Extraction of Valuable Compounds from Ginja Cherry By-Products: Effect of the Solvent and Antioxidant Properties

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Abstract

Purpose To study the composition of extracts of Ginja cherries stems and leaves obtained after extraction using different solvents and to evaluate their antioxidant activities. Stems and leaves are by-products of the Ginjinha cherry liquor production; extracting valuable compounds with the most appropriate solvent would valorise these wastes.

Method The extraction was performed using different solvents (acetone, ethanol, methanol, ethyl acetate, water, 2-propanol); liquid chromatography-electro spray ionization-mass spectrometry (LC-ESI/MS) was utilized to identify the predominant phenolic acids and flavonoids present in the resulting extracts. The Total Phenolic Content was determined with the Folin-Ciocolteau method. The antioxidant activity was also tested using the $ABTS^{+}$ essay.

Results Stems extracts showed a higher concentration in polyphenols than those from leaves. The solvent affected remarkably the extracts compositions: considering the polyphenols content ethanol and water gave the best results for stems and leaves, respectively. A good correlation was established between the antioxidant activity of the extracts and their polyphenolic composition.

Keywords Cherry by-products · High added-value compounds extraction · Polyphenols · Antioxidant activity

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Introduction

One of the problems facing the western world today is the production of increasing amount of waste, from different and varied sources. A high proportion of this waste is linked to the food industry, as the production processes of many food products is normally associated with the generation of large quantities of waste and/or by-products.

For the vegetable and fruit industries, for instance, a waste or by-products production between 30 and 50% is reported, the values depending on the kind of fruit or vegetable considered [1]. In Europe only, these industries generate about 30 million tones of waste per year [2].

The majority of this waste is land filled, burned or used as low quality compost or animal feed without segregation or significant treatment. In many cases these waste contain compounds with interesting properties; recovering bioactive phytochemicals that could be used in different sectors, such as pharmaceutical, cosmetic and food industries, is potentially valuable.

Polyphenols are one of the most prominent classes of phytochemicals compounds [3]. Their anti-cancer, anti-allergic, anti-mutagenic and anti-ageing activities were previously reported [4–6]. The extraction of phenolic compounds from different parts of plants, fruit and vegetables has been widely studied [7, 8 and reference therein]. Furthermore, the use of food by-products as a polyphenols source has been also considered: the use of agricultural waste [3, 9, 10] and waste from fruit juices [11] are some of the examples reported in literature.

Phenolic compounds, extracted from plant materials, have been shown to have anti-oxidative and pro-oxidative properties in various model systems. Their antioxidant activity can be performed according to different mechanisms: via free-radical scavenging, hydrogen donation,

singlet oxygen quenching, metal-ion chelation or as substrates for attack by superoxide [12]. For all these properties, polyphenols are finding increasing use in the development of functional foods [13].

Portuguese Ginjinha is a traditional sour cherry liqueur, produced from Ginja sour cherries (*Prunus cerasus*, L. Rosaceae), which are native to Portugal. Stems and leaves of these cherries are pre-by–products of the Ginjinha production line. They could be valorised, by employing them to extract high added-value compounds.

Preliminary work was previously performed on this topic, with promising results: extracts from both stems and leaves were prepared using ethanol as solvent and their antioxidant properties were measured [14]. Furthermore, the antibacterial properties of these ethanol extracts were also analysed [15]; the results showed that both stems and leaves extracts presented antimicrobial activity.

In this paper we present a more complete and systematic investigation about the extraction process from these by-products. To study the effect of the solvent on the extracts composition, several solvents of different polarity were considered, for both stems and leaves. A complete characterization of the extracts was performed: their polyphenols content and antioxidant activity were determined, and a correlation between the concentration and the properties of the extracts was established.

Materials and Methods

Samples

Ginjinha pre-by-products were provided by a Ginjinha company (Frutóbidos) located in Óbidos (Portugal), in July 2009. Stems and leaves were separated from each other manually. As the fresh raw materials can be susceptible to spoilage, the stems and leaves were dried at 45°C in a tray drier for 24 h; subsequently they were kept in a cool and tightly closed box for further analysis. By-products were ground prior to extraction for 5 min. using a Ciatronic (Germany) kitchen grinder at the highest mixing rate (level 2), in order to reduce the particle size and increase the surface area.

