UNIVERSIDADE FEDERAL DO ESTADO DO RIO DE JANEIRO

(UNIRIO)

FERNANDA DE SOUSA BEZERRA

A clean extraction of phenolic compounds from sunflower meal with NaDES and its application in the nutraceutical and food industries

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Thesis presented to the Food and Nutrition Graduate Program at the Federal University of the State of Rio de Janeiro, as a partial requirement for obtaining the degree of Doctor in Food Science.

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Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição da Universidade Federal do Estado do Rio de Janeiro, como requisito parcial para a obtenção do grau de Doutora em Ciência de Alimentos.

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Para minha *mom* e seu primogênito,

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I can do it with a broken heart

Taylor Swift

RESUMO

Extração limpa de coproduto industrial e sua aplicação na saúde. O girassol (*Helianthus annuus* L.) é uma planta amplamente cultivada, cujas sementes são utilizadas para obtenção de óleo, com uma produção mundial de 23 milhões de toneladas de sementes em 2024. O farelo de girassol, um coproduto da extração de óleo, é uma fonte abundante de compostos fenólicos, especialmente da família dos ácidos clorogênicos, que têm grande potencial para aplicações na indústria alimentícia e farmacêutica, devido às suas propriedades bioativas, como atividade antioxidante, antimicrobiana e anticancerígena. O objetivo desta tese foi desenvolver e avaliar metodologias de extração de compostos fenólicos do farelo de girassol, utilizando solventes eutéticos profundos naturais (NaDES) e técnicas de extração líquido-líquido (LLE), além de estudar a estabilidade e a citotoxicidade dos extratos obtidos. A extração com NaDES ofereceu vantagens como a sustentabilidade, baixa toxicidade e a capacidade de solubilizar uma ampla gama de compostos. A valorização de um coproduto pela aplicação de NaDES associa a química verde à economia circular. A combinação de NaDES LA:G se mostrou mais eficiente na extração de compostos fenólicos do farelo de girassol com 1786 CGA $mg.L^{-1}$, seguido pelo extrato hidroetanólico com 1305 CGA $mg.L^{-1}$. A estabilidade dos extratos foi avaliada sob diferentes condições de temperatura (40°C, 60°C e 80°C) e armazenamento (-18°C, 8°C e 25°C). Os resultados mostraram que os extratos em NaDES mantiveram mais de 85% dos ácidos clorogênicos após 30 dias de armazenamento a 8°C. Na extração líquido-líquido, a acetonitrila foi o solvente mais eficaz, com uma recuperação de 80% do conteúdo fenólico total em NaDES CC:GL e 63% em U:GL. Os extratos obtidos mostraram uma significativa redução na viabilidade celular em linhagens de câncer de mama, com redução maior que 70% nas células cancerígenas MCF-7 e MDA-MB-231. NaDES puros e com extrato fenólico também foram eficazes contra cepas de microrganismos patogênicos, a menor inibição foi observada no extrato fenólico NaDES CC:GL contra *Pseudomonas aeruginosa*, com MIC de 7,7 mg CGA L^{-1} e a maior em *Salmonella enterica* com 62,1 mg CGA L^{-1}. Os NaDES apresentaram eficiência como solventes verdes para extração de compostos fenólicos e na sua estabilidade ao armazenamento e ainda apresentaram atividade citotóxica em células de linhagem humana de câncer MCF-7 e MDA-MB-231 e cepas bacterianas causadoras de doenças transmitidas por alimentos.

Palavras-chave: NaDES, compostos fenólicos, farelo de girassol, ultrassom, atividade citotóxica, atividade antimicrobiana, extração líquido-líquido.

ABSTRACT

A clean extraction of an industrial by-product and its application on health. The sunflower (*Helianthus annuus* L.) is a widely cultivated plant, whose seeds are used to obtain oil, with a global production of 23 million tons of seeds in 2024. Sunflower meal, a by-product of oil extraction, is an abundant source of phenolic compounds, particularly from the chlorogenic acid family, which have great potential for applications in the food and pharmaceutical industries due to their bioactive properties, such as antioxidant, antimicrobial, and anticancer activities. The aim of this thesis was to develop and evaluate methodologies for the extraction of phenolic compounds from sunflower meal using natural deep eutectic solvents (NaDES) and liquid-liquid extraction (LLE) techniques, as well as to study the stability and cytotoxicity of the obtained extracts. Extraction with NaDES offered advantages such as sustainability, low toxicity, and the ability to solubilize a wide range of compounds. The valorization of a by-product through the application of NaDES links green chemistry to the circular economy. The combination of NaDES LA:G proved to be more efficient in the extraction of phenolic compounds from sunflower meal with 1786 CGA $mg.L^{-1}$, followed by the hydroethanolic extract with 1305 CGA $mg.L^{-1}$. The stability of the extracts was evaluated under different temperature conditions (40°C, 60°C, and 80°C) and storage conditions (-18°C, 8°C, and 25°C). The results showed that the extracts in NaDES retained more than 85% of the chlorogenic acids after 30 days of storage at 8°C. In liquid-liquid extraction, acetonitrile was the most effective solvent, with a recovery of 80% of the total phenolic content in NaDES CC:GL and 63% in U:GL. The obtained extracts showed a significant reduction in cell viability in breast cancer cell lines, with a reduction greater than 70% in MCF-7 and MDA-MB-231 cancer cells. Both pure NaDES and those with phenolic extracts were also effective against pathogenic microorganism strains, with the lowest inhibition observed in the phenolic extract NaDES CC:GL against *Pseudomonas aeruginosa*, with a MIC of 7.7 mg CGA L^{-1}, and the highest against *Salmonella enterica* with 62.1 mg CGA L^{-1}. NaDES demonstrated effectiveness as green solvents for the extraction of phenolic compounds, their stability during storage, and also exhibited cytotoxic activity in human cancer cell lines MCF-7 and MDA-MB-231 and bacterial strains responsible for foodborne diseases.

Keywords: NaDES, phenolic compounds, sunflower meal, ultrasound, cytotoxic activity, antimicrobial activity, liquid-liquid extraction.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

SUMMARY

Introduction

Phenolic compounds are molecules found in various plant tissues such as fruits, vegetables and their by-products, leaves, roots, skins, among others. They are considered specialized metabolism substances, responsible for protecting plants against pests and aiding their survival against environmental factors, such as exposure to UV rays and climate change. Additionally, they are among the compounds that contribute to the flavor, odor, and color of foods. More than 8.000 phenolic compounds, containing at least one aromatic ring with a hydroxyl group, have been identified and can be classified in four different ways: as flavonoids and non-flavonoids (Figure 1); by the number of aromatic rings, which may include one, two, three or more rings; by the carbon skeleton, describing how the carbon atoms are arranged in the molecule; and by the basic chemical structure, such as the presence of functional groups like aromatic rings, single or double bonds, and the types of bonds between molecules (Table 1) (INAMUDDIN; ALTALHI, 2023; NOLLET; GUTIERREZ-URIBE, 2018). Although they may not be essential for sustaining human life, phenolic compounds offer several health benefits, including antioxidant, antimicrobial, and antihypertensive activities, among others (LÓPEZ-SALAS et al., 2024; MIR-CERDÀ et al., 2023). Consequently, they are highly valuable products, yet they are not fully used, leading to the generation of tons of agri-food waste (AFW) annually in the juice (GÓMEZ-URIOS et al., 2022a; RASHID; ANWAR; ARIF, 2009; VIÑAS-OSPINO et al., 2023), wine (CALDAS et al., 2018; DABETIC et al., 2022) and oil industries (ALAÑÓN et al., 2020; BEZERRA et al., 2024; DABBOUR et al., 2021; JIA et al., 2021).

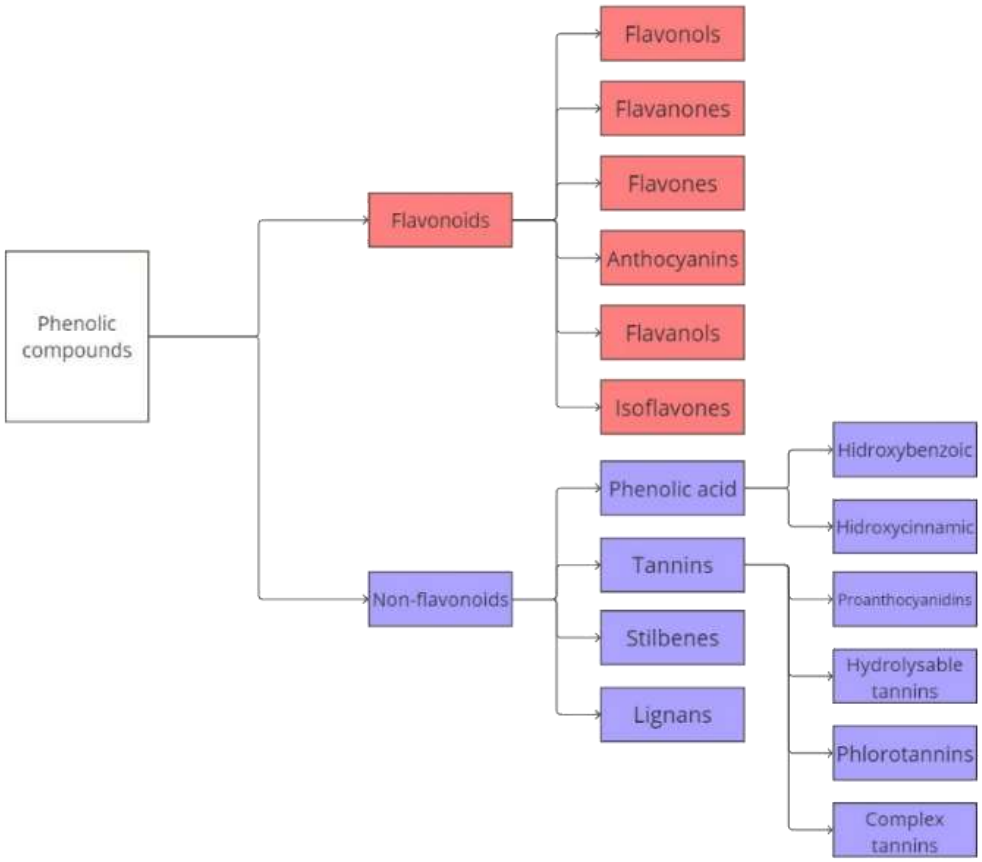
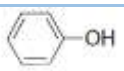
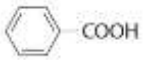
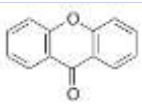
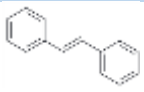
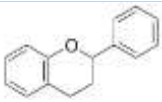
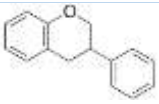


Figure 1. Classification of phenolic compounds into flavonoids and non-flavonoids.

Table 1. Examples of phenolic compounds classified by the number of aromatic rings, carbonic skeleton and basic chemical structure.

Number of aromatic rings	**Carbon skeleton**	**Basic chemical structure**
One	C_6	 Simple phenols
	C_6-C_1	 Phenolic acids
Two	C_6-C_1-C_6	 Xanthones
	C_6-C_2-C_6	

		Stilbenes
Three	C_6-C_3-C_6	 Flavonoids
	C_6-C_3-C_6	 Isoflavonoids
Three or more	$(C_6$-$C_3)_2$	Have multiple chemical structure. Example: Lignans
	$(C_6$-$C_3)_n$	Have multiple chemical structure. Example: Lignins

Humanity has finally realized that most natural resources are finite and that, up to the present moment, we have not adequately managed the waste we produce (UNITED NATIONS ENVIRONMENT PROGRAMME, 2024). The circular economy emerged to rethink waste generation, reevaluating its destination in order to reduce the waste of by-products and give new meaning to their use (MESKERS; WORRELL; REUTER, 2024). This practice is based on the application of the 4Rs—reduce, reuse, recycle, and recover—which can be expanded to the 10Rs by adding: rethink, repair, recondition, remanufacture, repurpose, and refuse (KIRCHHERR et al., 2023). The concept of a circular economy is a powerful tool to achieve several UN Sustainable Development Goals, among which the reduction of poverty (1st) and world hunger rates (2nd) stand out, which consequently support the 3rd goal of promoting health and well-being. Proper waste treatment reduces pollution of water sources, promoting a higher percentage of drinking water on the planet (6th), and also encourages sustainable cities (11th) and the conscious use of packaging, reusing whatever is possible (12th), thereby improving the quality of marine (14th) and terrestrial (15th) life (ONU, 2024). The participation of the agroindustry in circular economy actions brings benefits to the global community in

several ways, including the revaluation of by-products rich in bioactive compounds, which have various properties that benefit health (RAȚU et al., 2023).

Sunflower can be used as an ornamental plant, for consuming the seed in human food in its raw form, or through the extraction of sunflower oil. This process generates by-products such as cake and sunflower meal, the former being the result of oil extraction by cold pressing, followed by the removal of residual oil with organic solvents, generating sunflower meal (BEZERRA et al., 2024; DUNFORD; MARTÍNEZ; SALAS, 2015). This by-product is rich in phenolic compounds, which, under alkaline conditions, form irreversible bonds with proteins, reducing their accessibility (JIA et al., 2021; KAREFYLLAKIS et al., 2017). In sunflower meal, 70% of the phenolic compounds belong to the chlorogenic acid family (ZHANG et al., 2022).

Chlorogenic acids belong to the group of hydroxycinnamates, which are generally abundant in everyday foods and drinks, most notably in coffee (OTEEF, 2022). Chlorogenic acid (CGA), also called 5-O-caffeoylquinic acid (5-CQA) (Figure 2), is one of the most abundant and functional polyphenolic compounds in the human diet (WIANOWSKA; GIL, 2019). CQAs are present in various plant sources such as apples, berries, and vegetables like artichokes. The content of CQAs can vary significantly between different plant sources, with 5-CQA as the most represented component (LU et al., 2020). In addition to 5-CQA, the 3-feruloylquinic acid, diCQAs, caffeic acid, ferulic acid, coumaric acid, and quinic acid are also part of the Chlorogenic Acid Family (CLIFFORD, 2000).

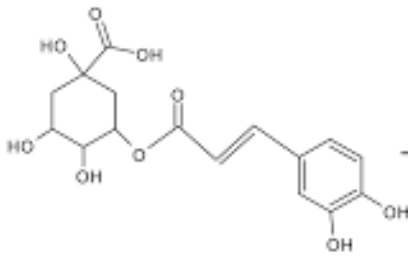


Figure 2. Structure of the 5-O-caffeoylquinic acid

The chlorogenic acid family is present in several foods, but its most extensively studied sources are green coffee and sunflower (GIL; WIANOWSKA, 2017; VÉLEZ; PEDROZA-DÍAZ; SANTA-GONZÁLEZ, 2023). Chlorogenic acids possess various beneficial properties for health, including antioxidant, immunomodulatory, anticancer,

and antimicrobial activities (GUPTA et al., 2022). The ability of chlorogenic acid to reduce cell viability has been studied in colon cancer cells (HCT-116), where a 40% reduction was achieved after 24 hours of treatment (ZHU et al., 2024).

The conventional techniques for extracting phenolic compounds from food matrices and their by-products typically use organic solvents such as ethanol and methanol, and involve the application of temperatures that are not consistent with the stability of these compounds, leading to their degradation (KOH et al., 2023). A "green" alternative to these organic solvents in the extraction of phenolic compounds is the use of Natural Deep Eutectic Solvents (NaDES). These solvents are sustainable, low-cost, non-toxic, non-flammable, and safe for application in products intended for human consumption (BEZERRA; DA COSTA; KOBLITZ, 2020; MEENU et al., 2023). Several methods of using NaDES to obtain and valorize phenolic compounds have been described, including ultrasound-assisted extraction (UAE) (CHAVES et al., 2024; FU et al., 2021b), microwave-assisted extraction (MAE) (FROSI et al., 2024; POPOVIC et al., 2022), pressurized liquid extraction (PLE) (BENVENUTTI; ZIELINSKI; FERREIRA, 2022; GRISALES-MEJÍA et al., 2024). Additionally, there are other extraction techniques, such as liquid-liquid extraction (LLE). This technique involves mixing two immiscible solvents, where one solvent has a greater affinity for the target compound, facilitating its transfer from one liquid phase to the other (PALOS-HERNÁNDEZ et al., 2022). No records were found on the use of LLE for the extraction of phenolic compounds from NaDES.

The cytotoxicity of NaDES, whether associated with phenolic extracts or not, has been a subject of recent study, as it can vary depending on the combination used for solvent preparation, molar ratio, pH, and form of exposure (SHARMA; LEE, 2024). In addition to composition, the type of cancer cell and/or target microorganism can also influence the cytotoxicity of these solvents (GONZALEZ–DIAZ; GARCÍA–NÚÑEZ, 2023).). NaDES formed by organic acids tend to be more toxic due to their lower pH values compared to combinations with sugars and polyols, affecting both human cancer cells and showing antimicrobial activity against pathogens (FOROUTANI et al., 2023).

General objective

The general objective of the present work was to obtain and evaluate phenolic compounds from sunflower meal using NaDES, focusing on the recovery of these bioactive compounds through the liquid-liquid extraction technique and the evaluation of their stability and cytotoxicity.

To achieve this general objective, the following specific objectives were listed, namely:

Specific objectives

• Extraction of phenolic compounds from sunflower meal using NaDES

• Evaluation of the stability of phenolic extracts from sunflower meal in NaDES media at room temperature, under refrigeration, and exposure to artificial light

• Recovery of phenolic compounds from the extract in NaDES by liquid-liquid extraction

• Assessment of the toxicity of extracts in NaDES and those obtained by liquid-liquid extraction on human cancer cells and pathogenic microorganisms.

Presentation of the chapters

To fulfill the objectives presented above, this doctoral thesis was composed of four chapters. In Chapter 1, a literature review was carried out and submitted to the journal Innovative Food Science and Emerging Technologies (IFSET), about the most common methods for extracting phenolic compounds from agro-industry by-products, using NaDES as solvents and applying different extraction methods. Studies that used these methods alone or in combination with each other were analyzed. Articles that had used the most common methods in the literature were searched: ultrasound assisted extraction, microwave assisted extraction and pressurized liquid extraction. All original scientific articles that used one or more of these methods in the extraction of phenolic compounds from by-products of food matrices were considered. It is known that the form of treatment that by-products undergo in the extraction of phenolic compounds can increase the final concentration of polyphenols in the extract and that these techniques enhance the acquisition of bioactive compounds. The objective of this review was to evaluate the types of assisted extractions in the acquisition of phenolic compounds from by-products. It became clear that the search for the valorization of these by-products, that would be discarded by the industry, is a current and recurring theme in literature, as they are rich in compounds that show therapeutic properties such as antioxidant, antimicrobial, antihypertensive, among others.

Chapter 2 presents a complementary review on topics relevant to this doctoral thesis, which were not covered in the literature review of the previous chapter. In this section, liquid-liquid extraction was discussed as a possible way to obtain bioactive compounds in NaDES media. The cytotoxic capacity of phenolic extracts obtained in NaDES media, with or without auxiliary methods, was also discussed. Since these extracts can, in most cases, be directly applied to products for health benefits, their cytotoxicity on tumor cells, non-tumor cells and antimicrobial activity were explored.

Chapter 3 presents an original scientific article, published in January 2024, in the journal ‘Sustainable Chemistry and Pharmacy’(BEZERRA et al., 2024). The article handles the acquisition of phenolic extracts from sunflower meal in NaDES media and using 40% ethanol as control. Different combinations of NaDES were compared in order to find the composition that had the best development in the extraction of phenolic compounds from sunflower meal. After the extraction and quantification of the phenolic compounds by chromatography and spectrophotometry, the stability of the phenolic

compounds to heat (40°C, 60°C and 80°C), storage (-18°C, 8°C and 25°C) and artificial light was also evaluated in the extracts in NaDES and hydroethanolic medium.

Chapter 4 presents an original scientific article, submitted to 'ACS Sustainable Chemistry & Engineering' journal. In this work, a study was conducted to recover phenolic compounds through liquid-liquid extraction, evaluating the capacity of organic solvents (heptane, hexane, ethyl acetate, acetone, dichloromethane, ethyl ether and acetonitrile) in removing the phenolic contents from sunflower meal in NaDES media. The extracts recovered by LLE, phenolic extract from sunflower meal in NaDES and the pure NaDES obtained in Chapter 3 underwent an evaluation of their cytotoxic capacity in human breast cancer cells (MCF-7 and MDA-MB-231) and antimicrobial activity in pathogens transmitted by food (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, Salmonella enterica*).

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CHAPTER 1

“Extraction of phenolic compounds from agro-industrial by-products using natural deep eutectic solvents: a review of green and advanced techniques”

Abstract

Due to the growing search for more sustainable practices, the Circular Economy has been gaining prominence by encouraging the reuse and recycling of resources. These by-products, such as fruit pomace and cereal bran, are rich in bioactive phenolic compounds that show antioxidant and anti-inflammatory properties. However, conventional extraction methods which use organic solvents are not compatible with the CE concept. Natural Deep Eutectic Solvents (NaDES) emerge as a more environmentally friendly alternative to extract these compounds from Agri-Food Waste (AFW). This review investigated ultrasound (UAE), microwave (MAE) and pressurized liquid (PLE) assisted extraction methods associated with the use of NaDES. The research included original articles published between 2020 and 2024, focused on the extraction of phenolic compounds from food by-products using NaDES. Among the 116 articles that were initially found, 19 were selected after careful analysis. The results showed that the UAE with NaDES were effective, especially for fruit and oilseed by-products, whereas MAE was useful for low-thermosensitive phenolic compounds. The PLE, although less explored, also demonstrated potential to increase the efficiency of the extraction of phenolic compounds. It was deduced that the combination of NaDES with assisted extraction techniques promoted a green and efficient approach for the recovery of bioactive compounds from food by-products by biorefinery, contributing to the valorization of these wastes in the circular economy.

Keywords: ultrasound, microwave, pressurized liquid, assisted extraction, food by-products

1. Introduction

The concept of circular economy (CE) is based on the "4Rs", which stands for: reduce, reuse, recycle and recover. The main definition is to manage waste and by-products giving them another use and stopping the "end-of-life" concept (KIRCHHERR et al., 2023). Any raw solid material that is generated by industry is considered by-product, while waste is any material that is discarded after human consumption, whether it is solid or liquid. The use of the concept of by-product intends to show that this type of product can still be reused and this is not the end of its useful life (CHIARALUCE et al., 2024). Not only does the food industry generate waste, but there is also significant generation in automotive parts, in the construction and demolition industries, as well as mining and textile (MESKERS; WORRELL; REUTER, 2024). According to a report by the United Nations Environment Program (UNEP), global waste production could reach 3.7 billion tons by 2050, if no control measure is taken. Currently, North America is the continent that produces the most solid waste *per capita* with more than 2kg/person/day and the one that produces the least is South and Central Asia with 0.5kg/person/day. The food and garden waste are responsible for more than 50% of global waste production, followed by paper and cardboard, plastic, other materials (textiles, wood, rubber, leather, household and personal hygiene products), glass and, ultimately, metal. Food and garden waste represent a little bit less than 60% of the waste generated in South America, just lower than Africa (> 65%) and Asia (> 60%) (UNITED NATIONS ENVIRONMENT PROGRAMME, 2024).

It is true to affirm that agriculture is one of the largest residue generators. The oil industry produces cake and meal from oilseeds and fruits rich in oil, such as sunflower (BEZERRA et al., 2024), but also from leaves, that are removed as a result of harvesting, such as date palm and olive (ABDELRAHMAN et al., 2023; ALAÑÓN et al., 2020; ZUROB et al., 2020). In cereal production, bran is generated as in rice (ZHENG et al., 2022) and in the production of juices, pomace like apple (Alchera et al., 2024) and jaboticaba (Benvenutti et al., 2022) are generated. Only in the former examples there are several phenolic compounds, such as chlorogenic acid, hydroxytyrosol, anthocyanins, ferulic acid, and oleuropein. Even though almost 1 billion tons of AFW are produced annually, these so-called "wastes", mainly rich in bioactive compounds, are not recovered from their matrices and are commonly used for animal feed (AWOGBEMI; KALLON, 2022; EZEORBA et al., 2024). By-products are the main source of bioactive compounds, such as phenolic compounds, which are known as secondary metabolites from plants, that

present an aromatic ring and at least one hydroxyl group. Their consumption is commonly associated with anti-inflammatory, antioxidant, anti-hypertensive effects (BEZERRA; DA COSTA; KOBLITZ, 2020; KOH et al., 2023).

The extraction of these bioactive compounds used to be performed mainly applying organic solvents like methanol, ethanol, acetone and others (MIR-CERDÀ et al., 2023), but these are not compatible with the 'green chemistry' appeal that the CE concept requires. Natural deep eutectic solvents (NaDES) are a much greener way of extracting bioactive compounds of AFW as they are natural extractors and a proven alternative to common organic solvents. NaDES are formulated with sugar, amino acids and/or organic acids, that are known as primary plant metabolites. Briefly, these solvents are made by a combination of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) under mixing (KOH et al., 2023). In the last few years, these greener solvents have been studied for their superior capacity of extracting phenolic compounds from by-products in comparison to conventional solvents (MARTINOVIĆ et al., 2022; POPOVIC et al., 2022; RATANASONGTHAM et al., 2024). NaDES align with the principles of 'green chemistry' preventing waste, promoting less hazardous syntheses, by being benign chemicals, reusable and reducing the risk of accidents for their no-flammability (AMERICAN CHEMICAL SOCIETY, 2013; ANASTAS; WARNER, 1998).

The conventional extraction of phenolic compounds has been based on liquid-liquid or solid-liquid or Soxhlet extraction and, depending on the form of presentation of the matrix, large amounts of organic solvents have been commonly used. Nevertheless, there are methodologies to assist the extraction of phenolic compounds, in order to increase their extraction from the raw material, which are used in association with organic solvents, but are also viable for NaDES media (ALARA; ABDURAHMAN; UKAEGBU, 2021). The choice of the ideal extraction method must consider the characteristics of the target compounds, the source matrix and the composition of NaDES (components, molar ratio, percentage of water added), since the viscosity of NaDES can be a problem for most extraction methods. Viscosity can affect mass transfer and hinder heat penetration as well as pressure application. However, these are parameters that can be adjusted by adding water and controlling the temperature, for example. Though, the combination of NaDES with techniques such as UAE, MAE and PLE provides a green and efficient alternative for the extraction of bioactive compounds, offering significant advantages in terms of sustainability and extraction efficiency (FOURMENTIN; COSTA GOMES; LICHTFOUSE, 2021; KOH et al., 2023). Table 1 presents the advantages and

disadvantages of using three methods commonly applied as auxiliary extraction methods of phenolic compounds. In this review we brought up these three methods: ultrasound assisted extraction (UAE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE). The objective of this research was to evaluate the types of assisted extractions associated with the use of NaDES in by-products and the effect of these auxiliary methodologies in the process of obtaining bioactive compounds.

Table 1.1. Advantages and disadvantages of the general use of UAE, MAE and PLE as auxiliary extraction methods.

Method	Advantages	Disadvantages	Reference
UAE	Facilitates solvent diffusion Easy-to-use Low cost Applicable to NaDES More effective than conventional extraction Reduced solvent volume Short extraction time	For water-bath ultrasound Long periods (>40 min) less reproducible than probe ultrasound Can negatively affect compounds For probe ultrasound Require small sample amounts No temperature control	(ALAÑÓN et al., 2020; ALARA; ABDURAHMAN; UKAEGBU, 2021; GIL-MARTÍN et al., 2022; LÓPEZ-SALAS et al., 2024)
MAE	Disrupts cell walls Easy-to-use Automated Multi samples Short extraction time High temperature Applicable to NaDES	Depends on concentration and cleaning steps for samples Uneven heating of the sample High energy consumption Can negatively affect thermosensitive compounds	(ALAÑÓN et al., 2020; GIL-MARTÍN et al., 2022; TAPIA-QUIRÓS et al., 2024)
PLE	High temperature and pressure Reduced solvent volume Easy-to-use Automated Accurate Applicable to NaDES	Time consuming Expensive instrumentation	(ALAÑÓN et al., 2020; ALARA; ABDURAHMAN; UKAEGBU, 2021; GIL-MARTÍN et al., 2022; MACHADO et al., 2024)

2. Materials and Methods

Research was carried out using Science direct, PubMed, and Scopus databases for original research papers that had studied the extraction of phenolic compounds from by-products using natural deep eutectic solvents. An initial search was carried out to define the most common extraction methodologies. The key words used were: “natural deep

eutectic solvents"; AND "by products" AND "phenolic compounds", in relation to the method the words were "extraction method" OR "liquid-liquid extraction" OR "UAE" OR "MAE" OR "PLE".

The following studies were included: papers published between 2020 and 2024, original research articles, dealing with matrices derived from food by-products, that applied NaDES as extraction solvent, which had as a goal to extract phenolic compounds. Any studies not featuring these attributes were excluded.

