Improving bioactive compounds extractability of *Amorphophallus paeoniifolius* (Dennst.) Nicolson

Anabela S.G. Costa^a, João C.M. Barreira^{a,b}, Adilson Ruas^a, Ana F. Vinha^{a,c}, Filipa B. Pimentel^a, Rita C. Alves^{a,d,*}, Isabel C.F.R. Ferreira^b, M. Beatriz P.P. Oliveira^a

^a REQUIMTE, LAQV/Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

^b Mountain Research Center (CIMO), ESA, Polytechnic Institute of Bragança, Apartado 1172, 5301-855 Bragança, Portugal

^c FCS-UFP/Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal

^d REQUIMTE, LAQV/Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

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ABSTRACT

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an underground, unbranched deciduous plant that produces a large tubercle (rhizome) with recognized health effects. In this study, the influence of solvent nature (water, water/ethanol (1:1) and absolute ethanol) and processing type (fresh, lyophilized and boiled) on the antioxidant activity and bioactive compounds extractability of elephant foot yam was evaluated. Extracts were compared for their contents in total phenolics, flavonoids and tannins. Moreover, their antioxidant capacity was assessed by the ferric reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging capacity assays. Phenolics (154 mg GAE/L) and tannins (109 mg GAE/L) were maximized in lyophilized samples extracted with the hydroalcoholic solvent, which attained also the highest FRAP value (711 mg FSE/L). In turn, flavonoids reached the highest yields in lyophilized samples (95 mg ECE/L) extracted with pure ethanol, as well as the highest DPPH• scavenging activity. These findings might have practical applications to define the best processing methodology regarding the enhancement of elephant foot yam, either for prompt consumption, as well as to develop food supplements or pharmaceutical related products.

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1. Introduction

Oxidative stress is involved in the etiology of various disorders and diseases, being reasonable to expect beneficial effects of antioxidants in maintaining our health and lowering disease risk (Kris-Etherton et al., 2002; Niki, 2010). Some antioxidants can be produced in the body, but the amounts maybe insufficient, particularly under conditions where production of free radicals is increased. Plants are natural alternative sources of antioxidants that might complement the production of these compounds in living organisms. The antioxidant activity in plants is often correlated to their phenolic contents (Cai et al., 2004; Razali et al., 2012). In addition, there has been a large volume of work aimed

at scientific validation of the efficacy of herbal drugs used in the traditional medicine. Furthermore, the preparation of dietary supplements/nutraceuticals and some pharmaceutical products is increasingly based on the extraction of bioactive compounds from natural matrices (Dai and Mumper, 2010).

Amorphophallus sp. are perennial herbaceous plants, growing in mountain or hilly areas in subtropical regions (Ishrud et al., 2001). Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an underground, unbranched plant with large stout mottled leaves. The leaf blade, which sits atop a thick fleshy stem, is divided into hundreds of leaflets, varying among 5 and 12.5 cm long, with highly ridge ovate or oblong shape. The plant is deciduous, dying back to a large tubercle (rhizome), weighing up to 8 kg and reaching up to 50 cm in diameter (Saha et al., 2013; Uprety and Poudel, 2010). Analgesic (Dey et al., 2010), antioxidant (Jayaraman et al., 2010), antibacterial, antifungal (Khan et al., 2008) and cytotoxic (Jayaraman et al., 2007; Khan et al., 2008) activities have been described. Therefore, it is assumable that elephant foot yam might be an important source of bioactive compounds since it is often

* Corresponding author at: REQUIMTE, LAQV/Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Fax: +351 22 018 49 58.

E-mail address: rita.c.alves@gmail.com (R.C. Alves).

used for the treatment of piles, dyspnea, splenomegaly, and cough (Rastogi and Mehrotra, 1995), being also recognized as analgesic, liver tonic, thermogenic, anthelmintic and diuretic (Arya, 1994).

The effectiveness of bioactive compounds extraction from plants, as well as their corresponding activity, is highly dependent on factors such as different types of solvent, solvent-to-solid ratios, extraction times and temperatures (Pinelo et al., 2005), and specially the solvent polarity (Razali et al., 2012). Accordingly, this work was designed to verify the influence of solvent nature (water, water:ethanol (1:1) and ethanol) and processing type (fresh, lyophilized and boiled) on the antioxidant activity and bioactive compounds extractability of elephant foot yam. Extracts were compared regarding their total phenolics, flavonoids and tannins contents. Moreover, their antioxidant capacity was assessed by two complementary procedures: the ferric reducing antioxidant power (FRAP) method and the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging capacity assay. The obtained results might have practical applications when deciding the best processing methodology regarding the enhancement of elephant foot yam extracts, either for prompt consumption as well as to develop food supplements or pharmaceuticals related products.

