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Optimization of the extraction of bioactive compounds from *Annona cherimola* Mill. bio-residues using different techniques

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List of Abbreviations

AChE: acetylcholinesterase

AVED: Ataxia with vitamin E deficiency

BHA: Butylated hydroxyl anisole

BO: Bioactive compounds

CAGR: Compound annual growth rate

CE: Circular economy

EAE: Enzyme-assisted extraction

EU: European Union

FAO: Food and Agriculture Organization

FVW: Fruit and vegetable waste

FW: Food waste

HAE: Heat-assisted extraction

LC-DAD-ESI/MSn: Liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry

MAE: Microwave-assisted extraction

NIH: National Institutes of Health

NO: Nitric oxide

PDO: Protected designation of origin

PEFE: Pulsed electric field extraction

PLA: Phenyllactic acid

BBD: Box–Behnken design

RSM: Response surface methodology

SFE: Supercritical fluid extraction

UAE: Ultrasound-assisted extraction

TCA: Trichloroacetic acid

SRB: Sulforhodamine B

TPC: Total phenolic compounds

DPPH: 2,2-diphenyl-1-picrylhydrazyl

RSA: Radical scavenging activity

TBARS: Thiobarbituric acid reactive substances

RP: Reducing power

TNF-α: Tumor necrosis factor alpha

IL-1β: leukocytic endogenous mediator

IL-6: Interleukin 6

AGS: Gastric adenocarcinoma

CaCo-2: Colorectal adenocarcinoma

MCF -7: Adenocarcinoma of the breast

Vero: African green monkey kidney

PLP2: Porcine liver primary cell culture

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Abstract

The study of biological waste from fruits and vegetables is of great interest, not only for the food industry for many reasons, including economic interests, but also for the scientific community, which, in addition to an interesting nutritional characterization, found that this type of waste is a very interesting source of bioactive compounds.

In recent years, *Annona cherimola* Mill. has been gaining great visibility worldwide due to its sensory properties, nutritional value and, consequently, its benefits for consumers' health. However, the epicarp of this fruit is discarded as worthless waste, despite several studies reporting a compelling nutritional, chemical and bioactive composition. Considering the amount of by-products produced annually, the main objective of this work is to optimize the extraction of an extract rich in phenolic compounds from the epicarp of *Annona cherimola* Mill. For that, two techniques were employed and compared to each other, heat-assisted extraction, and ultrasound-assisted extraction, to obtain extracts with potential interest for the food industry, namely as a natural preservative. The optimal conditions that guarantee the maximization of the extraction of phenolic compounds from *Annona* peel was determined using the response surface methodology (RSM). Phenolic compounds were analysed by HPLC-DAD-(ESI-)MS/MS. After obtaining the optimal extraction points by RSM, the bioactivity of the *Annona* peel was evaluated; the antioxidant capacity was assessed by colourimetric assays; the antimicrobial activity was evaluated against several food and clinical bacterial strains and fungi; the cytotoxic activity was tested on human tumor cell lines and non-tumor cell cultures, and the anti-inflammatory activity was tested in RAW 264.7 cells. The optimal peel extracts obtained by HAE and UAE presented the highest concentration of phenolic compounds (23.23 ± 0.92 and 11.75 ± 0.67 $mg.g^{-1}$ extract dw, respectively). Hydroxytyrosol-hexoside, B-type procyanidin dimer, epicatechin were among the most abundant compounds.

The maceration's extract of *Annona* peel presented the highest antioxidant capacity as measured by TBARS and DPPH since they showed lower EC_{50} values comparing to the extract using ultrasound, while they presented equal results by the reducing power assay. Promising antibacterial activity was noticed for *Annona*'s peel phenolic compounds extracted using Maceration that was proven to have a better bacterial inhibition against Gram-positive bacterial strains especially against the foodborne bacteria. Overall, it can be concluded that *A. cherimola*

peel, is a source of polyphenolic compounds with significant antioxidant and antimicrobial activities. The choice of extraction method and solvent greatly influenced the composition and bioactivity of the extracts. These findings highlight the potential of *Annona*'s peel for the development of natural additives, while also emphasizing the importance of sustainable utilization of food waste materials. Further research is warranted to optimize extraction techniques and explore additional applications for these valuable bioactive compounds.

Keywords: *Annona*, bio-residues, heat-assisted extraction, ultrasound-assisted extraction, phenolic compounds, bioactivity, natural ingredients.

Resumo

O estudo de resíduos biológicos de frutas e hortaliças é de grande interesse, não só para a indústria alimentar por diversas razões, incluindo interesses económicos, mas também para a comunidade científica, que, para além de uma interessante caraterização nutricional, verificou que este tipo de resíduos é uma fonte muito interessante de compostos bioativos.

Nos últimos anos, a *Annona cherimola* Mill. tem vindo a ganhar grande visibilidade a nível mundial devido às suas propriedades sensoriais, valor nutricional e, consequentemente, aos seus benefícios para a saúde dos consumidores.

No entanto, o epicarpo deste fruto é descartado como um resíduo sem valor, apesar de vários estudos relatarem uma composição nutricional, química e bioativa convincente. Considerando a quantidade de subprodutos produzidos anualmente, o principal objetivo deste trabalho é otimizar a extração de um extrato rico em compostos fenólicos do epicarpo de *A. cherimola*. Para isso, foram utilizadas e comparadas duas técnicas, a extração assistida por calor e a extração assistida por ultrassons, para obter extratos com potencial interesse para a indústria alimentar, nomeadamente como conservante natural. As condições ótimas que garantem a maximização da extração de compostos fenólicos da casca de Annona foram determinadas utilizando a metodologia de superfície de resposta (RSM). Os compostos fenólicos foram analisados por HPLC-DAD-(ESI-)MS/MS.

Em seguida, e após a obtenção dos pontos ótimos de extração por RSM, foi avaliada a bioatividade da casca de *Annona*; a capacidade antioxidante foi avaliada por ensaios colorimétricos; a atividade antimicrobiana foi avaliada contra várias estirpes bacterianas e fungos alimentares e clínicos; a atividade citotóxica foi testada em linhas celulares tumorais humanas e culturas de células não tumorais, e a atividade anti-inflamatória foi testada em células RAW 264.7. Os extratos de casca ótimos obtidos por HAE e UAE apresentaram a concentração mais elevada de compostos fenólicos (23,23 ± 0,92 e 11,75 ± 0,67 mg.g-1 de extrato dw, respetivamente). O hidroxitirosol-hexosídeo, o dímero de procianidina do tipo B e a epicatequina estavam entre os compostos mais abundantes.

O extrato de maceração da casca de *Annona* apresentou a capacidade antioxidante mais elevada, medida por TBARS e DPPH, uma vez que apresentaram valores EC_{50} mais baixos em comparação com o extrato utilizando ultrassons, enquanto apresentaram resultados iguais no

ensaio de poder redutor. Foi observada uma atividade antibacteriana promissora para os compostos fenólicos da casca de *Annona* extraídos por maceração, que provou ter uma melhor inibição bacteriana contra estirpes de bactérias Gram-positivas, especialmente contra as bactérias de origem alimentar (*Bacillus cereus*, *Staphylococcus aureus* e *Listeria monocytogenes*). Em geral, pode concluir-se que a casca de *Annona cherimola* Mill. é uma fonte de compostos polifenólicos com atividades antioxidantes e antimicrobianas significativas. A escolha do método de extração e do solvente influenciou grandemente a composição e a bioatividade dos extratos. Estas descobertas destacam o potencial da casca de *Annona* para o desenvolvimento de aditivos naturais ao mesmo tempo que, enfatizam a importância da utilização sustentável de materiais de resíduos alimentares. É necessária mais investigação para otimizar as técnicas de extração e explorar outras aplicações para estes valiosos compostos bioativos.

Palavras-chave: *Annona*, resíduos, extração assistida por calor, extração por ultrassons, compostos fenólicos, bioatividade, ingredientes naturais.

I.Introduction

Food consumption is no longer considered a simple way to satisfy hunger. It is now associated with the intake of nutrients and bioactive compounds directly impacting consumers' health and well-being. In this context, consumers are increasingly aware of their food choices and are more engaged in label analysis, preferring healthier and "more natural" labelled products. Therefore, the food industry, always attentive to consumer demands, constantly seeks to launch innovative products that meet new market trends (Alaya, 2020).

Over the years, nature has been a source of food and bioactive compounds, constituting the starting point for developing bio-based ingredients for incorporation into new functional foods, nutraceuticals, and pharmaceutical and cosmetic formulations (Sharma et al., 2021). Therefore, different industries, namely the food sector, are investing in searching for and characterizing new natural bioactive compounds to be used as functional ingredients, additives, or nutraceuticals. Although highly interesting, natural resources are finite and should not be over-exploited. Consequently, bio-residues have been identified as an excellent source of interesting bioactive compounds, promoting the circular economy and sustainability of processes and products (Ueda et al., 2022). Many bio-residues are thrown away annually by the food industry, which is of great concern not only from an economic point of view but also from an environmental point of view. Although considered waste not being used by the industry, these residues are often rich in nutrients, and their chemical composition indicates the presence of bioactive compounds of great interest (Giordano, 2021). During the last few years, the efficient extraction of compounds from bio-residues has been extensively studied (Sagar et al., 2018). Determining the type of extraction and the solvent(s) to be used is very important to guarantee a rich and pure extract. In addition, ensuring that it is possible to obtain the highest yield in the shortest time, avoiding excessive consumption of time and energy, is essential to safeguard the economic interest of the process for the industry.

Annona cherimola Mill. fruits have acquired a high value worldwide due to their sensory properties and nutritional value. Several studies have shown that *Annona* contains significant amounts of polyphenolic compounds that help prevent oxidative stress-related diseases such as cancer, atherosclerosis, and neurodegenerative diseases (Perrone et al., 2022). Since this fruit is perishable, approximately half of the total production is lost, and 500 tons of waste are produced

yearly, usually disposed of or used as fertilizer (Branco et al., 2010). Recently, this problem has led to the development of studies that nutritionally and chemically characterize different species of *Annona*. Some research works have identified the phytochemicals and bioactive compounds that can be extracted from plant organs, verifying their antioxidant, anti-degenerative disease, anticancer, anti-chronic diseases, and antimicrobial properties (Perrone et al., 2022).

Bearing these concepts in mind, the present work aimed to optimize the extraction of bioactive compounds from the bio-residues of *A. cherimola*, namely the peel, using different techniques, heat (HAE) and ultrasound-assisted extractions (UAE), to develop an extract with potential application in industry. This optimization was based on establishing an experimental *design* obtained through Response Surface Methodology (RSM). Moreover, the bioactive properties of the optimal point for both methodologies were studied. The antioxidant potential of the obtained extracts was evaluated by different *in vitro* methodologies, namely the DPPH radical-scavenging assay, the Ferricyanide/Prussian blue (or reducing power) assay, and the thiobarbituric acid reactive substances (TBARS) assay. The antimicrobial activity of the extracts was assessed, testing them against a panel of different pathogenic Gram-positive and Gram-negative bacteria and two different pathogenic fungi. The cytotoxic properties were evaluated by testing the extracts against a panel of human tumour cell lines, performing the sulforhodamine B (SRB) assay. Finally, the anti-inflammatory activity was assessed using a mouse macrophage-like cell line (RAW 264.7) to evaluate nitric oxide production.

1. Bio-residues in the food industry as a global challenge

1.1. Concepts of sustainability and circular economy

Research shows that global food waste (FW) is estimated at $1 trillion and increases to $2.6 trillion when social and economic costs are considered. According to the Food and Agriculture Organization (FAO), each year, about 1/3 of the food produced is lost on its way from field to plate (FAO, 2018). This waste is not only undesirable from an ethical and social perspective but also leads to the loss of resources such as water, energy, and fertilizers that are necessary for food production and processing (Provin et al., 2021). The population will be estimated to grow to 9 billion by 2050, and our natural resources are limited. As a result of demographic and economic trends and changing consumption patterns, the use of the world's resources has increased significantly. The 21st century faces ever greater and more complex challenges such as biodiversity loss, climate change, resource depletion, water scarcity, population growth, and economic problems (Hamam et al., 2021). These circumstances have led production systems toward more sustainable approaches from linear to circular economies. The circular economy (CE) concept aims to transform traditional production and economic growth models, considered linear systems, into a circular dynamic that links resource use and waste to prevent residue generation and pollution. In the last decade, the CE has received increased attention worldwide to overcome the current production and consumption model, characterized by increasing resource use and depletion. CE is then defined as "a model of production and consumption that involves sharing, renting, reusing, repairing, renovating, and recycling existing materials and products over the longest possible period of time, minimizing waste" and is a better alternative to the current "take, make, and throw away" from the standpoint of economic, environmental, and social sustainability.