3. Article search results

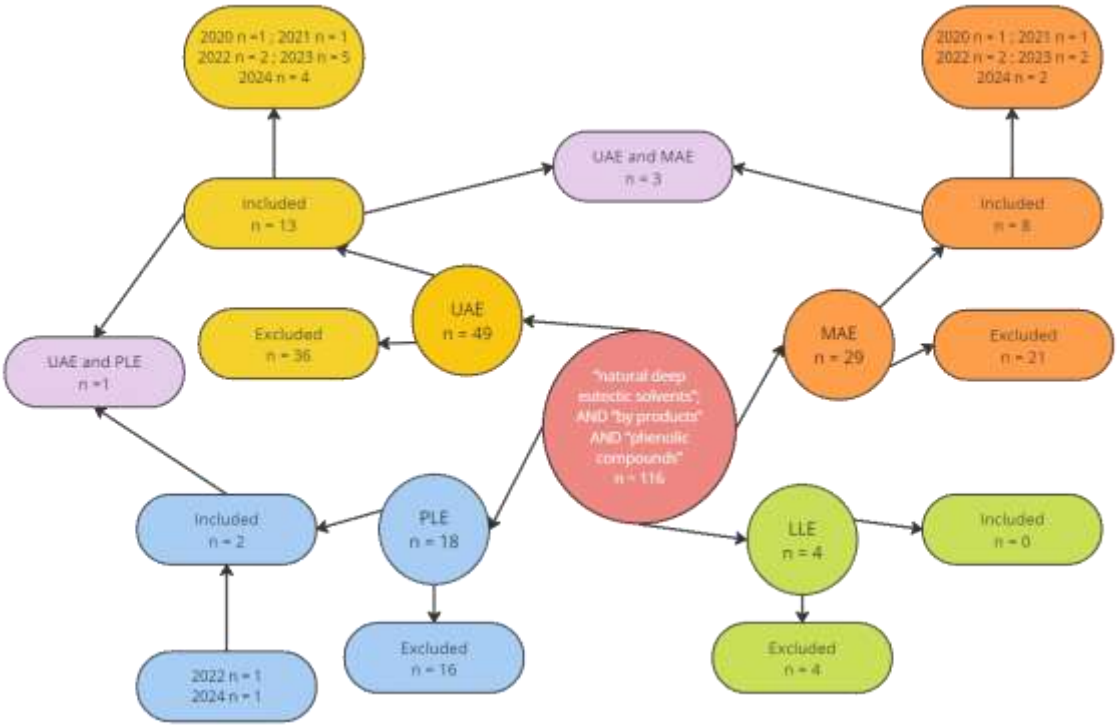

Figure 1.1. Search results. UAE (yellow) – Ultrasound Assisted Extraction; MAE (orange) – Microwave Assisted Extraction; PLE (blue) – Pressurized Liquid Extraction; LLE (green) – Liquid-Liquid extraction. Lilac – studies that used two methods.

A total of 116 scientific articles were found using the keywords. To filter the articles by extraction type, an extra keyword was added to the search, related to each specific auxiliary method, which resulted in the exclusion of 20 articles. A total of 96 studies were found among the researched methods. Posterior to evaluating each result, 56 studies that were unrelated to the topic or were review papers were excluded, and 19 were selected. Among the included articles, 3 evaluated UAE and MAE and 1 UAE and PLE

(Figure 1). No article that used the liquid-liquid extraction methodology to obtain phenolic compounds from by-products of food matrices was found. It was observed that the application of NaDES accompanied by auxiliary extraction methods to obtain phenolic compounds from waste food matrices has been a rising topic, as 56% of the articles found were published in the last 2 years of the studied range (2020 – 2024). The articles were mostly produced in the Asian continent, followed by Europe and ultimately, the American continent.

4. Ultrasound Assisted Extraction

Ultrasound-assisted extraction (UAE) is a technique that uses high-frequency sound waves to extract compounds from a sample. Ultrasound waves create cavitation, which causes local heating, high pressure and disruption of the sample matrix (ALARA; ABDURAHMAN; UKAEGBU, 2021). The mechanism that makes UAE an excellent option to obtain bioactive compounds is the vibration produced by the equipment that disrupts the cell walls of the sample, helping the solvent penetrate it and enhancing the transfer from the plant sample to the solvent (GIL-MARTÍN et al., 2022; LÓPEZ-SALAS et al., 2024).

4.1 UAE extraction of phenolic compounds with NaDES

The use of NaDES as solvent for the extraction of phenolic compounds has been largely studied on fruits, vegetables and non-food matrices, but also on by-products. The use of NADES coupled with UAE has shown promising results for the extraction of polyphenols, as NADES can effectively solubilize a wide range of polyphenolic compounds (KOH et al., 2023). UAE is one of the most commonly employed auxiliary extraction technique with NaDES. It is a simple, inexpensive, and efficient alternative to conventional extraction methods and often enhances the yield when associated with NaDES rather than using water, ethanol, or other organic solvents. Factors like ultrasound conditions (temperature, power and time), NaDES composition, and sample characteristics need to be optimized to maximize polyphenol recovery (FOURMENTIN; COSTA GOMES; LICHTFOUSE, 2021). On Table 2 the studies chosen under the inclusion criteria from the use of UAE to obtain phenolic compounds from AFW using NaDES combinations are presented.

Table 1.2. Phenolic compounds recovery in NaDES using UAE

NaDES components		By-product	Extraction	Results	Reference
Lactic acid*	**Glucose**	Sunflower meal	Ultrasonic probe 1 min Maximum power	Lactic acid NaDES showed 26% higher extraction power than Ethanol 40%, used as control	Bezerra et al., 2024
Choline chloride	Glycerol				
Choline chloride	Malic acid	Broccoli leaves	Probe cell crusher 20kHz Optimization of time, solvent:sample ratio, temperature, ultrasonic power	Optimized conditions were 36.35 mL/g; 49.5°C, 31.4 min; 383 W	Cao et al., 2023
	l-lactic acid				
	Glucose				
	Oxalic acid				
	1,2-Propylenglykol				
	1,3-Butandiol				
	Glycerol				
	Citric acid				
	D-Sorbitol				
	Urea				
Choline chloride	**Acetic acid**	Lemon peel	Ultrasonic probe 160, 240, 320 and 400 W 2, 4, 6, 8 and 10 min	Best results: 320 w; 6 min, 40% water	Chaves et al., 2014
Choline chloride	**Malic acid**	Peels of *Carya cathayensis* Sarg	Ultrasonic probe 20kHz Optimization of time, solvent:sample ratio, temperature, ultrasonic power	Improvement of more than 120% after the ultrasound treatment Optimized conditions were 15 min; 40 mL/g; 80°C; 460 W	Fu et al., 2021
Choline chloride	Glycerol		Ultrasonic probe		Tzani et al., 2023

	Lactic acid	Spent coffee	15 min 160 W – 9s pulse and 3s off Optimization: Solid:liquid ratio; % water; time; power	75% v/v with 25% water, 30 min, 3% solid-to-liquid ratio, and 127.5 W (ultrasonic power) Improvement of 1.5 times over the initial extraction.	
	Citric acid				
Betaine	Citric acid				
	Lactic acid				
	Glycerol				
Choline chloride	**Glycerol**	Date palm leaves	Water bath ultrasound – 40°C 40 and 60 W 30 and 60 min	UAE treatment had 500 GAE µg/100 mg, while aqueous samples without UAE approximately 400 GAE µg/100 mg	Abdelrahman et al., 2023
	Oxalic acid				
	Citric acid				
	Malic acid				
	Xylose				
	Glucose				
Choline chloride	Glycerol	Avocado epicarp and seed	Ultrasound bath – 28°C 40kHz 30 min	In comparison with PLE, the UAE had higher extraction power in seeds. Best combination: Bet:Fru in epicarp (~150mg GAE/g DS) and ChCl:Fru in seeds (~60mg GAE/g DS)	Grisales-Mejía et al., 2024
	Lactic acid				
	Glucose				
	Fructose				
	Citric acid				
Betaine	Glycerol				
	Lactic acid				
	Glucose				
	Fructose				
	Citric acid				

Choline chloride	Malic acid Urea **Fructose**	Sour cherry pomace	Ultrasonic bath 30 min at 40°C	Increase from <2000 µg/g TPC to >2500 µg/g TPC after UAE, in all samples	Popovic et al., 2022
Choline chloride	**Glycerol** Lactic acid Citric acid	Apple pomace	Optimization: 10 to 50 min; 20 to 70°C Sample:solvent ratio; 50 a 120 W/cm²; 20 to 100% - Duty cycle	Only time, sample:solvent ratio and duty cycle had influence in higher amounts of phenolic contents. Glycerol NaDES had 1.9 times more chlorogenic acid than conventional extraction. Optimized conditions were 40 min; 30% water; 1:30 ratio; 40°C; 83.2 W/cm²; 75% duty cycle	Rashid et al., 2023
Choline chloride	**1,6-hexanediol**	Coffee silverskin	Ultrasonic bath – 30°C 30 min 40kHz	An initial screening obtained 8mg GAE/g CS,	Taweekayujun et al., 2023

	Lactic acid		Optimization: Time, temperature, liquid:solid ratio, % water	after optimization 19.19 mg GAE/g CS. Optimized conditions were : 30% water; 45mL/g liquid/solid ratio, 90 min, 85°C	
	Glycerol				
Choline chloride	Oxalic acid	Rice bran	Ultrasonic bath – 50°C 37kHz 50 min Optimization: Time, sample:solvent ratio, % water	ChCl:EtG combination was 33% more effective than methanol extraction. 20% water; 40 min; 1:6 g mL^{-1} Higher yield of 26.49mg GAE/g DW	Ratanasongtham et al., 2024
	Glycerol				
	Ethylene glycol				
	Urea				
	Fructose				
Choline chloride	Oxalic acid	Foxtail millet bran	Ultrasonic bath – 50°C 30 min 250 W Optimization: % water, time, temperature, power	The best combination was Gly:CA, extraction 2 times higher than the control with 80% methanol. 29% water; 247 W; 61°C; 31 min	Zheng et al., 2022
	Lactic acid				
	Glycerol				
Proline	Glycerol				
	Lactic acid				
Glycine	Lactic acid				
	Glycerol				
Glycerol	**Citric acid**				
	Sodium acetate				
Choline chloride	Fructose	Olive leaves	Ultrasonic bath – 35°C 24h	CA:Glc (87 ppm) and LA:G (74 ppm) had better affinity with the sample,	Zurob et al., 2020
	Citric acid				
	Lactic acid				

	Glucose	therefore better extraction amounts than organic solvents and water – lower than 40 ppm.
Lactic acid	Glucose	
Citric acid	**Glycine**	

*** In bold are the NaDES combinations that showed the best results in each study, when applicable.**

4.1.1 Ultrasonic probe extraction

4.1.1.1 From fruit and vegetable by-products

Six different NADES compositions were investigated as extraction solvents for spent coffee grounds, and the betaine-based NADES were found to be more effective than the ones based on choline chloride or conventional hydroethanolic solutions. Probe ultrasound extraction for 15 minutes resulted in 15.99 mg GAE/g spent coffee, after an optimization, there was a final recovery of 21.99 mg GAE/g spent coffee. Optimized conditions were 30 min, 127.5 W and 25% water addition to the NaDES, which reduced its viscosity (TZANI et al., 2023). Chaves et al. (2024) also tested the samples with probe ultrasound to recover phenolic compounds from lemon peels. A first screening showed best affinity with choline chloride:acetic acid (ChCL:AA), so this NaDES was tested for different molar ratios, ultrasonic power and extraction time to obtain hesperidin and narirutin using a probe ultrasound equipment. The initial ratio (1:2) proved to be the best for both target compounds, time and ultrasonic power were optimized at 5 minutes and 400 W, although 320 W also achieved the maximum yield of the target compounds (5.25 mg hesperidin/g biomass and 0.21 mg narirutin/g biomass). The UAE outperformed conventional techniques like maceration and magnetic stirring in terms of extraction efficiency (CHAVES et al., 2024). An optimization was also carried out to evaluate the broccoli leaves phenolic composition, with similar power (383 W), longer time (31 min), at up to 49°C. Neochlorogenic acid was the most abundant phenolic compound extracted, followed by ferulic acid, quinic acid, chlorogenic acid, and caffeic acid. Total phenolic compound (TPC) value of the extract reached 4.91 mg/g of broccoli leaves (CAO et al., 2023).

4.1.1.2 From oilseed by-products

Lactic acid:glucose NaDES was the most efficient solvent, extracting 1786 mg of chlorogenic acid per liter of extract from sunflower meal, better than 40% ethanol with 1305 mg/L, after 1 minute treatment at probe ultrasound (Figure 2), without temperature control or any previous heat and stirring extraction (BEZERRA et al., 2024). Integrating the chlorine chloride:malic acid solvent with ultrasonication technique resulted in a synergistic effect, significantly enhancing the extraction yield and bioactivities of the phenolic extracts from Chinese nut (*Carya cathayensis* Sarg) peels compared to conventional extraction methods. The experimental TPC value, under the optimal

conditions, was 60.84 ± 0.48 mg gallic acid equivalents (GAE)/g dry weight (DW), an improvement of 120% after UAE (FU et al., 2021b).

Figure 1.2. Probe ultrasound apparatus used in Bezerra et al., (2024)

4.1.2 Ultrasonic bath extraction

4.1.2.1 From fruit and vegetable by-products

In Abdelrahman et al. (2023), choline chloride combinations were used for date palm leaves' phenolic compounds recovery. The water content of the NaDES helped a higher extraction yield, as water reduced the viscosity of the solvents. In a previous screening, the authors performed a conventional extraction and the choline chloride:glycerol combination showed the highest yield with 40% water. After optimization optimization, this NaDES was able to extract approximately 500 µg GAE/100 mg of date palm leaves with UAE after 30 min, which was lower than the extraction performed without UAE (600 µg GAE/100g). While on coffee silverskin, an initial screening in an ultrasonic bath, at 30°C, for 30 min, tested the molar ratio and water content of three combinations of NaDES, the choline chloride:1,6-hexanediol showed the best efficiency with 1:7 ratio and 30% water, resulting in a 23.6 mg GAE/g coffee silverskin. Lower water content and higher ratio lead to lower extraction power (TAWEEKAYUJAN; SOMNGAM; PINNARAT, 2023).

Grisales-Mejía et al. (2024) tested two types of hydrogen bond acceptor - chlorine chloride and betaine - for the treatment of avocado epicarp and seed, in an ultrasound bath (Figure 3) for 30 min, at 28°C. Each by-product resulted in a different best combination: betaine:fructose for the epicarp (150 mg GAE/g DS) and choline chloride:fructose for the seed (65 mg GAE/g dry sample), although the betaine:fructose showed no significant

difference for the latter. Other fruits' by-products have also been studied, like sour cherry pomace that showed significantly better results using NaDES than conventional solvents. Heat and stirring extraction on NaDES medium showed better results than UAE, especially with choline chloride:malic acid combination (3238 µg/g lyophilized sour cherry pomace) (POPOVIC et al., 2022).

Figure 1.3. Ultrasonic bath used in Grisales-Mejía et al., (2024)

According to Gil-Martín et al. (2022), temperatures between 45°C to 55°C should be ideal for phenolic compounds extraction, as they may prove thermosensitive. On the other hand, Rashid et al. (2023), when optimizing the extraction of polyphenols from apple pomace using between 20 and 70°C, found that temperatures higher than 40°C lead to significant decrease in TPC. This study determined that choline chloride:glycerol was a better extractor than ethanol 70%, at 40°C and 40 min.

4.1.2.2 From grain and oilseed by-products

Ratanasongtham et al. (2024) applied longer extraction times. Rice bran samples were treated for 50 min and 50°C had better results than 30 min because of the longer exposure to the treatment. Optimized extraction conditions were, however, 40 min with 20% water, resulting in a TPC value of 26 mg GAE/g DW. This study pointed out that UAE in NaDES resulted in significantly higher results compared to conventional solvent extraction. For the extraction of foxtail millet bran, different NaDES formulations were tested in an ultrasonic bath, at 50°C, for 30 min and 250 W. The control with 80% methanol resulted in 2 times lower TPC extracts than glycerol:citric acid and betaine:glycerol. Optimized conditions were 29% water, 247 W, at 61°C for 31 min, that yielded a TPC value of 7.8 mg ferulic acid equivalent/g with (ZHENG et al., 2022). Zurob et al. (2020) extracted olive leaves samples for 24h in an ultrasonic bath at 35°C to obtain

hydroxytyrosol. A combination of citric acid:glycine was able to extract 87 ppm of the target compound, 4 times higher than water.

Most of the studies that tested UAE applied ultrasonic baths, which demand longer exposure of the samples, when compared to probe ultrasonic devices. Higher temperatures helped to reduce the NaDES viscosity but could negatively affect the stability of the target phenolic compounds. The combination of UAE and NaDES provided a green, sustainable, and effective extraction method leading to high recoveries of bioactive substances from AFW. The cavitational, mechanical, and thermal effects of ultrasound, combined with the optimization of operating parameters of the process, contributed to the enhanced extraction of phenolic compounds in the by-products.

5. Microwave assisted extraction

Microwave-assisted extraction (MAE) involves the use of microwave radiation energy to heat up the solute-solvent mixture. The generated heat facilitates the solvents' diffusivity into the sample to improve the diffusion of the target phytochemicals out of the sample (ALARA; ABDURAHMAN; UKAEGBU, 2021). To extract phenolic compounds, MAE uses electric and magnetic fields to disrupt the hydrogen bonds of the sample, dissolving the target compounds on the solvent and obtaining higher extraction yields in a shorter time when compared to other extraction methods (GIL-MARTÍN et al., 2022; LÓPEZ-SALAS et al., 2024). MAE is useful in the extraction of some types of polyphenols like phenolic acids and flavonoids, but it should be taken into consideration that anthocyanins and tannins are more heat-sensitive and might be destroyed during microwave extraction, as a result of the high temperatures applied (ALARA; ABDURAHMAN; UKAEGBU, 2021).

5.1 MAE extraction of phenolic compounds with NaDES

The combination of MAE and NaDES is a promising approach for the recovery of polyphenols from AFW (TAPIA-QUIRÓS et al., 2024). MAE is an effective auxiliary extraction technique that has been combined with NaDES solvents. The extraction yields obtained with MAE-NaDES were often higher than those from maceration or UAE with NaDES. Optimal MAE conditions in terms of microwave power (W), temperature, and NaDES composition need to be determined through experimental design approaches (FOURMENTIN; COSTA GOMES; LICHTFOUSE, 2021). Overall, the MAE-NaDES approach represents an environmentally friendly strategy that combines the extraction

efficiency of NaDES with the speed of MAE (TAPIA-QUIRÓS et al., 2024). Table 3 presents the papers found in the search for studies that used MAE.

Table 1.3. Phenolic compounds recovery in NaDES using MAE

NaDES components		By-product	Extraction	Results	Reference
Choline chloride	**Glycerol**	Date palm leaves	Microwave – 40°C Optimization: Time, power, % water	MAE treatment had higher extraction (~800 GAE µg/100 mg), In comparison to samples of ethanol and methanol 70%. 49% water, 800W for 0.84 min	Abdelrahman et al., 2023
	Oxalic acid				
	Citric acid				
	Malic acid				
	Xylose				
	Glucose				
Choline chloride	**Acetic acid**	Palm pressed fiber	Microwave 40-80°C 3-15 min	Higher amount of ferulic acid was obtained at 15 min, 60°C (1.123 mg/g)	Ng & Nu'man, 2021
Choline chloride	Lactic acid	Olive leaves	Microwave extractor Optimization: 40-80°C; 10-40 min; water 0-70%	ChCL:EtG had the higher extraction power in initial screening with >25 mg/g dw. After optimization maximum yield of 32 mg/g was reached. 79.6°C; 16.7 min; 43.3% water	Alañón et al., 2020
	Oxalic acid				
	Tartaric acid				
	1,4-butanediol				
	Ethylene glycol				
	Xylitol				
	1,2-propanediol				
	Maltose				
	Urea				

Glucose	**Glycerol**	**Lactic acid**	Blueberry by products	Optimization: 60-70°C; 15-30 min; sample:solvent ratio; heating ramp:isotherm ratio	Sugar NaDES showed better results compared to ethanol and ChCl NaDES. 60°C; 30 min; 2min/min heating ramp/isotherm ratio; 20 mL/g sample:solvent	Alchera et al., 2024
Choline chloride	Glycerol	Citric acid				
Choline chloride	Malic acid		Sour cherry pomace	Microwave 90 w 3 cycles of 5s	All samples had greater phenolic compounds amounts (>2500 µg/g) than the conventional solvents (<2000 µg/g) when under MAE.	Popovic et al., 2022
	Urea					
	Fructose					
Choline chloride	1,2-butandiol		Turkish hazelnut by-products	Microwave Optimization: Time, temperature, % water	The ChCl:PG combination was the best and got twice the amount of quinic acid in the hazelnut pomace (17.9 mg/kg DS) than ethanolic extracts. 92°C; 38 min; 24% water	Bener et al., 2022
	1,2-propylene glycol					
	Glycerol					
	Malic acid					
Sucrose	Lactic acid					
Fructose						
Sucrose	Choline chloride					
Fructose						
Choline chloride	Glycerol		Spent coffee	Optimization:	The best NaDES were the ones with Betaine as HBA. In post	Tzani et al., 2023
	Lactic acid					
	Citric acid					

Betaine	Citric acid		30-90 min; sample:solvent; 30-60°C	optimization, Bet:Gly had 1.6 times fold the initial concentration. 60°C; 1% solid:liquid ratio; 30 min; 50% water	
	Lactic acid				
	Glycerol				
Choline chloride	Lactic acid	Corn cob	Optimization: 50-90°C; 20-50% water	MAE with solvent was more inefficient in comparison to the MAE NaDES extraction. 88°C; 5 min; 62.4% ethanol	Frosi et al., 2024
	Glycerol				
	1,2-propanediol				
	Urea				

*** In bold are the NaDES combinations that showed the best results in each study, when applicable.**

5.1.1 Extraction from fruit and vegetable by-products

Date palm leaves associated with choline chloride:glycerol NaDES was treated for 2 min, 30% water and 400 W in MAE and recovered approximately 800 µg/100 mg, that was twice the amount obtained with water extraction and 30% of this value corresponded to ferulic acid. After optimization with 49% water, 800 W for 0.84 min a maximum yield of 762 µg/100 mg was obtained (ABDELRAHMAN et al., 2023). Another by-product from palm, the pressed fiber, was also rich in ferulic acid. Ng & Nu'man (2021) treated these latter samples with choline chloride:acetic acid, testing temperature ranging from 40 to 80°C and treatment time from 3 to 15 min. Temperatures higher than 70°C were found to reduce the yield and between 12 and 15 min no significant difference was observed. The amount of ferulic acid extracted under microwave heating at 60°C for 9 minutes was 0.609 mg/g to 0.617 mg/g, and this increased up to 1.123 mg/g when the heating duration was extended to 15 minutes. The MAE-NaDES extraction method was more efficient compared to conventional solvent extraction for recovering ferulic acid from palm pressed fiber.

Nine combinations of NaDES were evaluated under MAE at 65°C, for 20 min for phenolic extraction of olive leaves. After a first screening the choline chloride:ethylene glycol had higher extraction power, without significant difference for methanol. Optimized conditions were 79.6°C, for 16.7 min and 43.3% water, and generated a TPC value of 32 mg GAE/g olive leaves (ALAÑÓN et al., 2020).

Popovic et al. (2022) chose shorter treatment times (15s) to extract phenolic compounds from sour cherry pomace, the choline chloride:malic acid combination was found to perform better, although all NaDES compositions had improved results in comparison to 50% ethanol and acidic methanol. However, in this study, conventional heat and stirring extraction had better results than the use of MAE. Alchera et al. (2024) compared choline chloride and sugar NaDES with organic solvents for the extraction of phenolic compounds from blueberry by-products. The optimized conditions were 30 min, at 60°C and the final yield with NaDES was 61% higher than with conventional solvents.

Among the surveyed studies using MAE, the longest treatment time applied was 90 minutes of microwave heating for polyphenol extraction from spent coffee, using choline chloride and betaine as HBA for NaDES formulations. Treatment time proved less significant than solvent:sample ratio and temperature. The betaine:glycerol combination was selected and optimized extraction conditions of 60°C, 30 min, 50% water and 50% ratio resulted in extracts with a TPC value of 30.9 mg GAE/g spent coffee.

(TZANI et al., 2023). Hazelnut by-products were treated under microwave (Figure 4) heating at 92°C, for 38 min, with 24% water in a NaDES of choline chloride:1,2-propylene glycol and resulted in extracts that were richer in caffeic acid, gallic acid and ferulic acid than ethanolic extracts obtained in the same conditions (BENER et al., 2022).

Figure 1.4. Microwave apparatus used in Bener et al. (2022)

5.1.2 Extraction from grain by-products

In Frosi et al. (2024), an optimization with choline chloride NaDES on corn cob was proposed. Conventional solvent extraction, MAE extraction using hydroethanolic solvent and MAE using NaDES were investigated, but none of the NaDES showed better yield results than the conventional solvents for this by-product. The highest yield was obtained with 62.4% ethanol, 88°C, 5 min and 42.8 mL/g solvent:solid ratio, after HPLC quantification MAE ethanolic extract showed 2.6 times the concentration of NaDES extract.

The microwave irradiation helped improve the extraction efficiency and reduce the phenolic compounds' extraction time and showed higher efficiency than UAE in some matrices. Optimizing the extraction medium helped to solubilize the target compounds, while the microwave heating improved their release from the AFW matrix.

6. Pressurized liquid extraction

Pressurized liquid extraction (PLE) is a technique that uses high temperature and pressure to efficiently extract compounds from a sample. The sample is placed in an extraction cell, sealed and pressurized, then the solvent is pumped into the cell. The pressure and temperature are the key parameters on this method and are applied to physically change the properties of the sample, keeping the solvent liquid regardless of

the temperature applied, disrupting the sample matrix, improving mass transfer and extracting the target compounds (LÓPEZ-SALAS et al., 2024; MACHADO et al., 2024; VIŠNJEVEC et al., 2024).

6.1 PLE extraction of phenolic compounds with NaDES

The use of PLE as an innovative and high-yield green technique for recovering phenolic compounds can be performed using green solvents like water and NaDES, which enhances the yield and bioactivity of the extracts. Implementing PLE with green solvents like NaDES has shown promising outcomes, but more research is needed on the effect of temperature on NaDES behavior, and strategies to reduce their viscosity (KOH et al., 2023; MACHADO et al., 2024). Table 4 presents the studies found in the search for articles that used PLE.

Table 1.4. Phenolic compounds recovery in NaDES using PLE

NaDES components		By-product	Extraction	Results	Reference
Choline chloride	**Propylene glycol** Malic acid	Jaboticaba by-products	Pressure 10 MPa 12 min 60, 90, 120°C Flow rate: 3, 4, 5mL/min 15, 30, 45% water	NaDES had significant better results in obtaining phenolic compounds by PLE (85 and 78 mg GAE/g DW) than water and acidified water samples (74 and 72 mg GAE/g DW) 90°C; 5.3 mL/min, 47% water	Benvenutti et al., 2022
Choline chloride	**Glycerol** Lactic acid Glucose	Avocado epicarp and seed	Stainless steel extraction cell 20 min 103.4 bar 100°C	All combinations had more than 160 mg GAE/g on epicarp. The best combination for seed was choline chloride:lactic acid with 60 mg GAE/g	Grisales-Mejía et al., 2024
Betaine	Glycerol Lactic acid Glucose				

*** In bold are the NaDES combinations that showed the best results in each study, when applicable.**

6.1.1 Extraction from fruit by-products

Only two studies were found using PLE and NaDES in AFW (Table 4). The first one used water and acidified water in comparison to choline chloride NaDES in jaboticaba by-products. The optimal conditions for the assay were 90°C, with 47% NaDES solution and a flow rate of 5.3 mL/min. Choline chloride:prolylene glycol achieved the highest yield, and was able to extract 85.68 mg GAE/g dw, while water and acidified water extracted at most 74.47 mg GAE/g dw and 72.97 mg GAE/g dw, respectively (BENVENUTTI; ZIELINSKI; FERREIRA, 2022).

PLE and UAE were compared for the extraction of avocado epicarp and seed using different compositions of choline chloride and betaine NaDES. The epicarp extracts obtained by PLE (Figure 5) using choline chloride NADES generated a TPC values up to 185 ± 9 mg gallic acid equivalent/g dw. Although PLE led to a general improvement in phenolic compound extraction compared to UAE, the differences were not considered substantial (GRISALES-MEJÍA et al., 2024), specially when differences in costs and apparatus are taken into account.

Figure 1.5. Pressurized liquid apparatus used in Grisales-Mejía et al., (2024)

7. Conclusion

NaDES are a sustainable alternative for the extraction of phenolic compounds, as they generally increase extraction yield when compared to traditional organic solvents. With the application of innovative auxiliary extraction methods such as UAE, MAE and PLE, this effect is even more significant.

Among the extraction methods discussed, the use of NaDES in combination with UAE proved to be highly effective for the solubilization of phenolic compounds, as a simple, low-cost and high-efficiency method compared to conventional methods, such as maceration and magnetic or shaker agitation. When using MAE, it is possible to carry out fast and high-yield extractions, although caution is required with thermosensitive compounds. PLE, although less studied in the extraction of phenolic compounds from by-products, demonstrated significant potential to increase the yield for the extraction of phenolic compounds, with the need for further investigations to optimize the processing conditions of temperature and pressure and minimize the viscosity of solvents.