2. Materials and methods

2.1. Reagents and standards

Gallic acid, epicatechin, Folin–Ciocalteu's phenol reagent, DPPH• (2,2-diphenyl-1-picrylhydrazyl radical), sodium nitrite, ferric chloride, aluminum chloride, TPTZ (2,4,6-tripyridyl-s-triazine) solution, and ferrous sulfate heptahydrate were all obtained from Sigma–Aldrich (St. Louis, U.S.A). Anhydrous sodium carbonate, sodium hydroxide and absolute ethanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used to prepare all aqueous solutions.

2.2. Samples and samples preparation

Elephant foot yam (*A. paeoniifolius* (Dennst.) Nicholson) was collected from Baucau, a district of East Timor, on the Northern coast in the Eastern part of the country. Voucher specimens were numbered and deposited in the local herbarium. Samples were submitted to different conservation processes (fresh, boiled and lyophilized). For the preparation of the cooked sample, cubes of peeled tubers were boiled at 100 °C for about 40 min, simulating the domestic cooking process. Afterwards, all visible water was drained out. To obtain lyophilized samples, fine-cut peeled tubers were frozen (−20 °C) and lyophilized (Telstar Cryodos-80 Terrassa, Barcelona). Samples obtained from different conservation processes were ground (Grindomix GM 200, Retsch, Haan, Germany) and used to prepare the extracts described in the next section.

2.3. Extracts preparation

Extracts were prepared using three different solvents: (i) ethanol, (ii) water and (iii) ethanol:water 50:50 (v/v). Three individual samples of fresh, boiled and lyophilized foot yam were tested with each solvent. Each sample (≈1 g) was extracted by stirring with 50 ml of the corresponding solvent, at 40 °C, 600 rpm, for 1 h and filtered through Whatman No. 4 paper. The residues were then extracted with additional portions of the corresponding solvents. The combined extracts of each solvent were evaporated (ethanol) under reduced pressure (Rotavapor® R-210, Büchi, Flawil, Switzerland) or frozen and lyophilized (water) and re-dissolved in the corresponding extract at an adequate concentration. Stock solutions were stored at 4 °C for further use; all the assays were

carried out in triplicate and the results were expressed as mean values ± standard deviations (SD).

2.4. Total phenolics

Total phenolic contents of diluted extracts (1:10 v/v) were determined according to Alves et al. (2010). Briefly, 500 µl of each extract were mixed with 2.5 ml of the Folin–Ciocalteu reagent (1:10) and 2 ml of a sodium carbonate solution (7.5% m/v). The mixture was first incubated at 45 °C, during 15 min, followed by 30 min incubation at room temperature before absorbance readings at 765 nm. Total phenolic contents were calculated from a calibration curve prepared with gallic acid (10–100 mg/L; $r = 0.9997$) and expressed as mg of gallic acid equivalents (GAE)/L of extract.

2.5. Total flavonoids

Total flavonoid contents were determined according to Soares et al. (2013). Aliquots of 1 ml of extract were mixed with 4 ml of distilled water and 300 µl of 5% sodium nitrite. After 5 min at room temperature, 300 µl of 10% AlCl₃ were added, followed (after 1 min) by 2 ml of sodium hydroxide (1 M) and 2.4 ml of ultrapure water. The absorbance was recorded at 510 nm. Total flavonoid contents were calculated through a calibration curve of epicatechin (50–450 mg/L; $r = 0.9998$) and expressed as mg of epicatechin equivalents (ECE)/L of extract.

2.6. Total tannins

Total tannins contents were determined according to Shad et al. (2012), with slight modifications. Briefly, 500 µl of extract (diluted at 1:10 when necessary) were mixed with 2.5 ml of the Folin–Ciocalteu reagent (1:10). After 3 min, 2 ml of sodium carbonate (7.5% m/v) were added. The mixture was kept in the dark for 2 h. Absorbance readings were carried out at 725 nm. Tannins content was calculated from a calibration curve prepared with gallic acid (10–100 mg/L; $r = 0.9997$) and expressed as mg of gallic acid equivalents (GAE)/L of extract.

2.7. Antioxidant activity

2.7.1. DPPH scavenging activity

The radical scavenging ability of extracts was analyzed according to the method described by Harini et al. (2012) with some modifications. Briefly, 14 µl of diluted extract (1:10 v/v) were mixed with 186 µl of a freshly prepared DPPH• solution (6.0×10^{-5} mol/L in ethanol). The absorbance decrease at 525 nm was measured in time intervals of 2 min, in order to observe the reaction kinetics. The reaction endpoint was attained in 40 min. The radical scavenging activity (RSA) was expressed as percentage of inhibition and calculated using the following equation:

$$\%RSA = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.7.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to Benzie and Strain (1996) with slight modifications. Briefly, 90 µl of diluted extract (1:10 v/v) were mixed with 270 µl of distilled water and 2.7 ml of the FRAP solution (containing 0.3 M acetate buffer, 10 mM TPTZ solution, and 20 mM of ferric chloride). After homogenization, the mixture was kept for 30 min at 37 °C protected from light. Absorbance was measured at 595 nm. A calibration curve was prepared with ferrous sulfate (50–450 mg/L, $r = 0.9998$) and ferric

Fig. 1. Interactions between solvent type (ST) and processing type (PT) effects on the bioactive compounds of *A. paeoniifolius* samples. Total phenolics (A), total tannins (B), total flavonoids (C).

