



Broccoli by-product extract as a functional ingredient: food application

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Abstract

Aim: Food production demand has been promoting an increase in the generation of agro-industrial waste. Food industry waste can contain compounds with added value that, if properly extracted and used, can be applied to the development of healthy foods (clean label), nutraceuticals, senior food, cosmetics, etc. The revaluation of by-products from the broccoli industry will make it possible to reduce the large volume of broccoli waste, reducing the cost of waste management and obtaining compounds of interest from them. The aim of this work is the extraction of compounds of interest by means of environmentally sustainable technologies and to characterize the freeze-dried broccoli extracts obtained in each green technology in terms of their characteristic compounds of interest and the study of antimicrobial and antioxidant capacity.

Methods: The methods of extraction of compounds of interest from broccoli used in this research are environmentally sustainable technologies, using water as the extraction solvent, including aqueous extraction (AE), enzymatic extraction (EE), and ultrasound-assisted extraction (UAE). After extraction, the freeze-dried broccoli extracts obtained were characterized and the antimicrobial capacity was studied with *Listeria* and *Salmonella* strains and the antioxidant capacity was studied with *Saccharomyces* strains, thus determining which of the techniques is most effective for obtaining a freeze-dried broccoli extract with a high concentration of bioactive compounds.

Results: As a result of the research, different products have been obtained from broccoli waste by means of three green extraction techniques, obtaining products with a high concentration of bioactive compounds with antioxidant and antimicrobial capacity against strains such as *Listeria* and *Salmonella*.

Conclusions: The waste generated in the broccoli industry has been revalued to obtain high added value products using environmentally sustainable techniques. Due to their high concentration of bioactive compounds, these products are effective as functional products due to their antioxidant and antimicrobial capacity.



Keywords

Green techniques, broccoli by-product, extraction, broccoli extract, ultrasound-assisted extraction, enzymatic extraction, phenolic compounds recovery

Introduction

The agricultural industry is known to be the main producer of waste each year. The generation and accumulation of waste from the food industry have a significant impact on biodiversity, human health, and climate change [1]. It is for this reason that by-products and waste from the agri-food industries are attracting international attention, mainly due to issues related to pollution and their mismanagement in the economy, as they produce losses of money at different levels of the supply chain. Therefore, a reduction in waste generation has the potential to generate considerable economic value [2].

The revalorisation of by-products has become the main objective of the agri-food industries; therefore, the implementation of the economy model makes possible the application of compounds of interest that can be used for application in different industries, due to their antioxidant, antimicrobial, and functional power [3, 4].

Recently, food by-products such as broccoli (genus *Perseus* and *Ares*) by-products have been studied for their content of other compounds of interest have been studied for their content of other non-nutritional compounds with potential biological properties, such as flavonoids, carotenoids, vitamins, and minerals, which may help reduce the risk of many chronic diseases (cardiovascular and age-related degenerative diseases) [5].

In recent years, several studies, both *in vitro* and *in vivo*, have focused on the bioactive compounds of brassicas and their potential to mitigate chronic diseases [6, 7]. Moreover, due to its composition rich in compounds beneficial to health, it can also be used to treat other problems such as hypercholesterolemia, cardiovascular diseases, diabetes or photosensitivity disorders [8, 9].

Thus, numerous studies have linked its richness in bioactive compounds to the properties associated with broccoli as an anticarcinogen [10], antioxidant [11], antimicrobial [12], anti-inflammatory [13], and antihypertensive [14]. The bioactive compounds in broccoli include glycosylates (and their hydrolysis products, isothiocyanates) and phenolic compounds (flavonoids and hydroxycinnamic acids), which have been shown to be antioxidants that may reduce the risk of certain types of cancer [15].

Conventional extraction using organic solvents, which have been used for many years but have many drawbacks, has led to the introduction of new and promising extraction techniques. These techniques are known as non-conventional or “green” extraction techniques [16]. Among the most common green extraction techniques for the extraction of bioactive compounds from by-products of the agri-food industry are enzymatic extraction (EE), ultrasound-assisted extraction (UAE) [17, 18], and microwave-assisted extraction (MAE) [19].

