ESCOLA
SUPERIOR
DE SAÚDE
POLITÉCNICO
DO PORTO

M

MESTRADO
BIOQUÍMICA EM SAÚDE

Ultrasound-assisted extraction of goji berries: Bioactive composition and pro-healthy properties

Filipa Marisa Ribeiro Teixeira

09/**2023**

Ultrasound-assisted extraction of goji berries: Bioactive composition and pro-healthy properties

Autor

Filipa Marisa Ribeiro Teixeira

Orientadores

PhD, Francisca Rodrigues, LAQV/REQUIMTE – Instituto Superior de Engenharia do Porto

MSc, Ana Margarida Silva, LAQV/REQUIMTE – Instituto Superior de Engenharia do Porto

PhD, Professor Ricardo Ferraz, Escola Superior de Saúde – Instituto Politécnico do Porto

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde – Ramo em Bioquímica Clínica e Metabólica pela Escola Superior de Saúde do Instituto Politécnico do Porto.

Financial Support

This work received financial support from project EXPL/BAA-GR/0663/2021 – Kiwi4Health – Exploring the Eco-Innovative Re-Use of Kiwiberry, supported by national funds by FCT/MCTES and by the projects UIDB/50006/2020 and UIDP/50006/2020 through national funds. This work was also financed by national funds from FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the project UIDP/04378/2020, UIDB/04378/2020 and the project LA/P/0140/2020.

The author would also like to express its gratitude to all the entities involved in this research work, both host and supply, for collaborating and making this work possible:

- Grupo de Reação de Análises Químicas (GRAQ), Instituto Superior de Engenharia do Porto (ISEP)
- Laboratório Associado para a Química Verde/Rede de Química e Tecnologia (LAVQ/REQUIMTE)
- Cooperativa de Ensino Superior Politécnico e Universitário (CESPU)

Agradecimentos

Depois de dois anos verdadeiramente desafiantes, é com imenso orgulho que finalmente apresento a minha dissertação de mestrado. Deste modo, gostava de expressar a minha gratidão por todos que, direta ou indiretamente, contribuíram para o sucesso deste trabalho.

Primeiramente, gostaria de deixar o meu sincero agradecimento à minha orientadora do Laboratório Associado para a Química Verde/Rede de Química e Tecnologia – Instituto Superior de Engenharia do Porto (LAQV/REQUIMTE – ISEP), Doutora Francisca Rodrigues. Agradeço por toda a disponibilidade, atenção e profissionalismo demostrados no decorrer deste trabalho. Obrigada por acreditar no meu trabalho e nas minhas capacidades, por toda a calma e energia positiva que sempre me transmitiu, e por toda a simpatia e bondade com que sempre me tratou. Da mesma forma agradeço também ao meu orientador da Escola Superior de Saúde do Instituto Politécnico do Porto (ESS – IPP), Professor Doutor Ricardo Ferraz, por toda a disponibilidade, simpatia e boa disposição.

À Ana Margarida Silva, por todos os ensinamentos, paciência, disponibilidade e companheirismo. Sem ti esta não teria sido a experiência incrivelmente positiva que foi. Obrigada por me acolheres e me fazeres sentir integrada, pela amizade e pelo apoio constante.

A todos os membros do Grupo de Reação de Análises Químicas (GRAQ) e da Cooperativa de Ensino Superior Politécnico e Universitário (CESPU) com quem tive o privilégio de trabalhar. Em especial, à Diana Pinto, Sofia Ferreira e Maria João Bessa, obrigada por toda a disponibilidade e por nunca terem exitado em ajudar-me.

Como não podia faltar, a toda a minha família. Aos meus pais, irmão, primos, padrinhos, tios e avós. Obrigada pelo apoio, carinho e amor. André, tiras-me do sério, mas és o melhor irmão que uma irmã mais velha podia ter. Mãe, sem ti não estaria onde estou hoje! Obrigada por nunca me deixares contentar-me com o que tenho e por me fazeres sempre querer lutar por mais e melhor.

A todos os meus amigos que, longe ou perto, estiveram presentes ao longo desta jornada. Em especial, à Eduarda, Joana e Sofia, agradecer-vos por todo o apoio e entreajuda, por todos os momentos partilhados e loucuras cometidas juntas. Mais virão nesta nova fase das nossas vidas!

E finalmente, ao Miguel, pelo companheirismo, compreensão, carinho, amor e apoio incondicional. Obrigado por estres ao meu lado todos os dias da minha vida e por acreditares em mim mesmo quando eu não acredito. Tenho a certeza de que juntos temos todo um futuro incrível pela frente e conto contigo para me fazeres querer ser todos os dias a melhor versão de mim mesma.

Abstract

Lycium barbarum L., also known as goji or wolfberries, have a remarkable chemical composition and extraordinary biological activities, allowing it to be classified as a "superfruit".

The main objective was to optimize the Ultrasound-Assisted Extraction (UAE) of goji berries using the Response Surface Methodology, and to characterize the optimal extract. The optimal UAE conditions established were a solid:liquid ratio of 8.75%, for 56.21 min and an intensity of 59.05 W/m^2. The optimal extract demonstrated a high total phenolic content (20.91 mg GAE/g dw) as well as exceptional antioxidant/antiradical properties (ABTS = 14.95 mg AAE/g dw; DPPH = 10.29 mg TE/g dw; FRAP = 106.79 µmol FSE/g dw). LC/DAD-ESI-MS analysis revealed a diverse phytochemical profile that included compounds such as feruloylquinic acid, 3,5-dicaffeoylquinic acid, rutin, and numerous derivatives.

Furthermore, the optimal extract demonstrated strong scavenging activities, particularly against HOCl (IC_{50} = 12.99 µg/mL). Cell viability studies on intestinal cell lines (Caco-2 and HT29-MTX) revealed that doses up to 500 µg/mL were safe, however 1000 µg/mL significantly reducing Caco-2 (60.00%) and HT29-MTX (17.02%) viability. Thus, a concentration of 500 g/mL was used in the intestinal co-culture experiment, which showed that most compounds from the optimal extract crossed the intestinal cell monolayer and exerted their pro-healthy properties.

Keywords: *Lycium barbarum*; response surface methodology; ultrasound-assisted extraction; antioxidant activity.

Resumo

Lycium barbarum L., também conhecido como goji, tem uma composição química notável e atividades biológicas extraordinárias, permitindo-lhe ser classificado como um "superfruto".

O principal objetivo foi otimizar a extração assistida por ultrassons (EAU) de bagas de goji utilizando a metodologia de superfície de resposta e caraterizar o extrato ótimo. As condições ótimas de EAU estabelecidas foram uma relação sólido:líquido de 8,75%, durante 56,21 min e uma intensidade de 59,05 W/m^2. O extrato ótimo demonstrou um elevado teor de fenólicos totais (20,91 mg GAE/g dw), bem como propriedades antioxidantes/antiradicalares excecionais (ABTS = 14,95 mg AAE/g dw; DPPH = 10,29 mg TE/g dw; FRAP = 106,79 μmol FSE/g dw). A análise LC/DAD-ESI-MS revelou um perfil fitoquímico diversificado que incluía compostos como o ácido feruloilquínico, o ácido 3,5-dicafeoilquínico, a rutina e numerosos derivados.

Além disso, o extrato ótimo demonstrou fortes atividades de captação, particularmente contra HOCl (IC50 = 12,99 μg/mL). Estudos de viabilidade celular em linhas de células intestinais (Caco-2 e HT29-MTX) revelaram que doses até 500 μg/mL eram seguras, mas 1000 μg/mL reduziram significativamente a viabilidade de Caco-2 (60,00%) e HT29-MTX (17,02%). Assim, foi utilizada uma concentração de 500 g/mL no ensaio de co-cultura intestinal, que mostrou que a maioria dos compostos do extrato ótimo atravessou a monocamada de células intestinais e exerceu as suas propriedades pró-saúde.

Palavras-chave: *Lycium barbarum*; metodologia de superfície de resposta; extração assistida por ultrassons; atividade antioxidante.

List of communications resulting from the research work developed

1. **Scientific Articles:**

- Filipa Teixeira, Ana Margarida Silva, Cristina Delerue-Matos and Francisca Rodrigues (2023). *Lycium barbarum* Berries (*Solanaceae*) as Source of Bioactive Compounds for Healthy Purposes: A Review, International Journal of Molecular Sciences, 24(5), 4777. https://doi.org/10.3390/ijms24054777.
- Filipa Teixeira, Ana Margarida Silva, Stefania Sut, Stefano Dall'Acqua, Oscar L. Ramos, Alessandra B. Ribeiro, Ricardo Ferraz, Cristina Delerue-Matos and Francisca Rodrigues (2023). Reviving the pro-healthy properties of goji berries: Ultrasound-assisted extraction optimization by response surface methodology and intestinal permeability assessment, *under review.*

2. **Book Chapter:**

- Ana Margarida Silva, Maria João Bessa, Filipa Teixeira and Francisca Rodrigues. Polyphenols in skin and hair health, "Bioactive Polyphenols for Health and Pathology Treatment" (Eds. Antonio Segura-Carretero, Maria de la Luz Cádiz-Gurrea, Javad Sharifi-Rad), Elsevier, in press, 2023.

3. **Oral Communications:**

- Filipa Teixeira, Ana Margarida Silva, Cristina Delerue-Matos and Francisca Rodrigues. Ultrasound-assisted extraction of goji berries: Bioactive composition and prohealthy properties, 16th Investigação Jovem da U.Porto, May 10th – 12th 2023, Porto, Portugal.
- Filipa Teixeira, Ana Margarida Silva, Stefania Sut, Stefano Dall'Acqua, Cristina Delerue-Matos, Ricardo Ferraz and Francisca Rodrigues. Bioactive composition and pro-healthy properties of goji berries: Ultrasound-assisted extraction optimization and intestinal permeability assessment, 5th PhD Day CESPU, October 26th 2023, Porto, Portugal.

4. **Poster Communications:**

- Filipa Teixeira, Ana Margarida Silva, Cristina Delerue-Matos and Francisca Rodrigues. Ultrasound-assisted extraction of goji berries: Bioactive composition and prohealthy properties, 2nd Dare2Change, March 21st 2023, Porto, Portugal.
- Filipa Teixeira, Ana Margarida Silva, Cristina Delerue-Matos and Francisca Rodrigues. Bioactive composition of goji berries extracted by Ultrasound-Assisted Extraction: Validation of a mathematical model, 5th Doctoral Congress in Engineering: Symposium on Chemical and Biological Engineering, June 15th and 16th 2023, Porto, Portugal.

Index

Index of Figures

Index of Tables

List of Abbreviations

3D	Three-dimensional
AAE	Ascorbic Acid Equivalent
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CAE	Catechin Equivalents
CAT	Catalase
CTC	Condense Tannin Content
DHR	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	dry weight
EAE	Enzyme-Assisted Extraction
EPR	Electron Paramagnetic Resonance
EU	European Union
Exp	Experimental
FAO	Food and Agriculture Organization of the United Nations
$Fe^{2+}E$	Fe^{2+} Equivalent
FRAP	Ferric Reducing Antioxidant Power
FSE	Ferrous Sulphate Equivalents
fw	fresh weight
GAE	Gallic Acid Equivalents
GPx	Glutathione Peroxidase
GSH	Glutathione
HBSS	Hank's Balanced Salt Solution
HE	Hyperoside Equivalent
HWE	Hot Water Extraction
IC_{50}	Half-maximal inhibition concentration
LBP	*Lycium barbarum* polysaccharides
LC-MS	Liquid Chromatography coupled with Mass Spectrometry

MAC	Monomeric Anthocyanin Content
MAE	Microwave-Assisted Extraction
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	β-nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
ORAC	Oxygen Radical Absorbance Capacity
PEFE	Pulsed Electric Field Extraction
PMS	Phenazine methosulphate
Pred	Predicted
QE	Quercetin Equivalents
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	rotations per minute
RSM	Response Surface Methodology
Rt	Retention time
SFE	Supercritical Fluids Extraction
SOD	Superoxide Dismutase
SWE	Subcritical Water Extraction
TBARS	Thiobarbituric acid reactive substance
TCC	Total Carotenoid Content
TE	Trolox Equivalent
TEAC	Trolox equivalent antioxidant capacity
TEER	Transepithelial electrical resistance
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TPTZ	2,4,6-Tri(2-pyridyl)-s-triazine
UAE	Ultrasound-Assisted Extraction
USA	United States of America
UV	Ultraviolet
UV/Vis	Ultraviolet–visible
VCE	Vitamin C Equivalent
WHO	World Health Organization

1. Introduction

Nowadays, the interest in natural matrices, such as exotic berry-type fruits and other botanical products, has been increasing worldwide (1, 2, 3, 4). The society has become more concerned with eating habits, mostly due to the reinforcement that exists a positive relation between good eating habits and the prevention of disease development, such as diabetes, cardiovascular, neurological pathologies or even cancer (5). Simultaneously, the emergent awareness about the worrying state of the planet, and the impact that various types of industries have on it, has left the population concerned and demanding greener formulations using bioactive ingredients recovered from natural sources (1, 6). Therefore, the consumption of natural matrices has increased in recent years, not only as a supplement for imbalanced diets, but also as an integral part of a normal healthy diet (3, 7).

Other than that, plants have been used for thousands of years as source of compounds for traditional medicine, aiming to prevent and treat health problems (8). Data from the World Health Organization (WHO) estimates that 80% of the world's population resorts largely to traditional remedies for health care and speculate that around two billion people heavily rely on medicinal plants (9). Examples of natural matrices used for pharmaceutical, medical, or healthcare proposes are *Lycium barbarum* L. (10), *Castanea sativa* (1), *Schisandra chinensis* (8), *Aloe vera* (11), *Zingiber officinale* (12), *Papaver rhoeas* (13) and *Sambucus nigra* L. (14). The remarkable phytochemical composition of *L. barbarum* berries (Figure 1) (10, 15, 16) have raised the interest of the science community in this fruit and, therefore, the number of studies focused in goji berries has increased drastically in recent years (16, 17).

Figure 1. *Lycium barbarum* berries.

1.1. *Lycium barbarum*

Lycium barbarum L. is one of the most common members of the *Solanaceae* family (16, 18), consisting of about 70 species distributed from temperate to subtropical regions (15, 19). The berries, also known as wolfberries, mostly grow in China and other parts of Asia (2, 16, 20). China, however, is the primary worldwide supplier (17, 21), with about 25000 – 30000 tons of dried fruit being produced annually in Ningxia, Xinjiang, Gansu, and Mongolia (22).

Due to the raising number of reports on the positive correlation between the consumption of natural matrices and the consumers health improvement (5), the production of fruits all over the world has been increasing in the last 20 years (Figure 2). Asia is the region with the highest production (71.2%), followed by Africa (15.8%), Americas (8.3%), Oceania (3.4%) and Europe (1.3%) (23). Goji berries is one of the fruits whose production have been growing in the last decades (24), particularly in Europe (Italy, Romania, Bulgaria, Portugal, Greece, Serbia), Northern America, and Australia.

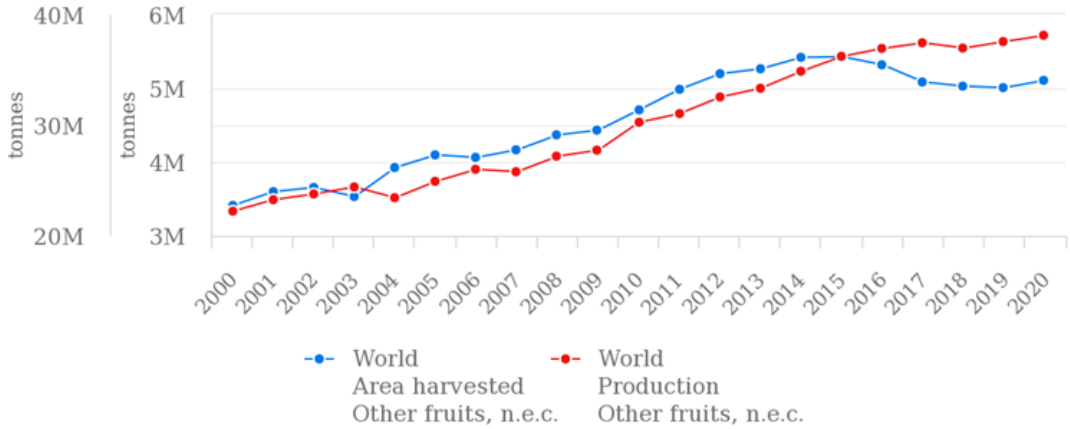

Figure 2. Annual production and harvested area of fruit in the last 20 years (23).

Goji berries can be divided into different classes, according to the ripening stage, dimension, weight, color, firmness, soluble solid content, pH, and titratable acidity (18). The mature fruit is between 1 and 2 cm long, presenting an ellipsoid shape and a bright orange-red color, and contains up to 40 tiny seeds per fruit (2, 16, 18). The berries are sweet (25) and widely used as a dietary supplement, food, and natural health product (15, 20, 21). Although mostly consumed fresh in the regions of its cultivation (5), in the rest of the world it is essentially consumed as a dried fruit (5, 26) or transformed into alimentary products, such as juices, herbal teas, yogurt products, granola, powders and tablets (Figure 3) (5, 10, 26).

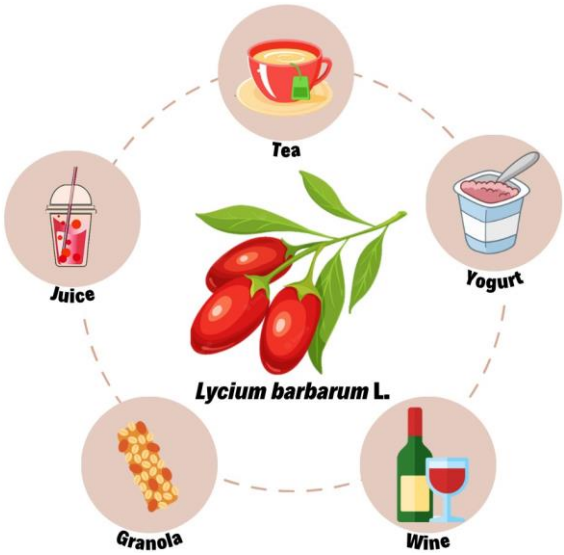

Figure 3. Food products containing *L. barbarum* berries.

For thousands of years, goji berries have been used in herbal medicine in Asian countries (17). Based on their rich nutritional value and medical properties, such as antioxidant, antimicrobial, immunomodulatory and anti-inflammatory effects (20, 27, 28), this fruit has been employed by local population as an anti-aging, tranquilizer, thirst quenching, blood nourishing, early onset diabetes and tuberculosis treatment, dizziness, chronic cough, and eye health protection (16, 28, 29). All these pro-healthy properties make this fruit more attractive to the general population, making goji berries one of the most popular functional food ingredients/supplements worldwide (16). Nevertheless, the consumption of natural supplements needs to be balanced to avoid negative effects related to their overuse or interaction with other medical treatments (3). Therefore, further risk/benefit evaluations are urgently needed in food or health-promoting formulations (15).

1.1.1. Bioactive compounds and chemical composition

Different authors have reported that goji berries have a remarkable concentration of phenolic compounds, organic acids, fat, dietary fibers, essential amino acids, valuable trace minerals, and vitamins (2, 10, 16, 27, 30).

Phenolic compounds (Figure 4) are plants secondary metabolites that possess different physiological activities associated with their chemical structures (27, 31, 32). They act as a defense mechanism to provide adaptation and survival capacity to plants in adverse environmental conditions, like protection or defense against ultraviolet radiation (UV),

pathogens aggression, parasites, and predators, along with contributing to plant parts colors (1, 31). These compounds usually add nutritional and functional value to fruits, contributing to their organoleptic characteristics, such as astringency, bitterness, and aroma (6, 31). Simultaneously, they guarantee outstanding biological activities and pro-healthy properties against oxidative stress (1, 16, 27), being capable of delay, prevent and inhibit oxidation by scavenging free radicals and, thus, reducing the oxidative stress (31).

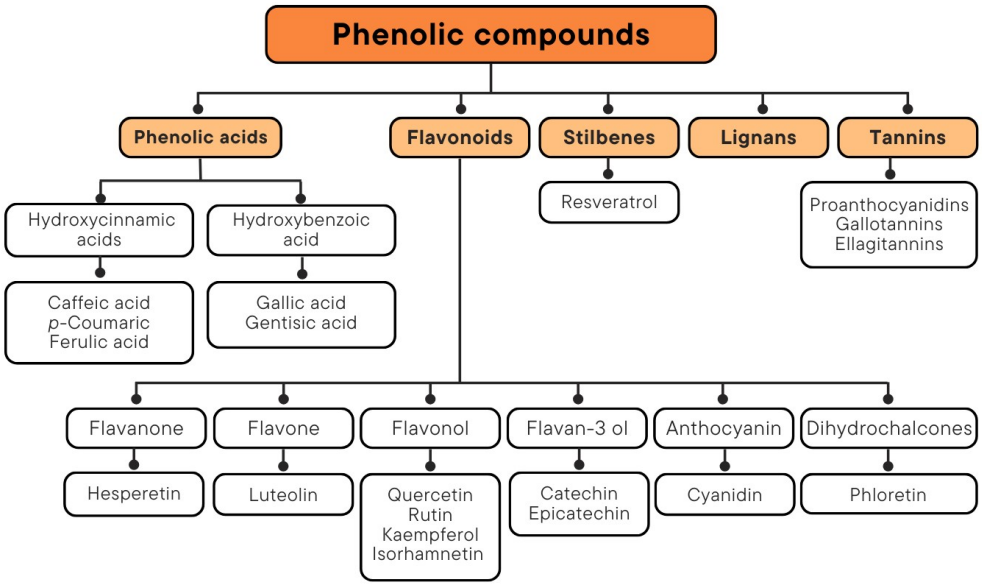

Figure 4. Categories and subcategories of phenolic compounds.

A way of preliminarily assessing the phenolic profile of a product is by evaluating their total phenolic content (TPC), total flavonoid content (TFC) and total carotenoid content (TCC) (33), which are intrinsically related to the fruit aspect as well as to its nutritional and health promotion capacity (24, 32, 34). Table 1 summarizes the TPC, TFC and TCC in goji berries, according to different authors.

Table 1. Total phenolic, flavonoid and carotenoid content in goji berries according to different studies.

TPC	TFC	TCC	Reference
449 – 778 mg GAE/100 g dw	-	400 - 950 mg/100 g dw	(24)
3000 mg GAE/100 g dw	2480 mg QE/100 g dw	-	(26)
31.6 mg GAE/100 g dw	28.3 mg CAE/100 g dw	23.30 mg CAE/100 g dw	(29)
145.2 mg GAE/100 g dw	74.5 mg QE/100 g dw	-	(30)
97.23 mg /100 g dw	-	212,94 mg/100 g dw	(34)
162.4 mg GAE/100 g fw	214.2 mg HE/100 g fw	41.71 mg/100 g fw	(35)

GAE – Gallic Acid Equivalents; CAE – Catechin Equivalents; HE – Hyperoside Equivalent; QE – Quercetin Equivalents; TPC – Total Phenolic Content; TFC – Total Flavonoid Content; TCC – Total Carotenoid Content; dw – dry weight; fw – fresh weight.

