Processing Cherry Waste with Supercritical Fluids to Develop a New Promising Nutraceutical

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ABSTRACT

Cherries are a rich source of bioactive compounds, such as polyphenols and perillyl alcohol, reported to be a potential anticancer and antiinflammatory agents.
In this work natural extracts were obtained by supercritical fluid extraction from cherries of Portuguese crops. Bioactivity of the extracts was evaluated in terms of antioxidant capacity and antiproliferative effect in human colon (HT29) and stomach (MKN45) cancer cells. Results obtained were compared with pure perillyl alcohol, and cherry supercritical extract showed to be a potential source of promising new nutraceutical formulation.

INTRODUCTION

Clinical trials and epidemiological studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular diseases, cancer and aged related disorders [1].
The formulation of new products by incorporation of natural extracts is nowadays widely in use, as the trend of the future is moving towards nutraceuticals and functional food with specific health effects. There is consolidated scientific evidence supporting the existence of a synergetic effect between the compounds present on a natural matrix, what is thought to be the main reason for an investigation directed towards in obtaining a natural bioactive concentrate instead of trying to synthesize or to isolate the pure compounds.
Cherries are a rich source of bioactive compounds such as polyphenols (quercetins, neochlorogenic acid, chlorogenic acid, ellagic acid, anthocyanins, etc) and perillyl alcohol (POH). POH is a monoterpene with well established chemopreventive activity in rodent mammary, skin, liver, lung, and colon cancers and also chemotherapeutic activity in pancreatic and mammary, and tumour models, leading to regression of existing malignant tumours [2,3]. Nevertheless, POH has been highly studied for its bioactivity in cancer but its extraction from natural matter is yet far from being considered thoroughly studied. Supercritical CO_2 extraction was already reported to be effective for the extraction of perillyl alcohol (POH) in Korean orange and citrus unshiu peel [4,5].
In this work, the potential of using supercritical CO_2 extraction as a method to develop a cherry bioactive concentrate for nutraceutical development was investigated.
The antioxidant activity of the extracts obtained was studied using two different complementary assays: i) ORAC -scavenging of peroxyl radicals, $ROO^{\bullet}$ and ii) EPR (electron paramagnetic ressonance) - scavenging activity toward hydroxyl radicals.
Finally, antiproliferative activities of the extracts were also studied in vitro, using HT29 and MKN45, colon and stomach cancer cell lines respectively, after previous evaluation of citotoxicity in Caco2 cell line model (mimetizing human intestinal epithelium).

MATERIALS AND METHODS

Chemicals

Perillyl alcohol [CAS 18457-55-1], was purchased from Extrasynthese and maltodextrin [CAS 9050-36-6] was supplied by Sigma Aldrich. Carbon dioxide (99.5%, industrial grade) was obtained from Air Liquide. All products were used with no further purification.

Cherry
The samples used in this work were from two typical Portuguese varieties: Saco cherry fruit and S. Julião cherry waste. Immediately upon arrival in the laboratory, the samples were crushed and stored at -20ºC, until the day of the assays.

Solubility measurements
The design of the supercritical extraction processes requires knowledge of the solubility of the bioactive compounds of the mixture in CO_2. These studies have been performed in a pressure range between 15-25 MPa and at 303, 313 and 323 K, using an apparatus and a procedure previously described by Matias et al [6]. Briefly, it consisted of loading a high-pressure visual cell with cherry sample and carbon dioxide and stirring the mixture for one hour, at certain pressure and temperature. After a 30 minutes pause for phase equilibrium, a sample from the gas phase was taken through a six-port sampling HPLC valve. Samples were collected by depressurization and expansion into a glass trap, and the amount of CO_2 is quantified by measurement of the resulting sub-atmospheric pressure increase in a calibrated volume upon expansion. Samples were analysed in terms of content of phenolic compounds. Solubility of phenolic compounds in CO_2 was calculated taking into account the second virial coefficients of carbon dioxide.

Supercritical fluid extraction
Extractions were performed in a high pressure apparatus schematically presented in the following picture.