Reagents and Standards

Methanol was HPLC grade and purchased from Romil (Cambridge, UK). Folin-Ciocalteu reagent and ascorbic acid were from Merck (Darmstadt, Germany). 2, 2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form, and isorhamnetin were obtained from Sigma–Aldrich Quimica (Alcobendas, Spain). Analytical grade phenolic standards (naringenin, catechin, quercetin, chlorogenic, protocatechuic, ferulic and p-coumaric acids) and analytical grade reagents ethanol, acetone, isopropanol and ethyl acetate were purchased from Merck (Darmstadt, Germany).

Solvent Extraction of the Phenolic Compounds

Different solvent systems were used to evaluate the effectiveness of the solvent on the extraction of phenolic compounds from stems and leaves of Ginja cherries: 70% aqueous mixtures of methanol, ethanol, ethyl acetate, 2-propanol, acetone and 100% pure water. The phenolic compounds were extracted from 0.5 g of dry samples using 20 mL of each solvent. The mixtures were shaken in a C24k refrigerated incubator (NJ, USA) at room temperature for 20 h. Then, the mixtures were centrifuged in a Universal 320r Hettich Zenrifugen (Tuttlingen, Germany) at 9,000 rpm for 5 min. at 4°C. The supernatants were recovered and used for the determination of total phenolic contents, antioxidant activities and LC–ESI/MS analysis. The supernatants were filtered through a 45 μm filter prior to analysis. All the extractions were performed in duplicate and resultant solutions were kept at −25°C under nitrogen until further analysis.

LC-ESI/MS Analysis

The instrument employed for the LC-ESI/MS analysis was a Prostar 210 LC pump (Varian, CA, USA) coupled with a Varian 1200 triple quadrupole mass spectrometer (Varian, CA, USA) with electrospray ionization in positive and negative modes. A 5 μm C18 column (4.6 × 100 mm, Merck) was used for the separation at a flow rate of 0.4 mL/min.

For the polyphenols separation, a LC/MS/MS method was developed. The separation was performed by gradient elution (eluent A, water with 0.1% formic acid; eluent B, 100% methanol) in 33 min. The ESI–MS was tried under negative ion mode, with different fragmentor voltage in the range between 10 and 120 V. MS/MS ions were produced by Electron Spray Ionisation; for the fragmentation, Argon atoms were used (pressure 1.20 mtorr; collision energy of 15 V). Data were acquired by Varian LC–MS 1200L Workstation.

Structural identification of the phenolic compounds present in the extracts was performed by comparison of the retention times with chromatographic profiles of phenolic standards. The quantification of these compounds was carried out by integration of the peaks using an external standard calibration method.

For flavonoid glycosides, the identification was performed by comparison with literature data obtained from previous studies [16, 17]. No quantification was done.

Phenolic Content Determination

The Total Phenolic Content (TPC) of extracts was determined by using the Folin-Ciocalteu reagent according to a modified Singleton's method (1965). Solvent extract (0.5 mL), 0.5 mL of Folin-Ciocalteu reagent, 10 mL of 75 g L^{-1} sodium carbonate and deionized water were added to a final volume of 25 mL. After 1 h, the absorbance of the sample was measured at 750 nm against a blank by using UV 1203 Shimadzu spectrophotometer (Tokyo, Japan). Gallic acid served as the standard for preparing the calibration curve; for this reason, the TPC values are expressed in milligram of Gallic Acid Equivalent (mg GAE) per gram of dry sample. All determinations were performed in triplicate.

Evaluation of Antioxidant Activity

$ABTS^+$ radical cation scavenging activity is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. Evaluation of antioxidant activity was carried out using an improved $ABTS^+$ decolourization assay, described in detail in the literature [18]. All determinations were carried out in triplicate. Ascorbic acid was used as the standard. Quantitative results were expressed in mg ascorbic acid equivalents (mg AAE) per g^{-1} of dry weight of sample, obtained through a calibration curve with ascorbic acid standard.

Statistical Analysis

Results for total phenolic contents by LC–ESI–MS and Folin Ciocalteu assay and antioxidant activities were reported as means ± SD for samples. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Tukey's test with $\alpha = 0.05$ by SPSS statistical package (ver.17.0).