Goji berries are renowned for their diverse array of phytochemicals that contribute to their numerous health benefits (36). These phytochemicals include phenolic acids, such as chlorogenic and *p*-coumaric acids, flavanols like rutin and quercetin, and flavan-3-ols such as catechin and epicatechin. Additionally, these berries are a source of essential vitamins like ascorbic acid and tocopherols, vibrant pigments including zeaxanthin, β-carotene, and cryptoxanthin, as well as sugars such as glucose, fructose, and sucrose. They also contain both soluble and insoluble fibers, organic acids, primarily quinic, tartaric, and malic acids, and essential minerals like potassium, sodium, and calcium. Furthermore, goji berries provide essential fatty acids, predominantly linoleic, oleic, and palmitic acids. Table 2 summarizes the recently reported data on the phytochemical composition of goji berries.

Table 2. Phytochemical composition of goji berries according to different authors.

Compounds	Amount	Reference
Phenolic compounds	12697.90 mg/100 g fw	(5, 10, 16, 30)
· **Phenolic acids**	· 32.70 mg/g	
· **Chlorogenic acid**	· 25.07 – 1.07 mg/g	
· ***p*-coumaric acid**	· 12.30 mg/g	
· **Caffeic acid**	· 0.93 – 8.99 mg/g	
· **Flavanols**	· 27.60 mg/g	
· **Rutin**	· 16.60 – 12.84 mg/g	
· **Quercetin**	· 10.23 – 9.41 mg/g	
· **Flavan-3-ol**	· 10.40 mg/g	
· **Catechin**	· 1.34 – 1.13 mg/g	
· **Epicatechin**	· 2.18 – 1.98 mg/g	
Organic acids	4461.02 mg/100 g fw	(16)
· **Citric**	· 254.09 mg/100 g fw	
· **Malic**	· 601.43 mg/100 g fw	
· **Quinic**	· 2011.73 mg/100 g fw	
· **Tartaric**	· 1580.35 mg/100 g fw	
Carotenoids	212.94 mg/100 g dw	(34)
· **Zeaxanthin**	· 84.54 mg/100 g dw	
· **β-Carotene**	· 19.35 mg/100 g dw	
· **Cryptoxanthin**	· 72,29 mg/100 g dw	

Compounds	Amount	Reference
Vitamins		(16, 34, 37)
· **Ascorbic acid**	· 2.39 - 48.94 mg/100 g fw	
· **Tocopherol**	· 0.33 mg/100 g dw	(10)
Carbohydrates	77.1 – 87 g/100 g dw	(10, 37)
· **Total sugars**	· 45.60 – 67.83 g/100 g dw	(22, 37)
· **Soluble sugar**	· 27.09 g/100 g dw	(10, 24)
○ **Glucose**	○ 14.40 – 17.32 g/100 g dw	
○ **Frutose**	○ 12.70 – 21.71 g/100 g dw	
○ **Sucrose**	○ 0.80 – 1.48 g/100 g dw	
Dietary fibers	3.63 – 16 g/100 g fw	(20, 35)
· **Soluble**	· 0.90 – 5.5 g/100 g dw	(10)
· **Insoluble**	· 2.73 – 11.7 g/100 g dw	
Proteins	5.3 – 14.3 g/100 g dw	(10, 37)
· **Essential aminoacids**	· 2.139 g/100 g dw	(37)
· **Non-essential aminoacids**	· 6.728 g/100 g dw	
Fatty acids	0.39 – 4.1 g/100 g dw	(10, 37)
· **Linoleic acid**	· 37.89 – 53.4%	
· **Oleic acid**	· 16.5 – 23.6%	
· **Palmitic acid**	· 12.77 – 21.79%	
Ash	0.78 – 3.21 g/100 g dw	(10, 37)
Minerals		(2, 20, 35)
· **Potassium**	· 434 – 1460 mg/100 g fw	
· **Calcium**	· 29 – 60 mg/100 g fw	
· **Sodium**	· 75 – 550 mg/100 g fw	
· **Iron**	· 5.4 mg/100 g fw	
· **Phosphor**	· 232 mg/100 g fw	

fw – fresh weight; dw – dry weight.

The different results obtained by the diverse authors may be explained by the extraction techniques employed as well as the extractor solvents used (5, 33). Moreover, the bioactive compounds present in plants are highly influenced by cultivation factors, namely variety, ripeness, geographic location, climatic conditions, among others (19, 27).

1.1.2. Biological activities of *L. barbarum* berries

As previously mentioned, goji berries have been used for thousands of years in herbal medicine due to their rich nutritional value, medical properties, and biological activities (16, 20, 27, 29). Several studies highlighted the pro-healthy effects of this fruit, particularly in what concerns to antioxidant, anti-tumor, antimicrobial, hypoglycemic, hypolipidemic, immunomodulatory anti-mutagenic, and prebiotic activities, alongside with anti-aging, anti-fatigue, neuroprotective events, and increased metabolism, cytoprotective and general well-being effects (6, 15, 16, 17, 20, 25, 26, 27, 28, 29, 38). These biological activities have been closely related to the fruit phenolic composition, particularly phenolic acids, flavonoids, carotenoids, and tannins (1, 20, 27, 29, 39). The following sections will highlight three of these biological activities: antioxidant, antimicrobial and antiproliferative.

1.1.2.1. Antioxidant activity

Oxidative stress is a phenomenon that occurs due to an imbalance between pro-oxidants and antioxidants, which can be a consequence of the excessive production of reactive oxygen species (ROS) (30). ROS naturally occur in living organisms, being involved in processes such as proliferation and apoptosis (21). However, when the quantities of ROS, such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot-}$), overcome the activity of endogenous antioxidant mechanisms (Figure 5) such as antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)) and nonenzymatic molecules (glutathione (GSH) and ascorbic acid), a state of oxidative stress is installed (6, 21, 31).

This stress phenomenon is implicated in the aging process as well as in several pathologies (cardiovascular dysfunction, various typologies of cancer, inflammation, rheumatism, diabetes, rheumatoid arthritis, pulmonary emphysema, dermatitis, cataract, neurodegenerative diseases, endothelial cell dysfunction, and several autoimmune diseases linked to degenerative processes of aging) that frequently lead to death or incapacitation (5, 16, 30). In some situations, an external antioxidant supplementation is required to reestablish the balance, and fruit phenolic compounds arise as an option (31). The main contributors to the antioxidant capacity of food, especially fruits and vegetables,

are phenolic compounds, such as phenolic acids and flavonoids, carotenoids, tocopherol, polysaccharides, anthocyanins, and condensed tannins (1, 6, 29, 34, 39). These molecules stimulate the antioxidant defenses by delaying, inhibiting, or preventing the free radicals from damaging proteins, deoxyribonucleic acid (DNA) and lipids, as well as by scavenging free radicals by hydrogen atom transfer or electron donation, or enhancing endogenous antioxidant defenses, such as antioxidant enzymes (SOD, CAT, GPx) (6, 16, 30, 31).

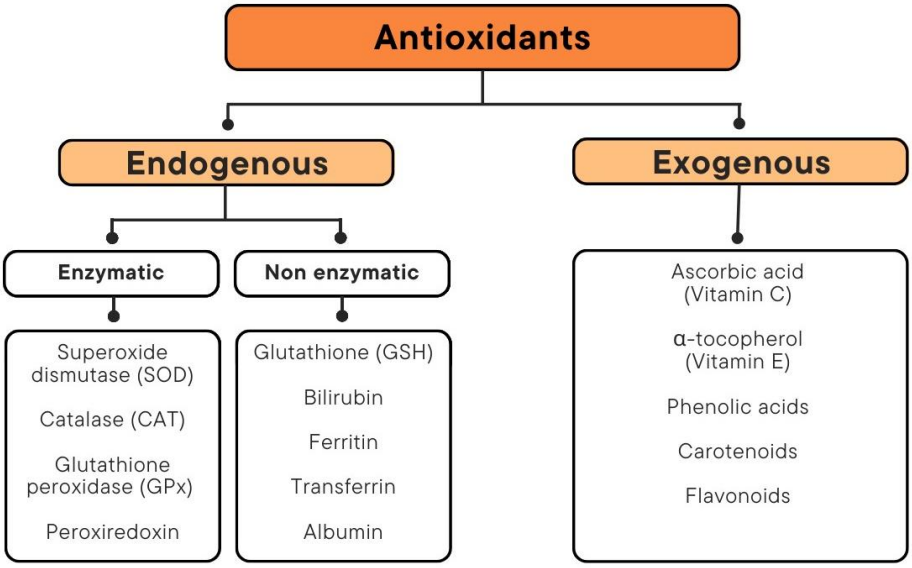

Figure 5. Endogenous and exogenous antioxidant molecules.

There are various ways to evaluate a product's antioxidant activity, and several methods have been used to test the antioxidant/antiradical properties of goji berries. The most frequently applied are the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) and 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical ($ABTS^{\cdot+}$) scavenging assays as well as ferric reducing antioxidant power (FRAP) assay (6, 33, 40). Table 3 summarizes the recently reported data on the antioxidant and antiradical activities of goji berries.

Table 3. Antioxidant and antiradical activities of goji berries according to different authors.

DPPH	ABTS	FRAP	References
18.5 – 13.9 µmol VCE/g dw	61 – 54 µmol VCE/g dw	–	(5)
8.79 – 9.35 mg TE/g dw	24.86 – 25.12 mg TE/g dw	16.91 – 19.52 mg TE/g dw	(15)
16.65 µmol TE/g dw	59.14 µmol TE/g dw	35.1675 mmol Fe^{2+}E/g dw	(29)
-	16.0 – 68.3 µmol TE/g dw	14.4 – 63.0 µmol TE/g dw	(34)
4.526 µmol TE/g fw	129 µmol TE/g fw	5.324 µmol TE/g fw	(35)

TE – Trolox equivalent; VCE – vitamin C equivalent; Fe^{2+}E – Fe^{2+} equivalent; dw – dry weight; fw – fresh weight.

A study conducted by Islam *et al.* (29) assessed the TPC (3.16 mg GAE/g dw), TFC (2.83 mg CAE/g dw), condense tannin content (CTC; 1.08 mg CAE/g dw) and monomeric anthocyanin content (MAC; 0.24 mg MAC/g dw), along with DPPH (16.65 µmol TE/g dw), ABTS (59.14 µmol TE/g dw) and FRAP (3516.75 mmol Fe^{2+}E/g dw) assays of a goji berries extract. The results attested a positive correlation between DPPH, ABTS and FRAP assays and the phenolic compounds (0.786, 0.643 and 0.856, respectively), flavonoids (0.857, 0,714 and 0.786, respectively), condensed tannin (0.429, 0.714 and 0.643, respectively) and anthocyanin content (0.643, 0.786 and 0.857, respectively), supporting the idea that phenolic compounds are the main contributors to the antioxidant activities of goji berries, which is in line with information previously reported (31).

1.1.2.2. Antimicrobial activity

Goji berries have already demonstrated antimicrobial activity according to different authors (10, 41). This activity is usually associated with the phytochemical profile of the fruit that includes polysaccharide, flavonoid, phenolic acids, and carotenoid (6, 41). These compounds exert antimicrobial effects by complexing with extracellular and soluble proteins, leading to a disruption of the microbial membrane, interaction with enzymes, or provoking metal ion deprivation (10, 41). Another way of explaining the antimicrobial activity of polyphenols is their structural features, as well as the pH and sodium chloride concentration, which results in physiological changes in the microorganisms and eventually leading to cell death (41).

A study carried out by Kabir *et al.* (2014) (41) confirmed that chlorogenic acid, one of the phenolic acids detected in greater amounts in goji berries, exhibited a bacteriostatic and bactericidal effect against the Gram-negative bacterium *Escherichia coli*. The bacteriostatic effect was assessed by measuring the optical density to determine the growth inhibitory effect of chlorogenic acid when added to a culture of *E. coli* in a concentration of 0.5 mM. As for the bactericidal effect, it was evaluated by using mid-logarithmic phase cell cultures (10^6 cells/mL) at different doses (2.5, 5.0, and 10 mM), treatment times (0, 1, 3, and 6 h), temperatures (20, 37, 45, and 50 °C) and pH conditions (8.0, 7.0, 6.0, 5.0, and 4.0). The overall results demonstrated a synergetic antimicrobial effect expressed by chlorogenic acid and related compounds, and a dose, temperature, and time-dependent bactericidal

effect. The authors stated that this effect may be related to the fact that chlorogenic acid is a phenolic acid, presenting the capacity of promoting physiological changes on the microbial cell membrane that result in cell death (41).

1.1.2.3. Anticancer activity

Despite the extensive research and advances in cancer treatments made in recent years, cancer is still a worldwide problem (38, 42), with breast, lung and colorectum cancer being the most mortal and persistent ones (Figure 6). Even though the human body exerts a protective effect against tumors development, for example, by modulating immune response (42, 43) or promoting apoptosis and cell cycle arrest (38), most of the times medical intervention is required. Currently, there are multiple cancer therapies available, including surgery, chemotherapy, radiotherapy, hormone therapy and immunotherapy, all of which have associated disadvantages (25).

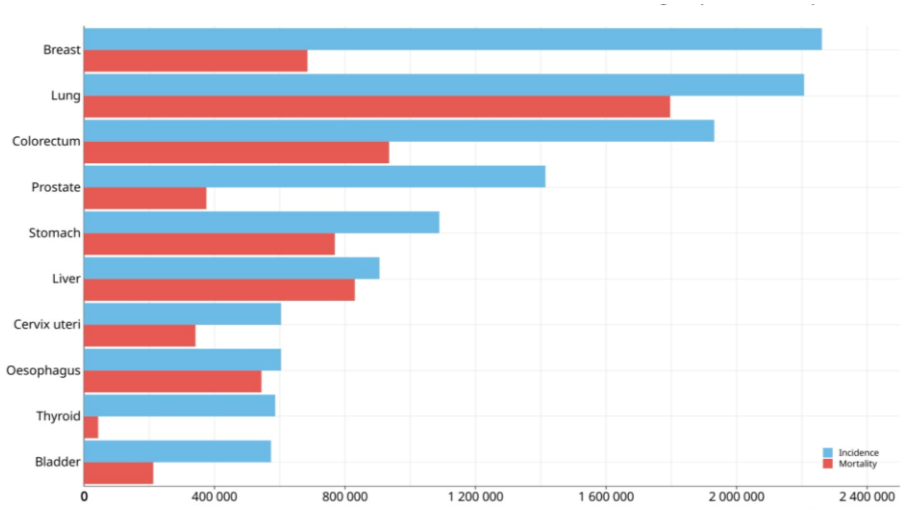

Figure 6. Estimated numbers of incidence and mortality of some types of cancer in the world, accounting for both sexes and all ages (44).

The knowledge that several polyphenols-rich extracts from natural matrices have been suggested as promising anti-cancer agents, with few side effects and are associated with a lower risk of cancer and cancer mortality, has increased the populations interest in the consumption of fruits and natural matrices (1, 2, 31). For example, phenolic compounds can act immediately before, during and even after the initiation of the carcinogenesis process (33, 40). These compounds exert an antioxidant effect through prevention of ROS attack by scavenging radicals, regulating molecular targets and signal pathways, suppressing

carcinogenic agents, inducing cell-cycle arrest, or exerting an antiproliferative, anti-inflammatory and apoptotic effect (25, 31, 33, 38, 40, 45).

A study with a goji berries ethanolic extract assessed the proliferation, apoptotic and necrotic effect of different extract concentrations in T47D human breast cancer cell line (45). The results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay attested a strong decrease of the cells proliferation (70, 55.7 and 51.4% after 24, 48 and 96 h, respectively) after exposure to the highest extract concentration (1 mg/mL). The bromodeoxyuridine cell proliferation assay supported these results, showing a sharp decrease in the proliferation of T47D cells, as well as the neutral red cell viability assay that demonstrated a slight decrease in the T47D cell viability. As for propidium iodide and Hoechst solutions staining, in T47D cells, it was detected an increase in the apoptotic percentage (37, 61.6 and 88% when treated with 0.1, 0.5 and 1 mg/mL of extract, respectively) and a significant necrotic change for the extract concentration of 0.5 mg/mL. These results suggest that the anti-cancer effect of goji berries extract is due to apoptotic effects through the mitochondrial pathway. To confirm this theory, by Western blotting, the authors (45) demonstrated a dose-dependent significant increase in pro-apoptotic Bax protein expression and a decrease in the anti-apoptotic BclxL protein expression after treatment of T47D cells with the extract for 48 h, in relation to control.

1.1.3. Potential applications of *L. barbarum* berries

Overall, considering the excellent nutritional profile and the positive health effects of goji berries, this fruit can be classified as a "superfruit" (16, 26) used in different areas, such as food, nutraceutical, or cosmetic industries.

Regarding the food industry, it has been reported that aqueous extracts can be used as potentially prebiotic food additives (6, 46). Goji berries extract has also been successfully used in meat products to improve the sensory properties and the oxidative stability during storage (47). The addition of 1.0% of goji berries extract and 1.0% of buckwheat flour improved the oxidative stability and quality of modified horse-meat products, resulting in a better smell, taste, and surface color properties after 21 days of storage (48). Another study determined the effect of the addition of goji berries or goji berries extracts on sausages (47),

reporting that the addition of 1% goji berries extracts effectively suppressed the lipolysis and the protein/lipid oxidation, reducing the microbial count during storage, and preserving the color, aroma and taste of the sausages. Antonini *et al.* (49) also evaluated the addition of goji berries to meet formulas and determined that the addition of chia seeds and *L. barbarum* puree (2.5 and 5%, respectively) to beef burgers promotes a higher TPC, an increase up to 70% of the total antiradical capacity (through Oxygen Radical Absorbance Capacity (ORAC), ABTS and DPPH assays) and a decrease of the lipid peroxidation up to 50%. The confectionery and bakery industries are also using goji berries and their compounds to improve the functional, sensory, or texture properties of various products (6). Muffins and cookies enriched with different shares of goji berries powder or by-products showed an increase in the TPC and insoluble and soluble fiber contents as well as good sensory properties (20).

Currently, nutraceuticals are being introduced into consumers' everyday diets. They are defined as foods, beverages, or supplements with high concentrations of bioactive chemicals that have excellent health-promoting effects on human body (5). Due to the bioactive compounds present in fruit, goji berries are being investigated as potential nutraceutical ingredients (1, 29, 30).

As previously stated, goji berries have been used in herbal medicine for thousands of years (17), along with hundreds of plants that are cultivated worldwide to be used in medicine and pharmaceutical formulations (50). Based on the long-term traditional use of goji berries, this fruit is now generally recognized as non-toxic (6). However, adverse effects can occur and, depending on the amount and how goji berries are consumed, they might pose a risk to the general public health (50). The presence of tropane alkaloids, chemical contaminants (such as pesticides and toxic elements) or some proteins that can cause allergic reactions in sensitive consumers may represent a threat (6). Therefore, safe consumption of natural matrices, such as goji berries, should be ensured through professional guidance to avoid adverse effects, toxicity, and allergies (51). Furthermore, the application of this fruit and its components at high levels in different industries, such as food, pharmaceutical, nutraceutical or cosmetic, can substantially deteriorate the sensory, textural, and quality of

the final products (20). More studies should be conducted in order to determine if the use of this fruit is still beneficial for consumers (15).

1.1.4. By-product valorization

During production and processing of food products, a huge amount of waste is generated, some of which may contain a significant load of bioactive compounds (7). Annually, in the European Union (EU) approximately 88 million tonnes of waste are generated, with associated costs estimated at 143 billion euros (39). The last data from the Food and Agriculture Organization of the United Nations (FAO) states that fruit and vegetables processing wastes, such as peels, pomace, flowers, stems, leaves, seeds and pulps, are the 5th highest contributor (representing 8% of total food waste) (32, 39). In the particular example of goji berries, approximately 10 kg of waste are generated per 90 kg of goji-based beverages (20). Besides the fact that the disposal of this waste can cost a lot of money to manufacturers, it has a serious negative impact on the environment (20, 32). As a result, the industries face the urgent and necessary challenge of implementing greener, more efficient, sustainable, and eco-friendly processing protocols in order to generate less waste, but also reduce, reuse and valorize agro-residues and industrial wastes (1, 32).

The by-products of *L. barbarum* include leaves, stems, young shoots, root bark, flowers, seeds, and pulps and can also be consumed as part of a traditional diet and used for medicinal purposes (35). For instance, a study developed in Portugal (10) compared the composition of goji stems and berries and reported similar values of phenolic compounds (71.9 *vs* 71 mg/g dw), organic acids (2.08 *vs* 2.07 g/100 g dw), energy (383 *vs* 408 kcal/100 g dw) and total carbohydrates (78.1 *vs* 87 g/100 g dw). Even more, stems showed higher antioxidant and antiradical capacities (DPPH scavenging activity (EC_{50} = 0.28 mg/mL), reducing power (EC_{50} = 0.23 mg/mL) and thiobarbituric acid reactive substance (TBARS) inhibition (EC_{50} = 0.07 mg/mL), as well as antibacterial activity against Gram-negative (*Escherichia coli*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*) and Gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*), than goji berries (10).

Another study, this time focusing on *L. barbarum* flowers (52), showed a major presence of chlorogenic, *p*-coumaric and ferulic acids, isoquercitrin, rutin and quercitrin (similar to what was stated in studies concerning goji berries (10, 27, 30)), while the TPC and TFC was very similar to *L. barbarum* fruits (3.75 and 0.61 mg/g dw *vs* 1.45 and 0.75 mg/g dw (30), respectively).

As for goji leaves (53), after an ultrasonic extraction with methanol/water (70:30, *v/v*), the phenolic profile revealed the presence of chlorogenic acid and rutin, as previously reported in goji berries (10, 27, 30). Regarding the antioxidant activity, trolox equivalent antioxidant capacity (TEAC) and electron paramagnetic resonance (EPR) spectrometry were performed, and the results showed a high antioxidant power (140 mg TE/g dw and 212 mg FSE/g dw, respectively). Aside from that, polyphenols from goji berries leaves were also encapsulated in liposomes to improve their delivery and successfully serve as carriers for polyphenols, demonstrating a cytoprotective effect on L-929 mouse fibroblast cells (6).

Based on the results detailed above, it can be stated that goji berries by-products are characterized by high amounts of dietary fiber, vitamins, minerals, phytochemicals and antioxidants, being valuable ingredients for food industry. Therefore, the incorporation of goji berries by-products, for example, in bakery industry, may provide benefits to consumers and manufacturers, not only due to the nutritional value, but also based on the economic advantages that comes from the elimination of the costs for its disposal (20).

1.2. Extraction techniques

A way of assuring the recovery of highest amounts of bioactive compounds is by choosing an efficient extraction technique (7) and the correct extraction conditions such as temperature, pressure, solvent, and time (32, 54). There are multiple techniques available in the market, from conventional to non-conventional ones.

