UNIVERSIDADE FEDERAL DO PARANÁ

ROSANA RABELO MANÇANO

QUÍMICA VERDE: VALORIZAÇÃO SUSTENTÁVEL DE PRÓPOLIS VIA EXTRAÇÃO

PALOTINA

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Dissertação apresentada ao curso de Pós-Graduação em Engenharia e Tecnologia Ambiental, Setor de Palotina, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia e Tecnologia Ambiental.

Orientadora: Prof(a). Dr(a). Leda Maria Saragiotto Colpini

Coorientadora: Prof(a). Dr(a). Tânia Forster Carneiro

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA E TECNOLOGIA AMBIENTAL da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **ROSANA RABELO MANÇANO**, intitulada: **Química Verde: Valorização Sustentável de Própolis Via Extração**, sob orientação da Profa. Dra. LEDA MARIA SARAGIOTTO COLPINI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestra está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Palotina, 25 de Fevereiro de 2025.

Assinatura Eletrônica
25/02/2025 19:31:19.0
LEDA MARIA SARAGIOTTO COLPINI
Presidente da Banca Examinadora

Assinatura Eletrônica
26/02/2025 20:34:32.0
RODRIGO BRACKMANN
Avaliador Externo (UNIVERSIDADE TECNOLóGICA FEDERAL DO PARANá - CAMPUS PATO BRANCO)

Assinatura Eletrônica
25/02/2025 22:23:45.0
GIANE GONÇALVES LENZI
Avaliador Externo (UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ)

Assinatura Eletrônica
27/02/2025 09:42:42.0
TÂNIA FORSTER CARNEIRO
Coorientador(a) (UNIVERSIDADE ESTADUAL DE CAMPINAS)

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RESUMO

Própolis, uma substância resinosa produzida por abelhas, é rica em compostos bioativos, como flavonoides e ácidos fenólicos, que contribuem para suas propriedades terapêuticas bem documentadas, incluindo efeitos antibacterianos e anti-inflamatórios. O interesse recente em própolis aumentou devido à identificação de novos compostos bioativos e avanços em técnicas de extração, que aumentaram sua aplicabilidade nas indústrias farmacêutica, agrícola e cosmética. Esta dissertação explora a extração de compostos bioativos de própolis marrom usando extração líquida pressurizada (PLE) em um sistema de fluxo semicontínuo. O estudo investiga o impacto da concentração de etanol, temperatura e pH na eficiência da extração, revelando condições ótimas para recuperar compostos fenólicos (90 °C, pH 7), flavonoides (120 °C) e açúcares (90 °C, pH 2). Além disso, a atividade antioxidante dos extratos foi avaliada, demonstrando bioatividade significativa, particularmente em ensaios DPPH e FRAP. As descobertas destacam a eficácia do PLE na recuperação seletiva de diferentes compostos bioativos, oferecendo um método sustentável com aplicações em vários setores. Além disso, a dissertação discute a crescente integração de própolis, extratos naturais e nanopartículas de prata (AgNPs) em aplicações industriais inovadoras. O potencial desses materiais, incluindo seu papel na medicina personalizada, tratamentos antimicrobianos e eletrônica avançada, é examinado, enfatizando seu impacto transformador nos setores de saúde, segurança alimentar e meio ambiente. Uma análise bibliométrica das tendências de pesquisa de 2010 a 2024 ressalta ainda mais a crescente relevância desses materiais no tratamento de preocupações globais de saúde e no avanço de soluções terapêuticas sustentáveis.

Palavras-chave: Hidrólise; Água subcrítica; Ácidos orgânicos; Atividade antioxidante; Análise bibliométrica; Nanotecnologia; Compostos bioativos.

ABSTRACT

Propolis, a resinous substance produced by bees, is rich in bioactive compounds such as flavonoids and phenolic acids, which contribute to its well-documented therapeutic properties, including antibacterial and anti-inflammatory effects. Recent interest in propolis has surged due to the identification of novel bioactive compounds and advancements in extraction techniques, which have enhanced its applicability in the pharmaceutical, agricultural, and cosmetic industries. This dissertation explores the extraction of bioactive compounds from brown propolis using pressurized liquid extraction (PLE) in a semi-continuous flow system. The study investigates the impact of ethanol concentration, temperature, and pH on extraction efficiency, revealing optimal conditions for recovering phenolic compounds (90 °C, pH 7), flavonoids (120 °C), and sugars (90 °C, pH 2). Additionally, the antioxidant activity of the extracts was evaluated, demonstrating significant bioactivity, particularly in DPPH and FRAP assays. The findings highlight the effectiveness of PLE in selectively recovering different bioactive compounds, offering a sustainable method with applications in various sectors. Furthermore, the dissertation discusses the growing integration of propolis, natural extracts, and silver nanoparticles (AgNPs) in innovative industrial applications. The potential of these materials, including their role in personalized medicine, antimicrobial treatments, and advanced electronics, is examined, emphasizing their transformative impact on the healthcare, food safety, and environmental sectors. A bibliometric analysis of research trends from 2010 to 2024 further underscores the increasing relevance of these materials in addressing global health concerns and advancing sustainable therapeutic solutions.

Keywords: *Hydrolysis; Subcritical water; Organic acids; Antioxidant activity; Bibliometric Analysis, Nanotechnology, Bioactive compounds*

SUMÁRIO

CHAPTER I

INTRODUÇÃO GERAL, OBJETIVOS E ESTRUTURA DA DISSERTAÇÃO

1.1 INTRODUÇÃO GERAL

A crescente busca por soluções sustentáveis e tecnologicamente avançadas tem impulsionado o desenvolvimento de novos métodos de extração e aplicação de compostos bioativos naturais. Nesse contexto, a própolis tem se destacado como um material de grande interesse científico e industrial devido à sua rica composição química, que inclui flavonoides e ácidos fenólicos. Esses compostos conferem à própolis diversas propriedades terapêuticas, como atividades antibacteriana, anti-inflamatória e antioxidante, tornando-a um ingrediente promissor para as indústrias farmacêutica, agrícola e cosmética (Bankova et al., 2021; Beserra et al., 2020).

Nos últimos anos, avanços significativos na biotecnologia têm permitido a otimização de técnicas de extração, visando maior eficiência na obtenção de compostos bioativos e a redução do impacto ambiental dos processos industriais. Métodos convencionais, como a maceração, apresentam limitações quanto ao tempo de extração e à degradação de compostos sensíveis ao calor (Chua & Rahaman, 2021). Por outro lado, técnicas inovadoras, como a extração líquida pressurizada (PLE), vêm se destacando por possibilitarem a extração seletiva de componentes bioativos de maneira mais eficiente e sustentável, sem a necessidade de grandes quantidades de solventes orgânicos (Perino-Issartier et al, 2011).

Além dos avanços na extração, a integração da própolis com a nanotecnologia tem aberto novas possibilidades para aplicações industriais. Em especial, a síntese de nanopartículas de prata (AgNPs) a partir de extratos naturais tem se mostrado uma alternativa promissora para o desenvolvimento de materiais com propriedades antimicrobianas e antioxidantes aprimoradas (Hernández-Morales et al., 2020). As AgNPs apresentam ampla aplicabilidade,

desde formulações farmacêuticas e biomédicas até o uso em embalagens ativas para a conservação de alimentos e na remediação ambiental (Rai, Yadav, & Gade, 2009). A combinação da biotecnologia com a nanotecnologia possibilita, assim, a criação de produtos inovadores, capazes de aliar eficácia terapêutica e sustentabilidade.

Dessa forma, compreender os mecanismos de extração dos compostos bioativos da própolis e suas possíveis aplicações no desenvolvimento de materiais avançados é essencial para expandir seu potencial de uso. A análise dos fatores que influenciam a extração, como temperatura, pH e solventes utilizados, permite aprimorar a recuperação seletiva de compostos de interesse, contribuindo para a formulação de novos produtos com valor agregado (Kim, et al., 2020). Paralelamente, a caracterização das nanopartículas obtidas a partir da própolis possibilita avaliar suas propriedades estruturais e funcionais, ampliando suas aplicações em diversas áreas estratégicas, como saúde, segurança alimentar e meio ambiente (Gutiérrez et al., 2022).

1.2 OBJETIVOS

1.2.1 Objetivos gerais

O objetivo geral deste trabalho foi avaliar o uso de um processo hidrotérmico de alta pressão semicontínuo para a recuperação de produtos de valor agregado da própolis bruta e obtenção de um extrato/hidrolisado com alta quantidade desses compostos.

1.2.2 Objetivos específicos

- Realizar análise bibliométrica dos últimos 14 anos afim de verificar as áreas de estudos utilizando própolis menos explorados e possibilidades de utilização;

- Estudar parâmetros operacionais de extração e hidrólise da própolis em reator com água subcrítica na pressão 200 bar e fluxo 5 mL/min, variando concentração de solvente (0 a 80%), temperatura (60 a 120 ºC) e pH (2 a 12);
- Caracterizar os extratos e hidrolizados quanto a cor, concentração de compostos bioativos, açúcares, inibidores e ácidos orgânicos.

1.3 ESTRUTURA DA DISSERTAÇÃO

Esta dissertação está organizada em capítulos, sendo que cada um deles aborda o tema central de cada estudo, os quais resultam em artigos submetidos em revistas científicas na área de Engenharia de Alimentos.

Capítulo 1: Estrutura da dissertação.

Capítulo 2: Esta seção apresenta o artigo intitulado "*Exploring methods for propolis extract production and its application in silver nanoparticle synthesis: a comprehensive review*", no qual foi realizada revisão bibliográfica, onde se explora o tema central do trabalho. A própolis, uma substância resinosa com composição química rica em flavonoides e ácidos fenólicos, tem sido amplamente valorizada por suas propriedades terapêuticas bem documentadas, como atividade antibacteriana e anti-inflamatória. Além disso, o interesse crescente por soluções naturais e inovadoras nas indústrias farmacêutica, agrícola e cosmética tem dado destaque à própolis e a substâncias como as nanopartículas de prata (AgNPs) e extratos naturais. Esta revisão oferece uma visão geral do estudo e estabelece as bases para a análise das aplicações de própolis e outros compostos bioativos em diversos setores.

Capítulo 3: Apresenta o artigo intitulado "*Enhanced Extraction Of Bioactive Compounds From Brown Propolis: Employing Pressurized Liquid Extraction In Semi-Continuous Flow-Through System*", no qual se investigou a influência da concentração de etanol, temperatura e pH na eficiência da especificação de produtos bioativos da própolis marrom, utilizando extração com líquido pressurizado (PLE) em um sistema de fluxo semicontínuo. O

estudo analisou a recuperação de compostos fenólicos, flavonoides, açúcares e ácidos orgânicos, além dos efeitos das condições de extração nas propriedades colorimétricas e na atividade antioxidante dos extratos obtidos.

Capítulo 4: Considerações finais e perspectivas futuras.

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Rai, M., Yadav, A., & Gade, A. (2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances, 27(1), 76-83.

CHAPTER II

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EXPLORING METHODS FOR PROPOLIS EXTRACT PRODUCTION AND ITS APPLICATION IN SILVER NANOPARTICLE SYNTHESIS: A COMPREHENSIVE REVIEW

ABSTRACT

Propolis, a resinous substance collected by bees from plant sources, has a diverse chemical composition rich in flavonoids and phenolic acids, which contribute to its well-documented therapeutic effects, including antibacterial and anti-inflammatory properties. Historically esteemed in traditional medicine, propolis is currently experiencing a resurgence of interest due to the recent identification of novel bioactive compounds and advancements in extraction techniques that enhance its utility in pharmaceutical and cosmetic products. The distinctive interactions of propolis with the human body suggest its potential as an agent for the prevention and treatment of infection and inflammation. The pursuit of natural and innovative solutions across a range of sectors, particularly in the pharmaceutical, agricultural, and cosmetic industries, has garnered significant interest in substances such as propolis, natural extracts, and silver nanoparticles (AgNPs). This review provides an overview of the key aspects of propolis, natural extracts, and silver nanoparticles (AgNPs), including their production, benefits, and applications across diverse sectors. A bibliometric analysis spanning the period from 2010 to 2024 reveals a growing interest in these areas, as evidenced by trends in publication and the emergence of key words. This underscores the relevance of these studies in addressing global health concerns. The analysis reveals a vast and evolving research landscape, with notable contributions to sustainable therapeutic options and innovative applications. It also portends a promising outlook for future exploitation in improving efficacy and safety across various sectors, particularly in health, food safety, and environmental applications. The emergence of new trends suggests a trajectory towards greater integration of natural substances with advanced technologies, which could facilitate advancements in the medical and agricultural fields.

Keywords: *Bibliometric Analysis, Nanotechnology, Bioactive compounds, Antimicrobial, Antioxidant, and Antifungal.*

1 INTRODUCTION

The growing demand for natural and innovative solutions in the pharmaceutical, agricultural, cosmetic, and food industries has increased interest in propolis, natural extracts, and silver nanoparticles (AgNPs). Derived from natural sources and enhanced through technological advancements, these materials offer a wide range of therapeutic and functional benefits, which are well-documented in scientific literature. Propolis, a resinous substance collected by bees from various plant sources, is distinguished by its unique chemical composition, primarily consisting of flavonoids, terpenoids, and phenolic acids. These compounds contribute to its antibacterial, anti-inflammatory, and antioxidant properties, making propolis a valuable component of traditional medicine for centuries [1,2]. Beyond its conventional applications, recent discoveries of new bioactive compounds and technological advancements have expanded its potential use in pharmaceuticals and cosmetics. Studies suggest that propolis interacts with biological systems in ways that may help prevent infections, reduce inflammation, and even combat certain types of cancer. The chemical diversity of propolis depends on the plant sources used by bees, resulting in variations in color and bioactivity [1,3].

Research on natural extracts derived from plants, animals, and microorganisms has also yielded promising results across multiple industries. These extracts are rich in bioactive compounds such as polyphenols, terpenes, and alkaloids, which exhibit therapeutic, antioxidant, and antimicrobial activities. The use of natural extracts in medicine dates back centuries and remains highly valued, especially as consumer demand for safer and more sustainable products continues to grow. Advances in extraction technologies have further enabled the incorporation of these compounds into cosmetics, food, and pharmaceuticals [4].

Alongside the use of propolis and natural extracts, nanotechnology has revolutionized the application of natural substances in medicine and other fields. AgNPs, in particular, have emerged as one of the most promising innovations due to their potent antibacterial properties. These nanoparticles have

demonstrated significant efficacy against infections, including antibiotic-resistant strains, one of modern medicine's most pressing challenges. Their applications extend beyond healthcare to agriculture and cosmetics, reflecting their versatility and effectiveness in various fields [5,6].

In agriculture, AgNPs have shown potential in enhancing plant resistance to pathogens and environmental stress. The development of controlled-release nanoparticle systems in pesticides and fertilizers could revolutionize agricultural practices by improving efficiency while minimizing environmental impact. Such advancements align with the growing demand for sustainable and safer food production, addressing the challenges posed by excessive chemical use in farming [7].

The increasing significance of propolis, natural extracts, and AgNPs highlights their potential in developing more effective and sustainable therapeutic solutions. The integration of natural substances with emerging technologies, such as nanotechnology, creates new opportunities for innovative, multifunctional treatments. For example, the combination of propolis and AgNPs could enhance their antibacterial and antioxidant properties, leading to therapeutic solutions that merge traditional knowledge with modern scientific advancements [8,9].

While previous reviews have addressed the individual properties and applications of propolis and AgNPs, this study provides a novel perspective by focusing on the synergistic effects when these two materials are combined. This approach adds new insights into their potential to enhance bioactive properties, particularly in antimicrobial, antioxidant, and anti-inflammatory applications. Additionally, the review examines different extraction methods and applications of AgNPs, highlighting recent technological advancements that may not have been fully explored in earlier works. By addressing the intersection of natural extracts and nanotechnology, this review emphasizes the cutting-edge developments in both fields and their future potential in sustainable and multifunctional therapeutic solutions.

Given the growing demand for sustainable and eco-friendly alternatives, particularly in the fight against antimicrobial resistance, the relevance of this

research is more pronounced than ever. This study aims to provide a comprehensive overview of the production, characterization, and applications of propolis and AgNPs, with an emphasis on their bioactive properties and emerging industrial uses.

2 METHODOLOGY FOR LITERATURE SEARCH AND BIBLIOMETRIC ANALYSIS

The bibliographic search was conducted on the Science Citation Index Expanded (SCI-E) platform - Clarivate Analytics' ISI - Web of Science©, using the advanced search and following search logic: TS = (("propolis") AND ("extraction" OR "nanoparticle")). TS is a search operator that allows searching by topic, encompassing specific terms in the article title, abstract, and keywords. The approach chosen was to search for "propolis" in general without restricting it to "brown propolis" due to the low number of publications focused exclusively on this type. It was decided to include all propolis to overcome this limitation and obtain a more comprehensive view of the research landscape. This strategy enabled it to get broader and more relevant insights, which can be directly applied to the study of brown propolis. The keywords were chosen to cover research related to propolis, considering both the extraction processes and the development of nanoparticles. Additionally, keywords plus were excluded, limiting the search to terms in the title, abstract, and author keywords. Two filters were applied to the search: the timespan from 2010 to 2024 (up to August 25th) and the document type, including only experimental and review articles. **Figure 1** illustrates the steps taken in the search process, which resulted in 493 documents, including 448 articles and 45 reviews. The systematic search enabled an analysis of publication patterns and research themes that received significant attention.

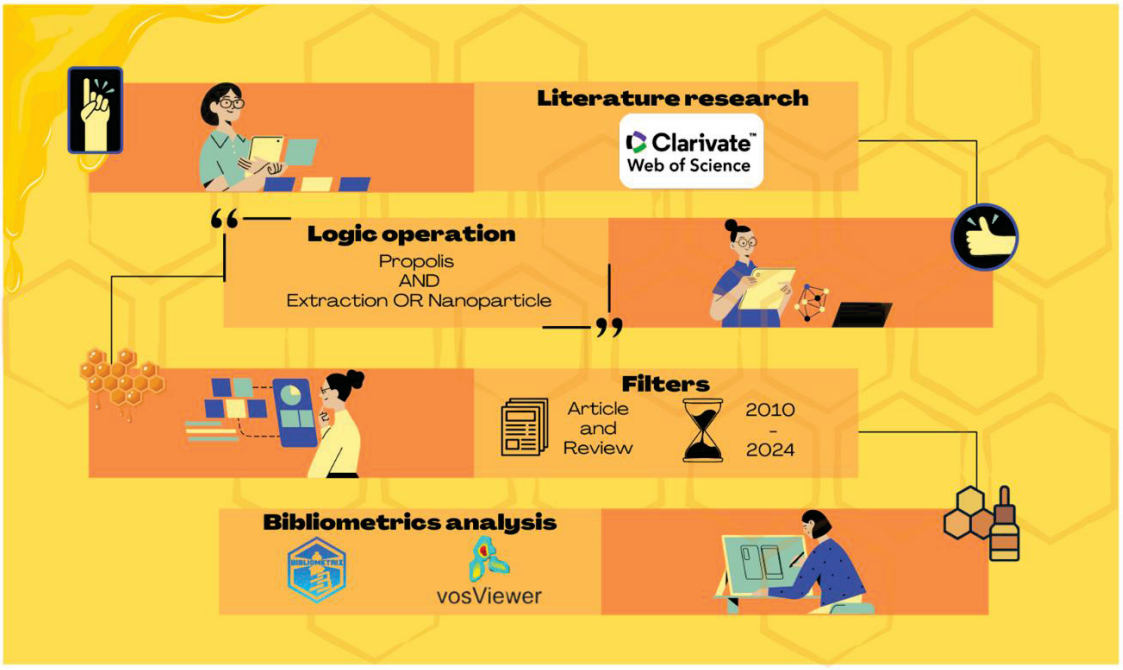

Figure 1. The methodological steps involved in conducting the bibliometric analysis.

The VOSviewer© software was used to investigate networks related to keywords and citations among studies from different countries. Bibliometrics was employed to construct a thematic map to visualize the most frequently used keywords and to create a figure highlighting the countries most actively involved in research, showing their interconnections through citations of relevant works. The **Web of Science (WoS)** is widely used in bibliometric analyses. Still, it has some limitations, such as its restricted coverage of high-impact journals and the predominance of texts only in English.

2.1 PUBLICATION EVOLUTION

As observed in **Figure 2**, there is a clear and continuous trend in the growth of propolis-related research over the years evaluated. The early years show some irregularity in publication numbers, followed by a significant increase in 2019, with 52 publications. A decline is noted in 2020, mainly reflecting broader trends in scientific research, likely influenced by the COVID-19 pandemic. However, the number of publications rebounded in the

subsequent years. For 2024, with data collected up to August, there are already 35 publications, indicating a likely increase in the coming months as the year progresses. This continuous trend in the growth of propolis-related research is a testament to the field's sustained effort and exploration of emerging and innovative applications rather than a singular spike of interest.

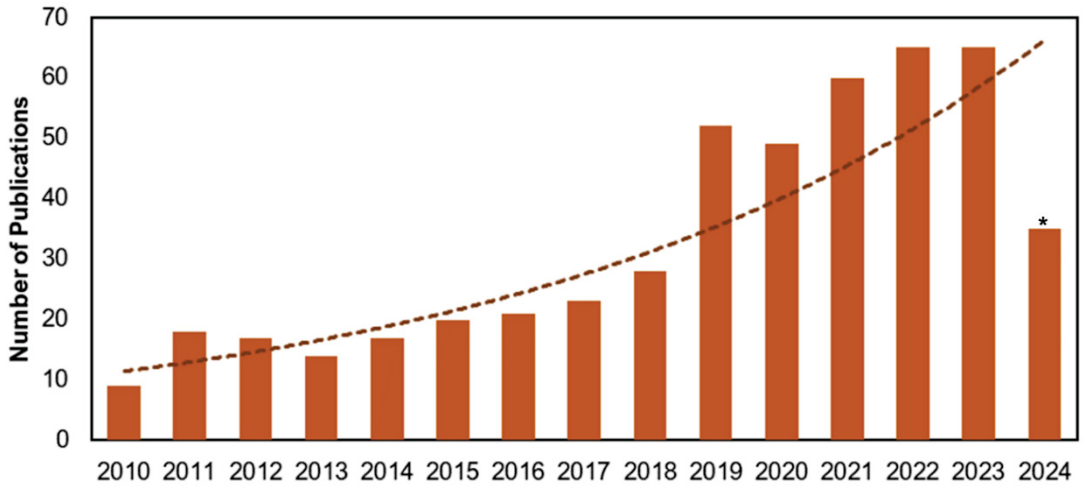

Figure 2. Annual evolution of scientific publications on propolis extraction and nanoparticle production over the past 14 years. (*) = Search carried out until August 2024.

2.2. KEYWORD ANALYSIS

Figure 3 presents a network map that groups authors' most frequently used keywords into seven clusters (**Table 1**). These clusters are organized based on their relevance and the connections between them. A total of 55 keywords, each with a minimum of five occurrences, were selected for inclusion. The network map shows the clusters and their interconnections. The red cluster (Number. 1) highlights properties associated with propolis, such as anti-inflammatory, antibacterial, anticancer, antimicrobial, and antioxidant. These themes are also reinforced in the green cluster (Number. 2), including terms like antibacterial, antifungal, and antioxidant activity. Additionally, classifications such as green and red propolis and techniques like ultrasound-assisted extraction, which indicate widely used extraction methods for propolis, appear. Other extraction techniques mentioned include maceration in cluster

number seven and supercritical extraction in cluster Number. 5. The network map also features keywords related to compound identification techniques, such as HPLC, GC-MS, and mass spectrometry, as well as experimental designs, such as central composite design. Furthermore, it mentions using alternative solvents for extraction, such as natural deep eutectic solvents.

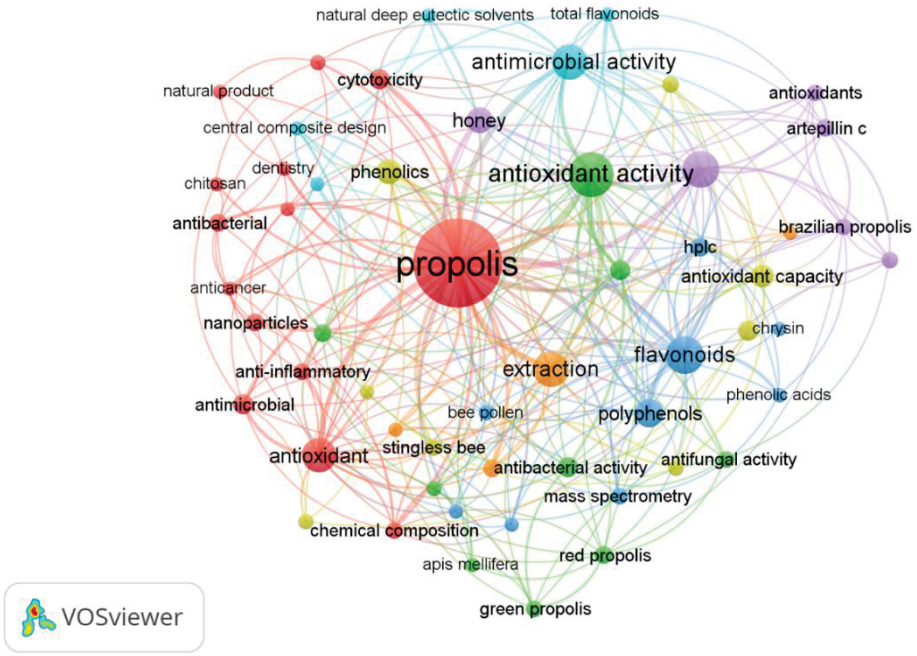

Figure 3. Classifying frequently employed keywords by authors into clusters. The area of the circle represents the frequency of term occurrence, while the connections indicate the co-occurrence of related terms.