The European Union (EU) estimates that full implementation of existing waste legislation could save €72 billion per year, create over 400,000 jobs, and increase the annual turnover of the waste management and recycling sector by €42 billion. However, to achieve the medium- and long-term goals, profound changes are needed that go beyond the waste sector and involve all stakeholders in creating an effective CE model. Minimizing waste generation or reducing its harmfulness depends on actions throughout the product's life cycle, which can also help create new markets for recycled materials. Therefore, using bio-waste or bio-residues is

considered an eco-friendly practice that generates new products that can benefit human health (Alaya, 2020).

1.2. Bio-residues from the fruit and vegetable industry

Fruits and vegetables are the most widely used commodities among all horticultural crops. They are consumed raw and processed (to a lesser extent) for their nutrients and health-promoting ingredients. With the growing population and changing dietary habits, the production and processing of horticultural crops, especially fruits and vegetables, has greatly increased to meet the growing demand. Significant losses and waste in the fresh producing and processing industries are becoming a severe nutritional, economic, and environmental concern. For example, FAO estimates fruit and vegetable losses and generated waste are the highest among all food types and can reach 60%. The processing of fruits and vegetables creates significant amounts of by-products, accounting for about 25-30% of the entire product group (Deng et al., 2015). A resume of the general nature of potential fruit and vegetable losses and waste is presented in **Table 1**.

Table 1. Nature of potential fruit and vegetable losses and waste.

Commodity	Nature of waste	Typical losses and waste (%)	References
Apple	Pomace, peel, and seeds	-	Gupta and Joshi, 2000
Banana	Peel	35	Gupta and Joshi, 2000
Citrus	Rag, peel, and seeds	50	Gupta and Joshi, 2000
Dragon fruit	Rind, seeds	30 to 45	Cheok et al., 2018
Durian	Skin, seeds	60 to 70	Siriphanich and Yahia, 2011
Grapes	Skin, stem, and seeds	20	Gupta and Joshi, 2000
Guava	Peel, core, and seeds	10	Gupta and Joshi, 2000
Jackfruit	Rind, seeds	50 to 70	Saxena et al., 2011
Mango	Peel, stone	45	Gupta and Joshi, 2000; Mitra et al., 2013
Mangosteen	Skin, seeds	60 to 75	Chen et al., 2011; Ketsa et al., 2011
Papaya	Rind, seeds	10 to 20	Parni and Verma, 2014
Passion fruit	Skin, seeds	45 to 50	Esquivel et al., 2014; Almeida et al., 2015
Pineapple	Core, skin	33	Choonut et al., 2014

Rambutan	Skin, seeds	50 to 65	Issara et al., 2014; Sirisompong et al., 2011
Tomato	Core, skin and seeds	20	Gupta and Joshi, 2000
Onion	Outer leaves	-	Gupta and Joshi, 2000
Peas	Shell	40	Gupta and Joshi, 2000
Annona muricata	Peel	7 to 20	Santos et al., 2023
Potato	Peel	15	Gupta and Joshi, 2000

(-) Data not available.

The fruit and vegetable residues and waste are mainly composed of seeds, peels, rinds, and pomace, where remaining compounds with nutritional and bioactive interest and, therefore, this discarded material could be an excellent source of added-value compounds, such as carotenoids, polyphenols, dietary fiber, vitamins, enzymes, oils, among others. These phytochemicals can be used in various industries, including the food industry, for developing functional or fortified foods, the pharmaceutical industry for drug and medicines manufacture, or even the textile industry. Using waste to produce various essential bioactive components is an important step towards sustainable development (Deng et al., 2015; Sagar et al., 2018).

1.3. Bioactive compounds from bio-residues

For a better understanding of the origin of bioactive compounds (BC), it should be kept in mind that the compounds of the biological plant system are divided into two classes. The first class includes primary metabolites, i.e., chemical substances that serve development and growth, such as carbohydrates, amino acids, proteins, and lipids. The second class comprises secondary metabolites that help the plant improve its overall survivability, thrive, and overcome local obstacles in its growing environment. BC are secondary molecules, metabolites, or chemical substances found in small amounts in plants and foods such as vegetables, fruits, oils, grains, and nuts. These molecules are usually known for their biological effects on human health, including antioxidant, anticarcinogenic, antimicrobial, and anti-inflammatory properties and can modulate metabolic processes. BC have then a positive impact on human health, as they can reduce the risk of many diseases, such as cancer, heart disease, stroke, and diabetes.

There are two main categories of BC: essential and non-essential. The first group includes vitamins and minerals that maintain the specific biochemical processes in the body. In

contrast, the non-essential group comprises phenols, flavonoids, carotenoids, phytosterols, glucosinolates, saponins, alkaloids, and essential oils, responsible for maintaining optimal cell conditions and enhancing the effects of other essential nutrients(Rodríguez-Núñez et al., 2021).

Of the most studied bioactive compounds present in the plant kingdom, the polyphenols (found in about 8000 species), alkaloids (found in approximately 12,000 species), terpenoids (found in approximately 25,000 species), and glucosinolates (found in nearly 200 species) could be highlighted (Rodríguez-Núñez et al., 2021).

Polyphenols are among the most studied compounds, given their bioactive potential and proven safe for consumption. Other organic acids, including vitamins such as C or E, are also sought compounds in various plant sources. These compounds will be described below, including their definition and importance, which make them some of the most sought-after to include in various functional products, thus reducing the use of artificial ingredients.

1.3.1. Phenolic compounds

Phenolic compounds are secondary metabolites of plants, usually involved in the defence system against ultraviolet radiation or pathogen attack. The main dietary sources of these compounds are fruits and plant beverages such as fruit juices, teas, coffee, and red wine; vegetables, cereals, chocolate, and legumes also contribute to the total intake of phenolic compounds (Alaya, 2020).

Although their structural diversity results in a wide range of phenolic compounds in nature, they can be divided into several classes. They range from simple molecules (such as phenolic acids with a single aromatic structure) to biphenols (such as ellagic acid) and flavonoids containing 2 to 3 aromatic rings to polyphenols with 12 to 16 rings. The main groups of polyphenols are flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes, and lignans (Oliveira et al., 2014). The presence of these compounds in various foods has been associated with sensory and health-promoting properties (Oliveira et al., 2014).

Since these molecules of plant secondary metabolism act as defense mechanisms against environmental stress and attacks by other organisms, several authors sought other bioactivities, describing their antioxidant, antimicrobial, anti-inflammatory, antitumour, and hepatoprotective effects, among others. The literature defines phenolic compounds as neutralising reactive

species and preventing oxidative stress, thus acting in various diseases. Some of these compounds exhibit high antioxidant activity as single compounds, while others rely on synergistic effects to exert bioactive effects (Carocho and Ferreira, 2013).

2. Bioactive compounds as natural ingredients

2.1 Consumer demand for healthy products

The use of artificial additives and some associated health concerns lead to a negative view of the consumer, who considers these compounds as having hazardous consequences, including potentially carcinogenic effects. For this reason, consumers are increasingly paying attention to the composition of food products, and therefore, they prefer healthier and more natural products containing little or no artificial additives. Natural products represent a substantial proportion of the composition of new drugs developed by the pharmaceutical industry. Some studies have shown that the anticancer effects of bioactive plant constituents on cell lines are even more efficient than those of some synthetic compounds (Alaya, 2020). This has led to a growing interest in the food industry, specifically the functional food sector, which has grown exponentially (Alaya, 2020). According to the literature, food can be defined as "functional" when, besides having adequate nutritional effects, it demonstrates beneficial effects on consumer health, namely in reducing the risk of disease. However, these beneficial effects should be demonstrated when ingested in amounts typically consumed in the usual diet (Diplock et al., 1999). In fact, the incorporation of several natural ingredients has been tested into foods to make available to the market new food products designed to improve consumer health. Some examples are presented in **Table 2**.

Table 2: Food products incorporated with natural ingredients (Nascimento et al., 2018).

Product	Study/Assay	Conclusion
Bakery products, flours or cereals	The fungicidal activity of phenyllactic acid (PLA) and growth inhibition by PLA were evaluated by using the microdilution test and 23 fungal strains belonging to 14 species of *Aspergillus*, *Penicillium*, and *Fusarium* that were isolated from bakery products, flours or cereals.	PLA delayed the growth of various fungal contaminants, providing new perspectives for possibly using this natural antimicrobial compound to control fungal contaminants and extend the shelf lives of foods.

Biscuits	The study focused on using fennel and chamomile extracts, rich in phenolic compounds, as natural antioxidants in biscuits. It compared their performance with a synthetic antioxidant widely used, the butylated hydroxyl anisole (BHA).	Both natural and synthetic additives conferred similar antioxidant activity to the biscuits. Therefore, natural additives are a more convenient solution for consumers who prefer foods "free" of artificial additives. Additionally, natural additives were obtained by aqueous extraction, an environmentally friendly and safe process.

It should be considered that these new foods must be strictly controlled, especially in terms of label claims. Different natural sources of plant, animal or mycological origin can be used for this type of fortification (Alaya, 2020).

As shown in **Figure 1**, food-derived bioactive compounds are a diverse group of molecules, including fat-soluble and water-soluble molecules, as well as macromolecules and micro molecules, and their health benefits depends on the body absorption (Luo et al., 2020).

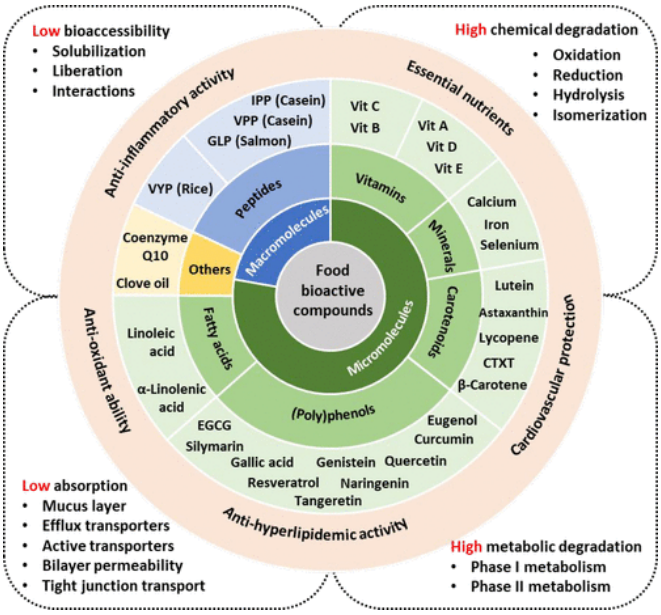

Figure 1. Health benefits of various bioactive compounds from food and the challenges in achieving their biological activities in the body (Luo et al., 2020).

A global overview of the types and applications of bioactive ingredients between 2014 and 2024 was conducted. In terms of applications, the focus is on functional foods and beverages, dietary supplements, clinical nutrition, personal care, and other products. According to the report from Markets and markets (2017), a global market research and advisory firm, the global bioactive ingredients market trends and forecasts are projected to grow from $23.8 billion in 2013 to $33.6 billion in 2018 at a CAGR of 7.2% (Fernandes et al., 2019). Global sales of naturally healthy foods totalled $253 billion in 2017, while functional/fortified foods totalled $247 billion. Organic foods and beverages led global retail sales with a compound annual growth rate of 7% from 2012 to 2017, followed by free-form products at just over 6%, genuinely healthy foods at 3%, and fortified/functional foods at 2%. Natural foods and beverages sales grew 9% in developing countries, and free-form product sales rose 5%. Consequently, searching for natural compounds that can exhibit biological activity remains a hot topic. To this end, the nutritional and bioactive characterization of products and their by-products or bio-residues as a source of natural additives and their use in the formulation of new foods are recommended, helping to reduce global concerns about food waste and safety while meeting consumer expectations concerning improving their well-being. Therefore, the European Commission has supported several measures against food waste, being one of the proposed solutions the use of by-products/bio-residues to develop new formulations for different industries (Silva et al., 2021).

2.2 New methodologies for extracting bioactive compounds

A wide variety of BC are present in foods and their bio-residues. It is essential to know the matrix and adapt the methodologies to the desired compounds for the separation, identification, characterization, and appropriate extraction process of these compounds.

Many factors can generally affect the extraction, such as pressure, temperature, matrix, and solvent used. BC can be extracted from fruit and vegetable waste (VFW) using either conventional (maceration, soxhlet extraction, and hydro-distillation) or non-conventional techniques (microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and pulsed electric field extraction (PEFE). Conventional extraction methods are considered classical methods. The

basic principle of these techniques is solvent extraction and heat application. The choice of solvent for the extraction is crucial. In conventional methods, alcohols (methanol and ethanol) are the most used solvents for BC extraction. Nevertheless, several other solvents may be employed, *e.g.*, chlorinated solvents, such as chloroform, carbon tetrachloride, and chlorobenzene, and non-chlorinated solvents, such as acetone and acetonitrile. The particle size also affects the efficiency of extraction. The smaller the particles, the better the solvents can penetrate them; however, if the particle size is too small, the subsequent filtration process becomes complicated.