The conventional extraction techniques, also known as solid-liquid extractions, require direct contact between solvent and solid sample (1) and are based on the principle that the solvent and the application of heat are enough to extract compounds (32). Some examples include Soxhlet extraction, hydro distillation, maceration, and solvent extraction (1, 32, 55). However, since the extraction process is a critical step in the isolation and purification of

bioactive compounds (7, 19, 54), some of these conventional techniques may not be the best option due to some limitations: reduced extraction efficiency, high energy employment, low selectivity and yields of extraction, time consuming, use of high temperatures and the associated compound degradation, and use of large volumes of solvents (19, 55, 56, 57). Based on that, industries have been focused on overcoming these limitations by developing new non-conventional or "green" extraction technologies (58, 59), such as Microwave-Assisted Extraction (MAE), Supercritical Fluids Extraction (SFE), Enzyme-Assisted Extraction (EAE), Subcritical Water Extraction (SWE), Pulsed Electric Field Extraction (PEFE) and Ultrasound-Assisted Extraction (UAE).

The MAE is based on the solvent penetration into the plant matrix and, due to electromagnetic waves, the components break down, and the solubilized compounds are transferred to the bulk solution. The combination of heat and mass gradients promote high extraction yields (1, 32). Other extraction technique commonly employed is SFE, which is based on the principle that when a material is exposed to a temperature and pressure above the critical limit, the supercritical condition takes place and the fluid acquires gas/liquid properties (1, 32, 56, 59). EAE consists in the addition of an enzyme to the extraction medium and the consequent break of the cell wall, allowing the intracellular materials to be extracted (32, 59). Regarding SWE, water is used as solvent at temperatures between 100 ºC and 374 ºC and the pressure generated is high enough (10 – 100 bar) to sustain water in the liquid state (1, 56). At this state, the polarity of water decreases, being capable of extracting polar and non-polar compounds (56). Concerning PEFE, it is a non-thermal extraction technique based on the principle that when subjected to que correct external electric field the cell membranes will rupture, releasing their content (32, 59).

Table 4 summarizes some of the advantages and disadvantages of the different green extraction techniques mentioned, according to different authors (1, 7, 32, 58, 59).

Table 4. Advantages and disadvantages of different green extraction techniques.

Technique	Advantages	Disadvantages
SWE	Energy and time saving; high selectivity; high extraction yields; eco-friendly solvents	High temperatures; inefficient in large-scale production; expensive equipment and maintenance
SFE	Time saving; easy to separate the extracted compounds; little amount of sample and solvent	Energy consuming; low selectivity; organic solvents; expensive equipment and maintenance
MAE	Easy; clean; little amount of solvent; energy and time saving; high extraction yields	High temperatures; organic solvents; expensive equipment and maintenance; difficult handling
EAE	Eco-friendly solvents; low temperatures; high extraction yields	Difficult to separate the extracted compounds; inefficient in large-scale production; time consuming.
PEFE	Non-thermal; clean; high selectivity; time and energy saving; high extraction yields	Hord to accurately regulate parameters; expensive equipment and maintenance
UAE	Eco-friendly solvents; non-thermal; time and energy saving; high extraction yields	Expensive equipment and maintenance; possible free radicals' generation

Even though all these sustainable extraction processes are good, UAE have been considered the most promising technique by the scientific community (40, 60), being selected for this study.

1.2.1. Ultrasound-assisted extraction

UAE is an advanced green extraction method that uses high intensity sound waves to increase the speed of molecular movements and cause the disruption of plant tissues through physical forces developed during acoustic cavitation (7, 21, 60). This process helps the release of extractable components in the solvent in less time by enhancing the mass transport (7, 55).

This extraction technique consists of a series of compression and rarefaction sound wave cycles that can be propagated through solid, liquid or gas medium, inducing displacement and dislodgement of molecules from their original positions (7). The ultrasound devices for extraction use a range of frequencies from 20 kHz to 2 MHz (59) that are capable of creating an acoustic cavitation effect (Figure 7), which is the main mechanism involved in the UAE (32, 59). The ultrasonic waves generate cavitation bubbles in the medium that grow and, upon collapsing, release energy and create localized high pressure and temperature zones that are capable of rupturing cell membranes and release intracellular content into the medium (7, 32, 59). However, other phenomena, alone or in combination, can be in the origin of the compounds release, such as fragmentation, localized erosion, pore formation (sonoporation), shear force, and increased absorption and swelling index in the cellular matrix of the plant (7, 32, 57).

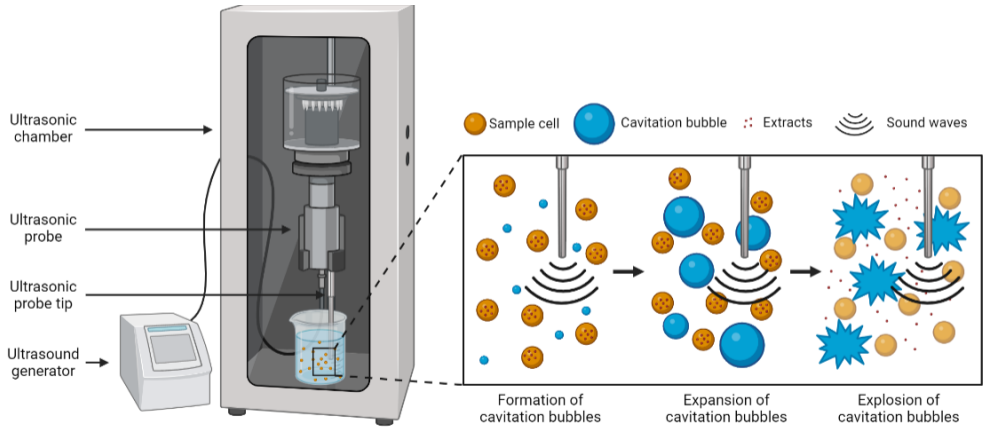

Figure 7. Scheme of a UAE and the acoustic cavitation effect created.

The UAE system can be performed through an ultrasonic bath or an ultrasonic probe. The ultrasonic bath consists of a stainless-steel tank connected to a transducer with the solid matrix and the solvent in it. Despite being more economic and easier to handle, it has a low reproducibility, distributes the energy in a non-uniform way and leads to a decrease of the ultrasonic power over time (7). Regarding the ultrasonic probe, a probe connected to a transducer is immersed in the extraction vessel and delivers ultrasound waves with minimum energy loss directly into the extraction medium (7, 32). This type of system is usually preferred because the energy is delivered in a specific zone, generating a more efficient cavitation effect (7, 40).

Compared to other methodologies, this technique offers better results. In fact, high temperatures are usually responsible for the degradation of compounds and, therefore, conduct to a reduced extraction efficiency (7, 40). Since this technique is a non-thermal extraction process, it is better equipped to retain the functionality of the bioactive compounds extracted, by preserving structural and molecular properties (7, 58, 60). On the other hand, many extraction processes require organic solvents to obtain a significant amount of bioactive compounds. Oppositely, UAE is able to extract components from the natural matrices using non-organic solvents such as water (40), which represents a huge advantage since it is more eco-friendly. However, as with every technology, UAE also has some disadvantages. Table 5 summarizes some of the advantages and disadvantages of this technique (7, 32, 40, 58, 60).

Table 5. Main advantages and disadvantages of UAE technique.

Advantages	Disadvantages
Requires less time, lower temperatures, and lower energy levels	Elevated cost associated with the maintenance of the equipment
Requires little amount of sample and solvent to generate high extraction yields	Using high sonication powers, free radicals can be generated
Maintaining the quality of the extract by preserving structural and molecular properties of the bioactive compounds	Using the probe system, contaminations can occur because the probe is in direct contact with the extraction medium
The equipment is easy to handle and allows application to large scale production	Requires centrifugation and filtration to separate the extracted compounds

Regarding the parameters that may influence the efficiency of UAE, the main ones are time, frequency, power, temperature, solvent, solid:liquid ratio and type of material (40, 60). Therefore, the optimization of UAE is required to assure that the maximum amount of bioactive compounds is extracted (40). The Response Surface Methodology (RSM) is a software commonly used to perform these optimizations (59). It consists in a collection of powerful mathematical and statistical techniques that allows the user to study the correlations between factors and to analyze the influence that independent variables have in the responses studied (1, 7, 58, 60). There is a panoply of experimental designs that can

be applied, such as factorial, central composite, face centered cube and Box-Behnken (7, 59). Considering UAE, this methodology represents a way of evaluating how variables such as time, temperature, and solvent, may influence, for example, the antioxidant activity of an extract (40), and how to optimize the process in order to maximize que antioxidant activity. This happens by reducing the number of required experimental points and through equations that relate to responses and experimental parameters, identifying the optimal conditions (7, 60).

1.3. Intestinal permeability

Intestinal permeability techniques are core to food and drug development industries since the small intestine is the primary site of absorption after oral administration (61, 62). Besides, regardless of the route of administration, it is imperative that a drug/compound permeates at intestinal level to reach bloodstream and, thus, exert its effect (63, 64). Therefore, the ability to evaluate the bioaccessibility and bioavailability of a compound in an early stage of its development represents a huge advantage (65). Moreover, *in vitro* techniques are not only cheaper and easier to manipulate than *in vivo* techniques, but also are less time consuming and involve less ethical issues (63, 65).

The gold standard for this type of assays is Caco-2 cells (66). Caco-2 is a human colorectal adenocarcinoma cell line that resemble mature enterocytes (63) and, despite the origin, simulate the morphology and biochemical environment of human small intestine epithelial cells (62) by expressing typical membrane transporters and tight junctions between adjacent cells (63). However, this model has some limitations, such as the fact that tight junctions expressed are tighter than the *in vivo* ones and the use of only one type of cells is inefficient to evaluate the intestinal permeability, since it neglects the role of non-absorptive cells (61, 62, 66).

The *in vivo* intestinal environment is complex, counting with the contribution of different epithelial cell types, including mucus-producing goblet and immune cells (62). Therefore, trying to create a three-dimensional (3D) intestinal environment and develop models that can better replicate the *in vivo* conditions to predict permeability outcomes is of extreme importance (61, 63, 66). Although it is a relatively recent topic, 3D models have been

explored in the past years, and different models have been developed, from models based on scaffolds, to decellularized tissue models, gut-on-a-chip models or even organoids (61). The advantages associated with these models are astronomic, however 3D models also have some drawbacks: the models are only able to mimic the *in vivo* environment and not replicate it and physiological factors such as age, sex, diseases, hepatic and renal disfunction, among others, are not considered (63).

One of the most used models is the co-culture of Caco-2 and HT29-MTX (63). This model is based on the principle that two compartments are created in each well using Transwell™ inserts (67), so that each well has an apical compartment, in which the cells are located, and a basal compartment (Figure 8). The enterocyte-like (Caco-2) and the goblet-like (HT29-MTX) cells mimic the intestinal conditions (68), presenting microvilli and tight junctions, as well as various transporters, enzymes, and nuclear receptors (63, 65, 66). Furthermore, this model allows to overtake some of the disadvantages associated with the use of only Caco-2 cell line: it is more heterogenic, the tight junctions are more similar to the ones observed *in vivo*, and the mucin properties of the intestine are represented (63, 64, 65). By collecting samples from the basolateral compartment at specific timepoints, it is possible to determine the compounds capable of passing though the cell monolayer and at what rate. This provides vital insights into the bioavailability and bioaccessibility of these compounds, shedding light on their potential to exert pro-health effects within the body (64). Additionally, this information helps to identify the absorption route since the co-culture model mimicked enterocytes (transcellular rout), tight junctions (paracellular route) and transporters (primary and/or secondary active transport and facilitated diffusion) (63, 68).

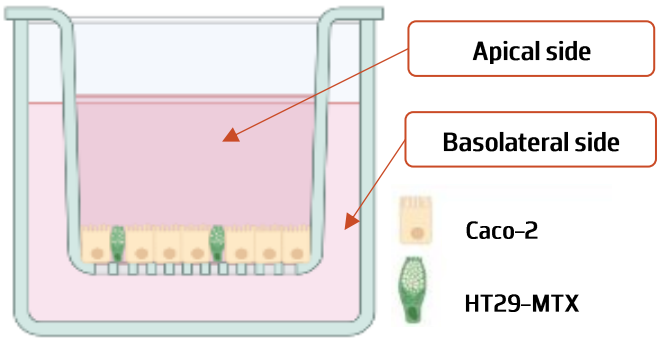

Figure 8. Scheme of an intestinal co-culture with Caco-2 and HT29-MTX cell lines.

2. Objectives

The proposed research work had three main objectives:

1) Selection of the optimal conditions of extraction (solid:liquid ratio (% *w/v*), ultrasonic time (min) and ultrasonic intensity (W/m^2)) of *L. barbarum* berries by UAE based on antioxidant and antiradical activities;
2) Study and characterization of the bioactivity and biological properties of the optimal *L. barbarum* berries extract;
3) Performance of *in vitro* cell-based studies.

For the first objective, an RSM approach was performed. The responses evaluated in this mathematical model were the TPC and the antioxidant/antiradical activities screened by the ABTS and DPPH radical scavenging assays and the FRAP assay.

The second objective involved the definition of the phytochemical profile of the optimal extract was assessed through Liquid Chromatography coupled with Mass Spectrometry (LC-MS). Further, the evaluation of ROS and radical nitrogen species (RNS) scavenging assays against superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\cdot}$), and peroxinitrite ($ONOO^-$) in the absence and presence of sodium bicarbonate ($NaHCO_3$) in the optimal extract was performed.

Finally, the third objective was reached by performing cell viability assays on HT29-MTX and Caco-2 cell lines, and a 3D co-culture model composed of the same cell lines previously mentioned. LC-MS was performed in the permeates to identify the compounds capable of crossing the cell layer and exerting health properties.

3. Material and Methods

The present research work was performed at Grupo de Reação e Análise Químicas (GRAQ), located at Instituto Superior de Engenharia do Porto (ISEP), and at Cooperativa de Ensino Superior Politécnico e Universitário (CESPU), between October 2022 and July 2023.

3.1. Chemicals, Reagents and Excipients

Most of the reagents were acquired from Sigma-Aldrich (Steinheim, Germany) and Sigma Chemical Co. (St. Louis, United States of America (USA)). For the LC/DAD-ESI-MS analysis, the solvents employed were supplied by Merck (Darmstadt, Germany). The human colorectal adenocarcinoma (Caco-2; passages 9-10) as well as the human intestinal epithelial cells (HT29-MTX; passages 47-52) were provided from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells reagents were supplied by Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain) and Biowest (Nuaillé, France).

3.2. Sample preparation

Organic dried goji berries were purchased from Naturefoods, in October 2022, at a local store located in Porto, Portugal. The berries were crushed (Figure 9) using a miller (Moulinex A320, France) and subsequently stored in the dark until further use.

Figure 9. Dehydrated and crushed *L. barbarum* berries.

3.3. Ultrasound-Assisted Extraction

The bioactive compounds from goji berries were obtained by UAE using distilled water as solvent. The extract was obtained using an ultrasonic probe processor (Sonic Vibracell, model VCX50, Newtown, CT, USA) associated with a probe tip No. 630-0219 with 13 mm

diameter and a frequency of 20 kHz. The extractions were carried out in duplicates, according to the RSM design described in Section 3.4. The samples were subsequently centrifuged (TermoFisher Scientific, Germany) at 5000 rpm, at a temperature of 20 °C for 45 min, filtered through a Whatman nº1 paper, and stored at -80 °C (Haier Biomedical, Qingdao, China) for subsequent lyophilization (Telstar, model Cryodos–80, Barcelona, Spain). Afterwards, samples were stored at room temperature until further analysis. The procedure is summarized in Figure 10.

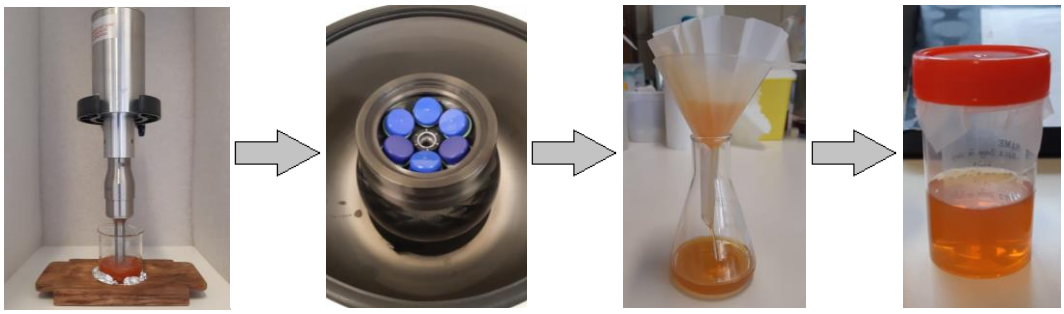

Figure 10. Scheme of the extraction process of *L. barbarum* berries.

3.4. Response Surface Methodology

Experimental designs from the RSM family are powerful mathematical and statistical techniques used to explore the interaction between factors when a response is influenced by several variables (7, 58, 60). In the present work, RSM was used to optimize the UAE in order to find favorable conditions to maximize the TPC and the antioxidant/antiradical activities of the extract. Therefore, a Box-Behnken design with five central points was constructed. The independent variables analyzed were solid:liquid ratio (X_1), ultrasonic time (X_2) and ultrasonic intensity (X_3) in three different ratios (2.5, 6.25 and 10 % *w/v*), ultrasonic times (20, 40 and 60 min) and ultrasonic intensities (30, 50 and 70 W/m^2). A total of seventeen experiments were conducted at random, as summarized in Table 6.

Table 6. Experimental extractions performed according to the RSM design.

Run	X_1 (solid:liquid ratio, % *w/v*)	X_2 (time, min.)	X_3 (intensity, W/m^2)
1	10.00	60	50
2	6.25	60	70
3	10.00	20	50
4	6.25	40	50
5	6.25	40	50
6	6.25	60	30
7	6.25	40	50
8	2.50	60	50
9	2.50	40	70
10	6.25	40	50
11	2.50	20	50
12	10.00	40	70
13	2.50	40	30
14	6.25	20	70
15	6.25	40	50
16	10.00	40	30
17	10.00	60	50

The response evaluated in the experimental designs were the TPC (Y_1) and the antioxidant/antiradical activities evaluated by ABTS (Y_2), DPPH (Y_3) and FRAP (Y_4) assays. The results were statistically analyzed using the software Design Expert Version 13 (Stat-Ease Inc., Minneapolis, MN, USA) with the intention of predicting the model fitting and ascertaining the optimal extraction conditions based on the desirability function combined response surfaces, contour plots and predictive regression equations. A denoting significance of $p < 0.05$ was accepted.

3.5. Preparation of the optimal extract

After analyzing the results of the RMS model, the extraction was carried out under the optimal extraction conditions predicted by the model. To this end, 4.375 g of sample were added to 50 mL of distilled water to obtain a solid:liquid ratio of 8.75%, and the mixture was then placed in an ultrasonic processor for 56.21 minutes at an amplitude of 59%. After centrifugation, filtration and lyophilization under the same conditions previously described

in Section 3.3, a new set of experiments (TPC, ABTS, DPPH and FRAP assays) were carried out with the new extract to appraise the validation of the model. The experimental values obtained under the optimal extraction conditions were compared with those predicted by the model using a *t*-test in the SPSS software (SPSS Inc., Chicago, IL, USA) and considering that a significant statistical difference exists when $p < 0.05$.

3.6. Determination of the Total Phenolic Content

The TPC was determined according to the Folin–Ciocalteu methodology described by Singleton & Rossi (69), with minor modifications. In a 96-well microplate, 30 μL of sample (1 mg/mL of distilled water) was added to 150 μL of Folin reagent (1:10, *v/v*) and 120 μL of sodium nitrate ($NaNO_3$) solution (7.5%). Then, the plate was incubated in a stove (POL-EKO Aparatura and Astell Scientific, Middlesex, England) at 45 °C for 15 min and placed 30 min at room temperature, protected from light. The assays were performed in triplicate and the absorbance was read at 765 nanometers (nm) using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Gallic acid was used as standard solution (curve linearity range = 5 – 100 μg/mL; $R^2 > 0.998$). The results were expressed as milligrams of gallic acid equivalents per gram of extract on dry weight (mg GAE/g dw).

3.7. *In vitro* antioxidant and antiradical activities

The antioxidant/antiradical activities were assessed by ABTS and DPPH radical scavenging assays and as well as the FRAP assay. The absorbance was read in a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) and the assays were performed in triplicate as described below.

3.7.1. ABTS radical scavenging assay

The ABTS radical scavenging assay was performed according to Re *et al.* (70) procedure, with minor modifications. Equal volumes of 7 mM solution of ABTS and 2.45 mM potassium persulfate stock solution ($K_2S_2O_8$) were mixed and left to react in the dark at room temperature for 16 hours. The $ABTS^{\cdot+}$ solution was then filtered using a nylon filter (0.22 μm) and diluted in ultrapure water (MIlipore Simplicity Personal Ultrapure Water System, Merck, Darmstadt, Germany). The absorbance of the solution was measured at 734 nm and

adjusted until 0.700 ± 0.02. In a 96-well microplate, 180 μL of $ABTS^{\cdot+}$ diluted solution was added to 20 μL of each extract (1 mg/mL). For 6 min the plate was kept in the dark and then the absorbance was read at 734 nm, after agitation for 30 seconds at medium speed. Ascorbic acid was used as a standard solution (curve linearity range = 5 – 100 μg/mL; R^2 > 0.988) and the results were expressed as milligrams of ascorbic acid equivalent per gram of extract of dw (mg AAE/g dw).

3.7.2. DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to Barros *et al.* (71), with minor modifications. In each well of a 96-well microplate was added 30 μL of each extract (1 mg/mL) and 270 μL of DPPH solution ($6x10^{-5}$ M). The microplate was incubated for 40 min at room temperature and the absorbance was measured at 535 nm. Trolox was used as standard solution (curve linearity range = 5 – 125 μg/mL; R^2 > 0.995) and the results were expressed as milligrams of trolox equivalents per gram of extract on dw (mg TE/g dw).

3.7.3. Ferric reducing antioxidant power assay

FRAP assay was performed according to Benzie & Strain (72), with minor modifications. To obtain the FRAP reagent, three different solutions were mixed: one part of 10 mM TPTZ; one part of 20 mM ferric chloride ($FeCl_3$); ten parts of 0.3 mM sodium acetate buffer. In a 96-well microplate, 35 μL of extract (1 mg/mL) was mixed with 265 μL of FRAP reagent in each well. The microplate was incubated for 30 min at 37 °C in a stove and the absorbance was measured at 595 nm. Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) was used as standard solution (curve linearity range = 25 - 500 μM; R^2 > 0.990). The results were expressed in micromole of ferrous sulphate equivalents per gram of extract on dw (μmol FSE/g dw), resulting from the reduction of a ferric complex to the ferrous form when antioxidants are present.

3.7.4. Reactive species scavenging capacity

Reactive species scavenging capacity was evaluated according to the procedure performed by Gomes *et al.* (73). The lyophilized sample was dissolved in phosphate buffer and tested

at different concentrations using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) equipped with a thermostat was used to perform fluorescence and ultraviolet–visible (UV/Vis) measurements. IC_{50} values (maximum inhibitory concentration, *in vitro*, to decrease 50% of the amount of reactive species in the media tested) were calculated from the percentage inhibition curves as a function of antioxidant concentration. IC_{50} values were calculated using Microsoft Office Excel (2016).

