FRACTIONED HPE FROM ELDERBERRY POMACE

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ABSTRACT

Commercially, elderberries (*Sambucus nigra* L. fruit) are mainly used to produce cordials, soft drinks, soft spreads, wine, tea and nutraceutical beverages. These berries are a well known rich source of phenolic compounds, namely anthocyanins, and have insulin-stimulating and anti-angiogenic properties, as well as anti-carcinogenic potential. Its pomace, which is constituted of fruit seeds and skins, accounts for almost 15 % of total fruit weight and contains higher amounts of polyphenolic compounds than those present in the edible fleshy parts of the fruits. In this study, polyphenolic-rich extracts were obtained by using a Fractioned High Pressure Extraction (FHPE) methodology. It was carried out, at 313 K and 20 MPa, in two sequential steps: i) first of all, elderberry byproduct was extracted with CO_2 to recover less polar compounds; ii) and subsequently, a second step was performed using CO_2 + EtOH/H_2O (8:2, v/v) to recover more polar compounds, like polyphenols. Different percentages of EtOH/H_2O mixtures (10-100 %, v/v) were employed and their effects on global yields, total phenols, total flavonoids and total anthocyanins (quantified by spectrophotometric assays) and on extracts antioxidant activities (measured by the DPPH assay), are discussed. A positive, significant linear relationship between the total extracted phenols, flavonoids and anthocyanins and the amount of EtOH/H_2O used in the 2nd FHPE step solvent mixture was observed. Higher EtOH/H_2O percentages also tended to originate extracts possessing higher antioxidant activities though there was not a direct relationship to their total phenols contents. Total flavonoids and total monomeric anthocyanins were not directly related with antioxidant activities although the extracts with higher antioxidant activities were amongst the ones with higher contents of these compounds. Elevated total phenolic contents were registered in the obtained extracts, showing that elderberry residue can be an excellent source of polyphenols.

INTRODUCTION

Elder (*Sambucus nigra* L.) is a shrub native to Europe, Asia and North Africa. In northern Portugal, the elder takes advantage of the excellent edaphoclimatic conditions and its intensive culture is presently growing [1]. Elderberries are a recognized rich source of phenolic compounds, namely anthocyanins [2], and various parts of the plant have been used as a food source, to make elderberry wine and pies, as a flavoring or a food dye, and also in traditional medicine. For example, Sambucol®, a syrup containing 38 % of standardized elderberry extract, proved to have several health promoting/protecting characteristics [3]. In those applications, elderberry processing generates high quantities of presscake residues, which contain high amounts of phenolic compounds [4], thus being a potential residue source of nutraceuticals. Moreover, and besides being a cheap, renewable and abundant source of

polyphenols, its reuse could also contribute to the maintenance of environmental equilibrium [5]. However, when compared to other fruit pomaces, elderberry pomace has not been conveniently recycled which is surprising due to its already referred high phenolic content [6]. High pressure extraction processes, like supercritical fluid extraction and enhanced solvent extraction, are very interesting alternatives to conventional solvent extraction processes, offering several advantages like extraction time, solvent consumption, extraction yields, reproducibility and automation [7].

The presence of undesired extractable compounds in several vegetal matrixes, which may be co-extracted or which may interfere negatively with the extraction of the desired substances, decreasing the extraction yields and selectivity, is a typical situation in natural products extraction methodologies. Fractioned extractions, using different solvents and cosolvents mixtures, at different steps of the extraction procedures, can be utilized to overcome the co-extraction of these undesired compounds. These extractive procedures have been applied to elderberry pomace [8], performing an initial extraction of low polarity CO_2 soluble compounds with ~17 % (w/w, db) of global yield, followed by a CO_2 + EtOH/H_2O (8:2, v/v) extraction of higher polarity compounds during 45 minutes. The 2nd step resulted in an anthocyanin rich extract with a global yield that varied between 4.0 % and 17.7 %, for 20 and 100 % (v/v) of EtOH/H_2O, respectively.

The main goal of this work was to study the relationship between the polar mixture composition of the 2nd step used in the fractioned high pressure extraction of elderberry pomace, by varying the percentage of EtOH/H_2O (8:2, v/v), and the composition of the obtained anthocyanin rich extracts, in terms of total phenols, flavonoids and anthocyanins. The correlation between composition of the extracts and their antioxidant activity, expressed as IC_{50} values, was also evaluated. Operational conditions (313 K and 20 MPa) were selected based on the literature information about anthocyanin stability and lipophilic compounds solubility.