Results

Composition of the Extracts: Qualitative and Quantitative Determination of Polyphenols

As described in the experimental section, a gradient elution-based LC-ESI/MS method was developed for the identification and the quantification of phenolic compounds in the different extracts. These results are summarized in Table 1, where the molecules and the corresponding retention times are listed.

Profiles of phenolic compounds, recovered from both by-products, were dominated by catechin and chlorogenic acid. On the other hand, naringenin, quercetin, ferulic, p-coumaric, and protocatechuic acids were also identified.

In the negative ion mode, protocatechuic, ferulic and p-coumaric acids produced a depronated $[M-H]^-$ molecule and a $[M-H-44]^-$ fragment ion via loss of a CO_2 group

Table 1 LC-ESI/MS spectral information of the identified peaks

Compound name	MW	$[M-H]^-$ (frag MS^2 *m/z*)	Rt (min)
Hydroxybenzoic acids			
Protocatechuic acid	154	153 (109)	8.4
Hydroxycinnamic acid			
p-coumaric acid	164	163 (119)	20.8
Ferulic acid	194	193 (134)	22.7
Chlorgenic acid	354	353 (191)	15.8
Flavan-3-ols			
(+)-Catechin	290	289 (245)	13.4
Flavonol and glycosides			
Quercetin	302	301 (151)	27.7
Quercetin-3-O-glucoside[a]	464	463 (301)	16.7
Quercitrin[a]	448	447 (284)	24.6
Flavonone and glycosides			
(+)-naringenin	272.3	271 (151)	27.6
Naringenin -7-O-glucoside[a]	434	433 (271)	25.1
Flavone and glycosides			
Apigenin-7-O-glucoside[a]	432	431 (269)	15.2
Apigenin-8-C-glucoside[a]	432	431 (311)	14.8
Luteolin-7-O-glucoside[a] or kaempferol-3-O-glucoside[a]	448	447 (285)	24.8
Kaempferol-3-O-rutinoside[a]	594	593 (285)	28.7

[a] Compounds not identified with a standard but by comparison with literature data

from the carboxylic acid moiety. Ferulic acid also showed the loss of the CH_3 group, providing an $[M-15]^-$ anion radical at *m/z* 178. Chlorogenic acid showed the $[M-H]^-$ depronated molecule (*m/z* 353) and the ion corresponding to the depronated quinic acid (*m/z* 191) which was consistent with a previous method [19].

Catechin ($[M-H]^-$ *m/z* 289) is in the subgroup of flavan-3-ols of phenolic compounds, yielding a fragment ion at *m/z* 245. The $[M-H-44]^-$ fragment ion at *m/z* 245 in (+)-catechin was produced by the loss of a $(CH)_2OH$ group, as described by Perez- Magariño [20]. The aglycone quercetin produced a fragment ion at *m/z* 151, which results from a cleavage of the heterocyclic C-ring by Retro-Diels–Alder (RDA), but the fragmentation mechanism remains unclear at present. Finally, naringenin produces ions with *m/z* 119 by retro-Diels–Alder fragmentation. For instance, the $m/z = 119$ ion is characteristic of naringenin and its derivatives.

The concentration of these molecules is reported in Table 2, for each of the extracts; it can be seen that stems and leaves exhibited different phenolic compositions.

In stems, the extracts in ethyl acetate and ethanol were the richest, as 7 compounds were identified. They were also those with the highest recovery yield: 1113.2 and 854.4 μg/g dry stems respectively. Considering the other solvents, some molecules were not detected: 70% acetone and methanol extracts did not contain quercetin, protocatechuic and ferulic acids. Moreover, in 2-propanol and in water extracts ferulic acid and quercetin were not identified, respectively. For all the solvents, the compound with the highest concentration was (+)-catechin; its content ranged from 103.6 to 767.8 μg/g dry weight of stems. Chlorogenic acid was the second prominent phenolic compound in stems and the highest concentration was found in 70% methanol extract. Quercetin had the lowest content, followed by protocatechuic acid.

In leaves extracts, catechin, protocatechuic, p-coumaric and chlorogenic acids were detected; ferulic acid, naringenin and quercetin, however, were not determined for none of the solvents employed. Pure water was found to be the most efficient solvent for extraction of phenolic compounds (166.72 μg/g dry leaves). Similarly to the stems, catechin was the major free phenolic compound found in leaves under our experimental conditions, the only exception being the extracts in ethyl acetate. Pure water showed the highest extraction capacity for p-coumaric and protocatechuic acids, while 70% 2-propanol solvent had the highest extraction capacity for chlorogenic acid.