Table 1Bioactive compounds contents obtained for the polar extracts of foot yam submitted to different processing types^a.

	Total phenolics (mg GAE/L)	Total tannins (mg GAE/L)	Total flavonoids (mg ECE/L)	
Solvent type (ST)	Water	43 ± 17	41 ± 16	18 ± 7
	Water:ethanol (1:1)	132 ± 79	129 ± 78	58 ± 32
	Ethanol	69 ± 71	29 ± 9	41 ± 39
	<i>p</i> value (<i>n</i> = 54)	<0.001	<0.001	<0.001
Processing type (PT)	Fresh	24 ± 11	26 ± 7	10 ± 5
	Lyophilized	154 ± 68	109 ± 83	70 ± 33
	Boiled	67 ± 47	64 ± 46	37 ± 23
	<i>p</i> value (<i>n</i> = 54)	<0.001	<0.001	<0.001
ST × PT	<i>p</i> value (<i>n</i> = 162)	<0.001	<0.001	<0.001

^a The results are presented as mean ± SD. GAE, gallic acid equivalents; ECE, epicatechin equivalents.

reducing antioxidant power was expressed as mg of ferrous sulfate equivalents (FSE)/L of extract.

2.8. Statistical analysis

All statistical tests were performed at a 5% significance level, using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA). For each processing type (PT) and solvent type (ST), three samples were analyzed, with all the assays being also carried out in triplicate. The results are expressed as mean value ± standard deviation (SD).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors PT and ST. In this case, when a statistically significant interaction (PT × ST) is detected, the multiple comparisons classification results cannot be considered, and the two factors should be evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction is verified, means might be compared using Tukey's honestly significant difference (HSD) multiple comparison test.

Further, a linear discriminant analysis (LDA) was used to compare the effect of the PT and ST on antioxidant activity and extracted bioactive compounds. A stepwise technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination processes, where the inclusion of a new variable is preceded by ensuring that all variables selected previously remain significant (Maroco, 2003; López et al., 2008). With this approach, it is possible to identify the significant variables obtained for each sample. To verify the significance of canonical discriminant functions, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

3. Results and discussion

In what regards to antioxidant activity, two assays were performed to evaluate different mechanisms of action: ferric reducing antioxidant power (FRAP), an electron transfer method, which cannot detect compounds that act by radical quenching, but detects compounds with redox potentials lower than 0.7 V (the redox potential of Fe³⁺-TPTZ); and the DPPH• scavenging assay, where the radicals may be neutralized either by direct reduction via electron transfers, or by radical quenching via H atom transfer (Prior et al., 2005). Also, three groups of compounds were quantified, namely total phenolics, total tannins and total flavonoids. Besides studying the effects of physical variables related with mass transfer kinetics (specifically the solvent type), it was also intended to verify if the way in which the studied matrices were processed exerted a significant effect.

The effects of solvent and processing were evaluated by fixing one of the factors, i.e., the results are presented as the mean value of each ST, including all the PT, and as the mean value of each PT, with the contribution of all ST. Hence, the standard deviation values should not be looked up as a measure of assays repeatability. As it can be concluded from Tables 1 and Table 2, each factor showed a significant effect *per se*, but the interaction among factors (ST × PT) was also a significant (*p* < 0.001) source of variation for all parameters, indicating a strong dependence between the solvent used and the way in which samples were processed. This significant interaction might be easily observed in the estimated marginal means (EMM), where the variation in total phenols, tannins and flavonoids (Fig. 1A–C) among fresh, lyophilized and boiled samples were clearly dependent on the solvent type. For instance, tannin levels are similar for fresh, lyophilized and boiled samples when extracted with water or ethanol, but completely different when extracted with the hydroalcoholic solvent (Fig. 1B). Likewise, while DPPH• was maximal in boiled samples when using water or water:ethanol, the highest activity in lyophilized samples was reached when the samples were extracted with ethanol

Table 2Antioxidant properties obtained for the extracts of foot yam submitted to different processing types^a.