Table 1. Clusters of critical terms identified using Vosviewer software.

Cluster	Citations	Keywords on VOSviewer Network
1	14	Anti-inflammatory, antibacterial, anticancer, antimicrobial, antioxidant, chemical composition, chitosan, cytotoxicity, dentistry, nanoparticle, nanoparticles, nanotechnology, natural product, and propolis.
2	9	Antibacterial activity, antifungal activity, antioxidant activity, *Apis mellifera*, bioactive compounds, green propolis, natural products, red propolis, and ultrasound-assisted extraction.

Table 1. Continued

3	9	Bee pollen, chrysin, flavonoids, HPLC, mass spectrometry, phenolic, phenolic acids, polyphenols, and stingless bees.
4	8	Antioxidant capacity, GS-MS, MS, PCA, phenolics, propolis extract, quercetin, and stingless bee.
5	6	Antioxidants, artepillin c, Brazilian propolis, honey, phenolic compounds, and supercritical extraction.
6	5	Antimicrobial activity, central composite design, natural deep eutectic solvents, response surface methodology, and total flavonoids
7	4	Extraction, maceration, poplar-type propolis, and ultrasound.

Additionally, **Table 2** ranks the authors' ten most frequently used keywords. Besides terms such as "propolis" and "extraction," which are derived from the search logic applied, there is notable interest in compounds to be extracted, such as flavonoids and polyphenols, as well as their antioxidant and antimicrobial effects. The absence of terms related to nanoparticles in the ranking indicates a significant gap in current research in this application field, representing an opportunity for future studies. Generally, the intense focus on extraction studies often paves the way for subsequent research on the application of extracts, whether in the form of nanoparticles or other modalities, potentially sparking interest in areas such as food, cosmetics, and pharmaceuticals.

Table 2. 10 most commonly used keywords in the field are ranked by their frequency of occurrence.

Ranking	Keywords	Occurrences	Total link strength
1	Propolis	202	252
2	Antioxidant activity	51	91
3	Flavonoids	36	69
4	Phenolics compounds	34	60
5	Extraction	32	59
6	Antimicrobial activity	32	52
7	Antioxidant	29	56
8	Polyphenols	20	33
9	Honey	17	17
10	Phenolics	15	22

Figure 4 presents a quadrant map that organizes keywords into groups based on relevance and development. These groups are classified as motor, basic, niche, and emerging or declining themes. **Motor themes** are highly relevant and developed, defining the field of study more precisely. These groups include keywords related to propolis's properties, such as antioxidant, antimicrobial, antibacterial, and antifungal activities, as well as commonly extracted compounds like flavonoids and phenolic compounds. Terms like "green propolis" and "red propolis" also appear, indicating the most studied topics. **Basic themes** are highly relevant but less developed. These themes have the potential to evolve into motor themes as research progresses. Keywords like "Brazilian propolis" and "artepillin C" (an active compound found in green propolis, known for its anti-inflammatory, antioxidant, antimicrobial, immunomodulatory, and gastroprotective properties Beserra, Gushiken, Hussni, Ribeiro, Bonamin, Jackson, Pellizzon e Bastos (2020) [[10]] are present. Additionally, terms synonymous with those already found among motor themes are included. **Niche terms** are those with advanced development but lower overall relevance, essential for specialized areas of study. These terms are predominant in more focused research groups, focusing intensely on specific subfields. In the current context, terms like "nanoparticles," "cytotoxicity," and "dentistry" exemplify these specialized fields. Finally, **emerging or declining themes** include terms that are decreasing in usage frequency, suggesting a possible decline, or those that are beginning to gain relevance and may grow in the coming years. Examples include "nanoparticles," "nanotechnology," and "quercetin." Understanding the growth in nanoparticle research, we can infer that their presence in this quadrant indicates an emerging theme. This trend highlights the increasing interest in nanotechnology applications for enhancing bioavailability, targeted delivery, and stability of bioactive compounds like quercetin. The growing focus on these topics suggests promising opportunities for future research and potential industrial applications, particularly in food technology, pharmaceuticals, and biotechnology.

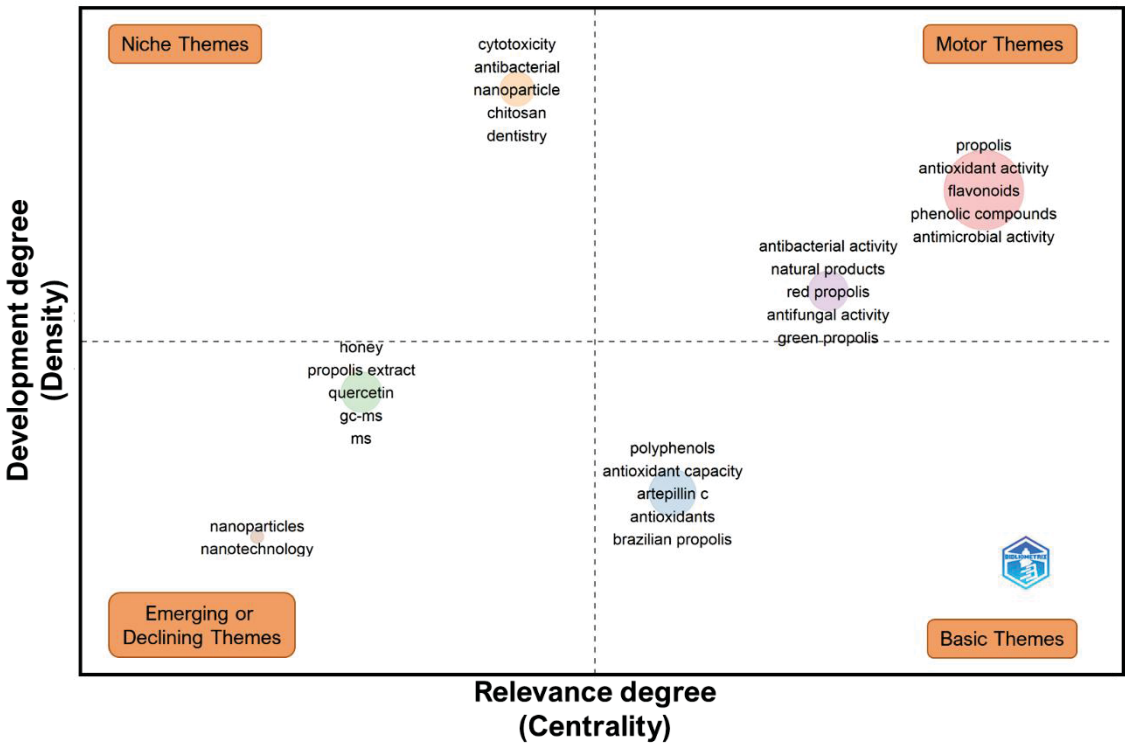

Figure 4. A thematic overview of frequently employed keywords. The classification of keywords based on Development Degree (Density) on the Y-axis and Relevance Degree (Centrality) on the X-axis allows for identifying research trends and priorities in the field.

2.3. STUDY OF RESEARCH AREAS, AFFILIATIONS, COUNTRIES, AND JOURNALS

The ranking of the top 10 study areas, affiliations, countries, and journals is presented in **Table 3**. Chemistry (29.006%) has the highest number of publications, followed by Food Science Technology (23.732%). These fields highlight chemistry's central importance in the analysis and extraction of propolis compounds. At the same time, food science and technology reflect the growing interest in food preservation and innovation applications. Other significant fields, such as Pharmacology, Pharmacy and Biochemistry, and Molecular Biology, indicate a diversification of research exploring both the therapeutic effects of propolis and its biotechnological applications. The top affiliations and countries in the ranking point to the dominance of specific institutions and regions that lead scientific production in this domain, driving continuous and significant advancements. Many Brazilian universities are

represented, reflecting Brazil's leadership, which accounts for 21.095% of the publications. The most prominent journal in this field is the Journal of Apicultural Research (3.854%), which is fitting given its focus on beekeeping, followed by Molecules (3.245%), demonstrating a strong interest in obtaining compounds from propolis.

Figure 5 presents a world map highlighting the countries with the highest number of publications and their level of interaction. This visual representation identifies geographical areas with the most significant scientific activity related to propolis, particularly in extraction and nanoparticle studies. The map illustrates how different regions contribute to the field and their collaborative dynamics, providing insight into global research trends and concentrations.

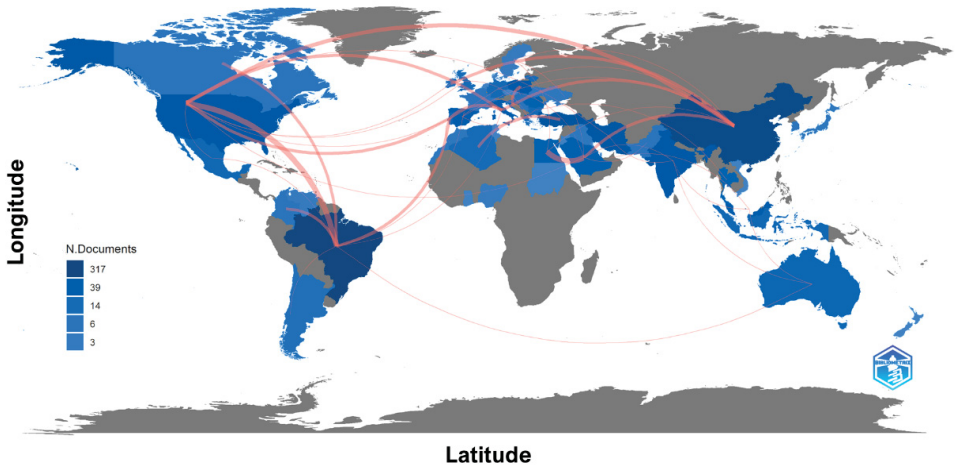

Figure 5. Insights at the country level, collaborative dynamics, and international partnerships in global propolis research over the past years.

Table 3. Top 10 study areas, affiliations, countries, journals, and authors in propolis research over the past 14 years, based on the quantity and impact of publications.

Ranking	Research areas	Number	%[1]
1st	Chemistry	143	29.006
2nd	Food Science Technology	117	23.732
3rd	Pharmacology Pharmacy	66	13.387
4th	Biochemistry Molecular Biology	63	12.779
5th	Science Technology Other Topics	46	9.331
6th	Engineering	34	6.897
7th	Agriculture	32	6.491
8th	Entomology	24	4.868

Table 3. Continued

9th	Materials Science	21	4.260
10th	Dentistry Oral Surgery Medicine	17	3.448
Ranking	**Affiliations**	**Number**	**%[1]**
1st	Universidade de Sao Paulo	20	4.057
2nd	Universidade Estadual de Campinas	18	3.651
3rd	Egyptian Knowledge Bank Ekb	15	3.043
4th	Universidade Federal da Bahia	13	2.637
5th	Jiangsu University	9	1.826
6th	Universidade Tiradentes	9	1.826
7th	University of Zagreb	9	1.826
8th	Chinese Academy of Agricultural Sciences	8	1.623
9th	Universidade Estadual de Maringa	8	1.623
10th	Universidade Tecnologica Federal do Parana	8	1.623
Ranking	**Countries**	**Number**	**%[1]**
1st	Brazil	104	21.095
2nd	Peoples'r China	59	11.968
3rd	Turkey	34	6.897
4th	Iran	28	5.680
5th	India	26	5.274
6th	USA	26	5.274
7th	Poland	24	4.868
8th	Italy	21	4.260
9th	Malaysia	21	4.260
10th	Spain	20	4.057
Ranking	**Journals**	**Number**	**%[1]**
1st	Journal of Apicultural Research	19	3.854
2nd	Molecules	16	3.245
3rd	Plos One	12	2.434
4th	Food Chemistry	9	1.826
5th	Antioxidants	7	1.420
6th	Food Analytical Methods	7	1.420
7th	LWT Food Science and Technology	7	1.420
8th	Chemistry Biodiversity	6	1.217
9th	Foods	6	1.217
10th	Journal of Food Processing and Preservation	6	1.217

Additionally, **Figure 6** depicts the relationships and collaborations between countries through a network map considering the co-occurrence of the countries and citations. From this map, three main clusters can be identified:

- **Cluster 1**: Australia, Brazil, Bulgaria, Chile, England, Greece, Hungary, Ireland, Mexico, the People's Republic of China, Portugal, Romania, Taiwan, Thailand, and the USA.
- **Cluster 2**: Algeria, Argentina, Croatia, Indonesia, Italy, Lithuania, Poland, Slovakia, and Turkey.
- **Cluster 3**: Egypt, Germany, India, Iran, Malaysia, Saudi Arabia, Slovenia, and Spain.

The network map illustrates how these countries are interconnected through collaborative research efforts and citation practices, highlighting regional patterns and global research dynamics.

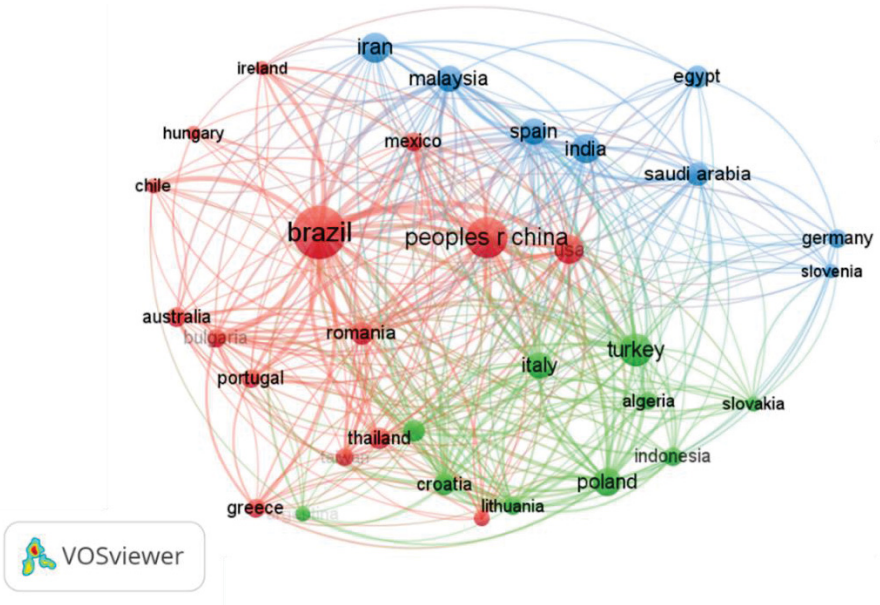

Figure 6. Co-occurrence network map between countries and citations in the study of propolis in extraction and nanoparticles. The circumference indicates the occurrence of publications, and the connections represent partnerships.

3. PROPOLIS AND NATURAL EXTRACTS

Propolis, also known as bee glue, is a resinous material produced by bees from wax and plant exudates used to seal openings and cracks in hives [11,12]. Its typical composition includes approximately 10% volatile substances, 50 to 55% resin, 30 to 40% beeswax, 10% essential oils, and 5 to 10% pollen, along with other substances in smaller proportions [11]. The composition of propolis is influenced by various factors, such as the plant source, season,

climate, bee species, and collection methods [13]. More than 300 compounds have been identified in propolis, including phenolic acids, flavonoids like flavones, flavanones, and flavanols, terpenes, aldehydes, aromatic alcohols, fatty acids, and stilbenes [12,14]. In general, propolis exhibits various bioactive properties, such as antibacterial [15] antifungal [16], anti- inflammatory [17], wound healing [18], antitumor [19], antiparasitic [19], and antioxidant [17] activities.

As the chemical composition of propolis largely depends on the flora surrounding the hives, propolis produced in specific geographic zones shows similarities in its composition. Temperate zone propolis is rich in flavonoids, phenolic acid esters, and compounds absent in tropical propolis. Tropical propolis contains prenylated coumaric acids, flavonoids, benzophenones, lignans, and terpenes, while propolis from cold regions and high altitudes contains phenolic glycerides and other acid combinations [20]. Mediterranean propolis is characterized by high concentrations of diterpenoids [21]. Due to the wide variety of propolis types and their different characteristics, chemical characterization is essential to identify the present compounds, as they can vary significantly depending on various factors.

Despite its diverse bioactive potential, the bioavailability of propolis and its key compounds remains a challenge. The poor solubility, low absorption rate, and rapid metabolism of its polyphenolic constituents can limit its effectiveness when consumed orally. Several factors influence its bioavailability, including the extraction method used and the formulation in which it is administered. Alcoholic extracts generally exhibit higher bioavailability compared to aqueous extracts due to the improved solubility of polyphenols in ethanol. Additionally, some flavonoids undergo extensive first-pass metabolism in the gastrointestinal tract and liver, altering their systemic availability and potentially modifying their biological effects. To address these limitations, recent advancements in nanotechnology have introduced innovative delivery systems such as nanoparticles, liposomes, and solid dispersions, enhancing the solubility, stability, and intestinal permeability of propolis bioactives. Encapsulation using biopolymeric matrices, such as chitosan or alginate, has also shown promise in

prolonging compound release and increasing bioefficacy. These strategies not only improve absorption but also ensure sustained therapeutic activity, maximizing the health benefits of propolis [22–24].

While propolis is generally recognized as safe, some adverse effects and potential toxicity have been reported, particularly in individuals with allergies or sensitivities to bee products. Allergic reactions, including dermatitis, oral mucositis, and respiratory symptoms, have been observed, especially in individuals with a history of hypersensitivity to pollen or bee venom. Prolonged or excessive use of propolis may also lead to hepatotoxicity or nephrotoxicity due to the accumulation of bioactive compounds such as flavonoids and cinnamic acid derivatives, which can exert cytotoxic effects at high concentrations. Additionally, certain components of propolis, such as caffeic acid phenethyl ester, have been associated with DNA damage and genotoxicity in some in vitro studies, although further research is needed to confirm their long-term safety in humans. Quality control and standardization are essential to minimize the risk of adverse effects, as variations in chemical composition between different types of propolis can influence both efficacy and toxicity. Individuals with pre-existing conditions, pregnant women, and those taking medications should consult a healthcare professional before using propolis to avoid potential interactions or side effects [23,25,26].

There are various classifications for the different types of propolis based on either the plant of origin or the color. In Brazil, the main classification is by color, resulting in categories of green, red, brown, yellow, and black propolis [27]. **Table 4** presents the classification by color, some identified compounds, and their biological activities. Green propolis is one of the most studied, characterized, and used due to its pharmaceutical properties, most related to Artepillin C, its main phytochemical marker [28]. It has a deep green color, resulting from the resin source used by the bees, mainly *Baccharis dracunculifolia* [29]. While Brazilian green propolis contains prenylated p-coumaric acid and diterpenic acids, the European version is rich in phenolic compounds such as flavonoid aglycones, hydroxycinnamic acids, and their

esters [30], highlighting the significant influence of geographic factors on propolis production.

Red propolis can be derived from the plant *Dalbergia ecastophyllum (L.)* Taub. (Fabaceae), rich in isoflavonoids, pterocarpans, and chalcones, or *Symphonia globulifera Lf, Clusiaceae*, which is rich in polyprenylated benzophenones [31]. It usually has a bright red color [32]. Brown propolis is obtained from various sources, such as *Luehea sp.* (Malvaceae), *Piptadenia falcata Benth* (Fabaceae), *Tabebuia spp.* (Bignoniaceae), *Tabebuia caraiba* (Mart.) *Bureau* (Bignoniaceae), *Vernonia spp.* (Asteraceae), and *Cecropia pachystachya Trécul* (Urticaceae) [33]. Due to the wide variety of sources, brown propolis exhibits different chemical profiles, resulting in varied compositions andiological activities [33,34].

On the other hand, yellow propolis has not been extensively studied, leaving gaps in its characteristics, such as the plant of origin, which has not yet been determined [35]. It has low concentrations of phenolic compounds, with ursadienol, lupenone, oleanone, and betulin as its main constituents, along with lanosterol and cycloartenol [35]. Finally, black propolis contains polyisoprenylated benzophenones, such as xanthochymol, with a chemical composition similar to Venezuelan and Cuban propolis, as they all have *Clusia* species as their plant source [35]. This type of propolis can also be obtained from *Mimosa hostilis Benth* [36] and, in Europe, from *Populus nigra L.* [37].

The extraction methods for obtaining propolis extracts can be divided into classical and modern methods [38] and are presented in **Figure 7**. The most common classical method is simple maceration, which involves adding a solvent to the solid sample, followed by a resting period. After this time, the mixture is filtered, and the obtained liquid is used to determine the bioactive compounds, with applications in the pharmaceutical, cosmetic, and food industries [39,40]. In studies using this method, a mixture of ethanol and water in the proportion of 70 to 80% ethanol is the most common solvent [41]. However, maceration has disadvantages, such as long extraction periods, large amounts of solvent use and the need for subsequent evaporation [42].

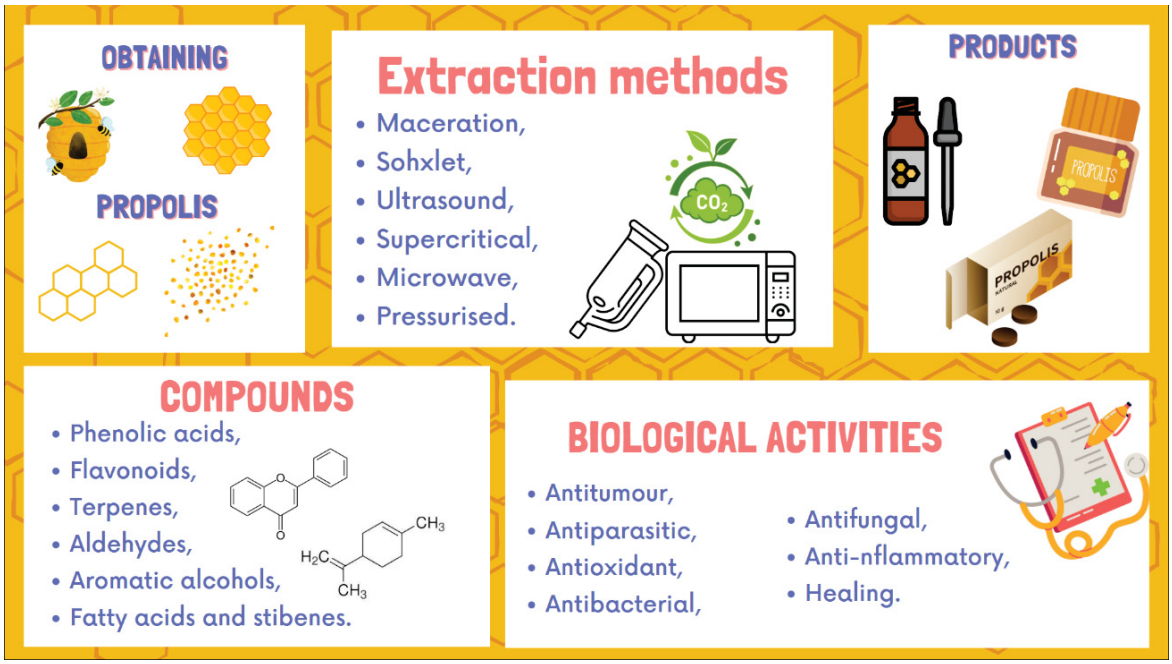

Figure 7. Propolis extraction: processes, bioactive compounds, and biological benefits.

Another widely used classical method is Soxhlet extraction, which stands out for using smaller amounts of solvent and reducing extraction time compared to maceration [42]. Studies indicate that using absolute ethanol at 60 °C for 4 to 6 h, in a 5:150 w/v ratio, offers the best results for the extraction of total phenolic compounds and flavonoids [39,43]. However, a disadvantage of this method is the need for high temperatures, which can lead to the degradation of thermosensitive compounds present in propolis, making it unsuitable in certain cases, depending on the type of propolis and the compounds of interest [39].

New extraction methodologies, known as modern methods, are increasingly being explored to obtain propolis extracts. Among them are supercritical fluid extraction [44], ultrasound-assisted extraction [45], high-pressure extraction [46], and microwave-assisted extraction [41]. These techniques are promising as they offer advantages such as reduced extraction time, less need for solvent evaporation, greater stability of thermosensitive compounds, and higher yields.

In addition to innovative techniques, the use of green and non-toxic solvents is also being studied. Currently, the most commonly used solvents

include water, methanol, ethanol, chloroform, dichloromethane, ether, and acetone [47]. Although hydroethanolic extracts are the most common form of commercial propolis, they have disadvantages, such as characteristic odor and taste, and are unsuitable for consumers intolerant to ethanol, children, and alcoholics [48].

The search for environmentally friendly solvents has been receiving increasing attention. Among the solvents studied, ionic liquids present high costs and significant toxicity potential [49], while deep eutectic solvents have shown satisfactory results, being cheaper and easier to synthesize [49,50]. Other green solvents under investigation include water in pressurized extractions and carbon dioxide in supercritical extractions, both facilitating the extraction of bioactive compounds and fitting the characteristics of green extractions [49].

Additionally, alternative solvents such as propylene glycol, honey brandy, mead [47], polyethylene glycol [51], glycerol, and vegetable oils are also being evaluated [52]. Finally, the development of extraction methods using alternative solvents to replace ethanol extracts represents a promising strategy for future commercial applications, broadening their use in industrial sectors such as food, cosmetic, and pharmaceutical industries.

Table 4. Characteristics and biological activities of different types of propolis.