Temperature also plays an important role: high temperatures improve solubility and diffusion but can also lead to solvent loss, resulting in the formation of impurities and possibly the decomposition of heat-sensitive compounds. Conventional methods have long processing times for extraction. However, extending the time does not affect extraction until solvent equilibrium is reached inside and outside the solid matrix (Rodríguez García and Raghavan, 2022). **Table 3** shows a brief description of the conventional extraction methods.

Table 3. Description of the conventional extraction methods.

Technique	Advantages	Disadvantages	BC extracted	References
Soxhlet	• Basic model technique for comparison and commonly used. • Best suited for small-scale industries.	• Large quantities of solvents. • Time consuming. • Low efficiency. • Not suitable for heat sensitive ingredients.	Essential oils, lipids, fats, phenolics.	Azmir et al., 2013; Garavand et al., 2019; Sagar et al., 2018
Hydro-distillation	• Simplest and oldest technique. • Different classifications: steam, water, or hydro-diffusion distillation. • Used in small-scale industries. • Uses water to facilitate the extraction.	• Not suitable for heat sensitive ingredients. • Time consuming • Consume high energy levels.	Essential oils and phenolics.	Azmir et al., 2013; Soquetta et al., 2018

Heat-assisted extraction	• Used in small-scale extractions. • Inexpensive method.	• Time consuming. • Requires large quantities of solvents.	Essential oils and phenolics.	Azmir et al. 2013; Garavand et al. 2019

To overcome the disadvantages of the conventional extraction methods, there are other extraction techniques that have shorter extraction times, high efficiency, and selectivity, and reduce the use of solvents. These techniques are referred to as non-conventional or green extraction techniques. The term "green extraction" is due to lower energy consumption, renewable natural products, reduction of hazardous substances, and less time required for the extraction process.

These extraction methods are the new trend for extracting BC from many plant sources, including fruits and vegetable waste, as they are a sustainable option compared to conventional extraction methods. The non-conventional extraction techniques follow several principles, such as innovative selection of renewable plant resources like VFW, use of alternative solvents, reduction of energy consumption, production of by-products, obtaining a biodegradable and pure extract, and reduction of the number of steps. Most organic solvents are volatile, flammable, and even toxic, and alternative solvents are an excellent way to replace petrochemical solvents. (Rodríguez García and Raghavan, 2022). A comparison of some extraction methods is shown in **Table 4.**

Table 4. Comparison of different extraction techniques for obtaining bioactive compounds (Rodríguez García and Raghavan, 2022).

Technique	Concept	Advantages	Disadvantages	BC extracted
Microwave assisted extraction (MAE)	Electromagnetic fields between 300 MHz to 300 GHz. The solvent penetrates the solid matrix by diffusion and the solute is dissolved.	• Better extract quality. • High selectivity. • High extraction yield and less extraction time. • Cost-effective in comparison with Soxhlet. • Simply operable in comparison with SFE. • Short extraction time in comparison with UAE.	• Expensive equipment. • Operation is difficult compared to UAE. • Use organic solvents. • Unsuitable for non-polar compounds. • Unsuitable for heat sensitive ingredients.	Polyphenol, flavonoids, phenolics, and carotenoids.

Ultrasound assisted extraction (UAE)	Sound wave between 20 kHz to 100 MHz. Cavitation to form pores that facilitate the leaching of organic compounds and inorganic plant matrix.	• Less energy and power usage. • Higher product yield. • Short processing time and less chemical usage. • Appropriated for thermos-sensitive ingredients.	• Proper optimization in ultrasound frequency. • Generation of free radicals at high sonication powers. • Solvent needed procedure. • Difficult to scale up for industrial uses.	Phenolics, lipids, and carotenoids.
Enzyme assisted extraction (EAE)	Use of various enzymes such as cellulases and pectinases that hydrolyze the cell wall components, increasing cell wall permeability, resulting in the extraction.	• Uses water as solvent. • Suitable to separate bound compounds. • High extraction rate.	• Enzyme sensitivity. • Difficult to scale up to industrial uses. • Expensive price of enzyme for large volume of samples. • High maintenance.	Essential oils and phytochemicals.
Pulsed electric field extraction (PEFE)	Use of two electrodes. The pulse varies between 100300 V/cm to 20-80 kV/cm. At room temperature.	• Short extraction time • High efficiency. • Low energy consumption. • High final yield. • Reduced environmental impact in comparison with Soxhlet.	• High maintenance. • Accurate control parameters.	Phytosterols and polyphenols.
High hydrostatic pressure extraction	Uses low or room temperatures, at pressure that ranges from 100 to 600 MPa.	• Accelerated mass transfer. • Can be use with polar and nonpolar compounds. • Do not utilize a heating process. • Low energy consumption. • High yield of extraction.	• Expensive equipment. • Difficulty of maintenance. • High pressure needed.	Polyphenols

3. *Annona cherimola* Mill.

3.1 Origin and distribution

The *Annonaceae* family includes over 100 genera and 2400 species (**Figure 2**). The cherimoya or *A. cherimola* is the most cultivated species of this family.

Figure 2. A characteristic plant of *Annona cherimola* Mill. (a) tree, (b) fruit, (c) leave, and (d) lower (Perrone et al., 2022).

A. cherimola is a fruit (**Figure 3**) with a thick green rind, whose pulp is creamy and sweet. It is known for its exceptional taste and is becoming increasingly important in tropical and subtropical regions due to its involvement in commercial and traditional medicine, especially for treating skin diseases. *A. cherimola* is widely distributed in America, Africa, Asia, and even southern Europe, where it is cultivated for its edible fruit.

Figure 3. Typical fruits of *Annona cherimola* Mill. (a) smooth skin with fingerprint-like markings (b) and (c) skin covered with conical or rounded protuberances (Perrone et al., 2022).

Annona is widely cultivated in Madeira Islands, and about 1000 tons per year are exported to the mainland, France, Spain and England. It is the most exported fruit after the banana on this Portuguese island, with excellent climatic conditions for producing some exotic and tropical fruits, such as avocado, cherimoya, banana and passion fruit. The production of this fruit has increased in recent years because of its attractive sensory properties and because it is

known to be a good source of vitamins and other bioactive compounds, such as polyphenols or carotenoids.

Since 2000, the European Union has granted Madeira cherimoya the Protected Designation of Origin (PDO), becoming the first regional fruit to receive this level of international protection (Albuquerque et al., 2016). This fruit is also cultivated in the United States, Chile, Peru, Bolivia, Ecuador, Mexico and Spain (Rodríguez-Núñez et al., 2021).

3.2 Phytochemical composition and bioactive properties of *Annona*

Annona cherimola Mill, commonly known as custard apple, has recently attracted the attention of researchers due to its delicious taste and sweet aroma, along with its richness in nutrients, including vitamins, carbohydrates, essential minerals, and fiber, and the presence of bioactive compounds.

Generally, the pulp of cherimoya is rich in water and has lower contents of proteins and total fat than other fruits. Therefore, it has a low energy content but is considered a good source of bioactive compounds such as phenols and carotenoids (Albuquerque et al., 2016). In addition, *Annona* fruits contain a significant amount of polyphenolic compounds in the pulp, rind and seeds that help prevent oxidative stress-related diseases such as cancer, atherosclerosis and neurodegenerative diseases.

Several chemical constituents have been isolated from the stem and seeds of *Annona*, including annocherin A, cherioin, annocherin B, aromin A, N-cis-caffeoyltyramine, dihydro-feruloyltyramine, and cherimoline. The seeds also contain cyclooctapeptides, cherimolacyclopeptide A, and cherimolacyclopeptide B. The bark contains the alkaloid annonain, to which numerous properties are attributed. Major volatile compounds detected in the fruit include methyl butanoate, butyl butanoate, 3-methyl butanoate, 3-methyl butanoate, and 5-hydroxymethyl-2-furfural. The leaves contain a large amount of rutin (Jamkhande et al., 2017). As shown in **Table 5,** this fruit is a good source of antioxidant molecules and contains reasonable amounts of vitamins C, E, and polyphenols.

Table 5. Categories and singular phytochemicals identified in different plant tissues of *Annona Cherimola* Mill. (Perrone et al., 2022).

Pulp	Seed	Leaf	Peel	Bark
• **Vitamin C** • **Vitamin E** • **Acetogenins** • **Chitinases** • **Polyphenolics compounds** (Rutin, Flavan-3-ols, Catechin, (epi)catechin, (epi)gallocatechin, (epi)afzelechin-(epi) catechin procyanidin, locatechin, procyanidins, proanthocyanidins,ent-kaurane diterpenoids, diterpenes, kauran 15-ol, kauran-16-ol gummiferolic acid) Volatile compounds (methyl butanoate butyf butanoate,3-methylbutyl butanoate, 3-methyl butyl 3 methyl butanoate. 5-hydroxymethyl-2-furfural) • **Fatty acids** • **Sterols**	• **Alkaloids** • **Acetogeins** • **Terpenes** • **Polyphenolic compounds** (Flavonoids, flavonoid glycosides, proantocvanidins, kaempferol, quercetin, nicotifionn, rutin, narcissin, chlorogenic acids)	• **Alkaloids** • **Acetogenins** • **Terpenes** • **Polyphenolic compounds** (Flavonoids, flavonoid glycosides, proanthocvanidins kaempferol, quercetin nicotifionn, rutin, narcissin, chlorogenic acids)	• **Alkaloids** • **Carotenoid** (Lutein) • **Vitamin C** (L-ascorbic acid) • **Polyphenolic compounds** • **Flavonoids**	• **Alkaloids** ((-)-Xylopin37) • **Acetogenins** (polykrtide)

Given the rich composition of the cherimoya, the use of its by-products could be a good option for the development of food or pharmaceutical products, while reducing the large amounts of waste generated by their industrial processing, thus reducing the negative impact on the environment (García-Villegas et al., 2022).

3.3 Exploitation of *Annona* peel as natural ingredients

Annona peel has been exploited as a source of interesting compounds. This seems to be rich in carotenoids (lutein) and vitamins (*L*-ascorbic acid). Some authors studied the peel of the Perry Vidal variety of *A. cherimola*, recording contents of *L*-ascorbic acid of 4.41 mg/100 g.

Also, *Annona* peel revealed higher quantities of polyphenolic compounds, compared with the other fruit parts, followed by seeds and pulp (Perrone et al., 2022).

Other authors studied and compared the pulp, peel and seeds of four cultivars of *A. cherimola* in terms of bioactive compounds and properties (Albuquerque et al., 2016 ; Perrone et al., 2022). The authors concluded that the peel of the Madeira cultivar has a higher antioxidant capacity and flavonoid than Funchal, Mateus II and Perry Vidal cultivars. Among carotenoids, lutein was the most abundant, with concentrations ranging from 129 to 232 μg/100 g. The peel of cherimoya fruit from Almunécar (Granada, southern Spain) showed reasonable quantities of K, P, C, and N, with considerable Ca and Mg contents. Moreover, cherimoya peel has been shown to strongly inhibit neurological disorders, probably due to the high content of alkaloids with acetylcholinesterase (AChE) inhibitory properties, this being a promising result for the development of new therapies for Alzheimer's disease and other neurological disorders. **Figure 4** illustrates the main components present in *Annona* peel.

Figure 4. Phytochemical composition of *Annona* peel (Perrone et al., 2022).

Overall, it is possible to find in the literature some studies that indicate the bioactive potential of *Annona* bio-residues, especially their antioxidant properties. In this way, the present work intends to go beyond the state-of-the-art insofar as it aims to optimize the extraction of compounds of interest from these wastes to obtain the purest extracts with the highest possible yields. It is intended as a last resort to offer new alternatives for the food, pharmaceutical or cosmetics industries.

II.Objetives

This work's objective focuses on optimizing the extraction of phenolic compounds from *Annona cherimola* Mill. residues using heat (HAE) and ultrasound-assisted extraction (UAE) to develop an extract with potential industrial application.

The specific objectives of this work are:

i) Optimization of the extraction conditions using HAE and UAE that lead to a higher yield and purity of phenolic compounds from *Anonna* residues;

ii) Chemical characterization of the optimized extract;

iii) Evaluation of the bioactive properties of the optimized extract.

III.Material and methods

3.1 Sampling of *Annona cherimola* Mill. peel

The fruits of *Annona cherimola* Mill. (about 1.5 kg) were purchased in a local supermarket in the Northeastern region of Portugal (**Figure 5**). Then, the fruits were peeled to perform a more detailed study, separating the seeds into bark, skin, and pulp with a knife (**Table 6**). Finally, the samples were frozen, lyophilized, crushed into a fine dried powder (~20 mesh) and stored in a place protected from light and temperature for subsequent analyses.

Figure 5. *Annona cherimola* Mill. Fruit.

Table 6. Information regarding the *Annona cherimola* Mill. parts.