MATERIALS AND METHODS

Raw material – Fresh elderberries were collected in Varosa Valley, Portugal, and stored under vacuum at 255 K, until further processing. Elderberry pomace was produced by mechanical pressing, to separate the juice, and then dehydrated in a fluidized bed dryer, at 308 K and in the absence of light, for 12 hours. Dried byproducts containing 6.1 ± 0.1 % (w/w, db) of humidity [8] were milled in a grinder for 2 minutes and conditioned under vacuum in plastic bags, at 255 K. Water content was determined gravimetrically by drying samples in an oven, at 378 K.

Chemicals - Carbon dioxide (99.998 %), ethanol (99.5 %) and distilled water were used for the extraction experiments. Chemicals and solvents employed for extract analysis were: ethanol, sodium carbonate, sodium nitrite, sodium hydroxide anhydrous, potassium chloride and sodium acetate 3-hydrate, all of them of P.A. degree, and also methanol (Lichrosolv), Folin-Ciocalteu's phenol reagent, aluminium chloride anhydrous (98 %), 2,2-diphenyl-1-picrylhydrazyl (DPPH radical) (~90 %) and bi-distilled water. Used standards were gallic acid and epicatechin for total phenols and flavonoids analysis, respectively.

Fractioned High Pressure Extraction (FHPE) – Extractions were carried out at 20 MPa and 313 K, using a HPE apparatus described by Seabra et al. [8]. A two-step FHPE methodology was employed, comprising: *i*) A first extraction step, in which elderberry byproducts were extracted with supercritical CO_2, in order to remove low polarity CO_2 soluble compounds, employing a 15 min static period followed by a 40 min dynamic period; *ii*) A second dynamic

extraction step was performed for 45 minutes, in order to extract polar compounds like anthocyanins, by using CO_2 and EtOH/H_2O (8:2, v/v). Different percentages of EtOH/H_2O (10, 20, 40, 50, 60, 70, 80, 90 and 100 %, v/v) were assayed.
A 30×10^{-6} m^3 stainless steel extraction cell was filled with ~1.5×10^{-3} kg of elderberry pomace and glass beads. CO_2 was delivered to the cell using a high pressure liquid compressor and EtOH/H_2O was delivered by a high pressure liquid pump (L-6200A, Hitachi, Merck, Darmstadt, Germany). To prevent line obstructions, cotton wool was placed on both endings of the cell, which was placed into a water bath. Extracts were recovered in two glass flasks, placed in an ice bath, and the expanded CO_2 was measured by a wet gas meter. Extracts were lyophilized (Labconco, model 77560, Kansas City, Missouri, USA) and kept at 255 K.
Quantification of phenols, flavonoids and anthocyanins - Total phenols in extracts were measured according to the Folin-Ciocalteu's method, and following the procedure proposed by Singleton and Rossi [9] with some modifications [10], and were expressed as gallic acid equivalents. Total flavonoids were determined using the modification of the $AlCl_3$ assay, proposed by Zhishen, Mengcheng and Jianming [11] and were expressed as epicatechin equivalents. Total anthocyanins, expressed as cyanidin 3-glucoside equivalents, were determined using the pH differential method, according to Lee, Durst and Wrolstad [12]. All these assays were, performed, at least, in triplicate, in a Jasco V-530 UV-vis spectrophotometer.
Antioxidant Activity (DPPH assay) - An adaptation of the method described by Blois [13] was employed. Samples reducing activities were estimated from the observed absorbance decrease and results were expressed as IC_{50} values, defined as the amount of extract (μg, db) that decreased, by 50 %, the initial absorbance of the DPPH radical solution. All assays were performed, at least, in triplicate.