Type of Propolis	Region	Main Compounds	Biological Activities	Reference
Green	Southeast Brazil, Europe, Taiwan	Cinnamic, caffeic, ferulic, chlorogenic, isochlorogenic a,b,c, and Artepillin C	Anti-obesity, anti-inflammatory, gastroprotective, immunomodulatory, antibacterial, antiviral, anticancer	[33,53]
Red	Brazil, Cuba, Venezuela, Mexico, China	Flavonoids (Isoflavones, flavanones, dihydroflavonoids), medicarpin, vestitol, formononetin, isoliquiritigenin, and benzophenones	Anti-obesity, antibacterial, anti-inflammatory, anticancer, antifungal	[33,53]

Table 4. Continued

Brown	Brazil, Cuba, Mexico, Europe	p-Coumaric acid, drupanin, artepillin C, baccharin, isocupressic acid, dihydro-p-coumaric acid, caffeic acid	Antioxidant, antibacterial, anti-inflammatory, cytotoxic, antileishmanial, antigenotoxic, anti-mycoplasma	[34]
Yellow	Brazil, Cuba	Triterpenic alcohols, flavonoids, polymethoxylated, lanosterol, germanicol, lupeol, and cycloartenol	Antimicrobial, antioxidant, anti-inflammatory, anticancer	[54,55]
Black	Brazil, Europe, Asia, North America	Hydroxycinnamic acid (caffeic acid and phenethyl ester of caffeic acid) and flavonoids (apigenin, quercetin, pinocembrin, galangin, chrysin)	Antioxidant, anti-inflammatory, antimicrobial, antidiabetic, antitumor, neuroprotective, gastroprotective, immunomodulatory	[17,36]

4. EXTRACTION METHODS FOR PRODUCTION OF PROPOLIS EXTRACTS

A comparative analysis of traditional and modern extraction methods is essential to highlight their respective advantages and limitations. Traditional techniques, such as cold pressing and enzyme extraction, are widely used due to their simplicity, low cost, and environmentally friendly nature, as they often avoid the use of organic solvents. Additionally, they help preserve thermolabile bioactive compounds. However, these methods typically suffer from lower extraction efficiency, longer processing times, and incomplete recovery of target compounds, which can limit their industrial applicability.

In contrast, modern extraction techniques, including supercritical CO_2 extraction, ultrasound-assisted extraction, pressurized liquid extraction, and reverse micelle extraction, have been developed to overcome these limitations. These methods offer higher yields, improved selectivity, and faster processing times while reducing solvent consumption, making them more sustainable

alternatives. Despite these advantages, modern extraction techniques often require specialized equipment, higher operational costs, and technical expertise to optimize parameters for maximum efficiency. Additionally, some methods still involve the use of organic solvents, which may pose environmental and safety concerns.

The choice of an extraction method depends on several factors, including the nature of the bioactive compounds, cost-effectiveness, environmental impact, and scalability. Understanding these differences is crucial for selecting the most appropriate technique to maximize the extraction of valuable compounds from propolis. The following sections will provide an in-depth discussion of each extraction method (**Figure 8)**, their mechanisms, and their applications in obtaining bioactive compounds.

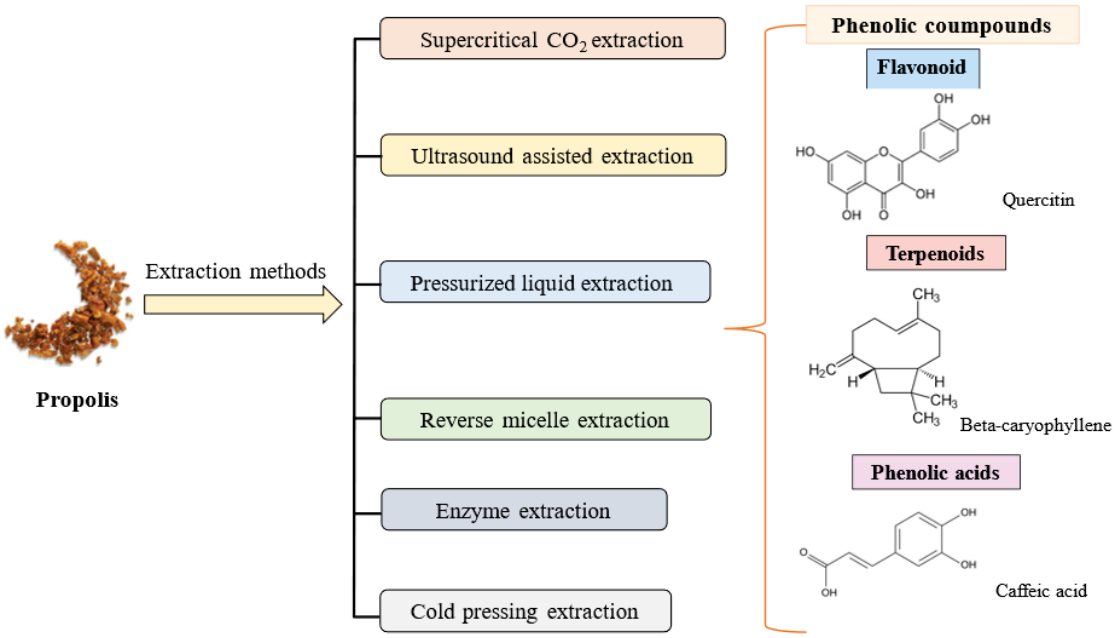

Figure 8. Overview of different extraction methods for propolis extracts obtaining.

4.1 SUPERCRITICAL CO_2 EXTRACTION

Supercritical carbon dioxide (CO_2) extraction is considered a green extraction method because it offers economic and environmental benefits [48, 49]. This technology has been widely used to recover natural products.

Supercritical fluids are obtained by raising the temperature and pressure above the critical point of the substances used [56]. The properties of a supercritical fluid combine characteristics of both liquids and gases, enhancing extraction efficiency compared to conventional solvents in terms of quality and quantity. Notable properties include low viscosity, a density close to a liquid, diffusion similar to that of a gas, and surface tension approaching zero [57,58]. Due to their high diffusivity, supercritical fluids easily penetrate solid materials, allowing for dissolution and making the substances accessible [59,60]. Furthermore, due to its near-zero surface tension, the supercritical fluid can easily penetrate materials with low porosity [61].

Supercritical CO_2 is the most widely used fluid due to its non-toxicity, ease of recovery, volatility, non-flammability, low critical temperature (31.1 ºC), and relatively low critical pressure (72.8 bar). These properties make it possible to recover CO_2 during the process, thus avoiding this greenhouse gas emission [55,56]. This prevents the thermal and chemical degradation of the compounds in the extracts, as CO_2 is easily removed by simply decompressing the extraction line [62,63]. This technology avoids the production of chemical solvent waste, which would otherwise need to be incinerated, a process that is both extremely dangerous and costly for the environment [64]. Another key sustainability aspect of supercritical CO_2 extraction is its energy efficiency. The process occurs at relatively low temperatures compared to other extraction methods, reducing energy demand and preventing thermal degradation of bioactive compounds [64]. Additionally, the selectivity of supercritical CO_2 enables precise extraction, minimizing the need for extensive downstream purification steps and reducing waste generation [65].

Several studies have validated the use of supercritical CO_2 extraction technology for producing propolis extracts **(Table 5).** In one study, the authors identified the optimal operational conditions for obtaining extracts with high antioxidant potential from red propolis. Using supercritical CO_2 technology and ethanol as a cosolvent, they found that conditions of 50 °C and 450 bar, with an S/F ratio of 131 and ethanol at a concentration of 4% (w/w), were ideal for obtaining extracts rich in phenolic compounds and antioxidant activity [65].

Another study evaluated extracts of green and red propolis obtained using ethanol, methanol, water, hexane, and supercritical CO_2 at 50 °C and 300 bar. The results showed that extracts obtained through supercritical CO_2 technology had the highest levels of flavonoids compared to those obtained with ethanol. This confirms that supercritical extraction tends to concentrate flavonoids, making it an effective method for fractionating these compounds. Flavonoids in propolis are present in higher quantities and exhibit significant biological activity [66].

4.2 ULTRASOUND ASSISTED EXTRACTION

Ultrasound consists of longitudinal sound waves that can rarefy and compress the medium along their propagation path, creating a non-uniform pressure field. This causes the medium, which is initially uniformly distributed, to vibrate rapidly [67]. Ultrasound waves have a frequency greater than 20 kHz [68]. The use of ultrasound in a medium induces vibration and consequently transfers energy. In this medium, cavitation occurs, which, during the propagation of the waves, causes the rupture of cell walls, reduces particle size, and enhances mass transfer [69].

When ultrasound is used in a liquid medium, thousands of cavitation bubbles are formed due to the longitudinal propagation of the ultrasound waves. As the waves propagate, these cavitation bubbles are continuously stretched and compressed due to pressure variations until they implode, releasing significant energy. This implosion generates shock waves, which, when they collide, create high shear forces that rupture cell walls. This implosion process leads to an increase in temperature and pressure, which accelerates the internal diffusion of particles. It also controls particle size and distribution due to the generation of hydroxyl radicals, microjets, and high-speed collisions between particles [67].

The use of ultrasound can increase extraction efficiency and reduce processing time. Compared to other methods, ultrasound-assisted extraction is the best alternative, as it does not require several preparatory operations for its application [70]. One of the key sustainability advantages of UAE is its potential

to minimize the use of organic solvents [67]. By improving solvent penetration and accelerating compound diffusion, UAE allows for the use of smaller solvent volumes or even the replacement of toxic solvents with greener alternatives such as water or ethanol [70]. This significantly reduces solvent waste and environmental pollution while also lowering health risks associated with hazardous chemicals [69].

The use of this technique for obtaining propolis extracts has been explored. In one study, the authors evaluated ultrasound-assisted extraction to obtain propolis extracts from stingless bees **(Table 5)**. They used aqueous and ethanolic media with varying pH levels (2, 6, and 9). The extraction parameters included a time of 15 min, a temperature of 70 °C, and a frequency of 40 kHz. The authors concluded that ethanolic extracts yielded higher amounts than aqueous extracts, although the difference was not statistically significant [71].

In another study, the authors compared different extraction methods for obtaining phenolic compounds from propolis, using double maceration (at room temperature with agitation for 24 h at 250 rpm), double microwave treatment (1 min at 140 W), and double ultrasound-assisted extraction (15 min at 20 kHz). The authors concluded that ultrasonic extraction achieved a higher yield compared to both microwave extraction and maceration methods [41].

4.3 PRESSURIZED LIQUID EXTRACTION (PLE)

Pressurized liquid extraction (PLE) is performed at room temperature to 200 °C and pressures between 10 and 15 MPa [72]. Furthermore, it is important to note that the pressure and temperature are always kept below the critical points to ensure that the solvent remains liquid during the extraction process. Under these conditions, the fluid exhibits significantly altered characteristics: the diffusion of the fluid is inversely proportional to its viscosity and surface tension. In other words, as diffusion increases, viscosity and surface tension decrease. This change facilitates the solvent's penetration into solid samples, reduces the interaction between the matrix and the compound of interest, and thus improves mass transfer [72,73]. These characteristics result in a faster extraction process with high yields and a low amount of solvent [74]. This facilitates the

development of methods that require less labor, thereby improving the reproducibility of extractions [75].

The PLE technique is similar to other common extraction methods, such as the Soxhlet extraction. Still, it offers the advantages of a shorter process time and using a smaller amount of solvents [76]. One of the key sustainability benefits of PLE is its ability to use environmentally friendly solvents such as water or ethanol instead of toxic organic solvents [74]. This reduces the risk of chemical exposure, lowers hazardous waste production, and decreases the environmental impact associated with solvent disposal. Additionally, the closed-loop system of PLE enables solvent recovery and reuse, further improving resource efficiency and reducing emissions [75].

The use of PLE technology to obtain propolis extracts has been relatively underexplored in the literature, and few studies have assessed the efficiency of this technique (**Table 5)**. In one study, the authors evaluated propolis extracts from Anatolian producers obtained through PLE. Several parameters were assessed during the process, and the authors concluded that the most favorable PLE conditions were 40 °C, 1500 psi, with a solvent mixture of ethanol, water, and HCl (70:25:5, v/v/v), containing 0.1% tert-butylhydroquinone, and using three extraction cycles with a cell capacity of 11 mL. The compounds gallocatechin, catechin, epicatechin gallate, caffeic acid, chlorogenic acid, and myricetin were identified in all samples [77].

4.4 REVERSE MICELLE EXTRACTION

The use of reverse micelles has advanced significantly in recent decades due to their versatile applications. Reverse micelles are formed by aggregates of surfactants arranged in an inverted orientation compared to a typical micelle. In a reverse micelle, the hydrophilic "head" of the surfactant comes into contact with the water inside the micelle core, while the hydrophobic "tail" interacts with the surrounding solvent. When water droplets are typically added to a reverse micelle solution, the surfactants arrange themselves into a spherical shape, creating a water reservoir within the sphere [78–80].

In reverse micelle extraction, the hydrophobic chains of the surfactants face outward and interact with the organic solvent phase, while the hydrophilic core of the reverse micelle is used for solubilizing biomolecules such as proteins and metabolites. Essentially, reverse micelles create an aqueous microenvironment within a bulk organic solvent, which helps isolate the organic reactants and products of the proteins, keeping them in the organic phase [78,80]. The reverse micelle system shows great potential for the solubilization of various biomolecules between the organic and aqueous phases. Among its many applications, reverse micelle technology can be used for extracting fats, proteins, and food enzymes, as well as for drug delivery systems. It can also serve as a nanocarrier for functional ingredients or nutraceuticals [80,81]. Furthermore, this technology offers several advantages, including low cost due to the potential recovery of surfactants and non-polar solvents. Reverse micelles can also protection molecules from denaturation because the aqueous microenvironments they form closely resemble physiological conditions [82]. Compared to traditional solvent-based methods, reverse micelles reduces solvent consumption and the need for energy-intensive downstream purification steps, thereby lowering the overall environmental footprint. Additionally, the ability to use biocompatible and biodegradable surfactants enhances the eco-friendliness of the process [80,81].

No studies were found in the literature using the reverse micelle method to obtain propolis extracts, suggesting that this extraction methodology could be explored in future research.

4.5 ENZYME EXTRACTION

Enzyme extraction is a viable alternative to conventional organic solvent extraction processes and pressing technologies, as it is efficient, sustainable, and environmentally friendly [83,84]. Enzyme extraction leverages the unique characteristics of enzymes to perform reactions with regioselectivity and specificity. Additionally, enzymes can conduct reactions under mild conditions, preserving the biological potential of the compounds present in the raw material [85]. The basic principle of enzyme extraction involves breaking down the cell

wall through hydrolysis, with the enzyme acting as a catalyst under optimal conditions to release intracellular components. The enzyme binds to its active site on the cell wall, facilitating maximum interaction. This interaction causes a conformational change in the enzyme, which leads to the disruption of cell wall bonds and the subsequent release of intracellular constituents [86].

The operational conditions for enzyme extraction technology are crucial and include factors such as temperature, extraction time, medium pH, enzyme concentration, and particle size. The applications of this technology are diverse and particularly useful for extracting heat-sensitive molecules such as flavors, pigments, and oils. Various enzymes are employed, including cellulases, glucoamylases, xylanases, amylases, papain, pectinase, and hemicellulose [87]. Enzyme extraction offers several advantages, including lower energy consumption, a higher extraction rate, and simpler recovery of the obtained products compared to conventional extraction methods [85]. One of the primary sustainability advantages of enzyme-assisted extraction is its ability to reduce the consumption of organic solvents, which are typically associated with environmental pollution and health risks [86]. In many cases, water or milder, less toxic solvents can be used in combination with enzymes, further minimizing the environmental impact of the process [87]. Additionally, enzymes are biodegradable, non-toxic, and often derived from renewable sources, contributing to the overall sustainability of the extraction process [85].

The literature lacks studies using enzymatic extraction to obtain propolis extracts. As previously explained, this technology could be an excellent alternative for obtaining extracts rich in phenolic compounds.

4.6 COLD PRESSING EXTRACTION

Most traditional extraction methods are time-consuming, expensive, and can destroy heat-sensitive compounds. This highlights the advantage of cold-press extraction, a mechanical technique that relies on applying pressure to the raw material [88]. Cold pressing can be performed using a screw press (for continuous pressing) or a hydraulic press (for batch pressing). It is a simple technology that does not require much energy or substantial investment [89,90].

It is considered an environmentally friendly technique because it requires no organic solvents. Additionally, extracts obtained through cold pressing generally have better physicochemical qualities compared to those obtained using other extraction methods [91]. One of the primary sustainability advantages of cold pressing extraction is that it does not require the use of chemical solvents, which can pose environmental risks and require expensive disposal processes [88]. By avoiding chemicals, cold pressing eliminates the need for post-extraction solvent recovery and waste treatment, reducing the generation of hazardous waste and lowering the environmental footprint of the process [91].

The main disadvantage of this method is the low yield of oil recovered from the raw material, although this can be improved with pre-treatments. Cold pressing has been used to obtain oils from various raw materials, such as peanuts, almonds, walnuts, cashews, and Brazil nut [89]. No studies were found in the literature that used cold pressing extraction to obtain propolis extracts. It is worth noting that this technique has significant potential and could be a valuable subject for future research.

Table 5. Overview of Propolis Extraction Techniques: Yields, Solvents, and Extraction Parameters.

Propolis	Extraction methods	Solvent	Solid liquid-ratio (m/v)	Extraction time	Operating parameters	Main results	Advantages	Disadvantages	Reference
Chinese	Supercritical CO_2 extraction	CO_2 with Ethanol 80% (cosolvent) 5% (m/v)	-	60 min	55 ºC and 30 MPa	Total flavonoids (2.17 mg g^{-1} propolis)	Green and environmentally friendly; Selective and efficient extraction; Preservation of extract quality; Solvent recovery and reusability; No residual solvents; Versatility in applications; Reduced chemical waste; Scalability and process integration;	High initial investment; Energy consumption; Limited to non-polar compounds; Pressure and temperature control complexity; Co-solvent requirement for some extracts; Maintenance and operational costs;	[92]
Brazilian red	Supercritical CO_2 extraction	CO_2	-	20 min	50 ºC and 300 bar	Total flavonoids (12.4 mg g^{-1} propolis red)			[66]
Italian	Supercritical CO_2 extraction	CO_2	-	30 min	Flow rate of 2 mL min^{-1}, Pressure: 82,3 to 317,7 bar, Temperature: 31,6 to 48,4 °C	Maximum yield extracted material (14.3%)			[93]

Table 5. Continued.

Turkish	Pressurized liquid extraction	Ethanol:water:HCl (70:25:5, v/v/v)	1:23	15 min – 3 cycles	40 ºC and 1500 psi	Phenolic recovery of 97.2 - 99.7%	Higher extraction efficiency; Reduced solvent consumption; Minimal thermal degradation; Increased selectivity; Scalability; Shorter extraction times; Compatibility with various solvents; Low residual solvents;	High equipment costs; Energy consumption; Complex process optimization; Solvent disposal issues; Complexity of equipment maintenance; Potential for co-extraction of unwanted compounds;	[77]

Table 5. Continued.

Romenian	Ultrasound-assisted extraction	Ethanol 50%	1:50	15 min	20 kHz	Extraction efficiency 31.9 - 95.8%	Improved extraction efficiency; Faster extraction process; Reduced solvent use; Preservation of heat-sensitive compounds; Energy efficiency; Selective extraction; Versatility; Scalability; Simplified set-up;	Limited penetration for dense materials; Risk of cavitation damage; Equipment costs; Heat generation; Limited effectiveness for non-polar compounds;	[41]
Malaysian	Ultrasound assisted extraction	Ethanol (96%) and water	1:10	15 min	70 ºC and 40 kHz	Yield of ethanol extraction (35.7-42.6%) / aqueous extraction (4.9-11.8%)			[94]
Polish	Ultrasound assisted extraction	Ethanol (70%)	1:10 1:5	10, 20 and 30 min	210 W and 20 kHz. Room temperature	Extraction yield (8.35-11.86%)			[94]
Egyptian	Ultrasound assisted extraction	Ethanol (96%)	1:5	35 min	1100 W and 28 kHz – 35ºC	Antioxidant activity (DPPH): 22.262-97.674 mg GAE g^{-1} of propolis			[95]

5. APPLICATION OF PROPOLIS EXTRACTS

Knowledge about propolis has advanced in recent years. Its anti-inflammatory, pharmacological, and antimicrobial properties, etc. [3] have led to the study of numerous possible applications for this natural substance. In terms of products, propolis has been studied in the production of capsules, lotions, mouthwashes, food preservatives, etc., and its application (**Figure 9**) stands out in various fields, including medicine, dentistry, pharmaceuticals, cosmetics, the food industry and agriculture [96–99].

Figure 9. Propolis extract applications.

5.1 MEDICINE AND HEALTH

Studies have linked propolis extract to significant health benefits, including anti-inflammatory, antifungal, antibacterial, antiviral, healing, and immunomodulatory [100–107]. It has been described in the literature as having the potential to activate the body's natural defenses. As a result, its use is increasing as its health benefits become more evident. Oral capsules containing

propolis, chewable tablets, drops, sprays, creams, mucoadhesive gels, lozenges, oral syrups, mouthwashes, and toothpaste are commercially available products [97].

In the medical field, as propolis compounds are known to reduce inflammation and impact platelet responses, their efficacy has been studied in the treatment of various infections and diseases, such as ulcers [108], respiratory tract infections [109,110], hypertension [111], and diabetes [112]. Propolis has been used to control inflammation in patients undergoing hemodialysis has been reported [113–115]. Properties such as antithrombotic, antiplatelet, antioxidant, and others have also been reported [112].

In addition, there is growing interest in the dental field in using natural medicines, such as propolis, as alternative antimicrobial agents since there is an eminent concern about the global threat caused by antimicrobial resistance in individuals [116]. The antimicrobial efficacy of propolis against oral pathogenic microorganisms has been widely evaluated, with solid overall efficacy reported [117]. Propolis is a promising alternative antimicrobial agent, but it also has several biological activities [118] that make its use in the prevention or treatment of various dental diseases and oral conditions attractive. Laboratory and clinical studies in humans report various dental-related applications, such as mouthwashes, toothpaste, and chewing gums containing natural propolis extract [97,119].

In addition to medical and dental applications, natural extracts are gaining ground in the pharmaceutical and cosmetics sectors. Studies report that propolis properties as an antiseptic, antifungal, bacteriostatic, astringent, anesthetic, anti-inflammatory, and antioxidant make the applications of this natural extract attractive as a cosmetic agent [120]. Its use as a cosmetic has been reported as beneficial when applied for hair health and growth [121], in healing skin wounds and cell regeneration [122], in reducing acne inflammation, and in sun protection [123,124].

5.2 AGRICULTURE AND THE ENVIRONMENT

As in medicine and pharmacology, research into the use of bioactive natural compounds and sustainable alternatives for agricultural pest control is increasing [125]. Natural extracts such as propolis, known for their therapeutic effects, are already used as pesticides, stimulants, and agricultural growth promoters. When used, they have no significant adverse impact on health and the environment [121–123]. The use of propolis in agriculture is still recent. Still, studies show the effectiveness of nanomaterials with propolis extract when used as an agricultural defense against some types of bacteria, fungi, and phytopathogenic diseases [126,127].

As demonstrated by research, this extract has been utilised as an herbicide against weeds and as an insecticide, proving effective against certain species of insects [128]. Its beneficial use in the post-harvest preservation of fruits and vegetables has been reported, with inactive biofilms resulting in greater utilisation of freshly harvested food [126,128].

Research has indicated the efficacy of employing aqueous propolis extract in preserving and protecting pine wood against fungi that cause rot (*Coniophora puteana*), with the application of the extract also resulting in a reduction in wood mass loss [129]. Furthermore, Corciovă et al. [130] demonstrated in their study that the synthesis of silver nanoparticles enhanced the antioxidant potential of propolis, and also reported positive effects of AgNPs coated with propolis in the photocatalytic process of malachite green dye under solar radiation.

5.3 FOOD AND BEVERAGE

Growing consumer interest in minimally processed foods without the addition of synthetic additives has led the food industry to adopt innovative approaches to food processing. These include using natural compounds as colorants, flavorings, and preservatives in foods because of their antioxidant properties [131,132]. These compounds are found in plants, edible vegetables, herbs, fruits, and spices [132].

Due to the wide range of properties and safety of using propolis as a natural extract, its applications in the food industry are varied and make this extract an excellent natural food preservative that, like synthetic preservatives, its application aims to extend the shelf life and guarantee the safety of food [98]. Propolis extract can be applied directly to the food by dipping or adding to the composition, together with polymer-based coatings such as biofilms, or even incorporated into bio-packaging for food [133]. In all cases, the goal is to reduce pathogens and ensure safe food preservation. A reduction in the number of bacteria, yeasts, and fungi, such as *Staphylococcus aureus* and *Listeria*, was achieved in diverse types of foods, such as meat and fish, milk, fruit juices, fruits, and vegetables, when propolis extract was used [99]. Mahdavi-Roshan et al. [134] added propolis extract to chicken breast marinade at different concentrations and studied the total yeast and mold counts, textural parameters, and sensory aspects under adequate refrigeration. They found a reduction in the microbial growth rate (*Staphylococcus aureus* and *Escherichia coli*) as the concentration of extract in the samples increased, and samples containing the extract showed less change in the quality parameters of texture and odor over the storage time. A study also showed a reduction in the deterioration of ground beef when ethanolic extract of propolis was added at different concentrations (3 to 7%) [135].

Chua et al. [136] added aqueous propolis extract to prepare jaboticaba juice and studied its degradation during storage at a controlled temperature. They demonstrated that the extract's performance was comparable to the commonly used chemical preservative, sodium benzoate, in preserving the quality of jaboticaba juice. In addition to the direct use of propolis extract in food products, studies show its application in coatings and packaging. Ezazi et al. [133] demonstrated that they developed an edible coating with chitosan and propolis extract in the formulation for coating fresh eggs. They found that in the eggs that received the optimized formulation, there was no detection of *S. enteritidis* on the shell and in the egg's contents, and there was high antibacterial activity against *Salmonella enteritidis*.