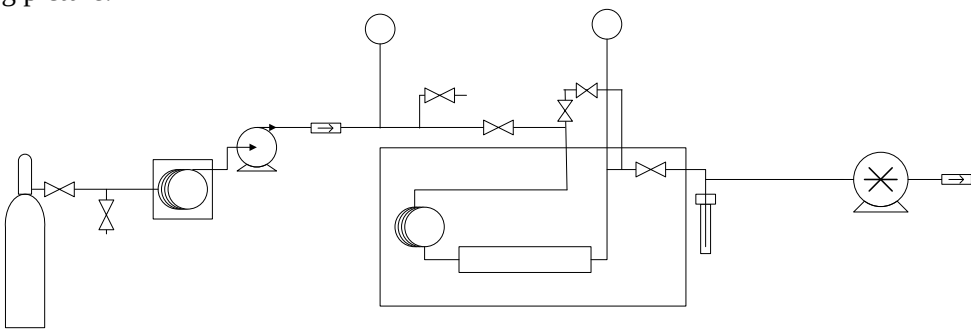

Figure 1 : Schematic diagram of the supercritical fluid extraction apparatus:1- CO_2 bottle; 2,3,6,7,8,9,12-Needle valves; 5- CO_2 compressor; 4-CO_2 Cooling loop; 10- Heating loop ; 11- Steel extraction vessel ;13- Oven;14-Glass trap;15-Flow meter

The extraction procedure consisted in heating a high-pressure extractor containing the matrix and adding carbon dioxide up to the desired pressure. Carbon dioxide was then continuously fed with an average flow rate of 0,03 m^3/h and the extracts obtained were collected in a glass trap filled with ethanol to entrap the most volatile compounds. The extraction was performed at 20 MPa and 323ºC, during 8 hours. This procedure was repeated for 6 days.

Analytical methods
Thin Layer Chromatography .TLC was performed in silica pre-coated plates (0.20-mm layer). Plaques were developed with chloroform [4] and detected by a 254nm UV lamp.

Total phenolic content. The total phenolic content of cherry extracts was measured using a colorimetric Folin-Ciocalteu assay [7]. The measurement was compared to a standard curve of gallic acid concentrations and results were expressed as milligrams of gallic acid equivalents per litre of extract.

Total anthocyanin content. Total anthocyanins was estimated by differential pH as described by Wroslstad [8].

Antioxidant Capacity. Two independent assays were used to access the antioxidant activity of the cherry extracts:
i)Oxygen radical absorbance capacity (ORAC), expresses the ability of the sample to scavenge peroxyl radical ($ROO^{\bullet}$) and was carried on following the modified method described by Ou et al, 2001[10].The standard used was Trolox and final results were expressed as μM of Trolox equivalents antioxidant capacity (TEAC);
ii) Electron paramagnetic resonance (EPR) shows the ability of the sample to scavenge hydroxyl radical ($HO^{\bullet}$). EPR measurements were conducted using an EPR spectrometer (Bruker EMX6/1, 1998) as reported by Leonard et al (2003) [11]. Final results were expressed as % of inhibition of $HO^{\bullet}$ radicals.

Antiproliferative Assays
Cell culture. HT29, human colorectal cancer cell line, was obtained from American Type Culture Collection (ATCC, USA). MKN45 human stomach cancer cell line was kindly provided by Dr. Celso Reis (IPATIMUP, Portugal). All cells lines were grown in RPMI 1640 (Gibco, Portugal) supplemented with 10% of fetal serum bovine (FBS) (Gibco, Portugal) and 2 mM of glutamine (Gibco, Portugal). MKN45 medium was also supplemented with 50μg/mL of gentamycin (Gibco, Portugal). Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC with 5% CO_2 in a humidified atmosphere.

Antiproliferative assays. Antiproliferative cell assays were executed in HT29 and MKN45 cell lines as previously reported[20]. Briefly, cells were cultured in 96-well microplates at a density of 1×10^4cells/well. After 24h, cells were incubated with cherry extract and POH diluted in 0.5% FBS culture medium for 4 hours. Afterwards medium was substituted by fresh medium and cells were allowed to proliferate for 20 hours. Cells viability was assayed by MTT assay and results were expressed in terms of % of cellular viability relative to control (cells without treatment). The amount of sample necessary to decrease 50% of the cellular viability, EC_{50}, was also calculated.