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CHAPTER 2

"Extração Líquido-Líquido, Estabilidade e Atividade Citotóxica de Compostos Fenólicos: Perspectivas e Aplicações com Solventes Eutéticos Naturais"

1. Stability of phenolic compounds

Stability is a critical factor to be considered when developing methods for extracting and processing phenolic compounds to preserve their beneficial properties. Careful control of pH, temperature and enzyme activity is necessary to ensure the stability and quality of phenolic extracts (ALARA; ABDURAHMAN; UKAEGBU, 2021)

Phenolic compounds are generally more stable at acidic pH values compared to neutral or alkaline conditions. Higher temperatures, above 80°C, can promote the degradation of phenolic compounds, especially for more heat-labile types, such as anthocyanins, which are also sensitive to light, pH variation and exposure to oxygen (BI et al., 2020). In the study of Zheng et al. (2022) the content of phenolic compounds and antioxidant activity remained more stable at lower temperatures (50 to 60°C). However, prolonged exposure to elevated temperatures led to significant accelerated degradation after 60 minutes at 40-80°C and within 20 minutes at 100°C (SZOPA; WRÓBEL; WITEK-KROWIAK, 2024). On that account, extraction methods that use moderate temperatures, such as pressurized liquid extraction or supercritical fluid extraction, can help preserve the stability of phenolic compounds (GIL-MARTÍN et al., 2022). The inactivation of enzymes in sample preparation is important to maintain the integrity of the phenolic fraction. Compounds with more complex structures, such as flavonoids and tannins, tend to be more stable than simpler phenolic acids. Storage conditions, such as temperature, light exposure and oxygen availability, can also impact the long-term stability of extracted phenolic compounds (BEZERRA et al., 2024).

1.1 Stability of phenolic compounds in NaDES

The main advantage of using NaDES for maintaining the stability of extracts is the ability of these solvents to form strong hydrogen bond interactions with unstable compounds such as natural pigments and bioactive compounds. Their high viscosity, especially in combinations with low water content, helps to reduce molecular movement and maintain stable interactions between the solute and NaDES components (PALOS-HERNÁNDEZ et al., 2022). This results in a significant improvement in the stability of phenolic compounds compared to conventional solvents such as water and ethanol. Viscosity can be adjusted by changing the amount of water when preparing the solvents,

allowing the optimization of the stabilization effect (DAI; VERPOORTE; CHOI, 2014; MUSTAFA et al., 2021).

1.1.1 Stability of fruit by-products

In orange by-products, after 30 days of storage at 4°C there was a loss of 15% and 35% of the total phenolic content in lactic acid:glucose and proline:malic acid extracts, respectively (GÓMEZ-URIOS et al., 2022). For mulberry anthocyanins, samples in NaDES choline chloride:lactic acid medium showed only 7% degradation after 90 days of storage at -20°C and the extract in acidified ethanol lost 20% in the same period. In storage at 4°C, the NaDES extract contained 1.2 times more anthocyanin after 3 months than the control extract (BI et al., 2020). For blueberry pomace samples, choline chloride:oxalic acid was the combination tested in comparison to acidified ethanol, samples at acidic pH (< 4) had good stability in both solvents, but at higher pH values (> 4) the degradation was slower in NaDES medium with a final maintenance of 90% while ethanol had 80% (FU et al., 2021a).

1.1.2 Stability in leaf and flower products

Bioactive compounds from *Rosmarinus officinalis* L. in NaDES media showed a greater capacity to stabilize phenolic compounds compared to the ethanolic extract, after 72 hours at 25°C. The half-life of 7 to 49 days for the NaDES extracts was calculated, while for the ethanolic extract it was only 6 days (BARBIERI et al., 2020).

The stability of cyanidin from *Catharanthus roseus* petals was studied in NaDES lactic acid:glucose, after storage for 3 months at 4°C and -20°C, the compound continued with the same absorbance at 520 nm, but the sample that was stored in acidified ethanol quickly degraded. At 80°C, a half-life of 111 minutes was calculated in NaDES extracts and 72 minutes in acidified ethanol extracts (DAI et al., 2016).

Safflower samples in NaDES medium in glucose:choline chloride and sucrose:choline chloride were 25% more stable than samples in 40% ethanol after 3 days of exposure to light, at room temperature (DAI; VERPOORTE; CHOI, 2014).

2. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a separation technique used to extract and purify compounds from liquid mixtures and was developed to recover high value compounds or remove impurities from biorefinery liquids. Usually, a mixture of organic solvents and aqueous phase and a mass transference occurs to the organic phase. Since these two liquids are immiscible, after a period of rest or centrifugation, two phases are formed again. This method is applied in petroleum, pharmaceutical, biochemical and waste treatment industries (BOKHARY; LEITCH; LIAO, 2021; CAÑADAS et al., 2020). The choice of solvents to LLE determines the selectivity and efficiency of the extraction, other factors like pH, temperature and the ratio between the sample and solvent also show marked influence (ALARA; ABDURAHMAN; UKAEGBU, 2021).

2.1 LLE extraction of phenolic compounds with NaDES

LLE studies using NaDES and by-products were searched, but no article was found under the key words used – "natural deep eutectic solvents"; AND "by products" AND "phenolic compounds" AND "LLE" OR "liquid-liquid extraction", even though this is a method that can be applied to this type of solvent and agri-food waste. NaDES low volatility may prove to be a disadvantage when there is a need to remove the phenolic compounds from these solvents, so the LLE can be a solution, despite requiring the use of organic solvents (FUNARI et al., 2019). There is a range of organic solvents that are able to form a two-phase mixture with NaDES. The aprotic solvents like hexane, heptane, acetonitrile, ethyl acetate are immiscible with NaDES, while protic solvents – ethanol, methanol, water are miscible, therefore not appropriated for this LLE (PALOS-HERNÁNDEZ et al., 2022; RUESGAS-RAMÓN; FIGUEROA-ESPINOZA; DURAND, 2017).

Hydrophobic NADES have shown to be promising in liquid-liquid extraction applications, as they can effectively extract analytes from aqueous samples. They have been used to extract volatile organic acids, antibiotics, dyes, and other compounds. The use of hydrophobic NADES in liquid-liquid extraction contributes to the principles of green analytical chemistry by providing a sustainable and environmentally friendly approach to sample preparation (CAÑADAS et al., 2020; FUNARI et al., 2019; MANSOUR et al., 2024). There are studies showing the use of hydrophobic NaDES (H-NaDES) for microextraction. The H-NaDES remove the target compound from water or

aqueous sample replacing the organic solvents. The most common combination to achieve H-NaDES use thymol, menthol and camphor (MANSOUR et al., 2024) and their applications are associated mostly with the removal of lipophilic samples, organic pollutants and metal ions from aqueous phases (TANG; AN; ROW, 2021), while hydrophilic NaDES and organic solvents are widely implemented in bioactive compounds recovery, as most of them are also hydrophilic (CAÑADAS et al., 2020).

3. Future perspectives for the use of phenolic extracts in NaDES media

In addition to common use to extract and detect phenolic compounds from by-product matrices of oils, fruits and other AFW, NaDES extracts have been used for various applications, such as: cosmetics and personal care products; foods and nutraceuticals; pharmaceuticals. These solvents can retain the antioxidant effect of phenolic compounds in cosmetic formulations; can be incorporated directly into food and nutraceutical products to provide antioxidant and health benefits of phenolic extracts without the need for additional purification (FOURMENTIN; COSTA GOMES; LICHTFOUSE, 2021).

3.1 Cytotoxicity of NaDES and extracts

Cytotoxic activity refers to the ability of a substance or compound to cause cell damage or death. This can occur through different mechanisms, such as induction of apoptosis, necrosis, oxidative stress or inhibition of essential cellular processes (GUPTA et al., 2022; INAYAT et al., 2023). The evaluation of cytotoxic activity is important to determine the toxicity of compounds, especially in contexts of pharmacological research and development of new drugs but also new foods and cosmetics. This information is crucial to understanding the biological effects of compounds and their therapeutic or toxicological potential use (KOH et al., 2023; MARTÍNEZ; TOWNLEY; MARTÍNEZ-ESPINOSA, 2022).

3.1.1 Effect on tumor cells

The cytotoxicity of NaDES depends on factors such as the cell lines used, the molar ratio of the components and the synergy between the cellular components and the NaDES. In human colorectal cancer cells (HT-29 and Caco-2) betaine:citric acid NaDES

showed higher cytotoxic effect, while breast cancer cells (MCF-7) suffered only moderate cytotoxic activity in combinations using choline chloride as acceptor of hydrogen bonds and ethylene glycol, acetamide and glycerol as hydrogen donors. In liver cancer cells (HepG2) there was low cytotoxicity when choline chloride was associated with organic acids such as lactic and malic acid (KOH et al., 2023).

3.1.2 Effect on non-tumor cells

In non-cancerous cells, HaCaT (epithelial cells) lactic acid: 1,2-propanediol and choline chloride: 1,2-propanediol showed low toxicity, when associated with propolis extract the cytotoxic effect was greater than the pure solvents (TRUSHEVA et al., 2024). NaDES containing organic acids such as malic acid, citric acid and oxalic acid had greater cytotoxic activity in fibroblast cells (L929), while sugar-based NaDES (glucose, fructose and sucrose) were less cytotoxic in these cells. NaDES composed of citric acid also showed greater toxicity in fish cells (CCO), along with NaDES of 1,2-propanediol. Also an organic acid, lactic acid had greater cytotoxicity in mouse cells (3T3). Meanwhile, NaDES composed of choline chloride:glycerol had low to moderate action on oral epithelial (OKF6), mouse (3T3) and fish (CCO) cells (JOARDER et al., 2023; KOH et al., 2023).

In summary, the studies so far indicate that NaDES based on sugars and polyols tend to be less toxic to healthy and cancerous cells than those containing organic acids, due to the impact of acidic pH on toxicity (GONZALEZ–DIAZ; GARCÍA–NÚÑEZ, 2023). The composition and proportion of NaDES components are important factors that influence their cytotoxicity, regardless of the cell type.

3.2 Antimicrobial activity

Antimicrobial activity refers to the ability of a substance or compound to inhibit the development or cause the death of microorganisms, such as bacteria and fungi. This function can be evaluated through *in vitro* tests such as: determination of the minimum inhibitory concentration (MIC), determination of the minimum bactericidal/fungicide concentration (MBC/MFC) or evaluation of the dehydrogenase activity (DEHA) of the treated microorganisms, through the MTT assay (MOHAMMADNEZHAD; VALDÉS; ÁLVAREZ-RIVERA, 2023). The antimicrobial activity of natural compounds can be

applied to the preservation of foods, preventing spoilage and the proliferation of harmful microorganisms (TRUSHEVA et al., 2024).

3.2.1 Antimicrobial effect of food products

Grape pomace extract dispersed in NaDES media, without any step for extracting phenolic compounds, was added to polypropylene food packaging films in order to test its antimicrobial activity against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria. The antimicrobial effect was attributed to the presence of gallic acid and quercetin that inhibit the synthesis of nucleic acids in microorganisms (MACHADO et al., 2024). The associated use of sour cherry pomace and NaDES, extracts based on choline chloride: malic acid, exhibited the lowest minimum inhibitory concentration values (0.781%) against different bacteria strains, including *Staphylococcus aureus, Escherichia coli, Salmonella enterica* and *Pseudomonas aeruginosa*. The antimicrobial activity of malic acid-based NADES has been attributed to its acidic nature (POPOVIC et al., 2022). Also in Popovic et al. 2022, the sour cherry pomace in choline chloride:fructose showed the highest antibacterial activity against P. *aeruginosa* with an MIC of 6.25% and choline chloride:urea was the least potent. In Trusheva et al. (2024) the propolis extract in NaDES, in lactic acid:fructose, was more efficient (10 µg mL^{-1}) in antimicrobial activity than the 70% ethanolic extract (443 µg mL^{-1}) in S. *aureus*, B. *cereus*, L. *monocytogenes* and C *albicans* (TRUSHEVA et al., 2024).

3.2.2 Antimicrobial activity of NaDES

The use of choline chloride:glycerol NaDES decreased the minimum inhibitory concentration (MIC) of four types of catechins against *Staphylococcus aureus* and *Pseudomonas putida*. When determining the inhibition halo, where the samples need to be diffused in agar, the authors reported difficulty in diffusing the samples in agar due to the viscosity of the NaDES. Compared to aqueous solutions of epicatechin gallate and the MIC was 10 mg mL^{-1}, while in NaDES 0.5 mg mL^{-1} for S. *aureus* and 4.5 mg mL^{-1} and 1.75 mg mL^{-1} between aqueous and NaDES, respectively, in P. *putida* (ZHOU et al., 2019).

In general, antimicrobial activity was influenced by the diffusion ability of the compounds, incubation temperature, in addition to the microorganism and composition of the NaDES, since the pH of the solvent varies according to the composition used.

NaDES also show antioxidant activity, which contributes to antimicrobial properties and improve the stability and bioavailability of bioactive compounds when compared to organic solvents (QUEFFELEC et al., 2024).

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CHAPTER 3

"Natural deep eutectic solvents characteristics determine their extracting and protective power on chlorogenic acids from sunflower meal"


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Natural deep eutectic solvents characteristics determine their extracting and protective power on chlorogenic acids from sunflower meal

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Abstract

Sunflower meal is a residue of the edible oil extraction and a promising source for the extraction of phenolic compounds, especially the chlorogenic acid (CGA) family. Their clean extraction and later conservation may be improved by the use of natural deep eutectic solvents (NaDES). This study evaluated the extraction and stability of phenolic compounds from sunflower meal using different formulations of NaDES as extractor, in contrast to ethanol (40%). The main goal was to find a clean extraction method for this rich bioactive compound by-product that would also enhance its stability and bioactivity. The best solvent was the NaDES formulated with lactic acid:glucose (LA:G - 5:1) that resulted in an extract containing 1786 mg CGA per liter of extract, followed by ethanol 40% (1305 $mg.L^{-1}$) and choline chloride:glycerol (807 $mg.L^{-1}$). The results for the antioxidant activity of the extracts did not follow the results for chlorogenic acid concentration, as they were higher in the ethanolic extract (102.59 and 90.35 mg/100g - ABTS and DPPH, respectively) than in the NaDES extracts (83. 36 and 66.56 mg/100g for LA:G and 48.26 and 26.17 mg/100g for CC:GL). In terms of stability, the acidic NaDES (LA:G) showed better protection against heat treatment (60ºC) and storage (30 days), while ethanol exhibited better stability under light exposure (7 days). The findings suggest that the NaDES composition, pH and other characteristics may influence both, extraction efficiency, antioxidant activity and stability, enabling the clean extraction and stabilization of phytochemicals from agro-industrial waste.

Keywords: phytochemicals; phenolic compounds; *Helianthus annuus*; green solvent; bioactive compounds; bioeconomy

1. Introduction

Sunflower seed is the third most produced oilseed in the world, with an output of 57.27 million tons per year. Its largest producers are Ukraine (30.5%), Russia (27.2%) and the European Union (17.9%) (USDA, 2023). The foremost purpose of the seed is the production of edible oil. From this process, 30% of the seed remains as meal, an unavoidable by-product of the extraction of sunflower oil that is mainly used as animal feed, with a high protein content. Based on the world production, an estimate of circa 17.2 million tons of sunflower meal is globally generated every year. This by-product is rich in phenolic compounds, especially those belonging to the chlorogenic acid (CGA) family, which represent more than 70% of the phytochemicals in the sunflower meal (KAREFYLLAKIS et al., 2017; ZHANG et al., 2022).

In the extraction of “green" products, some of the desirable characteristics of the solvents are to be nontoxic, non-flammable and non-explosive, biodegradable, non-polluting, with the possibility of reuse or recycling, among others (BUBALO et al., 2016). With the growing demand for clean label products, natural deep eutectic solvents (NaDES) have emerged as an alternative to organic solvents. NaDES are formed by mixing at least two components, a hydrogen donor, and an acceptor that, when mixed under specific conditions, form a eutectic mixture, which is liquid at low temperature (BASHIR et al., 2023). These solvents show no flammability or volatility and little toxicity, unlike conventional organic solvents, characteristics that are among the main advantages of their use. In addition, NaDES have been used for the extraction of phenolic compounds due to some of their properties - viscosity and polarity, mostly - which, when optimized, show higher efficiency then the regularly used organic solvents or water (PAIVA et al., 2014). It has been demonstrated that NaDES plant and seaweed extracts do not pose health risks when ingested or topically applied (SHIKOV et al., 2022a, 2022b) and NaDES have already been successfully applied for the extraction of polar and non-polar compounds such as coumarins (ARYATI et al., 2020), isoflavones (DURU et al., 2022), saponins (PETROCHENKO et al., 2023), polysaccharides (NICOLAE et al., 2019), phlorotannins (OBLUCHINSKAYA et al., 2023), anthocyanin (ASLAN TÜRKER; DOĞAN, 2022).

The extraction of phenolic compounds has been studied using different techniques. For some raw materials, time consuming methods were used, such as extraction by mechanical agitation or using the Soxhlet apparatus, applying organic

solvents such as ethanol and methanol (ALARA; ABDURAHMAN; UKAEGBU, 2021; MIR-CERDÀ et al., 2023). However, such methods can lead to hydrolysis and oxidation of phenolic compounds due to their susceptibility to thermal degradation (MIR-CERDÀ et al., 2023). Ultrasound (ZARDO et al., 2017) or microwave (CALDAS et al., 2018) extraction methods have also been studied as alternatives for faster extractions, with better yield, quality, and purity, especially from fruits and their by-products.

The storage stability of phenolic compounds depends on temperature and time, in addition to extraction and storage conditions (CHENG et al., 2017). Different studies have evaluated the stability of phenolic compounds using NaDES as solvent e.g., for the extraction of *Catharanthus roseus*, an ornamental plant (DAI et al., 2016), turmeric (JELIŃSKI; PRZYBYŁEK; CYSEWSKI, 2019) and safflower (DAI; VERPOORTE; CHOI, 2014), compared to conventional organic solvents or water. The extracts that used NaDES as solvent showed greater stability, which was attributed to the strong hydrogen bonds between the solutes and the solvent. The high viscosity of these solvents also seemed to contribute to the stabilization of these molecular interactions (DAI; VERPOORTE; CHOI, 2014).

The aim of the present study was to investigate the extraction of phenolic compounds from sunflower meal using two different NaDES formulations, with the aid of ultrasound, and to assess the stability of the extract over time – after heat treatment, at different storage temperatures, and exposure to light. This work hypothesized that the use of NaDES would be more efficient to extract phenolic compounds from sunflower meal, in addition to improving stability, when compared to a hydroethanolic solution. This is, to the extent of our knowledge, the first time this approach has been applied to the valorization of this residue and promising CGA source (ZHANG et al., 2022).

2. Materials and Methods

2.1 Vegetable raw material

The sunflower meal used in this work was provided by the Caramuru company, located in Itumbiara - GO, Brazil. The meal was supplied desolventized and pelletized. Oil extraction was achieved by a 2-step process, namely screw-press and hexane extraction, of the dehulled and cooked seeds. Solvent removal was carried out by a steam desolventizer-toaster, immediately followed by pelletization of the material.

2.2 NaDES

The components and the different proportions and combinations for preparing the solvents are shown in Table 1.

The components were weighed directly into hermetic flasks and placed under magnetic stirring at 50ºC (Gehaka, model AA2050LED, São Paulo, Brazil), until a transparent and homogeneous liquid was formed. Ultra-pure water was added to the solvents to reach 30 g.100g^{-1} of NaDES, according to Radosevic et al (2016), who recommended this amount of water as safe to improve the characteristics of NaDES without destabilizing the desired macromolecular structure. The amount of water in the components used to produce the NADES were considered in the NaDES formulation.

Table 3.1. Composition and characteristics of NaDES.

NaDES	**Composition**		**Molar ratio**	**pH NaDES**	**Aw**	**Viscosity mPa.s**	**Density g.mL^{-1}**
LA:G	Latic acid	Glucose	5:1	0.73	0.68	79.24	1.222
CC:GL	Choline chloride	Glycerol	1:1	6.35	0.08	84.11	1.138
Et	Ethanol	Water	0.4:1*	5.54	0.92		0.930

*(v/v). Aw – water activity.

2.3 Extraction of phenolic compounds

Sunflower meal (4.50 g) was weighed and 30 mL of NaDES or hydroethanolic solution (40% ethanol - used as a control) were added. All samples were heated under agitation up to 45°C, followed by sonication by a probe ultrasound equipment (Ultronique, Desruptor, São Paulo, Brazil) for 1 minute, at maximum power (99%), according to Zardo et al (2017). After extraction, the extracts were filtered through polyester cloth, to eliminate solid particles, and stored at -80ºC in an ultra-freezer (INDREL, IULT 335 D, São Paulo, Brazil) until analysis.

2.4 Identification and quantification of phenolic compounds by HPLC

The extracts prepared in NaDES, and the control were analyzed by HPLC, for quantification of CGA and evaluation of the general profile of phenolic compounds. A high-performance liquid chromatograph with PDA detector and column oven (Perkin Elmer, Flexar, Connecticut, USA) was used. Other analysis conditions were: reversed phase column (Kromasil - 100-5-C18 4.6 × 250 mm), at 40 ºC, injection volume of 20 µL, flow rate of 0.8 mL/min. Mobile phase: 0.3% formic acid in water (A); 100% methanol (B); 100% acetonitrile (C). Elution gradient: 0.1 min, 85% A, 14.5% B and 0.5% C; 7 min, 55% A, 43.5% B and 1.5% C; 14 min, 5% A 93% B and 2% C; 20 min, 1% A, 97% B and 2% C; 23 min, 15% A, 83% B and 2% C; and 23-33 min, 85% A, 14.5% B and 0.5% C. Chlorogenic acid (1,4,5-trihydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)), with purity level ≥ 95% (Sigma-Aldrich), was used as standard. A water: methanol (97:3, v/v) solution was used to dilute the standard and all samples. Analyzes were performed in duplicate at 260, 280 and 320 nm. The identification was performed by retention time comparison and the quantification was calculated according to a standard curve in the concentration range of 0.5 to 20.0 $mg.L^{-1}$.

2.5 Stability

The extracts in NaDES and the hydroethanolic extract, used as a control, were evaluated for stability of the CGAs to heat, exposure to light and storage for 30 days at room temperature, under refrigeration and freezing, according to Dai et al (2014). For analysis, samples kept at low temperature were heated to 25ºC in a water bath. All stability tests were performed in triplicate and the results were evaluated by analysis of variance (two-way ANOVA), using the GraphPad Prism software (version 5.0) with a confidence level of 95%.

To evaluate the thermal stability, the extracts were placed in sealed cryogenic tubes and submitted to a metabolic bath (Marconi, MA093, São Paulo, Brazil) preheated at 40, 60 and 80°C. Samples were removed from the bath after 10, 30, 60 and 120 minutes, and immediately cooled in an ice bath to 25°C.

The effect of light incidence was evaluated on the extracts, in sealed cryogenic tubes, exposed to white light (LED 12 W OSRAM) or protected by aluminum foil

(control), at room temperature. Samples were collected after 0, 3, 7 and 15 days for analysis.

To assess the effect of storage under refrigeration (8ºC ± 2ºC), freezing (-18ºC ± 2ºC) and at room temperature (25ºC ± 3ºC), the samples were kept in microcentrifuge tubes and samples were taken after 0, 3, 7, 15 and 30 days, for analysis.

During the stability assays quantification of CGAs, in NaDES and hydroethanolic extracts, was estimated by evaluating the total content of phenolic compounds by the method proposed by Wang et al. (2019), using CGA as a standard. Briefly, the samples were homogenized in vortex and 200 µL aliquots were taken and added to 200 µL of a Fe^{III} solution (3.0 mM) – composed of Ferric Chloride (1.5 mM) and Potassium Ferricyanide (1.5 mM) – vortexed and reserved for 10 minutes. After that time, 200 µL were transferred to a 96-well microplate and the samples were read at 790 nm in a plate reader (PerkinElmer, VICTOR Nivo, Massachusetts, USA). The samples were analyzed in quadruplicate.

2.6 Determination of antioxidant activity

To evaluate the antioxidant activity of solvents and extracts, two different methods were used, and the results were expressed in Trolox equivalents. The DPPH (2,2-diphenyl-1-picrylhydrazyl) method was performed according to Pires et al. (2017). The microplate was incubated in a dark environment for 30 minutes and the absorbance was read at 517 nm. The ABTS (2,2'-azino-bis 3-ethylbenzothiazolin 6-sulfonic acid) method was adapted from Torres et al. (2017). The microplate was incubated for 20 minutes in the dark and the absorbance was read at 734 nm. Blanks were performed by replacing the sample volume with methanol.

3. Results

3.1 Extraction

As can be observed in Table 2, the solvent that extracted the highest concentration of CGA was the NaDES containing lactic acid and glycerol (LA:G), followed by the hydroethanolic solution (Et), with 26% less than LA:G and, lastly, the NaDES containing

choline chloride and glucose (CC:GL), with a final concentration of CGA, 54% lower than that of LA:G and 38% lower than that of the Et extract.

Table 3.2. Concentration of total phenolic compounds in extracts.

NaDES	pH extracts	[CGA]* mg.L^{-1}
LA:G	1.3	1786
CC:GL	5.7	807
Et	5.8	1305

* quantified by HPLC

Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol; [CGA] – chlorogenic acid concentration.

The chromatograms (Figure 1) show that the major compound extracted had an elution time of 8.5 min and maximum absorption at the wavelength of 320 nm. It was identified as CGA by comparison with the standard. It is also possible to verify that the 3 solvents extracted similar compound profiles, as observed by their absorbance of the 320 nm wavelength. Chromatographic analyzes of a standard curve made of chlorogenic acid (Supplementary Figure 1) and the pure solvents (blanks) were performed. The latter showed just the peaks corresponding to the solvents' components (Supplementary Figures 2 and 3).

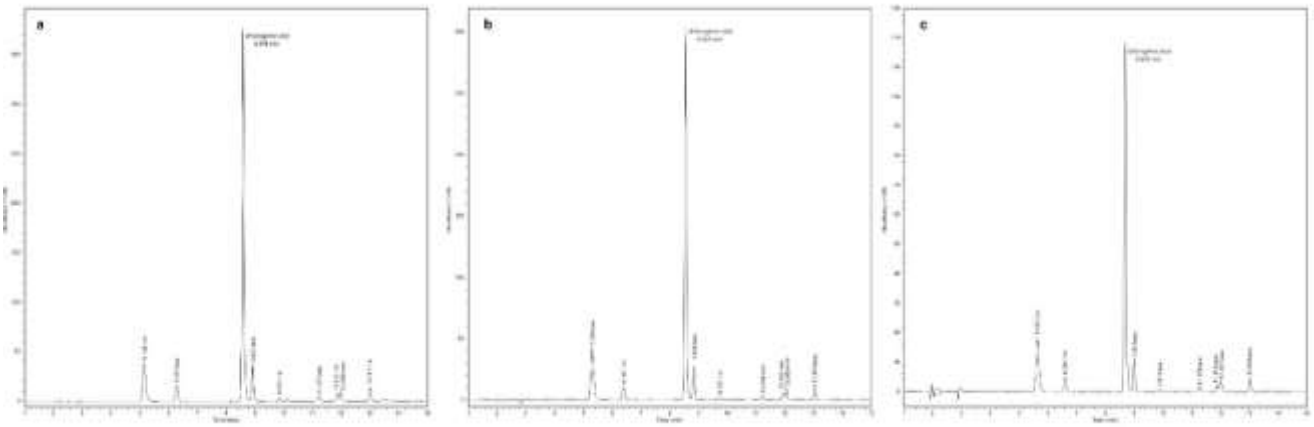

Figure 3.1. Example of chromatograms of sunflower meal extracts at 320nm. (a) Et; (b) LA:G; (c) CC:GL. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol.

3.2 Antioxidant activity

Tables 3 and 4 present the results for antioxidant activity of the solvents and the extracts, respectively. It is clear from the observation of the results that pure solvents, either NaDES or ethanol, present no measurable antioxidant activity in the tested conditions, and that the extracts show considerable antioxidant activity, which may be completely attributed to the phytochemicals extracted from the sunflower meal. However, some influence of the solvent seems to occur in the measurement of this activity, since the highest extraction of chlorogenic acid was achieved using LA:G followed by ethanol and CC:GL, but antioxidant results were higher for the ethanolic extract, followed by LA:G and CC:GL.