RESULTS

The solvent mixture compositions, at the extraction cell conditions and for the 2nd step HPE, are presented in **Table 1**, according to the CO_2-EtOH-H_2O equilibrium data reported by Durling et al. [14], at 313 K and 20 MPa. There were two phases in the cell for the 10-60 % EtOH/H_2O mixtures and one liquid phase for the 70–100 % EtOH/H_2O mixtures.
With the increment in the EtOH/H_2O percentage from 10 to 60 %, there was an increment in the amount of the liquid phase in the cell. As expected, the EtOH mole fraction of the high pressure liquid phase increased with the increase in EtOH/H_2O added to the solvent mixture, for all assays.
The quantification of total phenols, total flavonoids and monomeric anthocyanins in 2nd step extracts is represented in **Figure 1**. A positive, significant linear relationship between the amount of extracted substances and the amount of EtOH/H_2O used in the solvent mixture was found (R^2 values of 0.966 for flavonoids, 0.954 for anthocyanins and 0.895 for phenols). This behaviour is probably related to the presence of higher amounts of EtOH and H_2O rich high pressure liquid phase, a high density and polarity phase, which presents a better capacity to dissolve polar substances. These conditions were favourable to higher concentration gradients of the substances being extracted between the matrix and the solvent, thus increasing extraction rate and yields. The increment in the EtOH mole fraction (**Table 1**) may also have a positive influence on the extraction of these substances.

Table 1: Solvent mixture composition for 2nd step HPE, at 313 K and 20 MPa, using CO_2 and EtOH/H_2O (8:2, v/v) as solvents [14].

EtOH/H_2O (%, v/v)	Liquid Phase			Gaseous Phase			$\frac{\text{Liquid Phase}}{\text{Gaseous Phase}}$ (mol/mol)
	x_{CO_2}	x_{EtOH}	x_{H_2O}	x_{CO_2}	x_{EtOH}	x_{H_2O}	
10	0.05	0.08	0.87	0.97	0.02	0.008	0.13
20	0.20	0.32	0.49	0.88	0.10	0.020	0.24
40	0.27	0.38	0.36	0.86	0.13	0.020	1.15
50	0.28	0.38	0.34	0.86	0.13	0.020	2.89
60	0.30	0.39	0.31	0.82	0.14	0.020	13.14
70	0.25	0.42	0.34	-	-	-	-
80	0.16	0.46	0.38	-	-	-	-
90	0.08	0.51	0.41	-	-	-	-
100	0.00	0.55	0.45	-	-	-	-

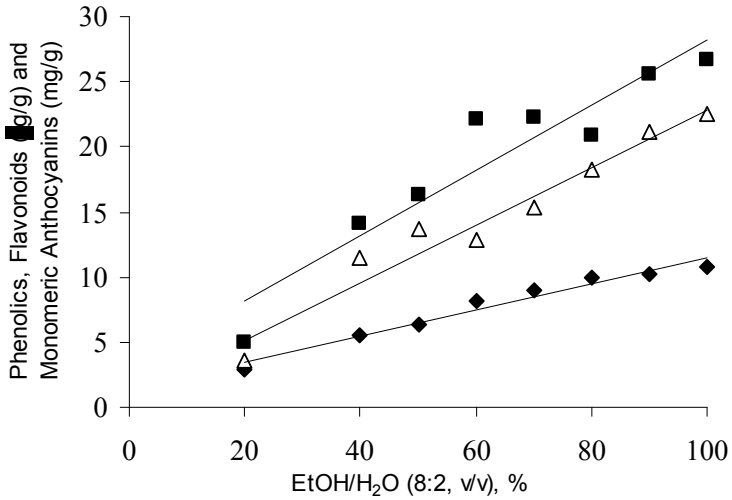

Figure 1: Total phenols (μg gallic acid equivalents/g pomace, db) (■), total flavonoids (μg epicatechin equivalents/g pomace, db) (◆) and monomeric anthocyanins (mg cyanidin 3-glucoside equivalents/g pomace, db) (Δ) of elderberry pomace extracts obtained in 2nd step HPE, at 313 K and 20 MPa, using CO_2 and EtOH/H_2O (8:2, v/v) as solvents.

Higher EtOH/H_2O percentages in the 2nd extraction step tended to originate extracts possessing higher antioxidant activities (lower IC_{50} values), though there was not an evident and direct relationship with the amount of total phenols present in extracts (**Figure 2 a**). For example, extracts with similar phenolic contents (50 % EtOH/H_2O – 0.146 mg/g and 100 % EtOH/H_2O – 0.151 mg/g) showed diverse IC_{50} values (65 and 50 μg, respectively). This is not surprising because there is a wide antioxidant activity variation for different phenolic compounds [15].