5.4 SYNTHESIS OF NANOPARTICLES

Incorporating natural extracts into formulations can be technologically challenging, and the desired effects of using these compounds can be altered during formulation processing. This challenge also applies to propolis applications. In addition to the potential degradation of its active ingredients, there are factors such as its low solubility in water and sensory characteristics such as a strong odor and taste.

As a result, creative technological solutions have been applied to overcome these challenges, such as the development of biofilms [137], microparticles, and nanoparticles [138], among others. Nanoparticles can be based on polymers, carbon, lipids, ceramics, or metals. Metallic nanoparticles can be made of silver, gold, copper, titanium, and other metals. AgNPs are one of the most capable due to good catalytic and conductive phenomena, which have proven to be particularly useful in photochemistry, biomedicine, and agriculture. In addition to their remarkable antimicrobial, antiviral, and biocompatible properties [138–140].

The synthesis of AgNPs can be chemical, physical, or green. The biosynthesis of green synthesis of AgNPs was developed using natural extracts as reducing and stabilizing agents [140]. Hernández-Morales et al. [141] report the development of green synthesis of silver particles using natural extract of dark and light chia seeds as a reducing and stabilizing agent. In their study, in addition to determining the optimal conditions for the formulation of the nanoparticles, they also observed the antimicrobial activities, which were found to be high against *E. coli* and *S. aureus*.

A plethora of studies have been conducted in various domains to explore the green synthesis of silver nanoparticles doped with propolis, their applications, and the outcomes observed. Islam et al. [142] have documented how the properties of AgNPs doped with propolis have been found to facilitate the wound healing process in animal models. Additionally, there are reports of the efficacious utilization of occlusive dressings comprising silver nanoparticles and propolis [143]. In the domains of food and environment, AgNPs doped with

propolis extract have been the focus of research as edible coatings and insecticides [144–146].

6. SILVER NANOPARTICLE SYNTHESIS TECHNIQUES

6.1 CHEMICAL REDUCTION METHOD

The chemical reduction method is one of the most widely used techniques for synthesizing AgNPs due to its simplicity, efficiency, and ability to produce nanoparticles with controlled size and shape. In this process, a silver salt, typically silver nitrate ($AgNO_3$), is reduced by a chemical reducing agent such as sodium borohydride ($NaBH_4$), hydrazine, or ascorbic acid, resulting in the formation of metallic AgNPs. Stabilizing agents, like polyvinylpyrrolidone (PVP) or citrate, are often added to prevent aggregation and maintain nanoparticle stability. This method allows for fine-tuning of nanoparticle properties by adjusting the concentration of the reducing and stabilizing agents, reaction temperature, and pH. The versatility and effectiveness of the chemical reduction method make it a preferred choice for producing AgNPs for various applications, including medicine, electronics, and catalysis.

Different compounds have been used as reduction agents in the literature. Khan et al. [147] synthesized AgNPs using aniline as a reducing and adsorbing agent in the presence of CT. UV-vis spectroscopy confirmed nanoparticle growth through a plasmon absorption band at 390–450 nm. Transmition Electron Microspy (TEM) analysis revealed well-dispersed, spherical nanoparticles ranging from 10 to 30 nm. The formation rate initially increased with aniline concentration but later declined, although aniline concentration had no significant impact on the nanoparticles' shape or size distribution. Wang et al. [148] produced AgNPs by reducing silver nitrate with glucose in a PVP-containing solution, with sodium hydroxide used to accelerate the reaction. Optimal stability was achieved with a NaOH to $AgNO_3$ mole ratio of 1.4 to 1.6. TEM analysis showed better particle dispersion with increasing PVP, with individual colloidal particles forming when the PVP to $AgNO_3$ weight ratio

was at least 1.5. X-Ray Diffraction Analysis (XRD) confirmed that the particles were pure silver when the reductant was sufficient and the mixing speed was slow. Suriati et al. [149] AgNPs were uniformly synthesized using a simple chemical reduction method involving trisodium citrate as a reducing agent and ascorbic acid as a surfactant. Characterization revealed AgNPs with sizes ranging from 35–80 nm, averaging 50 nm. The study showed that higher trisodium citrate concentrations led to smaller, more uniform quasi-spherical nanoparticles while increasing ascorbic acid concentrations resulted in larger, slightly polygonal particles.

One of the best applications of AgNPs is antibacterial activity. Lee et al. [150] synthesized AgNPs by chemically reducing silver nitrate with sodium borohydride in water, using SDS as a stabilizer. The AgNPs exhibited antibacterial activity against both Gram-positive *S. aureus* and Gram-negative *E. coli*, with effectiveness influenced by the degree of particle aggregation. Kim et al. [151] tested the antimicrobial activity of AgNPs against yeast, *E. coli*, and *S. aureus*, revealing inhibitory solid effects on yeast and *E. coli* even at low concentrations. In contrast, the effects on *S. aureus* were milder. Characterization confirmed stable nanoparticles with defined shape and size distribution. The study also suggested that the growth inhibition of microorganisms by AgNPs might be linked to free-radical generation, highlighting their potential use in medical devices and antimicrobial control systems. Finally, Thiruvengadam and Bansod [152] AgNPs were synthesized using $NaBH_4$ and ethanol as a reductant and stabilizer. Analysis revealed an average particle size of 18.31 nm, with a crystalline morphology and face-centered cubic structure. These nanoparticles demonstrated antibacterial activity, producing inhibition zones of 19 mm against *Bacillus subtilis* and 17 mm against *Pseudomonas aeruginosa*, highlighting their potential in medical applications.

6.2 RADIATION REDUCTION METHOD

The radiation reduction method is an advanced technique for synthesizing AgNPs by utilizing high-energy radiation to reduce silver ions to metallic silver [153]. This method involves irradiating a silver precursor solution with radiation sources such as gamma rays, X-rays, or ultraviolet (UV) light. The energy from the radiation induces the reduction of silver ions and promotes the nucleation and growth of nanoparticles [154]. This approach offers precise particle size and morphology control by adjusting radiation parameters and conditions. It also minimizes the use of chemical reductants, making the process environmentally friendly [153]. The resulting AgNPs are typically well-dispersed, uniform in size, and exhibit enhanced properties suitable for various applications in catalysis, medicine, and electronics [155].

A wide range of radiation sources has been used in the literature to synthesize AgNPs. For instance, Shaheen et al. [156] highlighted the potent antimicrobial and anticancer properties of differently shaped AgNPs produced using ionizing radiation, demonstrating significant antifungal, antibacterial, antiviral, and cytotoxic effects against various pathogens and cancer cell lines. Saion et al. [157] successfully synthesized size-controlled, monodispersed AgNPs using a radiolytic method, where particle size is inversely correlated with radiation dose due to the dominance of nucleation over ion association. The resulting nanoparticles displayed sharp absorption spectra, with quantum physics calculations suggesting their absorption behavior may be linked to intra-band excitations of conduction electrons. Kang et al. [158] used e-beam treatment to obtain AgNPs, finding that lower e-beam energy produced smaller nanoparticles, while higher beam current and absorbed dose led to agglomeration. The dispersing agents like PVA effectively controlled particle size and uniformity, making this e-beam method ideal for applications in electronics, catalysts, and photonics due to its high productivity, chemical-free process, and eco-friendly nature.

6.3 PRECIPITATION METHOD

The precipitation method is widely used for the synthesis of AgNPs due to its simplicity, efficiency, and scalability [159]. In this process, silver ions (Ag^{+})

are reduced to form solid AgNPs, precipitating out of the solution. The method typically involves using a reducing agent, such as hydrazine or sodium borohydride, to convert Ag^{+} into AgNPs (Ag^{0}). Various stabilizing agents or surfactants can be added to control the nanoparticles' size, shape, and dispersion, preventing them from agglomerating [160]. The precipitation method produces AgNPs with uniform morphology and size distribution, making it a valuable approach for applications in analysis, electronics, and medicine [161]. Dasaradhudu and Srinivasan [162] prepared Ag-NPs using the coprecipitation method with $AgNO_3$ and trisodium citrate, resulting in nanoparticles with an average size of approximately 5.5 nm. The Ag-NPs have a zeta potential of -44.6 mV, demonstrating high stability and mobility. These properties suggest that the synthesized Ag-NPs are suitable for applications in cancer treatments, including brain and breast cancer. Chou et al. [163] used continuous precipitation of AgNPs at room temperature using sodium borohydride as the reducing agent. The nanoparticles varied in size from 13 to 130 nm. Size control was effectively managed through the PVP/AgNO3 weight ratios ranging from 0.05 to 1.5. Sobhani-Nasab et al. [160] utilized a precipitation method to synthesize AgO nanostructures from silver nitrate in aqueous solution. The AgO nanostructures exhibited ferromagnetic behavior, as shown by the hysteresis loop at room temperature. Additionally, the photocatalytic properties of the AgO nanoparticles were evaluated through the degradation of rhodamine-B under visible light.

6.4 MICROEMULSION METHOD

Microemulsion synthesis is an innovative and adaptable method for producing AgNPs with precise control over their size and morphology. This technique creates a microemulsion, a thermodynamically stable mixture of water, oil, and surfactants [164]. Silver ions are reduced to form nanoparticles within this microemulsion, with surfactants stabilizing the particles to prevent aggregation. The microemulsion's unique environment facilitates uniform nucleation and growth, resulting in highly monodisperse and well-defined AgNPs [165]. This method is particularly beneficial for catalysis, medicine, and

electronics application where high-quality, consistently sized nanoparticles are crucial [166].

The most common microemulsion technique for AgNP synthesis is oil-in-water based. Rivera-Rangel et al. [167] developed a green synthesis approach using a low-toxicity microemulsion system with castor oil and Geranium leaf extract, producing AgNPs ranging from 25 to 150 nm. This sustainable method has potential applications for other metals. Zhang et al. [168] used $AgNO_3$ and hydrazine hydrate in separate microemulsions with dodecane and AOT, finding that increasing $AgNO_3$ concentration accelerated nanoparticle growth. At the same time, a higher water-to-surfactant ratio led to larger particles and broader size distributions. These nanoparticles were spherical, stable, and low toxicity, suitable for direct antibacterial applications.

A few authors had approached a different way to produce AgNPs using the reverse microemulsion technique. Nourafkan and Alamdari [169] explored the reverse microemulsion technique, using $AgNO_3$ and hydrazine to synthesize spherical AgNPs with an average size of 7.1 nm. Surfactant hydrophile-lipophile balance and molecular structure significantly influenced nanoparticle morphology and size. Wani et al. [170] investigated inverse microemulsions with surfactants CTAB, Tergitol, and Triton X-100, producing nanoparticles in various shapes (spheres, cubes, discs) and sizes (8 to 40 nm) with distinct surface plasmon resonance peaks and excellent antimicrobial activity.

Recent advancements include the use of ionic liquid microemulsions. Althobaiti et al. [171] synthesized extremely small, monodispersed AgNPs using benzyl alkyl imidazolium ionic liquids (BAIILs), demonstrating minimal agglomeration and potent antibacterial activity. Li et al. [172] developed a method for continuous, controllable AgNP synthesis using quaternary ionic liquid microemulsions, with sizes ranging from 2 to 13 nm, tunable by adjusting synthesis conditions. Patil et al. [173] introduced a one-phase method using 1-(dodecyl) 2 amino-pyridinium bromide, producing uniform, monodispersed crystalline AgNPs with significant antibacterial effects.

6.5 LASER-ASSISTED SYNTHESIS METHOD

Laser-assisted synthesis of AgNPs is an advanced technique that leverages the precision and energy of lasers to produce high-quality nanoparticles [174]. This method involves irradiating a silver precursor or target material with a laser, inducing nanoparticle formation through a combination of photothermal and photochemical effects [175]. The controlled energy input from the laser allows for precise manipulation of particle size, shape, and distribution, resulting in well-defined and uniform nanoparticles. This technique offers advantages such as high purity, reduced chemical waste, and the ability to tune nanoparticle properties for specific applications [176]. Laser-assisted synthesis is particularly valuable in electronics, catalysis, and biomedical fields, where high-performance AgNPs are required [177].

Due to the wide applications of laser-assisted synthesis of AgNPs, the literature is rich with diverse methods and applications of these nanoparticles. Ognjanovic et al. [177] utilized pulsed laser ablation in liquid to prepare AgNPs from a pure silver plate iN-dimethylformamide, which were then used to modify screen-printed carbon electrodes (SPCE). The modified SPCE was employed for gallic acid detection, demonstrating practical application in measuring gallic acid in biological fluids and estimating antioxidant capacity for food quality. In another study, Yu et al. [176] produced a hybrid material for optical limiting by combining reduced graphene oxide functionalized with AgNPs using femtosecond laser ablation in liquids. This hybrid material showed enhanced nonlinear absorption and excellent optical limiting properties with a low activation threshold of about 0.38 J cm^{-2}. It highlights its potential for solid-state optical limiters and practical applications in the optical limiting field.

A recent trend in nanoparticle synthesis involves combining laser-assisted processes with supercritical deposition techniques. Arakcheev et al. [174,175] synthesized AgNPs in the pores of silica aerogel using supercritical deposition. The sample was impregnated with the precursor Ag(hfac)COD dissolved in supercritical carbon dioxide, and conversion was achieved via laser irradiation at 405 nm, matching the plasmon band of AgNPs. The concentration

of AgNPs could be adjusted by the exposure time, and the method was effective both under supercritical conditions and after depressurization. In another study, AgNPs were synthesized in the pores of Vycor glass, and the influence of irradiation wavelength on nanoparticle properties was analyzed. The synthesis was performed using laser irradiation at three different wavelengths: two resonant wavelengths matching the plasmon bands of spherical and elongated AgNPs, and one off-resonant wavelength red-shifted from the former two. The findings revealed that the irradiation wavelength significantly affects both the synthesis rate and the homogeneity of the AgNP ensemble, with resonance wavelengths increasing the mass fraction of non-spherical particles.

6.6 ELECTRODEPOSITION METHOD

Electrodeposition synthesis is a highly effective technique for producing AgNPs with precise control over their size, shape, and distribution. This method involves reducing silver ions onto a conductive substrate using an electric current [178]. By adjusting parameters such as voltage, current density, electrolyte composition, and deposition time, researchers can fine-tune the properties of the resulting nanoparticles. Electrodeposition offers simplicity, cost-effectiveness, and the ability to produce uniform and well-dispersed nanoparticles [179]. This method is particularly suitable for electronics, catalysis, and biomedical applications because it can generate high-purity AgNPs with excellent functional properties [180].

In antibacterial and biomedical applications, electrodeposition-synthesized AgNPs have shown promising results. For example, Mayouf et al. [180] presented a rapid double-pulse electrodeposition method for AgNPs on a thin polypyrrole film, enhancing electrical conductivity and antibacterial activity. The resulting AgNPs demonstrated uniform size and distribution, achieving a 100% bacterial kill rate against *E. coli* and 99.99% against *S. aureus*. Similarly, Singaravelan and Alwar [179] developed a rapid electrochemical technique for synthesizing silver nano dendrites, which exhibited significant particle

aggregation and exceptional antibacterial activity against multidrug-resistant strains, including *S. aureus* and *E. coli*, particularly in combination with Streptomycin.

Other studies have explored different applications of electrodeposition-synthesized AgNPs, such as improving materials' mechanical and structural properties. Pan et al. [181] introduced carboxylated chitosan for synthesizing AgNPs and creating AgNPs/carboxylated chitosan nanocomposite films. The carboxylated chitosan acted as both a green reducing and stabilizing agent and formed the main component of the nanocomposite film, which was smooth, homogeneous, and detachable from the substrate. The films exhibited favorable antibacterial properties. Yin et al. [174] also developed a novel electrochemical method for synthesizing size-controlled spherical AgNPs using poly(N-vinylpyrrolidone) as a stabilizer. This method facilitated the production of monodispersed nanoparticles and enabled their use in creating silver-doped tin electrodeposited nanocomposite coatings.

6.7 SOL-GEL METHOD

Sol-gel synthesis is a versatile and efficient method for producing AgNPs with controlled size and morphology. This technique involves the transition of a system from a liquid "sol" into a solid "gel" phase, providing a low-temperature process for the formation of nanoparticles. In the sol-gel process, silver precursors are typically hydrolyzed and condensed to form a gel-like network, which is then subjected to thermal treatment to produce AgNPs [182]. This method allows for precise control over the particle size, distribution, and surface properties, making it ideal for applications in catalysis, antimicrobial treatments, and electronics [183]. The sol-gel technique's adaptability and simplicity have made it a popular choice for synthesizing AgNPs with tailored characteristics [184].

Ahlawat et al. [185] worked with AgNPs that were successfully prepared using the sol–gel method by annealing the sample at 550 °C for 30 min. The presence of silver metal in the silica matrix was confirmed, with an average

nanoparticle size of 10.2 nm, an absorption peak around 375 nm, and a particle size distribution from 8 nm to 25 nm. FTIR spectroscopy identified different chemical groups in samples prepared at room temperature, 450 °C, and 550 °C, confirming that silver nanoparticle formation depends on the annealing temperature.

Shahjahan et al. [182] describe a simple and convenient procedure for preparing crystalline AgNPs using the sol-gel technique with CH_3COONa and hydrazine as reducing agents in water at room temperature. The nanoparticles, averaging 11 nm, show homogeneity, uniform size, and a regular granular shape without impurities. The study concluded that the synthesized particles were pure and suitable for large-scale production with applications in electronics and catalysis.

Some other works in the literature have tested different applications of AgNPs synthesized by sol-gel with biomedical applications. Patil et al. [183] report the rapid one-pot synthesis of AgNPs at room temperature using hydrazine hydrate as the reducing agent and polyvinyl alcohol as the stabilizing agent. Characterization reveals spherical nanoparticles with diameters ranging from 10 to 60 nm, a surface plasmon resonance at 410 nm, and a face-centered cubic structure. The synthesized AgNPs demonstrated antimicrobial activity against *B. cereus, E. coli, S. aureus,* and *P. vulgaris*, indicating potential applications in biotechnology and biomedical science. Ahmed et al. [186] report the formation of new conjugates comprising single-wall nanotubes and multi-wall nanotubes doped with silver-doped titanium dioxide, exhibiting outstanding antimicrobial and toxic properties. The SWNTs–TiO_2/Ag and MWNTs–TiO_2/Ag conjugates exhibited significant antibacterial effects against *E. coli* and *S. aureus* and selectively killed uterine cancer cells (~60-40%) while minimally affecting normal cells (~10%).

6.8 GREEN SYNTHESIS METHOD

The use of microorganisms as eco-friendly precursors for nanoparticle production, including silver and gold, has garnered significant interest [187].

Bacteria and fungi are particularly important in reducing metal ions, aiding in the remediation of toxic metals [188]. *Pseudomonas stutzeri*, known for its silver resistance, accumulates intracellular silver crystals approximately 200 nm in diameter with specific composition and shape [189]. Bacterial synthesis is superior to fabricating eco-friendly and cost-effective AgNPs [190]. Optimized cultures of *Bacillus sp.* have demonstrated rapid and high-yield synthesis of AgNPs [190].

Solis-Sandi et al. (2023) [191] noted that AgNPs were biosynthesized using the supernatant and intracellular extract of *Cupriavidus necator, Bacillus megaterium, and Bacillus subtilis*. AgNPs showed particle sizes ranging from 20.8 to 118.4 nm. This study concluded that bacterial species, temperature, pH, and the type of extract (supernatant or intracellular) significantly impact the biosynthesis process. The synthesis method is simple, environmentally friendly, and cost-effective, making it suitable for producing AgNPs for antibacterial applications.

Research on prokaryotic synthesis of metallic NPs is extensive due to bacteria's abundance, adaptability to extreme conditions, cost-effectiveness, and controllable growth conditions [192]. However, fungi are preferred for metallic NP synthesis because they secrete more proteins, facilitating higher nanoparticle production and offer easier scale-up and downstream processes [193]. Çiğdem et al. (2024) [194] demonstrated that *Chroococcus sp.* cell extracts can reduce aqueous Ag^{+} ions to synthesize stable, non-toxic, cost-effective, and environmentally benign AgNPs. Thirumurugan et al. (2024) [195] reported the extracellular synthesis of AgNPs by marine actinobacteria, specifically *Streptomyces parvisporogenes* KL3. The AgNPs showed a mean diameter of 23-27 nm and demonstrated broad-spectrum antibacterial activity, inhibiting *E. coli, B. subtilis*, and *K. pneumoniae*.

The current trend in nanoparticle production emphasizes green synthesis using plant extracts, which serve as multifunctional agents for reducing and stabilizing nanoparticles, thereby promoting green chemistry principles [138]. This method is cost-effective compared to microbial isolation, as non-toxic plant extracts act as natural capping agents. The increasing

interest in 'green' methods for metal nanoparticle synthesis has led to successful trials with various plant extracts [196]. Plant-mediated AgNP synthesis is preferred due to its local availability, eco-friendliness, cost-effectiveness, high yield, and rapid synthesis compared to microbial methods. Abada et al. [138] reviewed various AgNPs synthesis methods, highlighting plant-assisted synthesis as an emerging area in nanotechnology. Tesfaye et al. [197] noted the compounds like alcohols, aldehydes, phenols, and flavonoids oxidize during plant-mediated synthesis, reducing metal ions to nanoparticles.

Melo et al. [198] revealed the essential thyme (*Thymus vulgaris*) oil can efficiently produce metal nanostructures through green methods, avoiding hazardous solvents and waste. Their study demonstrated that biosynthesized AgNPs, prepared at different pH levels (7, 8, 9, and 10), exhibited excellent physicochemical stability over 90 days at 6 °C and 25 °C. Characterization techniques, including UV–visible spectroscopy, TEM, and Dynamic Light Scattering (DLS), confirmed the formation of nanoparticles with an average diameter of 40 nm and a homogeneous size distribution with an average particle diameter of around 90 nm for all pH levels tested. The AgNPs showed high antibacterial activity against *Escherichia coli and Staphylococcus aureus*.

Using rosemary essential oil, Sganzerla et al. [192] produced eco-friendly nanocomposite films with AgNPs. The AgNPs, confirmed by UV–vis spectroscopy, TEM, SEM, and XRD, had a size below 50 nm and demonstrated strong antimicrobial activity. The nanocomposites, functionalized with 15, 30, and 50% AgNPs, showed enhanced mechanical properties, making them a sustainable material for biological applications. Maciel et al. [199] presented an environmentally friendly approach to synthesizing AgNPs using essential oils as natural reducing agents. Essential oils from oregano (Origanum vulgare), thyme (*Thymus vulgaris*), clove (*Syzygium aromaticum L.*), rosemary (*Rosmarinus officinalis L*.), and Poiretia latifolia were screened for their effectiveness in AgNP synthesis. Clove essential oil, containing 80% eugenol, produced the best results. UV–Vis spectrophotometry confirmed the formation of AgNPs, and further characterization using TEM and DLS showed predominantly spherical nanoparticles. Small-Angle X-ray Scattering (SAXS) provided detailed insights,

revealing particle sizes between 18.6 nm (AgNP-Eugenol) and 22.4 nm (AgNP-Clove). The AgNPs exhibited significant antimicrobial activity against *S. aureus* at various concentrations (40–100 µL mL^{-1}), with a Minimum Inhibitory Concentration of 40 µL mL^{-1}, indicating strong bactericidal properties. This study highlights that AgNP-Clove possesses similar characteristics to AgNP-Eugenol, making it a cost-effective and reliable alternative for producing AgNPs, particularly for applications in food packaging.

Recent studies on the green synthesis of AgNPs using various natural sources have highlighted their potential environmental and biomedical applications. Aloe vera-derived AgNPs demonstrated strong antimicrobial properties against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, showcasing their ability to degrade organic pollutants and treat microbial infections [200]. A microalga *Scenedesmus sp.* Study produced a biomass-AgNPs composite that effectively reduced the industrial pollutant p-nitrophenol and exhibited antimicrobial activity against multiple pathogens, making it suitable for industrial effluent treatment [201]. The phytochemical-rich extract of red seaweed *Champia parvula* was used to synthesize stable, bioactive AgNPs with significant antioxidant, antimicrobial, and anticancer properties [202]. Furthermore, *Artemisia absinthium callus* cultures treated with silver and copper nanoparticles synthesized from *Moringa oleifera* leaves exhibited enhanced biomass accumulation, increased production of antioxidative enzymes, and improved antioxidant activity [203]. These findings underscore the versatility and effectiveness of using natural sources for the green synthesis of AgNPs, offering sustainable alternatives for environmental remediation and biomedical applications.

The critical factors for the green synthesis of AgNPs include selecting suitable solvents, reducing agents, and non-toxic materials. AgNPs have shown excellent antibacterial performance and synergistic effects when combined with antibiotics [204,205]. Singh et al. [206] successfully extracted promising AgNPs from the propolis of *Apis mellifera*, which is rich in antioxidant compounds and polyphenolics, making it useful for drug delivery, free radical scavenging, and cytoprotective and genoprotective activities.

Karimitabar et al. [207] prepared a hydroalcoholic propolis extract to synthesize AgNPs through green methods. These nanoparticles exhibited low toxicity to cells and effectively inhibited the growth of various bacteria, including both Gram-positive and Gram-negative strains.

Recent studies have expanded AgNP synthesis using various plant extracts, such as M*aclura pomifera*, *Picea*, *Ginkgo biloba* needles [208], *Thymus vulgaris*, *Ficus pomifera* wall, *Strobilanthes flaccidifolius* nees, *Crassocephalum crepidioides* [209], *Moringa peregrina* [210], *Lallemantia royleana* [211], *Punica granatum* fruit peels [212], *Salvia officinalis* [213], *and Eupatorium adenophorum* leaf [214]. The use of plant extracts in green AgNP synthesis significantly enhances environmental and economic feasibility, as many plant extracts have effectively synthesized AgNPs with consistent sizes and diverse applications. **Table 6 s**ummarizes the methods presented, the characteristics of the process, and the products obtained.