Table 3.3. Antioxidant activity of pure solvents by DPPH and ABTS methods

Solvents	ABTS (mg 100 mL^{-1})*	DPPH (mg 100 mL^{-1})*
LA:G	0.04 ± 0.1^{a}	-0.07 ± 0.2^{a}
CC:GL	-0.06 ± 0.1^{b}	-0.15 ± 0.2^{a}
Et	-0.76 ± 0.2^{c}	0.17 ± 0^{a}

* mean and SD in milligrams of Trolox per 100 mL of solvent. Different lowercase letters represent a significant difference between solvents. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol

Table 3.4. Antioxidant activity of sunflower meal extracts by DPPH and ABTS methods

Extracts	ABTS (mg 100 g^{-1})*	DPPH (mg 100 g^{-1})*
LA:G	83.36 ± 2.81^{c}	66.56 ± 0.8^{b}
CC:GL	48.26 ± 1.99^{a}	26.17 ± 0.7^{c}
Et	102.59 ± 1.14^{b}	90.35 ± 0.2^{a}

* mean and SD in milligrams of Trolox per 100 g of sunflower meal. Different lowercase letters represent a significant difference between extracts. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol

3.3 Stability

3.3.1 Heat stability

Figure 2 presents the results obtained for the heat stability tests of the 3 evaluated extracts. It is possible to observe that the heat treatment at 40ºC and 60ºC, regardless of their duration, caused a significant reduction in the content of phenolic compounds in both the hydroethanolic (Figure 1A) and the CC:GL extracts (Figure 1C). In the

hydroalcoholic extract, these losses ranged from 30.6% (10'; 80°C) to 100% (60'; 40°C and 60°C), while in CC:GL the losses were 40.7% (10'; 80°C) to 100% (120'; 60°C). For treatments at these same temperatures, LA:G proved to be significantly more protective than the other extractors, showing no loss after treatment at 60°C (Figure 1B). At 80ºC, this last extractor (LA:G) did not maintain its protective capacity, presenting a loss of 96.9% after 30 minutes of treatment, similar to those suffered in CC:GL at 80ºC, and hydroethanolic extract at 60°C.

However, in this extract (LA:G), at a temperature of 60ºC, there seems to have been an unexpected increase in the concentration of phenolic compounds in relation to the initial extract, regardless of treatment duration. In this same unforeseen way, treatment at 80ºC seems to have been less harmful to the phenolic compounds in the hydroethanolic extract than the lower temperatures tested.

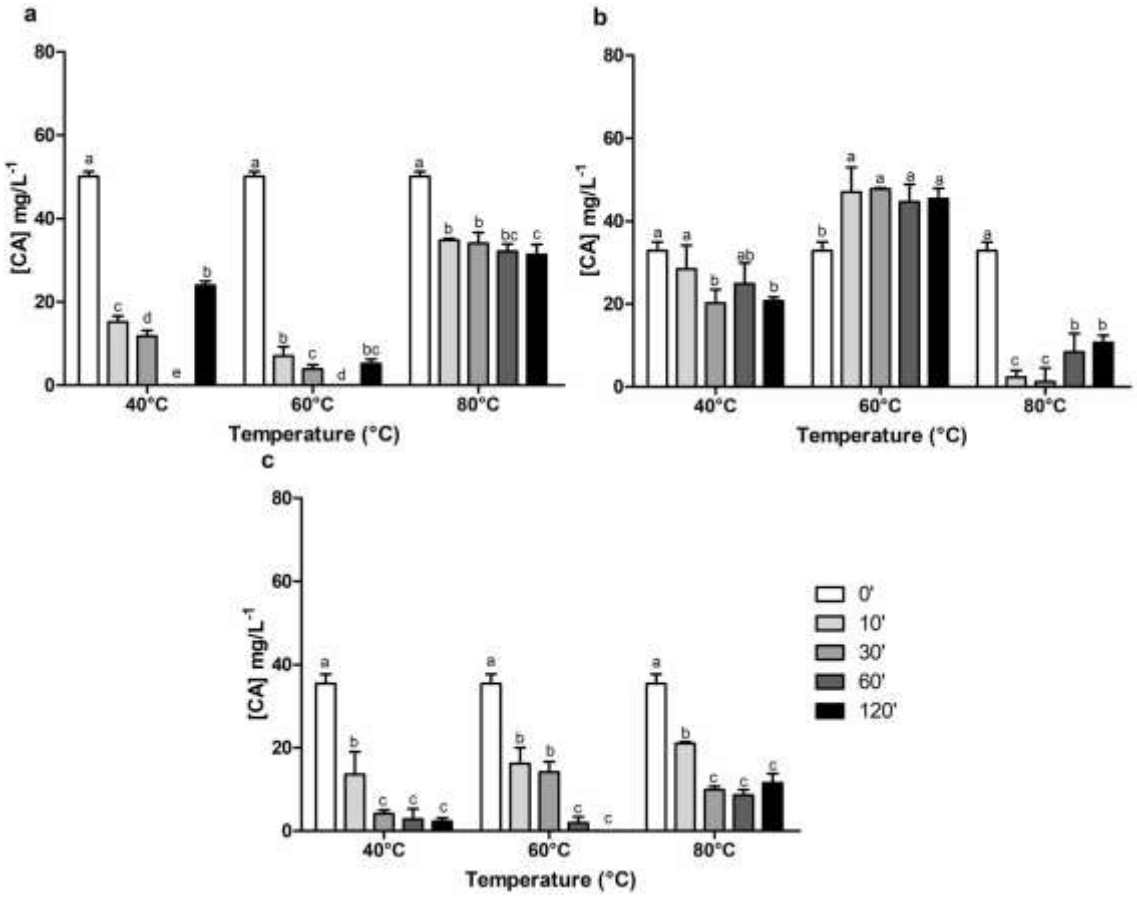

Figure 3.2. Heat stability of sunflower meal phenolic extracts. (A) Et; (B) LA:G; (C) CC:GL. Different lowercase letters represent a significant difference between heat treatment duration in the same solvent. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol; [CA] mg/L^{-1} – concentration of chlorogenic acid in milligram per liter of extract. The apostrophe on 1C legend image stand for "minutes"

3.3.2 Storage stability

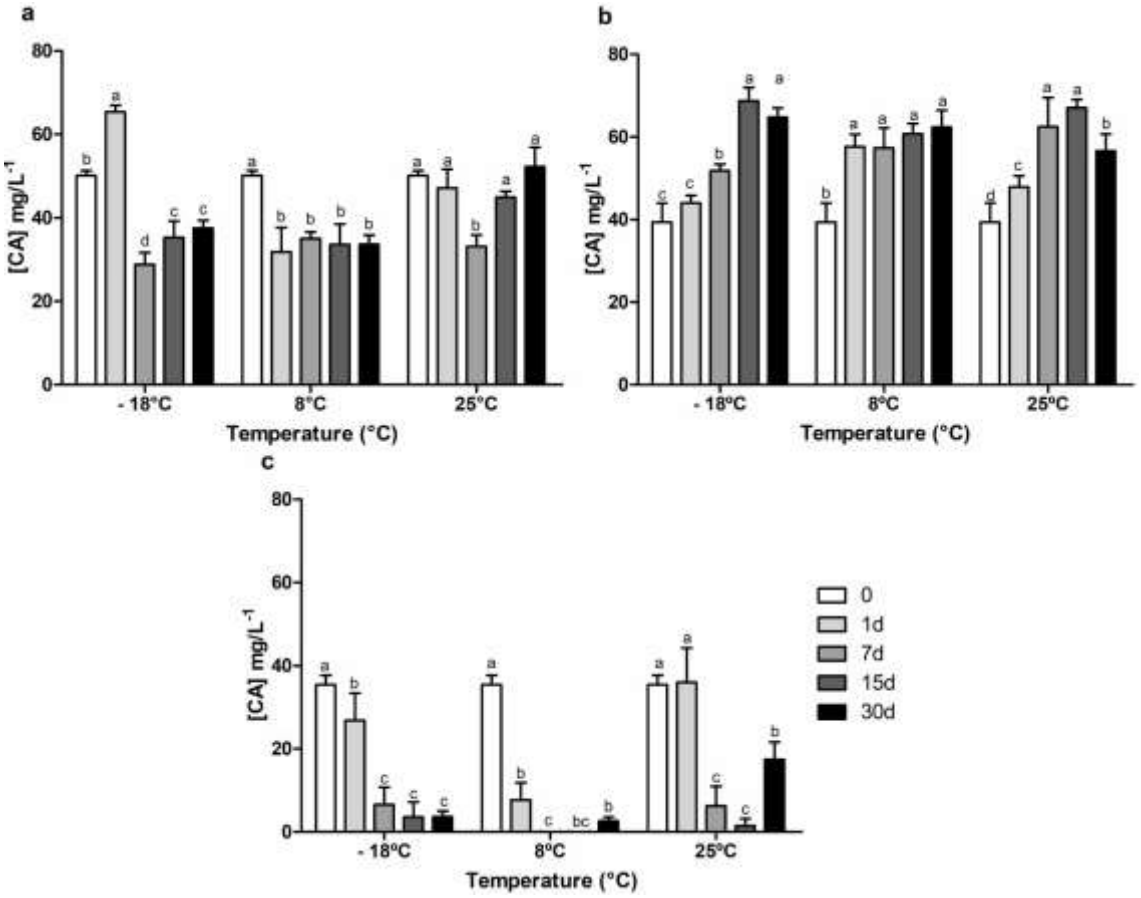

Figure 3.3. Storage stability of sunflower meal phenolic extracts. (A) Et; (B) LA:G; (C) CC:GL. Different lowercase letters represent a significant difference between storage times in the same solvent. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol; [CA] mg/L^{-1} – concentration of chlorogenic acid in milligram per liter of extract. The letter d on 2C legend image stand for "days"

The results obtained after the storage stability study are shown in Figure 3. In the hydroethanolic extract (Figure 2A), storage at -18°C showed the greatest loss, with a maximum of 42.5% (7d). The lowest loss in this extract was 10.4% after fifteen days at 25°C, similarly to what was observed in the heat treatment, when higher temperatures unexpectedly resulted in higher final concentrations of CGA in this extract. In CC:GL (Figure 2C) a loss variation of 24.2 to 95.9%, in 1d at 25°C and 15d at 25°C, respectively, was found. The loss reached 100% in the 7d to 8°C. This extract also behaved similarly to what was observed in the heat treatment, with a constant reduction in the concentration of CGA over time, accelerated with the increase in the storage temperature. In LA:G

(Figure 2B), there was an increase in the concentration of total phenolic compounds at all storage temperatures, ranging from an addition of 21.5 (1d to 25°C) to 74.5% (15d to -18 °C). This behavior was also similar to that observed during heat treatment, when the phenolic compounds content in this solvent was higher after treatment than before. It was observed that the increase in the concentration of phenolic content in the LA:G extract stabilized after day #1 at 8°C. In this solvent there was also a formation of granules (Supplementary Figure 2) from day #7 in the samples stored at -18°C and 8°C and on day #30 in the extracts at 25°C.

3.3.3 Light stability

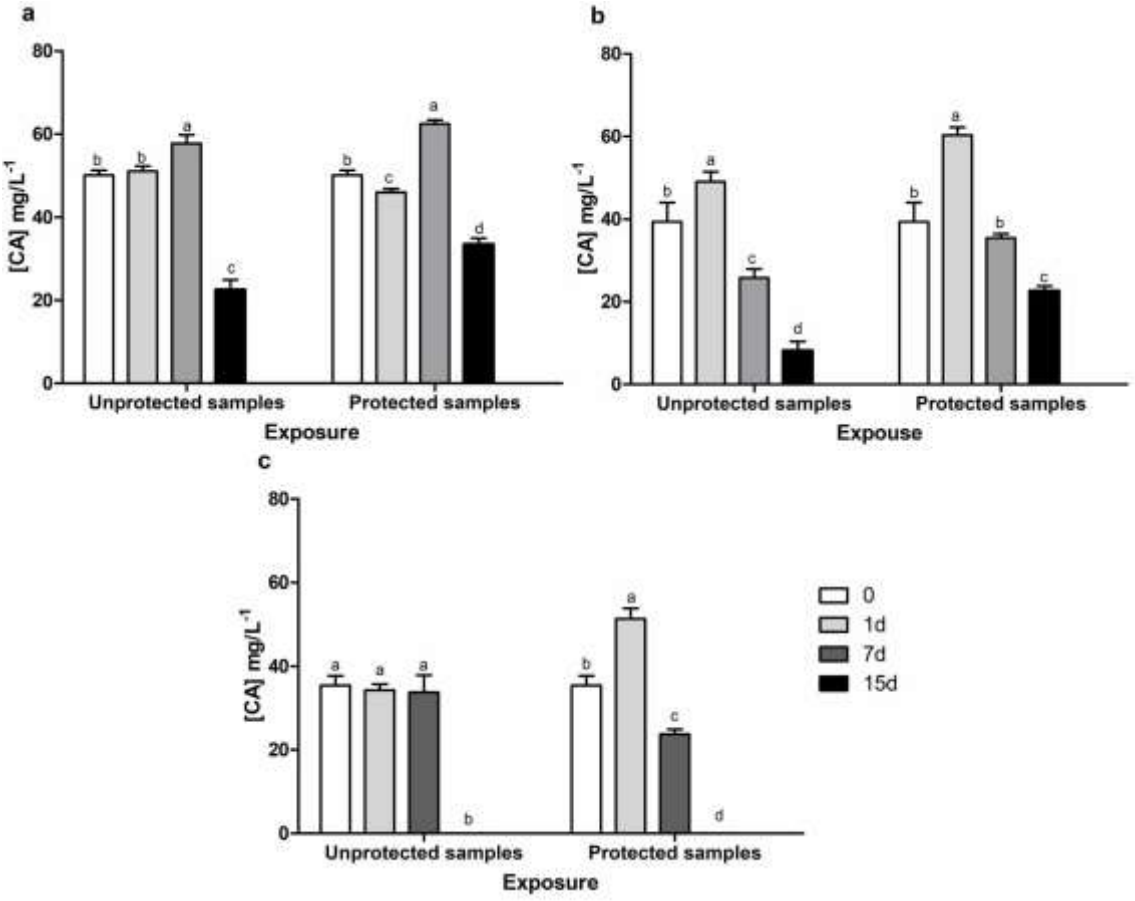

Figure 3.4. Light stability of sunflower meal phenolic extracts. (A) Et; (B) LA:G; (C) CC:GL. Different lowercase letters represent a significant difference between treatment times in the same solvent. Et – ethanol; LA:G – NaDES made of lactic acid and glucose; CC:GL – NaDES made of choline chloride and glycerol; [CA] mg/L^{-1} – concentration of chlorogenic acid in milligram per liter. [CA] mg/L^{-1} – concentration of chlorogenic acid in milligram per liter of extract. The letter d on 3C legend image stand for “days”

The results of stability to exposure to white light are presented in Figure 4. In the hydroethanolic extract (Figure 3A), the greatest loss was 55.1% after 15 days of direct exposure to white light. This was the only treatment after which the ethanol extract retained a higher CGA content than LA:G. In LA:G (Figure 3B), there was a maximum loss of 79.2% after 15 days of light exposure. The protected samples of this extract showed a similar pattern of loss of phenolic content, but the maximum loss in these was 42.6% (15d). As in the storage stability study, CC:GL (Figure 3C) showed total CGA loss after 15 days when exposed to or protected from artificial white light. Also as with storage stability, there was an increase in phenolic content in the LA:G extracts after 1 day of exposure in all samples. However, after exposure to white light, this increase was also observed in hydroethanolic extracts and in CC:GL. A discoloration of the extracts was also observed, a little less pronounced in the samples with the reflective protection, except for LA:G, in which there seemed to be no difference between the two samples (Supplementary Figure 3).

4. Discussion

4.1 Extraction

The main phenolic compounds of sunflower and its meal are the family of chlorogenic acids (CGAs), esters of quinic acid and trans-cinnamic acid, which may represent up to 70% of their total phenolic compounds (CLIFFORD, 2000; KAREFYLLAKIS et al., 2017; NASCIMENTO et al., 2023). The extraction of CGAs usually applies methanol, ethanol, or acetone in association with water, which improves solubility. Among the above, ethanol may be considered a GRAS solvent, but it is still flammable, unlike NaDES. The concentration of organic solvent for extraction often depends on the food matrix and acidification may also be required (FROSI et al., 2021). The NaDES formulations tested in the present study were designed based on two main parameters: efficiency reported in the literature and estimated cost. NaDES formulations containing choline chloride as a hydrogen acceptor are among the most used in studies for the extraction of phenolic compounds from plant material (HIKMAWANTI et al., 2021). However, this reagent is expensive and may have application limitations in cosmetic products in Europe (RENTE et al., 2022). The designed alternative was based on edible, low-cost compounds that are commonly used in the food industry.

Furthermore, it was also desired that the resulting NaDES presented an acidic pH, related to higher extraction efficiency of phenolic compounds (FROSI et al., 2021).

The use of different extractors, in the present work, resulted in extracts with very similar characteristics when evaluated by HPLC, with a major chlorogenic acid peak and some minor peaks. These peaks, although not identified by comparison with standards, were quite similar in all extracts, regardless of the extractor used (Supplementary Figure 1). This may indicate a similar affinity of the two NaDES and the hydroethanolic solution tested for the phenolic compounds present in the sunflower meal, despite the significant differences in pH, density, and viscosity among them. It could also be due to the uniform composition of the phenolic fraction in sunflower meal, as according to the work of Nascimento et al. (2023), of the 10 most abundant secondary metabolites obtained from sunflower meal, six belonged to the CGA family. Another study that used different NaDES formulations to extract phenolic compounds and also obtained a similar peak pattern for different extractors was the work by Souza et al. (2022). In this study, *Eugenia uniflora* leaves extracted with different compositions of NaDES (malic acid:sorbitol, malic acid:glucose:fructose, lactic acid:sucrose, choline chloride:glucose and choline chloride:lactic acid) showed mainly gallic acid, acid ellagic acid and quercetin in all extracts. The same seems to have also been verified in the extraction of sunflower disks with NaDES (choline chloride:1,4-butanediol) and ethanol, where CGA was identified and in the extraction of tomato by-product with NaDES (choline chloride:lactic acid, choline chloride:1,2-propanediol) and ethanol where gallic acid, caffeic acid and CGA were identified by HPLC . Thus, in the present study, the only difference between the extracts obtained seems to have been the concentration of CGA extracted from the initial raw material (LA:G > Et > CC:GL).

Regarding the relative extraction efficiency, the recent literature on the subject presents quite varied results when comparing similar NaDES formulations with ethanol. Barbieri et al. (2020) evaluated the extraction potential of four different NaDES formulated with choline chloride, compared to ethanol (100%). All NaDES showed better results than ethanol in the extraction of phenolic compounds from rosemary, except of the one containing glycerol (CC:GL), also used in the present study. Another study also obtained low concentrations of phenolic compounds from kale by-product using a glycerol:betaine (3:1) NaDES formulation. In this case, however, the result obtained with NaDES was still superior to the ethanolic and aqueous extracts tested (LEE et al., 2023).

In the extraction of phenolic compounds from orange peel, there was no significant difference between NaDES composed of lactic acid:glucose (LA:G), L-proline:malic acid and 50% ethanol (GÓMEZ-URIOS et al., 2022). The extraction of chlorogenic acid from *Artemisiae scopariae* was studied by Yue et al. (2021), two formulations stood out when compared to ethanol and water, sorbitol:choline chloride and lactic acid:glucose (LA:G).

The performance of NaDES in the extraction of phenolic compounds may be influenced by their viscosity. Higher viscosities are due to a greater number of H bonds, which increases NaDES solubilization capacity. In contrast, excessively high viscosities hinder the diffusion of the desired compounds, decreasing the extraction (LIU et al., 2019; VIÑAS-OSPINO et al., 2023). In the present study, the solvent with the highest viscosity was CC:GL, which was also the least efficient extractor of chlorogenic acid from sunflower meal. These results agree with the findings of Alam et al. (2012), who stated that preparations with choline chloride tend to show higher viscosity and reduce the transfer of the target compound from the solid matrix. It is possible that the high viscosity of this NaDES has hindered the extraction of phenolic compounds from the raw material. If this was the case, it is also possible to infer that the small difference in viscosity between the tested NaDES (see Table 1) was already enough to affect their extraction ability.

Another factor that may influence the extraction of CGAs is the pH of the solvent. In the present study, all solvents were acidic (LA:G >> Et > CC:GL - Table 1), and the most acidic NaDES showed higher extraction efficiency. On the process of obtaining phenolic compounds from hazelnuts skin, choline chloride:lactic acid extracts yielded 170% more when compared to water . Two other extraction studies also used NaDES composed of choline chloride:lactic acid as solvent and, in both, this composition was superior for the extraction of phenolic compounds, including CGAs, from tomato pomace and coffee pulp. In coffee pulp, extraction with betaine:glycerol (pH 8.78) was 60% lower when compared to choline chloride:lactic acid, and lower even than ethanolic extract (RUESGAS-RAMÓN et al., 2019). In tomato pomace the NaDES preparation using choline chloride:propanediol (pH 2.2) extracted approximately 30% less when compared to choline chloride:lactic acid (pH 0.5). A different amount of water in the preparation of the acid NaDES in these two studies caused a pH value shift from 0.50 to 0.78 (RUESGAS-RAMÓN et al., 2019). A study by Friedman; Jürgens (2000) evaluated the stability of CGAs and showed that pH values close to 7 tended to cause irreversible structural alterations. On the other hand, in the work of Mellinas et al. (2020), for the

extraction of phenolic compounds from cocoa shells, alkaline extractors formed pores enabling the extraction, while acidic or neutral solvents did not show the same efficiency.

When the use of hydroethanolic solutions was considered, the work of Zardo et al. (2017) evaluated ultrasound amplitude, temperature, and ethanol concentration. Ethanol concentrations higher than 45% lead to a decrease in the CGA extraction. The effect of ethanol concentration, temperature and pH was evaluated on the extraction of phenolic compounds from defatted sunflower seed husks. The best results were obtained at neutral pH, 50°C and at ethanol concentrations below 60% (JIA et al., 2021). In the present study, the hydroethanolic solution (40% ethanol) showed the lowest viscosity and intermediary pH and achieved 1.6 times higher extraction of phenolic compounds than CC:GL, whereas LA:G resulted in concentrations of CGA 1.3 higher than the Et extract. This NaDES showed the more acidic pH and intermediate viscosity.

4.2 Antioxidant activity

Several forms of bioactivity have already been attributed to compounds from the chlorogenic acid family: anticarcinogenic activity; anti-inflammatory activity; antibacterial activity and metal chelation. However, its most studied activity is as antioxidant. Like many phenolic compounds, CGAs are capable of donating electrons or hydrogens, neutralizing free radicals and generating low-energy radicals (ERCOLI et al., 2021). Abdalla et al. (2021) tested the antioxidant activity of the methanolic extracts of 7 different lineages of sunflower seeds. The results found (between 22.6 and 40.42 mg Trolox/g sample for DDPH and between 20.7 and 30.7 mg Trolox/g sample for ABTS) were higher than the values found in the present study, from sunflower meal.

Although CGA extraction was more efficient by LA:G followed by Et and CC:GL, the antioxidant activity of the extract did not follow the same trend, as the highest activity, by both methods tested, was found in Et. These results are not in line with the studies by Martinović et al. (2022) and Rukavina et al. (2021) that found a positive correlation between antioxidant activity and total phenolic content in aqueous and hydroethanolic extracts as well as in different NaDES formulations.

4.3 Stability

In the present work, the protective capacity of three different extractors on phenolic compounds extracted from sunflower meal was evaluated when exposed to heat treatment, storage in freezer, under refrigeration and at room temperature, and exposure to light, with and without reflective protection. In general, it may be said that LA:G showed the greatest protection to the phenolic compounds against heat treatment, especially at temperatures up to 60ºC, and during storage for 30 days, regardless of the temperature. The ethanol extract granted the lowest decomposition of phenolic compounds after exposure to light for up to 7 days.

4.3.1 Heat stability

Phenolic compounds in general tend to be sensitive to heat and decompose at temperatures above 60°C (DAI et al., 2016; DAI; VERPOORTE; CHOI, 2014; ZANNOU et al., 2020). In the present study, at treatment temperatures of 40 and 60ºC, LA:G showed a protective capacity against the thermal degradation of the phenolic compounds in the extract, while Et and CC:GL did not. NaDES protection against thermal degradation of phenolic extracts has already been described by Yue et al. (2021). In their study, chlorogenic acid from *Artemisiae scopariae* was stable at 85°C, showing less than 5% loss, after 30 minutes, in both proline:malic acid and lactic acid:glucose (LA:G) formulations. In another study, which evaluated the heat stability of curcuminoids, the degradation at 50°C was similar in all extracts, but at 80°C, the thermal degradation in the ethanolic and methanolic extracts was higher when compared to NaDES of citric acid:glucose (LIU et al., 2019). Dai et al. (2016) observed that there was little degradation of anthocyanins in all extracts tested at 40°C, but that these compounds were more stable in lactic acid:glucose (LA:G) than in acidified ethanol, at 60°C. This same group (DAI; VERPOORTE; CHOI, 2014) evaluated the heat stability of carthamin, which showed a half-life twice as high in xylitol:choline chloride than in water, at 60°C. In both studies, the greatest loss of phenolic compounds was recorded at 80°C, as well as in the present study.

The higher stability of phenolic compounds in DES media is associated with the binding between hydrogens of the target compound and the DES components, which is believed to decrease the oxidation and increase the solubility of phenolic compounds in these solvents (LIU et al., 2019). In the present work, although one NaDES promoted

greater thermal protection than the organic solvent, the other formulation tested did not behave in the same way. It is possible that, despite the formation of H bonds in NaDES, the little acidic pH (6.35 – Table 1) of this formulation contributed to the degradation of the CGA. The stability of CGA was pH dependent in the study by Narita and Inouye (2013). Stability increased as the pH was reduced, and CGA showed significant degradation in aqueous solution with pH values greater than 5, at 37°C. Krungkri and Areekul (2019) also confirmed the low stability of phenolic compounds at pH 6. In their work, the stability decreased as the treatment temperature increased, in 95% ethanol, 60% acetone and water, reaching maximum degradation at 80 °C, in an ethanolic solution at pH 6. However, the opposite was observed in the work by Zhou et al. (2022), where catechins were more stable to exposure at 80°C in NaDES composed of choline chloride:glycerol than in choline chloride:lactic acid.

Another unexpected behavior was also observed during the evaluation of the stability of the extracts to heat treatment and no similar results were found in the available literature. Both Et and LA:G showed higher concentrations of phenolic compounds after thermal treatment at 80ºC and 60ºC, respectively. Although all treatments were carried out in airtight tubes, several samples showed a reduction in volume after the treatment time, indicating a possible evaporation, either of the organic solvent or of the aqueous fraction (addition of 30% water) of the NaDES. In all cases, when a reduction in volume was verified, the sample was reconstituted with pure solvent, up to the initial volume. Even so, it is possible that the higher content of phenolic compounds detected in Et samples was a consequence of a partial concentration, by evaporation of the solvent during the thermal treatment at higher temperatures. When LA:G was heated to 60ºC, regardless of the treatment time, there was a significant increase in the content of phenolic compounds in the samples. This NaDES consisted of a liquid of high viscosity and density, which made it very difficult to separate the solid (sunflower meal) and liquid (NaDES) fractions after the extraction process. Thus, a residue of finely particulate solid material remained in suspension in the extract. It is possible that, with heating, changes occurred in the density, viscosity, and surface tension of the solvent, as described by Alam et al. (2021), thus promoting an improvement in the interaction with the phenolic compounds of the residues, generating an extraction of these compounds during the thermal treatment, which more than compensated for the thermal degradation that occurred over the treatment time. Following the same reasoning, it is possible that this

same extraction occurred at 80ºC, but the degradation that occurred at that temperature was greater than this additional extraction. The other formulation tested (CC:GL) was even more viscous and also contained suspended solid residue. However, apparently, the degradation of CGA that occurred in the little acidic pH of this solvent was greater than the possible additional extraction that may have occurred during the thermal treatment. Furthermore, this solvent was less efficient in extracting CGA under the initial extraction conditions, and may also have been less efficient during heating, when compared to LA:G.

4.3.2 Storage stability

In the present study, extracts with significant differences in pH were obtained, as shown in Table 2. LA:G, the most acidic of all, granted higher stability at different storage temperatures, while CC:GL, the closest to neutrality, did not. Et showed intermediate results, as well as pH value. According to Friedman and Jürgens (2000), the stability of phenolic compounds is associated with three main factors: pH, storage time and structure of phenolic compounds. In their study, alkaline media caused irreversible structural changes in caffeic, chlorogenic and gallic acids. Catechin, epigallocatechin and rutin were comparatively more stable, which was attributed to their greater number of aromatic structures, that provided higher stability at alkaline pH.