Fruit part	Fresh parts (g)	Lyophilized parts (g)
Peel	262	89.7
Seed	121.2	71.3
Pulp	601	169.7

3.2 Experimental design for extraction optimisation

To optimize the extraction of phenolic compounds, two different techniques were performed and compared, namely heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). An independent quadratic Box–Behnken design (BBD) model was used for both methods. The BBD is a three-level-three-factor system through which it was possible to determine the best combination of extraction factors to lead to desired responses. The used function was

"maximize" to understand the conditions of each parameter that return the highest yield in each specific phenolic compound. The response surface methodology was carried out using Design-Expert 11 (Stat-Ease, Minneapolis, MN, USA).

3.3 Heat-assisted extraction

A maceration using temperature (**Figure 6**) was performed according to Lopes et al. (2018), using an ethanol/water solution as solvent (80:20 v/v), which was added to the sample at a ratio of 600 mg/20 mL and submitted to constant stirring according to **Table 7**.

Figure 6. Heat equipment used for HAE extraction.

Various parameters were considered in the preparation of the experimental design: the percentage of solvent (ethanol), the temperature and the duration of the extraction. After extraction, the resulting suspension was filtered through filter paper (Whatman No. 4), and the organic solvent was evaporated in a rotary evaporator (R-210 rotary evaporator, Buechi, Flawil, Switzerland; **Figure 7**) under reduced pressure. All aqueous extracts were frozen and lyophilized for further analysis.

Table 7. Experimental design for HAE.

Run	Time (min)	Temperature (◦ C)	Solvent (Ethanol, %)
1	120	20	50
2	62.5	50	50
3	120	50	0
4	5	20	50
5	62.5	80	100
6	5	50	0
7	5	80	50
8	62.5	50	50
9	62.5	20	100
10	120	80	50
11	62.5	20	0
12	5	50	100
13	62.5	50	50
14	62.5	50	50
15	62.5	80	0
16	62.5	50	50
17	120	50	100

Figure 7. Rotary evaporator equipment.

3.4 Ultrassound-assisted extraction

The ultrasound-assisted extraction (UAE) of the powdered samples was performed according to **Table 8** using an ultrasound machine (QSonica Sonicators, model CL -334,

Newtown, CT, USA), which has an ultrasound power ranging from 50 to 500 W at a frequency of 20 kHz and is equipped with a digital timer (**Figure 8**).

Figure 8. Ultrasound equipment used for UAE extraction.

Several parameters were considered in the development of the experimental design: solvent content (ethanol), ultrasonic power and extraction time. Following the literature, a solvent/solid ratio of 75% was used and maintained for all extracts. According to table 8, the solvent was added to the sample at a ratio of 1.5 g/50 mL. After extraction, the resulting suspension was filtered through filter paper (Whatman No. 4), and the organic solvents were evaporated in a rotary evaporator previously mentioned. All aqueous extracts were frozen and lyophilized for further analysis.

Table 8. Experimental design for UAE.

Run	Power (W)	Time (min)	Solvent (Ethanol, %)
1	250	11	50
2	250	20	0
3	250	11	50
4	100	11	0
5	250	11	50
6	100	20	50
7	250	11	50
8	100	2	50
9	400	20	50
10	400	11	0
11	250	11	50
12	400	11	100
13	250	2	100
14	250	2	0
15	250	20	100
16	400	2	50
17	100	11	100

Afterwards, the dried extracts obtained were dissolved in water:ethanol (20mg/mL; 20:80, v/v). Then, they were filtered through a 0.2 μm disposable nylon disk filter LC, and transferred to an injection vial for HPLC-DAD analysis.

3.5 Chromatographic analysis of phenolic compounds

Extracts were analyzed in a Dionex Ultimate 3,000 series high-performance liquid chromatograph (HPLC, Thermo Fisher Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series with a linear ion trap mass spectrometer LTQ XL (MS, Thermo Fisher Scientific - San Jose, CA, USA). Compounds were separated in a Spherisorb S3 ODS -2 C18 column (3 μm, 4.6 x 150 mm, Waters - Milford, USA) at 35 ºC under a gradient of 0.1% (v/v) formic acid in ultrapure water (A) and acetonitrile (B). The elution gradient was set as follows: 15% B (5 min), 15% B to 20% B (5 min), 20–25% B (10 min), 25–35% B (10 min), 35–50% B (10 min), followed by re-equilibration of the column at a flow rate of 0.5 ml/min. UV-Vis spectra were recorded between 180 and 700 nm, and chromatograms were processed at 280,

330, and 370 nm for the different classes of phenolic compounds. The compounds were ionised using an electrospray ion source (ESI) operating in negative mode.

The full MS /MS and MS scans covered the mass range of *m/z* 100–1800, and the collision energy was set to 35 (arbitrary units). Data acquisition was performed using the Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). Compounds were identified based on the interpretation of the elution order on the reversed phase column and the characteristics of the UV-Vis and mass spectra compared to standards (when available) and literature data. Quantification of phenolic compounds was performed using external calibration curves of commercial standards, and results were expressed in mg per g of freeze-dried extract ($mg.g^{-1}$, dry weight, dw).

3.6 Bioactivity evaluation

Since the phenolic compounds, the studied target in this work, are recognized for their bioactive potential, the extracts' antioxidant, antimicrobial, cytotoxic and anti-inflammatory properties corresponding to both techniques (HAE and UAE) optimal points were evaluated.

The bioactivity of the extracts was then evaluated through different *in vitro* assays.

3.6.1. Antioxidant capacity

The antioxidant capacity of both extracts of *Annona* peel was evaluated using three different methods, namely the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, the Ferricyanide/Prussian blue assay, and the thiobarbituric acid reactive substances (TBARS) assay.

DPPH radical scavenging activity. The DPPH radical is an organic nitrogen radical with a deep purple colour. In the performed method, the purple chromogen radical is reduced by the antioxidants or reducing compounds to the corresponding pale-yellow hydrazine. This methodology was performed according to the method of Karra et al. (2020).

Basically, the reaction mixture on the 96-well plate consisted of a solution by well of the extract solutions with different concentrations (30 µL) and the methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 1 hour (room temperature, light protected), and the absorbance was measured in a SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenberg, Germany) at 515 nm (**Figure 9**). The radical

scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation (1):

$$\%\ \text{RSA} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100$$

Where A_{DPPH} is the absorbance of the DPPH solution, and A_S is the absorbance of the solution containing the sample.

Trolox was used as standard.

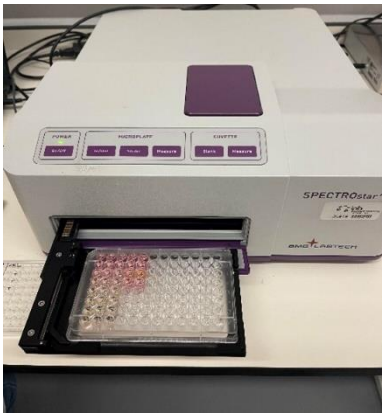

Figure 9. SPECTROstar equipment used for absorbance measurements.

Ferricyanide/Prussian blue assay. This assay is based on the ability of phenolics to reduce yellow ferric form to blue ferrous form by the action of electron-donating antioxidants. In this assay, the extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min to promote the reaction. Then, trichloroacetic acid (10% w/v, 0.5 mL) was added to stop the reaction. The mixture (0.6 mL) was poured into the 48-well plate, as also deionised water (0.6 mL) and ferric chloride (0.1% w/v, 0.12 mL), and the absorbance was measured at 690 nm using a microplate reader (SpectroStar nano, Labtech, Ortenberg, Germany). The reducing power was obtained directly from the absorbances. Trolox was used as standard.

TBARS assay. The thiobarbituric acid reactive substances (TBARS) assay was performed according to the procedure described by (Pinela et al., 2012). In this assay, lipid peroxidation is assessed by the products of the oxidation that react with thiobarbituric acid (TBA), namely malondialdehyde, giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS).

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (100 μL) of the supernatant was incubated in a 48-well microplate with the different concentrations of the extract's solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL). The microplate was incubated at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), and thiobarbituric acid (TBA, 2%, w/v, 350 μL) was added. The plate was then heated at 80 ºC for 20 min, and the contents of each well were transferred to 2 mL tubes that were centrifuged (3000*g*, 5 min). After centrifugation, 300 μL of the supernatant from each tube was transferred to a 96-well plate, and absorbance of the malondialdehyde (MDA)-TBA complexes formed was measured at 532 nm using the same spectrophotometer as for the DPPH assay. The inhibition ratio (%) was calculated using the following equation (2):

$$\text{Inhibition ratio (\%)} = \frac{(\text{A} - \text{B})}{\text{B}} \times 100$$

Where A and B were the absorbance of the control and the sample solution, respectively. Trolox was used as standard.

All the results for the antioxidant activity were expressed as EC_{50} values, *i.e.*, the effective concentration causing a half-maximal antioxidant response (μg/mL), or in other words, the extract concentration corresponding to 50% of antioxidant activity, or 0.5 of absorbance for the Ferricyanide/Prussian blue (reducing power) assay.

3.6.2. Antimicrobial activity

Antibacterial activity. To evaluate the antimicrobial activity, the studied extracts were re-dissolved in water to obtain a stock solution of 20 mg/mL and, subsequently, submitted to further dilutions.

The clinical isolates used in this study were obtained from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro, Vila Real, Portugal. The bacterial strains used were a total of five Gram-negative bacteria: *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from skin wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration), and *Morganella morganii* (isolated from urine), and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from sputum). Prior to analysis, all microorganisms were incubated for 24 hours at 37 ℃ in a fresh medium that ensures an exponential growth phase of bacterial cells.

The extracts were also tested against a total of eight food contaminants (bacteria) purchased from Frilabo in Porto, Portugal. Five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 9027), *Salmonella enterica* subsp. (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *L. monocytogenes* (ATCC 19111) and *S. aureus* (ATCC 25923). The bacterial strains were incubated at 37 ºC for 24 hours in a suitable fresh medium prior to analysis to obtain the exponential growth phase.

Two negative controls, one with MHB and Tween 80 and another with the extract, and two positive controls, one with MHB and Tween 80 and each inoculum and one with culture medium, antibiotics, and bacteria, were prepared. Ampicillin and streptomycin were used for all bacteria tested, and methicillin was used for *Staphylococcus aureus*.

To determine the minimum inhibitory concentration (MIC), the microdilution method and the dye *p*-iodonitrotetrazolium chloride (INT) were used, allowing colorimetric measurement as described by Pires et al. (2018), with some adjustments. For this purpose, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT), an indicator dye used as a microbial growth indicator, was added to the wells and the microplates were then incubated at 37 ℃ for 30 min. The minimum inhibitory concentration (MIC) of the samples was defined as the lowest concentration required to

inhibit bacterial growth, indicated by a colour change from yellow to pink upon two-electron capture, when the microorganisms are viable (Owuama, 2017). To determine the minimum bactericidal concentration (MBC), defined as the lowest concentration required to kill a bacterium, 10 µL of liquid from each well that showed no colour change was plated on a solid medium, blood agar (7% sheep blood), and incubated for 24 h at 37 ℃, measuring the lowest concentration that yielded no growth.

Antifungal activity. The antifungal activity was performed as described by Pires et al. (2018) with some rearrangements. The fungal strains used were obtained from Frilabo, Porto, Portugal, and were: *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). Fungi were maintained on malt agar and cultures were stored at 4℃ before being transferred to new medium and incubated at 25℃ for 72 hours. To examine the antifungal activity, fungal spores were removed from the surface of the agar plates with 0.85% sterile serum containing 0.1% Tween 80 (v/v) wash. The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 to a final volume of 100 µl per well. Samples were first dissolved in Mueller-Hinton Agar (MHA) medium containing Tween 80. Next, 10 µL of each sample was added in duplicate to the first well (96-well microplate), followed by 190 µL of malt extract medium (MEB). 90 µL of MEB was added to the remaining wells. Samples were then serially diluted to obtain a concentration range of 2.5% to 0.01%. Minimum inhibitory concentrations (MICs) were determined by serial dilution in 96-well microplates. Minimum fungicidal concentration (MFC) was determined by serially subculturing 2 µL of the extracts dissolved in the medium and inoculated into microplates containing 100 µL MEB per well for 72 hours, followed by incubation at 26 ℃ for 72 hours. The minimum fungicidal concentration was defined as MFC, indicating 99.5% killing of the original inoculum. The commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as a positive control.

3.6.3. Cytotoxic activity

The potential hepatotoxicity of the studied extracts was tested in a primary cell culture from porcine liver, designed as PLP2 (Abreu et al., 2011). The effects of the extracts on the cell growth were evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screen,

which uses the sulforhodamine B (SRB) assay to assess cell growth inhibition (Monks et al., 1991; Vichai & Kirtikara, 2006). PLP2 cells were cultured and maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin. Cells were plated in 96-well plates at an appropriate density (1.0×10^4 cells/well), treated with the different diluted sample solutions, left 1h at room temperature to attach without temperature oscillations that could interfere with this step, and placed at 37 °C under 5% CO_2, in humidified air for 72h.