Table 6. Methods of AgNPs synthesis and products.

Method	Silver precursor	Synthesis conditions	Stabilization system	Product	Reference
Chemical reduction	$AgNO_3$	Mixtures containing sodium citrate, PVP, and H_2O_2 were added to the $AgNO_3$ solution while stirring. Varying volumes of $NaBH_4$ solution were used for each mixture, which was stirred for 3 h before being filtered through a 0.2 μm membrane filter	Sodium citrate	Triangle, spherical, rod-shaped, and disc-shaped AgNPs, with diameters ranging from 3 to 30 nm and varying aspect ratios.	[215]
	$AgNO_3$	A mixture containing sodium citrate and tannic acid was added to the $AgNO_3$ solution in a molar ratio of 1:7:2, respectively, and stirred for 15 min at room temperature	Sodium citrate	Homogeneous, spherical shape with an approximate diameter of 30 nm and a monodisperse size distribution.	[216]
	$AgNO_3$	$AgNO_3$ was added to a cetyltrimethylammonium bromide (CTAB) solution under stirring, followed by the addition of $NaBH_4$ solution. The resulting seeds were kept in the dark for 1 h, after which they were added to a freshly prepared solution of $AgNO_3$, ascorbic acid, and CTAB	CTAB	Triangular silver nanoplates with 45 nm of edge length.	[217]
	$[Ag(NH_3)_2]^+$	Sequential adding of sodium citrate, $AgNO_3$, and NaCl to water under stirring at room temperature	Citrate	Monodisperse and quasispherical, with sizes ranging from 40 to 300 nm.	[218]
	$[Ag(NH_3)_2]^+$	A mixture of $AgNO_3$, ammonium hydroxide, and silica in water was stirred and sonicated for 2 h in a dark environment overnight at room temperature	Mesoporous silica	AgNPs deposited on mesoporous silica.	[219]
Radiation reduction	$AgNO_3$	Mixture of Poly(vinyl pyrrolidone, $AgNO_3$, n-butanol, and water was subjected to different irradiation doses using a Co-60 γ-cell-220	Poly(vinyl alcohol)	AgNPs with hexagonal shape with minor nanorods shapes.	[154]

	$AgNO_3$	Mixture of $AgNO_3$, polyvinyl alcohol, isopropanol and water, agitated for 3h and nitrogen bubbling to remove oxygen and irradiated with doses ranging from 10 to 70 kGy using a 1.25-MeV ^{60}Co γ-ray source	Poly(vinyl alcohol)	AgNPs with size varying from 10-50 nm.	[157]
	$AgNO_3$	A mixture of $AgNO_3$, polyvinyl alcohol, isopropyl alcohol and irradiated with electron beam varying from 10-300 kGy	Poly(vinyl alcohol)	AgNPs homogeneous with average size of < 10 nm.	[158]
	Ag_2SO_4, $AgClO_4$, and $AgNO_3$	Radiolytic synthesis was performed using a panoramic γ-ray source with Co-60 to irradiate a mixture of polyvinyl alcohol, 2-propanol, and various silver salts. The mixture was added to water and stirred at 100 ºC for 1 h	Poly(vinyl alcohol)	Colloidal AgNPs with high purity and homogeneous sizes (diameter ~20 nm).	[220]
	$AgClO_4$	Radiolytic reduction using an electron beam from a Cs_2Te photocathode to irradiate a mixture of silver perchlorate, water, and 2-propanol	Poly(acrylic acid)	Silver nanoclusters and nanoparticles under 2 nm of diameter.	[221]
Precipitation	$AgNO_3$	A solution of trisodium citrate was added drop by drop to a mixture of $AgNO_3$ and water at 50 ºC until a yellow color was obtained and silver precipitated	Citrate	AgNPs with an average diameter of 5.5 nm, highly stable and mobile	[162]
	$AgNO_3$	A mixture of $AgNO_3$, sodium borohydride, and poly(vinyl pyrrolidone) underwent a reaction in a microchannel reactor for continuous precipitation of AgNPs	Poly(vinyl pyrrolidone)	Colloidal AgNPs with average size ranging from 13 to 130 nm, and uniform size distribution.	[163]
	$AgNO_3$	A mixture of $AgNO_3$, different surfactants (glucose, SDBS, SDS, and CTAB), NaOH, and $K_2S_2O_8$ was stirred for 1 h at 50 ºC until a gray powder precipitated	Surfactants	AgO nanostructures with an average size of 32 nm, composed of agglomerated nanosheets and nanoparticles	[160]
Microemulsion	$AgNO_3$	A mixture of $AgNO_3$, sodium bis(2-ethylhexyl) sulfosuccinate, dodecane, and hydrazine hydrate	AOT	Colloidal AgNPs are spherical with a very	[164]

		was stirred vigorously for 2 h at room temperature, maintaining a water-to-AOT molar ratio of 7.5, until a stable light-yellow colloidal dispersion formed		narrow size distribution and a mean diameter of 1.6 nm.	
	$AgNO_3$	Mixture of $AgNO_3$, hydrazine, cyclohexane, and different surfactants. The mixture was stirred for 30 min at room temperature. The microemulsion was initially transparent but gradually became milky because of nucleating the solid phase	Surfactants	Spherical AgNPs, with average particle size of 7.1 nm, with triangular or spherical morphology.	[169]
	$C_{18}H_{36}AgO_2$	Silver stearate was solubilized in oil, geranium leaf extract was added to a surfactant/oil/metallic precursor mixture. The reaction was maintained at 25 °C for 24 h. Nanoparticles were then separated by centrifugation and washed with acetone, ethanol, and water	Surfactants	AgNPs sizes varying from 25 to 150 nm.	[167]
Laser-Assisted	Ag plate	AgNPs were synthesized by pulsed laser ablation of a silver plate in N,N-dimethylformamide citric acid solution. A 15 mJ Nd:YAG picosecond laser (1064 nm, 10 Hz) was used to irradiate the silver plate at a ~90° incident angle	-	AgNPs suspension with average particle size of 220 nm and uniform size.	[177]
	Pure Ag	A pure silver target was irradiated using a laser ablation technique in deionized water and other organic solvents, with radiation at a wavelength of 532 nm and a fluence ranging from 26.3 J/cm² to 47.4 J/cm² in steps of 5.3 J/cm², at a repetition rate of 10 Hz	-	AgNPs exhibit tunable size, stability, and viability	[222]
	Ag plate	A pure silver plate was placed in a glass vessel filled with deionized water. A Q-switched Nd laser with a 5 Hz repetition rate and 20 ns pulse width was used as the irradiation source	-	AgNPs with typical diameter of about 8.5 nm, with high colloidal stability	[223]
Electrodeposition	$AgNO_3$	Electrolyte solution containing $AgNO_3$, an	-	AgNPs with cubic	[180]

		electrode containing glassy carbon and other silver metal. The silver ions were reduced at room temperature		nanocrystals with dendritic structures and average crystal size of 33.3 nm	
	Ag plate	A Ag plate was used as the anodic electrode and a platinum foil as the cathodic electrode. A constant voltage of 2 V was applied for 10 min in the electrodeposition solution, which was made from a carboxylated chitosan solution	-	Smooth and homogeneous electrodeposited films on the silver plates, AgNPs with an average size of 10 nm	[181]
	$AgNO_3$	A two-electrode cell was constructed using platinum and silver with an EG&G M173 potentiostat. The electrolytic solution consisted of KNO_3, $AgNO_3$, and poly(N-vinylpyrrolidone). Electrolysis was carried out potentiostatically at room temperature under mechanical stirring	PVPK30	Spherical monodispersed AgNPs with average size of 16.6 nm.	[224]
Sol-gel	$AgNO_3$	$AgNO_3$ and TEOS were used as precursors. TEOS was hydrolyzed in a water-alcohol solution with HNO_3 as a catalyst, resulting in SiO_2 sols. An aqueous solution of $AgNO_3$ was prepared by dissolving $AgNO_3$ in water under gentle heating. TEOS and ethanol were mixed and stirred, followed by the slow addition of HNO_3 and water to initiate hydrolysis and polycondensation. The resulting solution was stirred until it became viscous, incorporating the silver nitrate solution to form the final product	-	Spherical AgNPs with size distribution varying from 8 to 25 nm	[185]
	$AgNO_3$	A chemical reduction process was used to prepare silver nanoparticles. Solutions of $AgNO_3$, citric acid, and NaOH were mixed and the pH was adjusted to 7 with ammonia. $N_2H_4 \cdot H_2O$ was added to the mixture, causing the solution to turn black, indicating the reduction of silver ions. The solution	Sodium acetate	AgNPs were homogeneous and had uniform size, with as average diameter of 11 nm.	[182]

		was stirred for 3 h at room temperature until it became transparent with silver particles visible. The nanoparticles were collected by filtration, washed with deionized water, and air-dried			
Green synthesis	$AgNO_3$	A mixture of aqueous algal extract was mixed with $AgNO_3$ solution. After 1 h of incubation at 37 °C, reduction of silver ions to silver nanoparticles was observed via color changes in the reaction solution. Finally, the nanocolloidal solution was centrifuged at 12,000 rpm for 20 min	Seaweed secondary metabolites	AgNPs with round shape with 79 nm of size with crystalline structure	[202]
	$AgNO_3$	The extract of Moringa oleifera leaves was used for the reduction and further capping of $AgNO_3$. The solutions were then reduced stepwise by adding the extract of M. oleifera with continuous boiling until the solution colour turned to brown	M. oleifera leaves extract	AgNPs showed surface plasmon resonance at 423-425 nm, with rectangular in shape	[203]
	$AgNO_3$	L. inermis extract was added to $AgNO_3$ solution under vigorous stirring, the reaction will be completed with the visualization of brownish yellow color from reddish orange	Leaf extract biomolecules	Silver nano-crystals with average size varying from 28 to 60 nm	[205]
	$AgNO_3$	Hydroethanolic extract of propolis was added to $AgNO_3$ solution and stirred for 1h at room temperature and colour change to brownish	Polyphenols presented in propolis	AgNPs showed a particle size of 43 nm with spherical shape and crystalline phase	[206]
	$AgNO_3$	Plant extracts was added to $AgNO_3$ stock solution dropwise at 80 ºC for 15 min with constant stirring	Metabolites present in the plant extract	AgNP colloidal solution with spherical nanoparticles ranging from 5 to 45 nm	[208]
	$AgNO_3$	Extracts of F. pomifera was added in a ratio of 1:3 to $AgNO_3$ precursor, the mixtures was heated on a steam bath till color change	Indigo	AgNPs with average size was 16 nm with globular morphology and spherical-shape	[209]

7. CHARACTERIZATION OF SILVER NANOPARTICLES (AGNPS): METHODS AND EVALUATION TECHNIQUES

The characterization of AgNPs is crucial for determining their physical, chemical, and morphological properties, which directly influence their behavior in various applications. This section will discuss the main methods and techniques used to characterize AgNPs, considering their morphological properties, particle size, chemical composition, crystal structure, optical and surface properties, and colloidal stability.

7.1 MORPHOLOGY, PARTICLE SIZE, AND DISTRIBUTION

The morphology of AgNPs can be evaluated using microscopy techniques, such as TEM and Scanning Electron Microscopy (SEM). TEM are widely used to provide high-resolution images, allowing for the observation of the shape and size of nanoparticles on a nanometric scale. Additionally, this technique can obtain detailed information about the internal structure of the nanoparticles. On the other hand, SEM is used to obtain high-resolution images of the surfaces of nanoparticles, enabling a three-dimensional analysis of their morphology.

SEM analysis is based on the interaction between the electron beam and the sample, producing particles and radiation that can be used to create a magnified image of the sample. The most important interactions between the primary electron beam and the solid species for the study of materials are those that provide information about the topography of the surface. This information is obtained by means of low-energy electrons (secondary or backscattering electrons), which provide photographic contrast and allow the study of the shallow relief of the surface. Among the possible applications of scanning electron microscopy in the field of catalysis is the study of morphology [225,226].

Determining the size of AgNPs and their size distribution is crucial for understanding their properties and behavior in practical applications. DLS is one of the most widely used for this purpose, allowing for the determination of the average particle size and evaluation of the particle size distribution in a suspension. This technology is essential because the uniformity of the nanoparticle size affects their optical and catalytic properties. In addition, image analysis using software that processes data obtained from TEM or SEM allows for a more precise distribution of nanoparticle sizes [227,228].

7.2 CHEMICAL COMPOSITION

The chemical composition of silver nanoparticles can be determined using analytical techniques such as MEV with energy-dispersive X-ray spectroscopy (EDX) and X-ray photoelectron spectroscopy (XPS). Often performed with TEM or SEM, EDX is used to identify the elements present in the nanoparticles, providing a semi-quantitative analysis of the composition. XPS is used to provide information about the chemical elements present on the surface of the nanoparticles and their oxidation states, providing an essential technique for the chemical characterization of surfaces [197].

7.3 CRYSTALLINE STRUCTURE

The crystalline structure of silver nanoparticles can be characterized by XRD and high-resolution transmission electron microscopy (HRTEM). X-rays are electromagnetic waves considered to be ionizing radiation. They are generated by elements that emit a certain number of photons, which are collimated and directed at the material to be characterized, which then diffracts them at a certain angle; these diffracted X-rays are detected and converted into signals. In the case of material identification, these signals are compared to the literature to confirm the presence of the desired phase and/or other phases. On the other hand, HRTEM provides detailed images of the atomic structure of the nanoparticles, enabling visualization of the crystalline orientation and identification of structural defects. These techniques are essential for understanding how the crystalline structure of silver nanoparticles affects their physical and chemical properties, such as thermal stability and catalytic reactivity [229].

7.4 OPTICAL PROPERTIES

The optical properties of silver nanoparticles are distinctive, resulting from surface plasmon resonance phenomena. These properties can be evaluated through the use of UV-Vis spectroscopy and in the near-infrared region (FT-IR). Photoacoustic UV-Vis spectroscopy (PAS) is a method for obtaining optical

absorption spectra of solids, semi-solids, liquids, and gases. The technique is versatile in that it can analyze both optically opaque and transparent samples. The scattering of light by the sample, which represents a significant challenge in other optical spectroscopy techniques, does not present a substantial issue in photo acoustics, as only the light absorbed by the sample is converted into the desired signal. Conversely, in most instances, this technique does not necessitate the rigorous sample preparation. Moreover, as a non-destructive technique, the same sample can be monitored when subjected to various chemical, thermal, and physical treatments, among other factors. The use of photoacoustic absorption spectra facilitates the study of energy bands in a given element, as it allows for the assignment of the optical transitions involved.

FT-IR enables the determination of molecular surface interactions. When subjected to a specific frequency within the infrared range, surface interactions absorb energy capable of inducing vibrations in chemical bonds. The capacity for this phenomenon varies depending on the type of chemical bond in question, with each bond exhibiting a distinct sensitivity to infrared radiation [230] [231].

7.5 COLLOIDAL STABILITY

The colloidal stability of silver nanoparticles in solution can be evaluated using DLS spectroscopy and zeta potential analysis. As mentioned earlier, DLS is used to monitor changes in the size of nanoparticles over time, indicating their stability under different conditions. On the other hand, Zeta potential analysis provides information about the surface charge of the nanoparticles, which is an essential indicator of colloidal stability, as similar surface charges between particles can prevent aggregation [232]. Checking the behavior of the surface of materials in an aqueous environment allows conclusions to be drawn about the surface species. The determination of the zeta potential (ZP) is a simple analysis that allows to verify the tendency of the surface to acidity or basicity, indicating that for pH values above the pH (ZP), the surface of the material is negatively charged and, consequently, for values below the pH (ZP), it is positively charged [232,233].

8. APPLICATIONS OF SILVER NANOPARTICLES

AgNPs are widely used due to their antimicrobial, catalytic, conductive, and photothermal properties, with significant applications in healthcare, electronics, water treatment, and cosmetics. Their unique characteristics enable the development of innovative solutions that have driven significant advancements in these fields.

8.1 ANTIMICROBIAL AND ANTIFUNGAL PROPERTIES

AgNPs are highly effective at inhibiting the growth of bacteria, fungi, and viruses due to the continuous release of silver ions, which bind to the cell membranes and DNA of microorganisms, inducing metabolic disruption and cell death. These properties make them essential in antibacterial coatings, textiles, and food packaging to prevent contamination by pathogenic agents [234].

8.2 CATALYST

AgNPs are also important catalysts due to their high specific surface area and chemical activity. They are used in synthesizing organic compounds and gas purification processes, such as converting carbon monoxide to carbon dioxide in emission control systems. Additionally, they find applications in fuel cells, enhancing the efficiency of electrochemical reactions for clean energy generation [235].

8.3 SENSORS

Due to their optical and electrical properties, AgNPs are widely used in sensors that detect ions and organic molecules with high sensitivity. These sensors are employed in the medical field for rapid diagnostics and environmental applications for pollutant monitoring. The interaction between light and the nanoparticles enhances optical signals, resulting in more precise and faster devices [236].

8.4 ELECTRONIC APPLICATIONS

In the field of electronics, AgNPs are used to manufacture printed circuits and touchscreen displays. Their excellent electrical conductivity and ability to be processed into thin films enable their application in flexible electronics and advanced memory devices. Additionally, AgNPs are being explored for the development of reusable masks with antimicrobial functions and real-time respiratory monitoring [225].

8.5 PHOTOTHERMAL THERAPY

In medicine, AgNPs are being studied for use in photothermal therapies for cancer treatment. This technique involves irradiating nanoparticles with near-infrared light, generating localized heat that destroys tumor cells without harming adjacent healthy tissues. This approach shows promise in targeted therapies, offering new opportunities for treating complex diseases [237].

8.6 FILTERS AND WATER PURIFICATION

AgNPs are incorporated into filters and water purification membranes due to their antimicrobial capacity, effectively eliminating pathogens such as bacteria and protozoa. They are widely used in water treatment systems and household filters, helping to improve water quality in various environmental contexts.

The diverse applications of silver nanoparticles (AgNPs) demonstrate their technological and scientific importance across various sectors. By combining unique chemical and physical properties, AgNPs represent a promising source of innovation in fields such as health, environment, and electronics [238].

Table 7 summarizes the applications presented, as well as process descriptions and practical applications.

Table 7. Applications of silver nanoparticles.

Application	Description	Practical Examples	Reference
Antimicrobial and Antifungal Properties	AgNPs release silver ions that interact with cell membranes, inhibiting the growth of bacteria, fungi, and viruses.	Wound dressings, antibacterial textiles, food packaging.	[234]
Catalyst	Due to their high surface area and reactivity, they are used to accelerate chemical reactions and gas purification.	Emission control, fuel cells, organic synthesis.	[235]
Sensors	AgNPs amplify optical and electrical signals, increasing the accuracy of sensors.	Medical sensors for rapid diagnostics and environmental monitoring of pollutants.	[236]
Electronic Applications	The conductive properties of AgNPs enable their use in electronic devices and printed circuits.	Touch screens, flexible electronics, masks with respiratory sensors.	[225]
Photothermal Therapy	They emit localized heat under infrared irradiation, destroying target cells, such as tumor cells.	Cancer therapies and treatments for other complex diseases.	[237]
Water Filters and Purification	They act as antimicrobial agents in purification systems, eliminating pathogens.	Household filters, water treatment systems, ultrafiltration membranes.	[238]

9. CONCLUSION

Research on propolis and AgNPs has shown substantial advancements, particularly in their antimicrobial properties, wound healing potential, and drug delivery applications. Progress in extraction methods has enabled more efficient isolation of bioactive compounds from propolis, while improved synthesis techniques have enhanced the specific properties of AgNPs for targeted uses. The combination of these materials presents an exciting opportunity for developing more effective and sustainable biomedical solutions.

Looking ahead, future research should focus on optimizing green extraction techniques to preserve the bioactivity of propolis while minimizing environmental impact. Additionally, the use of propolis as a natural stabilizer for AgNPs could enhance their biocompatibility, reduce toxicity, and improve their therapeutic effectiveness. Further efforts are needed to refine the scalability and reproducibility of nanoparticle synthesis, ensuring their reliable use in industrial applications. Investigating the potential of propolis-based AgNPs in areas such as medical device coatings, controlled drug release, and functional

biomaterials could significantly expand their utility in healthcare and beyond. Moreover, exploring the long-term stability and interactions of these nanoparticles in biological systems will be crucial for ensuring consistent, safe, and effective therapeutic outcomes.

These findings have the potential to influence industry practices by offering sustainable, biocompatible alternatives to traditional materials used in antimicrobial treatments and drug delivery. The combination of propolis and AgNPs could drive innovation across healthcare, agriculture, and materials science. Regulatory frameworks may need to adapt to support the use of natural, eco-friendly materials like propolis, which could foster the development of green nanotechnologies and encourage policies that prioritize biocompatible, non-toxic nanomaterials. As such, these advancements could lead to more sustainable practices in manufacturing, particularly within the pharmaceutical and biotechnology industries.

In conclusion, while the integration of propolis and AgNPs offers tremendous potential for improving medical treatments and industrial applications, continued interdisciplinary collaboration, optimization of synthesis methods, and regulatory support are essential to fully realizing their benefits. By focusing on green synthesis, environmental sustainability, and safety, propolis-based nanoparticle formulations could become foundational in future biomedical solutions, offering safer and more effective alternatives in medicine, biotechnology, and material science.

CrediT authorship contribution statement

Rosana Rabelo Mançano: Writing - original draft, Writing - review & editing. **Larissa Resende Matheus:** Writing - original draft, Writing - review & editing. **Luiz Eduardo Nochi Castro:** Writing - original draft, Writing - review & editing. **Tiago Linhares Cruz Tabosa Barroso**: Writing - review & editing. **Rafael Gabriel da Rosa:** Writing - review & editing. **Vanessa Cosme Ferreira:** Writing - review & editing. **Tânia Forster-Carneiro:** Conceptualization, Project administration, Supervision, Writing - review & editing. **Leda Maria Saragiotto Colpini:** Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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CHAPTER III

ENHANCED EXTRACTION OF BIOACTIVE COMPOUNDS FROM BROWN PROPOLIS: EMPLOYING PRESSURIZED LIQUID EXTRACTION IN SEMI-CONTINUOUS FLOW-THROUGH SYSTEM

ABSTRACT

This study examined the influence of ethanol concentration (0–80%), temperature (60–120 °C), and pH (2–12) on the extraction efficiency of bioactive compounds from brown propolis using pressurized liquid extraction (PLE) in a semi-continuous flow system. The findings demonstrated that the maximum total phenolic content (144.55 ± 8.73 mg GAE g^{-1}) was attained at 90 °C and pH 7, while the optimal flavonoid yield (56.01 ± 3.37 mg CE g^{-1}) was achieved at 120 °C. Furthermore, the highest sugar concentration (445.27 mg g^{-1}) was achieved at 90 °C and pH 2, whereas acetic acid production peaked at 62.77 ± 3.44 mg g^{-1} under 90 °C and pH 10. Higher temperatures and more basic pH conditions promoted the formation of Maillard reaction products, influencing the colorimetric properties of the extracts, with lower luminosity (L*) values and increased chroma (C*). The antioxidant activity, evaluated by DPPH and FRAP assays, reached 304.82 ± 0.40 and 385.37 ± 18.88 μmol TEAC g^{-1}, respectively. These findings suggest that PLE can be optimized to selectively recover different bioactive compounds while minimizing degradation. In conclusion, the extraction conditions at 90 °C and pH 7 were optimal for phenolic compound recovery, while higher temperatures (120 °C) and basic pH (12) maximized flavonoid extraction. The findings of this study demonstrate that PLE is a highly effective and sustainable method for extracting bioactive compounds from brown propolis, with significant potential applications in the pharmaceutical, food, and cosmetic industries.

Keywords: *Hydrolysis; Subcritical water; Organic acids; Antioxidant activity*.

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1 INTRODUCTION

Propolis, a resinous substance collected by bees from plant exudates, is subsequently combined with salivary secretions and wax. This material plays a fundamental role in protecting the hive, acting as a natural antimicrobial agent and a physical barrier against predators and pathogens. Beyond its biological function, propolis has been the focus of extensive research due to its complex chemical composition, which includes flavonoids, phenolic compounds, terpenoids, and other biomolecules with bioactive properties. These characteristics give propolis significant value for various applications, particularly in the pharmaceutical, cosmetic, and food industries [1–5].

Among the different types of propolis found worldwide, brown propolis stands out due to its rich chemical composition and widespread availability. Recent studies have highlighted the complex phytochemical profile of this variety, with a high concentration of antioxidant, antimicrobial, and anti-inflammatory compounds. The presence of these bioactive compounds gives brown propolis promising therapeutic properties, justifying the growing interest in its scientific and industrial exploration. However, for these bioactive compounds to be effectively utilized, it is essential to develop efficient extraction methods capable of preserving their properties and optimizing their use [5–10].

The extraction of bioactive compounds from propolis involves various techniques, each with its particularities. Conventional methods, such as maceration, percolation, and extraction with organic solvents, are widely used but present significant limitations. The main challenges associated with these methods include excessive use of chemical solvents, long extraction times, low selectivity, and the risk of thermal degradation of sensitive compounds. Additionally, conventional extraction often results in complex mixtures containing impurities that may compromise the effectiveness of the obtained extracts. These difficulties have driven the search for innovative strategies that not only enhance process efficiency but also reduce environmental impact [10–14].

In this context, advanced extraction techniques, such as pressurized liquid extraction (PLE), have been explored as promising alternatives. PLE utilizes solvents under controlled high-pressure and temperature conditions, promoting the selective

extraction of bioactive compounds while reducing the need for large volumes of organic solvents. Compared to traditional approaches, this method offers several advantages, including higher yield, shorter processing time, and reduced degradation of target compounds. The application of PLE in obtaining secondary metabolites from plant matrices has been extensively studied, demonstrating superior efficiency in extracting flavonoids and phenolic compounds compared to conventional methods [14–18].