The protection of phenolic compounds in acidic NaDES was also observed by other authors. In the work by Gómez-Urios et al. (2022) the phenolic compounds from orange by-products in formulations containing lactic acid showed better upkeep at lower temperatures (4ºC > 25ºC). Phenolic compounds extracted from by-products of olive, onion, tomato and pear seeds with lactic acid:glucose (LA:G) remained stable for two months at -18°C and 4°C, but there was a loss of 90% of apigenin and quercetin in the aqueous extract in the same conditions (FERNÁNDEZ et al., 2018). The authors attributed the greater conservation in NaDES to the lower mobility of the compounds in these media, that might reduce contact with oxygen and delay oxidative degradation. The study by Aslan et al. (2022) evaluated the stability of anthocyanins from purple carrots in choline chloride:citric acid, at 4, 25 and 37°C, protected from light, for 90 days. Highest upkeep was achieved at 4°C, with a retention of 70% total phenols. In the study by Lee et al. (2023) 90% of the phenolic compounds from hazelnut skin were maintained in the

NaDES sample after 30 days of storage. Recently, Obluchinskaya et al. (2021) evaluated the ability of two different NaDES formulations containing lactic acid and of ethanol to preserve the total phenolic content of a seaweed (*Fucus vesiculosus*). After a 360 days period, the ethanolic extract lost almost 70% of its initial concentration whereas NaDES of lactic acid:choline chloride and lactic acid:glucose presented a much lower loss of 20% and 40%, respectively (OBLUCHINSKAYA et al., 2021). The results of the present study, as well as those from the literature mentioned above, point to the acidic pH as an extremely important factor in the storage of phenolic extracts. However, other studies obtained better upkeep in NaDES (acidic or not) than in acidified ethanol, indicating a possible joint or synergistic effect between the H bonds of NaDES and their pH. This may be demonstrated by the following examples: Dai et al. (2016) evaluated the storage of anthocyanin extracts in NaDES and ethanol for three months. In their study, the concentration of total phenolic compounds remained stable in NaDES, as opposed to acidified ethanol, which lost approximately 40% and 60% total phenols when stored at 4°C and 25°C, respectively. In Dai et al. (2014), carthamin was storage-stable in all NaDES tested for 7 days at -20°C, however, in choline chloride:sucrose, the stability extended to the 15th day. Contrary to these studies, NaDES containing choline chloride, in the present study, was not as protective to sunflower meal extract after day #1, regardless of the temperature of storage. A recent report by Zhu et al. (ZHU et al., 2023) concluded that both, the acidic pH and the high-strength H-bonding of some NaDES may be responsible for the stabilization of CGA.

As in the study of thermal stability, during storage there was also an increase in the concentration of CGAs in LA:G, which appears to have occurred earlier at higher temperatures and later at lower temperatures. This occurrence might also be attributed to continued extraction from residues of the sunflower meal that remained in suspension after filtration. Likewise, it can be assumed that the process was not repeated in the other extractor (CC:GL) due to its lower extracting capacity.

4.3.3 Light stability

CGAs were found to be sensitive to visible and ultraviolet light, undergoing trans to cis 5-caffeoylquinic acid isomerism. The degree of degradation varied according to

exposure time, temperature, and wavelength (ERCOLI et al., 2021; GALMARINI et al., 2013; GIL; WIANOWSKA, 2017).

In the present study, attention is drawn to the fact that there was little or no difference between the samples protected by aluminum foil and those exposed without protection to artificial white light. To avoid the evaporation of solvents that occurred during the thermal treatment, these tests were conducted using cryogenic vials with screw caps. These flasks are made of polypropylene, which allows the passage of light above 290 nm, but absorbs shorter wavelengths, especially below 250 nm (MONTAGNA; SANTANA, 2012), while the reflective covering should protect the sample from all wavelengths emitted by the lamp. The lack of significant difference between protected and unprotected samples indicates that exposing the extracts to wavelengths above 290 nm had the same effect as completely protecting the samples. It is possible that the degradation observed in the samples was mainly due to the period of exposure to a temperature slightly higher than the ambient temperature, an increase caused by the presence of the light bulb. However, the behavior of the extracts was quite different from the behavior observed for other tests involving storage or heat treatment. In the light stability test, Et and CC:GL promoted better conservation of phenolic compounds than LA:G, at least for 7 days of exposure to the test conditions. It is also possible that the aluminum foil was ineffective in providing protection to the vials, as this was the only assay after which the samples showed marked visible discoloration, that may be observed in Supplementary Figure 3.

The stability of phenolic compounds under white light is little studied and assays evaluating the role of NaDES on the stability of CGAs under white light or sunlight are rare. In the few works available, as a rule, the phenolic compounds showed greater stability in NaDES than in organic solvents. Jeliński et al. (2019) tested the stability of curcumin in choline chloride based NaDES after exposure to artificial light for 2 hours. Only 5% of curcumin remained after treatment in methanolic solution and 19% in the turmeric powder. In NaDES composed of choline chloride:glycerol (CC:GL), curcumin remained stable throughout the period of exposure to light. In the present work, CC:GL also granted stability of the CGAs in solution for up to 7 days. Dai et al. (2014) tested the stability of carthamin under white light, in acidic NaDES (lactic acid:glucose, proline:malic acid) and in 40% ethanol. Light accelerated the degradation process in all solvents, but NaDES were more protective than the organic solvent. In this same study,

low-acidic NaDES (sucrose:choline chloride, glucose:choline chloride) and water ensured complete stability of carthamin under the test conditions. Blueberry pomace anthocyanins were extracted with acidified ethanol and acid NaDES (choline chloride:oxalic acid) and kept under artificial light, sunlight or in the dark. The extracts behaved similarly in the absence of light, but exposure to sunlight showed the greatest loss of phenolic compounds, approximately 50% and 15% in the ethanolic extract and NaDES, respectively. Under artificial light, the loss was 20% in ethanol and around 10% in NaDES. Ferreyra et al. (2023) recently evaluated the exposure to light of hydroethanolic (50%) extracts of grape by-products. After 2 weeks, a 43% reduction in total phenolic content was observed, similar to the obtained in the present study. Based on the results of the present test and the data available in the literature, apparently the acidic pH of the solvent negatively influenced the stability of the phenolic compounds exposed to light, in opposition to what occurred during heat treatment and the storage protected from light.

5. Conclusion

The results obtained in the present work indicate that the pH was the determining factor in the ability of LA:G to extract and protect the sunflower meal CGAs, promoting greater extraction and greater protection against heating and storage over time when compared to CC:GL, a less acidic and more viscous NaDES formulation. On the other hand, these conditions were reversed when the extracts were exposed to light. When compared to the organic solvent used as control (40% ethanol), the acidic NaDES formulation was more efficient in extracting CGAs and the less acidic NaDES formulation was less efficient, although the extracted compound profile was the same for all extractors.

The unexpected behavior of the extracts after heat treatment (especially at 60ºC) or prolonged storage suggests that a significantly higher extraction of phenolic compounds (CGA) might be achieved if the contact of NaDES with sunflower meal is maintained for a longer time or at higher temperatures, especially for LA:G. This is in accordance with a peculiar characteristic of NaDES, its high viscosity, which may hinder the partitioning of the compounds, but whose effects may be minimized by increasing the temperature and/or increasing the contact time. For practical purposes, these phenomena

can be applied in the highly efficient and clean extraction and recovery of bioactive compounds from sunflower meal, generating NaDES extracts with ready application in foods, pharmaceuticals and cosmetics. The efficient removal of chlorogenic acids, through the application of NaDES, may also enable the recovery of the protein fraction of the meal, for food uses, generally hampered by the interaction between phenolic acids and proteins during this extraction.

This study succeeded in the endeavor to shed light on the behavior of important phenolic compounds in natural deep eutectic solvents, at the same time that it indicates an alternative to add value to sunflower meal and avoid the waste of tons of bioactive phytochemicals annually.

6. Author contributions:

FSB - Formal analysis; Data curation; Writing - original draft; GSMR - Formal analysis; MGOC - Formal analysis; Data curation; MGBK – Conceptualization; Funding acquisition; Project administration; Supervision; Writing - review & editing.

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9. Data availability:

Data will be available upon request.

10. Ethical Approval:

Not applicable

11. Competing interests:

There are no conflicts of interest to be declared

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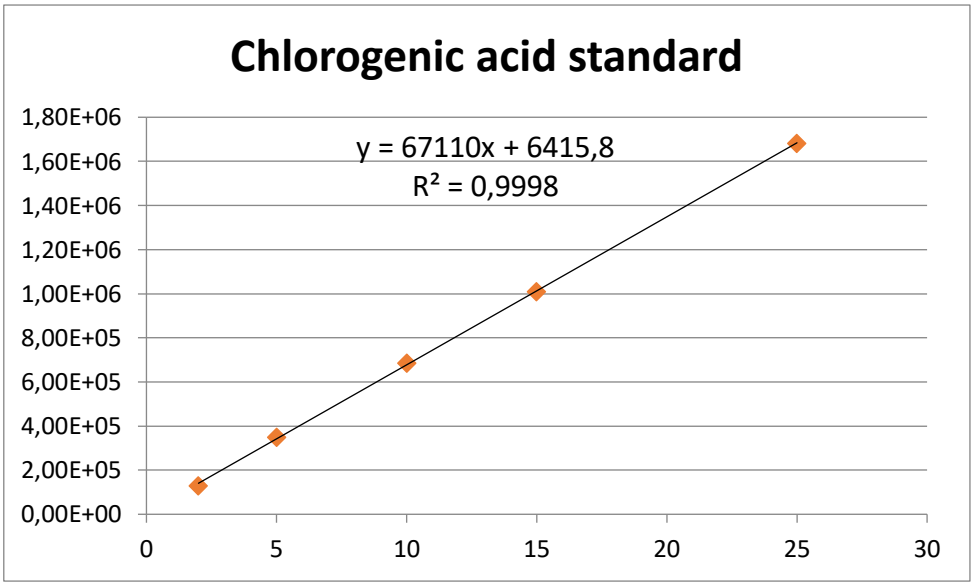
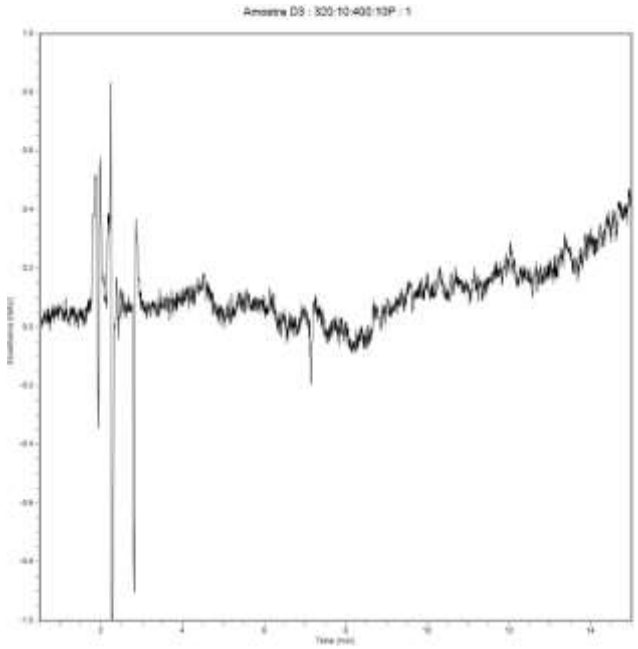
Supplementary Material

Supplementary Table 3.1. Chromatographic analysis of the extracts in different solvents

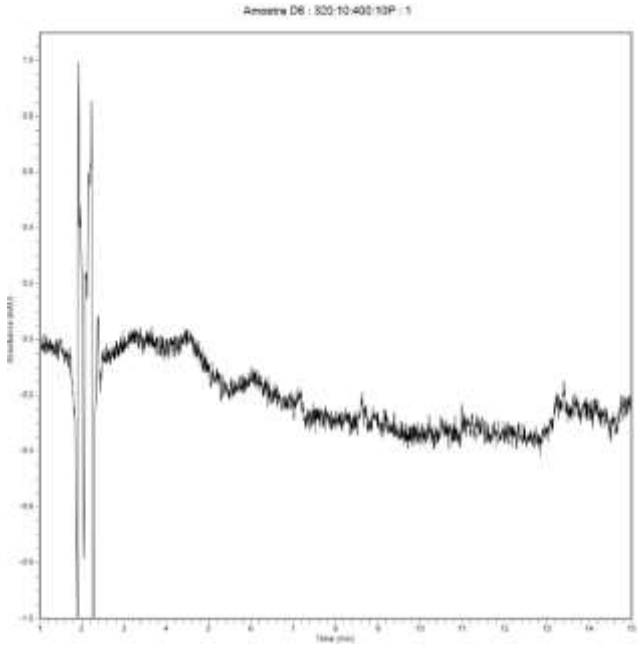
Sample (solvent)	Retention time for CGA (min)	Peak Area	Dilution factor	[CGA] mg L^{-1}
Et	8.593	2437968	50	**1305**
LA:G	8.694	1951807	50	**1786**
CC:GL	8.593	80223.9	50	**807**



Supplementary Figure 3.1. Chlorogenic acid standard curva for HPLC

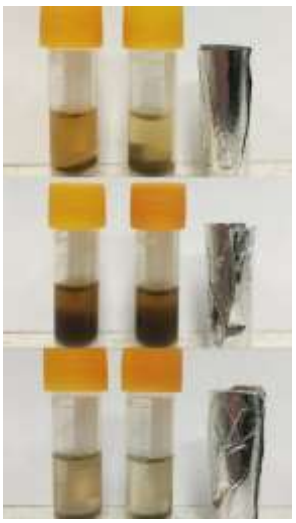


Supplementary Figure 3.2. Example of chromatogram of pure LA:G NaDES at 320 nm.



Supplementary Figure 3.3. Example of chromatogram of pure CC:GL NaDES at 320 nm.

Supplementary Figure 3.4. Granules formed after 7 days of storage at 8°C 9 (LA:G).



Supplementary Figure 3.5. Discoloration after 15 days of white light exposure with (right) and without (left) aluminum protection. From top to bottom: Et, LA:G and CC:GL.

CHAPTER 4

“Cytotoxic Potential of Sunflower Meal NaDES and Liquid-Liquid extracts”

Abstract

Sunflower meal, a by-product rich in proteins and phenolic compounds, has great potential for applications in the food industry and healthcare due to its bioactive properties. However, the efficient extraction of these compounds faces challenges, such as the formation of phenol-protein complexes that can compromise digestibility and nutritional value. In this study, phenolic compounds were extracted from sunflower meal using NaDES and hydroethanolic extract, followed by a recovery step of the target compounds using liquid-liquid extraction (LLE) with organic solvents. To evaluate the cytotoxicity of natural solvents and phenolic extracts, cytotoxicity assays were performed on breast cancer cell lines, Gram-positive and Gram-negative bacteria. In LLE, acetonitrile was the solvent with the best recovery in NaDES CC:GL and U:GL, with a total phenolic content removal of 80% and 63%, respectively. These same combinations demonstrated a reduction in cell viability of up to 78.4% in MCF-7 cells and 74% in MDA-MB-231 cells. In the antimicrobial activity analysis, the hydroethanolic extracts showed the highest inhibitory effect, with up to 100% reduction in bacterial viability at 0.78% concentrations. The results of this study not only confirm the potential of NaDES as effective green solvents for the extraction of phenolic compounds but also highlight their bioactivity when using pure NaDES, emphasizing the importance of optimizing parameters such as pH and polarity during the extraction process.

Key words: liquid-liquid extraction; natural deep eutectic solvents; by products; antimicrobial; cancer cells

1. Introduction

Sunflower (*Helianthus annuus* l.) is a plant with a short cultivation cycle and the ability to adapt to various climates. Sunflower seeds contain up to 42% lipids and approximately 20% protein (FRIOLLI et al., 2023; SARICAOGLU et al., 2023). After oil extraction from the seeds, initially by cold pressing and subsequently by organic solvents, a by-product called sunflower meal, rich in proteins and phenolic compounds, is produced (BEZERRA et al., 2024).

In 2024, global sunflower meal production reached 23 million metric tons. Russia and Ukraine are the largest producers and importers, with 17.1 and 14.5 million tons, respectively, together representing 57% of total global production (USDA, 2024a, 2024b). Currently, this by-product is primarily used for animal feed, despite being rich in phenolic compounds, with a variation of 1% to 4% in content, of which 70% belongs to the chlorogenic acid family. The protein content in sunflower meal is also high, reaching up to 20%. However, under alkaline conditions, these phenolic compounds form an irreversible phenol-protein complex that results in a green color (FRIOLLI et al., 2023; LO VERDE et al., 2022). Over the years, methodologies have been researched to remove phenolic compounds and/or proteins from food matrices without forming the phenol-protein complex that affects digestibility and the nutritional value of proteins, limiting the application of phenolic and protein compounds in the food industry (ALARA; ABDURAHMAN; UKAEGBU, 2021).

The extraction of phenolic compounds from plant sources is a process influenced by various conditions, such as pH, temperature, and time, which determine the final yield of the process. Acidic pH is ideal for this extraction as it facilitates the breaking of hydrogen bonds, releasing them. The optimal temperature for polyphenol extraction is between 45°C and 55°C; temperatures above 60°C can degrade phenolic compounds, and prolonged times can oxidize or volatilize them (GIL-MARTÍN et al., 2022). The type of solvent used is also crucial, with organic solvents such as ethanol, methanol, and acetone being the most common. Conventional extraction methods include maceration and Soxhlet, which can be improved with ultrasound or microwave-assisted techniques (ALARA; ABDURAHMAN; UKAEGBU, 2021; BRGLEZ MOJZER et al., 2016).

Natural deep eutectic solvents (NaDES) are an emerging green alternative for the extraction of bioactive compounds. They are formed by two or more components, such

as organic acids, sugars, and quaternary ammonium salts, which interact to form a eutectic mixture with a melting point significantly lower than that of the individual components (ALAÑÓN et al., 2020). This characteristic makes NaDES excellent solvents for a wide range of applications, including the extraction of bioactive compounds. Additionally, NaDES are generally non-toxic, biodegradable, and low-cost, making them a sustainable alternative to traditional organic solvents (GIL-MARTÍN et al., 2022; KOH et al., 2023). Although the high viscosity of NaDES can affect mass transfer during extraction, they have proven effective in extracting polyphenols, flavonoids, and other bioactive compounds. Optimizing this process with NaDES is essential to select the most favorable parameters, such as the composition, molar ratios, and concentration of NaDES, to maximize the polyphenol content and minimize the number of experiments (MANSOUR et al., 2024; SZOPA; WRÓBEL; WITEK-KROWIAK, 2024). The pH of NaDES varies according to the combination used, particularly in relation to the hydrogen bond donor. Since the hydroxyl groups of polyphenols are excellent hydrogen donors, NaDES are a good option for the extraction of phenolic compounds of varying polarities (FOURMENTIN; COSTA GOMES; LICHTFOUSE, 2021).

Liquid-liquid extraction is an effective technique for recovering phenolic compounds from aqueous matrices, using organic solvents such as diethyl ether, ethyl acetate, and chloroform. This technique involves transferring the compound of interest from the aqueous phase to the organic phase, followed by the separation of the phases (BOKHARY; LEITCH; LIAO, 2021). The efficiency of extraction depends on factors such as solvent polarity, solution pH, phase ratio, and the number of extraction steps. After extraction, the organic phase containing the phenolic compounds can be subjected to purification and concentration techniques, such as solvent evaporation (CAÑADAS et al., 2020). The recovery of polyphenols can reach 95% when extraction parameters such as pH, time, sample-to-solvent ratio, and the number of extractions are adequately optimized (LLANO et al., 2015; SAS et al., 2018).

Phenolic compounds found in various food sources and food waste have numerous health benefits, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Studies have shown that extracts rich in phenolic compounds, such as anthocyanins, flavonoids, and phenolic acids, exhibit antiproliferative and pro-apoptotic effects in cancer cell lines, including colon, breast, lung, and liver cells, as well as

antimicrobial effects against Gram-positive and Gram-negative bacteria (FOROUTANI et al., 2023; QUEFFELEC et al., 2024).

2. Materials and Methods

2.1 Plant material and sample preparation

The sunflower meal used in this study was provided by Caramuru company (Itumbiara - GO, Brazil).

All NaDES and sunflower meal extracts were prepared according to Bezerra et al. (2024) and are presented in Table 1. Briefly, NaDES components were mixed with magnetic stirring (Gehaka, model AA2050LED, São Paulo, Brazil) at 50°C until a transparent and homogeneous liquid was formed. For extraction, the sunflower meal (4.50 g) was added to the NaDES or 40% hydroethanolic solution (30 mL), heated for 15 min at 45°C and sonicated (Ultronique, Desruptor, São Paulo, Brazil) for 1 min, at maximum power (99%). All extracts were filtered and stored at -80°C in an ultra-freezer (Glacier Ultralow temperature freezer, model NU9483GC, Minnesota, USA) until use.

Table 1. NaDES composition and initial CGA concentration in sunflower meal extract

Solvent	Composition		Molar ratio	solvent pH	extract pH	[CGA] mg L^{-1}
	HBA[1]	HBD[2]				
LA:G	Lactic acid	Glucose	5:1	0.73	1.4	99.0
CC:GL	Chlorine chloride	Glycerol	1:1	6.35	5.7	59.6
U:GL	Urea	Glycerol	1:1	8.82	8.5	124.3
B:LA	Betaine	Lactic acid	1:2	3.00	3.1	114.8
Et	Ethanol	Water	0.4:1*	5.54	5.8	147.9

* (v/v); [1] Hydrogen Bond Acceptor; [2] Hydrogen Bond Donor

2.2 Liquid-liquid extraction

Preliminary tests were conducted to evaluate the extraction conditions, considering the target product: phenolic compounds, especially from the chlorogenic acid family (KLEMZ et al., 2021). The following extract and solvent ratios were tested: 1:1 and 1:2; stirring times: 0.5, 1, and 5 minutes; phase separation: static at 25°C, 8°C, and -18°C for 24 hours or by centrifugation at 4500 rpm for 7 minutes (Jouan Centrifuge B4i, Massachusetts, USA) (TAN et al., 2016). The solvents tested were heptane (Hp), hexane (Hx), ethyl acetate (EtA), acetone (Ac), dichloromethane (DiCl), ethyl ether (EEt), and acetonitrile (ACN). Aprotic solvents, which are immiscible with NaDES, were preferred

(RUESGAS-RAMÓN; FIGUEROA-ESPINOZA; DURAND, 2017). The stirring was performed using a vortex (Kasvi, K45-2820, Paraná, Brazil), and the pH of the samples was not adjusted for these tests.

2.3 Assessment of extraction efficiency

Liquid-liquid extraction was evaluated by the spectrophotometric quantification of residual CGA in the NaDES phase using the Folin-Ciocalteu reagent method to all samples except those with CC:GL, to which the Fast Blue BB salt method was applied. CGA standard curves were constructed in each NaDES (Supplementary Figures 1 to 5). Results are presented as means of at least 4 replicates followed by standard deviation (Mean±SD) and the statistical analysis was performed through two-way analysis of variance followed by the Bonferroni post-test at 95% confidence.

2.3.1 Quantification of CGA

2.3.1.1 Folin-Ciocalteu reagent method

Aliquots of 50 μL were collected and 3.95 mL of ultrapure water, 200 μL of Folin-Ciocalteu reagent and 250 μL of sodium carbonate solution (20%) were added. The mixture was then vortexed and kept in the dark for 2 hours. Aliquots were transferred to a 96-well microplate and read at 765 nm (Biotek Epoch Microplate Reader, Massachusetts, USA - Gen5 Software 3.12).

2.3.1.2 Fast Blue BB salt method

Aliquots of 200 μL were collected, 100 μL of the Fast Blue BB solution (0.1% w/v) were added and the mixture was taken to an ultrasound bath (Unique USC-2500, São Paulo, Brazil) for 30 s, then 100 μL of 5% sodium hydroxide solution was added and the mixture was vortexed and set aside for 90 minutes. Aliquots were transferred to a 96-well microplate and read at 420 nm.

2.4 Cytotoxicity assay

2.4.1 Sample preparation

To perform the cytotoxicity assays the liquid-liquid samples (LLE) were first neutralized with HCl (0.1 g 100 mL^{-1}) or alcoholic NaOH solutions (0.5 g 100 mL^{-1}) and then the solvents were evaporated in a centrifugal evaporator (SAVANT SPD131DDA-

115, Massachusettes, USA) at 45°C, 20 torr pressure, for 2 hours. CC:GL and U:GL solvents and extracts were neutralized with HCl or NaOH solutions (0.1 g 100 mL^{-1} and 1 mol L^{-1}). For comparison purposes, two samples were prepared according to Nascimento et al. (2023), these samples were called Et Ch (active charcoal extract) and W (water extract). The hydroethanolic (Et) and Et Ch samples were first evaporated in a centrifugal evaporator and then freeze dried, W samples were also freeze dried (Terroni, LD 3000, São Carlos, Brazil). Other samples did not need previous preparation. All samples tested are described in Table 2. For use, the samples were resuspended in DMEN (Dulbecco's Modified Eagle's Medium) or TSB (tryptone soy broth) to the initial concentration.

Table 2. Description of the samples used on the cytotoxicity and antimicrobial assays

Solvent	Sample	Organic solvent for LLE†	Sunflower meal	pH
LA:G	LLE	Ethyl acetate	✓	-
CC:GL	NaDES	-	×	7.2
	Extract	-	✓	7
	LLE	Hexane	✓	-
U:GL	NaDES	-	×	7.1
	Extract	-	✓	7
	LLE	Ethyl acetate	✓	-
B:LA	LLE	Ethyl acetate	✓	-
Et	FD*	-	✓	7
Et Ch	FD*	-	✓	7
W	FD*	-	✓	7

* FD – freeze dried sample, † LLE – liquid-liquid extract

2.4.2 Human cancer cells assay

2.4.2.1 Cell culture

The human breast epithelial carcinoma cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM containing 4.5 g L^{-1} glucose supplemented with 10% fetal bovine serum and 1% of penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.4.2.2 Cell treatment

Breast cancer cells were subcultured into 96-well plates for at least 24 h until reaching 60% confluency. In order to evaluate cytotoxicity, cells were exposed, for 24 h, to 50%, 25%, 12.5% and 6.25% of samples in DMEN medium. Control cultures were performed without any treatment.

2.4.2.3 Cell viability

After treatment, cells were exposed to culture medium containing 10% Alamar's reagent, for 3 h, at 37°C, in a humidified atmosphere containing 5% CO_2. Plates were evaluated at 570 and 600 nm (Victor X5, Massachusetts, USA – PerkinElmer 2030 Manager), and the data were expressed as percentage of cell viability compared to the control.

2.4.3 Pathogenic microorganism assay

2.4.3.1 Microorganism culture

Three Gram-positive bactéria (MRC *Bacillus cereus* - NCTC® 10320 (WDCM 00001), MRC *Staphylococcus aureus* - NCTC® 12493 (WDCM 00212) and MRC *Listeria monocytogenes* - NCTC® 11994 (WDCM 00019) and four Gram-negative bacteria (PCC *Shigella flexneri* - NCTC® 12698 (WDCM 00126), MRC *Escherichia coli* - NCTC® 9001 (WDCM 00090/WDCM 00155), MRC *Pseudomonas aeruginosa* - NCTC® 12903 (WDCM 00025) and MRC *Salmonella enterica* - NCTC® 12023 (WDCM 00031) were selected for antimicrobial activity assays. All microorganisms were maintained on tryptone soy agar (TSA) medium at 37°C.

2.4.3.2 Microorganisms' treatment

After 24 hours of bacterial growth, suspensions of each microorganism were prepared in sterile 0.45% saline solution to the 0.5 point of the McFarland scale, using a turbidimeter (Turbidímetro Digital Portátil, MCF-500, Sinergia Cientifica, Campinas, SP). The samples were diluted in tryptone soy broth (TSB) from 50% to 0.78% of the initial concentration of each sample by serial dilution. The following controls were performed: pure TSB, pure sample, pure chlorhexidine digluconate (10 mg mL^{-1}); negative control – inoculum in TSB; and positive control – inoculum in TSB with chlorhexidine digluconate. Plates were incubated at 37°C for 24 hours.

2.4.3.3 Antimicrobial activity

Antimicrobial activity was determined by the microdilution method. After the incubation period, 0.125% 2,3,5-triphenyl tetrazolium chloride (TTC) was added to the samples, incubated for two hours in the dark, followed by reading at 540 nm in a microplate reader (VICTOR Nivo, PerkinElmer, Massachusetts, USA). The result was expressed as a percentage of bacterial growth reduction, according to Equation 1.

$$\%\ reduction = 100\ - \left[\frac{(Abs\ 1 - Abs\ 2) \times 100}{(Abs\ 3 - Abs\ 4)}\right] [1]$$

Where: ABS 1 is the absorbance of the sample

ABS 2 is the absorbance of the pure sample control

ABS 3 is the absorbance of the negative control

ABS 4 is the absorbance of the pure TSB control

3. Results

3.1 Liquid-liquid extraction

3.1.1 Preliminary tests

For the application of the liquid-liquid extraction methodology, a 1:1 molar ratio was adopted for all tests to minimize the use of organic solvents, as no significant difference was observed between using 1:1 or 2:1 ratios. Regarding the agitation time, durations longer than 30 seconds generated emulsions between extracts and organic solvents, making separation difficult (Supplementary Figure 6). Therefore, agitation was performed using a vortex for 30 seconds in all tests.

Due to the high viscosity of NaDES, prolonged resting was necessary for phase separation. Extended resting periods (24 hours) at 25°C (room ambient), 8°C (refrigerator), and -18°C (freezer) led to visible degradation of the samples—color changes varied depending on the NaDES medium, from different shades of brown to greenish (Supplementary Figure 7). Thus, accelerated separation by centrifugation was adopted.