In this study, different green extraction technologies, including the use of enzymes, thermal extractions, and UAE, alternatives to the conventional ones in which large amounts of organic solvents are used, have been developed to obtain extracts rich in compounds of interest from broccoli. The lyophilized broccoli extracts obtained with different methods have been compared in terms of their characterization of compounds of interest such as chlorogenic acid, caffeoylquinic acid, Di caffeoylquinic acid, and total polyphenols and their measurement of antioxidant and antimicrobial capacity *in vivo*.

Materials and methods

Plant material

Broccoli by-products (*B. Oleracea* Var. *Italica*) of the *Blanca Tudela* variety are composed of broccoli stem and leaf were supplied by several companies in Murcia-Spain during the months of September and October

2022. Broccoli leaves and stems were transported directly to the laboratory within 1 h after generation and stored at $-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$ until the extraction experiments were carried out.

Experimental design

In this study, four techniques were applied to investigate the impact of broccoli polyphenolic compounds, including chlorogenic acid, caffeic acid, caffeoylquinic acid, and Di caffeoylquinic acid. Concentrations of the broccoli polyphenolic compounds in the extracts were compared for the identification of suitable conditions for maximum recovery yield from broccoli by-products. Figure 1 shows the experimental scheme.

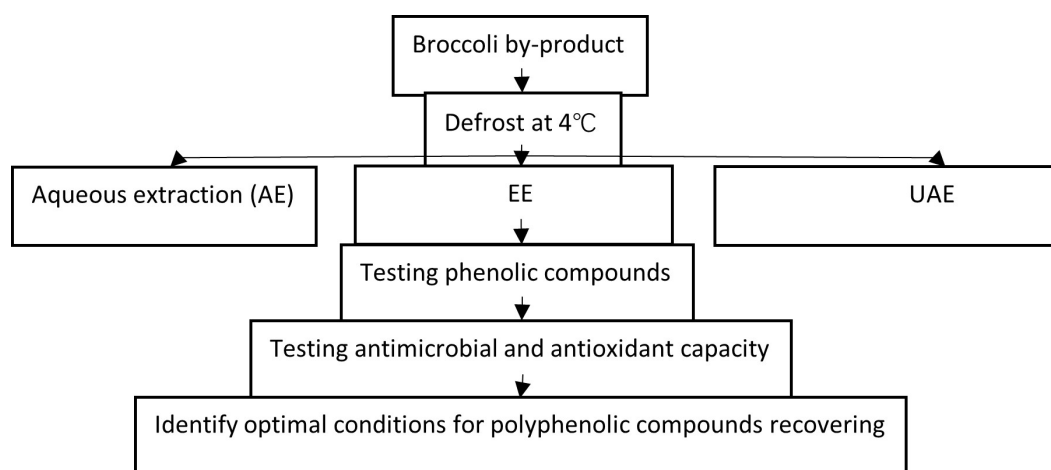


Figure 1. Experimental scheme

Extraction of phenolic compounds using different techniques

Extracts of interest were prepared with three different extraction methods as follows:

- AE: Water was added to broccoli by-product in a 1:3 (*w/w*) proportion. It was then heated up to 100°C and constantly stirred for 60 min.
- EE: The broccoli by-product was mixed with water 1:3 (*w/w*). A cellulase enzyme (Validase[®] TRL, Sigma) was added at 0.01% of the weight of the broccoli by-product and the mixture was kept stirred in constant agitation at 50°C for 60 min. Then the enzyme was deactivated heating the mixture at 100°C . This method was described by the enzyme supplier.
- UAE: According to the procedure described by Saifullah et al. [20] (2020), the broccoli by-product was mixed with water 1:3 (*w/w*). UAE in a UIP500hdT equipment (Hielscher, Germany) with a power of 164 W, amplitude of 100% with constant agitation at 75°C for 60 min was applied to the mixture.

All the samples obtained through the AE, EE, and UAE extraction were centrifuged (Centrifuge Heraeus Labofuge[®] 200, Thermo Scientific, Massachusetts, EEUU) at 11,200 *g* for 20 min, filtered (Whatman[®] Grade. 42) and the supernatant liquid was lyophilised using Coolvacuum Lyoepic-85 (Coolvacuum Technologies, Granollers, Spain). All the experimental processes were conducted in triplicate.