A screening of the growth inhibitory activity was also performed in a normal cell line from *Cercopithecus aethiops* (African green monkey) designed as Vero (kidney epithelial cells). The cells were cultured and maintained in DMEM medium supplemented with 10% heat-inactivated FBS, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air. These cells were also plated in 96-well plates at an appropriate density (1.9×10^4 cells/well) and treated with the different diluted sample solutions for 72h, as previously described.

Finally, a screening of the growth inhibitory activity in human tumour cell lines was performed. For that, the procedure referred above for the cytotoxicity effects (SRB assay) was followed. The extracts were tested against different human tumour cell lines: AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), MCF -7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS. Cells were plated in 96-well plates at an appropriate density (1.0×10^4 cells/well) and treated with the different diluted sample solutions for 72h, as previously described.

Cells were not used until they showed 70% to 80% confluence. A known amount of each extract was dissolved in H_2O at a concentration of 8 mg/mL to obtain stock solutions. Successive dilutions were then prepared from this to obtain the concentrations to be tested (0.125 - 8 mg/mL). Each of the extract concentrations (10 μL) was incubated with the cell suspension (190 μL) of the tested cells in 96-well microplates for 72 hours. The microplates were incubated at 37 ºC and 5% CO_2 in a humid atmosphere. All cell cultures were tested at a concentration of 10,000 cells/well, except for Vero, which used a density of 19,000 cells/well. Following 72 h of incubation with the extract, plates were fixed by adding ice-cold 10% trichloroacetic acid (w/v, final concentration; 100 μL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.057%, m/v; 100 μL) was then added to each plate well and incubated

for 30 min at room temperature. Unbound SRB was removed by washing with acetic acid (1% v/v). Plates were air-dried, and the bound dye was solubilised by adding 10 mM Tris base and the absorbance was measured at 540 nm (Monks et al., 1991) in a Biotek ELX800 microplate reader (**Figure 10**). The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

Figure 10. Microplate reader used to evaluate the cytotoxicity.

3.6.4. Anti-inflammatory activity

The extracts were dissolved in H_2O at a final concentration of 8 mg/mL. From this, successive dilutions were made to obtain the concentrations to be tested (0.125 - 8 mg/mL).

The anti-inflammatory activity was evaluated in a mouse macrophage-like cell line, designed as RAW 264.7. The assay was performed following a procedure described by Taofiq et al. (2015). The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, and antibiotics at 37 °C under 5% CO_2, in humidified air. Cells were seeded in 96-well plates at 1.5×10^5 cells/well and allowed do attach to the plate overnight. Then, cells were treated with the different concentrations of each one of the extracts for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM. For the determination of nitric oxide, Griess Reagent System kit was used, which contains sulphanilamide, N-(1-napthyl)ethylenediamine

hydrochloride (NED) and nitrite solutions. A reference curve of the nitrite (sodium nitrite 100 µM to 1.6 µM; $y = 0.0066x + 0.1349$; $R^2 = 0.9986$) was prepared in a 96-well plate. The cell culture supernatant (100 µL) was transferred to the plate and mixed with sulphanilamide and NED solutions, 5–10 min each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader ELX800 Biotek; **Figure 11**), and by comparison with the standard calibration curve. The results were calculated by graphing the percentage inhibition of nitric oxide production as a function of sample concentration and expressed in terms of the concentration of each of the extracts that causes 50% inhibition of nitric oxide production (IC_{50}).

Figure 11. Microplate reader equipment to evaluate the anti-inflammatory activity.

3.7 Statistical analysis

The results obtained throughout the different evaluation studies were analysed by applying statistical tools, selected according to the results, and considering the defined research purposes. The software used was Statistics Package for Social Sciences (SPSS) version 22 (IBM Corporation, New York, USA). All results were expressed as mean values ± standard deviations (SD), maintaining the decimal places allowed by the magnitude of standard deviation. All tests were performed at a 5% significance level. For all methods, three samples were mixed to have a representative pool and all assays were carried out in triplicate. Since the factors studied used only two levels, the results of each parameter were compared through a Student's *t-test* to determine the significant difference among samples, with $\alpha = 0.05$.

IV.Results and discussion

4.1. Phenolic compound profile from *Annona cherimola* Mill. peel

The phenolic profile of *A. cherimola* peel extracts, obtained both by heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE), was carried out by HPLC-DAD connected in series to a Linear Ion-Trap LTQ XL mass spectrometer.

A representative chromatogram of the chromatographic analysis is depicted below (**Figure 12**).

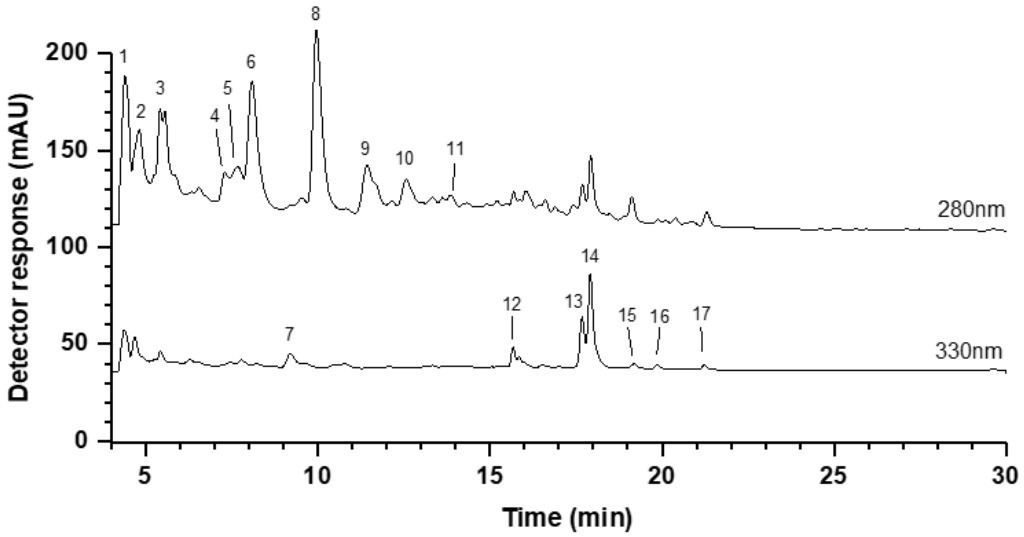

Figure 12. HPLC-DAD-ESI/MSn chromatogram of phenolic compounds from *Annona* peel extracts obtained by ultrasound-assisted extraction (UAE).

Phenolic compounds are important phytoconstituents, abundantly produced by plants. They are potential substitutes for bioactive agents in several sections to promote human health and prevent and cure various diseases (Sun and Shahrajabian, 2023).

Results revealed the presence of 17 phenolic compounds. Different classes of simple and complex phenolic compounds were identified such as phenolic acids, Procyanidins (condensed tannins) and flavonoids. Hydroxytyrosol-hexoside, A-type procyanidin dimer, ferulic acid derivative, hydroxytyrosol, B-type procyanidin dimer, epicatechin, B-type procyanidin trimer, B-type procyanidin tetramer, quercetin-rutinoside-pentoside, quercetin-*O*-rutinoside isomer, quercetin-3-*O*-rutinoside are among identified phenolic compounds in *Annona* peel. Epicatechin was the main phenolic compound identified. (**Figure 12, Table 9**).

Table 9. Chromatographic and spectroscopy characteristics of phenolic compounds in *Annona* peel extract

Peak[a]	Rt (min)[b]	UV-Vis (λ max)[c]	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Tentative identification
1	4.37	279	315	153, 123	Hydroxytyrosol-hexoside
2a	4.68	281, 320sh	575	449, 423, 287, 285	A-type procyanidin dimer
2b			153	123	Hydroxytyrosol
2c			373	193, 178, 149, 135	Ferulic acid derivative
3a	5.4	279	577	559, 451, 425, 407, 289, 287	B-type procyanidin dimer
3b			363	269, 175	ni
4	7.2	280	289	245, 205	Catechin
5	7.63	280	1153	1027, 865, 576, 425, 287	B-type procyanidin tetramer
6	8.07	280	577	425, 407, 289, 287	B-type procyanidin dimer
7a	9.2	281, 327	577	425, 407, 289, 287	B-type procyanidin dimer
7b			355	209, 191	Coumaroylglucaric acid
7c			863	711, 693	A-type procyanidin trimer
7d			1153	1027, 865	B-type procyanidin tetramer
8a	9.94	279	289	289, 245, 203, 125, 109	Epicatechin
8b			179	135	Caffeic acid
9	11.4	280	865	739, 713, 695, 577, 287	B-type procyanidin trimer
10	12.55	280	1153	1027, 863, 557, 575, 407	B-type procyanidin tetramer
11	13.84	280	1441	1315, 1153, 865, 575	B-type procyanidin pentamer
12	15.67	265, 354	741	609, 301, 300(100), 271, 179, 151	Quercetin-rutinoside-pentoside
13	17.67	256, 267sh, 354	609	300, 301, 271, 179, 151	Quercetin-*O*-rutinoside isomer
14	17.91	256, 356	609	300, 301, 271, 179, 151	Quercetin-3-*O*-rutinoside
15	19.1	280, 350	463	301, 179, 151	Quercetin-3-*O*-glucoside
16	19.86	279, 355	593	285, 255	Kaempferol-3-*O*-rutinoside
17	21.22	279, 353	593	285, 241	Kaempferol-*O*-rutinoside isomer

[a] Peaks numbered according to the chromatogram shown in Figure 1. [b] Retention time on C_{18} column. [c] Gradient of 0.1% formic acid and acetonitrile. ni: not identified.

Among the polyphenols found in the extracts, 10 were identified as procyanidins. These molecules comprise the so called group of condensed tannins as they are polyhydroxyphenols, polyflavonoids, which are soluble in water, alcohols and acetone and can coagulate proteins (Ghahri et al., 2021). 8 flavonoids were found in the extracts which were mainly represented by quercetin and epicatechin.

The presented statements are also confirmed by the study conducted by (García-Villegas et al., 2022) on the characterization of Custard Apple seed and peel extracts which had identified fifty-five compounds, some of which were identified for the first time in both custard apple by-products. Both seed and skin extracts showed a diverse phenolic composition. The main compounds identified were organic acids, terpenoids, phytohormones, flavones, glycosylated flavan-3-ols, flavanones, isoflavans and lignans. Many compounds, such as poncirin (flavanone), miconoside A (flavanone), kaempferol rutinoside, rutin (flavan-3-ol), and chemical and citric acids, among others, had been previously identified.

4.2 Experimental data for extraction optimization by RSM

As mentioned above, the phenolic content obtained with the 17 runs of the experimental design matrix for the *A. cherimola* extracts was used as the response variable for the RSM optimisation of both processes. **Table 10** and **11** present the quantification of the phenolic compounds found in both extracts, obtained by HAE and UAE, respectively.

4.2.1. Quantification of phenolic compounds

Total phenolic compounds (TPC, computed as the sum of all individual compounds separated by HPLC) data showed that Heat extraction procedure consistently provided the highest polyphenol content or recovery (**Table 10; Table 11**).

Table 10. Quantification of phenolic compounds in *Annona* peel extract obtained by maceration.