Among the solvents tested, acetone and acetonitrile were incompatible with the LA:G; B:LA and Et samples. Initially, when added to the samples, they indicated the formation of two phases, but after agitation, they mixed into a single phase that remained even after centrifugation (Supplementary Figure 8).

3.1.2 Evaluation by Residual CGA Concentration in Extracts

Table 3 shows the results obtained for the quantification of residual CGA concentration in all tested extracts after liquid-liquid extraction using all solvents compatible with these extracts—those that, after homogenization, were separable into two phases.

Table 3. Spectrophotometric quantification of residual phenolic compounds of NaDES phase after liquid-liquid extraction

	[CGA] mg L^{-1}				
	LA:G	**CC:GL**	**U:GL**	**B:LA**	**Et**
Initial concentration	99 ± 3.2^{Da}	59.6 ± 2.2^{Ea}	124.3 ± 11^{Ba}	114.8 ± 2.2^{Ca}	147.9 ± 3.5^{Aa}
Hp	46.5 ± 3.3^{Db}	12.0 ± 5.5^{Ec}	55.8 ± 1.7^{Cc}	61.1 ± 5.0^{Bbc}	91.3 ± 0.8^{Ac}
Hx	47.4 ± 1.6^{Db}	9.8 ± 0.3^{Ec}	55.1 ± 1.7^{Cc}	60.8 ± 0.9^{Bbc}	91.3 ± 1.7^{Ac}
EtA	32.6 ± 0.6^{Dc}	13.1 ± 1.9^{Ecd}	52.8 ± 0.9^{Ccd}	62.8 ± 0.6^{Bb}	78.9 ± 0.6^{Ae}
Ac	-	19.2 ± 2.0^{Bb}	59.8 ± 1.4^{Ab}	-	-
DiCl	41.6 ± 1.6^{Db}	11.4 ± 0.5^{Ec}	50.5 ± 1.7^{Cd}	58.9 ± 1.9^{Bc}	94.9 ± 1.5^{Ad}
EEt	48.1 ± 1.2^{Db}	16.2 ± 1.1^{Ebd}	53.8 ± 0.6^{Ccd}	63.4 ± 7.8^{Bbc}	103.2 ± 0.7^{Ab}
ACN	-	11.4 ± 1.2^{Bc}	45.4 ± 0.7^{Ae}	-	-

- samples unavailable. Different lowercase letters indicate significant difference among solvents in the same extract. Different capital letters indicate significant difference in the same solvent among different extracts.

Since the extracted amount is not a good basis for comparison because each extract had a different initial CGA concentration, from this point onward, the data will be presented as a percentage of removal, considering the initial concentration of each extract, as shown in Table 3.

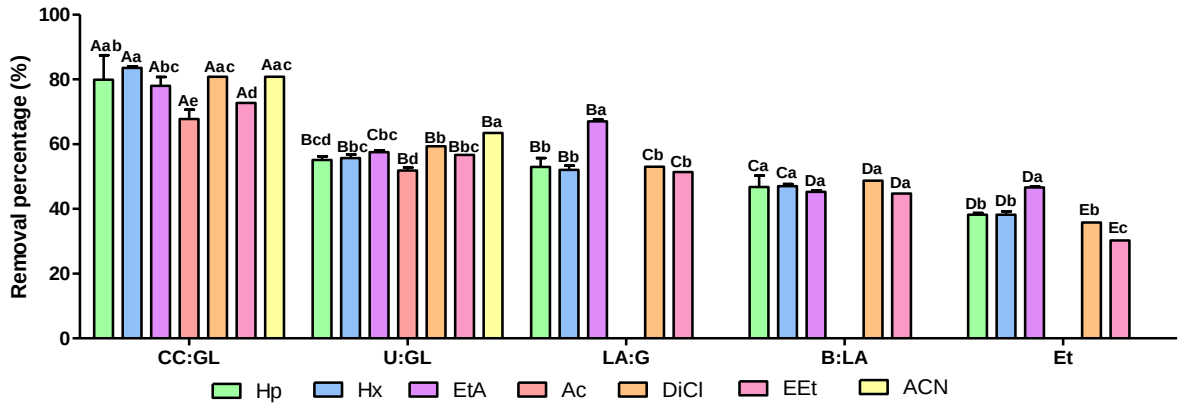


Figure 1. Chlorogenic acid removal percentage from the NaDES/Ethanol phase after liquid-liquid extraction from sunflower meal extracts.

Different lowercase letters indicate significant difference among solvents in the same extract. Different capital letters indicate significant difference in the same solvent among different extracts. Hp – heptane; Hx – hexane; EtA – ethyl acetate; Ac – acetone; DiCl – dichloromethane; EET – ethyl ether; ACN - acetonitrile.

In the analysis of NaDES CC:GL, the efficiency of the extracting solvents followed the order: ACN = EtA = DiCl = Hx = Hp > EEt > Ac, with a minimum extraction of 67.7% using acetone (Figure 1). Acetonitrile (ACN) was the most efficient solvent, achieving a total removal of 80% of the TPC from NaDES CC:GL, with no significant difference compared to heptane, hexane, ethyl acetate, and dichloromethane. Acetonitrile also stood out as the most effective solvent in the NaDES U:GL medium, with 63% removal, showing significant differences compared to the other solvents. The order of TPC extraction efficiency in NaDES U:GL was ACN > EtA = DiCl = Hp = Hx = EEt > Ac, with acetone again being the worst solvent, with 51.8% removal.

In samples where acetonitrile and acetone were not used due to the absence of two distinct phases typical of liquid-liquid extraction, ethyl acetate stood out as the best-performing organic solvent for TPC removal. NaDES LA:G chieved 67% removal, and the final order of extraction efficiency was EtA > DiCl = Hp = Hx = EEt, with diethyl ether removing 51.3%. The hydroethanolic extract removed 46.6% of the TPC (EtA > DiCl = Hp = Hx > EEt), and from NaDES B:LA, 45.2%, although in the latter, there was no significant difference between the solvents tested (EtA = DiCl = Hp = Hx = EEt).

Dichloromethane (DiCl), although not the most effective in any sample, performed well in all cases, ranking as the third most efficient solvent after acetonitrile (when used) and ethyl acetate. In samples where acetonitrile was not used, the organic solvents showed little or no significant difference from each other.

3.2 Cell viability

Figures 2 and 3 present the results obtained for the reduction of cell viability in human breast cancer cell lines MCF-7 and MDA-MB-231, respectively. The extract and NaDES samples of lactic acid:glucose (LA:G) and betaine:latic acid (B:LA) were not viable for cytotoxic evaluation due to their acidic pH, which could not be neutralized and remained stable even when large amounts of alkaline solution were added. Thus, only the liquid-liquid extraction samples from these matrices were evaluated for cytotoxic activity. There was no significant difference between the treated samples and the control; therefore, no reduction in cell viability was observed in the B:LA LLE sample (Supplementary Tables 2 and 3). There was only a 53% reduction in the highest concentration of LA:G LLE (Supplementary Tables 2 and 3), possibly related to the pH

of the sample, which, even after neutralization (pH 7) and solvent removal, altered to pH 6 when resuspended in DMEM medium.

In Figure 2, the results for cell viability in the MCF-7 cell line after treatment with neutralizable samples are shown. Overall, better cytotoxicity results were observed in pure NaDES samples, followed by sunflower extract in NaDES, compared to LLE samples and conventional extraction samples (Et, Et Ch, and W).

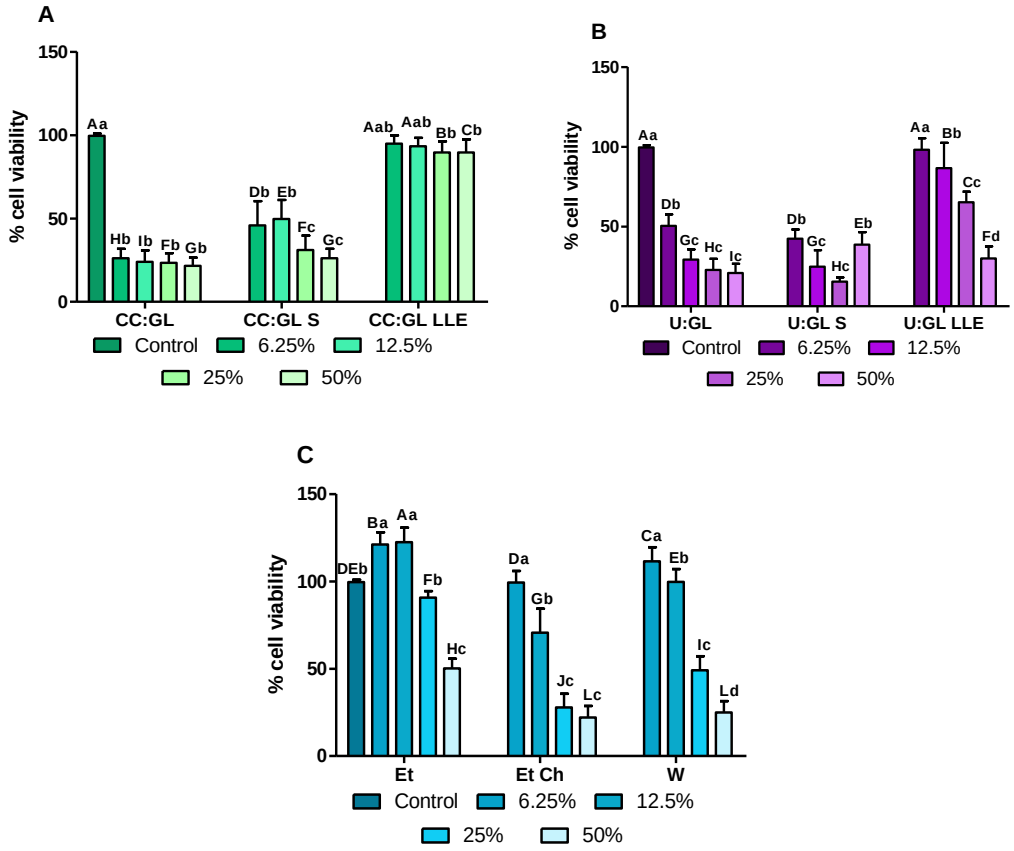


Figure 2. Cytotoxic activity in human cancer cells MCF-7 in NaDES CC:GL (A), NaDES U:GL (B) and conventional extraction (C) samples.
Lower case letters indicate significant difference between concentrations of the same sample. Upper case letters indicate significant difference between samples and the same concentration. CC:GL S – sample with sunflower meal; CC:GL LLE – sample from liquid-liquid extraction; U:GL S – sample with sunflower meal; U:GL LLE – sample from liquid-liquid extraction; W – aqueous extraction.

A dose-dependent effect was observed in all samples, except those containing CC. The results using pure NaDES achieved the greatest reduction in cell viability at the lowest concentrations (6.25% for CC:GL and 12.5% for U:GL), maintained even with an increase in the tested concentration. The addition of sunflower meal phenolic extract significantly reduced the cytotoxicity of CC:GL, but did not alter the results for U:GL. Conventional extraction samples (Et, Et Ch, and W) also showed a protective effect from

the presence of sunflower extract at lower concentrations and cytotoxic effects at higher concentrations.

In the MCF-7 cell line, a maximum reduction of 78.4% in cell viability was observed with 50% NaDES CC:GL with no significant difference for the lower concentrations (Figure 2A). This initially indicates that this is the maximum effect that the natural solvent can achieve among the tested concentrations. In sunflower extract in NaDES (CC:GL S) at the same concentration, there was a 73.9% reduction in cell viability, corresponding to 29.8 mg CGA L^{-1} (Supplementary Table 1), with no significant difference from the CC:GL. he same pattern was observed at 25% concentration: in this case, the phenolic extract sample had a 69% reduction, while the pure NaDES sample had 76.6%. The liquid-liquid extract (CC:GL LLE) maintained cell viability at 89.6% at the highest sample concentration, making it the sample with the lowest cytotoxic potential in this group.

Figure 2B presents the results for cell viability of U:GL samples in MCF-7 cells, all of which showed dose-dependent behavior. In this group, the sunflower in NaDES sample (U:GL S) had the best cytotoxic potential and the lowest cell viability (24.8%), representing a 75.2% reduction when using 12.5% (15.5 mg CGA L^{-1}) of the sample, with no significant difference from the 25% concentration. The same sample containing only NaDES U:GL also showed good results, achieving a 70.7% reduction in viability. A dose-dependent effect can be seen in the U:GL LLE sample, with a maximum reduction of 70% in the MCF-7 cell line.

In the MCF-7 cell line, conventional extraction samples also showed a more visible dose-dependent effect than those in pure NaDES or phenolic extract in NaDES (Figure 2C). The activated charcoal desorption sample (Et Ch) had the best results, reducing MCF-7 cell viability by 77.9% at 50%, a similar result to the aqueous extraction (W) at the same concentration (75.1%). In the hydroethanolic sample (Et), there was initially an increase in cell viability at lower concentrations and a significant reduction only at 50% concentration. After incubation for 24 hours in an oven, a color change from pink to green was observed in this sample (Supplementary Figure 10).

Therefore, it is possible to point out that the best results in terms of greater reduction in human breast cancer cell viability in the MDF-7 cell line were: U:GL (12.5%)

= U:GL S (12.5%) > CC:GL (6.25%) > W (50%) > Et Ch (25%) > CC:GL S (12.5%) > U:GL LLE (50%) > CC:GL LLE (25%) > Et (50%).

Figure 3 presents the results for cell viability in the MDA-MB-231 cell line after treatment with neutralizable samples. The results obtained for the cells in this line were quite similar to those obtained for the MCF-7 line (Figure 2), i.e., the greatest cytotoxicity was presented by pure NaDES samples, followed by NaDES samples containing sunflower extract. This also showed some protective effect, especially in the U:GL S and Et samples.

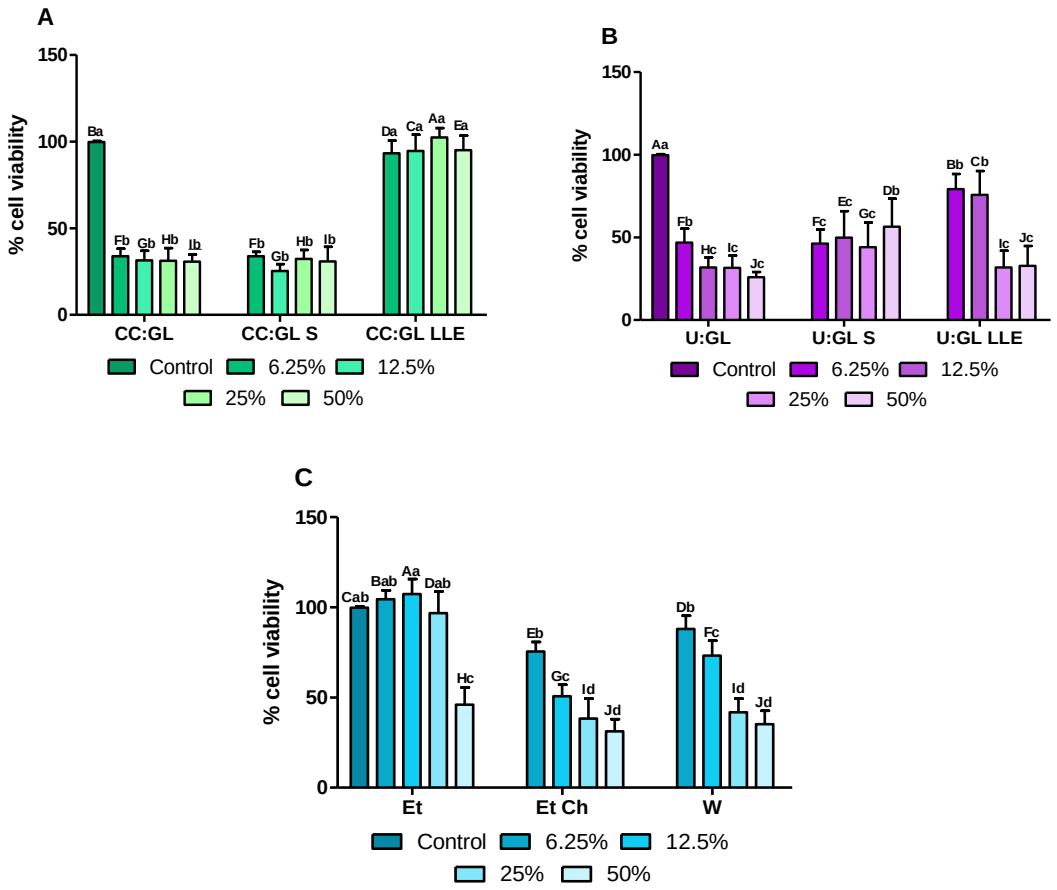


Figure 3. Cytotoxic activity in human cancer cells MDA-MB-231 in NaDES CC:GL (A), NaDES U:GL (B) and conventional extraction (C) samples.
Lower case letters indicate significant difference between concentrations of the same sample. Upper case letters indicate significant difference between samples and the same concentration. CC:GL S – sample with sunflower meal; CC:GL LLE – sample from liquid-liquid extraction; U:GL S – sample with sunflower meal; U:GL LLE – sample from liquid-liquid extraction; W – aqueous extraction

The CC:GL LLE samples did not exhibit cytotoxic activity in MDA-MB-231 cells, nor was there a significant difference compared to control cells (Figure 3A), similar

to the MCF-7 cell line. The results between NaDES CC:GL and NaDES with sunflower meal extract (CC:GL S) were similar, showing a 66% reduction in both samples at the first concentration (6.25%), with no significant difference at other concentrations for both samples—thus, the phenolic extract did not affect cytotoxicity. As with the MCF-7 samples, the samples reached their maximum effect at low concentrations, and no dose-dependent effect was observed. CC:GL LLE maintained cell viability between 93% and 100%, so it cannot be concluded that this sample had a cytotoxic effect on the cells.

The NaDES of U:GL sample was the most effective in reducing cell viability in the MDA-MB-231 cell line at a concentration of 12.5%, reducing viability by 68.2%, with no significant difference between extract concentrations at 25% and 50%; in the same concentration (12.5%) in the U:GL S sample, the maximum reduction was 53.8%. The dose-dependent effect is more subtle in the first two samples, but in U:GL LLE , a variable concentration effect can be seen between 12.5% and 25% — the latter showed a 67% reduction in cell viability (Figure 3B). Once again, as with the CC:GL extract, the phenolic extract did not seem to assist in cytotoxic activity.

In the control samples from conventional extraction (Figure 3C), Et showed a significant difference from the control only at 50% concentration, reducing cell viability by approximately 54%; in addition, a color change to green was observed after 24 hours of treatment (Supplementary Figure 11). Similar to the MCF-7 cell line, the dose-dependent effect was more noticeable in the Et Ch and W samples, with no significant difference between the two highest concentrations (25% and 50%) and between extracts, with a maximum reduction of 61.7% and 58.3% at the 25% concentration.

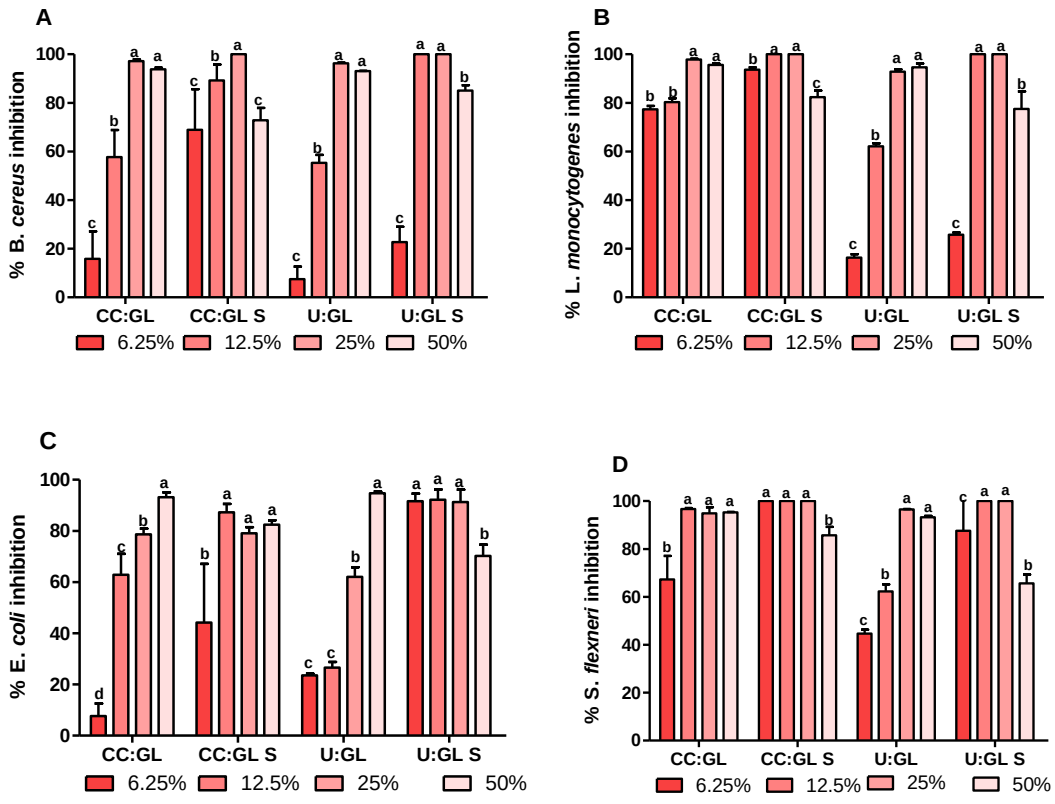
The best samples for MDA-MB-231, in order of greatest to least reduction in cell viability, were: CC:GL S (6.25%) = CC:GL (6.25%) > U:GL (12.5%) > Et Ch (25%) > U:GL LLE (25%) > W (25%) > U:GL S (12.5%) > Et (50%). The CC:GL LLE sample showed no significant difference between concentrations.

Finally, conventional extraction samples, such as Et Ch and W, showed a more pronounced dose-dependent effect compared to the others, suggesting that traditional extraction methods still have relevance, especially in contexts where pH stability and solvent composition are critical. However, these same extracts required higher doses to achieve effects similar to those of pure NaDES or NaDES with phenolic extract. The similarity between the results of Et Ch and W samples with those of MCF-7 and MDA-MB-231 cells highlights the consistency of these methods in different cellular contexts.

Despite presenting positive effects, the results were not as effective at low doses as in some NaDES samples with or without extract.

3.3 Antimicrobial activity

Figure 4 shows the antimicrobial activity results of sunflower phenolic extract samples in NaDES medium and pure NaDES. In this evaluation, the same sample preparation used for cytotoxic activity in cancer cells was followed, including the precaution of using neutralized samples to eliminate the possibility of pH interference in the assays. Thus, non-neutralizable samples were not evaluated. The liquid-liquid extraction samples of LA:G LLE and B:LA LLE (Supplementary Table 4) showed total inhibition of pathogens. As observed in cancer cells, this effect may be associated with the sample's pH, even after neutralization, while CC:GL LLE and U:GL LLE did not show inhibition of microorganisms and, therefore, do not have a MIC value.



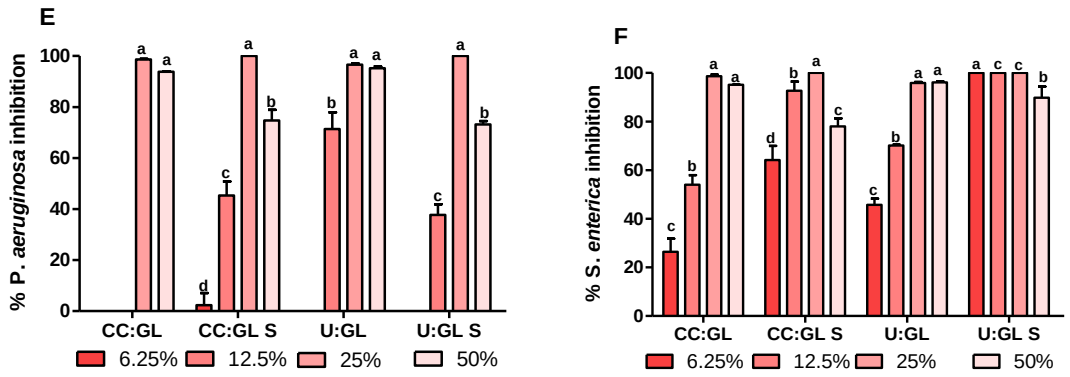
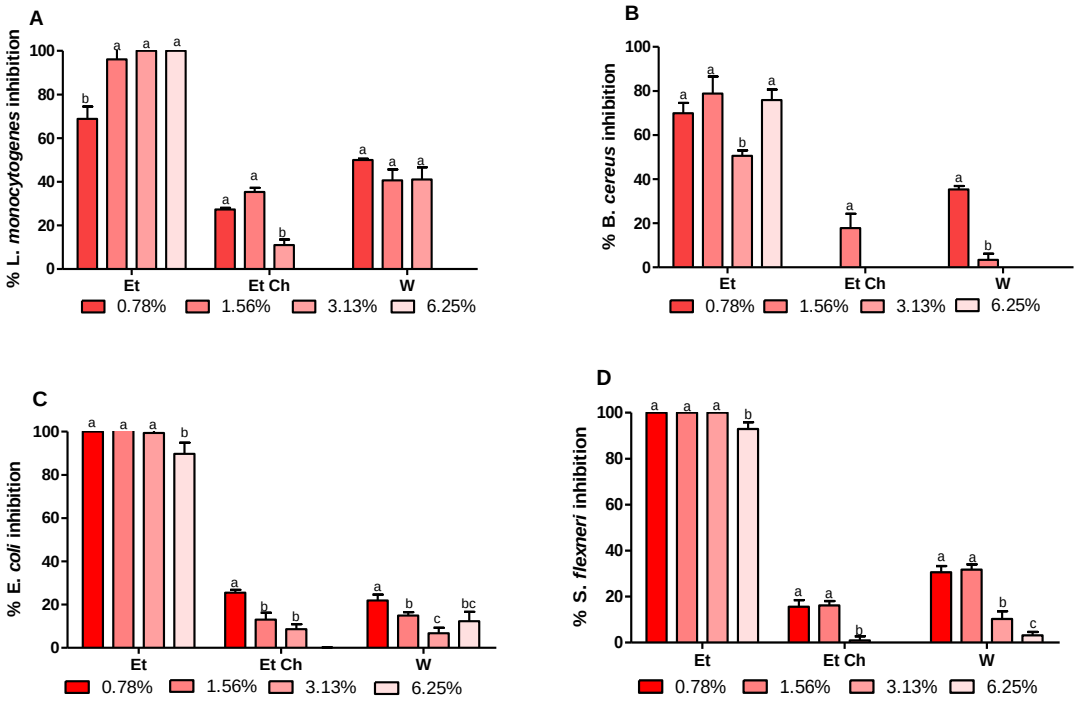


Figure 4. Percentage of microbial inhibition in NaDES samples extraction in Gram-positive and Gram-negative pathogens.

Lowercase letters indicate significant difference between concentrations of the same sample. CC:GL S – sample with sunflower meal; U:GL S – sample with sunflower meal

Figure 5 presents the antimicrobial activity results for samples obtained by alternative extraction. It is observed that, in this case, a lower extract concentration (6.25% to 0.78%) was required to verify antimicrobial effects compared to those used in NaDES samples. In general, the highest inhibitions were obtained with the hydroethanolic extract, although some concentrations did not show inhibition.



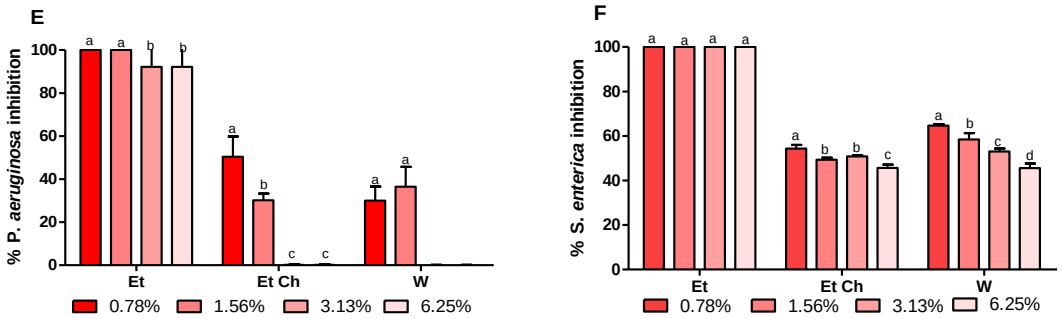


Figure 5. Percentage of microbial inhibition in conventional extraction samples extraction in Gram-positive and Gram-negative pathogens.
Lowercase letters indicate significant difference between concentrations of the same sample.
Et – extração hidroetanólica; Et Ch – extração com carvão ativado; W – extração aquosa

It was observed that samples in NaDES media—both pure and sunflower meal extract—achieved better antimicrobial activity at higher concentrations (50% to 6.25%), while those from conventional extraction showed better results at lower concentrations (0.78% to 6.25%) (Figure 6). In general, the NaDES sample containing sunflower meal had more effect on microorganisms than the pure solvent.

