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EVALUATION OF THE ANTIOXIDANT ACTIVITY OF EXTRACTS OBTAINED FORM CHERRY SEEDS

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Abstract

Annual cherry production in Portugal is around 19,000 tonnes, in an area of about 6,450 ha and covering about 11,100 farms, concentrated in some northern and central interior territories. It is also in these regions that in recent decades there has been a significant increase in farms specialized in the production of cherry, using new cultivars and new technologies in a business production model. Apart from being consumed in fresh form, cherries are used for many food preparations, like sweets, jellies or confectionary. In the plants that transform cherries, a significant amount of cherry seeds (also called cherry pits) is generated as residue or waste. The possible usage of these residues as raw material for extraction of compounds with antioxidant properties is beneficial in term of economic value as well as environmental impact. Hence, the objective of this work was to obtain extract rich in compounds with antioxidant activity from cherry seeds.

The cherry seeds were obtained from a local waste management company, Nutrofertil, located in Tondela, in the district of Viseu (Portugal). They were grinded and then submitted to extraction procedures testing different operating conditions: magnetic stirrer versus ultrasound, different solvents (methanol, ethanol, water) and temperatures (from 35 to 80 ^{0}C). For the obtained extracts antioxidant activity was evaluated through spectrophotometric methods, using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′ -azino-di-(3-ethylbenzthiazoline sulfonic acid)) radicals, and also the Ferric Reducing Antioxidant Power Assay (FRAP). All measurements were replicated at least trice, and were expressed as mg Trolox equivalents per gram (mg TE/g).

The results obtained for the different experimental conditions indicated that least efficient extractions at ambient temperature were obtained with methanol using magnetic stirrer and with water using ultrasounds, for which the antioxidant activities measured by the DPPH method were 0.26 and 0.33 mg TE/g and by the ABTS method were 0.82 and 0.86 mg TE/g, respectively. Most efficient methods were water : ethanol (at 50% concentration) and water (100%), using magnetic stirrer in both cases. Highest antioxidant activity was obtained for water : ethanol by the DPPH method (0.72 mg TE/g) and for water (10%) by the ABTS method (1.25 mg TE/g). Tests with different concentrations for the aqueous solutions of ethanol and at different temperatures revealed that with increasing concentration of water the antioxidant

diminished, from 0.62 to 0.27 mg TE/g at 35 ^{0}C using the DPPH method. Additionally, the variation in temperature allowed reaching a maximum extraction of compounds with antioxidant activity at 70 ^{0}C and decreasing thereafter. The maximum values obtained were registered at 70 ^{0}C for all cases and were 0.74 mg TE/g for the water : ethanol 50 : 50 (v/v) by the DPPH method, 2.16 mg TE/g for the water : ethanol 60 : 40 (v/v) by the ABTS method and 3.43 mg TE/g for the water : ethanol 60 : 40 (v/v) by the FRAP method. The results obtained by the different methods were concordant in terms of the observed trends but giving different values of the measured antioxidant activity, which is a common characteristic observed in these types of evaluation techniques.

This research allowed establishing some operational conditions that should be selected in order to maximize the extraction of compounds with antioxidant activity from cherry seeds. The use of ultrasounds was not found beneficial and the magnetic stirrer technique revealed to be more useful. Also the use of methanol was not found suitable, which is a good point given that this solvent is more pollutant and has more problems of toxicity. With respect to temperature, it was found that temperatures higher than 70 ^{0}C are not beneficial because they induce the degradation of some bioactive compounds thus reducing the antioxidant activity of thee extracts.

Keywords: *Extraction, Cherry, DPPH method, ABTS method, FRAP method, Optimization.*

1. Introduction

Cherries are one of the most consumed fruits and are considered one of the healthiest due to their high levels of phenolic compounds. These phenolic compounds are not only found in the fruit but also in the pit. The disposal of considerable quantities of residues from cherry processing industries to the environment occurs due to an increase in the rate of production of cherries and cherry products. Before being sold on the market, most of the cherries are processed, which leads to large quantities of cherry pits being produced by industry each year and discarded as waste, instead of being considered as raw material for possible new chemicals for food or pharmaceutical products [1].