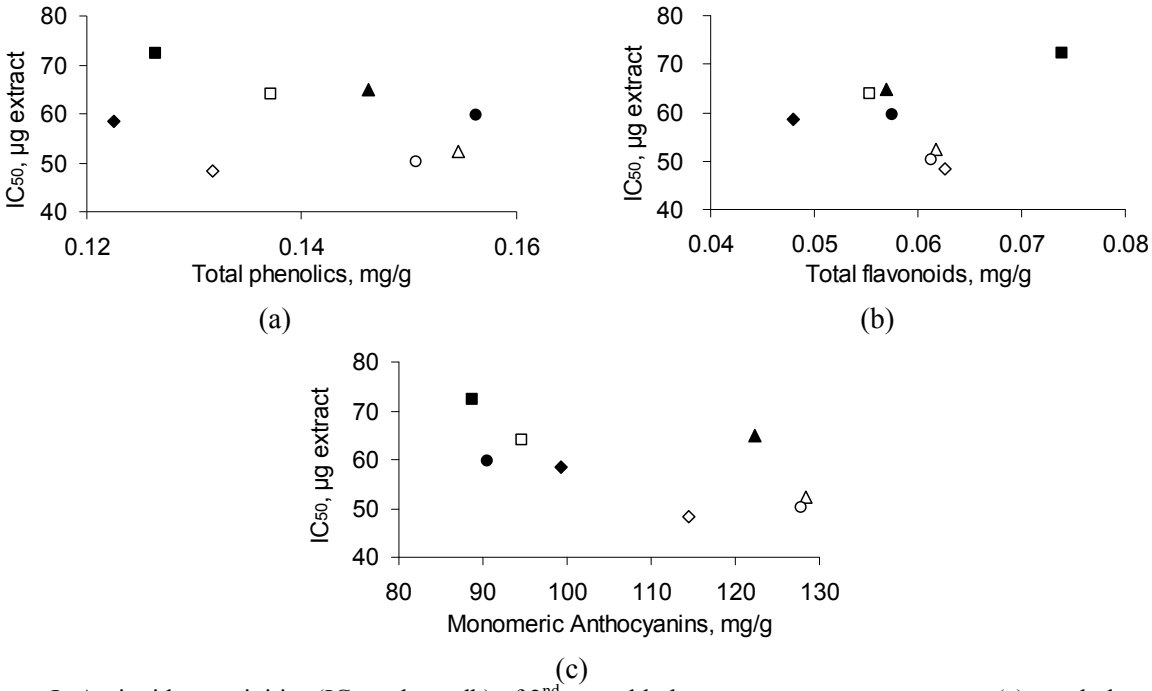

Figure 2: Antioxidant activities (IC_{50} values, db) of 2nd step elderberry pomace extracts versus: (a) total phenols (mg gallic acid equivalents/g extract, db); (b) total flavonoids (mg epicatechin equivalents/g extract, db); and (c) total monomeric anthocyanins (mg cyanidin-3-glucoside/g extract), obtained at 313 K and 20 MPa and using CO_2 + EtOH/H_2O in the following percentages: ■ 20, ♦ 40, ▲ 50, • 60, □ 70, ◊ 80, Δ 90, o 100.

Total flavonoids and monomeric anthocyanins (**Figure 2 b** and **c**) were also not directly related to antioxidant activities, though the extracts with lower IC_{50} values (52 and 50 µg, obtained with 90 and 100 % EtOH/H_2O, respectively) were among the ones with higher contents of these substances (~0.061 mg epicatechin equivalents/g and 128 mg cyanidin equivalents/g, for both extracts). A probable explanation for these results can be that, among phenolic compounds, flavonoids and namely anthocyanins are known to be very strong antioxidants [16].

In general, these results are in accordance with the fact that the relationship between antioxidant activity of berry extracts and their phenolic composition is quite complex [17] and, in addition, synergist and antagonist effects may as well have a significant effect on the antioxidant response of plant extracts.

Extracts total phenolic and anthocyanin contents were relatively high when compared to extracts obtained, for example, from winery byproducts [18] showing that elderberry pomace can be an excellent source of these substances.

CONCLUSION

This study demonstrated a positive, significant linear relationship between total extracted phenols, flavonoids and anthocyanins and the amount of EtOH/H_2O in the solvent mixture used for the 2nd step FHPE of elderberry pomace. Antioxidant activities of the obtained extracts were not directly related with total phenols, flavonoids and anthocyanins extract contents, though the extracts with lower IC_{50} values were amongst the ones with higher flavonoid and anthocyanin contents. These results suggest that elderberry residue may be an excellent source of antioxidant compounds, namely of polyphenols.

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