Peak	Tentative identification	Quantification (mg/g extract dw) [a]																
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**	**M14**	**M15**	**M16**	**M17**
1	Hydroxytyrosol-hexoside	0.09 ± 0.0003	0.09 ± 0.016	1.27 ± 0.008	1.07 ± 0.002	0.002 ± 0.0003	1.29 ± 0.01	1.34 ± 0.006	0.05 ± 0.001	0.04 ± 0.0001	1.28 ± 0.02	1.06 ± 0.001	0.06 ± 0.002	1.45 ± 0.04	0.03 ± 0.003	1.32 ± 0.11	0.04 ± 0.0008	0.06 ± 0.004
2a	A-type procyanidin dimer	0.03 ± 0.0002	0.03 ± 0.0003	0.08 ± 0.004	0.07 ± 0.004	0.03 ± 0.00007	0.08 ± 0.004	0.08 ± 0.001	0.03 ± 0.00004	0.04 ± 0.0001	0.09 ± 0.004	0.06 ± 0.0008	0.03 ± 0.0005	0.1 ± 0.005	0.03 ± 0.003	0.07 ± 0.00009	0.03 ± 0.0002	0.03 ± 0.0003
2b	Hydroxytyrosol																	
2c	Ferulic acid derivative																	
3a	B-type procyanidin dimer	0.38 ± 0.024	0.24 ± 0.005	1.16 ± 0.02	1.57 ± 0.008	0.12 ± 0.005	2.10 ± 0.14	2.50 ± 0.08	0.17 ± 0.008	0.21 ± 0.002	1.71 ± 0.007	0.98 ± 0.04	0.24 ± 0.02	2.41 ± 0.18	0.39 ± 0.004	1.22 ± 0.03	0.18 ± 0.01	0.3 ± 0.01
3b	ni																	
4	Catechin	0.29 ± 0.04	0.23 ± 0.038	0.09 ± 0.006	0.53 ± 0.01	0.13 ± 0.008	0.34 ± 0.02	0.47 ± 0.04	0.11 ± 0.005	0.17 ± 0.003	0.81 ± 0.02	0.15 ± 0.004	0.3 ± 0.02	0.75 ± 0.04	0.28 ± 0.02	0.37 ± 0.04	0.19 ± 0.005	0.44 ± 0.02
5	B-type procyanidin tetramer	0.61 ± 0.032	0.40 ± 0.05	0.05 ± 0.001	2.25 ± 0.05	0.26 ± 0.02	0.09 ± 0.008	1.11 ± 0.04	0.18 ± 0.002	0.31 ± 0.01	1.05 ± 0.01	0.06 ± 0.005	0.73 ± 0.03	2.74 ± 0.08	0.65 ± 0.07	0.11 ± 0.01	0.38 ± 0.005	0.77 ± 0.02
6	B-type procyanidin dimer	0.85 ± 0.027	0.55 ± 0.01	0.12 ± 0.005	4.50 ± 0.04	0.34 ± 0.01	0.44 ± 0.01	2.19 ± 0.02	0.27 ± 0.005	0.37 ± 0.006	2.93 ± 0.03	0.46 ± 0.005	0.84 ± 0.04	5.79 ± 0.22	0.87 ± 0.06	0.46 ± 0.02	0.45 ± 0.008	1.09 ± 0.02
7a	B-type procyanidin dimer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
7b	Coumaroylglucaric acid																	
7c	A-type procyanidin trimer																	
7d	B-type procyanidin tetramer																	

8a	Epicatechin	0.02 ± 0.0068	tr	tr	2.54 ± 0.06	tr	0.72 ± 0.006	2.41 ± 0.03	tr	tr	3.34 ± 0.02	tr	0.47 ± 0.05	3.9 ± 0.2	tr	0.73 ± 0.06	tr	0.68 ± 0.03
8b	Caffeic acid																	
9	B-type procyanidin trimer	0.55 ± 0.002	0.36 ± 0.009	0.33 ± 0.002	2.29 ± 0.02	0.21 ± 0.006	0.29 ± 0.01	0.58 ± 0.03	0.18 ± 0.001	0.27 ± 0.008	0.28 ± 0.005	0.23 ± 0.009	0.47 ± 0.04	3.09 ± 0.08	0.56 ± 0.02	0.29 ± 0.01	0.29 ± 0.02	0.62 ± 0.007
10	B-type procyanidin tetramer	0.33 ± 0.003	0.23 ± 0.01	0.26 ± 0.001	1.51 ± 0.03	0.11 ± 0.003	0.13 ± 0.02	0.53 ± 0.02	0.07 ± 0.002	0.19 ± 0.01	0.17 ± 0.001	0.11 ± 0.003	0.24 ± 0.02	1.86 ± 0.03	0.35 ± 0.01	0.1 ± 0.004	0.18 ± 0.02	0.34 ± 0.01
11	B-type procyanidin pentamer	0.16 ± 0.025	0.11 ± 0.007	0.07 ± 0.007	0.58 ± 0.01	0.05 ± 0.0005	0.22 ± 0.01	0.18 ± 0.01	0.03 ± 0.002	0.04 ± 0.003	0.32 ± 0.005	0.16 ± 0.003	0.08 ± 0.006	0.63 ± 0.03	0.15 ± 0.007	0.14 ± 0.009	0.10 ± 0.007	0.12 ± 0.006
12	Quercetin-rutinoside-pentoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
13	Quercetin-*O*-rutinoside isomer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
14	Quercetin-3-*O*-rutinoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
15	Quercetin-3-*O*-glucoside	0.47 ± 0.0009	0.47 ± 0.001	0.46 ± 0.00003	0.49 ± 0.003	0.46 ± 0.0001	0.46 ± 0.0001	0.48 ± 0.001	0.46 ± 0.0004	0.46 ± 0.0001	0.48 ± 0.001	0.46 ± 0.001	0.47 ± 0.0003	0.5 ± 0.01	0.47 ± 0.0007	0.47 ± 0.0001	0.46 ± 0.0003	0.47 ± 0.002
16	Kaempferol-3-*O*-rutinoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
17	Kaempferol-*O*-rutinoside isomer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
	Total phenolic compounds	**3.78±0.11**	**2.72±0.07**	**3.92±0.02**	**17.56±0.23**	**1.75±0.05**	**6.12±0.07**	**11.89±0.07**	**1.58±0.03**	**2.11±0.02**	**12.46±0.004**	**3.78±0.05**	**3.93 ± 0.22**	**23.23 ± 0.92**	**3.79 ± 0.17**	**5.28 ± 0.08**	**2.30 ± 0.06**	**4.93 ± 0.14**

[a] Quantitative data expressed as mg/g of the freeze-dried extract are presented as mean±standard deviation. More than one row per peak indicates coelution. tr: traces; nd: not detected.

Table 11. Quantification of phenolic compounds in *Annona* peel extract obtained by UAE.

Peak	Tentative identification	Quantification (mg/g extract dw) [a]																
		U1	**U2**	**U3**	**U4**	**U5**	**U6**	**U7**	**U8**	**U9**	**U10**	**U11**	**U12**	**U13**	**U14**	**U15**	**U16**	**U17**
1	Hydroxytyrosol-hexoside	0.10 ± 0.005	0.56 ± 0.03	0.12 ± 0.01	1.65 ± 0.07	0.79 ± 0.01	0.11 ± 0.002	0.06 ± 0.003	0.02 ± 0.0002	0.58 ± 0.002	0.12 ± 0.01	0.004 ± 0.0003	0.03 ± 0.001	0.01 ± 0.0008	1.17 ± 0.005	0.05 ± 0.003	0.11 ± 0.005	0.14 ± 0.009
2a	A-type procyanidin dimer	0.03 ± 0.0004	0.06 ± 0.0003	0.03 ± 0.0008	0.08 ± 0.003	0.04 ± 0.0001	0.04 ± 0.0007	0.03 ± 0.003	0.04 ± 0.0002	0.06 ± 0.001	0.03 ± 0.00008	0.03 ± 0.0001	0.03 ± 0.0002	0.03 ± 0.0005	0.08 ± 0.006	0.03 ± 0.001	0.031 ± 0.0002	0.03 ± 0.00002
2b	Hydroxytyrosol																	
2c	Ferulic acid derivative																	
3a	B-type procyanidin dimer	0.25 ± 0.01	0.26 ± 0.004	0.31 ± 0.03	1.82 ± 0.12	0.31 ± 0.005	0.31 ± 0.02	0.28 ± 0.03	0.39 ± 0.008	0.60 ± 0.04	0.22 ± 0.009	0.30 ± 0.02	0.25 ± 0.005	0.24 ± 0.02	1.23 ± 0.06	0.33 ± 0.02	0.24 ± 0.002	0.30 ± 0.05
3b	Ni																	
4	Catechin	0.22 ± 0.003	0.47 ± 0.01	0.23 ± 0.03	0.5 ± 0.02	0.09 ± 0.005	0.31 ± 0.009	0.31 ± 0.03	0.36 ± 0.005	0.25 ± 0.02	0.10 ± 0.004	0.23 ± 0.01	0.28 ± 0.01	0.28 ± 0.02	0.08 ± 0.002	0.40 ± 0.04	0.24 ± 0.002	0.37 ± 0.007
5	B-type procyanidin tetramer	0.50 ± 0.02	0.41 ± 0.04	0.67 ± 0.01	0.79 ± 0.05	2.85 ± 0.007	0.83 ± 0.07	0.69 ± 0.03	0.76 ± 0.007	0.08 ± 0.003	0.10 ± 0.003	0.59 ± 0.03	0.64 ± 0.05	0.60 ± 0.03	0.13 ± 0.009	0.89 ± 0.001	0.49 ± 0.027	0.79 ± 0.006
6	B-type procyanidin dimer	0.61 ± 0.009	0.48 ± 0.05	0.85 ± 0.01	2.83 ± 0.17	0.03 ± 0	0.85 ± 0.02	0.82 ± 0.02	0.80 ± 0.003	0.20 ± 0.007	0.26 ± 0.002	0.83 ± 0.06	0.89 ± 0.05	0.68 ± 0.05	0.18 ± 0.007	1.04 ± 0.06	0.71 ± 0.04	0.90 ± 0.07
7a	B-type procyanidin dimer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
7b	Coumaroylglucaric acid																	
7c	A-type procyanidin trimer																	
7d	B-type procyanidin tetramer																	
8a	Epicatechin	tr	0.98 ± 0.01	tr	1.80 ± 0.15	0.85 ± 0.002	0.004 ± 0.002	tr	0.15 ± 0.01	0.05 ± 0.005	tr	tr	0.04 ± 0.01	0.08 ± 0.009	tr	0.50 ± 0.04	tr	0.47 ± 0.01
8b	Caffeic acid																	
9	B-type procyanidin trimer	0.42 ± 0.02	0.25 ± 0.01	0.54 ± 0.01	1.03 ± 0.09	0.66 ± 0.009	0.55 ± 0.007	0.52 ± 0.006	0.62 ± 0.004	0.13 ± 0.02	0.20 ± 0.002	0.49 ± 0.03	0.54 ± 0.02	0.42 ± 0.02	0.28 ± 0.003	0.61 ± 0.05	0.49 ± 0.004	0.62 ± 0.03
10	B-type procyanidin tetramer	0.26 ± 0.003	0.26 ± 0.01	0.39 ± 0.008	0.61 ± 0.01	0.58 ± 0.006	0.41 ± 0.03	0.38 ± 0.004	0.40 ± 0.005	0.08 ± 0.003	0.10 ± 0.006	0.38 ± 0.03	0.37 ± 0.009	0.28 ± 0.02	0.13 ± 0.007	0.41 ± 0.01	0.35 ± 0.003	0.36 ± 0.008
11	B-type procyanidin pentamer	0.12 ± 0.008	0.18 ± 0.009	0.20 ± 0.009	0.17 ± 0.005	0.27 ± 0.009	0.23 ± 0.03	0.18 ± 0.003	0.17 ± 0.007	0.08 ± 0.007	0.05 ± 0.002	0.18 ± 0.007	0.16 ± 0.01	0.01 ± 0.004	0.23 ± 0.009	0.16 ± 0.004	0.16 ± 0.01	0.11 ± 0.008
12	Quercetin-rutinoside-pentoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
13	Quercetin-*O*-rutinoside isomer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
14	Quercetin-3-*O*-rutinoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
15	Quercetin-3-*O*-glucoside	0.47 ± 0.0006	0.46 ± 0.004	0.47 ± 0.0005	0.47 ± 0.0004	0.48 ± 0.0008	0.47 ± 0.002	0.46 ± 0.002	0.47 ± 0.0002	0.46 ± 0.0007	0.46 ± 0.0002	0.47 ± 0.001	0.47 ± 0.00009	0.46 ± 0.0003	0.46 ± 0.001	0.47 ± 0.001	0.52 ± 0.07	0.47 ± 0.0003
16	Kaempferol-3-*O*-rutinoside	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr

17	Kaempferol-*O*-rutinoside isomer	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
	Total phenolic compounds	**2.98 ± 0.06**	**4.37 ± 0.09**	**3.82 ± 0.06**	**11.75 ± 0.67**	**6.95 ± 0.01**	**4.10 ± 0.2**	**3.74 ± 0.04**	**4.21 ± 0.04**	**2.56 ± 0.08**	**1.64 ± 0.004**	**3.52 ± 0.2**	**3.70 ± 0.13**	**3.19 ± 0.17**	**3.98 ± 0.06**	**4.89 ± 0.22**	**3.35 ± 0.09**	**4.56 ± 0.17**

[a] Quantitative data expressed as mg/g of the freeze-dried extract are presented as mean±standard deviation. More than one row per peak indicates coelution. tr: traces; nd: not detected.

Comparing to literature, the concentration of total phenolics in *A. cherimola* peel extract through Maceration was 14.6 mg/100 g fresh weight (Loizzo et al., 2012) which is about 2 times higher than our optimal result by maceration. In another study, Aguilar-Villalva et al. (2021) reported that Ultrasound samples of *A. cherimola* have the highest values of total phenolics than the Maceration samples which disagrees with our findings.

Since the main compounds found in the extracts were condensed tannins, namely *b*-type procyanidin dimer, and flavonoids, namely catechin, these were used as the response model. The results for both extraction procedures are discussed below.

Table 12. Experimental runs, varying time in minutes (A), temperature in ºC (B) and solvent % of ethanol (C) for the heat-assisted extraction, and respective responses in mg/g of freeze-dried extract.