There is a great interest in determining the role of bioactive compounds of plant origin, phytonutrients, in promoting health. The natural antioxidants in food may play an important role in preventing the formation of lipid peroxides [2], which affect the integrity and function of cell membranes. On the other hand, antioxidants protect cellular defense systems from damage caused by free radicals and other oxidizing agents that are the pivotal in the mechanism of action of many toxins. These radicals induce oxidative damage in biomolecules, such as lipids, carbohydrates, proteins, and nucleic acids, and can cause cell death [3]. Phenolic compounds have antioxidant properties and can protect against degenerative diseases in which reactive oxygen species (superoxide anion, hydroxyl radicals, and peroxy radicals) are involved [2]. In fact, the antioxidant activity of cherry pit extract can contribute to the prevention of certain diseases caused by free radicals produced in our body, such as cardiovascular, rheumatic, neurological, psychiatric, premature aging, neoplasms, osteoporosis and diabetes [3].

According to Barbosa *et al.*, [4], the extraction efficiency depends mainly on the choice of solvent. The polarity of the target compound is the most important factor in this decision. Molecular affinity between solvent and solute, mass transfer, environmental safety, toxicity and financial viability should also be considered in solvent selection for the extraction of bioactive compounds [5]. The use of temperature may be favorable to the extraction of bioactive compounds. Heat can make cell walls permeable, increasing solubility and diffusion of the compounds to be extracted and decreasing the viscosity of solvents, thus facilitating extraction. However, extreme temperatures can degrade the substances under study [4].

Although the cherry fruit is a rich source of polyphenols and bioactive components, there is little information on the antioxidant activity of the residues such as cherry pits [6]. The objective of this work was to identify the best combination of solvent and extraction temperature to obtain cherry pit extract containing higher antioxidant capacity concentration. To measure the total antioxidant capacity of the extracts, three methods ABTS, FRAP, and DPPH were chosen that utilize the same single electron transfer mechanism. These methods were chosen for comparison because they are commonly used to evaluate plant materials [7], and all three methods are relatively simple to conduct, and cost-effective.

2. Materials and Methods

2.1 Samples

The sweet cherry seeds (SCS) for this study were obtained in Nutrofertil, a waste management company, located in Tondela, in the district of Viseu (Portugal). The samples were pre-dried in the sun, milled in a Retsch SMI mill (Retsch GmbH, Haan, Germany) and sieved afterwards using a Retsch AS200 sieve (Retsch GmbH, Haan, Germany) for 30 min at 50 rpm. The > 35 mesh (> 0.425 mm) fraction was dried in an oven for 24 hours at 40 ^{0}C, for better conservation during storage until further usage.

2.2 Preparations of extracts

The cherry pit extracts were obtained by weighing 5 g of the material and extracted for 40 minutes with 50 mL of different solvents and under several experimental conditions, as described in Figure 1. A preliminary experiment (phase 1) was done in order to determine the best extraction method for further testing. The preliminary extractions were made at room temperature under magnetic or ultrasonic bath stirring for different solvents. The extracts were filtered to a 50 mL volumetric flask using Whatman No. 3 paper filter and allowed to stand in the dark at room temperature until use. Based on the results of phase1, the experiments of the core work (phase 2) were made using similar methodology, but different processing conditions, namely the extraction solvent was now reduced to mixtures of water : ethanol at various ratios (water : ethanol ratios of 50 : 50; 60 : 40; 80 : 20; 100 : 0 (%, v : v)) and the extraction was carried out at different temperatures of: 35, 50, 70, and 80 ^{0}C, all under magnetic stirring for 40 minutes. All experiments were made to confirm results for the antioxidant activity as depicted in Figure 1.