For Gram-positive microorganisms, the best effects were observed in samples containing sunflower meal, with total inhibition when exposed to 25% CC:GL S and U:GL S in *Bacillus cereus* and *Listeria monocytogenes* with a minimum inhibitory concentration (MIC) of 3.7 mg CGA L^{-1} and 7.7 mg CGA L^{-1}, respectively, for both pathogens (Table 4). In pure NaDES medium, the activity was dose-dependent, showing only a 15% and 7% reduction at 6.25% concentration in the B. *cereus* strain (Figure 4A) and 16% at the same concentration for L. *monocytogenes* (Figure 4B). However, at concentrations starting from 12.5%, total inhibition of the pathogens occurred. In the *Staphylococcus aureus* strain, antimicrobial activity was only observed in NaDES U:GL S, with an MIC of 7.7 mg CGA L^{-1}; and W, with an MIC of 0.55 mg GAE L^{-1} (Table 4).

For Gram-negative microorganisms, the same pattern can be observed. CC:GL and U:GL showed a more visible dose-dependent behavior on most pathogens, and samples containing sunflower meal completely prevented microbial growth. For E. *coli*, only 7% and 6% of microorganisms remained viable after 24 hours of treatment at 50% CC:GL e U:GL concentrations, respectively (Figure 4C). In the S. *flexneri* (Figure 4D), treatment at 50% CC:GL S and U:GL S allowed 15% and 35% of the pathogen to grow, with MICs of 29.8 mg CGA L^{-1} and 62.1 mg CGA L^{-1}, respectively. In P. *aeruginosa*, only the 50% and 25% CC:GL samples (Figure 4E) were effective; CC:GL S at 6.25%

maintained over 90% of microorganisms viable, being more effective at 25% (MIC 7.4 mg CGA L^{-1}). The 6.25% concentration also did not show positive results in U:GL and U:GL S. In S. *enterica*, the phenolic extract U:GL medium was 100% effective at all tested concentrations, with an MIC of 25% NaDES of CC:GL (Figure 4F).

The results of *L. monocytogenes* inhibition are shown in Figure 5A. The hydroethanolic extracts exhibited a minimum reduction of 68.8% at a concentration of 0.78%, while the highest concentrations reached 100% inhibition of this pathogen. In contrast, Et Ch and W samples were less effective, with 35% inhibition at 1.56% extract, corresponding to an MIC of 2.31 mg CGA L^{-1}. The W extract showed 50% inhibition, with no significant difference between concentrations, with an MIC of 1.1 mg CGA L^{-1}.

For the Gram-positive bacterium *B. cereus* (Figure 5B), inhibition was observed in only one Et Ch sample (1.56%), with 17% total inhibition. Two samples showed inhibition of 35% (0.78% concentration) and 3% (1.56% concentration) in the aqueous extract (W). However, the Et sample, with an MIC of 1.1 mg CGA L^{-1}, achieved a 69% reduction, with an MIC of 0.78%. The dose-dependent effect was observed only in the Et sample in *L. monocytogenes* between the two Gram-positive pathogens.

In Gram-negative bacteria, the dose-dependent effect was reversed, with greater inhibition observed at lower extract concentrations. This effect was more clearly observed in Et samples in Gram-negative bacteria, specifically in *S. flexneri* (Figure 5D), *S. enterica* (Figure 5F) in W extract samples, and *E. coli* (Figure 5C), *P. aeruginosa* (Figure 5E) and *S. enterica* in Et Ch extract samples. All hydroethanolic extract (Et) samples achieved 100% inhibition in Gram-negative bacteria at a concentration of 0.78% and an MIC of 1.1 mg CGA L^{-1}. The most significant results for Et Ch and W were observed in antimicrobial activity against the S. enterica pathogen, where inhibition of 54% for Et Ch and 64% for the W extract was recorded, as well as 50% for P. aeruginosa. Despite this, the minimum concentrations for inhibition of these two samples were 1.1 mg CGA L^{-1}.

Table 4 presents the minimum inhibitory concentration (MIC) values for all samples evaluated for each tested microorganism.

Table 4. Minimum Inhibitory Concentration (MIC).

Microorganism / MIC	CC:GL %	CC:GL S mg CGA L^{-1}	U:GL %	U:GL S mg CGA L^{-1}	Et mg CGA L^{-1}	Et Ch mg GAE L^{-1}	W mg GAE L^{-1}
B. cereus	50	3.7	50	7.7	1.1	1.0	0.55
L. monocytogenes	50	3.7	50	7.7	1.1	2.1	0.27
E. coli	50	3.7	50	62.1	9.2	1.0	0.27
S. flexneri	50	29.8	50	62.1	9.2	1.0	0.55
P. aeruginosa	25	3.7	50	7.7	9.2	1.0	0.27
S. entérica	50	3.7	50	62.1	-	1.0	0.27
S. aureus	-	-	-	7.7	-	-	0.55

CC:GL S – sample with sunflower meal; U:GL S – sample with sunflower meal; CGA – chlorogenic acid; GAE – gallic acid equivalents; Et – extração hidroetanólica; Et Ch – extração com carvão ativado; W – extração aquosa.

4. Discussion

This study tested the use of organic solvents for recovering phenolic compounds extracted from sunflower meal in NaDES medium through liquid-liquid extraction (LLE). To date, no published studies have been found on this topic. The use of LLE in NaDES medium is possible due to the hydrogen bonds that allow the formation of two phases with aprotic solvents, such as hexane, ethyl acetate, acetonitrile, among others (PALOS-HERNÁNDEZ et al., 2022; RUESGAS-RAMÓN; FIGUEROA-ESPINOZA; DURAND, 2017). Aprotic solvents are those that do not have acidic hydrogen atoms capable of forming hydrogen bonds, while protic solvents can donate protons and form hydrogen bonds. However, solvents considered aprotic can behave in a protic manner under alkaline conditions (REICHARDT; WELTON, 2011). The use of hydrophobic DES (HDES) was evaluated for the removal of phenol, guaiacol, pyrocatechol, and eugenol from aqueous solutions and showed greater efficiency compared to some organic solvents and ionic liquids, in addition to their low cost and toxicity (CABLÉ; LE BRECH; MUTELET, 2022).

4.1 Liquid-liquid extraction

4.1.1 Preliminary tests

LLE involves the transfer of compounds between two immiscible liquids, where the target compound shows a preference for one of the components in the mixture. This process is known as partitioning and depends on favorable conditions for its effectiveness: partition coefficient (the ratio of a solute's concentration in the organic phase to the aqueous phase at equilibrium), high selectivity, rapid phase separation, and the chosen

solvents, which should have a difference in polarity and the solubility, molecular weight, and acidity of the target compound (BOKHARY; LEITCH; LIAO, 2021; REICHARDT; WELTON, 2011). After agitation and resting, the phases separate, allowing the collection of the desired phase. This methodology is interesting for recovering phenolic compounds as it does not rely on high temperatures, reducing losses due to heating (BOKHARY; LEITCH; LIAO, 2021; LLANO et al., 2015).

The phenolic compounds in sunflower meal degraded during storage at room temperature or above (>60°C), despite NaDES increasing their stability, as seen in Bezerra et al. (2024). However, phenolic compounds from the chlorogenic acid family have a particular degradation characteristic, acquiring a greenish color when stored under inadequate temperature conditions, exposed to oxygen, or in alkaline conditions, causing an oxidation reaction that leads to structural changes and results in compounds that reflect light at a different wavelength, producing a green color (LIANG; WERE, 2018; LU et al., 2020; WIANOWSKA; GIL, 2019).

The viscosity of NaDES, due to the strong hydrogen bonds between their components, can be a hindrance for analyses at room temperature (OMAR; SADEGHI, 2022; TIAN; SUN; ZHU, 2022). Given the risk of phenolic compound degradation at room temperature and the viscosity of the samples, rapid phase separation was necessary, leading to the adoption of centrifugation instead of the commonly used separation funnels in batch liquid-liquid separation.

Solvent polarity significantly impacts extraction efficiency. In the LA:G, B:LA and Et samples, no two phases formed when using acetone and acetonitrile—this may be related to the polarity of these two solvents. Although classified as aprotic—characteristics that, in theory, allow the formation of two phases after mixing with polar NaDES—acetone and acetonitrile are polar solvents, different from the other tested organic solvents, which are apolar or dipolar (REICHARDT; WELTON, 2011). The polarity of a NaDES is determined by the type of hydrogen bond donor used—those based on acids tend to be more polar than those based on sugars or polyalcohols, while the presence of glycerol and the amount of added water increase polarity (BASHIR et al., 2023; RENTE; PAIVA; DUARTE, 2021). Based on this information, it is possible to say that LA:G e B:LA are more polar combinations due to a higher amount of organic acid in their composition, as well as the hydroethanolic extract (ethanol and water). Acetone and acetonitrile, although aprotic solvents, are polar, and when mixed with other polar

solutions, they were unable to form the two characteristic phases of LLE. If we organize the organic solvents used from most polar to least polar, the order is as follows: acetonitrile > acetone > dichloromethane > ethyl acetate > diethyl ether > hexane > heptane. The NaDES used in this study and 40% ethanol can be organized from least polar to most polar as follows: U:GL > CC:GL > B:LA > LA:G > Et (BASHIR et al., 2023; HAYNES, 2014; REICHARDT; WELTON, 2011) This explains why ACN and Ac could not form two phases after mixing with LA:G; B:LA and Et.

4.1.2 LLE in hydrophilic NaDES

In this study, the natural solvents used and their respective extracts were paired based on pH value—two acidic NaDES combinations and two basic combinations—and composition—containing lactic acid or glycerol (Table 1). It was observed that these factors produced similar results, and the NaDES composition influenced the efficiency of the solvent used. According to LLANO et al. (2015), ethyl acetate is an excellent choice for its ability to extract phenolic compounds. The LA:G, B:LA (both containing lactic acid and acidic pH), and Et extracts showed similar results, particularly when using ethyl acetate. Similarly, MATEO et al. (2013) conducted a liquid-liquid extraction of an oily by-product derived from olive trees, in which ethyl acetate outperformed hexane and chloroform—24% and 30%, respectively—achieving up to 49% phenolic compound removal from the evaluated matrix.

Several parameters can determine better results in LLE, including the pH of the samples. In this study, four samples had an acidic pH, two were extremely acidic (< 3.0), and one was alkaline. For the extraction of phenolic compounds, it is necessary to carefully choose the ideal solvent, which should provide good solubilization—by penetrating the plant matrix, forming hydrogen bonds with the phenolic compounds, and the pH of the medium affecting the ionization of the compounds, influencing their solubility and extraction capacity (ALARA; ABDURAHMAN; UKAEGBU, 2021). Initially, acidic samples tended to improve the extraction of phenolic compounds from the original matrix (sunflower meal) by forming hydrogen bonds between the solution and the solvent (SAS et al., 2018) —a result contrary to that found in the LLE presented. The U:GL extract had a pH of 8.5 and achieved the best extraction values with acetonitrile. Chlorogenic acid (CGA) is more soluble in polar solvents such as ethanol, methanol, and acetonitrile, or in mixtures of these solvents with water, due to its structure composed of hydroxyl and carboxyl groups, which have polar properties. These solvents

favor the formation of hydrogen bonds with CGA, facilitating its extraction (LI et al., 2023; SINGH; SINGLA; PANDEY, 2023). The pH control also influences CGA ionization; in an alkaline medium, hydrolysis can occur, leading to the separation of caffeic and quinic acid and the formation of oxidation products. In an acidic medium, CGA is more stable, preserving its original structure and reducing the risk of hydrolysis (LI et al., 2023; LU et al., 2020; TARAHI et al., 2024).

In general, organic compounds are more strongly extracted by apolar solvents than by polar solvents. A polar solvent will have a higher affinity for polar compounds, while a apolar solvent will have a stronger relationship with apolar compounds. This choice affects extraction selectivity—more polar solvents will preferentially extract more polar compounds, while less polar solvents will better extract apolar compounds. Moreover, polarity influences the solvent's ability to displace previously partitioned compounds: thus, the solvent's polarity must be compatible with the polarity of the compounds to be extracted to maximize extraction efficiency (FUAD et al., 2021; REICHARDT; WELTON, 2011). Generally, phenolic compounds are polar, with their polarity varying depending on the number and position of hydroxyl groups, as well as the presence of other functional groups. Those with more free hydroxyl groups are also more soluble in water: the addition of hydroxyls to the aromatic ring increases polarity, while the methoxy group reduces it, for example (ALARA; ABDURAHMAN; UKAEGBU, 2021). Flavonoids and tannins, more complex phenolic compounds, can have varying polarity depending on their specific structure, while simpler compounds like phenol and phenolic acids tend to be more polar (ALARA; ABDURAHMAN; UKAEGBU, 2021; SAS et al., 2018).

Polarity influences the solubility of phenolic compounds—the more polar ones are also more soluble in polar solvents, such as water and ethanol, while the less polar ones are soluble in organic solvents—and it affects both their interactions with other molecules and their biological activity. Thus, methods for extracting and separating phenolic compounds must consider their polarity to optimize the process (ALARA; ABDURAHMAN; UKAEGBU, 2021; SAS et al., 2018). Combinations of apolar solvents, such as heptane, hexane, and diethyl ether, and dipolar solvents, such as ethyl acetate and dichloromethane, with potentially polar NaDES resulted in the formation of two phases and yielded good results. Although these combinations are not as polar as

acetonitrile, they still showed effectiveness, given that acetonitrile did not form two phases in the LA:G and B:LA, extracts or in the hydroethanolic extract.

Overall, LLE samples in glycerol showed better results in the percentage of phenolic compound removal, especially in extractions with acetonitrile, heptane, and hexane for the CC:GL extract, and with acetonitrile for the U:GL extract. These results indicated two extremes, as CC:GL, heptane, and hexane are more apolar compared to acetonitrile, which has a polar nature (REICHARDT; WELTON, 2011). Chlorogenic acid is a polar compound due to the presence of hydroxyl groups, making it soluble in polar solvents such as water and ethanol (LU et al., 2020) , and its solubility increases at higher pH levels. This suggests that slightly alkaline conditions could potentially improve the efficiency of LLE extraction. However, very high pH levels should be avoided, as they can lead to the degradation of chlorogenic acid (NGUYEN et al., 2024). These factors explain why there was better affinity with the U:GL, extract, as well as with the LA:G, B:LA and Et extracts with ethyl acetate, which is also more polar than apolar. Acetonitrile also proved to be a good solvent for the CC:GLextract, indicating that the polarity of the solvent is important for the success of the extraction.

Ethyl acetate and dichloromethane are classified as dipolar solvents, and certain factors determine whether these solvents will behave as polar or apolar, including the interactions between the solute and solvent and the polarizability of the solvent molecules affecting their ability to interact with solutes. The polarity of the NaDES samples may have assisted in hydrogen donation to the organic solvents, causing them to behave in a apolar manner.

4.2 Cytotoxicity assays

Two types of assays were conducted to evaluate the cytotoxicity of the samples: cell viability in human tumor cell lines and antimicrobial activity with pathogens.

The Alamar Blue method, a low-cost and non-toxic assay, is a fluorometric test that evaluates cell viability and proliferation. In this methodology, resazurin, a blue compound, is converted into resorufin, a fluorescent pink compound. When metabolically active cells are present, the mitochondria of viable cells can perform this conversion (SANJAI et al., 2024). The TTC method (2,3,5-triphenyltetrazolium chloride), which has

a lower cost and greater sensitivity in detecting bacterial growth compared to other colorimetric indicators, is a colorimetric test used to assess microbial susceptibility by adding the TTC color indicator before reading the results. In the presence of bacterial growth, TTC is reduced to an insoluble red compound called formazan (SOUZA et al., 2024).

Microdilution is a standardized technique for evaluating natural extracts, antibiotics, and other compounds, allowing serial dilutions to be performed, facilitating the determination of MIC and cell viability. This is a more economical methodology because, in addition to using small volumes of sample and culture medium, it provides quantitative results (BALOUIRI; SADIKI; IBNSOUDA, 2016; FERRARO, 2005).

4.2.1 Cancer cells

Sunflower meal extract is rich in chlorogenic acid (CGA), which can induce apoptosis by increasing the production of reactive oxygen species, reducing mitochondrial membrane potential, and thus suppressing tumor growth in human cancer cells and animal models, such as mice and rats. CGA did not affect normal cells, such as breast cells (MCF-10A) and human hepatocytes (QSG-7701) (GUPTA et al., 2022). However, when combined with conventional chemotherapeutic agents such as doxorubicin in osteosarcoma cells (U2OS and MG-63), CGA suppresses metabolic activity and cell growth, demonstrating synergistic effects (NGUYEN et al., 2024). Therefore, the use of sunflower meal is valued, as it is rich in CGA and is currently considered waste and/or used for animal feed. It is important to remember, however, that CGA is a sensitive compound, as mentioned earlier in section 4.1.1: exposure to oxygen and ambient or higher temperatures can lead to CGA degradation, resulting in a green coloration, as seen in the hydroethanolic extract samples after cell treatment. The CC:GL LLE samples did not significantly reduce cell viability, possibly due to insufficient concentrations of bioactive compounds to cause a cytotoxic effect on the cells.

The pH of the samples was not considered in the application of solvents and extracts in most of the studies found, even those that used organic acids for NaDES composition. It is possible to state that combinations containing choline chloride and urea, together or with other HBDs—as in this study—are good combinations, as they can have their pH neutralized and reduce the viability of human cancer cell lines. In Radošević et

al. (2018), 10 pure NaDES combinations were tested on two cancer cell lines (HeLa and MCF-7) and one non-cancerous line derived from embryonic kidney (HEK293T). The cytotoxic activity of choline chloride:oxalic acid (CC:OX) NaDES showed more significant results in HeLa and MCF-7 cell lines, while choline chloride:urea (CC:U) was effective only in MCF-7. Combinations containing organic acids showed higher toxicity, especially those with pH <3. NaDES of betaine:malic acid:proline exhibited a dose-dependent pattern indicating a proliferative effect on both tumor and non-tumor lines tested (RADOŠEVIĆ et al., 2018).

Twelve choline chloride-based pure NaDES combinations were tested on three tumor cell lines (HT-29, Caco-2, and MCF-7) and one normal fibroblast cell line (MRC-5). The choline chloride:citric acid and choline chloride:malic acid NaDES combinations showed the best results, with 5.54 mg mL-1 required to inhibit 50% of cell growth (IC50) in citric acid samples. These values were associated with the pH of the samples, showing that more acidic combinations (pH 1.5) had higher cytotoxicity (POPOVIĆ et al., 2023). In the present study, the best reduction in viability of a pure NaDES required only 6.25% of the neutralized urea:glycerol combination. Popović et al. (2023) and Radošević et al. (2018) also correlated the acidity of NaDES and/or extracts with cytotoxic activity in cells, although they did not neutralize the extracts to evaluate them without this factor, which could cause effects solely due to the acidity of the sample.

In another breast cancer cell line, MDA-MB-231, cytotoxicity was found to be dose- and time-dependent through the inhibition of the activation pathway of cells that contribute to tumor progression and resistance to therapies (NGUYEN et al., 2024). In the present study, the pure CC:GL NaDES sample had the highest cytotoxicity on this cell line, along with the sunflower meal phenolic extract in the same NaDES (CC:GL S), —minimum doses of 6.25% and 3.7 mg CGA L^{-1}, respectively, reduced cell viability by 80%.

Thus, it can be stated that pure NaDES already possess some cytotoxic activity on cancer cells, even when associated with the pH of the NaDES combination. Consequently, when extracting phenolic compounds in a NaDES medium, an associated effect of the NaDES cytotoxic activity with the phenolic extract is expected. Studies have evaluated the cytotoxic activity of extracts from various sources extracted with NaDES: the extract of a medicinal plant, *Sideritis scardica*, associated with choline chloride:glycerol NaDES, showed a more potent antiproliferative effect on CCL-1 cells

(mouse fibroblasts), requiring only 12.2 μg mL^{-1} of TPC to reach IC50. In comparison, the natural solvent alone required 1.18% after 24 hours of treatment. Pure NaDES samples were less cytotoxic after 72 hours compared to 24 hours (GROZDANOVA et al., 2020). As also observed in the results of the sunflower meal phenolic extract in NaDES presented in this study, the solvents were dose-dependent on the S. *scardica* extract.

Using lower concentrations of cocoa by-products than those of sunflower meal in the present study, Manuela et al. (2020) observed a reduction in cell viability of HeLa cells (cervical cancer) by approximately 40% when extracted in choline chloride-based NaDES at concentrations of 2 to 5%, and 30% in betaine-based NaDES at concentrations of 2.5 to 5%. However, these indices were less efficient than the results presented in this study, as both the pure NaDES samples and the sunflower meal extract samples showed reductions greater than 50% in cell viability in almost all MCF-7 and MDA-MB-231 samples. In anthocyanins from black carrots extracted with choline chloride:citric acid NaDES, tested on human colon carcinoma (Caco-2) and human hepatoma (HUH7) cells, as observed in other studies, the reduction in cell viability was more significant at higher concentrations, with the highest toxic effect observed at 150 μg mL^{-1} for both cell lines tested (ASLAN TÜRKER; DOĞAN, 2022).

Chlorogenic acid (CGA) alone or in infusion with Artemisia annua in breast adenocarcinoma cells (MCF-7) reached IC50 at a concentration of 127 μM—there was DNA damage, cell cycle arrest, and apoptosis of treated cells (DEKA et al., 2017; SUBERU et al., 2014). In this study, at least one sample from each set achieved 50% or more inhibition of cell viability: efficacy was seen in solutions from the lowest concentrations of NaDES with extract (3.7 mg CGA L^{-1} and 7.7 mg CGA L^{-1}, for CC:GL and U:GL, respectively); in conventional extractions, the ability to inhibit microbial growth was observed only at the highest concentrations. CGA is a phenolic compound commonly recognized for its antioxidant activity, protecting cells from oxidative stress caused by free radicals and normal cells through modulating inflammation signaling and preferentially causing the death of cancer cells (CHEN et al., 2021; LU et al., 2020).

In non-cancerous cells, a 60 to 85% cell proliferation was observed at the tested concentrations of betaine NaDES in HaCaT cells (normal keratinocytes), compared to untreated cells, indicating selective cytotoxicity against cancer cells while promoting the growth of normal epithelial cells (MANUELA et al., 2020). Tests were not conducted on non-cancerous cells, but this is an indication that, depending on the combination and with

attention to the pH of NaDES, applications aiming at the proliferation of normal cells are also feasible.

4.2.2 Antimicrobial activity

Antimicrobial activity occurs due to the binding of phenolic compounds to bacterial cell membranes, disrupting membrane protein synthesis and inhibiting microbial growth (CAO et al., 2023). showed antimicrobial activity against *Staphylococcus aureus*, with MICs higher than those found by Grozdanova et al. (2020), who used Plantago major extract in NaDES based on citric acid and 1,2-propanediol and reported MICs of 1.99 to 3.98 μg mL^{-1} of TPC against Gram-positive bacteria. Additionally, the 70% ethanol extract did not exhibit antimicrobial activity against most of the microorganisms tested (S. *pyogenes*, E. *coli*, S. *aureus*, P. *aeruginosa*, B. *cereus*, and C. *albicans*), with an MIC of 69.3 μg mL^{-1} of TPC for those that showed inhibition. In the present study, conventional extracts (Et, Et Ch, and W) showed the lowest MICs, ranging from 0.27 mg GAE L^{-1} for W in Salmonella enterica to 9.2 mg CGA L^{-1} in the Et extract applied to *Shigella flexneri*.

Overall, samples obtained through conventional extraction presented lower MICs (0.27 - 9.2 mg CGA L^{-1}) compared to NaDES, both pure and phenolic extracts (Supplementary Table 2). This phenomenon may be attributed to the viscosity of NaDES, which can hinder sample homogenization and interfere with results, requiring higher doses to achieve an antimicrobial effect. On the other hand, conventional extraction samples are resuspended in culture medium and are, therefore, not viscous, eliminating this potential limiting factor.

The chlorogenic acid present in sunflower meal has already shown inhibitory effects on foodborne pathogens, as it affects metabolic enzymes, inactivates bacterial cells, and consequently interferes with their growth and promotes cell death (WANG et al., 2022). A chlorogenic acid solution dissolved in dimethyl sulfoxide (DMSO) was applied to various pathogens, resulting in MICs of 8 μg mL^{-1} for *E. coli*, 8 μg mL^{-1} for *P. aeruginosa* and 16 μg mL^{-1} for *S. aureus* (ÖZÇELIK; KARTAL; ORHAN, 2011). These values are lower than those obtained in this study for hydroethanolic extract, which showed 9.2 mg CGA L^{-1} for *E. coli* and *P. aeruginosa*. Moreover, the S. *aureus* strain remained 100% viable after treatment.

The antimicrobial activity of other parts of the sunflower was tested: an ethanolic extract of sunflower leaves (Helianthus annus) was highly effective against S. *aureus* and E. *coli*, with MICs of 3 mg mL^{-1} and 4 mg mL^{-1} of extract, respectively, compared to the aqueous extracts of sunflower leaves (CHANDRAMOORTHY, 2016). Sunflower seed oil also showed antimicrobial activity against E. *coli* (MIC 36.3 μg mL^{-1}) and S. *aureus* (15.9 μg mL^{-1}) (ELIUZ, 2021). The ethyl acetate extract of sunflower flowers, at a concentration of 500 μg mL^{-1}, had the largest inhibition halo against *S. aureus*, measuring 20 mm (AL-SHUKAILI; HOSSAIN, 2019).

The phenolic extract of broccoli leaves, obtained by ultrasound-assisted extraction in NaDES of choline chloride and propylene glycol, showed an MIC of 0.25 mg mL^{-1} for *E. coli* and 0.125 mg mL^{-1} for *S. aureus* (CAO et al., 2023). In the present study, these two microorganisms exhibited higher MICs, 3.7 mg CGA L^{-1}, for the phenolic extract of sunflower meal in NaDES CC:GL for the E. *coli* strain. Furthermore, the minimum inhibitory concentration for L. *monocytogenes* in the sunflower phenolic extract sample in NaDES U:GL was slightly more than half (7.7 mg CGA L^{-1}) of that reported by Jovanović et al. (2022) in bilberry extract (MIC de 15 mg mL^{-1}). The authors also reported that the extract was more effective against Gram-positive bacteria than against Gram-negative ones.

Radošević et al. (2018) tested the antimicrobial activity of pure NaDES using the disk diffusion method, which quantifies the size of the inhibition halo formed by the samples on the inhibition plate. They found that combinations with citric acid showed the largest inhibition halo, with 50 mm for *E. coli* and 51 mm for *P. aeruginosa* and *S. aureus* in the combination of citric acid, fructose, and glycerol. In the results presented in this work, NaDES composed of organic acids were not tested due to the potential interference of the pH of these solvents in the analysis. The extracts derived from these NaDES, which underwent LLE, showed 100% inhibition of all microorganisms evaluated, but, as in the cell viability study, the pH of the extract may have interfered.

Similarly, Trusheva et al. (2024) valuated the antimicrobial activity of pure NaDES and propolis extract in a NaDES medium. The authors observed that when pure NaDES were applied, there was an inhibitory effect from these solvents, but when associated with the propolis extract, the inhibition was greater than that observed in the pure solvents. Choline chloride and 1,2-propanediol NaDES showed an MIC of 6.25% for *L. monocytogenes*, while the extract required only 96.41 μg of TPC mL^{-1}

(TRUSHEVA et al., 2024). For the phenolic extract of sunflower meal, choline chloride NaDES required 50% and 3.7 mg de CGA L^{-1} to inhibit the same microorganism. However, a similar reduction in MIC was not observed between pure NaDES and sour cherry pomace extract, except for *P. aeruginosa* and *E. coli*, where the MIC decreased from 25% to 12.5% only with the choline chloride and urea combination. The other combinations of choline chloride with malic acid and fructose showed no change between the pure sample or with extract (POPOVIC et al., 2022). This pattern was not observed in the samples from this study, where the phenolic extract resulted in a lower MIC when associated with NaDES.

The phenolic compounds in sunflower increase the permeability of bacterial cytoplasmic membranes, causing leakage of intracellular components and cytoplasmic coagulation, and consequently, cell death (AMIRUL, 2020). The microorganisms tested in this study are bacteria associated with food poisoning, present in contaminated food or water. It is possible to suggest that sunflower extracts in NaDES have the potential to be used as natural preservatives for food.

5. Conclusion

The results indicated that despite variations in the polarity and properties of the solvents used, NaDES, when combined with phenolic extracts from sunflower meal, demonstrated significant efficiency in both the extraction and biological activity of the compounds. The U:GL and CC: NaDES combinations proved particularly effective in reducing the viability of human cancer cell lines (MCF-7 and MDA-MB-231), with studies needed on non-tumor cells to determine whether there is potential for developing anticancer therapies. Additionally, the observed cytotoxicity was strongly influenced by the pH of the samples, suggesting that pH optimization is crucial to maximizing the effectiveness of the extracts. Regarding antimicrobial activity, the extracts obtained by conventional extraction methods, such as hydroethanolic extraction, showed the lowest minimum inhibitory concentration (MIC) values, outperforming NaDES samples in some cases. This may be attributed to the viscosity of NaDES, which can hinder sample homogenization and, consequently, reduce their antimicrobial activity.