3.7.4.1 Superoxide radical scavenging assay

The $O_2^{\cdot-}$ scavenging capacity was evaluated according to Gomes *et al.* (73). NADH/PMS non-enzymatic system generated the $O_2^{\cdot-}$, and the ability to capture $O_2^{\cdot-}$ was determined spectrophotometrically in a 96-well microplate, by monitoring the reduction of NBT, induced by $O_2^{\cdot-}$, into diformazan, which presents a purple color. In each well was added 50 µL of NADH 166 µM, 150 µL of NBT 43 µM, 50 µL of PMS 2.7 µM and 50 µL of samples (31.25 – 1000 µg/mL). Gallic acid and catechin were used as positive controls and the assay were performed at room temperature. The absorbance was read at 560 nm for 6 minutes, at 37 °C, and the results were expressed as the inhibition (IC_{50}) of NBT reduction to diformazan.

3.7.4.2 Hypochlorous acid scavenging assay

The HOCl radical scavenging assay followed the methodology described by Gomes *et al.* (73) that is based on the HOCl-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123. The determination of HOCl concentration was performed spectrophotometrically, at a wavelength of 235 nm (ε 100 M^{-1} cm^{-1}). HOCl was prepared by adjusting the pH of a 1% (*w/v*) NaOCl solution to 6.2 with the dropwise addition of 10% (*v/v*) H_2SO_4. This methodology was carried out in a 96-well microplate, where 150 µL of 100 mM phosphate buffer, 50 µL of solvent (phosphate buffer) or sample (3.91 – 125 µg/mL), 50 µL of 5 µM DHR and 50 µL of 5 µM HOCl were added to each well. Gallic acid and catechin were used as positive controls. Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 minutes. Finally, the IC_{50} was calculated.

3.7.4.3. Peroxyl radical scavenging assay

The peroxyl radical ($ROO^{\cdot}$) was generated by thermol decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C and pH 7.4. The fluorescence decay (oxidation of the fluorescent probe fluorescein) was monitored to measure the ROO• scavenging capacity according to the methodology described by Gomes *et al.* (73), named oxygen radical absorbance capacity (ORAC). In each well of a 96-well microplate was added 25 µL of sample (1.95 – 62.5 µg/mL), 150 µL of fluorescein 61.2 nM and 25 µL of AAPH 19.1 mM. The samples were previously prepared in 75 mM phosphate buffer with a pH of 7.4. The microplate was then incubated for 2 hours in a microplate reader at 37 °C where the fluorescence intensity was measured at each minute at the excitation wavelength of 485 ± 20 nm, with emission at 528 ± 20 nm, until total decay of florescence. Gallic acid (0.0469 – 1.5 µg/mL) and catechin (0.0625 – 0.2 µg/mL) were used as positive controls and Trolox (0.469 – 1.5 µg/mL) as standard. The results were expressed as micromole of trolox equivalents per milligram of extract on dw (µmol TE/mg dw).

3.7.4.4. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity was determined by a chemiluminescent methodology (73), where the effect of samples on the oxidation of lucigenin by induction of H_2O_2 was measured. The reaction mixture was performed in a 96-well microplate. In order to obtain a final volume of 250 µL, it was added 91.5 µL of 50 mM Tris buffer (with a pH of 7.4) to 50 µL of solvent (phosphate buffer) for the blank and control or sample (1000 µg/mL). Next, 100 µL of 800 µM lucigenin (which was previously dissolved in 50 mM Tris-HCl buffer) and 8.5 µL of 30% H_2O_2. Gallic acid and catechin (1000 µg/mL) were used as positive controls. The chemiluminescent signal was detected immediately after the reaction mixture was complete, for 5 minutes. The results were expressed as percentage of inhibition (%).

3.7.4.5. Peroxinitrite in the absence of $NaHCO_3$ scavenging assay

The scavenging capacity of $ONOO^-$ was measured according to Gomes *et al.* (73). The determination of peroxinitrite concentration was performed spectrophotometrically, at a wavelength of 302 nm (ε 1670 M^{-1} cm^{-1}). This methodology was carried out in a 96-well

microplate, where 240 µL of 7.5 µM DHR, 50 µL of solvent (phosphate buffer) or sample (31.25 – 1000 µg/mL) and 10 µL of 600 nM $ONOO^-$ were added to each well. Gallic acid and catechin were used as positive controls. Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 minutes. Finally, the IC_{50} was calculated to express the results in the percentage of $ONOO^-$–induced oxidation of DHR when in the absence of $NaHCO_3$.

3.7.4.6. Peroxinitrite in the presence of $NaHCO_3$ scavenging assay

The scavenging capacity of $ONOO^-$ was also assessed in the presence of $NaHCO_3$ in order to simulate the conditions of the human body fluids, according to Gomes *et al.* (73) methodology. The determination of peroxinitrite concentration was performed spectrophotometrically, at a wavelength of 302 nm (ε 1670 M^{-1} cm^{-1}). This methodology was carried out in a 96-well microplate, where 200 µL of 7.5 µM DHR, 40 µL of 187.5 mM $NaHCO_3$, 50 µL of solvent (phosphate buffer) or sample (31.25 – 1000 µg/mL) and 10 µL of 600 nM $ONOO^-$ were added to each well. Gallic acid and catechin were used as positive controls. Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 minutes. The IC_{50} was calculated to express the results in the percentage of $ONOO^-$–induced oxidation of DHR when in the presence of $NaHCO_3$.

3.8. Phytochemical profile of *L. barbarum* berries extract

The phytochemical profile of the optimal *L. barbarum* berries extract was determined by liquid chromatography equipped with triple quadruple mass spectrometry (LC/DAD-ESI-MS), according to the procedure described by Silva *et al.* (65). The stationary phase used was an Agilent Eclipse XDB C-18 (3.0 × 150 mm) 3.5 µm column and the mobile phase consisted of a gradient of three compounds: water 1% formic (A), acetonitrile (B) and methanol (C). The gradient started at 95% A, 5% B and 0% C and continued to 0% A, 90% B and 10% C, over 30 min. The flow rate was 0.4 mL/min, with the column temperature stable at 30 °C. The sample injection volume was 20 µL. The sample was diluted 10 times in methanol:water (50:50, *v:v*) and centrifuged at 13300 rpm for 15 min. MS spectra were recorded in negative ion mode in 150 – 2000 m/z range. For quantification purposes, the following standard calibration curves used were:

Chlorogenic acid curve: y = 198.01 x + 20.138 (R^2 = 1)

Rutin curve: y = 58.564 x + 41.752 (R^2 = 0.9998)

The results obtained were expressed as milligram of each compound per 100 grams of extract on dw (mg compound/100 g dw).

3.9. Cell viability assays

Two intestinal cell lines, namely Caco-2 (cell line of human colorectal adenocarcinoma) and HT29-MTX (intestinal epithelial cell), were used to perform an MTT assay and evaluate the effect of the optimal *L. barbarum* berries extract on their viability. Passages 9-10 and 47-52 were, respectively, used for Caco-2 and HT29-MTX. Both cell lines grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum, 1% essential amino acids and 1% antibiotic. The cells were maintained at a temperature of 37 °C with 5% CO_2 and the culture medium was changed every two days until reached confluence. In each well, the cell line was seeded at a concentration of 5×10^4 cells/mL and incubated for 24 hours at 37 °C with 5% CO_2 to provide exponential growth. After this period, the medium was removed, and the cells were washed with 200 µL of Hank's Balanced Salt Solution (HBSS). Then, cells were incubated with different concentrations of optimal goji berries extract (0.1 – 1000 µg/mL) dissolved in the respective medium, for 24 hours in the same conditions. After the incubation period, the extracts were removed, the cells were washed with HBSS and 120 µL of MTT reagent were added to each well, being incubated for 3 hours at 37 °C with 5% of CO_2. Afterwards, 200 µL of dimethylsulfoxide (DMSO) were added and stirring was carried out for 10 minutes in an orbital shaker (Digisystem Laboratory Instruments Inc., Taipei City, Taiwan) to solubilize the MTT crystals. The absorbance reading was performed at 570 nm and 690 nm to obtain a background. DMEM culture medium was used as positive control and Triton X-100 1% (*w/v*) as negative control. The results obtained were expressed in percentage of cell viability.

3.10. Intestinal permeability assay

The intestinal permeability was carried out in a co-culture model composed of Caco-2 and HT29-MTX in a proportion of 90:10, according to González *et al.* (64). The assay was

performed in a 12-well Transwell™ plate (well diameter of 22.1 mm, 3.8 cm^2) and Caco-2 and HT29-MTX cells were seeded on the apical side of the Transwell™ inserts (insert diameter of 11.9 mm, 1.1 cm^2), achieving a final density of 1.1×10^5 cells/cm^2 in each insert. The cells were maintained in the same conditions as the monocultures and were allowed to grow for 14 days, with medium changes every two days. After that, until the 21st day of the assay, only the apical medium was changed every day. After the 21st days of seeding, the cell monolayers were pre-equilibrated with fresh HBSS (pH 7.4) at 37 °C and 5% CO_2 for 30 min. Then, 0.4 mL of goji berry extract (500 µg/mL) prepared in HBSS was added to the apical side of the co-culture monolayers and 1.2 mL of HBSS to the basolateral side. A sample (0.2 mL) from the basolateral side was collected at different timepoints (15, 30, 45, 60, 90, 120, 180 and 240 min). After each sampling time, the basolateral side was replaced with the same HBSS volume. The collected samples were conserved at -20 °C for subsequent analysis. The transepithelial electrical resistance (TEER) of the model was also evaluated before, during and at the end of the permeability assay using an EVOM Epithelial Volthometer Instrument equipped with a chopstick electrode (World Precision Instruments, Sarasota, FL, USA).

3.10.1. Quantification of permeation by LC/DAD-ESI-MS

The compounds from the optimal goji berries extract that potentially crossed the co-culture intestinal model were determined by LC/DAD-ESI-MS, following the procedure described in section 3.8. The results were expressed as permeation percentage (%) calculated by the ratio of the mass of each compound that permeated the cell monolayer at each timepoint and the mass of each compound present in the optimal extract that was initially added in the apical side of the co-culture monolayer.

3.11. Statistical analysis

Results were expressed as mean ± standard deviation of, at least, triplicate experiments. IBM SPSS statistics 27.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis of the data. To investigate the differences between samples for all assays and comparison of the means were applicated the one-way ANOVA test and the Turkey's HSD test, respectively. For p-value < 0.05, samples were considered statistically

significative. The SPSS software was also applied to verify if there were significant statistical differences between the data collected from the RSM experimental design to validate the model. Microsoft Office Excel (2016) was used to calculate the IC_{50} values, as well as the standard error or mean values of antiradical assays. GraphPad Prism 9.0.2 software (GraphPad Software, La Jolla, CA, USA) was used to determine the area under the curve on the ORAC assay. Design Expert version 13 (Stat-Ease Inc, Minneapolis, MN, USA) allowed the obtention of the response surface analysis, points of prediction of the variables under study and the statistical analysis of the RSM design.

4. Results and Discussion

4.1. Validation of the RSM model

RSM is a powerful mathematical and statistical technique that helps to understand the impact of dependent variables on independent ones (58). In this work, RSM was applied to optimize the extraction process using UAE conditions as independent variables, namely solid:liquid ratio (%*w/v*), ultrasonic time (min) and intensity (W/m^2), and TPC (mg GAE/g) and antioxidant/antiradical activities of the extracts (ABTS (mg AAE/g), DPPH (mg TE/g) and FRAP (μmol FSE/g)) as the dependent variables. By reducing the number of required experimental points and through equations that correlate the responses and the experimental parameters, this methodology helps to optimize the extraction process, identifying the conditions that lead to better antioxidant/antiradical capacity (7, 60).

The predicted and experimental values of TPC, ABTS, DPPH and FRAP assays for all points predicted by the RSM software are summarized in Table 7.

According to the results, TPC ranged between 17.75 mg GAE/g dw (run 16: X_1: 10%; X_2: 40 min; X_3: 30 W/m^2) and 20.91 mg GAE/g dw (run 1: X_1: 10%; X_2: 60 min; X_3: 50 W/m^2). Regarding ABTS, the values varied from 9.06 mg AAE/g dw (run 17: X_1: 10%; X_2: 60 min; X_3: 50 W/m^2) to 14.95 mg AAE/g dw (run 4: X_1: 6.25%; X_2: 40 min; X_3: 50 W/m^2), while the DPPH results ranged between 3.43 mg TE/g dw (run 3: X_1: 10%; X_2: 20 min; X_3: 50 W/m^2) and 10.29 mg TE/g dw (run 5: X_1: 6.25%; X_2: 40 min; X_3: 50 W/m^2). In what concerns to the FRAP assay, the results ranged between 68 μmol FSE/g dw (run 16: X_1: 10%; X_2: 40 min; X_3: 30 W/m^2) and 106.79 μmol FSE/g dw (run 4: X_1: 6.25%; X_2: 40 min; X_3: 50 W/m^2).

Table 7. Experimental and predicted values of TPC, ABTS, DPPH and FRAP for all prepared extracts.

Point	Independent Variables			Dependent Variables							
	Extraction Conditions			Y_1, TPC (mg GAE/g dw)		Y_2, ABTS (mg AAE/g dw)		Y_3, DPPH (mg TE/g dw)		Y_4, FRAP (µmol FSE/g dw)	
Run	X_1 (solid:liquid ratio, % *w/v*)	X_2 (time, min)	X_3 (intensity, W/m²)	Exp *	Pred	Exp *	Pred	Exp *	Pred	Exp *	Pred
1	10.00	60	50	20.91 ± 1.36	20.72	14.56 ± 1.20	13.91	7.85 ± 0.80	7.12	106.00 ± 10.07	99.88
2	6.25	60	70	20.52 ± 0.49	20.52	10.09 ± 0.69	11.04	7.09 ± 0.35	7.98	95.00 ± 8.86	98.15
3	10.00	20	50	19.81 ±1.22	19.68	10.50 ± 1.96	11.24	3.43 ± 0.21	3.88	73.85 ± 17.68	75.65
4	6.25	40	50	19.16 ± 1.19	19.17	14.95 ± 0.95	13.85	9.84 ± 1.00	9.07	106.79 ± 11.25	105.24
5	6.25	40	50	19.01 ± 1.15	19.17	14.29 ± 0.84	13.85	10.29 ± 1.10	9.07	105.09 ± 9.33	105.24
6	6.25	60	30	18.91 ± 0.69	18.98	9.93 ± 1.16	10.31	5.80 ± 0.37	6.08	83.28 ± 2.35	88.05
7	6.25	40	50	19.24 ± 1.39	19.17	13.55± 1.09	13.85	9.25 ± 0.54	9.07	104.17 ± 7.98	105.24
8	2.50	60	50	20.18 ± 1.42	20.31	10.00 ± 0.42	9.33	6.09 ± 0.45	5.64	83.86 ± 6.23	82.06
9	2.50	40	70	18.68 ±1.71	18.56	12.95 ± 0.96	12.68	8.24 ± 1.63	7.79	85.00 ± 8.72	83.65
10	6.25	40	50	19.04 ± 1.15	19.17	14.16 ± 0.96	13.85	7.89 ± 0.29	9.07	104.30 ± 7.30	105.24
11	2.50	20	50	20.15 ± 0.62	20.34	10.20 ± 1.13	10.85	7.06 ± 0.58	7.79	88.70 ± 2.89	94.82
12	10.00	40	70	18.97 ± 0.83	19.17	14.55 ± 1.20	14.26	6.96 ± 0.67	6.80	97.00 ± 10.39	99.98
13	2.50	40	30	18.94 ± 0.65	18.74	9.13 ± 1.03	9.42	5.60 ± 0.59	5.76	90.00 ± 10.92	87.03
14	6.25	20	70	19.10 ± 1.65	19.03	12.46 ± 1.54	12.08	8.08 ± 0.81	7.79	100.72 ± 11.23	95.95
15	6.25	40	50	19.40 ± 1.12	19.17	12.30 ± 0.88	13.85	8.10 ± 0.55	9.07	105.83 ± 6.49	105.24
16	10.00	40	30	17.75 ± 1.36	17.88	12.54 ± 1.12	12.81	3.87 ± 0.43	4.32	68.00 ± 5.84	69.35
17	10.00	60	50	19.46 ± 0.97	19.46	9.06 ± 0.33	8.12	6.08 ± 0.55	5.18	81.93 ± 8.16	78.78

TPC – Total phenolic content; ABTS – ABTS radical scavenging assay; DPPH – DPPH radical scavenging assay; FRAP – Ferric reducing antioxidant power; Exp – Experimental; Pred – Predicted.
* Results are expressed as mean ± standard deviation ($n = 3$).

Table 8 summarizes the adequacy and significance of the obtained values by RSM, according to the ANOVA test analysis that allowed the application of the quadratic model. The following parameters must be confirmed in order to ensure the veracity of the model: adequate precision higher than 4, significant *p*-value (< 0.05) and R^2 value close to 1 (74).

The adequate precision was higher than 4 in all responses (Y_1, Y_2, Y_3 and Y_4), which support an adequate model fitting and signal-to-noise ratio. TPC (Y_1) showed a high R^2 value and adjusted R^2 (0.9644 and 0.9186, respectively), similarly to FRAP (Y_4, 0.9275 and 0.8343, respectively), reenforcing the adequacy of the model to the dependent variables. Besides the low adjusted R^2 values obtained for the ABTS (Y_2) and DPPH (Y_3) response, the experimental model was successfully validated based on the non-significance ($p > 0.05$) of the lack of fit and adequate precision above 4 for this response.

Regarding the independent variables (X_1, X_2 and X_3), it is possible to observe that X_1 had a significant effect ($p = 0.0139$) on ABTS (Y_2), while variable X_2 significantly affected ($p = 0.0137$) TPC (Y_1). In what concerns to variable X_3, it significantly affected ($p < 0.05$) all responses (Y_1, Y_2, Y_3 and Y_4). The "lack of fit" values did not present significant effects ($p > 0.05$) on any response, except for Y_4 ($p = 0.0014$). The R^2 value (0.9275) of this response validates the experimental model, as well as the adequate precision (9.4147) and the significance of the module ($p = 0.0031$). Also, it is relevant to highlight that all the variables evaluated (Y_1, Y_2, Y_3 and Y_4) were significant ($p < 0.05$) for the model applied, reinforcing the validation and effectiveness of the experimental design.

The following quadratic equations (Equation 1 to 4), in terms of codded factors, exhibit the relation between the dependent variables, namely TPC (Y_1), ABTS(Y_2), DPPH (Y_3) and FRAP (Y_3), and the independent variables, namely solid:liquid ratio (X_1), time (X_2) and intensity (X_3). These equations allow the prediction of the response of each variable (74).

Equation 1: $Y_1 = 19.17 - 0.0638\,X_1 + 0.2500\,X_2 + 0.2762\,X_3 + 0.2675\,X_1X_2 + 0.3700\,X_1X_3 + 0.4925\,X_2X_3 + 0.0900\,X_1^2 + 1.00\,X_2^2 - 0.6750\,X_3^2$

Equation 2: $Y_2 = 13.85 - 1.24\,X_1 + 0.2862\,X_2 + 1.17\,X_3 + 1.05\,X_1X_2 - 0.4525\,X_1X_3 - 0.8100\,X_2X_3 - 0.3050\,X_1^2 - 2.21\,X_2^2 - 1.25\,X_3^2$

Equation 3: $Y_3 = 9.07 - 0.6100\,X_1 + 0.2725\,X_2 + 1.13\,X_3 + 1.35\,X_1X_2 + 0.1125\,X_1X_3 - 0.1775\,X_2X_3 - 1.78\,X_1^2 - 1.19\,X_2^2 - 1.13\,X_3^2$

Equation 4: $Y_4 = 105.24 - 0.3388\,X_1 + 2.87\,X_2 + 6.81\,X_3 + 9.25\,X_1X_2 + 8.50\,X_1X_3 - 1.77\,X_2X_3 - 11.18\,X_1^2 - 5.95\,X_2^2 - 9.05\,X_3^2$

Table 8. RSM model analysis through ANOVA statistical test of TPC, ABTS, DPPH and FRAP of the prepared extracts.