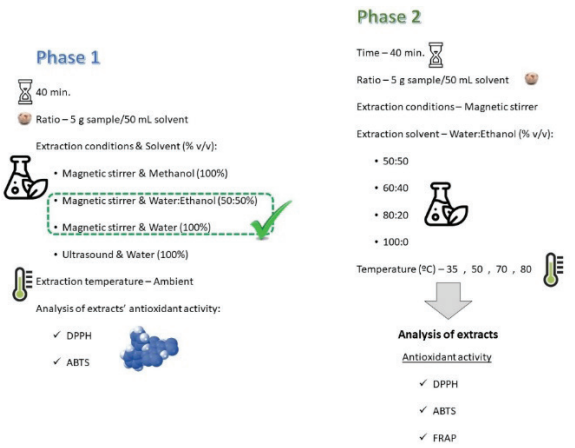

Figure 1. Schematic representation of the experimental conditions tested

2.3 Antioxidant capacity

2.3.1 ABTS assay

The antioxidant capacity was measured using the method based on the abilities of different substances to scavenge the ABTS^{+} radical compared with a standard antioxidant (Trolox) in a dose response curve, adapted from Santos *et al.*, [5], and Kelebek *et al.*, [8]. For the experiment, an ABTS^{+} radical stock solution (7 mM in water) was prepared by mixing with 2.45 mM potassium persulfate. This mixture was allowed to stand for 12 - 16 h at room temperature in the dark until reaching a stable oxidative state. Afterwards, 1 mL of this solution was diluted with 80 mL of ethanol (or solution of extract) before use. In the spectrophotometer, the autozero was performed at 734 nm with ethanol and the absorbance of the ABTS^{+} solution adjusted (Abs ≈ 0.700). In a tube 2.0 ml of ABTS^{+} solution were added and 0.1 mL of sample or standard solution and shaked. The solution was kept in the dark for 15 min. and then the absorbance was read at 734 nm. The antioxidant capacity determined by this method was expressed as Trolox equivalent antioxidant capacity per gram of sample (TE/g).

2.3.2 DPPH assay

For the evaluation of antioxidant activity by the DPPH$^{\cdot}$ method, 2,2-diphenyl-1-picrylhydrazyl hydrate radical - DPPH was used. This method is based on the measurement of the ability of antioxidants to donate hydrogen atom, thus, reducing free radicals in the solution. A methanolic solution (6 x 10^{-5} M) of the DPPH radical was prepared daily and protected from light in accordance to Kelebek *et al.*, [8]. In the spectrophotometer, the autozero was performed at 515 nm with methanol, adjusting the absorbance of the DPPH solution to Abs ≈ 0.700. Then 100 µL of sample (control solution or standard solution) were placed in a test tube and 2 mL of DPPH solution were added. After that, the mixtures were shaken and left for 30 min at room temperature in the dark [9]. The absorbance of the remaining DPPH$^{\cdot}$ was measured at 515 nm and converted into the percentage of antioxidant activity (AA) using the following formula:

$$\%\ \text{inhibition (AA)} = \frac{Abs_0 - Abs_{30}}{Abs_0} \times 100$$

Where: the Abs_0 is absorbance of the control, Abs_{30} is absorbance of the sample measured after 30 minutes. The standard curve was established using various concentrations of Trolox in methanol. The antioxidant capacity of DPPH scavenging for each sample was shown as mg Trolox equivalent per gram of sample (TE/g).

2.3.3 FRAP assay

The iron reduction power antioxidant assay, Ferric Reducing Antioxidant Power (FRAP), is based on the production of Fe^{2+} ion (ferrous form) from the reduction of Fe^{3+} ion (ferric form) present in 2,4,6-tripiridil-s-trialine (TPTZ) complex. When the reduction occurs, there is a change in the tone of the reaction mixture, from light purple to an intense purple, whose absorbance can be measured at the wavelength of 595 nm. The higher the absorbance or intensity of the staining, the greater the antioxidant potential [10]. The FRAP assay evaluates the capacity of the antioxidants in a sample to reduce the ferric-tripyridyltriazine complex (Fe^{3+}-TPTZ) to the colored ferrous-tripyridyltriazine complex form (Fe^{2+}-TPTZ).

Antioxidant activity was determined using the FRAP method reported by Urrea-Vitoria et *al.*, [11]. Working

solution was prepared as follows: 25 mL of 0.3 M acetate buffer solution at pH 3.6 (12.72 mL acetic acid/700 mL of mixed water with sodium acetate solution 7.38 g/300 mL of water) were combined with 2.5 mL of iron chloride solution ($FeCl_3$) at 20 mM with 2.5 mL of TPTZ 10 mM solution (31.2 mg of TPTZ (2,4,6-tris-(2- pyridil)-s-trialine)/10 mL HCl 40 mM) and 3 mL of water. Then 30 µL sample were added to 900 µL of buffer solution, kept at 37 ^{0}C for 30 min and the absorbance was measured at 595 nm wavelength. All measurements were performed in triplicate. The ferric reducing - antioxidant power of each sample under evaluation was determined according to the equation of the calibration curve (obtained from the standard points). The results were expressed as Trolox equivalent in milligrams per gram of sample (mg TE/g).

3. Results and Discussion

3.1 Preliminary tests - Phase 1

Preliminary results for DPPH method showed that the antioxidant capacity was higher for the extraction with water:ethanol 50 : 50 mixtures (0.72 ± 0.04 mg TE/g), rather than with methanol or water stirred by ultrasounds or magnetic stirrer, with 0.26 ± 0.01 mg TE/g and 0.33 ± 0.01 mg TE/g, respectively (Figure 2).

