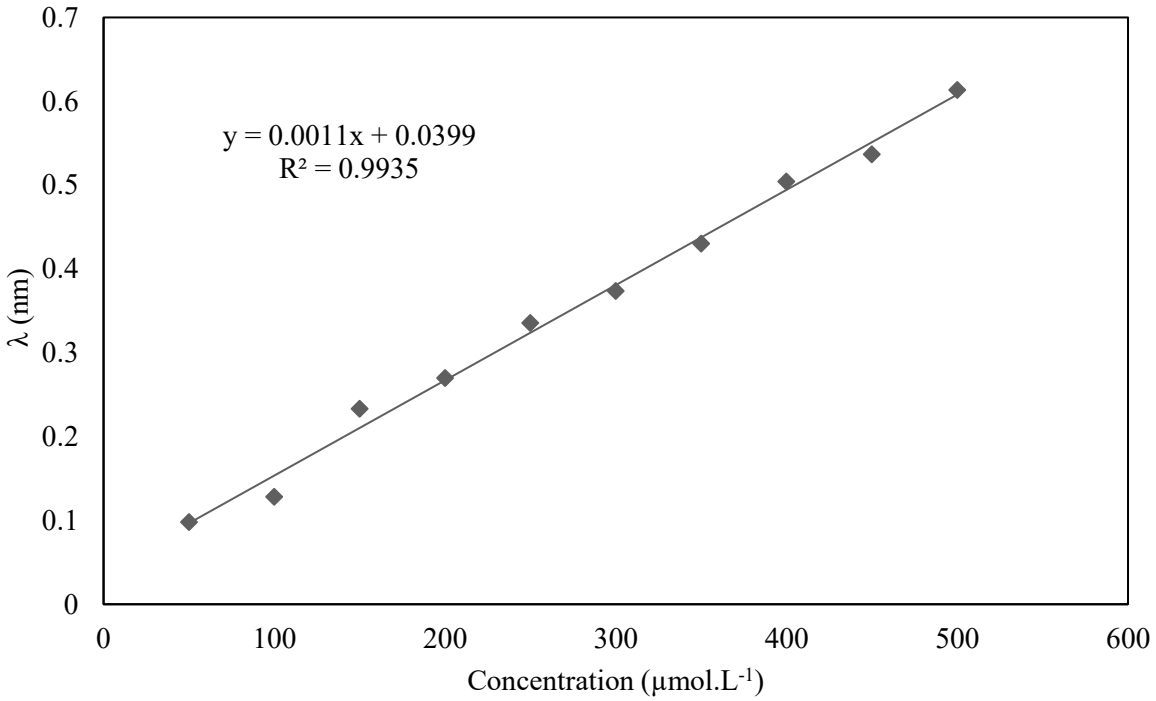


Figure B3 – Calibration curve of bovine serum albumin protein (BSA) used in Bradford's total soluble protein content determinations.