These conditions were defined in preliminary tests, considering the sample characteristics and the limitations of the apparatus. Moreover, the recommendations of Saifullah et al. [20] (2020), Lu et al. [21] (2021), and Barbosa et al. [22] (2021) were followed.

Determination of total polyphenols

The total polyphenols content was determined using the Folin-Ciocalteu method. Folin-Ciocalteu reagent (FCR) was prepared from water (90 mL) and Folin-Ciocalteu stock solution (10 mL). A dilution was made by mixing with FCR (5 mL), 5% sodium carbonate solution (4 mL), and the dissolved sample and kept in the

dark for 1 h. After 1 h, the sample was measured in a spectrophotometer (UVmini 1240 Shimadzu, Kyoto, Japan) with a wavelength of 765 nm. The units used to express the total polyphenols were mg gallic acid equivalents (GAE) per 1 g dry matter (DM; mg GAE/kg DM). [23].

Determination of chlorogenic acid, caffeic acid, caffeoylquinic acid and dicaffeoylquinic acid

Fifty milligrams of each sample were extracted in 1.5 mL methanol at 70°C for 30 min with vortex mixing every 5 min to facilitate the extraction. Samples were centrifuged at 13,000 *g*, 15 min, 4°C). The supernatants were collected, and methanol was completely removed using a rotavapor (R-210 Buchi). The dry material obtained was redissolved in 1 mL of ultrapure water and filtered through 0.22 µm Polyvinylidene Difluoride (PVDF) (Millipore) and measured with high performance liquid chromatography diode array detection (HPLC-DAD) Agilent Serie 1200 (Agilent Technologies) [24].

Measurement of *in vivo* antimicrobial capacity by electrical impedance

Measurement of *in vivo* antimicrobial capacity was performed by direct application of extracts on bacterial growth. Strains of *Salmonella Enterica* subsp. *Enterica*, serovar Typhimurium (CECT 4594), and *E. coli* (*Escherichia coli* CECT 515), from the Spanish Type Culture Collection, with a concentration of 10⁶ colony forming unit (cfu)/mL, were used as culture medium 001A (Sy-Lab, Austria), performing the impedance measurements at 30°C.

The lyophilized strain is placed in tryptic soy broth (TBS) for 24 h at 37°C, where a concentration of approximately 10⁹ cfu/mL is reached. After that, 9 mL of Sy-Lab001A medium was added to the tubes to be introduced into the BacTrac apparatus, inoculating each tube with 0.1 mL of strain at a concentration of 10⁶ cfu/mL and the antimicrobial extract to be tested at a concentration of 5 g/kg. The tubes were then placed in the apparatus and left to incubate for 24 h.

The changes in impedance or resistance of the culture medium due to the growth of microorganisms were measured automatically with the BacTrac 4300 equipment (Gomensoro, Spain).

Measurement of *in vivo* antioxidant capacity by electrical impedance

The method is based on the ability of CO₂ (released by the cells during cell respiration) to collapse potash (potassium hydroxide). In this sense, if the potash collapses, it is because there is cell growth, with the CO₂ concentration being directly proportional to the number of cells.

To measure the antioxidant capacity by electrical impedance, the BacTrac 4000 Series was used. For this, the cell is subjected to oxidative stress using 1 mmol/L hydrogen peroxide (H₂O₂). After subjecting the cells to oxidative stress, the antioxidant agent (final extracts) is added and left to stand for 1 h at room temperature for the antioxidant agent to counteract the effect of the peroxide, subsequently detecting the growth of the cell. After 1 h, 0.1 mL of each vial is added to cells containing 3 mL of yeast nutrient broth. The cells are placed in plastic tubes containing 2 mL of 2% KOH, and the equipment detects changes in conductivity by means of electrodes immersed in the vials.

The prepared vials were made in triplicate in all cases, starting from a yeast culture (*Saccharomyces Cerevisiae*) at a concentration of 10⁶ cfu/mL:

- (a) Vial 1: Medium control (2 mL potash). Potash is left open for atmospheric O₂ saturation.
- (b) Vial 2: Strain control (800 µL strain + 100 µL H₂O).
- (c) Vial 3: Atmospheric O₂ saturation control for oxidative stress (800 µL strain + 100 µL H₂O₂ + 100 µL H₂O).
- (d) Product vial: Antioxidant extract control (800 µL strain + 100 µL H₂O₂ + 100 µL extract with concentration 5 g/kg).