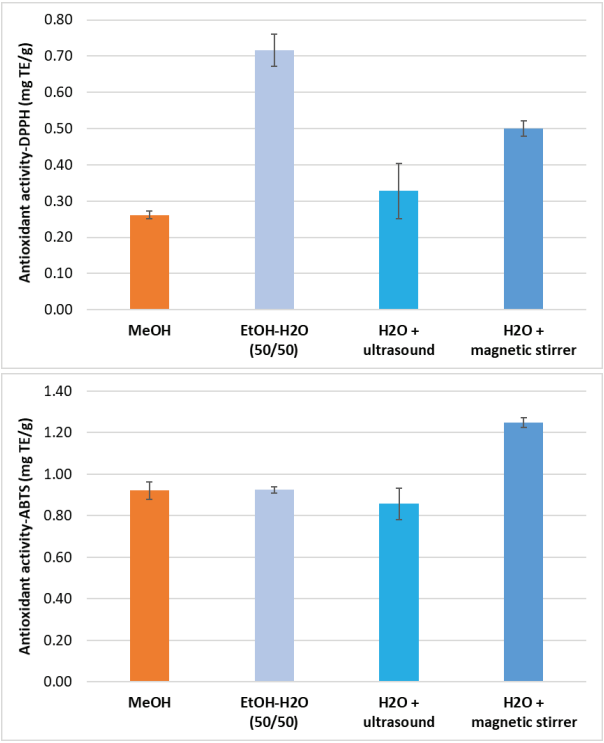

Figure 2. Antioxidant activity in the extracts obtained in the experiments of phase 1

The highest antioxidant activity determined by ABTS method was obtained in the water extract with magnetic stirrer (extracting 1.25 ± 0.02 mg TE/g), followed by methanol and water : ethanol 50 : 50 (v : v) (0.92 ± 0.02 mg TE/g in both cases). The lowest antioxidant activity was found for the extraction procedure with water using ultrasound stirring with a value of 0.86 ± 0.02 mg TE/g (Figure 2).

These results indicated the most suitable conditions to be used in the subsequent experiments, as indicated in Figure 1, comprising water : ethanol mixtures varying from a concentration of 50% up to 100% water and using magnetic stirring.

3.2 Core experiments - Phase 2

Figure 3 presents the antioxidant activity by DPPH method according to extraction conditions (variable temperature and water : ethanol ratio). The results of DPPH tests show that the highest results were achieved with the 50 : 50 water : ethanol solution for all temperatures. For this solution, the values were 0.62 ± 0.00 and 0.65 ± 0.03 mg TE/g at 35 ^{0}C and 50 ^{0}C, respectively, reaching the maximum peak at 70 ^{0}C with 0.74 ± 0.04 mg TE/g, which was the highest even when compared with other water : ethanol ratios.

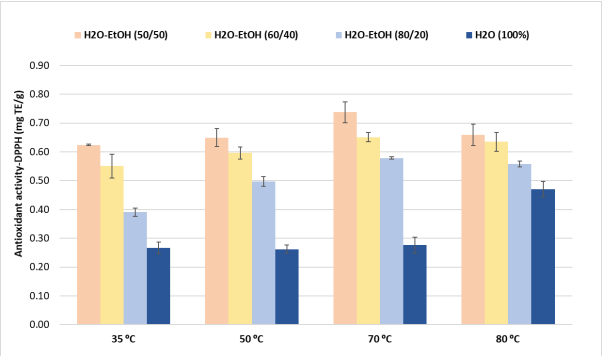

Figure 3. DPPH Antioxidant activity in the different extracts according to type of solvent and extraction temperature

When the temperature increased to 80 ^{0}C the antioxidant activity decreased in practically all extracts under study, except for the solution 100% water (0.47 ± 0.03 mg TE/g).

As for the study of the influence of increase in the percentage of water in the solution, a continuous reduction of the antioxidant capacity was observed, down to a minimum value of 0.26 ± 0.01 mg TE/g at 50 ^{0}C or 0.27 ± 0.02 mg TE/g at 35 ^{0}C.

The range of values obtained in the present work is higher than the results obtained by Afonso *et al.*, [12], who presented total amounts of DPPH antioxidant activity ranging from 0.30 ± 0.01 to 1.17 ± 0.06 µgTrolox/g for sweet cherry seeds by extracting with a water : ethanol solution.

Similar to the DPPH method, the results obtained by the ABTS method show that the increase of water in the ethanolic solution results in a continuous decrease in antioxidant capacity for each temperature tested, just except for the solution with 100% water (Figure 4). The highest antioxidant capacity was obtained again at 70 ^{0}C with values of 2.16 ± 0.01 and 2.14 ± 0.06 mg TE/g for 60 : 40 and 50 : 50 water : ethanol solutions, respectively. It is important to note that also high values of antioxidant activity were obtained at 50 ^{0}C using 50 : 50 and 60 : 40 water : ethanol solutions (1.94 ± 0.01 and 1.86 ± 0.05 mg TE/g, respectively).