In addition to PLE, another relevant technological innovation is the use of semi-continuous flow systems in extraction processes. Unlike static methods, where the solvent remains in contact with the solid matrix for a fixed period, continuous flow systems allow for the constant circulation of the solvent, promoting more efficient and homogeneous extraction. This approach enables more precise control of process variables such as temperature, pressure, and flow rate, which can result in higher selectivity and better utilization of the target compounds. Furthermore, semi-continuous flow systems facilitate industrial scalability, making the process more economically viable and environmentally sustainable [19,20].

The growing demand for natural and functional products reinforces the need to improve extraction techniques, particularly concerning bioactive compounds with therapeutic potential. Flavonoids, for example, are widely recognized for their antioxidant properties, acting in the neutralization of free radicals and protection against oxidative damage in human cells. Similarly, phenolic compounds have demonstrated significant antimicrobial activity, being used in food preservation and pharmaceutical formulations. The efficiency with which these compounds can be extracted from brown propolis directly impacts their application in final products, highlighting the importance of innovative methods to ensure their stability and bioavailability [21,22].

Another crucial aspect to consider in extracting bioactive compounds from brown propolis is the sustainability of the process, as traditional extraction methods often generate chemical waste and require significant amounts of energy and solvents. The adoption of more sustainable approaches, such as PLE combined with semi-continuous flow systems, represents a significant advancement in this regard. Reducing organic solvent consumption and optimizing process yield, promoted by these technologies, contribute to minimizing the environmental impact associated

with the production of plant extracts. Additionally, the development of more efficient processes can result in lower production costs, making propolis-derived products more accessible and competitive in the market [23–26].

In this context, the present study aims to investigate the optimized extraction of bioactive compounds from brown propolis using pressurized liquid extraction (PLE) in a semi-continuous flow system. The choice of this approach is based on the need to optimize process efficiency, ensuring a high yield of bioactive compounds without compromising their chemical integrity. Moreover, this study seeks to contribute to technological innovation in the field of secondary metabolite extraction, exploring strategies that can be applied on an industrial scale and meet sustainability requirements.

The research presented in this article not only expands knowledge about the bioactive compounds of brown propolis but also proposes a more efficient extraction model aligned with the needs of modern industry. The expected results include a detailed characterization of the obtained compounds, an evaluation of the efficiency of the proposed technique, and a comparative analysis with traditional methods. Thus, this study aims to contribute to the development of more sustainable extraction processes, providing insights for the industrial application of brown propolis extracts in different sectors.

Beyond the direct impacts on the pharmaceutical, food, and cosmetic industries, this work may also have significant implications for environmental science. The reduction in the use of organic solvents and the optimization of extraction yields align with the principles of green chemistry, promoting more responsible alternatives from an ecological perspective. By proposing an innovative method for obtaining bioactive compounds, this study reinforces the importance of integrating science, technology, and sustainability in the development of new natural products.

In conclusion, considering the growing importance of bioactive compounds in health promotion and disease prevention, optimizing extraction processes is a crucial factor in advancing this field of research. The combination of advanced techniques, such as PLE and semi-continuous flow systems, represents a promising strategy to optimize the use of natural resources, ensuring greater efficiency and sustainability. Therefore, this study not only deepens the understanding of brown propolis but also

opens new perspectives for its large-scale application, benefiting both industry and society.

2 MATERIALS AND METHODS

2.1 RAW MATERIAL

The raw propolis was obtained from a producer in the northern region of the state of Paraná, Brazil. The samples were subjected to a drying process at 35 °C for 24 hours in a forced ventilation oven (Lucadema, model LUCA-82/250, São Paulo, SP, Brazil). Thereafter, the samples were stored in a dark and dry environment until use.

2.2 SEMI-CONTINUOUS HIGH PRESSURE HYDROTHERMAL PROCESS

The extractive process used was semi-continuous hydrothermal at high pressure (**Figure 1).** The reactor used for hydrolysis has a capacity of 110 mL and was equipped with a water pump capable of providing high pressure. Initially the water was heated in a preheated preheated followed by a heat exchanger and later inserted into the reactor. During the hydrolysis process, pressure and temperature were monitored with pressure gauges, which had a measurement range of 0 to 7,500 psi and an accuracy of 0.1%, and K-type thermocouples.

The parameters used were determined by previous studies [27,28]. The hydrothermal process was carried out at a pressure of 200 bar, at a flow rate of 5 mL/min during a period of 30 minutes,6 where aliquots were collected every 5 minutes. The ratio between the solvent and the feed (S/F) was 60 g of water per gram of propolis. The propolis hydrolysis process was carried out by means of a central composite design of three factors containing three levels: high (+1), medium (0) and low (−1). The variables studied were ethanol concentration (0 to 80%), temperature (60 to 120 ºC) and pH (2 to 12). **Table 1** presents the sixteen (19) treatments performed.

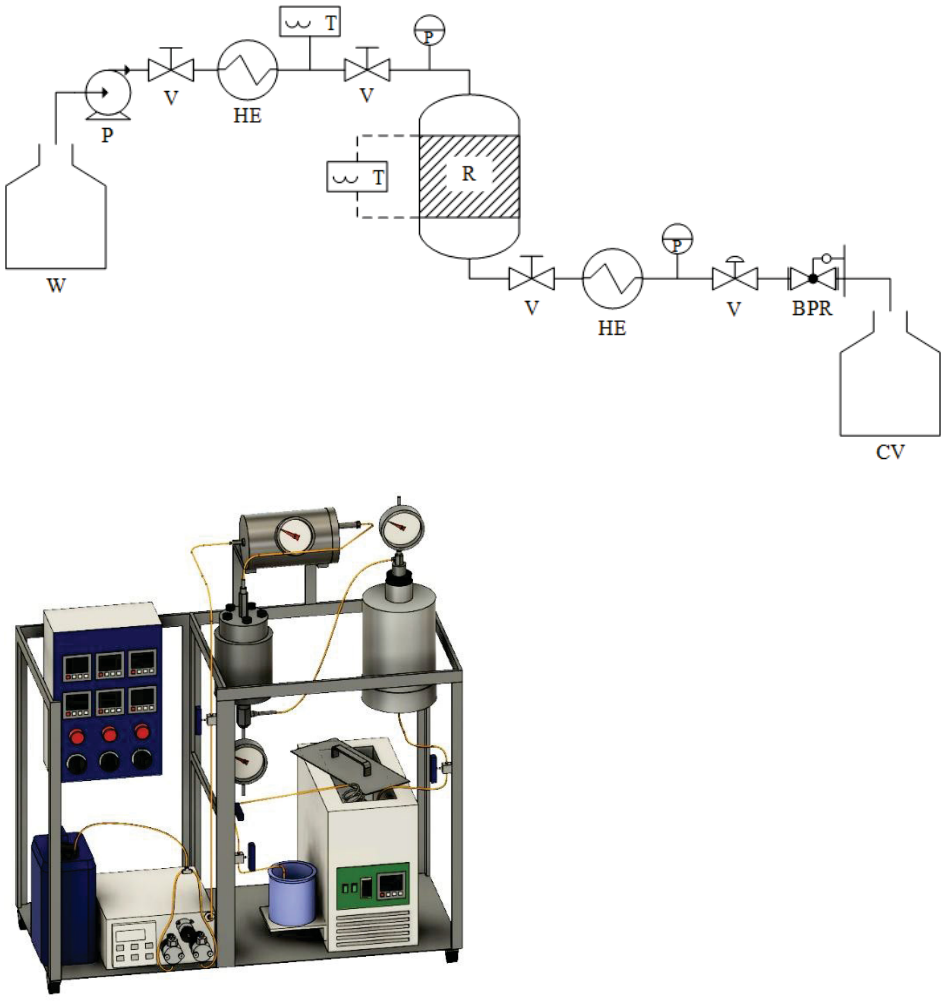

Figure 1. Schematic diagram of the experimental apparatus for the semi-continuous subcritical water hydrolysis of raw propolis. Reproduced from Barroso et al. [29], with permission from Elsevier.
*Label: W, water tank; P, high-pressure pump; V, block valves; P, manometer; T, thermocouples; R, subcritical water hydrolysis reactor; HE, heat exchanger; MV, micrometric valve; CV, collecting vessel.

TABLE 1. EXPERIMENTAL CONDITIONS FOR PRESSURIZED LIQUID EXTRACTION OF BROWN PROPOLIS.

Treatments	Codified variables			Non-codified variables		
	X_1	X_2	X_3	EtOH (%)	Temperature (ºC)	Ph
PLE - 1	-1	-1	-1	0	60	2
PLE – 2	-1	-1	+1	0	60	12
PLE – 3	-1	+1	-1	0	120	2
PLE – 4	-1	+1	+1	0	120	12
PLE – 5	+1	-1	-1	80	60	2
PLE – 6	+1	-1	+1	80	60	12

PLE – 7	+1	+1	-1	80	120	2
PLE – 8	+1	+1	+1	80	120	12
PLE – 9	-1	0	0	0	90	7
PLE – 10	+1	0	0	80	90	7
PLE – 11	0	-1	0	40	60	7
PLE – 12	0	+1	0	40	120	7
PLE – 13	0	0	-1	40	90	2
PLE – 14	0	0	+1	40	90	12
PLE – 15	0	0	0	40	90	7
PLE – 16	0	0	0	40	90	7

2.3 CHARACTERIZATION OF EXTRACTS AND HYDROLYSATES

2.3.1 Color parameters

The colorimetric parameters of the CIELab system (L* (luminosity), a* (red/green) and b* (blue/yellow)) were obtained through transmittance values in the range of 340 nm to 830 nm with the readings being performed every 5 nm in UV-Vis spectrophotomrto (Model UV-M51, Bell Photonics). The parameter Hº (hue angle) was calculated using the following formula:

$$h = tan^{-1}\left(\frac{b^*}{a^*}\right)$$

2.3.2 Total phenolic compounds

The total phenolic content was quantified using an adapted Folin-Ciocalteu method [30]. In the procedure, 60 µL of the sample was mixed with 300 µL of Folin-Ciocalteu reagent and 3 mL of distilled water. The resulting mixture was stirred, then allowed to sit in the dark for 3 minutes. Afterward, 0.9 mL of a 15% sodium carbonate solution and 1.74 mL of distilled water were added. The mixture was allowed to react for 2 hours, after which the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Model UV-M51, Bell Photonics). The phenolic content was determined by comparing the absorbance to a gallic acid standard curve, with concentrations ranging from 0 to 1000 µM. The final results were expressed in terms of milligrams of gallic acid per gram of propolis extract.

2.3.3 Total flavonoids compounds

The total flavonoid content of the hydrolysates will be analyzed following the methodology outlined by Ozsoy et al. [31] with certain modifications. The absorbance will be measured at 510 nm using a UV–vis spectrophotometer. The total flavonoid content of the samples will be determined using rutin (RE) as a standard, and the analysis results will be expressed as milligrams of rutin equivalents per gram of the dried sample (mg RE g^{-1}).

2.3.4 Antioxidant activity by DPPH and FRAP assays

The evaluation of antioxidant activity was performed in vitro by the DPPH and FRAP methods. To identify DPPH free radical inhibition, the method proposed by Brand-Williams was used [32]. 150 µL of the samples were mixed with 5850 µL of a DPPH solution (0.06 mmol/L). After 30 minutes, the absorbance was recorded at 515 nm in a spectrophotometer (Hach, model DR 4000U, São Paulo, SP, Brazil). The standard used was the Trolox, with the standard curve expressed by the equation y = 0.0006x + 0.5776, with R^2 = 0.9837. The results were expressed in µg of Trolox equivalent antioxidant capacity (TEAC) per gram of propolis (µg/g). The antioxidant activity was investigated through the ferric-reducing antioxidant power (FRAP) assay, according to the procedures of Benzie and Strain [33], with some adaptations. The reaction involved 100 µL of the sample, 100 µL of ferric chloride (3 mmol/L) and 1800 µL of the TPTZ solution (2,4,6-Tris(2-pyridyl)-1,3,5-triazine). The samples were incubated at 37 °C for 30 minutes. Absorbance measurement was performed at 620 nm using a spectrophotometer (Hach, model DR 4000U, São Paulo, SP, Brazil). The Trolox pattern was used, with the standard curve represented by the equation y = 0.0015x + 0.0171, with R^2 = 0.9805. The results were expressed as µg of Trolox-equivalent antioxidant capacity (TEAC) per gram of propolis (µg/g).

2.3.5 Maillard reaction products

The potential formation of Maillard reaction products during the extraction and hydrolysis processes was investigated by measuring the absorbance at 294 nm, corresponding to the intermediate state, and at 420 nm, which represents the final stage of the products of this reaction.

2.3.6 Sugar and Organic acids

The analysis of sugars and organic acids was performed by means of high-performance liquid chromatography (HPLC), using a refractive index detector (RID). The separation of the compounds was done using a Rezex column (Phenomenex, model ROA-Organic Acid H+ (8%), 8 µm, 300×7.8 mm, Torrance, CA, USA), with an isotropic flow of H_2SO_4 (5 mmol/L) at 60 °C, configured for 0.6 mL/min. The RID detector was set to a temperature of 40 °C. Prior to the analysis, the extracts and hydrolysates were subjected to centrifugation at 10,000×g for 15 minutes and filtered with a 0.22 µm nylon filter. After this procedure, 10 µL of the sample was injected into the system and the analysis lasted 50 minutes. The concentrations of arabinose, cellobiose, xylose and citric acid were computed based on calibration curves for each corresponding standard. Results were reported as mg g^{-1} of dry propolis.

2.3.7 Soluble proteins

The soluble protein content was quantified according to the method proposed by Bradford [34]. 100 µL of the dilute protein sample, 1 mL of the Coomassie Brilliant Blue G-250 solution, dissolved in phosphoric acid, and finally 900 µL of deionized water were added. Then, the mixture was homogenized and allowed to rest for 5 minutes at room temperature to allow the dye to bind to the proteins. After the incubation period, the absorbance of the solution was measured at 595 nm using a spectrophotometer. Protein concentration was determined by comparing absorbance with a standard curve obtained from BSA (bovine serum albumin) solutions at known concentrations, ranging from 0 to 1000 µg/mL.

2.4 STATISTICAL ANALYSIS

The results obtained will undergo analysis of variance (ANOVA) to evaluate the statistically significant factors and interaction effects among variables. Significant differences will be determined using Tukey's test ($p \leq 0.05$), and the results will be obtained in triplicate for all assays.

3 RESULTS AND DISCUSSION

3.1 VISUAL APPEARANCE AND COLOR PARAMETERS

The visual appearance of hydrolysates 1 and 14 is shown in **Figure 2(a)**. In general, it can be seen that all treatments produced a hydrolysate with a brownish color (**Figure 2(b)**), indicating the possible presence of phenolic compounds and flavonoids. As the hydrolysis time progressed, it can be seen that in the samples with a more basic pH, the color of the hydrolysates changed to a darker brown. The brown color developed in the hydrolysates can be related to the products of the Maillard reaction, due to the caramelization of sugars and the degradation of amino acids.

The colorimetric parameters (**Table 2**) confirm these visual observations. The luminosity (L*) values ranged from 70.49 ± 3.34 to 90.68 ± 4.20, with lower values associated with darker hydrolysates, especially at higher temperatures and basic pH conditions. The a* values, representing the red-green spectrum, varied between -4.43 ± 0.54 and 10.26 ± 3.37, with higher values indicating a shift towards a more reddish hue, which was particularly noticeable in samples subjected to extreme extraction conditions. Similarly, the b* values, corresponding to the yellow-blue spectrum, ranged from 29.43 ± 1.40 to 61.36 ± 7.15, reinforcing the dominance of brownish tones in all samples.

The chroma (C*) values followed a similar trend, with the highest intensity (62.80 ± 9.31) observed under more severe extraction conditions, suggesting an increased presence of pigments and possibly degradation products. The hue angle (Hº) varied from -3.06 ± 0.12 to 1.87 ± 0.11, indicating subtle shifts in coloration that may be attributed to differences in chemical composition among hydrolysates.

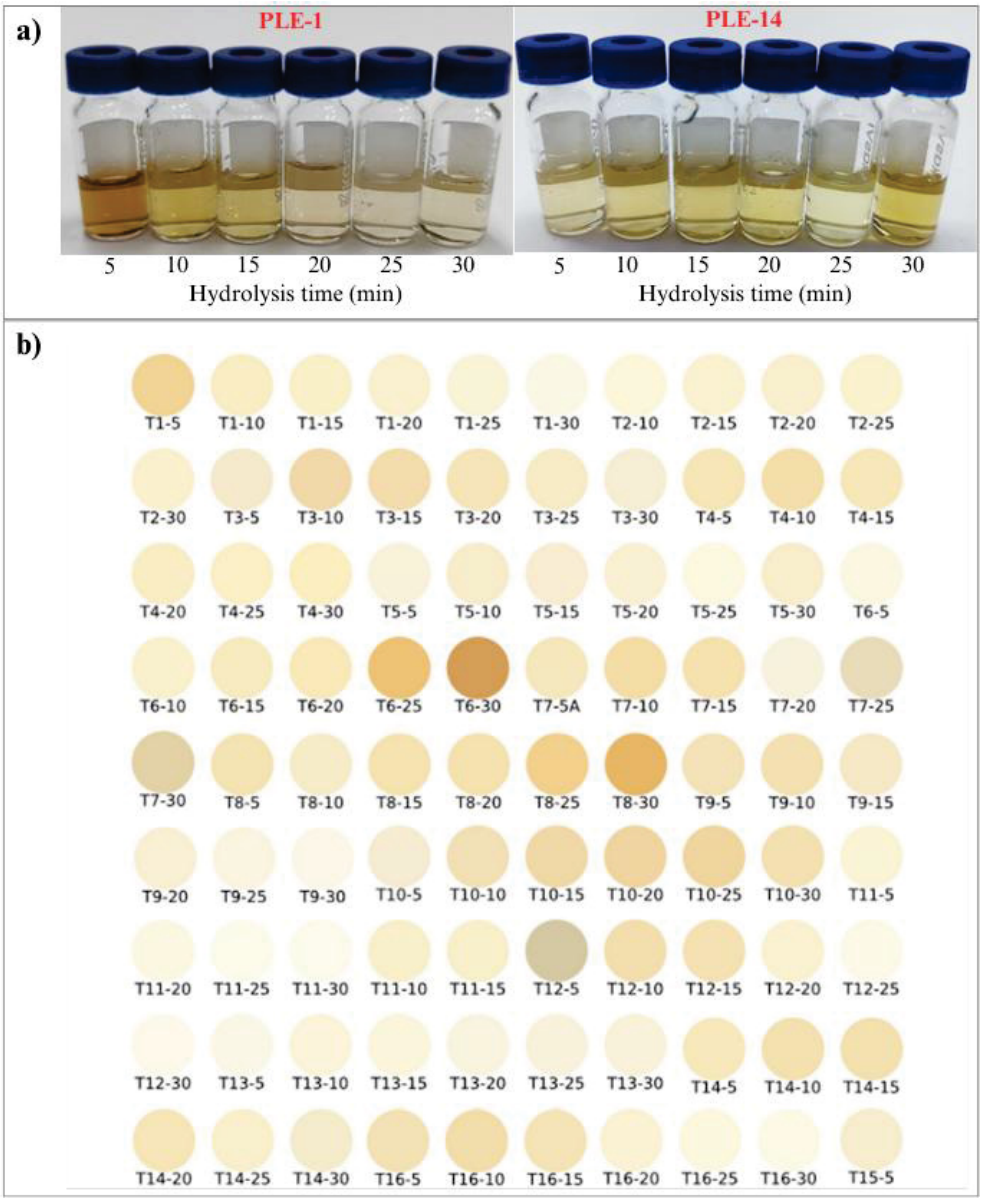

Figure 2. Hydrolysates obtained after pressurized liquid extraction of brown propolis: a) Visual appearance of treatments 1, and 14; b) Color kinetic profile of the brown propolis extracts.

These findings highlight the influence of processing conditions on the visual and colorimetric properties of the extracts, reinforcing the importance of optimizing hydrolysis parameters to control both the aesthetic and compositional attributes of the final product.

Table 2. CIELAB color parameters of the accumulated hydrolysates obtained from pressurized liquid extraction of brown propolis at a solvent-to-feed ratio of 60 g solvent g^{-1} propolis.

Parameter	PLE 1	PLE 2	PLE 3	PLE 4	PLE 5	PLE 6	PLE 7	PLE 8	PLE 9	PLE 10	PLE 11	PLE 12	PLE 13	PLE 14	PLE 15	PLE 16
L*	83.7 ± 6.01	87.90 ± 1.07	77.85 ± 4.85	81.04 ± 3.82	86.83 ± 2.72	72.05 ± 1.49	74.45 ± 1.74	70.49 ± 3.34	83.54 ± 6.24	72.31 ± 1.76	90.68 ± 4.20	79.92 ± 6.10	89.93 ± 4.20	79.55 ± 3.80	83.73 ± 4.35	83.33 ± 4.15
a*	-0.56 ± 0.06	-2.93 ± 0.66	4.99 ± 0.74	0.85 ± 0.06	-0.56 ± 0.03	8.67 ± 0.54	3.66 ± 1.22	10.26 ± 3.37	1.52 ± 0.30	7.75 ± 1.11	-4.43 ± 0.54	0.85 ± 0.14	-1.92 ± 0.23	1.57 ± 0.13	1.05 ± 0.05	-0.27 ± 0.55
b*	46.01 ± 3.23	43.22 ± 2.75	47.92 ± 1.87	56.37 ± 3.50	37.57 ± 1.47	53.4 ± 2.30	49.51 ± 3.12	61.36 ± 7.15	40.22 ± 2.09	54.33 ± 3.53	34.59 ± 2.80	40.35 ± 2.57	29.43 ± 1.40	52.88 ± 5.65	40.28 ± 2.17	45.45 ± 1.08
C*	46.39 ± 4.68	43.33 ± 2.73	48.31 ± 4.17	56.47 ± 3.57	37.61 ± 1.83	55.73 ± 5.79	49.73 ± 4.20	62.80 ± 9.31	40.33 ± 1.19	54.96 ± 7.81	34.92 ± 2.41	40.56 ± 3.96	29.52 ± 1.83	53.01 ± 1.76	40.38 ± 7.85	45.73 ± 2.11
Hº	0.03 ± 0.01	1.87 ± 0.11	-2.31 ± 0.25	1.52 ± 0.03	-0.34 ± 0.02	-2.24 ± 0.19	1.74 ± 0.16	0.64 ± 0.09	-3.06 ± 0.12	-0.65 ± 0.04	-0.15 ± 0.05	1.26 ± 0.18	-2.61 ± 0.21	-0.79 ± 0.10	-2.77 ± 0.26	-0.40 ± 0.05

The results are expressed as mean ± standard deviation. Analysis conducted in triplicate.

3.2 TOTAL PHENOLIC COMPOUNDS

The results obtained for the total phenyl compounds in the propolis extracts are shown in **Figure 3** and **Table 3**. The values ranged from 58.71 ± 1.74 mg GAE g^{-1} to 144.55 ± 8.73 mg GAE g^{-1}, with the lowest result recorded for the PLE 7 treatment (80% etOH - 120 ºC - pH 2) and the highest result for the PLE 9 treatment (0% etOH - 90 ºC - pH 7). These results are consistent with those documented in the extant literature, wherein a study employing 80% ethanolic extraction of brown propolis (1:25 m/v) yielded phenolic contents ranging from 48.5 to 238.9 mg GAE g^{-1} in samples from diverse regions of South Korea. In addition, the study reported concentrations of phenolic compounds ranging from 126.8 ± 4.12 mg GAE g^{-1} in Brazilian propolis to 142.4 ± 3.61 mg GAE g^{-1} in Australian propolis, with Chinese propolis exhibiting a concentration of 132.1 ± 3.28 mg GAE g^{-1} [35]. Another study developed solutions of Lithuanian propolis with different solvents and concentrations, and the results ranged from 85.4 ± 1.65 mg GAE g^{-1} to 175.6 ± 1.89 mg GAE g^{-1} [36].

Figure 3. Kinetic profile of the accumulated bioactive compounds obtaining during the pressurized liquid extraction of brown propolis: a) total phenolic compounds; b) total flavonoids content; c) DPPH; d) FRAP; and e) soluble proteins.