Source	Sum of squares				Mean squares				F value				*p*-value			
	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4
Model	8.86	61.43	48.50	2212.73	0.9848	6.83	5.39	245.86	21.06	5.87	4.63	9.95	0.0003 *	0.0146 *	0.0278 *	0.0031 *
X_1	0.0325	12.35	2.98	0.9180	0.0325	12.35	2.98	0.9180	0.6952	10.63	2.56	0.0372	0.4319	0.0139 *	0.1536	0.8526
X_2	0.5000	0.6555	0.5940	65.78	0.5000	0.6555	0.5940	65.78	10.69	0.5640	0.5110	2.66	0.0137 *	0.4771	0.4979	0.1467
X_3	0.6105	11.02	10.17	371.42	0.6105	11.02	10.17	371.42	13.05	9.48	8.75	15.04	0.0086 *	0.0178 *	0.0212 *	0.0061 *
X_1. X_2	0.2862	4.39	7.26	342.07	0.2862	4.39	7.26	342.07	6.12	3.78	6.25	13.85	0.0426 *	0.0931	0.0410 *	0.0074 *
X_1. X_3	0.5476	0.8190	0.0506	289.00	0.5476	0.8190	0.0506	289.00	11.71	0.7047	0.0435	11.70	0.0111 *	0.4289	0.8406	0.0111 *
X_2. X_3	0.9702	2.62	0.1260	12.50	0.9702	2.62	0.1260	12.50	20.75	2.26	0.1084	0.5059	0.0026 *	0.1766	0.7516	0.4999
X_1^2	0.0341	0.3917	13.35	526.57	0.0341	0.3917	13.35	526.57	0.7292	0.3370	11.48	21.32	0.4214	0.5797	0.0116 *	0.0024 *
X_2^2	4.23	20.61	5.92	149.09	4.23	20.61	5.92	149.09	90.48	17.74	5.09	6.04	< 0.0001 *	0.0040 *	0.0586	0.0437 *
X_3^2	1.92	6.61	5.34	345.08	1.92	6.61	5.34	345.08	41.02	5.68	4.59	13.97	0.0004 *	0.0486 *	0.0694	0.0073 *
Residual	0.3274	8.14	8.14	172.92	0.0468	1.16	1.16	24.70								
Lack of fit	0.2270	4.14	3.69	168.12	0.0757	1.38	1.23	56.04	3.01	1.38	1.11	46.68	0.1571	0.3691	0.4441	0.0014 *
Pure error	0.1004	3.99	4.45	4.80	0.0251	0.9980	1.11	1.20								
Total	9.19	69.57	56.64	2385.64												

R^2 (Y_1) = 0.9644	R^2 pred (Y_1) = 0.5878	R^2 adjust (Y_1) = 0.9186	Adeq Precision (Y_1) = 17.1301
R^2 (Y_2) = 0.8831	R^2 pred (Y_2) = −0.0425	R^2 adjust (Y_2) = 0.7327	Adeq Precision (Y_2) = 7.4276
R^2 (Y_3) = 0.8563	R^2 pred (Y_3) = −0.1655	R^2 adjust (Y_3) = 0.6716	Adeq Precision (Y_3) = 6.2837
R^2 (Y_4) = 0.9275	R^2 pred (Y_4) = −0.1307	R^2 adjust (Y_4) = 0.8343	Adeq Precision (Y_4) = 9.4147

Y_1 – TPC (mg GAE/g dw); Y_2 – ABTS (mg AAE/g dw); Y_3 – DPPH (mg TE/g dw); Y_4 – FRAP (µmol FSE/g dw); X_1 – solid:liquid ratio (%w/v); X_2 – ultrasonic time (min); X_3 – ultrasonic intensity (W/m²).
* significance at $p < 0.05$

4.1.1. Response surface analysis

The graphic relationship between the dependent (TPC, ABTS, DPPH and FRAP assays) and independent variables (solid: liquid ratio, time, and intensity) is represented in the 3D graphs obtained by the RSM experimental design (Figure 11). Intensity was fixated on 50 W/m^2

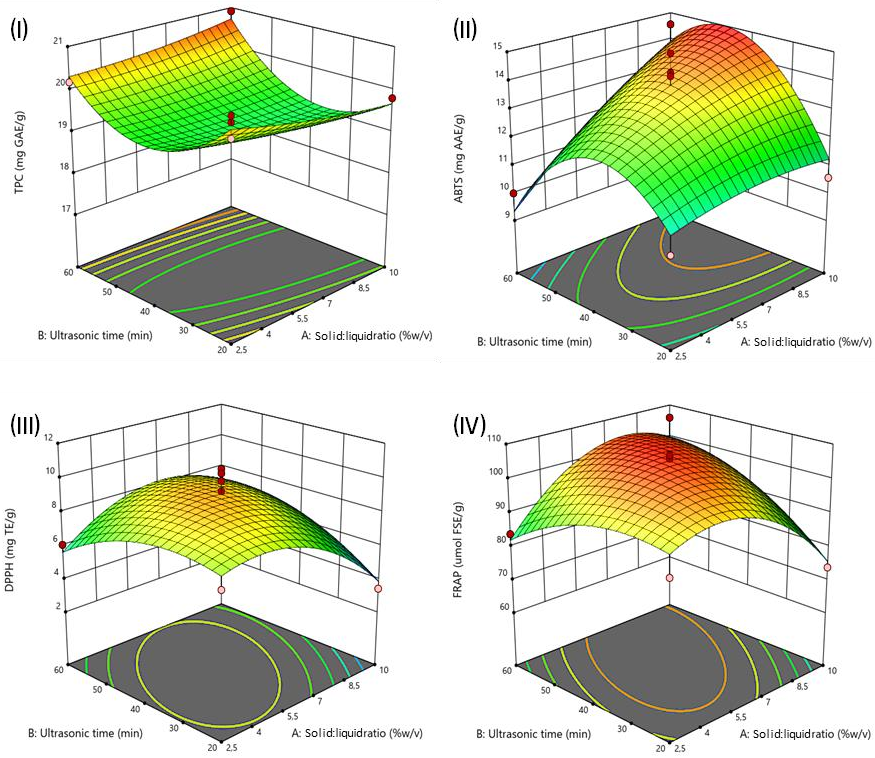

Figure 11. RSM 3D graphics for the interaction between independent (solid:liquid ratio and ultrasonic time) and dependent variables (I: TPC, II: ABTS, III: DPPH, IV: FRAP).

According to Figure 11, the TPC response differed notably from the other responses determined by the ABTS, DPPH, and FRAP experiments. In fact, when the extraction time and the solid:liquid ratio varied between the maximum values assessed (40 and 60 min and 5 and 10% *w/v*, respectively), a stronger antioxidant (FRAP = 106.79 mol FSE/g dw) and antiradical (ABTS = 14.95 mg AAE/g dw; DPPH = 10.29 mg TE/g dw) activity was observed. This data suggests that under these UAE conditions more bioactive compounds with antioxidant activity are extracted from the natural matrix. This hypothesis is reaffirmed by the TPC achieved (TPC = 20.91 mg GAE/g dw) when the maximum time and solid:liquid ratio (60 min and 10% *w/v*, respectively) were used.

Therefore, in general, higher extraction times (40 – 60 min) and solid:liquid ratios (6.25 – 10 % *w/v*) lead to better responses, which is corroborated by the desirability graph (Figure 12).

Higher ultrasonic times promote indirect heating of the extraction solution, leading to an enhanced solubility of specific compounds and, consequently, more phenolic compounds are effectively extracted from the source material (40). Noteworthy, the intensity impact was negligible on all responses studied. Still, it is anticipated that the employment of higher sonication intensities will result in the extraction of a greater number of compounds and, consequently, a higher biological activity. This occurs since a higher sonication intensity generates an increased formation of cavitation bubbles and elevated pressure, leading to a more efficient cell rupture and the subsequent extraction of intracellular compounds (58).

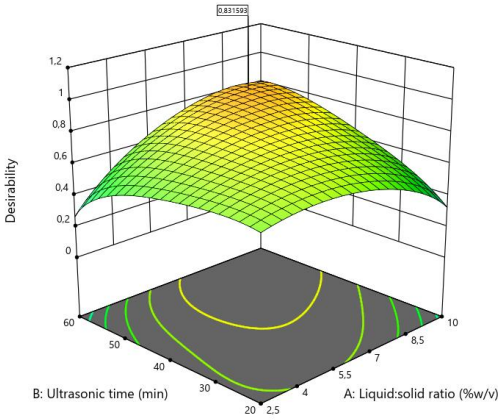

Figure 12. RSM 3D graphic for the desirability index.

The desirability index (Figure 12) shows the predicted optimal extraction conditions that allow to maximize the response variables (TPC, ABTS, DPPH and FRAP). The predicted optimal UAE conditions are a solid:liquid ratio of 8.75%, for 56.21 min through an ultrasonic intensity of 59.05 W/m² (R^2 = 0.831593).

After performing the UAE under the predicted optimal conditions, the dependent variables (TPC, ABTS, DPPH and FRAP) were evaluated once again. As can be observed in Table 9, no significant differences (p > 0.05) were observed between the predicted values and the experimental ones, validating the RSM model used in this work.

Table 9. Experimental and predicted value of TPC, ABTS, DPPH and FRAP for optimal goji berries extract.

	TPC (mg GAE/g)	ABTS (mg AAE/g)	DPPH (mg TE/g)	FRAP (µmol FSE/g)
Experimental value*	23.87 ± 1.47	15.15 ± 1.13	10.25 ± 0.81	105.97 ± 8.20
Predicted value	20.45	13.73	8.29	106.60
***p*-value**	0.066	0.067	0.258	0.266

* Results are expressed as mean ± standard deviation (n = 3).

4.2. Characterization of the optimal *L. barbarum* berries extract

4.2.1. Bioactive compounds and biological activity *in vitro*

Given its association with a number of health benefits (36), phenolic compounds — one of the most abundant and widely distributed groups of natural phytochemicals (54) — have recently gained substantial relevance in diets (3). Aside from the anti-inflammatory, antimicrobial, anti-viral, cardioprotective and neuroprotective effects (33, 35, 54), the antioxidant/antiradical activity is one of the most renowned capacities of phytochemicals.

The TPC was measured in the current investigation in a straightforward and repeatable spectrophotometric approach (75). In the presence of the Folin-Ciocalteu, polyphenols, cyclic derivatives of benzene with one or more hydroxyl groups associated with the aromatic ring (54), act as reducing agents, forming a bluish complex with a maximum absorbance at 765 nm (69). However, due to the reagent chemical structure, interferences from substances like sugars, ascorbic acid, sulphur dioxide, and aromatic amines and aromatic amines may occur (76). As can be observed in Table 9, the optimal extract demonstrated a considerable TPC (23.87 mg GAE/g dw), which is in accordance with studies conducted by other authors. For instance, Mocan *et al.* (15) reported a TPC of 15.70 mg GAE/g dw for a goji berries extract prepared in a sonication bath with methanol/water (70:30, *v:v*) as solvent during one hour, with a solid:liquid ratio of 10% (*w/v*). On the other hand, Benchennouf *et al.* (77) reported a TPC of 14.13 mg GAE/g dw for goji berries extracted through Soxhlet using water as solvent. Moreover, a TPC of 14.118 mg GAE/g dw was achieved for goji berries extracted by ultrasound at 65 °C for 1 h using 75% of ethanol and a solid:liquid ratio of 1:10 (*w/v*) (78). In addition, Rocchetti *et al.* (79) and Ceccarini *et al.* (80), also evaluated the TPC of goji berries extracted by ultra-turrax and reported lower results than the ones obtained in the present work (8.94 and 12.78 mg GAE/g dw, respectively).

As for the antioxidant/antiradical activities of the optimal extract (Table 9), three different methods were employed: ABTS, DPPH and FRAP assays. In what concerns to the ABTS assay, the optimal extract achieved an inhibition value of 15.15 mg AAE/g dw. Mendes *et al.* (81) also evaluated the ABTS scavenging capacity of a *L. barbarum* berries extracts

obtained by MAE at 100 °C for 8 min, using 25% of methanol as solvent, and reported values between 5.5 and 7.6 mg AAE/g dw.

Regarding the DPPH assay, the optimal extract demonstrated a scavenging capacity of 10.25 mg TE/g dw, being in concordance with the results reported for an extract obtained by sonication bath for 1 h at room temperature and using methanol/water (70:30, *v/v*) as solvent (9.35 mg TE/g dw) (15). Nevertheless, the results obtained in the present work are higher than the ones described by Fatchurrahman *et al.* (18) (3.12 mg TE/g fw) for a extraction performed with a ultra-turrax for 1 min using methanol/water (80:20, *v/v*) as solvent. Oppositely, Feng *et al.* (78) reported a DPPH scavenging capacity of 36.80 mg TE/g dw for the extract of goji berries obtained by ultrasounds with 75% of ethanol and a solid:liquid ratio of 1:10 (*w/v*), performed at 65 °C for 1 h.

The optimal extract demonstrated a strong antioxidant capacity for the FRAP assay, achieving values of 105.97 µmol FSE/g dw. In the study conducted by Benchennouf *et al.* (77) the authors reported values of 80.00 µmol FSE/g dw, being the extract prepared by a conventional solid-liquid extraction with water and orbital shaking of the extraction solution for 3 hours at 300 rpm.

4.2.2. Phytochemical profile

The identification and quantification of phytochemical compounds in natural matrices is an essential step to understand their potential beneficial properties (33, 54). Notably, the promising outcomes achieved in the previous assays may be attributed to the phytochemical profile of goji berries (82), which are known for their extremely rich composition in phenolic, flavonoid, carotenoid, and anthocyanin compounds (16, 34). Therefore, LC/DAD-ESI-MS analysis was carried out to identify and quantify the compounds present in the optimal goji berries extract. Table 10 summarizes the compounds identified in the optimal goji berries extract based on the retention time and the fragment ions detected by the mass spectrometer, while Figure 13 presents the chromatogram obtained for the optimal extract.

Table 10. List of phytochemical compounds identified and quantified in the optimal *L. barbarum* berries extract through LC/DAD-ESI-MS analysis.

Peak	Compound	Rt (min)	$[M-H]^-$ (m/z)	Fragments (m/z)	Quantification (mg/100 g dw) *
	Phenolic acids				
1	Feruloylquinic acid	1.23	377	377, 191	0.20 ± 0.01
2	3,5-dicaffeoylquinic acid	3.46	515	515, 353, 191	5.76 ± 0.29
	Σ Phenolic acids				**5.96 ± 0.30**
	Flavonoids				
11	Rutin hexoside	7.09	771	771, 609, 301, 179, 151	1.39 ± 0.07
12	Rutin	8.20	609	609, 301, 179, 151	19.27 ± 0.96
13	Kaempferol-3-*O*-rutinoside	10.65	593	593, 285, 257, 229	0.90 ± 0.04
14	Isorhamnetin-3-*O*-rutinoside	11.52	623	623, 316, 300, 271	1.60 ± 0.08
8	Rutin exoside	4.51	771	771, 609, 301, 179, 151	12.85 ± 0.64
	Σ Flavonoids				**36.01 ± 1.80**
	Others				
3	3-Glu-kukoamine	3.54	1015	1015, 853, 690, 529	17.63 ± 0.88
4	2-Glu-kukoamine (isomer 1)	3.62	853	690, 529	10.22 ± 0.51
5	2-Glu-kukoamine (isomer 2)	3.79	853	853, 690, 529	104.96 ± 5.25
6	Glu-lycibarbarspermidine F (isomer 1)	4.15	956	956, 795, 470	15.18 ± 0.76
7	Glu-lycibarbarspermidine F (isomer 2)	4.50	956	956, 795, 470	126.72 ± 6.34
9	Lycibarbarspermidine B	4.80	632	632, 470, 334	17.07 ± 0.85
10	Spermidine	5.53	470		0.96 ± 0.05
15	Corosolic acid	22.60	471	471, 453	83.23 ± 4.16
	Σ Others				**375.97 ± 18.80**

Rt – Retention time. * Results are expressed as mean ± standard deviation ($n = 3$).

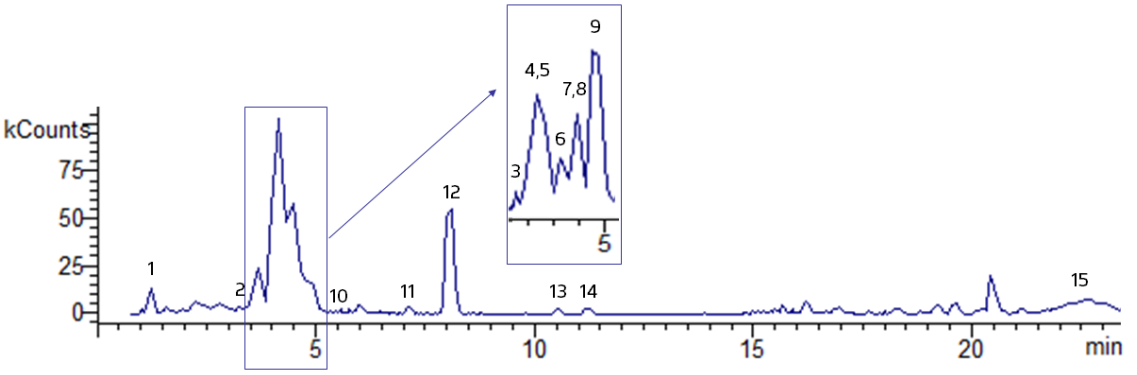

Figure 13. LC/DAD-ESI-MS chromatogram for the optimal *L. barbarum* berries extract; peak identification is presented in Table 10.

It is noteworthy that cultivation factors, namely variety, geographic location, and climatic conditions, play a crucial role in the definition of the phytochemical profile of a plant (19, 27). Depending on these intrinsic and extrinsic factors, the plants metabolism tends to produce different bioactive compounds (83). Moreover, the extraction techniques, and conditions employed, especially the solvent used, influence the extracts phytochemical profile (5, 33).

As can be observed in Table 10, a total of fifteen phytochemical compounds were identified in the optimal goji berries extract: two phenolic acids (5.96 mg/100 g dw), five flavonoids (36.01 mg/100 g dw), and eight other compounds (375.97 mg/100 g dw). Among these categories, flavonoids (peaks 8, 11, 12, 13 and 14) were the most abundant phenolic compounds (36.01 mg/100 g dw), with rutin (19.27 mg/100 g dw) being the major one, followed by their various derivatives, such as rutin hexoside (1.39 mg/100 g dw) and rutin exoside (12.85 mg/100 g dw). The second category of phenolic compounds detected in greatest amount (5.96 mg/100 g dw) were phenolic acids (peaks 1 and 2), comprising feruloylquinic acid (0.20 mg/100 g dw) and 3,5-dicaffeoylquinic acid (5.76 mg/100 g dw). These phenolic compounds (Figure 14) hold significant potential for therapeutic administration (33). For instance, the predominant phenolic compound detected in the optimal extract, rutin, a natural flavonoid compound, have demonstrated a strong antioxidant potential, acting as a powerful free-radical scavenger (6, 31). This holds true for its several derivatives, including rutin hexoside, rutin exoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside, which are present in the optimal goji berries extract (84). Similarly, feruloylquinic acid and 3,5-dicaffeoylquinic acid, derivatives of caffeoylquinic acid, have also demonstrated strong antioxidative properties (85).

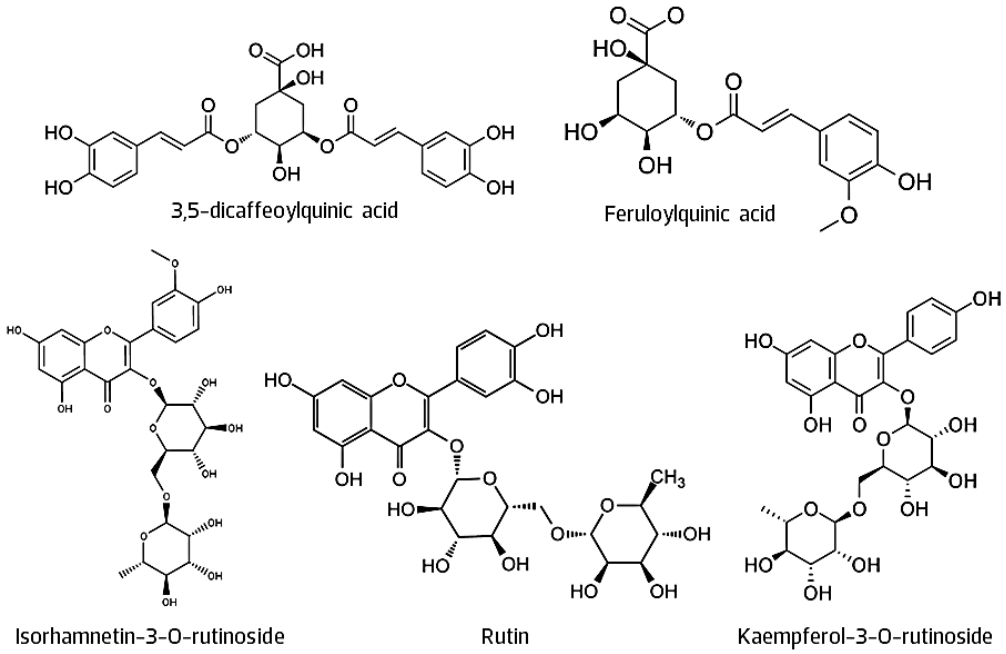

Figure 14. Chemical structures of the phenolic compounds identified and quantified through LC/DAD-ESI-MS analysis in the optimal *L. barbarum* berries extract.

Based on the retention time and the fragments detected by the mass spectrometer, peaks 3, 4, 5, 6, 7, 9, 10 and 15 were identified as 3-glu-kukoamine, 2-glu-kukoamine (isomer 1), 2-glu-kukoamine (isomer 2), glu-lycibarbarspermidine F (isomer 1), glu-lycibarbarspermidine F (isomer 2), lycibarbarspermidine B, spermidine and corosolic acid, respectively. Some of these compounds, namely spermidines and their derivatives, have been identified as characteristic chemical markers of *Lycium* fruits (15). Similarly to the present work, other studies have identified some of these compounds in *L. barbarum* berries. For instance, Xiao *et al.* (86) reported the presence of rutin, spermidine, 3-glu-kukoamine, 2-glu-kukoamine, glu-lycibarbarspermidine F and lycibarbarspermidine B in a goji berries extract prepared by ultrasound using ethanol and water (70:30, *v/v*) as solvent. Various glycosylated kukoamines, lycibarbarspermidines and glycosylated lycibarbarspermidines were also detected in *L. barbarum* berries extracts obtained in the study conducted by dos Santos *et al.* (87).

These compounds, which are represented in Figure 15, also have valuable biological activities (87, 88). Corosolic acid, commonly known as plant insulin, is a triterpenoid found in a variety of traditional Chinese medicines and is well known for its antidiabetic properties (89, 90). The remaining compounds may fall under the category of phenylamides, often

referred to phenolamides or hydroxycinnamic acid amides, which are generated when hydroxycinnamic acids are conjugated with amines (91). These compounds exhibit similar bioactivity to the unconjugated compounds since they retain the properties of their parent molecules (91). Spermidines, characteristic chemical markers of *Lycium* fruits (15), consist in a polyamine alkaloid with a carbon/nitrogen backbone (N-C_3-N-C_4-N) and show pro-healthy properties (87), namely antiaging, antihypertension, antidiabetic, and immune modulation (91). Their derivatives, such as lycibarbarspermidines and kukoamines, demonstrated similar activities (91). For instance, lycibarbarspermidines, also referred to as dicaffeoylspermidine, present a potent antioxidant potential as well as the ability to improve short-term memory in Alzheimer's patients (87). Lycibarbarspermidines consist of caffeic and/or hydrocaffeic acids and spermidine is connected by an amide bond (92). Kukoamines are phenylamines composed of a polymethylenepolyamine backbone, which includes molecules like putrescine, spermidine, and spermine, and at least one phenolic acid, such as dihydrocaffeic and caffeic acid (33, 87, 93). These compounds and their derivatives exhibit a wide range of bioactivities, making them versatile compounds with health benefits, including antihypertensive, antilipidic peroxidation and lipoxygenase effect, antisepsis, and neuroprotection (93).

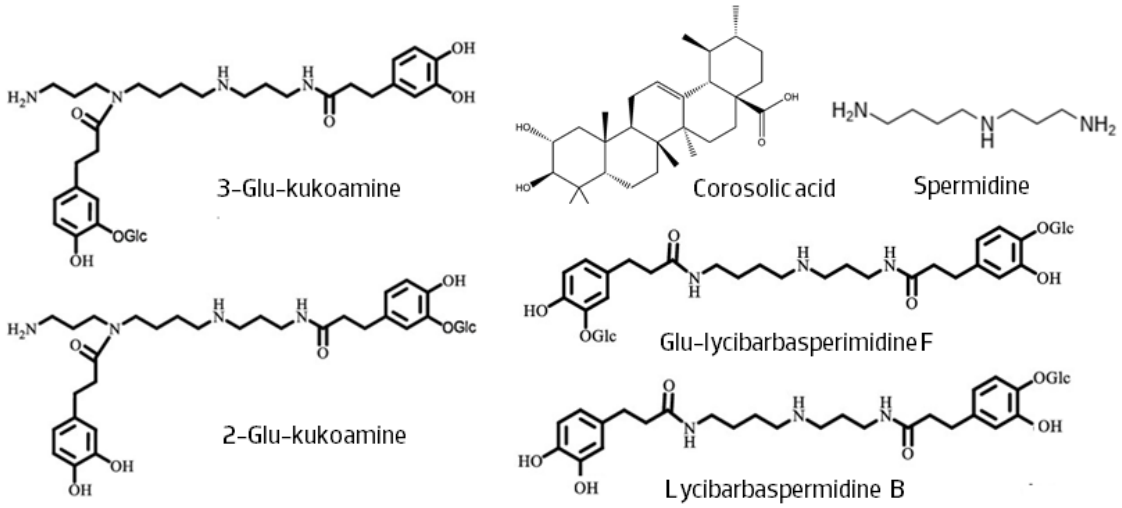

Figure 15. Example of chemical structures of other compounds identified and quantified through LC/DAD-ESI-MS analysis in the optimal *L. barbarum* berries extract.