For all temperatures was obtained a decrease in antioxidant capacity down to 0.60 ± 0.03 mg TE/g for the 00% water solution at 70 ^{0}C, due to the increase of water in ethanolic solution, thus neutralizing the positive influence of temperature in the extraction of compounds with antioxidant activity. Spychaj *et al.*, [13], obtained for cornelian cherry fruit stones values of antioxidant activity varying from 301.92 to 509.74 µmol TE/g.

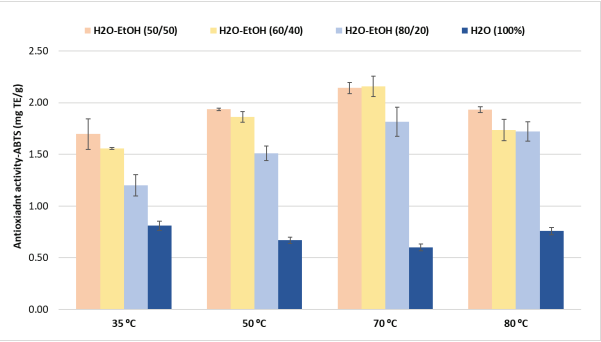

Figure 4. ABTS Antioxidant activity in the different extracts according to type of solvent and extraction temperature

Figure 5 presents the concentrations of FRAP antioxidant capacity according to extraction conditions such as temperature and water : ethanol ratio in the extracting solution.

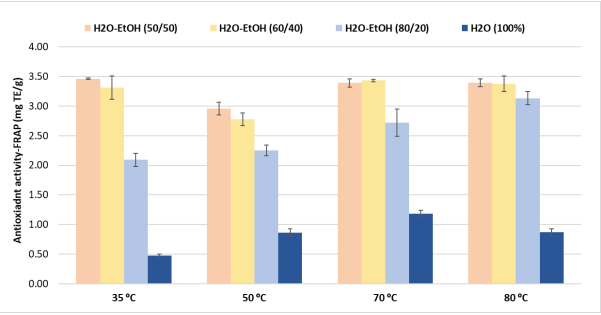

Figure 5. FRAP Antioxidant activity in the different extracts according to type of solvent and extraction temperature

Through the analysis of the graph it was noticed that the FRAP method presents a different trend compared to the two previous analyzed methods, ABTS and DPPH, whose general trend was similar. The best result for ferric reducing antioxidant power of 3.46 ± 0.01 mg TE/g was recorded at a lower temperature, 35 ^{0}C in 50 : 50 water : ethanol solution, although the result was very similar to 3.43 ± 0.02 mg TE/g which was obtained at the temperature of 70 ^{0}C and 60 : 40 water : ethanol solution. Chaovanalikit *et al.*, [14], presented amounts of reducing antioxidant power (FRAP) varying between 200 to 848 µmoles Trolox equivalent/100 g for sweet cherry seeds extracted with 70% (v/v) acetone.

Although with differences from FRAP to ABTS and DPPH methods, a similar observation was found, i.e., that each of the three tests demonstrated that 100% H_2O lead to a significant reduction in antioxidant activity in cherry seeds extracts.

4. Conclusions

- This work showed that sweet cherry seeds are by-products which have high antioxidant activity. The potential of different extraction conditions was investigated to optimize the extraction parameters, such as solvent and temperature for the maximum yield of antioxidant activity from the extracts of cherry pit, which was determined by DPPH, ABTS and FRAP. For DPPH method higher antioxidant activity was found at 70 ^{0}C and a 50 : 50 water : ethanol solution. For ABTS method higher antioxidant activity was obtained at the same temperature of 70 ^{0}C and a 60 : 40 ratio. The best result of ferric reducing antioxidant power was registered at 35 ^{0}C and a 50 : 50 ratio. The best temperatures for extraction of compounds with high antioxidant activity stand in a range from 35 to 70 ^{0}C, while the most appropriate solutions are water : ethanol with ratios varying from 50 to 60% water.
- From these results were obtained favorable conditions to optimize the extraction of compounds with antioxidant activity from sweet cherry seeds, thus allowing to add value to an otherwise discarded residue from the food industry. The compounds recovered from the cherry seeds can be used in the food, pharmaceutical or cosmetic industries.

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