Data processing

The experiments were performed in triplicate as were the analytical determinations. The values presented are the mean values obtained together with the standard deviation (SD). The statistical analysis to obtain the SD was carried out using Statistical Package for Social Sciences (SPSS) 12.0 (SPSS Sciences, Chicago, USA).

Results

Effect of the AE, EE, and UAE methods in the extraction of Polyphenols from the broccoli by-product

The values of polyphenols in the three lyophilized broccoli extracts are reported, and the concentration of compounds of interest is illustrated: total polyphenols, chlorogenic acid glycoside I, caffeoylquinic acid, and Di caffeoylquinic acid (Table 1). Highlighting that the highest concentration of chlorogenic acid glycoside I (3.24 g/kg) and caffeoylquinic acid (9.07 g/kg) were obtained when the EE extraction was used, and the highest concentration of total polyphenols (21.32 g GAE/kg) and Di caffeoylquinic acid (10.04 g/kg) was obtained when the AE extraction was used.

Table 1. Determination of compounds of interest (g/kg). Effect of AE, EE, and US methods in the extraction of polyphenols for the broccoli by-product. Results are expressed as mean values of triplicates \pm SD

Compound	AE	EE	UAE
Total polyphenols (g GAE/kg DM)	21.32 \pm 0.42	20.95 \pm 0.31	3.91 \pm 0.09
Chlorogenic acid glycoside	2.26 \pm 0.05	3.24 \pm 0.06	1.36 \pm 0.07
Caffeoylquinic acid	8.47 \pm 0.20	9.07 \pm 0.13	1.64 \pm 0.03
Di caffeoylquinic acid	10.04 \pm 0.24	7.99 \pm 0.10	0.76 \pm 0.01

The ability to extract polyphenols by enzymatic methods in vegetable products is corroborated by different articles [25]. As with the results of the study, other comparative studies of extraction methods claim that the results obtained after UAE are less effective [26].

Measurement of *in vivo* antimicrobial capacity by electrical impedance

A study was carried out to measure the antimicrobial and antioxidant capacity of the lyophilized extracts obtained by the different extractive techniques by *in vivo* electrical impedance, in this study *Listeria* and *Salmonella* strains were used as controls. The results obtained using the extract with a concentration of 5 g/kg against the different strains in this study are shown in the following figures, which represents in the vertical axe the changes in impedance (M%) of the culture medium due to the growth of microorganisms at different times (hrs, horizontal axe).

The growth time of the *Salmonella* strain is 1.24 h, when broccoli extract obtained by UAE is added, this growth slows down to 2.04 h, since the slowing down time is low, we cannot assure that these extracts have antimicrobial capacity against *Salmonella*.

The growth time of the *Listeria* strain is 7.95 h, when broccoli extract obtained by AE is added, this growth slows down to 40.08 h and when broccoli extract obtained by EE is added, the growth of the strain is completely inhibited, so we can assure that these extracts have antimicrobial capacity against *Listeria*, being the EE the one with the highest antimicrobial capacity against the *Listeria* strain. This is because the freeze-dried broccoli extract obtained by EE has the highest concentration of chlorogenic acid, followed by the aqueous broccoli extract (AE).

Measurement of *in vivo* antioxidant capacity by electrical impedance

The analysis of antioxidant capacity by *in vivo* electrical impedance consists of measuring the growth of a *Saccharomyces* strain and a *Saccharomyces* strain subjected to oxidative stress (H_2O_2 1 mmol/L), causing the growth of the strain to be retarded. To this strain subjected to oxidative stress, the different extracts mentioned above are added to see if they have the opposite effect on oxidation.

When subjected to oxidative stress the *Saccharomyces* strain has a growth of 10.00 h, if we add to these conditions broccoli extract obtained by enzymatic technology (EE) the growth is 8.25 h and if broccoli extract obtained by AE is added to these conditions the growth is 8.30 h, compared to broccoli extract obtained by UAE which reduces the time to 9.36 h. This is because the broccoli extract (UAE) has the least total polyphenols, so it has the least antioxidant capacity.