Xiao *et al.* (86) analyzed a goji berries extract prepared by ultrasound and detected the presence of rutin by UPLC-Q-Orbitrap-MS/MS in lower quantities than in the present study (9.3 mg/ 100 g dw *vs* 19.27 mg/100 g dw). The same occurred with Wu *et al.* (84) study

were the extract prepared by UAE presented 3,5- dicaffeoylquinic acid (3.70 mg/100 g dw), kaempferol-3-*O*-rutinoside (0.30 mg/100 g dw) and isorhamnetin-3-*O*-rutinoside (0.70 mg/100 g dw) in lower amounts than in the present study (5.76, 0.90 and 1.60 mg/100 g dw, respectively). All the studies mentioned above consistently report lower quantities of phenolic compounds in goji berries extracts. Given that none of them used the same UAE conditions as the ones employed in the present study, the data leads to the belief that the UAE conditions predicted by the RSM model have a significant impact on the recovery of bioactive compounds from goji berries. Yet, it is important to point out that the variations between the present study and the other authors may not solely be the result of the various extraction conditions or methods used. As previously stated, the phenolic profile of goji berries is also significantly influenced by the cultivation regions and seasons, along with the harvesting and transportation process (21, 88).

Besides that, most of the compounds extracted from goji berries in the present work have glycosyl residues. It has been previously reported that some phytochemical compounds exist in nature linked to other structures, such as proteins, fibers, or sugars (94, 95). In some cases, treatments such as alkaline or acid hydrolysis, employed alongside the extraction process, may lead to the break of bonds, transforming complex molecules into less complex ones (94), namely simple phenolic compounds. For example, the use of ethanol as solvent in the extraction of glycosylated compounds, such as kaempferol-3-*O*-rutinoside, can conduct to the breaking of glycosidic bonds, releasing the simple phenolic compound, kaempferol, and the rutinose residue (96). Since in the present study water was used as solvent, and no thermal or hydrolysis treatments were applied, this information suggests that the compounds extracted from goji berries may contain more complex structures due to the absence of hydrolysis treatments during the extraction step.

The overall presence of the phytochemical compounds detected In the *L. barbarum* berries optimal extract play a vital role in the bioactivity of the extract, namely combating the oxidative stress, a factor associated with various health conditions, including aging and degenerative diseases (33, 87). Hence, the phytochemical profile of the optimal extract is a preliminary indication that goji berries may indeed hold considerable value within the nutraceutical or pharmaceutical industries.

4.2.3. *In Vitro* Scavenging Capacity of ROS and RNS

ROS and RNS are physiologically generated in low concentrations promoting important processes in organisms, such as homeostasis and cell signaling (21, 83). However, if at any point the concentration of these species exceeds the antioxidant/antiradical capacity of the cellular systems, a state of oxidative stress occurs (31). The oxidative stress is associated with the development of diseases such as cardiovascular dysfunction, various typologies of cancer, neurodegenerative diseases and several autoimmune diseases linked to degenerative processes of aging (5).

Plants produce polyphenols as antioxidant/antiradical mechanisms to prevent oxidative stress (31) due to the capacity of these compounds to neutralize reactive species (30) and, therefore, prevent cell damage. Hence, consuming natural matrices rich in polyphenol compounds can bring numerous health benefits to consumers. In this sense, it is important to assess the capacity of the optimal extract to scavenge ROS and RNS (Table 11).

Table 11. Superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\cdot}$), and peroxinitrite ($ONOO^-$) in the absence and presence of sodium bicarbonate ($NaHCO_3$) scavenging capacities of the optimized *L. barbarum* berries extract.

Sample	ROS				RNS	
	$O_2^{\cdot -}$	H_2O_2	HOCl	$ROO^{\cdot}$	$ONOO^-$	$ONOO^-$ w/ $NaHCO_3$
	(IC_{50} (μg/mL))			(μmol TE/mg dw)	(IC_{50} (μg/mL))	
Optimized extract	225.25 ± 53.26 [b]	n.d.	12.99 ± 1.13 [c]	0.15 ± 0.01 [c]	57.78 ± 4.26 [b]	93.51 ± 19.65 [b]
Positive controls						
Catechin	18.01 ± 0.77 [a]	n.d.	0.20 ± 0.03 [a]	6.37 ± 2.32 [a]	0.19 ± 0.03 [a]	0.29 ± 0.07 [a]
Gallic acid	6.34 ± 0.53 [a]	193.50 ± 17.68	2.60 ± 0.14 [b]	2.45 ± 0.54 [b]	0.20 ± 0.004 [a]	0.28 ± 0.05 [a]

IC_{50} - *in vitro* concentration required to decrease in 50% the reactivity of the studied reactive species; n.d. – IC_{50} was not determined up to the highest tested concentration (1000 μg/mL). Results are expressed as mean ± standard deviation ($n = 3$). Different letters ([a,b,c]) in the same column mean significant differences ($p < 0.05$).

The results obtained attest that goji berries possess a great capacity for scavenging ROS and RNS and highlight the potential of goji berries as potent antioxidants. Yet, it is essential to recognize that variations in the units in which the results are expressed, extraction

methods, and the utilization of extract fractions in different studies create difficulties when attempting to directly compare results.

$O_2^{\cdot -}$ and H_2O_2 play a crucial role in multiple physiological processes, such as promoting a normal vascular physiology (97). While these species are not considered potent pro-oxidants, they are a starting point for oxidative stress (98) due to their capacity of acting as precursors of powerful po-oxidants (83). Regarding the $O_2^{\cdot -}$, the radicals were generated by the NADH/PMS system and their scavenging was measured by the reduction of NBT to diformazan (73). The optimal extract (IC_{50} = 225.25 μg/mL) demonstrated a significant lower ($p < 0.05$) quenching potential than the positive controls, catechin and gallic acid (IC_{50} of 18.01 and 6.34 μg/mL, respectively), being necessary a higher concentration of extract to promote a 50% decrease in the reactivity of $O_2^{\cdot -}$ when compared to the positive controls. As for the H_2O_2 scavenging capacity of the optimal extract, it was assessed by applying a procedure based on the luminescence of lucigenin (99). As can be seen in Table 11, it was not possible to determine the IC_{50} for the concentrations of the optimal extract. Therefore, the extract H_2O_2 scavenging capacity was expressed in terms of percentages of inhibition for the maximum concentration tested (1000 μg/mL). The optimal extract achieved an inhibition of 16.60%, while catechin showed a percentage of inhibition of 31.40% and gallic acid reached an IC_{50} value of 193.50 μg/mL.

HOCl is a powerful oxidant that is produced physiologically by active neutrophils and monocytes as a defense mechanism against different threats (96, 100). Its generation usually happens by the combination of $O_2^{\cdot -}$ and H_2O_2 (83) or H_2O_2 and Cl^- (98). The optimal extract exhibited a good scavenging capacity of HOCl (IC_{50} = 12.99 μg/mL). Still, the positive controls achieved best results, namely IC_{50} of 0.20 μg/mL for catechin and 2.60 μg/mL for gallic acid.

Concerning the scavenging capacity of $ROO^{\cdot}$, the ORAC assay was employed. In the present work, it was not possible to calculate the IC_{50} value at the maximum concentration of the goji berries extract tested (1000 μg/mL), being the results expressed as micromole of trolox equivalents per milligram of extract on dw (μmol TE/mg dw). The optimal extract demonstrated a good scavenging capacity (0.15 μmol TE/mg dw), while the positive controls, catechin and gallic acid, achieved scavenging capacities of 6.37 and 2.45 μmol

TE/mg dw, respectively. These results are consistent with previous studies reporting $ROO^{\cdot}$ scavenging capacities that ranged from 0.01 μmol TE/mg dw (79) to 0.24 μmol TE/mg dw (78) for goji berries extracts prepared by different techniques (ultra-turrax and ultrasound, respectively) and under different extraction conditions (10 mL of 1% formic acid in an 80:20 methanol/water solution at 25000 rpm for 3 min and 75% ethanol with a solid:liquid ratio of 1:10 (*w/v*) at 65 °C for 1 h, respectively).

In what concerns to the scavenging capacity of RNS, specifically $ONOO^-$, a powerful pro-oxidant and detrimental compound (98), the optimal goji berries extract achieved a noteworthy result in the absence of $NaHCO_3$, with an IC_{50} value of 57.78 μg/mL. The same happen to the positive controls, catechin and gallic acid, accomplishing a better scavenging capacity in the absence of $NaHCO_3$ (IC_{50} = 0.19 and 0.20 μg/mL, respectively). It has previously been noted that the presence of $NaHCO_3$ allows a closer approximation to physiological conditions (99). However, the presence of sodium bicarbonate may also lead to the formation of $ONOO^-$ derivatives, which affects the reactivity of the $ONOO^-$ scavengers, possibly decreasing their effects (73, 99). This phenomenon has been verified in studies using other natural matrices such as *Actinidia arguta* leaves (99), *Arrabidaea chica* leaves (96), *Castanea sativa* and *Quercus robur* leaves (98), *Bixa orellana* L. seeds (101), and *Psidium cattleianum* pulp and skin (102). Thus, a higher concentration of goji berries extract is required to inhibit half of the $ONOO^-$ radical activity in the presence of $NaHCO_3$ than in its absence.

It is conceivable that the phenolic compounds found in the goji berry extract (Table 10) — such as feruloylquinic acid, 3,5-dicaffeoylquinic acid, rutin, and its derivatives — are responsible for the ROS and RNS scavenging activity observed, since they show to be effective neutralizers of ROS and RNS (6, 31).

For instance, it has been noted in previous studies that the scavenging efficacy of flavonoids is positively influenced by an increased number of hydroxyl (OH) groups substitution in the C-ring of the phenolic structure (98, 100, 103). OH groups contribute to the compounds' ability to donate hydrogen atoms or electrons, thereby neutralizing reactive species and preventing oxidative damage (100, 103). Additionally, the location of these substitutions also plays a significant role in enhancing the antioxidant effect of the compounds.

Specifically, OH groups substitutions at position 3 of the C-ring have been established to contribute to improve the antioxidant activity (100). Therefore, rutin and its derivatives, given their number and location of OH substitutions (33, 65), may have a strong influence in the scavenging capacity of the optimal extract.

Regarding phenolic acids, it has been established that the CH=CH-COOH group, which is present in the hydroxycinnamic acids, greatly contributes to the antioxidant efficiency of these compounds (95, 104). Therefore, given that both phenolic compounds identified in the optimal goji berries extract are hydroxycinnamic acids, feruloylquinic acid and 3,5-dicaffeoylquinic acid (Figure 14), their contribution for the free-radical scavenging potential of the extract should be considered.

Nevertheless, one of the great advantages of natural plant extracts in the synergic effect that all the compounds exert together (98). Hence, the overall phytochemical compounds present in the optimal extract likely account for the demonstrated ROS and RNS scavenging capacity of the goji berries extract.

4.3. *In vitro* cell studies

In vitro assays are an accurate, fast, and reproducible method to evaluate the effect of bioactive compounds in living cells (60). Therefore, to determine if bioactive compounds can positively affect cell proliferation or exert a cytotoxic effect, and therefore be applied in the nutraceutical industry, the performance of cell viability assays are extremely important (64, 105). In this work, the safety of the optimal goji berries extract was assessed by an MTT assay on intestinal cell lines, namely Caco-2 and HT29-MTX. These lines were selected as intestinal cell models to evaluate the potential effects of the optimal extract on small intestine, the principal place where the absorption of bioactive compounds occurs (62). The extract was tested in a concentration range of 0.1 – 1000 μg/mL and Figure 16 summarized the results obtained.

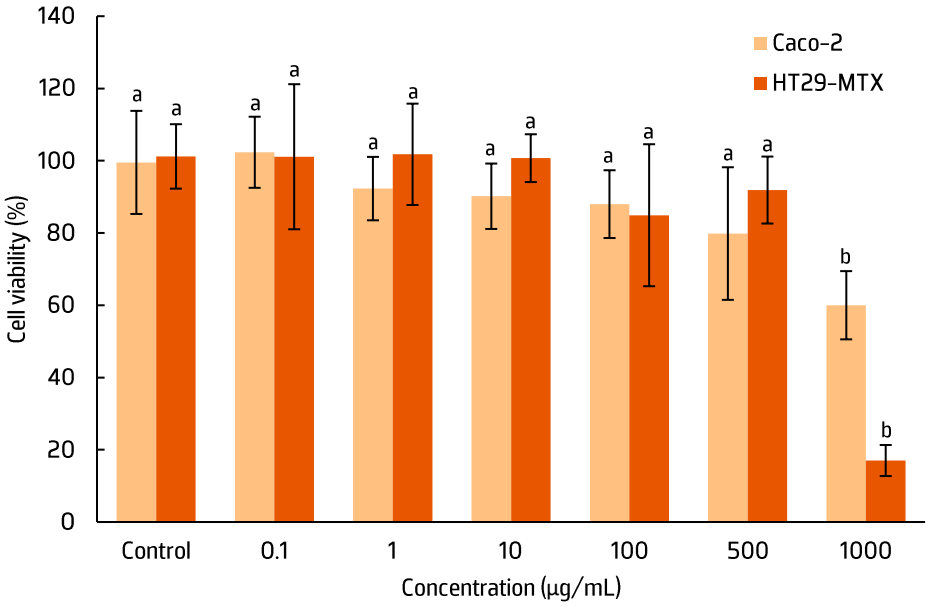

Figure 16. Effects of the exposure of the optimal *L. barbarum* berries extract on Caco-2 and HT29-MTX viability at different concentrations (0.1 – 1000 µg/mL). Results are expressed as mean ± standard deviations ($n = 3$). Different letters ($^{a, b}$) indicate significant differences between different concentrations of the same cell line ($p < 0.05$).

According to the obtained results, the optimal extract did not affect the viability of any of the cell lines in concentrations lower than 500 µg/mL, presenting results between 80% and 100%. However, the highest tested concentration, 1000 µg/mL, caused a significant decrease in the viability of both cell lines (60.00% and 17.02% in Caco-2 and HT29-MTX, respectively). Regarding Caco-2, the viability ranged between 60.00% and 102.34%, respectively for the concentrations of 1000 µg/mL and 0.1 µg/mL, with significant differences ($p < 0.05$) between the highest concentration and all other concentrations tested. Concerning HT29-MTX, the viability ranged between 17.02% and 101.10% for the concentration of 1000 µg/mL and 0.1 µg/mL, respectively. According to these results, the optimal extract did not affect the cells viability in concentrations between 0.1 µg/mL and 500 µg/mL, achieving viabilities of almost 80% and 100%. However, the highest concentration tested (1000 µg/mL) led to a significant ($p < 0.05$) decrease in the HT29-MTX viability (17.02%). Therefore, this study emphasizes the non-cytotoxic effect of the optimal goji berries extract in concentrations below 500 µg/mL in intestinal cell lines.

Several studies conducted with goji berries extracts focused on purifying the compounds that constitute the fruit in higher proportions. Wang *et al.* (106) tested the influence of *L. barbarum* polysaccharides (LBP) on Caco-2 cell viability across a concentration range

between 10 and 100 μg/mL. In all cases, the viability results were above 90%, suggesting the absence of toxicity. Likewise, Li *et al.* (107) assessed the effect of LBP on the same cell line and reported viabilities around 100% for LBP concentrations ranging from 0 to 400 μg/mL. In another study, Mao *et al.* (108) screened the effect of LBP in a wider range of concentrations (0 to 1000 μg/mL) and with 5 days of exposure. The authors reported that after 5 days, LBP inhibited the cell viability in concentrations between 200 and 1000 μg/mL, attesting a cell viability decrease in 50% for the concentration of 900 μg/mL (2 days of exposure) or 600 μg/mL (4 days of exposure).

To the best of our knowledge, this is the first study that explores the effects of the whole extract in Caco-2 and HT29-MTX cell lines.

4.3.1. 3D intestinal permeability

In recent years, cell-based methods have gained popularity among the scientific community (63). Since the effectiveness of a compound orally ingested depends on their absorption through the gastrointestinal tract, measuring the permeability of bioactive compounds at intestinal level is crucial (63, 94). The co-culture of Caco-2 and HT29-MTX is one of the most used models since it closely mimics *in vitro* the *in vivo* intestinal environment (67) in a simpler, less expensive, with fewer ethical issues, and highly reproducible way (63). Caco-2 cells simulate the human colon due to the presence of microvilli and tight junctions as well as various transporters, enzymes, and nuclear receptors, while HT29-MTX is able to simulate the goblet cells, allowing to evaluate the muco-adhesion of carrier systems (65, 66).

In this work, a co-culture intestinal model was performed to assess the permeability of the compounds present in the optimal goji berries extract. Based on the MTT assay results, the co-culture model was exposed to the optimal extract in a concentration of 500 μg/mL.

The various phytochemical compounds detected and quantified following the assay at the different time points are described in Table 12 and Figure 17. Figure 18 represent the acquired chromatograms after 240 min. To the best of our knowledge, this is the first investigation that was performed with goji berries extract on an intestinal model.

Table 12. Quantification of the optimal *L. barbarum* berries extract compounds after the intestinal co-culture permeation assay at different time points.

	Time (min)							
Permeability (%) *	15	30	45	60	90	120	180	240
Feruloylquinic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3,5-dicaffeoylquinic acid	16.56 ± 0.83 ^{a}	19.33 ± 0.97 ^{a,b}	22.70 ± 1.14 ^{a,b}	24.95 ± 1.25 ^{a,b}	28.32 ± 1.42 ^{b}	39.78 ± 1.99 ^{c}	47.75 ± 2.39 ^{c,d}	52.66 ± 2.63 ^{d}
Rutin hexoside	0.00 ± 0.00 ^{a}	11.12 ± 0.56 ^{b}	12.67 ± 0.63 ^{b}	18.96 ± 0.95 ^{c}	21.44 ± 1.07 ^{c}	24.40 ± 1.21 ^{c,d}	28.23 ± 1.41 ^{d,e}	32.31 ± 1.62 ^{e}
Rutin	4.98 ± 0.25 ^{a}	8.61 ± 0.43 ^{a,b}	9.95 ± 0.50 ^{a,b}	11.22 ± 0.56 ^{b}	13.85 ± 0.69 ^{b}	26.24 ± 1.31 ^{c}	30.93 ± 1.55 ^{c,d}	34.72 ± 1.74 ^{d}
Kaempferol-3-*O*-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-3-*O*-rutinoside	0.56 ± 0.03 ^{a}	2.34 ± 0.12 ^{a,b}	9.20 ± 0.46 ^{b}	19.12 ± 0.96 ^{c}	20.63 ± 1.03 ^{c}	34.67 ± 1.73 ^{d}	42.93 ± 2.15 ^{e}	49.31 ± 2.47 ^{e}
Rutin exoside	2.90 ± 0.14 ^{a}	3.42 ± 0.17 ^{a,b}	4.80 ± 0.24 ^{a,b,c}	5.41 ± 0.27 ^{b,c}	6.05 ± 0.30 ^{c}	9.30 ± 0.47 ^{d}	11.04 ± 0.55 ^{d,e}	12.24 ± 0.61 ^{e}
3-Glu-kukoamine	15.20 ± 0.76 ^{a}	16.73 ± 0.84 ^{a,b}	22.75 ± 1.14 ^{b,c,d}	24.09 ± 1.20 ^{c,d}	19.19 ± 0.96 ^{a,b,c}	25.42 ± 1.27 ^{c,d}	28.38 ± 1.42 ^{d,e}	34.45 ± 1.72 ^{e}
2-Glu-kukoamine (isomer 1)	9.06 ± 0.45 ^{a}	15.15 ± 0.76 ^{a,b}	18.98 ± 0.95 ^{b}	21.75 ± 1.09 ^{b,c}	27.07 ± 1.35 ^{c,d}	30.44 ± 1.52 ^{d}	34.29 ± 1.71 ^{d,e}	37.73 ± 1.89 ^{e}
2-Glu-kukoamine (isomer 2)	1.08 ± 0.05 ^{a}	2.77 ± 0.14 ^{b}	2.32 ± 0.12 ^{a,b}	3.49 ± 0.17 ^{b}	6.36 ± 0.32 ^{c}	6.38 ± 0.32 ^{c}	7.82 ± 0.39 ^{c,d}	8.74 ± 0.44 ^{d}
Glu-lycibarbarspermidine F (isomer 1)	2.28 ± 0.11 ^{a}	10.76 ± 0.54 ^{b}	13.22 ± 0.66 ^{b}	13.53 ± 0.68 ^{b}	15.01 ± 0.75 ^{b,c}	17.88 ± 0.89 ^{c,d}	19.39 ± 0.97 ^{d}	21.78 ± 1.09 ^{d}
Glu-lycibarbarspermidine F (isomer 2)	3.93 ± 0.20 ^{a}	22.61 ± 1.13 ^{b}	61.00 ± 3.05 ^{c}	61.00 ± 3.05 ^{c}	65.74 ± 3.29 ^{c}	67.22 ± 3.36 ^{c}	72.31 ± 3.62 ^{c}	73.70 ± 3.69 ^{c}
Lycibarbarspermidine B	1.55 ± 0.08 ^{a}	16.20 ± 0. 81 ^{b}	24.50 ± 1.23 ^{c}	24.50 ± 1.23 ^{c}	23.68 ± 1.18 ^{c}	24.84 ± 1.24 ^{c}	28.96 ± 1.45 ^{c}	37.27 ± 1.86 ^{d}
Spermidine	0.00 ± 0.00 ^{a}	6.38 ± 0.32 ^{b}	10.44 ± 0.52 ^{b,c}	11.63 ± 0.58 ^{c}	16.50 ± 0.83 ^{d}	19.69 ± 0.98 ^{d,e}	20.06 ± 1.00 ^{d,e}	22.75 ± 1.14 ^{e}
Corosolic acid	0.54 ± 0.03 ^{a}	0.77 ± 0.04 ^{a,b}	1.01 ± 0.05 ^{b,c}	1.33 ± 0.07 ^{c,d}	1.33 ± 0.07 ^{c,d}	1.47 ± 0.07 ^{d}	1.43 ± 0.07 ^{d}	1.55 ± 0.08 ^{d}

n.d. – not detected. * Results are expressed as means ± standard deviations ($n = 3$). Different letters (^{a,b,c,d,e}) in the same line mean significant differences ($p < 0.05$).