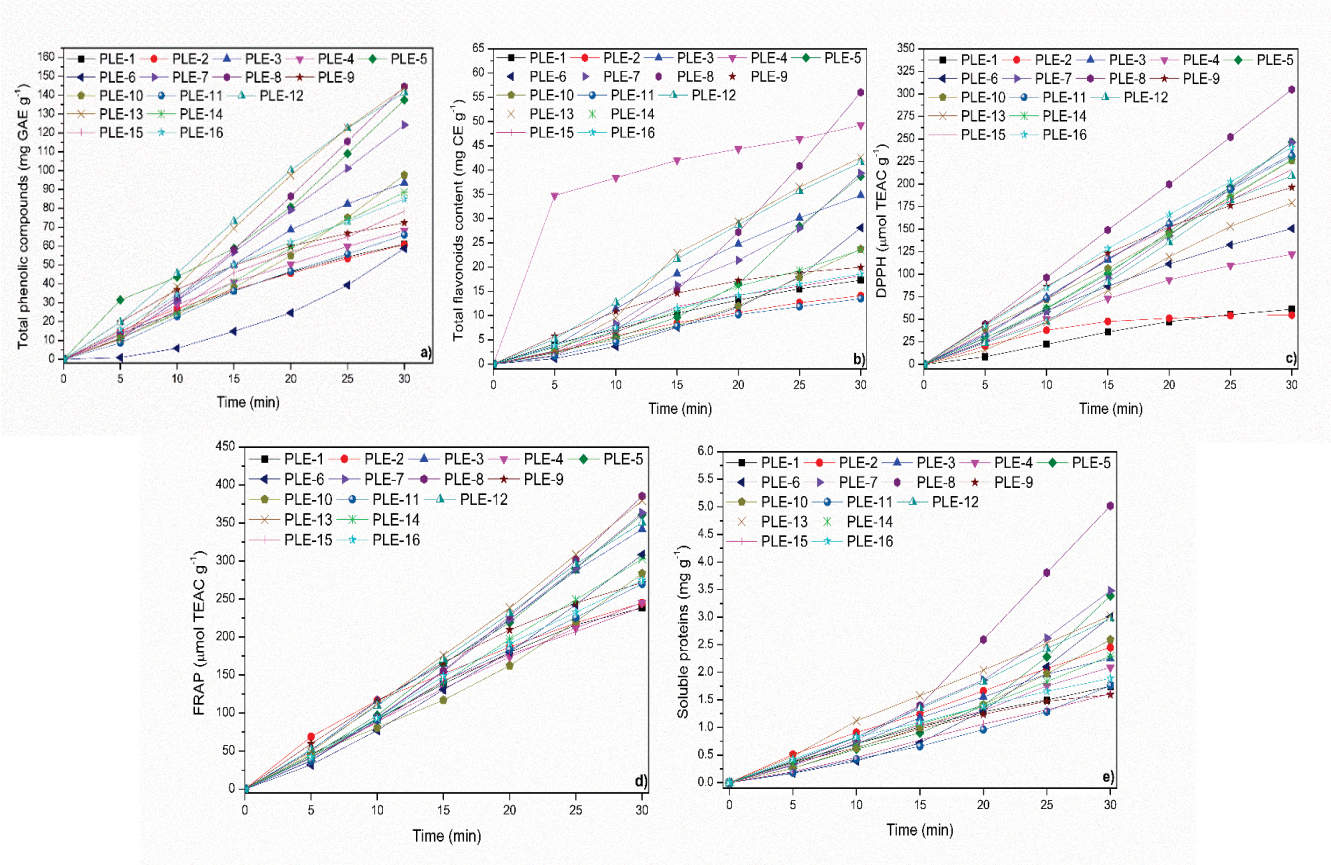

Table 3. Bioactive compounds composition of the accumulated hydrolysates obtained from pressurized liquid extraction of brown propolis at a solvent-to-feed ratio of 60 g solvent g^{-1} propolis.

Parameter*	PLE 1	PLE 2	PLE 3	PLE 4	PLE 5	PLE 6	PLE 7	PLE 8	PLE 9	PLE 10	PLE 11	PLE 12	PLE 13	PLE 14	PLE 15	PLE 16
TPC (mg GAE g^{-1})	60.95 ± 0.82	60.75 ± 1.07	93.51 ± 2.44	68.25 ± 1.73	68.56 ± 0.82	137.45 ± 14.49	58.71 ± 1.74	124.19 ± 3.34	144.55 ± 8.73	72.35 ± 1.76	65.90 ± 4.20	141.46 ± 6.15	143.94 ± 4.27	88.55 ± 2.26	78.54 ± 4.35	84.94 ± 4.15
TFC (mg CE g^{-1})	17.28 ± 0.69	14.08 ± 0.70	31.17 ± 1.53	18.08 ± 1.66	38.70 ± 1.33	28.09 ± 0.54	39.35 ± 1.22	56.01 ± 3.37	19.94 ± 0.30	23.70 ± 1.11	13.47 ± 0.54	41.56 ± 1.04	45.59 ± 1.03	23.53 ± 1.03	18.20 ± 1.05	18.55 ± 0.92
DPPH (µmol TEAC g^{-1})	61.07 ± 3.23	54.66 ± 3.16	232.82 ± 4.87	122.10 ± 2.10	246.34 ± 1.47	150.61 ± 2.30	246.25 ± 3.12	179.15 ± 2.15	196.23 ± 2.09	226.36 ± 1.93	231.10 ± 2.80	209.03 ± 2.97	304.82 ± 0.40	227.0 ± 2.62	216.2 ± 2.17	240.6 ± 1.58
FRAP (µmol TEAC g^{-1})	238.4 ± 24.66	244.81 ± 21.03	341.82 ± 24.17	245.45 ± 11.53	360.69 ± 21.83	308.25 ± 13.79	363.32 ± 4.20	385.37 ± 18.88	271.76 ± 11.19	283.69 ± 19.81	269.96 ± 2.41	350.41 ± 3.96	379.60 ± 12.54	302.9 ± 13.76	239.72 ± 14.85	274.7 ± 2.25
SP (mg g^{-1})	1.74 ± 0.25	2.45 ± 0.21	2.25 ± 0.45	2.09 ± 0.33	3.39 ± 0.32	3.00 ± 0.19	3.48 ± 0.16	5.01 ± 0.39	1.59 ± 0.12	2.59 ± 0.14	1.75 ± 0.25	2.97 ± 0.18	3.02 ± 0.21	2.29 ± 0.30	1.61 ± 0.26	1.89 ± 0.15
MRP (nm) A_{294}	1.11 ± 0.10	0.99 ± 0.08	0.87 ± 0.11	0.99 ± 0.18	1.20 ± 0.05	1.12 ± 0.03	0.97 ± 0.07	0.98 ± 0.13	1.07 ± 0.09	1.02 ± 0.14	1.09 ± 0.18	1.04 ± 0.05	0.79 ± 0.31	0.93 ± 0.09	0.91 ± 0.07	0.96 ± 0.11
MRP (nm) A_{420}	0.17 ± 0.03	0.15 ± 0.01	0.13 ± 0.01	0.16 ± 0.03	0.14 ± 0.04	0.15 ± 0.02	0.20 ± 0.03	0.25 ± 0.08	0.23 ± 0.05	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.01	0.17 ± 0.03	0.16 ± 0.02	0.16 ± 0.05	0.10 ± 0.02

*TPC = total phenolic compounds; TFC = total flavonoid compounds; DPPH = 2,2-difenil-1-picrilhidrazil radical scavenging; FRAP = ferric reducing antioxidant power; SP = soluble proteins; MRP = Maillard reaction products. The results are expressed as mean ± standard deviation. Analysis conducted in triplicate.

The results of the present study may not be entirely consistent with those reported in the extant literature. Such discrepancies can be attributed to the influence of various factors, including extraction methods, temperature, and the choice of solvent. It is imperative to consider the geographical origin of the raw propolis samples, as numerous studies have indicated that this factor can also influence the composition of propolis [37].

An evaluation of the statistical modeling and optimization for total phenolic compounds reveals that all factors (EtOH%, temperature, and pH) have a statistically significant effect, as illustrated in **Figure 4(a)** (Pareto chart). **Figure 5(a)** shows that when evaluating the combined effects of ethanol concentration and pH, the amount of total phenolic compounds (TPC) produced is significantly higher. Specifically, a decrease in pH accompanied by an increase in EtOH concentration results in a higher amount of TPC. Conversely, the effect of EtOH concentration and temperature demonstrates a divergent pattern; it was observed that a combination of a higher temperature together with an increase in EtOH%, specifically 120 °C and 80% EtOH, produced the highest concentration of TPC. Finally, the effect of temperature and pH on TPC recovery exhibited a pattern analogous to that observed with EtOH concentration and pH: a decrease in pH and an increase in temperature resulted in a greater amount of TPC recovered.

In consideration of the level of significance as determined by the analysis of variance (ANOVA) test, at a 5% level (see **Table 4**), the factors of ethanol percentage, temperature, and pH demonstrate a p-value less than 0.05, thereby indicating that there is no significant difference between them. The generation of response surface graphs (**Figure 6(a)**) further substantiates this finding, demonstrating the viability of recovering a quantity of TPC > 125 mg GAE g-1 at temperatures close to 100°C and concentrations above 20% EtOH, along with an acidic pH ≤ 2.

Derivation of the equation (**Eq. 1**) and the coefficients of determination for the regression model adjusted for the variables in the second-order functions was also possible. This estimation of TPC was found to be satisfactory, with a model showing a good fit, reaching an R^2 value above 0.6. This indicates that the model can predict the amount of TPC that can be recovered in the PLE process of brown propolis.

$$TPC\ (mg\ GAE\ g^{-1}) = 122.2 - 1.21 \times A - 0.54 \times B - 15.88 \times C - 0.01 \times A^2 + 0.003 \times B^2 + 0.58 \times C^2 + 0.007 \times A \times B - 0.02 \times A \times C + 0.06 \times B \times C$$

$$\left(S = 19.73;\ R^2 = 0.733;\ R^2_{adj} = 0.623\right) \qquad \textbf{(Eq.1)}$$

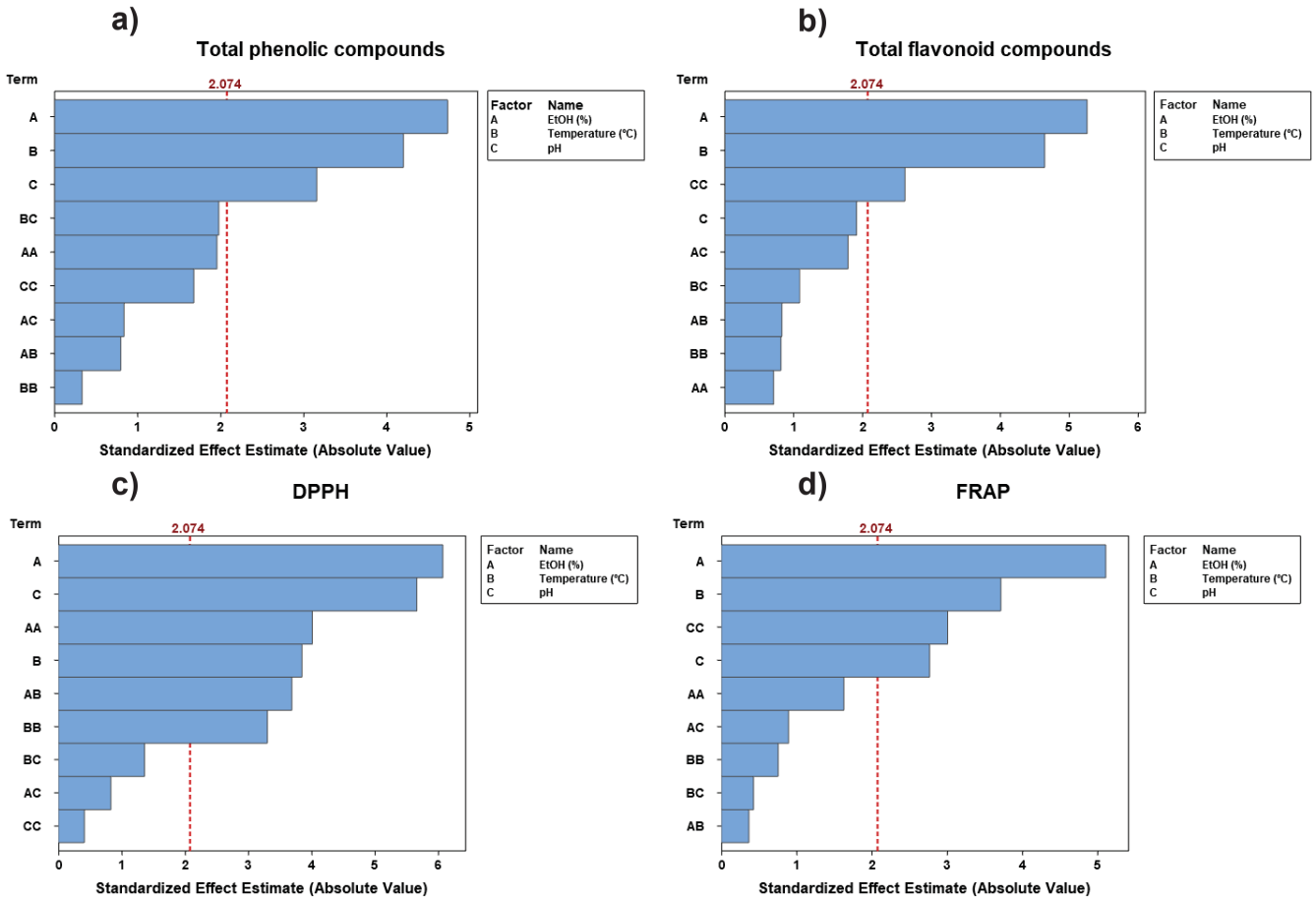

Figure 4. Pareto chart for the RSM design response variables: a) Total phenolic compounds; b) Total flavonoid compounds; c) DPPH; and d) FRAP.

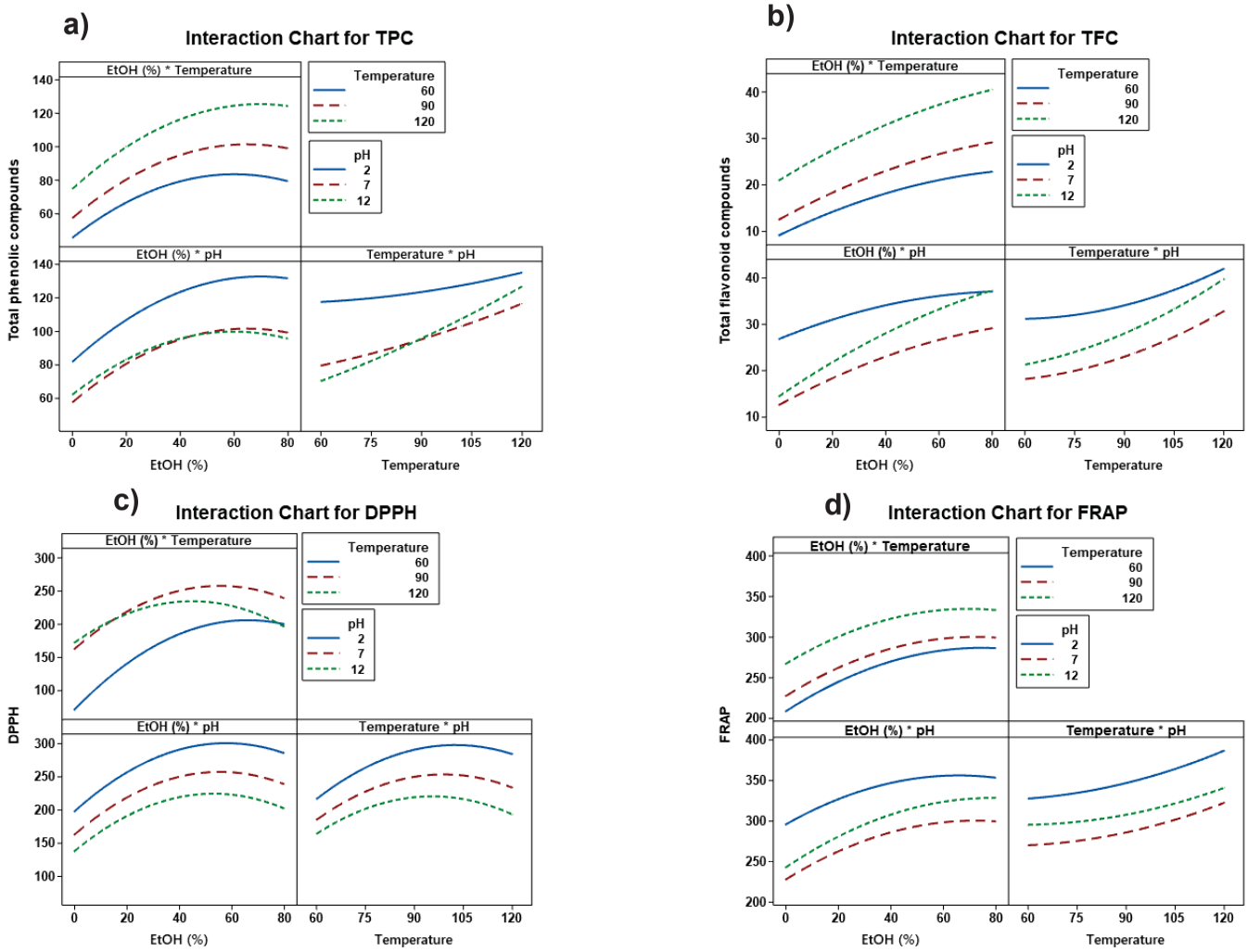

Figure 5. Interaction chart for the RSM design response variables: a) Total phenolic compounds; b) Total flavonoid compounds; c) DPPG; and d) FRAP.

Table 4. Analysis of variance (ANOVA) for various responses (TPC, TFC, DPPH, and FRAP). *TPC = total phenolic compounds;

Source	*Df*	TPC (mg GAE g^{-1})				TFC (mg CE g^{-1})				DPPH (µmol TEAC g^{-1})				FRAP (µmol TEAC g^{-1})			
		SS	MS	f-value	*p*-value	SS	MS	*f*-value	*p*-value	SS	MS	*f*-value	*p*-value	SS	MS	*f*-value	*p*-value
A	1	8,721.80	8,721.84	22.39	< 0.001	1,387.62	1,387.62	27.65	< 0.001	29,694.00	29,693.50	36.81	< 0.001	26,214.30	26,214.30	26.04	< 0.001
B	1	6,862.70	6,862.70	17.62	< 0.001	1,082.19	1,082.19	21.57	< 0.001	11,915.00	11,914.50	14.77	0.001	13,855.10	13,855.10	13.76	0.001
C	1	3,875.20	3,875.18	9.95	0.004	183.49	183.49	3.66	0.069	25,810.00	25,810.00	31.99	< 0.001	7,693.70	7,693.70	7.64	0.011
A^2	1	1,488.20	1,488.23	3.82	0.063	25.04	25.04	0.50	0.487	12,960.00	12,959.70	16.06	0.001	2,671.50	2,671.50	2.65	0.118
B^2	1	42.60	42.63	0.11	0.744	33.31	33.31	0.66	0.424	8,780.00	8,780.20	10.88	0.003	576.10	576.10	0.57	0.457
C^2	1	1,095.00	1,094.96	2.81	0.108	342.80	342.80	6.83	0.016	135.00	135.20	0.17	0.686	9,078.80	9,078.80	9.02	0.007
A×B	1	247.80	247.82	0.64	0.434	34.20	34.20	0.68	0.418	10,945.00	10,944.80	13.57	0.001	135.30	135.30	0.13	0.717
A×C	1	271.40	271.40	0.70	0.413	160.52	160.52	3.20	0.087	557.00	557.10	0.69	0.415	801.40	801.40	0.80	0.382
B×C	1	1,521.20	1,521.18	3.91	0.061	59.20	59.20	1.18	0.289	1,489.00	1,489.40	1.85	0.188	185.60	185.60	0.18	0.672
Error	22	8,568.70	389.49	-	-	1,104.01	50.18	-	-	17,748.00	806.70	-	-	22,145.20	1,006.60	-	-
Lack of fit	5	8,525.50	1,705.09	670.40	0.000	1,080.89	216.18	158.94	0.000	17,132.00	3,426.30	94.50	0.000	20,794.30	4,158.90	52.34	0.000
Pure error	17	43.20	2.54	-	-	23.12	1.36	-	-	616.00	36.30	-	-	1,350.90	79.50	-	-
Total	31	32,061.50	-	-	-	4,562.37	-	-	-	140,231.00	-	-	-	83,414.80	-	-	-

TFC = total flavonoid compounds; DPPH = 2,2-difenil-1-picrilhidrazil radical scavenging; FRAP = ferric reducing antioxidant power; A – EtOH (%), B – Temperature (ºC), C - pH; *df* = degree of freedom; SS = sum of squares; MS = mean square.

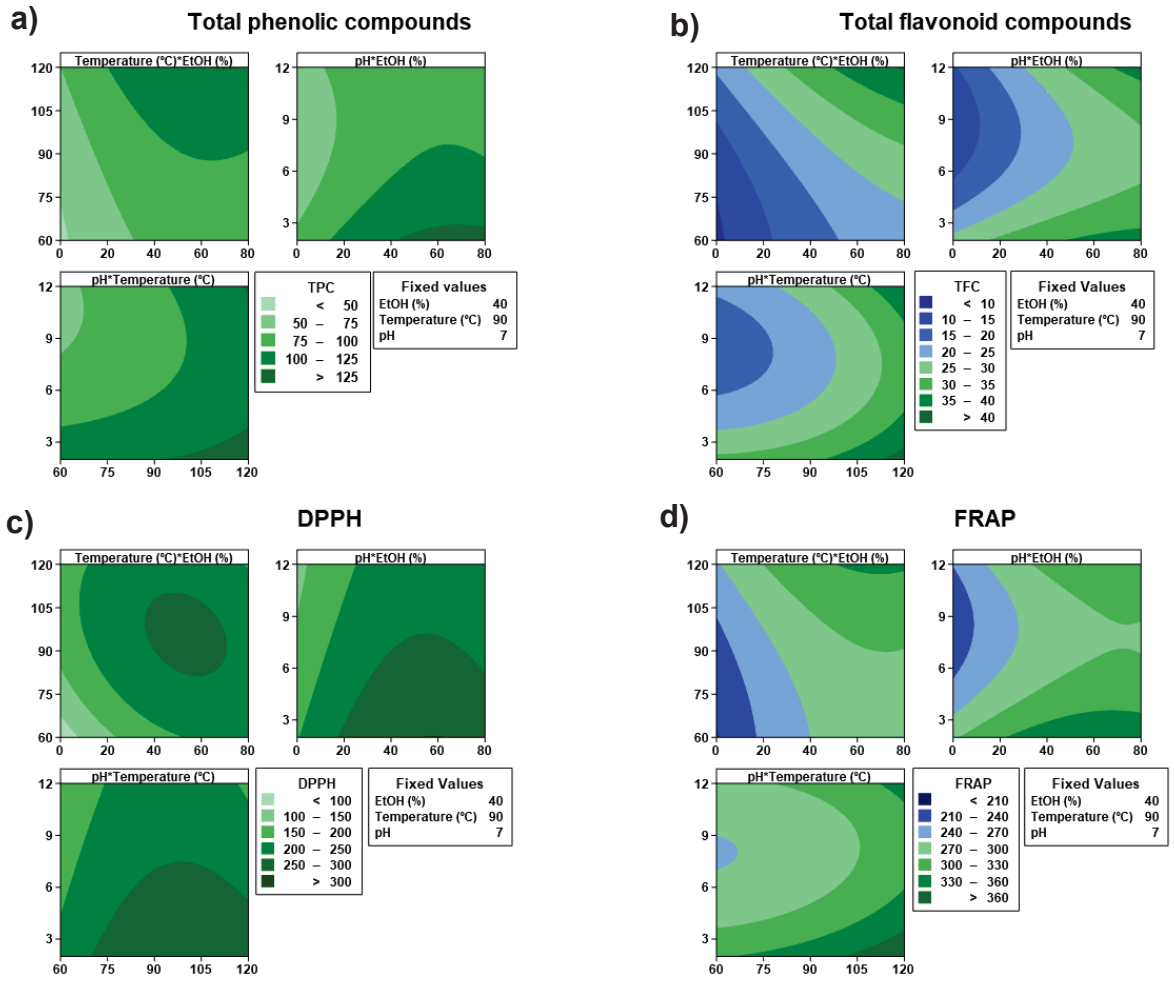

Figure 6. Response surface plots of the statistical analysis: a) 2D – level curve for total phenolic compounds; b) 2D – level curve for total flavonoid compounds; c) 2D – level curve for DPPH; and d) 2D – level curve for FRAP.

3.3 TOTAL FLAVONOIDS COMPOUNDS

Flavonoids are organic chemical compounds that contain polyphenols in their structure and are obtained from foods such as wine and honey. These natural compounds belong to the class of secondary metabolites and have therapeutic effects on health, acting as antioxidants, anti-inflammatory agents, antimutagenic agents, and demonstrating anticancer activity [38]. Due to their versatility, flavonoids are of great importance in the pharmaceutical, nutraceutical, medicinal, and cosmetic sectors [39].

The results obtained for total flavonoids compounds in brown propolis extracts are presented in **Table 3**. The values ranged from 13.47 ± 0.54 mg CE g^{-1} to 56.01 ±

3.37 mg CE g^{-1}, with the lowest result recorded for treatment PLE 11 (40% etOH - 60 ºC - pH 7) and the highest result for treatment PLE 8 (80% etOH - 120 ºC - pH 12). These results are similar to those reported in the literature, where a quantity of 19.34 mg CE g^{-1} was obtained from Anatolian propolis using 70% ethanolic extraction [40]. Can et al. [41], In another study using brown propolis from Romania, 95% ethanolic extraction (1:25 m/v) yielded phenolic contents ranging from 53.72 to 97.65 mg CE g^{-1} in samples from different regions. Ultrasound extraction technology was also employed to extract compounds from brown propolis, using an 80% ethanolic solution, acetone, and ethyl acetate for 7 hours. The authors obtained 220.19 ± 0.26 mg CE g^{-1} from the ethanolic extract, 269.74 ± 0.55 mg CE g^{-1} from the ethyl acetate extract, and 124.67 ± 0.54 mg CE g^{-1} from the acetone extract [42].

The results obtained in this experiment varied from those found in the literature. It is worth noting that these differences may be influenced by the extraction method and solvent used. Additionally, variations in geographic origins and the type of vegetation where the propolis was produced can be major contributing factors. Several studies suggest that the composition of propolis varies according to the available flora [37]. Therefore, the observed difference was expected, as the chemical composition of propolis undergoes significant changes depending on geographical location.

When evaluating the statistical modeling and optimization for total flavonoid compounds, it can be seen in **Figure 4 (b),** which shows the Pareto chart, that all factors (EtOH%, temperature, pH) have a significant statistical effect. **Figure 5 (b)** illustrates that when assessing the combined effects of EtOH concentration and temperature, the amount of total flavonoid compounds (TFC) produced is significantly higher. Specifically, a temperature of 120 °C combined with an EtOH concentration of 80% allows for the recovery of the highest concentration of TFC. In contrast, when evaluating the effect of EtOH concentration and pH, the behavior differs; lowering the pH while increasing the EtOH concentration results in a greater amount of TFC. Finally, regarding the effect of temperature and pH, the same pattern is observed as with EtOH concentration and pH: by decreasing the pH and increasing the temperature, a greater amount of TFC can be recovered.