In summary, this study reinforces the potential of NaDES as sustainable and effective solvents for extracting bioactive compounds, while also highlighting the

importance of parameters such as pH and polarity in optimizing extraction processes and the biological efficacy of the obtained extracts. Continued research in this field could deepen the understanding of interactions between solvents and bioactive compounds and promote advances in the application of these extracts in anticancer and antimicrobial therapies.

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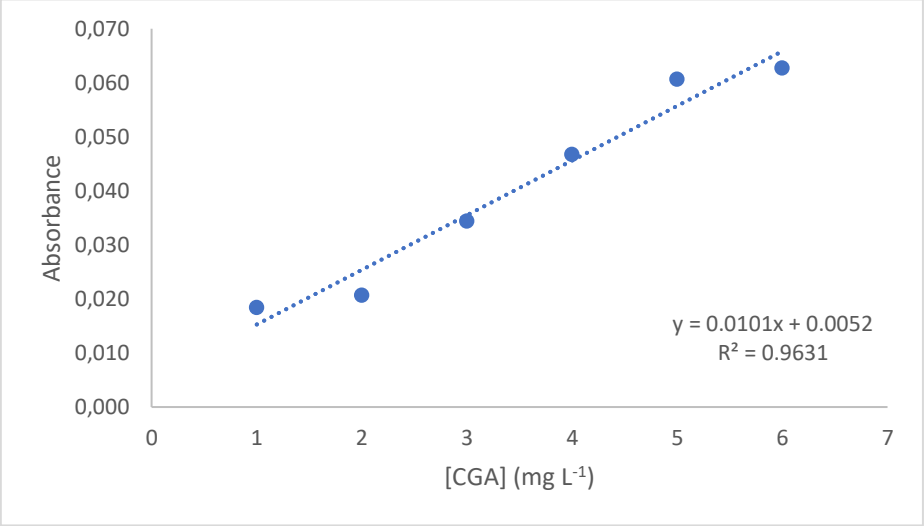
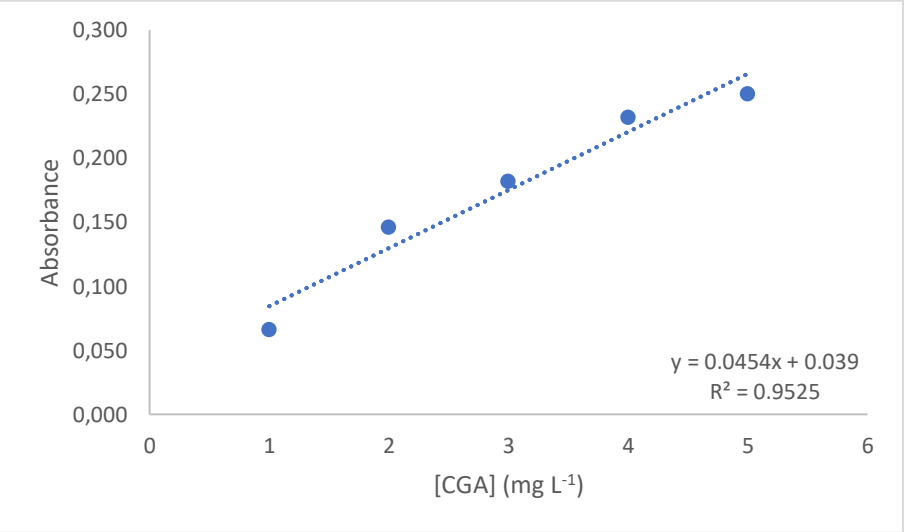
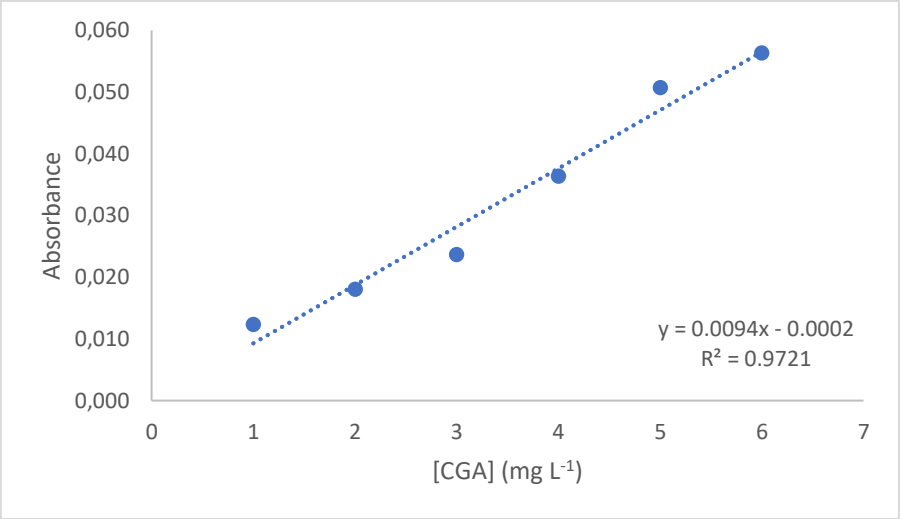
Supplementary material



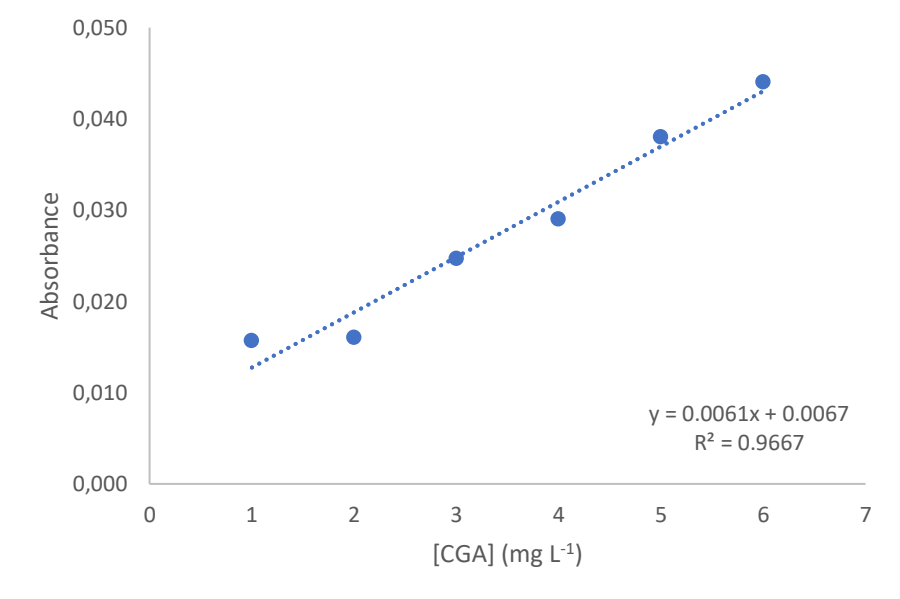
Supplementary figure 1. Standard curve for LA:G NaDES samples with equation and coefficients of determination (R^2)



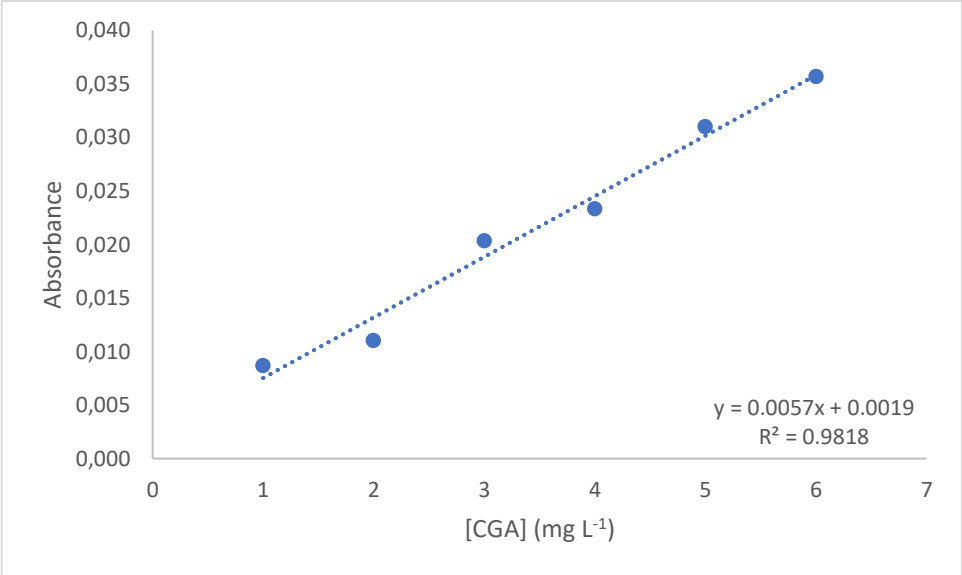
Supplementary figure 2. Standard curve for CC:GL NaDES samples with equation and coefficients of determination (R^2)



Supplementary figure 3. Standard curve for U:GL NaDES samples with equation and coefficients of determination (R^2)

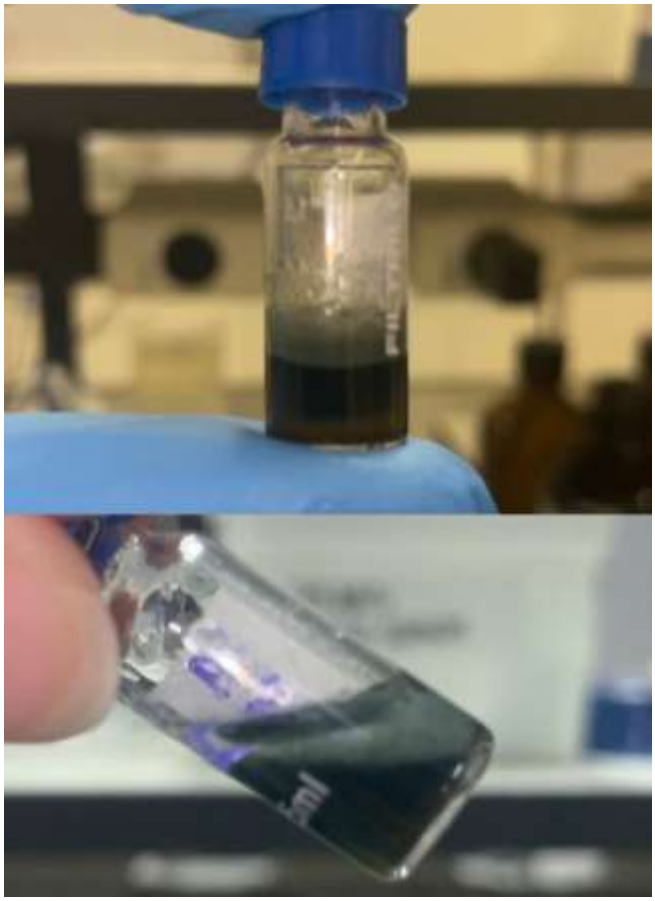


Supplementary figure 4. Standard curve for B:LA NaDES samples with equation and coefficients of determination (R^2)

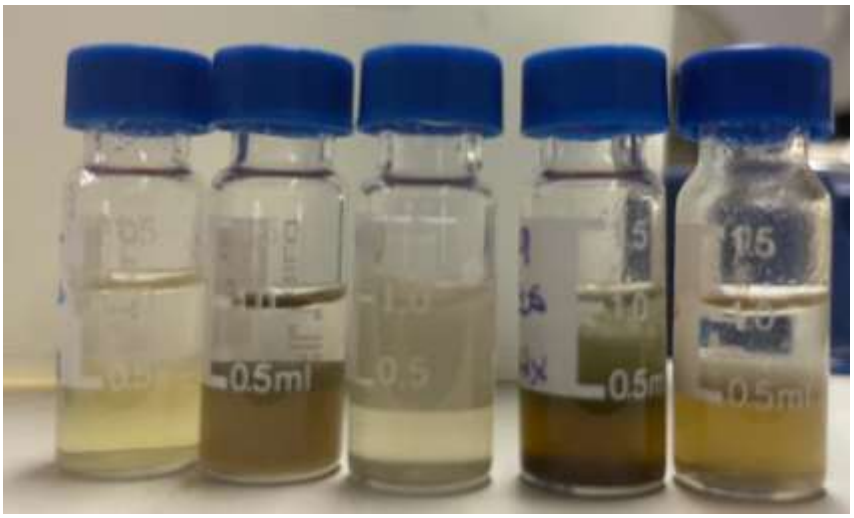


Supplementary figure 5. Standard curve in water for Ethanol samples with equation and coefficients of determination (R^2)

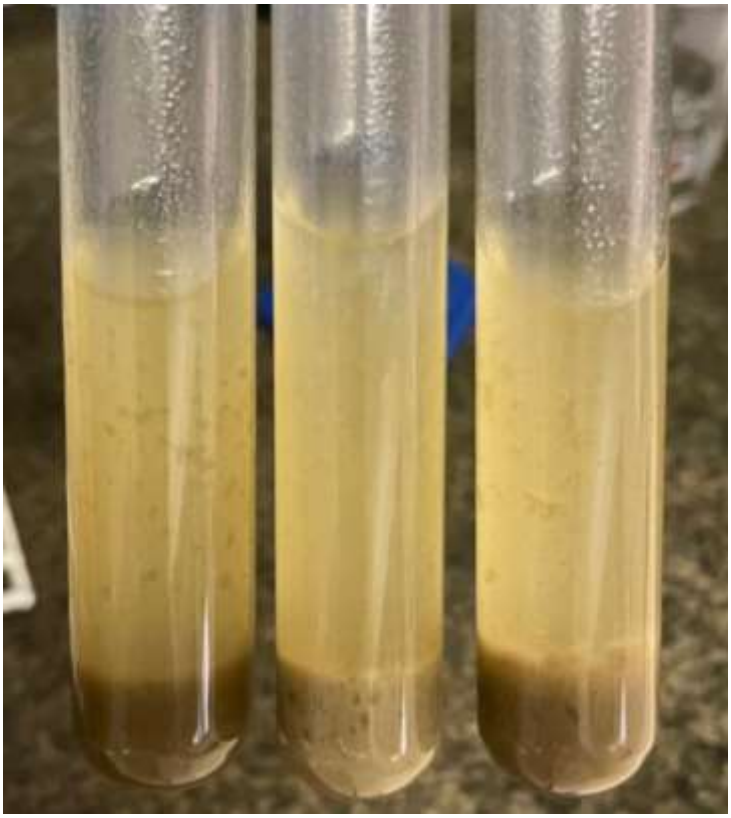
Supplementary figure 6. Samples of CC:GL in hexane after 1 and 5 minutes in vortex



Supplementary figure 7. Sample of U:GL after resting at 25°C for 24 hours



Supplementary figure 8. Samples of LA:G, CC:GL, U:GL, B:LA and Et after resting at 18°C for 24 hours



Supplementary figure 9. Samples of LA:G, B:LA and Et after mixture with Acetone

Supplementary table 1. Valores correspondentes as concentrações utilizadas no tratamento de citotoxicidade em células tumorais e antimicrobiano em patógenos em mg CGA L^{-1}

	50%	**25%**	**12.5%**	**6.25%**
CC:GL S	29.8	14.9	7.5	3.7
CC:GL LLE*	23.8	11.9	5.9	2.9
U:GL S	62.1	31.0	15.5	7.7
U:GL LLE*	35.7	17.8	8.9	4.4
Et	73.9	39.9	18.5	9.2
Et Ch†	67.3	33.6	16.8	8.4
W†	17.8	8.9	4.4	2.2
LA:G LLE*	33.2	16.6	8.3	4.1
B:LA LLE*	26	13	6.5	3.2

*valores calculados a partir do valor residual após extração líquido-líquido; †baseados nos valores obtidos por Nascimento et al. (2023)

Supplementary table 2. Mean (%) and standard deviation (SD) from the cytotoxicity essay in MCF-7 cancer cells

	Control	**50%**	**25%**	**12.5%**	**6.25%**
CC:GL	100.136 ± 0.942	21.631 ±4.909	23.409 ± 5.797	24.025 ± 6.765	26.162 ± 5.598
CC:GL S	99.994 ± 0.898	30.880 ± 17.214	19.718 ± 15.250	29.683 ± 20.069	34.067 ± 15.490
CC:GL LLE	99.612 ± 1.262	99.131 ± 10.507	99.921 ± 9.057	98.822 ± 9.184	98.528 ± 7.210
U:GL	100.136 ± 0.942	20.829 ± 5.833	22.762 ± 7.011	29.307 ± 6.350	50.392 ± 7.232
U:GL S	99.994 ± 0.898	29.403 ± 16.088	14.977 ± 6.736	16.088 ± 14.274	41.143 ± 11.172
U:GL LLE	99.722 ± 1.260	50.834 ± 34.391	78.495 ± 20.580	97.369 ± 18.972	104.345 ± 10.076
Et	99.994 ± 0.898	67.292 ± 20.987	93.718 ± 7.415	119.650 ± 9.265	125.779 ± 10.994
Et Ch	100.136 ± 0.942	24.974 ± 6.486	49.050 ± 8.093	99.756 ± 7.226	111.429 ± 8.049
W	99.953 ± 0.819	20.836 ± 5.720	26.567 ± 7.313	67.845 ± 11.742	98.783 ± 6.943
LA:G LLE	99.596 ± 1.345	46.564 ± 5.764	93.235 ± 9.708	106.839 ± 14.777	107.981 ± 12.191
B:LA LLE	99.596 ± 1.345	100.024 ± 4.739	99.355 ± 7.972	103.405 ± 8.286	107.708 ± 5.242

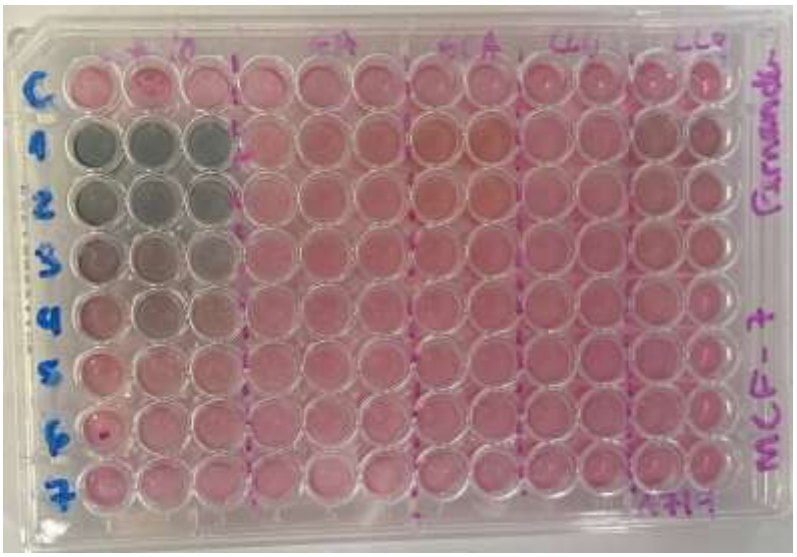
Supplementary table 3. Mean (%) and standard deviation (SD) from the cytotoxicity essay in MDA-MB-231 cancer cells

	Control	**50%**	**25%**	**12.5%**	**6.25%**
CC:GL	99.543 ± 0.779	27.225 ± 8.874	29.511 ± 9.563	29.376 ± 8.083	31.246 ± 7.359
CC:GL S	99.546 ± 0.703	24.074 ± 12.117	32.192 ± 14.029	30.362 ± 7.944	32.678 ± 2.797
CC:GL LLE	98.996 ± 2.791	95.804 ± 8.749	103.012 ± 5.409	100.051 ± 8.132	97.970 ± 8.233
U:GL	99.543 ± 0.779	22.935 ± 7.538	28.873 ± 10.360	35.314 ± 9.298	53.911 ± 15.094
U:GL S	99.546 ± 0.703	40.040 ± 29.517	31.636 ± 25.153	40.905 ± 28.015	41.541 ± 16.886
U:GL LLE	99.270 ± 2.414	50.824 ± 29.138	63.270 ± 36.837	70.280 ± 31.801	86.752 ± 15.263
Et	99.546 ± 0.703	41.374 ± 21.694	99.897 ± 11.926	113.930 ± 12.074	110.125 ± 8.375
Et Ch	99.543 ± 0.779	35.194 ± 7.412	44.636 ± 7.946	76.297 ± 5.049	94.536 ± 6.065
W	99.572 ± 0.827	30.162 ± 7.137	32.708 ± 8.554	47.610 ± 6.276	73.870 ± 4.651
LA:G LLE	99.191 ± 2.236	51.138 ± 8.858	86.779 ± 9.099	97.005 ± 8.238	103.303 ± 11.696
B:LA LLE	99.191 ± 2.236	101.376 ± 7.261	103.985 ± 8.778	103.693 ± 13.117	107.909 ± 9.424

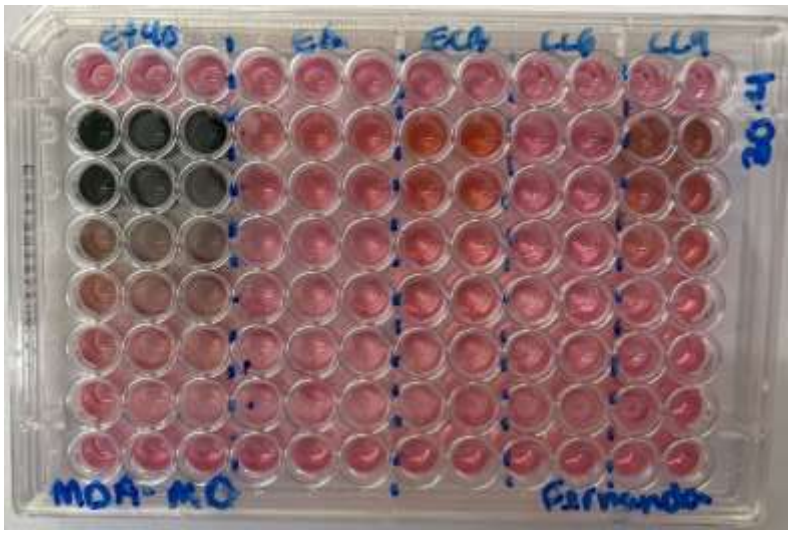
Supplementary table 4. Minimum inibitory concentration dos extratos líquido-líquido

Microrganismo / MIC	LA:G LLE mg CGA L^{-1}	B:LA LLE mg CGA L^{-1}
B. cereus	1	3.2
L. monocytogenes	4.2	3.2
E. coli	4.2	6.5
S. flexneri	2	6.5
P. aeruginosa	1	6.5
S. entérica	2	6.5
S. aureus	2	6.5

Supplementary figure 10. MCF-7 samples after 24h treatment



Supplementary figure 11. MDA-MB-231 samples after 24h treatment



Conclusion

The results of this thesis have opened new possibilities for the utilization of agro-industrial by-products, such as sunflower meal, in the production of functional ingredients and the formulation of value-added products, promoting sustainability and innovation in the food and pharmaceutical industries.

The studies conducted demonstrated that NaDES were effective green solvents for the recovery of bioactive compounds, offering a sustainable alternative to traditional organic solvents. The assessment of the stability of the extracted phenolic compounds showed that NaDES preserved the integrity of bioactive compounds for extended periods, particularly under controlled storage conditions, such as lower temperatures. These results reinforce the potential application of NaDES in preserving sensitive compounds, broadening their possibilities of use in the food and pharmaceutical industries.

The application of the LLE technique as a complementary method for the purification of phenolic extracts in NaDES proved to be a feasible technique, although dependent on applied conditions, such as the composition of the NaDES, the polarity of the solvents, and target compounds, which influenced the removal of the compounds of interest.

The evaluation of the cytotoxicity of the extracts demonstrated that NaDES exhibited significant bioactivity, particularly in reducing cell viability in breast cancer cell lines. However, further studies are needed to evaluate the cytotoxicity of NaDES and phenolic extracts in normal cells. Finally, antimicrobial activity studies revealed that phenolic extracts in NaDES exhibited potential as antimicrobial agents, with efficacy against a variety of Gram-positive and Gram-negative pathogens. These findings highlight the versatility of NaDES, not only as extraction solvents but also as vehicles for delivering bioactive compounds with potential applications in anticancer and antimicrobial therapies.

ATTACHMENT

1. Chromatographic analyses

A high-performance liquid chromatograph with photo diode array (PDA) detector and column oven at 30°C (Shimadzu LC-MS 2020, São Paulo, Brazil), equipped with a reversed phase column (C18 150 × 4.6 × 5µm – Phenomenex Luna), was used. The injection volume was 20 µL and the flow rate was 0.8 mL/min for 20 minutes. The mobile phase comprised: 80% H_2O and 20% methanol. A solution of ultrapure water:methanol (80:20, v/v) was used to dilute standards and samples. Analyses and injections were carried out in duplicate, spectral scan between 190 and 500 nm and detection were performed at 320 nm. Identification was achieved according to ultraviolet absorption spectrum and retention times and quantification based on a standard curve with concentrations ranging from 5 to 100 mg CGA.mL-1.

2. Results

2.1 HPLC analysis

Table A1 presents the quantification of phenolic compounds in chlorogenic acid equivalents from the extract phase in NaDES or hydroethanolic medium by HPLC. Due to the viscosity of the LA:G sample, only one phase was suitable for analysis: the remaining phase after extraction with dichloromethane (DiCl). Analyses with acetone and acetonitrile were not available because they did not form two phases in the LA:G sample, only one phase was suitable for analysis: the remaining phase after extraction with dichloromethane (DiCl). Analyses with acetone and acetonitrile were not available because they did not form two phases in the LA:G; B:LA and Et samples.

Due to variations in the initial concentrations of CGA in each extract, the amount extracted does not serve as a suitable basis for comparison. Therefore, from this point onward, the data will be expressed as a percentage of removal, adjusted according to the initial concentration of each extract, as shown in Table A1.

Table A1. Chromatographic quantification of residual phenolic compounds of NaDES phase after liquid-liquid extraction

[CGA] mg L^{-1}					
	LA:G	**CC:GL**	**U:GL**	**B:LA**	**Et**
Initial concentration	99 ^{Da}	59.6 ^{Ea}	124.3 ^{Ba}	114.8 ^{Ca}	147.9 ^{Aa}
Hp	×	21.1 ^{Db}	47.3 ^{Bb}	33.5 ^{Cc}	59.3 ^{Ac}
Hx	×	6.0 ^{Dh}	39.5 ^{Bd}	20.3 ^{Cf}	58.1 ^{Ad}
EtA	×	18.2 ^{Dc}	41.9 ^{Bc}	20.7 ^{Ce}	55.3 ^{Ae}
Ac	-	14.3 ^{Bg}	24.5 ^{Ah}	-	-
DiCl	21.6 ^{Cb}	17.3 ^{Ed}	38.6 ^{Be}	20.9 ^{Dd}	46.9 ^{Af}
EEt	×	15.8 ^{De}	34.4 ^{Cf}	49.4 ^{Bb}	89.0 ^{Ab}
ACN	-	15.4 ^{Bf}	27.1 ^{Ag}	-	-

× samples unable to analysis; - samples unavailable. Different lowercase letters indicate significant difference among solvents in the same extract. Different capital letters indicate significant difference in the same solvent among different extracts.

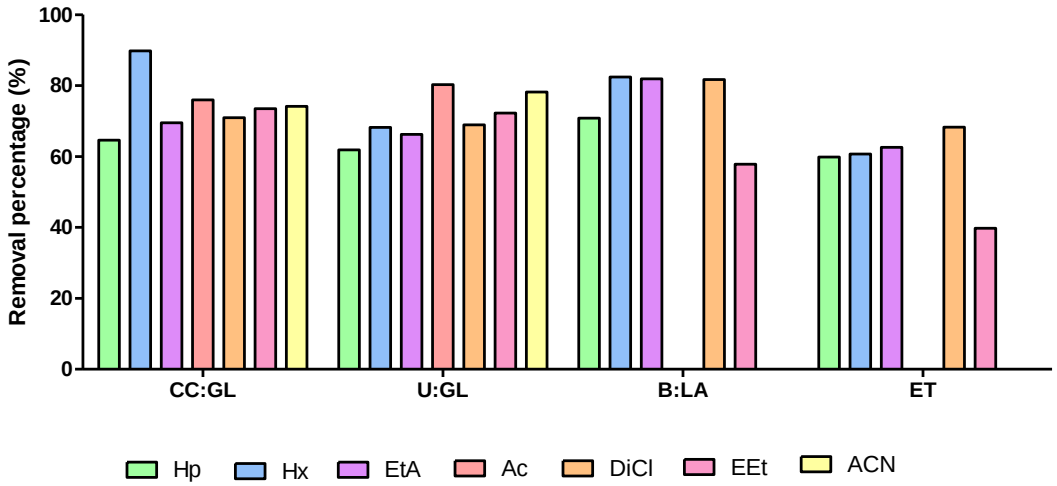


Figure A1. Chromatographic percentage of removed chlorogenic acid in the NaDES/Ethanol phase after liquid-liquid extraction from sunflower meal extracts.

Hp – heptane; Hx – hexane; EtA – ethyl acetate; Ac – acetone; DiCl – dichloromethane; EET – ethyl ether; ACN - acetonitrile.

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