Composition of the Extracts: Total Polyphenolic Content

The Total Phenolic Content (TPC) of all the extracts was determined using the Folin-Ciocalteu method; these measurements were previously performed in samples of natural origin [21], as the phenols are one of the major groups of

Table 2 Quantification of phenolic compounds by LC-ESI/MS

	Protocatechuic acid	p-coumaric acid	Ferulic acid	Naringenin	(+)-catechin	Cholorogenic acid	Quercetin	TPC
Stems								
Acetone	–	19.9 ± 0.65	–	21.76 ± 2.81	456.46 ± 15.56	61.64 ± 0.60	–	559.15
Methanol	–	8.37 ± 0.5	–	10.12 ± 0.16	103.66 ± 6.69	135.51 ± 3.42	–	257.66
Ethanol	36.53 ± 0.67	21.82 ± 0.63	46.70 ± 0.77	30.61 ± 6.03	613.03 ± 8.10	101.42 ± 9.08	4.36 ± 0.66	854.47
Ethyl acetate	15.40 ± 4.15	37.34 ± 0.55	218.39 ± 4.11	48.98 ± 12.57	767.87 ± 0.23	7.02 ± 0.22	18.21 ± 1.21	1113.21
2-Propanol	37.70 ± 0.66	20.20 ± 0.05	–	37.22 ± 0.51	538.26 ± 14.03	101.12 ± 0.12	2.91 ± 0.54	737.41
Water	37.93 ± 3.41	63.45 ± 12.3	129.16 ± 0.83	12.04 ± 0.12	181.24 ± 9.43	–	–	517.32
Leaves								
Acetone	16.45 ± 0.13	21.76 ± 1.06	–	–	69.78 ± 4.79	27.89 ± 1.16	–	135.88
Methanol	21.64 ± 0.09	20.50 ± 0.73	–	–	76.84 ± 1.64	24.99 ± 2.54	–	143.97
Ethanol	14.49 ± 2.31	22.74 ± 1.62	–	–	78.70 ± 1.44	24.79 ± 1.42	–	140.72
Ethyl acetate	–	1.82 ± 0.02	–	–	–	–	–	1.82
2-Propanol	–	11.90 ± 0.17	–	–	43.12 ± 1.75	49.16 ± 1.16	–	108.18
Water	27.44 ± 5.15	28.21 ± 3.43	–	–	96.30 ± 0.99	14.77 ± 1.44	–	166.73

All solvents are in proportion of 70:30 with distilled water

TPC total phenolic content. Units: μg/g of dry sample

compounds present in plants. The results are shown in Fig. 1.

For the stems, ethanol was the most effective solvent, followed by the 2-propanol (23.5 and 20.6 mg GAE/g dry stems respectively). Acetone extracts were those with the lowest concentration (2.6 mg GAE/g dry stems).

For the leave extracts, the highest concentration of TPC corresponded to the acetone extracts while the lowest one to the ethyl acetate. The values were between 11.9 and 3.8 mg GAE/g dry leaves, for acetone and ethyl acetate respectively.

Composition of the Extracts: Qualitative Determination of Flavonoid Glycosides

In addition to seven phenolic compounds, some flavonoid glycosides were also identified in both by-product extracts: apigenin-7-O-glucoside, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside or luteolin-3-O-glucoside and naringenin-3-O-glucoside (see Table 1). As reported above, these compounds were identified only by comparison to literature data, and no quantification was performed.

Properties of the Extracts: Antioxidant Activity

Figure 2 shows the antioxidant activity of the extracts from both leaves and stems. Considering the stems extracts, those from ethanol exhibited the highest antioxidant activity (24.0 mg AAE/g dry stems), while acetone showed the lowest (2.9 mg AAE/g dry stems). The other extracts showed activities decreasing in this order: 2-propanol, ethyl acetate, water and methanol. Their antioxidant activities were all significantly different; the only exceptions were the extracts in ethyl acetate and 2-propanol, whose difference was not significant according to the Tukey's test.