RESULTS

Samples characterization

Total phenols, total anthocyanins and antioxidant capacity of two cherry samples are presented in Table 1.

Table 1 : Total polyphenols, total anthocyanins and antioxidant capacity of cherry samples.

Cherry	Polyphenols (mg GAE/100g)	Anthocyanins (mg/100g)	$ROO^{\bullet}$ scavenging (uM TEAC)
Saco	255	78	6 081
S. Julião (waste)	138	32	3 608

Note: Samples characterization was performed with methanolic extracts of cherries as described by Gonçalves et al [12].

Between the two samples, Saco cherry contained the higher concentration of bioactive compounds and antioxidant capacity. Also, the results obtained for this variety, were similar with those reported by Gonçalves et al (2004) [12]. Concerning S.Julião cherry waste, this residue had only 2 times less polyphenols, anthocyanins and antioxidant capacity than Saco cherry.

Solubility of cherry's polyphenols in CO_2

Solubility assays were performed in a pressure range between 15-25 MPa and at 303, 313 and 323 K, in order to achieve the operational conditions where the solubility of phenolic compounds of cherry in carbon dioxide is maximized.
These measurements were done with Saco cherry, because it is the sample that contained the higher content of polyphenols. In Figure 2, the solubility of the phenolic compounds is plotted as a function of pressure (A) and temperature (B).

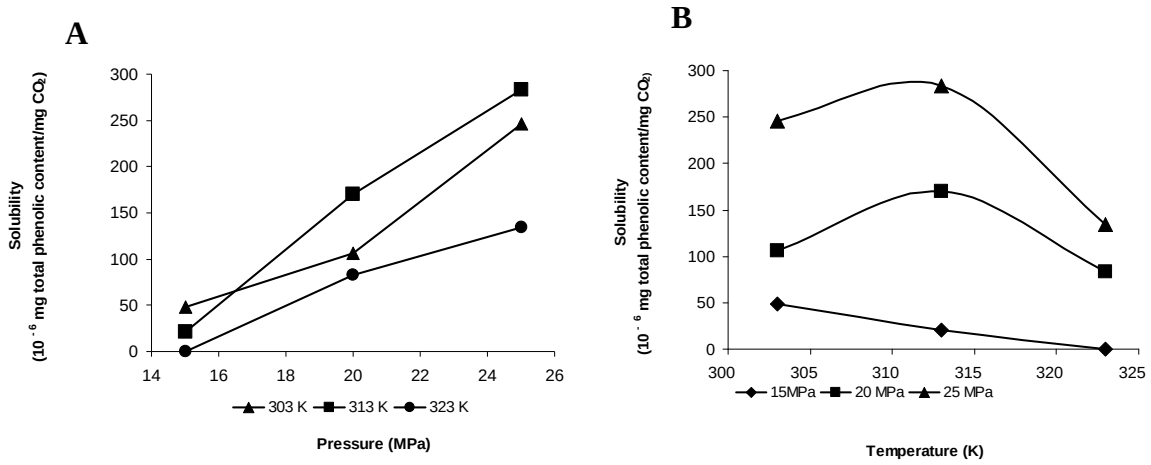

Figure 2 : Solubility of phenolic compounds of cherry as a function of pressure (A) and temperature (B).

Results obtained shows that to all temperatures studied the solubility of the phenolic compounds present in Saco cherry in supercritical CO_2 increased with the pressure (Figure 2A). According to the temperature, maximum of solubility is reached at 313 K, decreasing afterwards (Figure 2B). Additionally, solubility increased with CO_2 density and it was higher above 800kg/m^3 were CO_2 is liquid like (data not shown).
According to the obtained results, the extraction conditions for polyphenols extraction seemed to be maximized for a temperature and pressure range between 303-313K and 20-25MPa, respectively.

Supercritical extraction of POH-containg extract from cherry waste

This extraction was performed in order to obtain a bioactive extract containing perillyl alcohol. For this reason, the extraction conditions adopted were the same as reported by Lee et al (2001) and Lee et al (2004) - these authors successfully isolated POH from citrus unshiu peel and Korean orange peel at operating conditions of 323K and 20MPa [4,5].