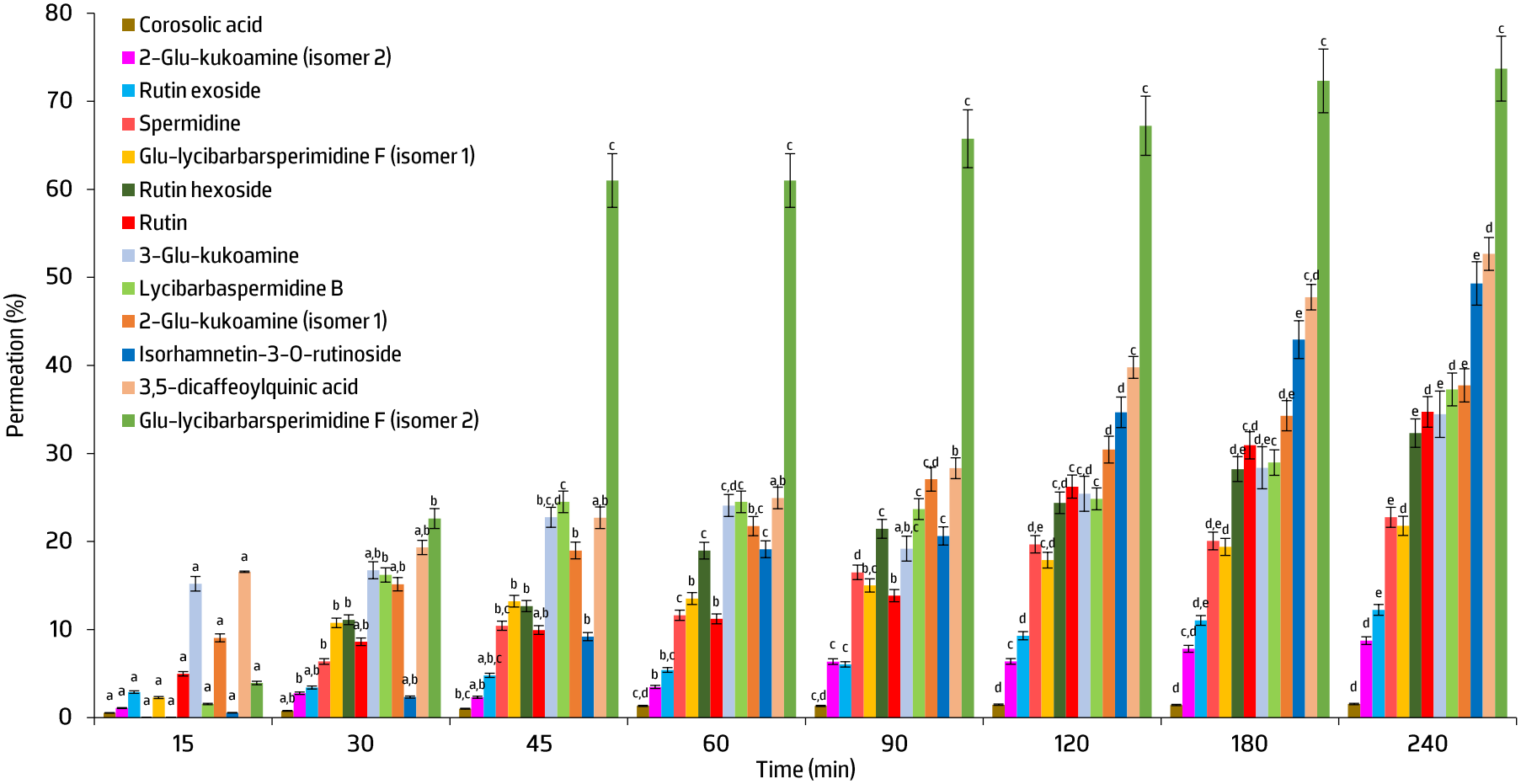

Figure 17. Permeation percentages of the compounds from the optimal *L. barbarum* berries extract detected in the intestinal permeation assay at different timepoints. Results are expressed as mean ± standard deviations ($n = 3$). Different letters ($^{a, b, c, d, e}$) indicate significant differences between different timepoints of each compound ($p < 0.05$).

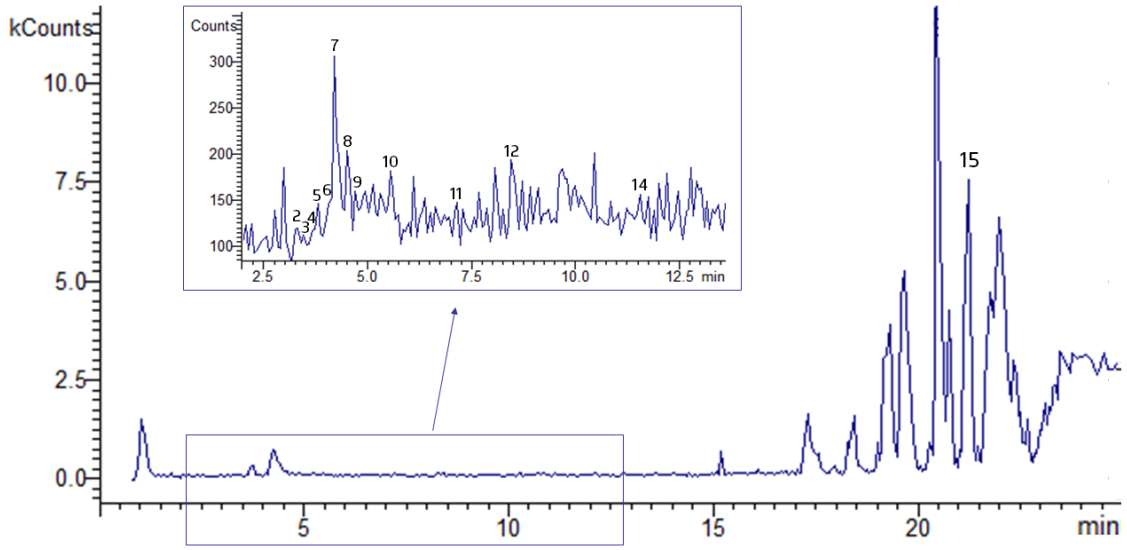

Figure 18. LC/DAD-ESI-MS chromatogram of the phytochemical compounds from the optimal *L. barbarum* berries extract that permeated the monolayer in the co-culture assay after 240 min; peak identification is presented in Table 10.

Glu-lycibarbarspermidine F (isomer 2) (Rt: 4.50 min; peak 7) was the compound detected in higher amounts in most of the timepoints, reaching an intestinal permeability of 73.70% at the final timepoint (240 min), followed by 3,5-dicaffeoylquinic acid (Rt: 3.46 min; peak 2; 52.66%) and isorhamnetin-3-*O*-rutinoside (Rt: 11.52 min; peak 14; 49.31%). Nevertheless, 3,5-dicaffeoylquinic acid (Rt: 3.46 min) achieved the highest permeation (16.56%) in the first timepoint (15 min). Overall, corosolic acid (Rt: 22.60 min; peak 15) was the compound detected in lower amounts in all timepoints, reaching an intestinal permeability that varied between 0.54% (15 min) and 1.55% (240 min).

Multiple parameters may influence the chemical intestinal permeation, such as the concentration, molecular size, compounds' structure and hydrophilicity, permeation time, and even the TEER (64, 94, 105). Moreover, different compounds require different times to reach equilibrium, which may justify the disparities in the permeation percentages among compounds. Similarly, the compound concentration used in the permeation assay deeply influences their permeation (94). For example, glu-lycibarbarspermidine F (isomer 2) was the compound quantified in greatest quantity in the optimal extract (126.71 mg/100 g dw) and showed the highest permeation (73.70%) by the end of the assay. Analogously, feruloylquinic acid and kaempferol-3-*O*-rutinoside, compounds detected in the optimal extract in lower amounts (0.20 and 0.90 mg/100 g dw), were not detected in the permeation assay.

The molecular size of a compound also greatly influences their permeability (64). For instance, 3,5-dicaffeoylquinic acid (515 m/z), isorhamnetin-3-*O*-rutinoside (623 m/z), and rutin (609 m/z), are relatively small. Their smaller size allows them to move more freely through the intestinal barrier (109), achieving higher permeations at the final timepoint (52.66, 49.31, and 34.72%, respectively). Yet, the compounds' molecular size present in the optimal extract depends on their glycosylation. The addition of sugar molecules increases the molecular weight and alters the compounds' three-dimensional structure and overall size (90). These modifications might prevent the molecules from passing through the monocellular layer by common molecular transport used (63), justifying the absence of kaempferol-3-*O*-rutinoside in the permeates.

Nonetheless, glycosylation often makes molecules more hydrophilic (90). This characteristic, alongside with the incorporation of HT29-MTX cell line in the co-culture model, is responsible for the improvement of the paracellular permeability of some compounds (64), such as glu-lycibarbarspermidine F (isomer 2), isorhamnetin-3-*O*-rutinoside, and 2-glu-kukoamine (isomer 1) that reached permeation percentages of 73.79, 49.31 and 37.73%, respectively, at the final timepoint. Oppositely, hydrophobic compounds, such as corosolic acid (90), displayed low permeations at the different timepoints (0.54 – 1.55%) despite the relatively high concentration in which it was applied to the assay (83.23 mg/100 g dw).

It should also be noticed the distinctive qualities of the several compounds present in the optimal extract along the intestinal permeability assay. Spermidine is an example since it has the ability to efficiently exploit specialized polyamine transporters found in intestinal cells to aid in its absorption (110). These transporters are specifically designed to identify and assist in the movement of polyamines (110), showcasing their effectiveness in facilitating the uptake of the bioactive substances within the intestinal environment. This may not only justify the spermidines' permeation percentage achieved at the end of the assay (22.75%), but also support the high permeation percentage of its' derivatives, such as glu-lycibarbarspermidine F (isomer 2) (73.70%).

There are also some phytochemical compounds that may act antagonistically and obstruct the absorption of other compounds (94). For example, kaempferol and kaempferol

glycosides, such as kaempferol-3-*O*-rutinoside, have been associated with antioxidant and anti-inflammatory capacities involved in the maintenance of enterocytes tight junctions (111). More specifically, kaempferol was found to strengthen the tight junctions' barrier in Caco-2 cells, notably elevating the TEER across the cell monolayers (112). Thus, these compounds may prevent others from permeating freely through the monolayer of cells in the co-culture model.

Another reason for some of the compounds identified in the optimal extract being detected in low permeation percentages is the possible entrapment inside intestinal cells (64, 65). For example, cells under oxidative stress have a greater tendency to take up bioactive compounds that possess antioxidant properties (64). Previous studies have demonstrated that specific compounds, including rutin, chlorogenic acid, caffeic acid, and their derivatives, are effective antioxidants in human colon cell lines such as Caco-2 and HT29-MTX (84, 104). This may be due to the OH groups of these compounds that are associated with antioxidant and radical scavenging activities (100, 103). Furthermore, since the intracellular conditions are different from the extracellular ones, some compounds may be metabolized inside the cells, not being detected in the permeation samples (65). This would also provide an explanation for the limited permeation observed for rutin exoside (12.24%), 2-glukukoamine (isomer 2) (8.74%), and corosolic acid (1.55%), despite their relatively high amounts in the optimal extract (12.85, 10.22, and 83.23 mg/100 g dw, respectively).

TEER is a non-invasive method used to monitor living cells during various stages of growth and differentiation (64). In this study, the TEER was measured for 21 days to guarantee the integrity and permeability of the model (65), as well as during the permeability assay to ensure the viability of the process (64). As observed in Figure 19 (I), the TEER values increased until the 10th day (198 ± 2 Ω/cm^2), supporting the cell growth, remaining stable until the 21st day (177 ± 10 Ω/cm^2). At the 21st day of the experiment, during the permeability assay, the TEER values ranged between 176 Ω/cm^2 and 197 Ω/cm^2 (Figure 19 (II)).

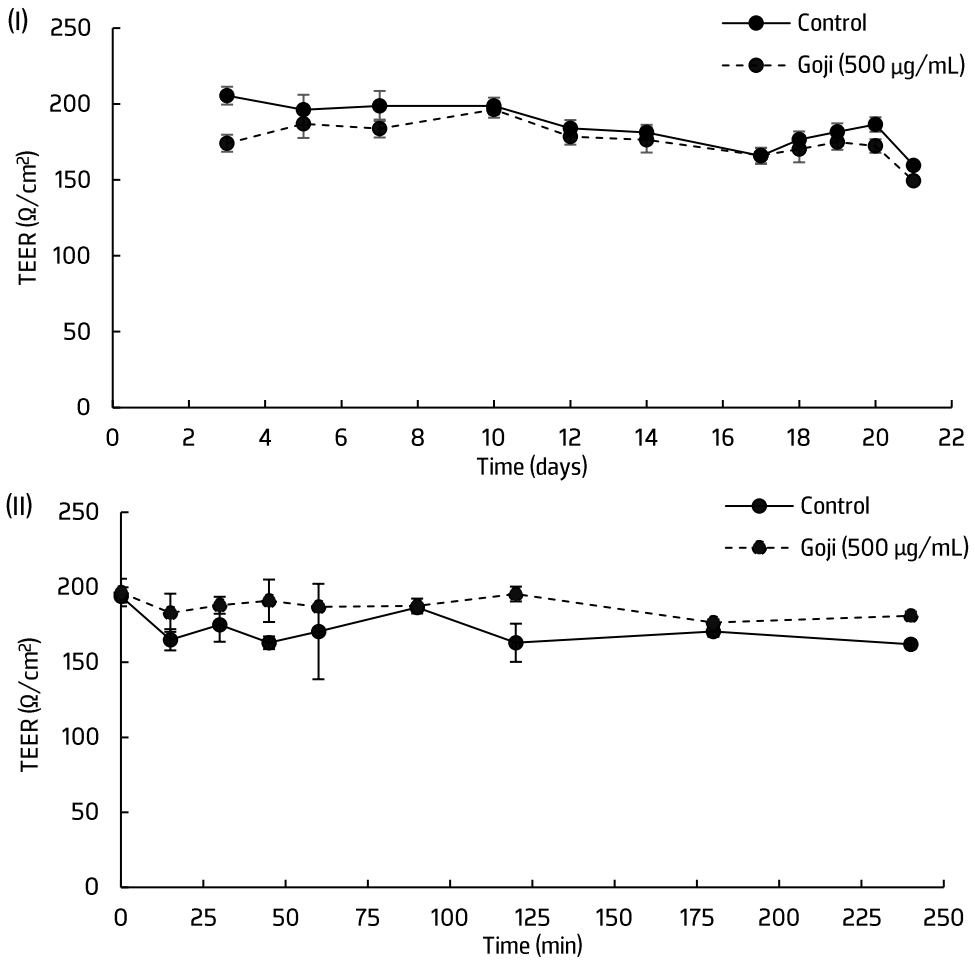

Figure 19. TEER measurements during 21 days of co-culture cell model in Transwell™ membranes (I) and during the 240 min of the permeability assay (II). Results are expressed as mean ± standard deviations (*n*=3) of two independent experiments.

Silva *et al.* (65) and Pinto *et al.* (113) obtained comparable results when evaluating the permeation of *Actinidia arguta* leaf extract and *Castanea sativa* Mill. shell extract, respectively, using a similar co-culture methodology. Nevertheless, as expected, these values are lower than other studies that only used Caco-2 (67, 113, 114) since the mucus-secreting HT29-MTX cells modulate the Caco-2 tight junctions and, consequently, allow larger intercellular spaces between Caco-2 and HT29-MTX (61, 67, 113).

5. Conclusion

Goji berries, mostly sold as dried fruits, gained popularity among consumers in recent years due to their nutritional value and association with health benefits. Moreover, since eco-friendly and sustainable processing methods are becoming popular, natural extracts, such as those from goji berries, have expanded the recognition as excellent alternative sources of bioactive compounds used in multiple industries, particularly the nutraceutical one.

The overall results obtained in this work made it possible to fulfill all the objectives set for this study. It was possible to successfully optimize the extraction of bioactive compounds from *L. barbarum* berries, obtaining a superb amount of phytochemical compounds (glu-lycibarbarspermidine F (isomer 2) (126.72 mg/100 g dw), 2-glu-kukoamine (isomer 2) (104.96 mg/100 g dw), 3,5-dicaffeoylquinic acid (5.76 mg/10 g dw) and rutin (19.27 mg/100 g dw)) in the optimal extract. These compounds were associated with antioxidant and antiradical activities, particularly regarding ROS and RNS. Moreover, the optimal extract was classified as safe in intestinal cell lines in concentrations under 500 µg/mL. The intestinal permeability assay attested the significant permeation of most of the compounds present in the optimal *L. barbarum* berries extract, with glu-lycibarbarspermidine F (isomer 2) (73.70%), 3,5-dicaffeoylquinic acid (52.66%), isorhamnetin-3-O-rutinoside (49.31%), 2-glu-kukoamine (isomer 1) (37.73%), lycibarbarspermidine B (37.27%) and 3-glu-kukoamine (34.45%) being the main ones.

Therefore, this work proved the benefits of employing *L. barbarum* berries extracts in nutraceutical industry.

6. Future perspectives

Even though this study provides substantial evidence of the potential and safety of goji berry extracts, further research is needed to obtain more accurate and comprehensive insights. Despite the antioxidant and scavenging ability of the optimal extract was assessed in this study, goji berries extracts have demonstrated other biological activities, such as antimicrobial, antidiabetic or anti-inflammatory activities that should be explored, particularly the pathways behind them.

Another aspect that could be explored in the future is the synergistic effect of the compounds present in goji berry extracts. This approach could potentially enhance and improve the desirable biological effects associated with goji berries, leading to a more comprehensive understanding of their therapeutic potential.

Regarding the toxic potential of the optimal extract, it would be useful to conduct studies to assess and detect the presence of pesticides or other potentially harmful contaminants that may arise from cultivation practices or industrial processing methods.

Additionally, given that phenolic compounds are abundant in goji berries and are known to be highly sensitive to environmental factors, it is imperative to investigate the stability of such compounds through time. Also, there are multiple technologies that allow the protection of these compounds. For example, nano or microencapsulation would be helpful since it might improve the desired biological effect/therapeutic action by increasing their bioavailability in organisms, while simultaneously reducing the degradation rate.

Finally, *in vivo* studies should be considered in a final stage since they play a crucial role in the assessment of safety, efficacy, and interactions with living organisms, validating health claims.

Bibliographic References

1. Pinto D, Cadiz-Gurrea ML, Vallverdu-Queralt A, Delerue-Matos C, Rodrigues F. *Castanea sativa* shells: A review on phytochemical composition, bioactivity and waste management approaches for industrial valorization. Food Research International. 2021;144:110364.
2. Llorent-Martínez EJ, Fernández-de Córdova ML, Ortega-Barrales P, Ruiz-Medina A. Characterization and comparison of the chemical composition of exotic superfoods. Microchemical Journal. 2013;110:444-51.
3. Attard E. History, Definition, and Legislation. In: Nabavi SM, Silva AS, editors. Nonvitamin and Nonmineral Nutritional Supplements: Academic Press; 2019. p. 3-8.
4. Garcia-Alvarez A, Egan B, de Klein S, Dima L, Maggi FM, Isoniemi M, et al. Usage of plant food supplements across six European countries: findings from the PlantLIBRA consumer survey. PLoS One. 2014;9(3):92265.
5. Protti M, Gualandi I, Mandrioli R, Zappoli S, Tonelli D, Mercolini L. Analytical profiling of selected antioxidants and total antioxidant capacity of goji (*Lycium* spp.) berries. Journal of Pharmaceutical and Biomedical Analysis. 2017;143:252-60.
6. Vidovic BB, Milincic DD, Marcetic MD, Djuris JD, Ilic TD, Kostic AZ, et al. Health Benefits and Applications of Goji Berries in Functional Food Products Development: A Review. Antioxidants. 2022;11(2):248.
7. Kumar K, Srivastav S, Sharanagat VS. Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. Ultrasonics - Sonochemistry. 2021;70:105325.
8. Mocan A, Schafberg M, Crișan G, Rohn S. Determination of lignans and phenolic components of *Schisandra chinensis* (Turcz.) Baill. using HPLC-ESI-ToF-MS and HPLC-online TEAC: Contribution of individual components to overall antioxidant activity and comparison with traditional antioxidant assays. Journal of Functional Foods. 2016;24:579-94.
9. Smith-Hall C, Larsen HO, Pouliot M. People, plants and health: a conceptual framework for assessing changes in medicinal plant consumption. Journal of Ethnobiology and Ethnomedicine. 2012;8(1):43.
10. Pires TCSP, Dias MI, Barros L, Calhelha RC, Alves MJ, Santos-Buelga C, et al. Phenolic compounds profile, nutritional compounds and bioactive properties of *Lycium barbarum* L.: A comparative study with stems and fruits. Industrial Crops and Products. 2018;122:574-81.
11. Naini MA, Zargari-Samadnejad A, Mehrvarz S, Tanideh R, Ghorbani M, Dehghanian A, et al. Anti-Inflammatory, Antioxidant, and Healing-Promoting Effects of *Aloe vera* Extract in the Experimental Colitis in Rats. Evidence-based Complementary and Alternative Medicine. 2021;2021:9945244.

12. Zhao L, Rupji M, Choudhary I, Osan R, Kapoor S, Zhang HJ, et al. Efficacy based ginger fingerprinting reveals potential antiproliferative analytes for triple negative breast cancer. Scientific Reports. 2020;10(1):19182.

13. Kim H, Han S, Song K, Lee MY, Park B, Ha IJ, et al. Ethyl Acetate Fractions of *Papaver rhoeas* L. and *Papaver nudicaule* L. Exert Antioxidant and Anti-Inflammatory Activities. Antioxidants. 2021;10(12).

14. Ferreira SS, Martins-Gomes C, Nunes FM, Silva AM. Elderberry (*Sambucus nigra* L.) extracts promote anti-inflammatory and cellular antioxidant activity. Food Chemistry: X. 2022;15:100437.

15. Mocan A, Moldovan C, Zengin G, Bender O, Locatelli M, Simirgiotis M, et al. UHPLC-QTOF-MS analysis of bioactive constituents from two Romanian Goji (*Lycium barbarum* L.) berries cultivars and their antioxidant, enzyme inhibitory, and real-time cytotoxicological evaluation. Food and Chemical Toxicology. 2018;115:414-24.

16. Donno D, Beccaro GL, Mellano MG, Cerutti AK, Bounous G. Goji berry fruit (*Lycium* spp.): antioxidant compound fingerprint and bioactivity evaluation. Journal of Functional Foods. 2015;18:1070-85.

17. Shah T, Bule M, Niaz K. Goji Berry (*Lycium barbarum*) — A Superfood. In: Nabavi SM, Silva AS, editors. Nonvitamin and Nonmineral Nutritional Supplements: Academic Press; 2019. p. 257-64.

18. Fatchurrahman D, Amodio ML, Valeria De Chiara ML, Mastrandrea L, Colelli G. Characterization and postharvest behavior of goji berry (*Lycium barbarum* L.) during ripening. Postharvest Biology and Technology. 2022;191:111975.

19. Skenderidis P, Lampakis D, Giavasis I, Leontopoulos S, Petrotos K, Hadjichristodoulou C, et al. Chemical Properties, Fatty-Acid Composition, and Antioxidant Activity of Goji Berry (*Lycium barbarum* L. and *Lycium chinense* Mill.) Fruits. Antioxidants. 2019;8(3).

20. Bora P, Ragaee S, Abdel-Aal E-SM. Effect of incorporation of goji berry by-product on biochemical, physical and sensory properties of selected bakery products. LWT. 2019;112:108225.

21. Skenderidis P, Kerasioti E, Karkanta E, Stagos D, Kouretas D, Petrotos K, et al. Assessment of the antioxidant and antimutagenic activity of extracts from goji berry of Greek cultivation. Toxicology Reports. 2018;5:251-7.