For the leaves, the extracts in pure water were those with the highest activity, while the ones in 2-propanol were the lowest (14.2 and 4.7 mg AAE/g dry leaves respectively); the other activities were ranked as follows: acetone, ethanol, methanol and ethyl acetate. The extract in acetone showed an activity slightly lower than those in water (13.3 mg AAE/g dry leaves); this difference, however, was not statistically significant.

Discussion

The solvent chosen to extract polyphenols had a clear effect on the final composition of the extracts; examples are reported in literature when other natural sources were used [7, 22–25]. Our results corroborate this, as extracts from the same starting material but prepared with different solvents show different composition—some compounds were detected in some extracts but not in others.

The choice of the solvent for the extraction process is not something easy and straightforward; for this reason, a systematic analysis like this is essential to determine the more appropriate solvent for a specific polyphenolic source. The profile of compounds obtained is different when using the same solvent for different sections of the plant. This was observed, for instance, for the ethyl acetate:water system 70:30: according to the LC-ESI/MS data (see Table 2), its use with the stems led to the maximum phenolic content; for the leaves, on the contrary, it was the less efficient one. The different performance is even more evident if we consider in particular the (+)-catechin: ethyl

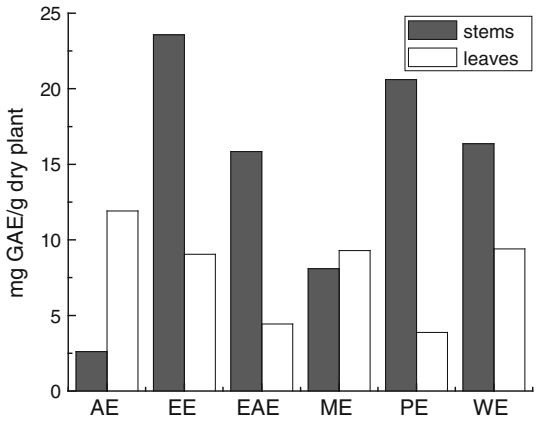

Fig. 1 Total Phenolic Concentration (TPC) for stems and leaves extracts determined with Folin-Ciocolteau method. *AE* 70% acetone extracts, *EE* 70% ethanol extracts, *EAE* 70% ethyl acetate extracts, *ME* 70% methanol extracts, *PE* 70% 2-propanol extracts, *WE* pure water extracts

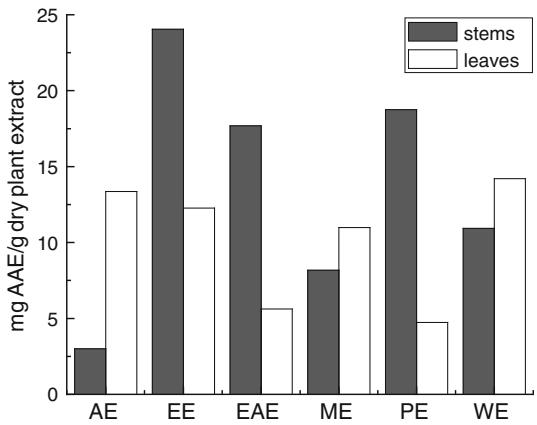

Fig. 2 Antioxidant activity for stems and leaves extracts. *AE* 70% acetone extracts, *EE* 70% ethanol extracts, *EAE* 70% ethyl acetate extracts, *ME* 70% methanol extracts, *PE* 70% 2-propanol extracts, *WE* pure water extracts

acetate showed the highest extraction capacity for such molecule in the stems, but the lowest in the leaves. In fact (+)-catechin was not detected in ethyl acetate leave extracts, despite the fact that the compound was detected in leaves extracts prepared with other solvents.

The effect of the starting material on the extraction process was previously reported in literature: Pinelo [26], for instance, reports about the influence of the molecular structure on the efficiency of the extraction for polyphenols. The interactions of polyhenols with other components or with parts of the plant were also described [27, 28]. According to these studies, there maybe a linkage between polyphenols and cell wall polysaccharide structure; this can affect the facility for the polyphenols to be released and absorbed in the extraction solvent. Considering the different nature of stems and leaves, it reasonable to think that a different matrix effect takes place; this can be due to different interactions between the polyphenols and the sample structure. The resulting composition of the extracts, therefore, will be different.