Discussion

As can be seen in [Table 1](#), UAE is the least effective for obtaining polyphenols and chlorogenic acid derivatives, obtaining less content in bioactive compounds, this is because these compounds tend to degrade when subjected to powerful frequencies. These results agree with the study carried out by Wianowska et al. [27], in which they investigated the stability of chlorogenic acid in pressurized liquid conditions, concluding that high extraction temperatures and pressures cause the degradation of these compounds, and consequently their low yield.

The antimicrobial activity of the aqueous extract and the enzymatic extract is due to the high concentration of polyphenols it possesses, the high concentration of chlorogenic acid, which is higher in the aqueous extract. Polyphenolic compounds are known for their antimicrobial capacity because they can increase the content of oxidative free radicals and cause endogenous oxidative stress in bacterial strains [28]. Experimental studies, such as the one conducted by Lou et al. [29] on the antimicrobial activity of chlorogenic acid obtained from burdock leaves (*Arctium lappa*), concluded that the molecular structure of this compound can bind to and permeabilise the bacterial membrane, causing damage to the integrity of the membrane, which could lead to the death of the bacteria. This experimental study showed that this type of compound acted remarkably on Gram-positive bacteria such as *Listeria* strains, while on Gram-negative bacteria such as *Salmonella* the antimicrobial activity was not so affected [29].

Polyphenolic compounds in general are also compounds with antioxidant capacity, which can act through two mechanisms of action, in the first one the polyphenol molecule reacts with the free radical, with the transfer of a hydrogen atom. In the second, the oxidant transfers a singlet electron [30].

Regarding the antioxidant activity of the extracts obtained, we observed that the extract obtained by UAE, having a low concentration of polyphenols, showed a lower antioxidant activity of the product. These results agree with the study carried out by López-Hernández et al. [31], in which they studied the antioxidant activity of broccoli liquid extracts using DPPH (radical scavenging capacity assay), ABTS (Trolox equivalent antioxidant capacity), and ferric reducing assay (FRAP), observing that the higher the concentration of total polyphenols, the higher the antioxidant activity of the extract [31].

With this research, another approach has been sought for the revalorisation of broccoli industry waste to obtain natural ingredients with antimicrobial and antioxidant capacity using environmentally sustainable techniques. The current need for a sustainable food chain requires the implementation of a circular economy approach in the processing industries. The focus of this is to revalorize the discarded parts of vegetables due to their high content of bioactive compounds.

Conclusion

In the present work, the efficiency of method with different green technologies for extraction of Di caffeoylquinic acid and polyphenols from broccoli wastes was studied. The technologies were based on AE, EE, and UAE. The AE showed the best result in terms of recovery total polyphenols and Di caffeoylquinic acid, with an optimum concentration of 21.32 g GAE/kg DM and 10.4 g GAE/kg DM respectively. In addition, extracts with the highest concentration of caffeoylquinic acid were obtained through EE, with a concentration of 9.07 g GAE/kg DM. The UAE was the technique with lower extractive efficiency in all compounds of interest. The main conclusion, this work has shown an optimal method of revaluation of by-products from the broccoli industry and extract rich in Di caffeoylquinic acid of high purity could be obtained.

The antimicrobial capacity by electrical impedance using *Salmonella* and *Listeria* as strains, was show an interesting difference depends on the green technology used. In *Salmonella* the result cannot assure that these extracts have antimicrobial capacity. Instead, could assure the *Listeria* inhibitory efficiency in broccoli extract obtained by EE. The objective of study the different green technologies is reduce waste of solvents and energy. This environmentally friendly method uses natural resources that companies have as waste and the final product with a quality concentration of interest component is obtained. In future we will improve the efficiency and the food industry must revalue their by-products.

Abbreviations

AE: aqueous extraction

cfu: colony forming unit

DM: dry matter

EE: enzymatic extraction

GAE: gallic acid equivalents

MAE: microwave-assisted extraction

SD: standard deviation

UAE: Ultrasound-assisted extraction

Declarations

Author contributions

MDF and FL: Conceptualization, Investigation, Writing—original draft, Writing—review & editing. DQM and PG: Validation, Writing—review & editing, Supervision. All authors read and approved the submitted version.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

Not applicable.

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