Regarding the significance level by the ANOVA test, at a level of 5% **(Table 4)** the EtOH % and temperature factors present a value of $p < 0.05$, demonstrating that

there is no significant difference between the EtOH concentration and temperature, but when it comes to the pH factor the value of $p > 0.05$ and therefore it has a significant difference. Response surface graphs were generated **(Figure 6 (b))**, it is possible to visualize that it is possible to recover an amount of TFC > 40 mg CE g^{-1}, at temperatures close to 120ºC and concentrations above 60% of EtOH, and with acidic pH < 2 and pH > 10.

It was also possible to derive the equation **(Eq. 2)** and the coefficients of determination for the regression model adjusted for the variables in the second-order functions to estimate the TFC. The model demonstrates a good fit, achieving an R^2 value above 0.6, indicating that it satisfactorily predicts the amount of TFC that can be recovered in the PLE process of brown propolis.

$$\text{TFC (mg CE g}^{-1}) = 49.9 + 0.097 \times A - 0.40 \times B - 6.91 \times C - 0.001 \times A^2 + 0.002 \times B^2 + 0.323 \times C^2 + 0.0012 \times A \times B + 0.016 \times A \times C + 0.013 \times B \times C$$

$$\left(S = 7.08; R^2 = 0.758; R^2_{adj} = 0.659\right) \qquad \textbf{(Eq 2.)}$$

3.4 SOLUBLE PROTEINS

The soluble protein results obtained are shown in **Table 3.** The soluble protein content found in the hydrolysates ranged from 1.61 ± 0.26 mg g^{-1} to 5.01 ± 0.39 mg g^{-1}. These results differ from those reported in the literature, where no significant values of soluble proteins were found in extracts obtained from propolis. The protein content of propolis may be attributed to the type of plant from which it is derived, as well as to the digestion and secretion processes carried out by the bees, given that the chemical composition of propolis varies according to geographical region and available flora [43–45].

3.2 3.5 ANTIOXIDANT ACTIVITY

3.5.1 DPPH

In **Table 3** and **Figure 3**, the results of the DPPH antioxidant activity of propolis extracts obtained through pressurized liquid extraction can be observed. The findings indicate that the DPPH antioxidant activity ranged from 54.66 ± 3.16 μmol TEAC g^{-1} in the PLE 2 treatment (0% EtOH, 60 ºC, and pH 12) to 304.82 ± 0.40 μmol TEAC g^{-1} in the PLE 13 treatment (40 % EtOH, 90 ºC, and pH 2).

In a study aimed at assessing the variability of propolis parameters from different apiaries in southeastern Mexico, the authors reported results ranging from 200 to 2710 μmol TEAC g^{-1}. In this study, the authors prepared extracts using 6 grams of propolis, which were mixed with 20 mL of 96 % EtOH. The mixture was agitated for 12 days at 100 rpm, kept in the dark, and maintained at room temperature (25 ºC). The extracts were then evaporated under reduced pressure and subsequently rediluted in 10 mL of 96 % EtOH [46]. In another study evaluating the DPPH antioxidant activity of 31 propolis extracts from northern Spain, the authors reported an average value of 1,114.28 μmol TEAC g^{-1} for the extracts. These extracts were obtained by adding 600 mL of a 70% hydroalcoholic solution to 10 g of propolis, carried out in two stages of 24 hours at 20 °C, with 300 mL of the hydroalcoholic solution added during each stage [47].

Extracts of brown propolis from Colombia were obtained using different techniques, including Soxhlet extraction, ultrasound-assisted extraction, and supercritical CO_2 extraction. The authors reported DPPH results of 86.63 ± 0.42 μmol TEAC g^{-1} for Soxhlet extraction, 99.26 ± 0.34 μmol TEAC g^{-1} for ultrasound-assisted extraction, and 101.79 ± 0.38 μmol TEAC g^{-1} for supercritical CO_2 extraction [18]. The results found in the literature vary significantly, with some values being similar to those obtained in this study. As previously mentioned, the geographic location and type of vegetation where propolis is produced greatly influence its characteristics.

The Pareto chart for DPPH analysis is shown in **Figure 4 (c)**, indicating that all the factors studied have a statistically significant influence on DPPH antioxidant activity. **Figure 5 (c)** illustrates the combined effects, demonstrating that a medium temperature (90 ºC) and an EtOH concentration of 80% produce extracts with higher DPPH antioxidant activities. Regarding the combination of pH and EtOH concentration, it can be concluded that lower pH (2) and an EtOH concentration between 60% and 70% result in greater DPPH antioxidant activity. For the

combination of temperature and pH, it is observed that a temperature range of 90 to 110 ºC with an acidic pH (2) yields the highest antioxidant activities of brown propolis.

The ANOVA test, conducted at a significance level of 5% **(Table 4)**, demonstrated that none of the factors studied presented statistically significant differences, as the *p-value* was <0.05. In **Figure 6 (c)**, the surface graphs of responses for DPPH antioxidant activity can be observed, showing that extracts with DPPH antioxidant activity greater than 300 µmol TEAC g^{-1} can be obtained at temperatures around 90 ºC, with EtOH concentrations above 60 %, and an acidic pH lower than 5.

Equation (3) presents the optimized equation along with its coefficients of determination for the regression model adjusted for DPPH antioxidant activity. The results indicate that the model is well adjusted, with an (R^2) value of 0.822, allowing for the prediction of the DPPH antioxidant capacity of the extract obtained from the pressurized liquid extraction (PLE) of brown propolis.

$$\text{DPPH (µmol TEAC g}^{-1}) = -346.0 + 5.61 \times A + 10.3 \times B - 3.05 \times C - 0.031 \times A^2 - 0.045 \times B^2 + 0.203 \times C^2 - 0.022 \times A \times B - 0.029 \times A \times C - 0.06 \times B \times C$$

$$\left(S = 28.40; R^2 = 0.873; R^2_{adj} = 0.822\right) \qquad \textbf{(Eq 3.)}$$

3.5.2 FRAP

The results for the FRAP antioxidant activity of propolis extracts obtained through pressurized liquid extraction are presented in **Table 3** and **Figure 3**. As shown, the DPPH antioxidant activity ranged from 238.4 ± 24.66 µmol TEAC g^{-1} in the PLE 1 treatment (0 % EtOH, 60 ºC, and pH 2) to 385.37 ± 18.88 µmol TEAC g^{-1} in the PLE 8 treatment (80 % EtOH, 120 ºC, and pH 12).

Propolis obtained from three regions of Iran was evaluated for its antioxidant activity. The ethanol extract was produced by cutting the propolis into very small pieces and adding 25 mL of 95 % ethanol, with continuous stirring for 24 hours. The authors obtained extracts that exhibited FRAP antioxidant activity ranging from 125.25 to 3381.64 µmol mL at propolis concentrations of 100 and 2000 µg mL^{-1} [48]. Another study evaluated the FRAP antioxidant activity of 19 samples from Turkey. The extracts were prepared by weighing one gram of propolis and extracting it with

10 mL of an 80 % EtOH solution, using a sonicator for 45 minutes. The results ranged from 62.3 to 1,396 µmol TEAC g^{-1} [49].

As with the DPPH antioxidant activity, the FRAP antioxidant activity is statistically influenced by all the factors studied, as shown in the Pareto chart in **Figure 4(d)**. The influence of the combination of factors is illustrated in **Figure 5 (d)**. When combining EtOH concentration (%) with temperature, it is demonstrated that using a high concentration of EtOH (80 %) in conjunction with a high temperature (120 ºC) result in FRAP antioxidant activity greater than 300 µmol TEAC g^{-1}. Evaluating the combination of EtOH concentration (%) with pH shows that an acidic pH (2) combined with a concentration ranging from 60 % to 80 % yields the highest FRAP antioxidant activity values. Finally, the interaction between temperature and pH indicates that extracts with greater FRAP antioxidant activity can be obtained at a temperature of 120 ºC and a pH of 2. It is also observed that high temperatures enhance antioxidant activity, regardless of the pH of the mobile phase.

In **Table 4**, it can be observed that none of the factors studied presents a statistically significant difference, as the *p-value <0.05*. In Figure g (d), the surface graphs of responses for FRAP antioxidant activity are shown, indicating that extracts with FRAP antioxidant activity greater than 360 µmol TEAC g^{-1} can be obtained at high temperatures close to 120 ºC, high EtOH concentrations (60-80 %), and an acidic pH near 2.

Equation (4) presents the optimized equation along with its coefficients of determination for the regression model adjusted for FRAP antioxidant activity. The results indicate that the model fits satisfactorily, with an R^2 value of 0.626, allowing for the prediction of the FRAP antioxidant capacity of the extract obtained from the pressurized liquid extraction (PLE) of brown propolis.

$$\text{FRAP } (\mu\text{mol TEAC } g^{-1}) = 338.0 + 2.00 \times A - 0.96 \times B - 26.53 \times C - 0.014 \times A^2 + 0.012 \times B^2 + 1.66 \times C^2 - 0.0024 \times A \times B + 0.035 \times A \times C - 0.023 \times B \times C$$

$$\left(S = 31.73; R^2 = 0.734; R^2_{adj} = 0.626\right) \quad \textbf{(Eq 4.)}$$

3.6 SUGAR AND MAILLARD REACTION PRODUCTS

The results obtained for sugars are presented in **Table 5**. The content of sugars of propolis can come from the pollen, the type of plant, the components of the exudates of surrounding plants, in addition to the digestion and secretion processes carried out by the bees [44]. The chemical composition of propolis varies depending on the available flora and the practices of beekeepers. This includes both simple sugars such as glucose and fructose, as well as more complex sugars derived from hemicellulose, such as xylose and arabinose, or from cellulose, cellobiose [50–52].

Table 5. Biocompounds composition of the accumulated hydrolysates obtained from pressurized liquid extraction of brown propolis at a solvent-to-feed ratio of 60 g solvent g^{-1} propolis.

Parameter	PLE 1	PLE 2	PLE 3	PLE 4	PLE 5	PLE 6	PLE 7	PLE 8	PLE 9	PLE 10	PLE 11	PLE 12	PLE 13	PLE 14	PLE 15	PLE 16
Sugars (mg g^{-1})																
Cellobiose	51.06 ± 1.25	40.34 ± 0.45	48.24 ± 0.32	41.78 ± 0.08	68.56 ± 0.82	40.91 ± 0.74	51.81 ± 1.66	49.53 ± 0.98	44.72 ± 2.45	58.19 ± 2.12	50.65 ± 0.32	38.62 ± 1.21	45.99 ± 0.99	44.0 ± 2.22	67.85 ± 0.66	47.7 ± 0.66
Xylose	127.88 ± 11.66	96.92 ± 5.66	139.49 ± 10.22	108.99 ± 5.33	155.96 ± 3.65	94.15 ± 2.47	117.84 ± 10.02	115.93 ± 3.65	133.32 ± 1.02	196.47 ± 3.55	104.75 ± 8.33	134.68 ± 0.99	94.89 ± 0.88	140.9 ± 6.02	112.25 ± 8.02	159.9 ± 8.22
Arabinose	147.12 ± 3.58	110.62 ± 1.47	194.10 ± 2.87	171.01 ± 2.44	150.72 ± 3.11	112.68 ± 1.45	142.58 ± 3.12	133.86 ± 2.01	158.78 ± 1.55	190.61 ± 4.20	42.68 ± 0.67	102.62 ± 5.11	168.85 ± 1.87	147.4 ± 0.68	155.37 ± 0.99	130.9 ± 4.10
Total	326.06 ± 5.50	247.88 ± 2.53	381.83 ± 4.47	321.78 ± 2.62	375.24 ± 2.53	247.74 ± 1.55	312.23 ± 4.93	299.83 ± 0.88	336.82 ± 1.68	445.27 ± 1.67	198.08 ± 3.11	275.92 ± 2.44	309.73 ± 1.24	332.3 ± 2.97	335.47 ± 3.22	338.5 ± 4.33
Organic acids (mg g^{-1})																
Acetic acid	31.78 ± 2.16	24.24 ± 0.55	59.78 ± 4.10	34.35 ± 2.11	28.52 ± 1.87	19.31 ± 2.54	37.67 ± 0.69	38.12 ± 1.86	35.95 ± 0.12	62.77 ± 3.44	37.82 ± 2.08	37.99 ± 3.17	36.01 ± 1.24	62.55 ± 0.89	37.82 ± 0.66	32.22 ± 1.74
Total	31.78 ± 2.16	24.24 ± 0.55	59.78 ± 4.10	34.35 ± 2.11	28.52 ± 1.87	19.31 ± 2.54	37.67 ± 0.69	38.12 ± 1.86	35.95 ± 0.12	62.77 ± 3.44	37.82 ± 2.08	37.99 ± 3.17	36.01 ± 1.24	62.55 ± 0.89	37.82 ± 0.66	32.22 ± 1.74

The results are expressed as mean ± standard deviation. Analysis conducted in triplicate.

In the hydrolysates, three sugars were identified: cellobiose, xylose and arabinose, which are present in the composition of different types of honey and can thus be transferred to the propolis [50–52]. Among the sugars identified, cellobiosis presented the lowest concentrations in all treatments, with values ranging from 38.62 to 68.56 mg/g. This variation suggests that experimental parameters, such as pH, temperature and pressure, directly influence the efficiency of obtaining this sugar, both to increase its concentration and to reduce its formation. Previous studies have proven that acidic pH can favor the release of cellobiosis [53,54]. The highest concentration was observed in the PLE 5 treatment (68.56 ± 0.82 mg/g) demonstrating that more acidic pH conditions, associated with moderate temperatures, are more effective for breaking glycosidic bonds. On the other hand, in treatments with severe temperature increase (PLE-3, PLE-7 and PLE-13), a reduction in cellobiose concentration was observed (**Figure 7 (a)**), which can be attributed to cellobiose degradation under high temperatures, especially under conditions of low pH and high pressures [55]. Xylose had the highest concentration in PLE 10 (196.47 ± 3.55 mg/g), while arabinose had its highest concentration in PLE 3 (194.10 ± 2.87 mg/g), which reflects the ideal extraction conditions for each type of sugar. The total sugar content, shown in **Figure 7 (d)**, is the sum of the concentrations of cellobiose, xylose and arabinose. These values ranged from 198.08 mg/g in PLE 11 to 445.27 mg/g in PLE 10. The large variation observed between the samples suggests that the extraction efficiency is directly related to the specific conditions of each treatment, such as temperature, pressure and pH. The optimized condition found in PLE 10 for the maximum release of sugars reflects the synergy between these variables and indicates that, under certain PLE conditions, it is possible to maximize the extraction of sugars, which has important implications for industrial processes that aim to obtain these compounds from agro-industrial residues.

The application of high pressure and temperature to extracts containing reducing sugars favors a number of chemical reactions, including the Maillard reaction. This reaction occurs between amino acids and reducing sugars, such as glucose, xylose, and arabinose, generating MRP, which evolve from intermediate compounds to final products [56,57]. During extraction in PLE, parameters such as pH, temperature, and the presence of solvents such as ethanol play a crucial role in the formation of these

compounds, altering characteristics such as color and molecular structure [56,58]. By analyzing the MRP under different PLE conditions, it is possible to identify the intermediate and final compounds formed and evaluate the conditions that optimize the production of these products (**Table 3**).

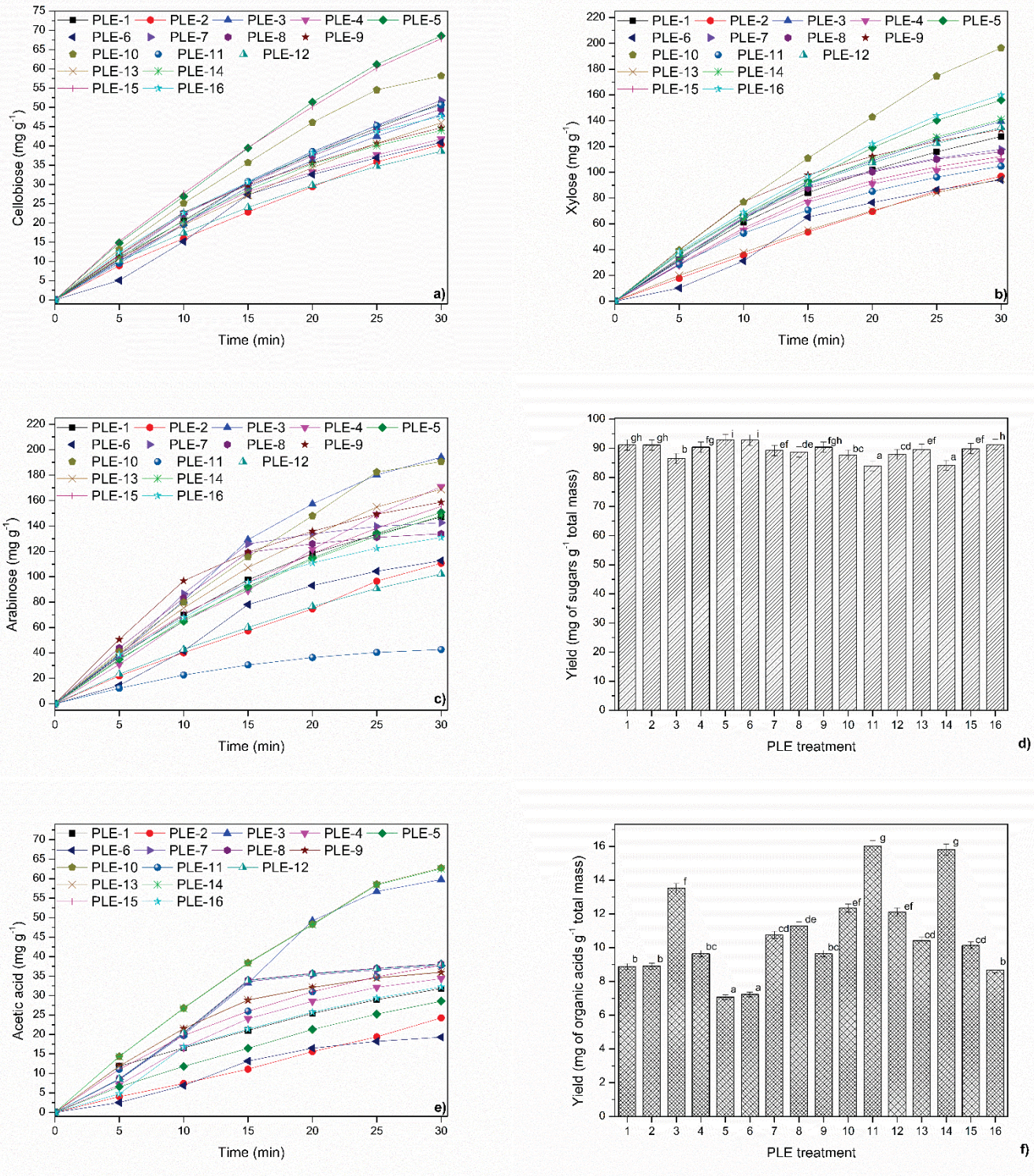

Figure 7. Kinetic profile of the accumulated biocompounds obtaining during the pressurized liquid extraction of brown propolis: a) cellobiose; b) xylose; c) arabinose; d) global sugars yield; e) acetic acid; and f) global organic acid yield. Different lower-case letters indicate significant differences by Tukey's test at $p \leq 0.05$.

In the absorbance values at 294 nm (A294), treatments such as PLE 5 and PLE 10 show high values (1.20 and 1.07 nm, respectively), indicating greater formation of reactive Maillard intermediates, such as amadori compounds [59]. These intermediate products tend to form more easily at moderate temperatures and acidic pH (pH 2) [59,60]. However, PLE 8, with an alkaline pH (pH 12) and high temperature (120°C), resulted in lower values of A294, suggesting a possible decomposition of the intermediates due to increased instability under these conditions. For the A420 values, which reflect the formation of final products of the Maillard reaction, such as melanoidins [56], an increase was observed in treatments using ethanol, such as PLE 8 (A420 = 0.25 ± 0.08). The presence of ethanol can favor the formation and stability of these compounds due to the stabilizing effect of alcoholic solvents on the reaction. However, PLEs with neutral pH and moderate to high temperature, such as PLE 9 and PLE 12, also demonstrated an increase in A420 values, suggesting that such conditions promote the formation of melanoidins in greater quantities.

3.7 ORGANIC ACIDS

Acetic acid was the only organic acid identified in the propolis extracts (**Table 5**). The concentration of acetic acid in the treatments ranged from 19.31 ± 2.54 mg/g (PLE 6) to 62.77 ± 3.44 mg/g (PLE 10), with the highest values observed in the treatments PLE 10 and 14 (Fig. The values obtained in this study were higher than the levels found by Pavlovic et al., 2020 [61] when analyzing crude propolis from hills and plains originating in Italy, where only substantial values ranging from 45.28 to 57.80 µg/g were found. The difference between the values can be explained by the difference in origin of the propolis analyzed, considering that several factors significantly influence their composition [62]. The result of total organic acids present in **Figure 7 (f)** corresponds only to the values presented by acetic acid, which was the only one to be identified.

Obtaining sugars and organic acids through pressurized liquid (PLE) extraction can be optimized by adjusting process variables to achieve specific objectives, such as maximizing the extraction of sugars or acids. For formulations that require simple

sugars, such as xylose and arabinose, PLE 10 conditions can be a reference, since they have the highest concentrations of these sugars. Similarly, for extracts with high acetic acid content, PLE 10 stands out, suggesting a favorable synergy between the extraction parameters and the release of this compound. In addition, the use of PLE in combination with other extraction techniques, such as extraction with organic solvents, can be an effective strategy to increase the overall efficiency of the process and maximize the recovery of bioactive compounds, such as sugars and organic acids, from agro-industrial residues [63].

4. CONCLUSION

The present study investigates the efficiency and sustainability of the removal of bioactive compounds from brown propolis by PLE in semi-continuous flow. The study demonstrates that the variation in operational parameters influences the recovery of phenolic compounds, flavonoids, sugars and organic acids, thus allowing the optimization of the process for different applications.

The highest concentrations of phenolic compounds (144.55 ± 8.73 mg GAE g^{-1}) were obtained under conditions of moderate temperature and pH (90 ºC - pH 7), and flavonoids (56.01 ± 3.37 mg CE g^{-1}) under conditions of elevated temperature and basic pH (120 ºC - pH 12). Conversely, the most efficient recovery of sugars (445.27 mg g^{-1}) was observed under moderate temperature and acid pH conditions (90 °C and pH 2), exhibiting a predominance of xylose and arabinose. The DPPH antioxidant activity ranged from 54.66 ± 3.16 to 304.82 ± 0.40 µmol TEAC g^{-1}, while the FRAP activity reached a maximum of 385.37 ± 18.88 µmol TEAC g^{1}. Acetic acid removal was optimized at 90 °C and pH 10, reaching 62.77 ± 3.44 mg g^{-1}.

This study underscores the potential of PLE for the valorization of brown propolis, offering a sustainable and efficient approach for the removal of bioactive compounds, with promising applications in the pharmaceutical, food and cosmetic industries.

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CHAPTER IV

CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Esta dissertação aborda de maneira abrangente a extração e as aplicações de compostos bioativos da própolis, com foco em sua utilização em diferentes setores, como o farmacêutico, alimentar e cosmético. O primeiro estudo analisou o crescente interesse na própolis, extratos naturais e nanopartículas de prata (AgNPs), destacando suas propriedades terapêuticas e a busca por soluções naturais e inovadoras. Os avanços nas técnicas de extração, como a extração com líquido pressurizado (PLE), têm permitido um aproveitamento mais eficiente dos compostos bioativos, o que potencializa seu uso em uma variedade de produtos industriais. A análise bibliométrica realizada demonstrou que há um grande potencial para futuras descobertas e aplicações dessas substâncias, principalmente na medicina personalizada e no desenvolvimento de terapias inovadoras.

O segundo estudo apresentou resultados detalhados sobre a extração de compostos bioativos da própolis marrom usando PLE em um sistema de fluxo semicontínuo, revelando a influência de variáveis como temperatura, pH e concentração de etanol na eficiência da extração. O estudo mostrou que condições otimizadas, como 90 °C e pH 7, proporcionaram a maior recuperação de compostos fenólicos, enquanto temperaturas mais altas (120 °C) e pH básico maximizaram a extração de flavonoides. Essas condições também afetaram as propriedades colorimétricas dos extratos e sua atividade antioxidante. A pesquisa conclui que o uso de PLE oferece uma abordagem eficiente e sustentável para a extração de compostos bioativos da própolis, com grandes perspectivas para sua aplicação em diversas indústrias.

Ambos os estudos destacam a importância das técnicas de extração aprimoradas e da aplicação de AgNPs e extratos naturais em uma variedade de indústrias. As inovações no processo de extração, como o uso de métodos verdes, têm o potencial de reduzir o impacto ambiental e melhorar a sustentabilidade dos processos. Além disso, a personalização das nanopartículas de prata, com ajustes em tamanho, forma e propriedades de superfície, pode resultar em avanços significativos em dispositivos biomédicos, tratamentos antimicrobianos e eletrônicos flexíveis.

A pesquisa sugere que, à medida que mais avanços forem feitos em técnicas de extração e otimização de propriedades materiais, as possibilidades de utilização desses compostos bioativos serão ampliadas, conduzindo a novas soluções sustentáveis e eficazes nos campos da saúde, segurança alimentar e ambientais. A integração de tecnologias avançadas com substâncias naturais promete um futuro promissor para inovações em diversos setores.