In the stems, the reason for higher extraction yield of phenolic compounds with 70% acetone, ethanol, ethyl acetate and 2-propanol aqueous solvent mixtures could be due to the nature of plant phenolics enhanced by the presence of an organic solvent, which facilitates diffusion through penetration in plant cell structure. These results are in agreement with literature: Yilmaz [24] also found that aqueous mixtures of either methanol, ethanol or acetone were better than a mono-component solvent for the extraction of phenolics from Muscadine seeds. Similar results were reported by Lapornik [7], who ascribed higher values of total polyphenols in ethanol and methanol extracts (70%) versus water extracts for several plants by-products.

The data regarding the TPC obtained with LC-ESI/MS do not agree completely with those obtained with the Folin-Ciocolteau method. For the stems extracts, for instance, the richest extracts are those in ethyl acetate with the first method; according the Folin-Ciocolteau method, however, the ones in ethanol and 2-propanol have a higher TPC concentration. Similar differences can be also observed for the leaves extracts. This discrepancy can be explained considering that the extracts contain other polyphenols which could not be identified with the LC-ESI/MS method, but that are detected with the Folin-Ciocolteau test. Literature reports that the reagents employed in this method are not specific and detect all the phenolic groups present in the extracts [13]. Both techniques show that stems extracts are richer in polyphenols than those from leaves, apart from the acetone and methanol extracts; hence, the results are in agreement for this.

Figure 3 shows the correlation between the antioxidant activity of the extracts and their TPC determined with the

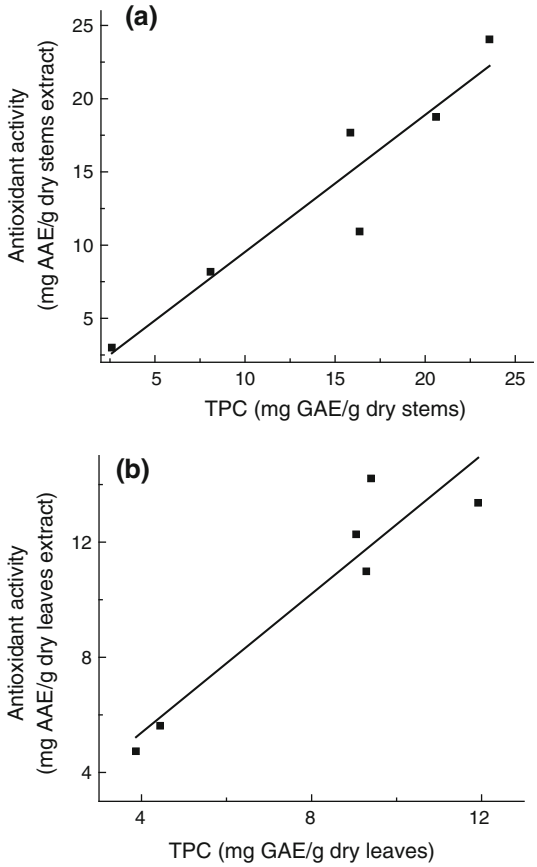

Fig. 3 Correlation between the Total Phenolic Content (TPC) and the antioxidant activity of (**a**) stems extracts, $R^2 = 0.88$; (**b**) leaves extracts, $R^2 = 0.86$

Folin-Ciocolteau method. A good correlation can be observed for both starting materials; the values of the correlation coefficient R^2 are 0.894 and 0.885 for the stems and the leaves respectively. The antioxidant properties are due to the activity of all polyphenols present in the extracts; it is, therefore, reasonable to have a correspondence between the TPC values determined by the Folin-Ciocolteau and the antioxidant activity itself. These results show that the stems and leaves are both good sources to obtain antioxidant compounds.

Conclusions

Stems and leaves from Ginja cherry, agricultural by-products, were successfully used for the extraction of polyphenols, a class of very valuable compounds. This study showed that the choice of solvent is crucial to obtain extract with high polyphenol content. 70% ethanol, 70% 2-propanol and 70% ethyl acetate were the most effective solvents for the extraction from the stems; for the leaves, on the contrary, 70% acetone and pure water gave the highest yields.

The antioxidant properties of the extracts were also tested; the results showed that the stems extracts in 70% ethanol were those with the highest antioxidant properties, while pure water was the most suitable solvent for the leaves.

This study shows that these by-products can be valorised effectively; giving extracts with antioxidant properties usable for several different applications.

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