The extraction was performed with S. Julião cherry waste due to the great availability and economic interest of this kind of crop residues.
The crushed cherries were freeze-dried with different amounts of maltodextrin for 24 hours (Edwards modulyo freeze-drier), in order to reduce the water content and better homogenise the sample. This operation was tested with different amounts of excipient and the best result was achieved for 30%.

A cumulative extraction of 3 to 5 grams of S. Julião cherry waste lyophilized with 30%w/w maltodextrin was performed at 20MPa and 323K for 8 hours in a continuous mode. This

procedure was repeated for 6 days. The average flow rate of CO_2 was 0,03 m^3/h and the total mass of feed used was 21,3g, which contains 53,4mg GAE of polyphenols. For each extraction experiment the samples were withdrawn every 2 hours and analysed for its total phenolic content. The extracts were transparent and had a strong cherry-like odour which might indicate the presence of volatile compounds. The cumulative extraction data is presented in Figure 3.

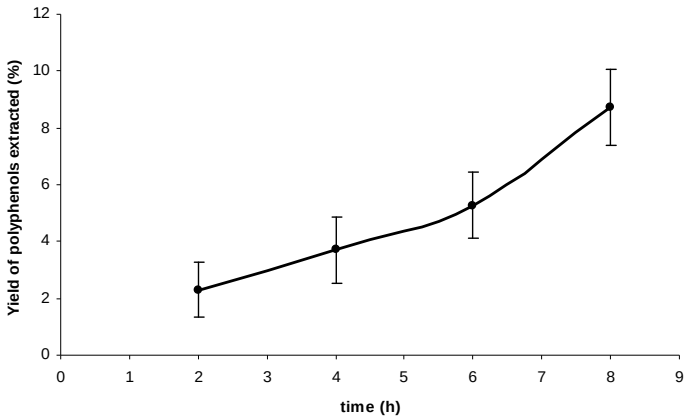

Figure 3 : Cumulative extraction of polyphenols from S.Julião cherry waste (20MPa and 323K).

Results obtained shows that the total yield of polyphenols extracted from S. Julião cherry waste was 8,73 %.

Total extract was concentrated 20 times (v/v) in a rotary evaporator, and then characterized in terms of bioactive compounds and antioxidant capacity (Table 2).

Table 2 : Characterization of cherry extract and POH

	Polyphenols (mg GAE/L)	Anthocyanins (mg/L)	POH	ROO• scavenging (uM TEAC)	HO• scavenging (%)
Cherry extract	113	0	present	21 936	8
POH				227 270	0

Note: The presence of POH in the extract was detected by TLC

Results obtained shows that cherry supercritical extract had high antioxidant potential, which could be do to the presence of bioactive compounds, namely polyphenols and POH. In comparison with standard POH, the natural extract contained only 10 times less activity towards ROO• radicals. Additionally, cherry extract was able to scavenging HO• radicals, whereas POH had no effect.

Finally, antiproliferative an activity of the extract was also studied in vitro, using HT29 and MKN45, colon and stomach cancer cell lines, respectively. Both cell lines proliferation was inhibited in a dose-dependent manner after exposure with various concentrations of natural cherry extract. EC_{50} values found for HT29 and MKN45 were 4,5 and 3,5% (v/v), respectively. Also, these concentrations were not cytotoxic which was previously evaluated in differentiated Caco2 cell line model.
In comparison, antiproliferative activity of cherry methanolic extract was also evaluated and for the same concentrations the growth of cancer cells was not affected.

CONCLUSION

Cherry is a fruit widely grown in Portugal and its known to be rich in bioactive compounds reported to have human health benefits. The recovery of these bioactive compounds from residues is of great importance, not only because of their biological activities but also as it could represent a significant advance in maintaining the environmental equilibrium- large quantities of residues presents problems of storage, transformation, or elimination, in both ecological and economic terms.

The results obtained in this work were encouraging as they proved that bioactive compounds of cherry, namely polyphenols and perillyl alcohol, can be extracted with supercritical carbon dioxide, at least when processed in this laboratory scale batch apparatus.

The extract obtained from cherry waste contains high antioxidant capacity and antiproliferative effect in human cancer cells. Due to bioactivity associated, this natural extract can be considered as a potential source for new promising nutraceuticals formulations.

ACKNOWLEDGMENTS

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