22. Lu Y, Guo S, Zhang F, Yan H, Qian D-w, Shang E-x, et al. Nutritional components characterization of Goji berries from different regions in China. Journal of Pharmaceutical and Biomedical Analysis. 2021;195:113859.

23. FAOSTAT: Crops and livestock products [Internet]. 2022 [cited 2022 Nov 29]. Available from: https://www.fao.org/faostat/en/#data/QCL/visualize.

24. Kafkaletou M, Christopoulos MV, Tsaniklidis G, Papadakis I, Ioannou D, Tzoutzoukou C, et al. Nutritional value and consumer-perceived quality of fresh goji berries (*Lycium barbarum* L. and *L. chinense* L.) from plants cultivated in Southern Europe. Fruits. 2018;73(1):5-12.

25. Tang WM, Chan E, Kwok CY, Lee YK, Wu JH, Wan CW, et al. A review of the anticancer and immunomodulatory effects of *Lycium barbarum* fruit. Inflammopharmacology. 2012;20(6):307-14.

26. Magalhães V, Silva AR, Silva B, Zhang X, Dias ACP. Comparative studies on the anti-neuroinflammatory and antioxidant activities of black and red goji berries. Journal of Functional Foods. 2022;92:105038.

27. Zhao W-H, Shi Y-P. Comprehensive analysis of phenolic compounds in four varieties of goji berries at different ripening stages by UPLC–MS/MS. Journal of Food Composition and Analysis. 2022;106:104279.

28. Hsu HJ, Huang RF, Kao TH, Inbaraj BS, Chen BH. Preparation of carotenoid extracts and nanoemulsions from *Lycium barbarum* L. and their effects on growth of HT-29 colon cancer cells. Nanotechnology. 2017;28(13):135103.

29. Islam T, Yu X, Badwal TS, Xu B. Comparative studies on phenolic profiles, antioxidant capacities and carotenoid contents of red goji berry (*Lycium barbarum*) and black goji berry (*Lycium ruthenicum*). Chemistry Central Journal. 2017;11(1):59.

30. Nardi GM, Farias Januario AG, Freire CG, Megiolaro F, Schneider K, Perazzoli MR, et al. Anti-inflammatory Activity of Berry Fruits in Mice Model of Inflammation is Based on Oxidative Stress Modulation. Pharmacognosy Research. 2016;8(Suppl 1):S42-9.

31. Vasantha Rupasinghe HP, Nair SVG, Robinson RA. Chemopreventive Properties of Fruit Phenolic Compounds and Their Possible Mode of Actions. In: Atta R, editor. Studies in Natural Products Chemistry. 42: Elsevier; 2014. p. 229-66.

32. Rodríguez García SL, Raghavan V. Green extraction techniques from fruit and vegetable waste to obtain bioactive compounds — A review. Critical Reviews in Food Science and Nutrition. 2022;62(23):6446-66.

33. Jiang Y, Fang Z, Leonard W, Zhang P. Phenolic compounds in *Lycium* berry: Composition, health benefits and industrial applications. Journal of Functional Foods. 2021;77:104340.

34. Wojdyło A, Nowicka P, Bąbelewski P. Phenolic and carotenoid profile of new goji cultivars and their anti-hyperglycemic, anti-aging and antioxidant properties. Journal of Functional Foods. 2018;48:632-42.

35. Ilić T, Dodevska M, Marčetić M, Božić D, Kodranov I, Vidović B. Chemical Characterization, Antioxidant and Antimicrobial Properties of Goji Berries Cultivated in Serbia. Foods. 2020; 9(11): 1614.

36. Inbaraj BS, Lu H, Kao TH, Chen BH. Simultaneous determination of phenolic acids and flavonoids in *Lycium barbarum* Linnaeus by HPLC–DAD–ESI-MS. Journal of Pharmaceutical and Biomedical Analysis. 2010;51(3):549-56.

37. FoodData Central Search Results [Internet]. 2022 [cited 2022 Nov 29]. Available from: https://fdc.nal.usda.gov/fdc-app.html#/food-details/173032/nutrients.

38. Gong G, Liu Q, Deng Y, Dang T, Dai W, Liu T, et al. Arabinogalactan derived from *Lycium barbarum* fruit inhibits cancer cell growth via cell cycle arrest and apoptosis. International Journal of Biological Macromolecules. 2020;149:639-50.

39. Silva AM, Costa PC, Delerue-Matos C, Latocha P, Rodrigues F. Extraordinary composition of *Actinidia arguta* by-products as skin ingredients: A new challenge for cosmetic and medical skincare industries. Trends in Food Science & Technology. 2021;116:842-53.

40. Dzah CS, Duan Y, Zhang H, Wen C, Zhang J, Chen G, et al. The effects of ultrasound assisted extraction on yield, antioxidant, anticancer and antimicrobial activity of polyphenol extracts: A review. Food Bioscience. 2020;35:100547.

41. Kabir F, Katayama S, Tanji N, Nakamura S. Antimicrobial effects of chlorogenic acid and related compounds. Journal of the Korean Society for Applied Biological Chemistry. 2014;57(3):359-65.

42. Kwasnik P, Lemieszek MK, Rzeski W. Impact of phytochemicals and plant extracts on viability and proliferation of NK cell line NK-92 – a closer look at immunomodulatory properties of goji berries extract in human colon cancer cells. Annals of Agricultural and Environmental Medicine. 2021;28(2):291-9.

43. Zhu PF, Zhao YL, Dai Z, Qin XJ, Yuan HL, Jin Q, et al. Phenolic Amides with Immunomodulatory Activity from the Nonpolysaccharide Fraction of *Lycium barbarum* Fruits. Journal of Agricultural and Food Chemistry. 2020;68(10):3079-87.

44. WHO: International Agency for Research on Cancer. Cancer Today [Internet]. 2022 [cited 2022 Dec 07]. Available from: https://gco.iarc.fr/today/online-analysis-multi-bars.

45. Wawruszak A, Czerwonka A, Okla K, Rzeski W. Anticancer effect of ethanol *Lycium barbarum* (Goji berry) extract on human breast cancer T47D cell line. Natural Product Research. 2016;30(17):1993-6.

46. Skenderidis P, Mitsagga C, Lampakis D, Petrotos K, Giavasis I. The Effect of Encapsulated Powder of Goji Berry (*Lycium barbarum*) on Growth and Survival of Probiotic Bacteria. Microorganisms. 2019;8(1).

47. Fadiloglu EE, ÇOban MZ. The Effects of Goji Berry (*Lycium barbarum* L.) Extract on Some Chemical, Microbiological and Sensory Characteristics of Liquid Smoked Common Carp (*Cyprinus carpio* L., 1758) Sausages. Yuzuncu Yıl University Journal of Agricultural Science. 2019;29(4):702-10.

48. Uzakov Y, Kaldarbekova M, Kuznetsova O. Improved technology for new-generation Kazakh national meat products. Foods and Raw Materials. 2020;8:76-83.

49. Antonini E, Torri L, Piochi M, Cabrino G, Meli MA, De Bellis R. Nutritional, antioxidant and sensory properties of functional beef burgers formulated with chia seeds and goji puree, before and after *in vitro* digestion. Meat Science. 2020;161:108021.

50. Salmerón-Manzano E, Garrido-Cardenas JA, Manzano-Agugliaro F. Worldwide Research Trends on Medicinal Plants. International Journal of Environmental Research and Public Health. 2020;17(10):3376.

51. Ferraz JdRS, Macedo JL, Silva DJS, Sampaio LVA. Goji Berry: nutritional properties and benefits for human health. Research, Society and Development. 2019;8(4):e284934.

52. Mocan A, Vlase L, Vodnar DC, Gheldiu AM, Oprean R, Crisan G. Antioxidant, Antimicrobial Effects and Phenolic Profile of *Lycium barbarum* L. Flowers. Molecules. 2015;20(8):15060-71.

53. Mocan A, Zengin G, Simirgiotis M, Schafberg M, Mollica A, Vodnar DC, et al. Functional constituents of wild and cultivated Goji (*L. barbarum* L.) leaves: phytochemical characterization, biological profile, and computational studies. Journal of Enzyme Inhibition and Medicinal Chemistry. 2017;32(1):153-68.

54. Ab Rahman Z, Abd Shukor S, Abbas H, Machap C, Alias M, Mirad R, et al. Optimization of Extraction Conditions for Total Phenolics and Total Flavonoids from *Kaempferia parviflora* Rhizomes. Advances in Bioscience and Biotechnology. 2018;09:205-14.

55. Pagano I, Campone L, Celano R, Piccinelli AL, Rastrelli L. Green non-conventional techniques for the extraction of polyphenols from agricultural food by-products: A review. Journal of Chromatography A. 2021;1651:462295.

56. Cvjetko Bubalo M, Vidović S, Radojčić Redovniković I, Jokić S. New perspective in extraction of plant biologically active compounds by green solvents. Food and Bioproducts Processing. 2018;109:52-73.

57. Deng Y, Wang W, Zhao S, Yang X, Xu W, Guo M, et al. Ultrasound-assisted extraction of lipids as food components: Mechanism, solvent, feedstock, quality evaluation and coupled technologies – A review. Trends in Food Science & Technology. 2022;122:83-96.

58. Skenderidis P, Petrotos K, Giavasis I, Hadjichristodoulou C, Tsakalof A. Optimization of ultrasound assisted extraction of of goji berry (*Lycium barbarum*) fruits and evaluation of extracts' bioactivity. Journal of Food Process Engineering. 2017;40(5):e12522.

59. Panja P. Green extraction methods of food polyphenols from vegetable materials. Current Opinion in Food Science. 2018;23:173-82.

60. Silva AM, Pinto D, Moreira MM, Costa PC, Delerue-Matos C, Rodrigues F. Valorization of Kiwiberry Leaves Recovered by Ultrasound-Assisted Extraction for Skin Application: A Response Surface Methodology Approach. Antioxidants. 2022;11(4):763.

61. Macedo MH, Martinez E, Barrias C, Sarmento B. Development of an Improved 3D *in vitro* Intestinal Model to Perform Permeability Studies of Paracellular Compounds. Frontiers in Bioengineering and Biotechnology. 2020;8.

62. Zhang J, Penny J, Lu JR. Development of a novel *in vitro* 3D intestinal model for permeability evaluations. International Journal of Food Sciences and Nutrition. 2020;71(5):549-62.

63. Sarmento B, Andrade F, Silva SBd, Rodrigues F, das Neves J, Ferreira D. Cell-based *in vitro* models for predicting drug permeability. Expert Opinion on Drug Metabolism & Toxicology. 2012;8(5):607-21.

64. González F, García-Martínez E, del Mar Camacho M, Martínez-Navarrete N, Sarmento B, Fernandes I, et al. Insights into the development of grapefruit

nutraceutical powder by spray drying: physical characterization, chemical composition and 3D intestinal permeability. Journal of the Science of Food and Agriculture. 2019;99(10):4686-94.

65. Silva A, Almeida A, Dall'Acqua S, Loschi F, Sarmento B, Costa P, et al. Insights into the 3D *In Vitro* Permeability and *In Vivo* Antioxidant Protective Effects of Kiwiberry Leaf Extract: A Step Forward to Human Nutraceutical Use. International Journal of Molecular Sciences. 2022;23:14130.

66. Pereira I, Lechanteur A, Sarmento B. 3D Model Replicating the Intestinal Function to Evaluate Drug Permeability. Methods in Molecular Biology. 2018;1817:107-13.

67. Araújo F, Sarmento B. Towards the characterization of an *in vitro* triple co-culture intestine cell model for permeability studies. International Journal of Pharmaceutics. 2013;458(1):128-34.

68. Lozoya-Agullo I, Araújo F, González-Álvarez I, Merino-Sanjuán M, González-Álvarez M, Bermejo M, et al. Usefulness of Caco-2/HT29-MTX and Caco-2/HT29-MTX/Raji B Coculture Models To Predict Intestinal and Colonic Permeability Compared to Caco-2 Monoculture. Molecular Pharmaceutics. 2017;14(4):1264-70.

69. Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. American Journal of Enology and Viticulture. 1965;16(3):144.

70. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine. 1999;26(9):1231-7.

71. Barros L, Baptista P, Ferreira IC. Effect of Lactarius piperatus fruiting body maturity stage on antioxidant activity measured by several biochemical assays. Food and Chemical Toxicology. 2007;45(9):1731-7.

72. Benzie IFF, Strain JJ. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Analytical Biochemistry. 1996;239(1):70-6.

73. Gomes A, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, et al. 2-Styrylchromones: Novel strong scavengers of reactive oxygen and nitrogen species. Bioorganic & Medicinal Chemistry. 2007;15(18):6027-36.

74. Stat-Ease. Response Surface [Internet]. 2022 [cited 2022 Dec 07]. Available from: https://www.statease.com/docs/v11/tutorials/multifactor-rsm/#mfrsm.

75. Prior RL, Wu X, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. Journal of Agricultural and Food Chemistry. 2005;53(10):4290-302.

76. Pinto D, Braga N, Rodrigues F, Oliveira MBPP. *Castanea sativa* Bur: An Undervalued By-Product but a Promising Cosmetic Ingredient. Cosmetics. 2017;4(4):50.

77. Benchennouf A, Grigorakis S, Loupassaki S, Kokkalou E. Phytochemical analysis and antioxidant activity of *Lycium barbarum* (Goji) cultivated in Greece. Pharmaceutical Biology. 2017;55(1):596-602.

78. Feng L, Tang N, Liu R, Nie R, Guo Y, Liu R, et al. Effects of different processing methods on bioactive substances and antioxidation properties of *Lycium barbarum* (goji berry) from China. Food Bioscience. 2021;42:101048.

79. Rocchetti G, Chiodelli G, Giuberti G, Ghisoni S, Baccolo G, Blasi F, et al. UHPLC–ESI–QTOF–MS profile of polyphenols in Goji berries (*Lycium barbarum* L.) and its dynamics during *in vitro* gastrointestinal digestion and fermentation. Journal of Functional Foods. 2018;40:564–72.

80. Ceccarini MR, Vannini S, Cataldi S, Moretti M, Villarini M, Fioretti B, et al. *In Vitro* Protective Effects of *Lycium barbarum* Berries Cultivated in Umbria (Italy) on Human Hepatocellular Carcinoma Cells. BioMed Research International. 2016;2016:7529521.

81. Mendes M, Carvalho AP, Magalhães JMCS, Moreira M, Guido L, Gomes AM, et al. Response surface evaluation of microwave-assisted extraction conditions for *Lycium barbarum* bioactive compounds. Innovative Food Science & Emerging Technologies. 2016;33:319–26.

82. Teixeira F, Silva AM, Delerue-Matos C, Rodrigues F. Lycium barbarum Berries (*Solanaceae*) as Source of Bioactive Compounds for Healthy Purposes: A Review. International Journal of Molecular Sciences. 2023;24(5):4777.

83. Almeida D, Pinto D, Santos J, Vinha AF, Palmeira J, Ferreira HN, et al. Hardy kiwifruit leaves (*Actinidia arguta*): An extraordinary source of value-added compounds for food industry. Food Chemistry. 2018;259:113–21.

84. Wu T, Lv H, Wang F, Wang Y. Characterization of Polyphenols from *Lycium ruthenicum* Fruit by UPLC-Q-TOF/MSE and Their Antioxidant Activity in Caco-2 Cells. Journal of Agricultural and Food Chemistry. 2016;64(11):2280–8.

85. Islam S, Yoshimoto M, Yamakawa O. Distribution and Physiological Functions of Caffeoylquinic Acid Derivatives in Leaves of Sweetpotato Genotypes. Journal of Food Science. 2006;68:111–6.

86. Xiao X, Ren W, Zhang N, Bing T, Liu X, Zhao Z, et al. Comparative Study of the Chemical Constituents and Bioactivities of the Extracts from Fruits, Leaves and Root Barks of *Lycium barbarum*. Molecules. 2019;24(8):1585.

87. dos Santos GS, de Almeida Veiga A, Carlotto J, Mello RG, Serrato RV, de Souza LM. Identification and fingerprint analysis of novel multi-isomeric Lycibarbarspermidines and Lycibarbarspermines from *Lycium barbarum* L. by liquid chromatography with high-resolution mass spectrometry (UHPLC-Orbitrap). Journal of Food Composition and Analysis. 2022;105:104194.

88. Li Y-Y, Di R, Hsu W-L, Huang Y-Q, Cheung H-Y. Quality control of *Lycium chinense* and *Lycium barbarum* cortex (*Digupi*) by HPLC using kukoamines as markers. Chinese Medicine. 2017;12(1):4.

89. Shi L, Zhang W, Zhou Y-Y, Zhang Y-N, Li J-Y, Hu L-H, et al. Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation. European Journal of Pharmacology. 2008;584(1):21–9.

90. Qian XP, Zhang XH, Sun LN, Xing WF, Wang Y, Sun SY, et al. Corosolic acid and its structural analogs: A systematic review of their biological activities and underlying mechanism of action. Phytomedicine. 2021;91:153696.

91. Wang W, Snooks HD, Sang S. The Chemistry and Health Benefits of Dietary Phenolamides. Journal of Agricultural and Food Chemistry. 2020;68(23):6248-67.

92. Zhou ZQ, Fan HX, He RR, Xiao J, Tsoi B, Lan KH, et al. Lycibarbarspermidines A-O, New Dicaffeoylspermidine Derivatives from Wolfberry, with Activities against Alzheimer's Disease and Oxidation. J Agric Food Chem. 2016;64(11):2223-37.

93. Li Y-Y, Wang H, Zhao C, Huang Y-Q, Tang X, Cheung H-Y. Identification and Characterization of Kukoamine Metabolites by Multiple Ion Monitoring Triggered Enhanced Product Ion Scan Method with a Triple–Quadruple Linear Ion Trap Mass Spectrometer. Journal of Agricultural and Food Chemistry. 2015;63(50):10785-90.

94. Pacheco-Ordaz R, Antunes-Ricardo M, Gutiérrez-Uribe JA, González-Aguilar GA. Intestinal Permeability and Cellular Antioxidant Activity of Phenolic Compounds from Mango (*Mangifera indica* cv. Ataulfo) Peels. International Journal of Molecular Sciences. 2018;19(2):514.

95. Kim K-H, Tsao R, Yang R, Cui SW. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chemistry. 2006;95(3):466-73.

96. de Siqueira FC, Barbosa-Carvalho APP, Costa Leitão DdST, Furtado KF, Chagas-Junior GCA, Lopes AS, et al. Scavenging Capacity of Extracts of *Arrabidaea chica* Leaves from the Amazonia against ROS and RNS of Physiological and Food Relevance. Antioxidants. 2022;11(10):1909.

97. Alfadda AA, Sallam RM. Reactive oxygen species in health and disease. J Biomed Biotechnol. 2012;2012:936486.

98. Almeida IF, Fernandes E, Lima JL, Costa PC, Bahia MF. Protective effect of *Castanea sativa* and *Quercus robur* leaf extracts against oxygen and nitrogen reactive species. Journal of Photochemistry and Photobiology B. 2008;91(2-3):87-95.

99. Marangi F, Pinto D, de Francisco L, Alves RC, Puga H, Sut S, et al. Hardy kiwi leaves extracted by multi-frequency multimode modulated technology: A sustainable and promising by-product for industry. Food Research International. 2018;112:184-91.

100. Firuzi O, Mladênka Pe, Petrucci R, Marrosu G, Saso L. Hypochlorite scavenging activity of flavonoids. Journal of Pharmacy and Pharmacology. 2010;56(6):801-7.

101. Chisté RC, Mercadante AZ, Gomes A, Fernandes E, Lima JLFdC, Bragagnolo N. *In vitro* scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species. Food Chemistry. 2011;127(2):419-26.

102. Ribeiro AB, Chisté RC, Freitas M, da Silva AF, Visentainer JV, Fernandes E. *Psidium cattleianum* fruit extracts are efficient *in vitro* scavengers of physiologically relevant reactive oxygen and nitrogen species. Food Chemistry. 2014;165:140-8.

103. Corrêa RCG, Peralta RM, Haminiuk CWI, Maciel GM, Bracht A, Ferreira ICFR. New phytochemicals as potential human anti-aging compounds: Reality, promise, and challenges. Critical Reviews in Food Science and Nutrition. 2018;58(6):942-57.

104. Jiménez-Moreno N, Cimminelli MJ, Volpe F, Ansó R, Esparza I, Mármol I, et al. Phenolic Composition of Artichoke Waste and Its Antioxidant Capacity on Differentiated Caco-2 Cells. Nutrients. 2019;11(8):1723.

105. Cuffaro D, Pinto D, Silva AM, Bertolini A, Bertini S, Saba A, et al. Insights into the Antioxidant/Antiradical Effects and *In Vitro* Intestinal Permeation of Oleocanthal and Its Metabolites Tyrosol and Oleocanthalic Acid. Molecules. 2023;28(13):5150.

106. Wang Y, Sun M, Jin H, Yang J, Kang S, Liu Y, et al. Effects of *Lycium barbarum* Polysaccharides on Immunity and the Gut Microbiota in Cyclophosphamide-Induced Immunosuppressed Mice. Frontiers in Microbiology. 2021;12.

107. Li W, Gao M, Han T. *Lycium barbarum* polysaccharides ameliorate intestinal barrier dysfunction and inflammation through the MLCK-MLC signaling pathway in Caco-2 cells. Food & Function. 2020;11(4):3741-8.

108. Mao F, Xiao B, Jiang Z, Zhao J, Huang X, Guo J. Anticancer effect of *Lycium barbarum* polysaccharides on colon cancer cells involves G0/G1 phase arrest. Medical Oncology. 2011;28(1):121-6.

109. Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke J-D, Serino M, et al. Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterology. 2014;14(1):189.

110. Uemura T, Stringer DE, Blohm-Mangone KA, Gerner EW. Polyamine transport is mediated by both endocytic and solute carrier transport mechanisms in the gastrointestinal tract. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2010;299(2):G517-G22.

111. Lee B, Moon KM, Kim CY. Tight Junction in the Intestinal Epithelium: Its Association with Diseases and Regulation by Phytochemicals. Journal of Immunology Research. 2018;2018:2645465.

112. Suzuki T, Tanabe S, Hara H. Kaempferol Enhances Intestinal Barrier Function through the Cytoskeletal Association and Expression of Tight Junction Proteins in Caco-2 Cells12. The Journal of Nutrition. 2011;141(1):87-94.

113. Pinto D, Silva AM, Dall'Acqua S, Sut S, Vallverdú-Queralt A, Delerue-Matos C, et al. Simulated Gastrointestinal Digestion of Chestnut (*Castanea sativa* Mill.) Shell Extract Prepared by Subcritical Water Extraction: Bioaccessibility, Bioactivity, and Intestinal Permeability by *In Vitro* Assays. Antioxidants. 2023;12(7):1414.

114. Wang Z, Zhang H, Shen Y, Zhao X, Wang X, Wang J, et al. Characterization of a novel polysaccharide from *Ganoderma lucidum* and its absorption mechanism in Caco-2 cells and mice model. International Journal of Biological Macromolecules. 2018;118:320-6.