Run	A: Time	B: Temperature	C: Solvent	R1: B-type procyanidin dimer	R2: Catechin
1	120	20	50	0.846	0.293
2	62.5	50	50	0.548	0.234
3	120	50	0	0.126	0.099
4	5	20	50	4.532	0.538
5	62.5	80	100	0.351	0.135
6	5	50	0	0.446	0.348
7	5	80	50	2.205	0.472
8	62.5	50	50	0.275	0.117
9	62.5	20	100	0.365	0.174
10	120	80	50	2.911	0.801
11	62.5	20	0	0.456	0.152
12	5	50	100	0.843	0.303
13	62.5	50	50	5.799	0.747
14	62.5	50	50	0.874	0.281
15	62.5	80	0	0.461	0.369
16	62.5	50	50	0.451	0.193
17	120	50	100	1.094	0.439

In terms of the heat assisted extraction (HAE), the 17 runs are shown in **Table 12**, along with the two optimized responses, namely the quantity of two specific phenolic compounds, b-type procyanidin and catechin. **Table 13** shows the runs for the ultrasound-assisted extraction

(UAE). The three factors varied between 5 and 120 minutes, 20 and 80 minutes of extraction, and 0 to 100% of ethanol.

Table 13. Experimental runs, varying ultrasonic power in watts (A), time in minutes (B) and solvent % of ethanol (C) for the ultrasound-assisted extraction, and respective responses in mg/g of freeze-dried extract.

Run	A: Power	B: Time	C: Solvent	R1: B-type procyanidin dimer	R2: Catechin
1	250	11	50	0.606	0.224
2	250	20	0	0.477	0.472
3	250	11	50	0.853	0.232
4	100	11	0	2.829	0.500
5	250	11	50	0.027	0.092
6	100	20	50	0.849	0.313
7	250	11	50	0.819	0.308
8	100	2	50	0.805	0.363
9	400	20	50	0.199	0.249
10	400	11	0	0.262	0.104
11	250	11	50	0.830	0.229
12	400	11	100	0.888	0.282
13	250	2	100	0.679	0.277
14	250	2	0	0.187	0.082
15	250	20	100	1.036	0.403
16	400	2	50	0.709	0.242
17	100	11	100	0.902	0.369

To optimize the two responses, the maximize function was used, showing the conditions for each extraction method that render the highest amount of each of these compounds. For HAE the optimal points were set at 5 minutes (A), 20 ºC (B) and 50.1% ethanol (C) for the type-b procyanidin dimer. In terms of the model, it showed a significant fit with an R^2 of 0.9261 and an Adjusted R^2 of 0.8152, predicting a maximum amount of this procyanidin of 4.136 mg/g. Run 13 was ignored due to its value constituting an outlier. The 3D plots for this polyphenol are shown in **Figure 13**.

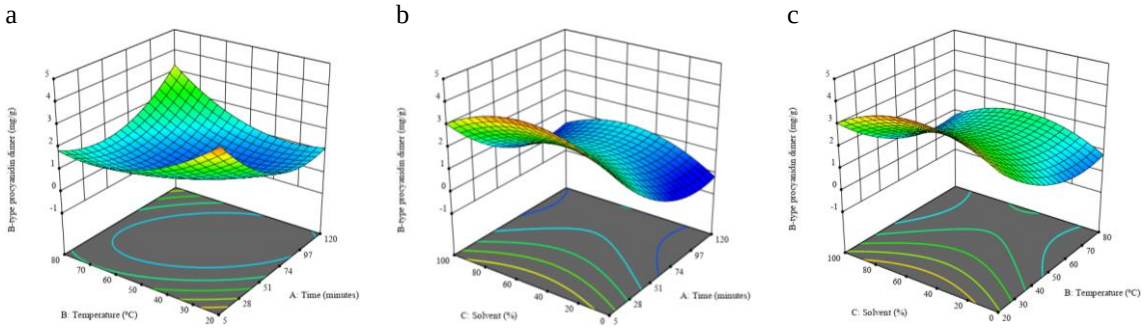

Figure 13. 3D plots of the optimization for maximum amount of the b-type procyanidin dimer using HAE.

By analyzing the coded equation (data not shown), the most important factor between the three for this polyphenol was the amount of ethanol used for the extraction. In **Figure 13b** and **c**, it is clear that the highest yields in the procyanidin are favored by intermediate amounts of ethanol, around 50%, while low temperatures, actually, the lowest temperature of 20 ºC favored this polyphenol. This could reveal a very low resistance to temperature. Furthermore, in terms of extraction time, the results were somewhat peculiar, with a clear favoring of extraction yield both at lower extraction times and the maximum time. This could either be due to most of the compound being extracted at the beginning of the extraction and residual amounts being scavenged near the end of the extraction time.

For catechin, the optimal point at which its recovery is maximized was set at 120 minutes (A), 80 ºC (B) and 60% of ethanol (C). For this model, run 13 was also ignored due to being an outlier. Overall, the model showed a significant fit, a R^2 of 0.8838 and an Adjusted R^2 of 0.7096, and predicted the maximum yield of catechin to be, at the conditions above mentioned, of about 0.747 mg/g. The coded equation showed that for catechin the most important factor was temperature.

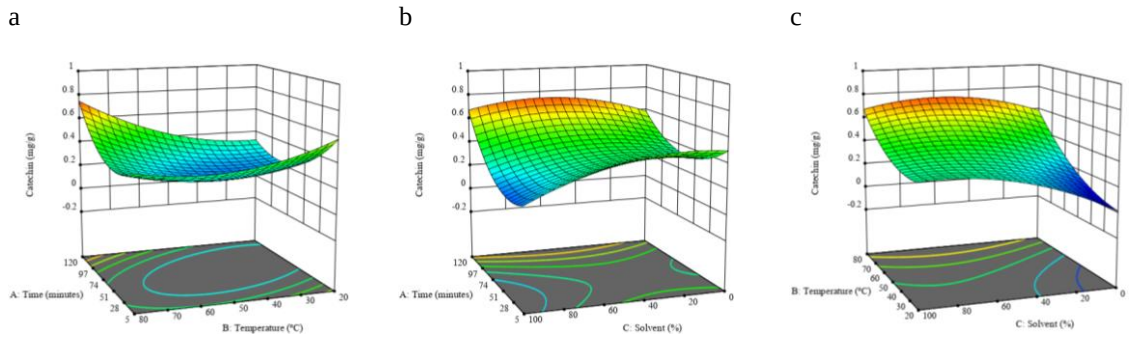

Figure 14. 3D plots of the optimization for maximum amount of catechin using HAE.

Regarding **Figure 14**, pertaining to catechin optimization using HAE, it is clear that longer extraction times and higher temperature favor the yield of this compound. **Figure 14a** shows this affinity to higher temperatures, as the red zones of the surface are close to the highest temperature and longer extraction time. In **Figure 14b**, intermediate amount of ethanol also seemed to promote the higher yield.

Overall, considering the HAE of these two compounds, higher temperatures and longer extraction times promote the extraction on catechin, while lower temperatures and short extractions promote higher yields of the procyanidin. Ethanol content in the extraction solution seemed to be quite similar for both, being adjusted at around 50 to 60%. For catechin, the factor that most influenced the extraction yields is temperature, while for the procyanidin, it was ethanol percentage.

The second analyzed extraction method, ultrasound assisted extraction (UAE), shown in **Table 11**, also relied on a model with 17 runs. The three factors used of the optimization were ultrasonic power (watts, factor A), extraction time (minutes, factor B), and ethanol percentage (factor C). These varied between 100 and 400 watts, 2 and 20 minutes and, 0 to 100% of ethanol. B-type procyanidin dimer modeling did not show a satisfactory R^2 for the model to be considered, thus, optimization was only carried out for catechin. This could be due to either operator errors in the extraction runs or an extreme degradation of this compound when subjected to ultrasonic waves, probably due to the high temperature occurring when ultrasound waves interact with the cells containing the polyphenols. In HAE, increasing heat showed a decrease in yield for this compound. Still, regarding the modelling of catechin, the optimal points for maximum yield were

set at 100 watts, 11 minutes and 0% of ethanol, which could render 0.5 mg/g of catechin. The 3D plots for catechin are shown in **Figure 15**.

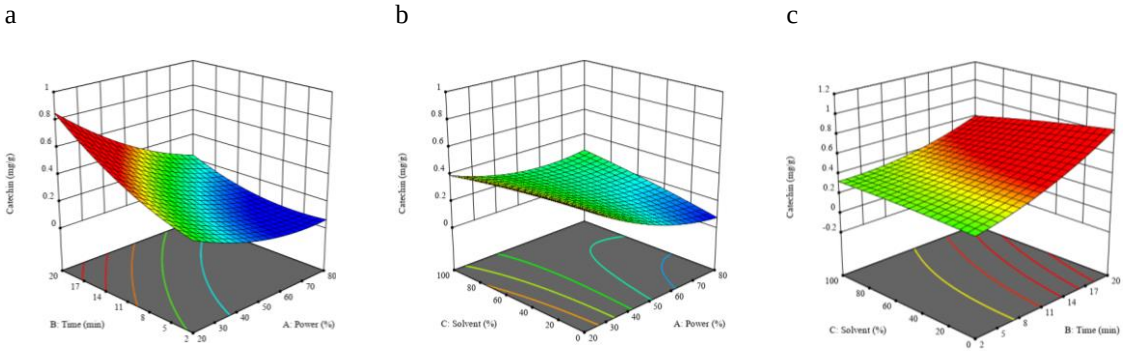

Figure 15. 3D plots of the optimization for maximum amount of catechin using UAE.

In terms of the optimization modeling for catechin, the R^2 was set at 0.9497, while de Adjusted R^2 was fixed at 0.8593, both within the frame of a significant model. The parameter with the most importance was the extraction time, being this result obtained from the coded equation (result not shown). In **Figure 15**, the importance of the extraction time can be identified, especially in **Figure 15a**, where the red zone of the surface, meaning higher yields, are found near the 20 minutes of extraction time, which recommends a further analysis of longer extraction periods with lower wattage. In fact, 100 watts showed better extractive potential than increasing watts, as can be seen in **Figure 15**. Finally, the percentage of ethanol did not show much influence on the yield in catechin. **Figure 15c** shows that despite the variation of ethanol its contribution was very slim, also seen on the left side of **Figure 15b**. Overall, long extraction times at low wattage and probably pure water are favored in terms of yields for catechin.

With regard of the whole design, some of the factors could be extended lower or higher due to no inflexion points being present in the surface models.

Regarding HAE, the extraction of procyanidins was favoured by combining the conditions of 5 minutes of extraction at 20 ºC and with 50.1% ethanol, while the extraction of catechin was favoured by combining the conditions of 120 minutes of extraction at 80 ºC with 60% ethanol, an intermediate extraction was carried out for the two compounds. So, the optimized extract obtained by HAE (OE_HAE) was obtained after 62.5 min at 50 ºC with 50% ethanol.

About the UAE, catechin recovery was maximized after 11 minutes of extraction at 100W and with 0% ethanol. Considering the b-type procyanidin dimer modelling did not show a satisfactory R^2 for the model to be considered, to optimize the extraction and obtain the optimized extract by UAE (OE_UAE), the previous conditions (for catechin) were applied.

4.3 Bioactivity of the most promising extracts obtained by heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE)

Antioxidant activity. As previously mentioned, the antioxidant properties of the studied samples, the optimized extract obtained by HAE (OE_HAE) and the optimized extract obtained by UAE (OE_UAE), were assessed through different in vitro assays, namely the DPPH radical scavenging activity, Ferricyanide/Prussian blue and TBARS assays. The results are presented in **Table 15**.

Table 14. Antioxidant activity (EC_{50}; mg/mL) of the studied extracts from *Annona cherimola* Mill. obtained by HAE and UAE (mean ± SD).

Activity	Assay	OE_HAE	OE_UAE	*t*-Students test *p*-value
Radical scavenging activity	DPPH scavenging activity	0.040 ± 0.001	0.127 ± 0.010	<0.001
Reducing power	Ferricyanide/Prussian blue	0.22 ±0.0111	0.22 ±0.0111	0.004
Lipid peroxidation inhibition	TBARS	0.0193 ± 0.0005	0.0247 ± 0.0005	<0.001

The antioxidant activity was expressed as EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. Trolox EC_{50} values: 0.043±0.002 mg/mL (DPPH scavenging activity), 0.029± 0.003 mg/mL (reducing power), and 0.0058±0.0006 mg/mL (TBARS inhibition).

Considering the antioxidant activity evaluated by the DPPH assay, the *Anonna* peel extract obtained after optimized conditions by HAE showed the lowest EC_{50} values (0.040mg/mL) compared with the extract obtained by UAE (EC_{50} of 0.127 mg/mL). Regarding the reducing power of the samples, evaluated through the Ferricyanide/Prussian blue assay, both *Annona*'s peel extracts presented similar antioxidant responses (EC_{50} of 0.22 mg/mL). Evaluating the lipid peroxidation inhibition through the TBARS assay, it was verified that the extract obtained from HAE showed the lowest EC_{50} value (0.0193 mg/mL).

Overall, the *A. cherimola* peel extracts obtained by HAE showed lower EC_{50} values than those obtained by UAE. Therefore, although both methodologies may have a similar phenolic

profile, extraction by HAE promotes the bioactivity of the extracts, namely their antioxidant potential.

Although the values obtained in the samples under study are generally higher than those for the standard trolox, they agree with other results obtained by different authors. Loizzo et al. (2012) studied the *A. cherimola* peel and evaluated its antioxidant potential using the DPPH assay. The authors reported an IC_{50} value of 57.7 μg/mL, which is higher than the value obtained in this study. Regarding the reducing power of the studied sample, Loizzo et al. (2012) found that the best results were obtained in the FRAP assay, with an IC_{50} value of 52.8 mg/mL.

To the best of our knowledge, there are no studies in the literature regarding the antioxidant properties of *Annona* peel using the TBARS assay. However, some reports are available on other *Annona* species, namely *Annona* crassiflora's peel (Roesler, 2011). The inhibition of lipid peroxidation using rat liver microsomes as an oxidative system was evaluated, and it was found that 50% inhibition of thiobarbituric acid reactive species formation in the microsomes required 4.44 $\mu g.mL^{-1}$ of the peel ethanolic extract. Our EC_{50} results for both extraction methodologies, namely HAE and UAE, are respectively about 4 and 6 times higher than this result.

The difference between the results obtained and those available in the literature is expected since the samples are from different locations, and the cultivation and storage conditions may also differ. In some cases, the species is also different, as are the extraction methods. All these conditions can interfere and lead to different chemical profiles and, consequently, different bioactivities.

In the food area, antioxidants are incorporated in the final products, mainly to extend the shelf-life of food and beverages, preventing the deterioration of their components by inhibiting, controlling, or delaying oxidation reactions. In other fields, such as pharmaceutical, nutraceutical or cosmetic sectors, antioxidants can be applied for similar purposes or bioactivity claims, functionalizing the product. (Rodrigues et al., 2023). Therefore, besides valuing *Annona* residues, the present work may be the basis for further studies trying to offer new alternatives to these sectors.

Antimicrobial activity. Another valuable property often associated with phenolic compounds is their antimicrobial activity. In this sense, the antimicrobial activity of the studied samples, the optimized extract obtained by HAE (OE_HAE) and the optimized extract obtained by UAE

(OE_UAE), was evaluated using the microdilution method. This method allowed us to assess the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), as well as the minimum fungicidal concentration (MFC). In these assays, different bacterial strains (clinical and food pathogens) and two fungal species were used to evaluate the antimicrobial potential of the tested extracts. The results are presented in **Tables 16**, **17** and **18**.

Table 16 presents the antibacterial results against food-pathogenic bacterial strains, showing that the tested extracts could inhibit the growth of most Gram-positive bacteria. The OE_HAE showed the highest inhibition capacity for the three tested bacteria compared to the extracts obtained by UAE. The lowest MIC value for this extract was 0.6mg/mL against *Bacillus cereus*.

Table 15. Evaluation of the antibacterial properties of the tested extracts against foodborne bacteria (MIC and MBC, $mg.mL^{-1}$).

	Extracts obtained after optimization				**Positive controls**					
	OE_HAE		**OE_UAE**		**Streptomycin (1mg/mL)**		**Methicillin (1mg/mL)**		**Ampicillin (10mg/mL)**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Enterobacter cloacae	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Escherichia coli	10	>10	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	5	>10	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria										
Bacillus cereus	0.6	>10	1.25	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	5	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	1.25	>10	5	>10	0.007	0.007	0.007	0.007	0.15	0.15

MIC-minimal inhibitory concentration; MBC-minimal bactericidal concentration; n.t.- not tested.

Regarding the Gram-negative bacteria, the extracts inhibited the growth of two tested strains, *Yersinia enterocolitica* (MIC values between 2.5 – 5 mg/mL) and *Escherichia coli* (MIC values of 10 mg/mL).

Analysing **Table 17**, both extracts generally did not reveal antibacterial capacity against all clinical bacterial strains except two Gram-negative bacteria, *Enterococcus faecalis* and *MRSA*. OE_HAE was most effective in inhibiting the growth of MRSA (MIC of 2.5 $mg.mL^{-1}$), although it was not able to kill the bacterium in the tested concentrations (MBC >10 $mg.mL^{-1}$). OE_UAE also inhibited MRSA's growth (MIC of 5 $mg.mL^{-1}$), with an MBC value >10 $mg.mL^{-1}$. For the *Enterococcus faecalis*, all of the tested extracts showed the same low inhibition capacity (MIC and MBC values of 5 and >10 $mg.mL^{-1}$, respectively).

Table 16. Evaluation of the antibacterial properties of the tested extracts against clinical bacteria (MIC and MBC, $mg.mL^{-1}$).

	Extracts obtained after optimization				**Positive controls**					
	OE_HAE		**OE_UAE**		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Escherichia coli	10	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>10	>10	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	10	>10	10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	>10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria										
Enterococcus faecalis	5	>10	5	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	2.5	>10	5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MIC-minimal inhibitory concentration; MBC-minimal bactericidal concentration; n.t.- not tested.

Overall, *Annona*'s peel phenolic extract obtained by HAE revealed the best results for bacterial inhibition against Gram-positive bacterial strains. In contrast, both extracts were less effective against Gram-negative bacterial strains. The exception was observed for *Yersinia enterocolitica*, which was sensitive to both extracts (MIC between 2.5 - 5 $mg.mL^{-1}$).

Thus, the bioactive compounds extracted by HAE seem to have more inhibiting capacity against Gram-positive foodborne bacteria, being an alternative as future substitutes of antimicrobial additives for food-preserving purposes. However, it is essential to highlight that further studies must be carried out to claim such potential.

Annona's peel extracts' antibacterial activity can be explained by its phenolic profile, which is rich in flavonoids and tannins. Indeed, the antibacterial mechanism of flavonoids may occur in several ways, including affecting the bacterial cytoplasmic membrane by forming pores and increasing membrane rigidity, inhibiting energy metabolism, and interfering with nucleic acid synthesis. Other mechanisms, such as inhibition of cell wall and cell membrane synthesis, have also been reported. In the case of tannins, which are water-soluble polyphenols, their antimicrobial mechanism of action may be attributed to inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth, inhibition of oxidative phosphorylation, which has a direct effect on microbial metabolism, or even deprivation of essential metal ions through their chelating effect on many metal ions (Ribeiro et al., 2018). The presented statements are also confirmed by the study of Aguilar-Villalva et al. (2021) on the antibacterial activity from *A. cherimola* leaves (aqueous extracts) against gram-positive and gram-negative bacteria. In this study, the antibacterial activity against the gram-negative *P. aeruginosa* bacterium was not observed in any evaluated extracts, suggesting that specific cell structure composition plays a key role in interacting with the compounds extracted. Pinto et al., (2017) have also confirmed the antibacterial activity of the water extract of *A. cherimola* against *S. aureus*, suggesting that the interaction with the peptidoglycan presented in the outer membrane allows the complexes formation, which can promote the destabilization of bacteria permeability.

Antifungal activity. The antifungal activity of the two studied extracts was assessed based on their inhibition capacity against two of the most common opportunistic fungal pathogens: *Aspergillus brasiliensis* and *Aspergillus fumigatus*. As shown in **Table 18**, the tested extracts

showed no remarkable antifungal activity against either fungal species (MIC and MFC of 10 and >10 $mg.mL^{-1}$).

Table 17. Evaluation of the antibacterial properties of the tested extracts against pathogenic fungi (MIC and MFC, $mg.mL^{-1}$).

	Extracts obtained after optimization				**Positive control**	
	OE_HAE		**OE_UAE**		**Ketoconazole (1 mg/mL)**	
	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	10	>10	10	>10	0.06	0.125
Aspergillus fumigatus	10	>10	10	>10	0.5	1

MIC-minimal inhibitory concentration; MFC-minimal fungicidal concentration.

In the literature, no studies of the antifungal activity of any species of *Annona*'s peel against *Aspergillus brasiliensis* and *Aspergillus fumigatus* exist. Nevertheless, Elhawary et al. (2013) compared the antifungal activity of three different *Annona* species, reporting significant antifungal activity of *A. cherimola* against *Candida albicans*. Data revealed that all *Annona* species were ineffective and had no antifungal activity against *Aspergillus flavus*.

Cytotoxic activity. The safe use of the studied extracts was proved by testing their potential hepatotoxicity using a cell culture designed as PLP2. Moreover, the extracts were also tested against a non-tumour cell line obtained from kidney epithelial tissue from African green monkey. The extract's cytotoxicity was also tested in human tumour cell lines from different models. The results of these assays are not shown since all extracts had an IC_{50} higher than the maximum tested concentration (>400 µg/mL). Although these results are acceptable for non-tumour cell cultures, they reveal a lack of bioactivity for tumour cells. Once again, to the best of our knowledge, there are no studies in the literature regarding the cytotoxic activity of either *A. cherimola* peel or other *Annona* species. Since this activity was performed for the first time in this research, we compared our results with a study on the cytotoxic activity of the leaf of another tropical fruit which is the Fig against the same studied cell cultures. Shiraishi et al., (2023) reported that in terms of cytotoxic activity, among the tumor cell lines tested, AGS had the highest susceptibility to fig leaf extract, followed by MCF-7, with no cytotoxicity observed in CaCo2 cells. As for antiproliferative activity

in non-tumor cells, no cytotoxicity was observed in the VERO cell line. Limited antiproliferative activity was observed in the PLP2 cell line, with only two of five varieties showing GI_{50} values of 248 ± 10 and 225 ± 11 μg/mL.

Anti-inflammatory activity. The anti-inflammatory activity of the studied extracts was tested in a mouse macrophage-like cell line (RAW 264.7) stimulated with LPS, verifying the changes in nitric oxide (NO) basal levels. As for the cytotoxicity, the extracts did not inhibit the nitric oxide production for the tested concentrations (GI_{50}>400 μg/mL). The only study found in the literature regarding the anti-inflammatory activity of *Annona* is from Laksmitawati et al. (2016), testing *Annona muricata*. The authors reported that *A. muricata* leaf extract has anti-inflammatory activity by inhibiting the inflammatory process mediators TNF-α, IL-1β, IL-6 and nitric oxide (NO).

V.Conclusions and future perspetives

5.1. Concluding remarks

This study aimed to value the bio-residues from *Annona cherimola* Mill. production, namely its peel, promoting sustainability and circular economy guidelines. It was intended to use the leftover materials, searching for natural compounds that could be an alternative for food, pharmaceutical or cosmetics applications while reducing the large amounts of waste generated by the production industries and its negative environmental impact.

Initially, the phenolic compounds extraction from *A. cherimola* peel was performed using heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE) as methodologies. Ethanol was selected as the extraction solvent since it is a green one.

After both extraction techniques, the identification and quantification of various phenolic compounds were performed by LC-DAD-ESI/MSn. Procyanidins (condensed tannins) were the most prominent group of phenolic compounds, followed by flavonoids (quercetin, epicatechin, catechin).

When analysing the phenolic compounds profiles recorded at 280 and 330 nm, there were no significant differences between HAE and UAE. But, regarding the phenolic compounds amounts obtained for all the extractions, HAE showed the best yields.

After obtaining the optimal points for both extraction techniques, the bioactivity of the extracts was tested.

Regarding the antioxidant assay, the tested extracts performed well as antioxidant substances both on chemical and cellular levels. So, these results could reveal that custard apple peel is an important source of antioxidant compounds. In general, the Maceration's extract exhibited higher antioxidant capacity than the Ultrasound's extract.

Concerning the antimicrobial activity, the phenolic compounds extracted by maceration have been proven to have a promising inhibiting capacity specially against gram-positive foodborne bacterial strains and are potent as antibacterial substances, making them a considerable option for food-preserving purposes. However, the two extracts from both techniques showed no remarkable antifungal activity against the two most common opportunistic fungal pathogens: *Aspergillus brasiliensis* and *Aspergillus fumigatus*.

In regard to the cytotoxic and the anti-inflammatory activities, no extracts inhibited the growth of the tested human tumour cell lines and the non-tumour cell cultures nor of the RAW 264.7 cells.

Overall, the results of this study could be highly interesting for several industrial applications such as in food, cosmetic, nutraceutical, and pharmaceutical.

5.2. Future perspectives

Future research should focus on optimizing the extraction of other green techniques.

The study of bioactive properties could be extended, predicting for example the enzymatic inhibition capacity.

It would be interesting to analyze other classes of compounds present in *Annona cherimola* Mill. peel, such as vitamins, acids, alkaloids, etc.

VI.References

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