	DPPH• scavenging activity (% of inhibition) ^b	FRAP assay (mg FSE/L)	
Solvent type (ST)	Water	26 ± 9	116 ± 20
	Water:ethanol (1:1)	57 ± 21	505 ± 248
	Ethanol	49 ± 35	287 ± 273
	<i>p</i> value (<i>n</i> = 54)	<0.001	<0.001
Processing type (PT)	Fresh	18 ± 8	97 ± 53
	Lyophilized	61 ± 29	506 ± 259
	Boiled	53 ± 18	306 ± 244
	<i>p</i> value (<i>n</i> = 54)	<0.001	<0.001
ST × PT	<i>p</i> value (<i>n</i> = 162)	<0.001	<0.001

^a The results are presented as mean ± SD. FSE, ferrous sulphate equivalents.^b Diluted extracts (1:10) were tested against a DPPH• solution of 6 × 10⁻⁵ mol/L.

Fig. 2. Interactions between solvent type (ST) and processing type (PT) effects on the antioxidant activity of *A. paeoniifolius* samples. DPPH• scavenging assay (A), FRAP assay (B).

(Fig. 2A). On the other hand, the ferric reducing power remained nearly the same for fresh, lyophilized and boiled samples when these were extracted with water, but the same samples gave significant differences if extracted with ethanol or the water:ethanol (Fig. 2B). Besides the individual variations, some general conclusions might also be drawn from the EMM: for instance, the highest amounts of total phenolics (132 mg GAE/L extract) and total tannins (129 mg GAE/L extract), as well as the most powerful FRAP (505 mg FSE/L) were achieved with the hydroalcoholic solvent; aqueous extracts revealed the lowest contents in total phenolics (43 mg GAE/L extract), total flavonoids (18 mg ECE/L extract) and also the weakest DPPH• scavenging activity (26%) and FRAP (116 mg FSE/L extract); total tannins content presented the least value (29 mg GAE/L extract) in ethanol extracts. In previous studies, ethanol extracts were reported as having the highest antioxidant efficiency along with the high content of phenolic compounds (Angayarkanni et al., 2010; Jayaprakasha et al., 2008). Herein, the best results were obtained with water:ethanol (1:1), potentially indicating the presence of phenolic acids (which are readily soluble in water) or a high percentage of glycosylated phenolics (it is a well-known fact that glycosylation increases the water solubility of phenolic compounds). Besides the polarity of extraction solvents and the solubility of phenolic compounds, differences might also be explained by changes in the rate of mass transfer (Bi et al., 2009). Regarding the type of processing, lyophilized samples allowed the best results for all assays, except for DPPH• scavenging activity, where it was not possible to differentiate from lyophilized and boiled samples. The

detected amounts of bioactive compounds are generally in agreement with previous results in alcoholic extracts of elephant foot yam (Angayarkanni et al., 2010; Nataraj et al., 2009).

Since the antioxidant activity is often correlated with the contents in total phenolics of a determined matrix (Razali et al., 2012), the correlation coefficients among bioactive compounds and antioxidant activity were also calculated. Despite the fact that the DPPH assay is representative of the capacity of test compounds to scavenge free radicals independently from any enzymatic activity, the detected correlations were higher for FRAP assay, especially with total phenolics ($y = 18.01 + 3.49x$, $R^2 = 0.91$) and total flavonoids ($y = 3.71 + 7.69x$, $R^2 = 0.95$).

In the following section the results obtained from the conjugated analysis of all parameters are comprehensively analyzed. This approach was followed using linear discriminant analysis in order to have an integrated perspective about the effect of solvent and processing on the antioxidant activity and bioactive compounds amounts. The significant independent variables (evaluated parameters) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

Starting with the ST effect, two significant functions were defined (plotted in Fig. 3), which integrated 100.0% of the observed variance (first, 80.3%; second, 19.7%). As it can be observed, the naturally occurring groups (each used solvent) were not completely individualized. However, the classification performance

Fig. 3. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds contents and antioxidant activity results according with solvent type (ST).

Fig. 4. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds contents and antioxidant activity results according with the processing type (PT).

Table 3

Contingency matrix obtained using LDA based on antioxidant activity and bioactive compounds contents of elephant yam extracts.

Predicted group membership	Total			Sensitivity (%)
	Water	Water:etanol (1:1)	Ethanol	
Water	54	0	0	54
Water:etanol (1:1)	18	36	0	54
Ethanol	18	0	36	54
Total	90	36	36	162
Specificity (%)	60	100	100	87

was satisfactory, resulting in 78% of correctly classified samples (sensitivity) and 87% of overall specificity within the leave-one-out cross-validation procedure (Table 3). The analysis kept all variables in the final discriminant model, being verified that total tannins and DPPH• scavenging activity (both higher in hydroalcoholic samples) were the variables with the highest correlation with function 1 and 2, respectively. The discriminant power proportion of the *n*th function may be estimated by the ratio among its own value and the sum of all discriminant functions values. The canonical discriminant function coefficients allowed obtaining the following model:

$$D_1 = -1.20 + 0.003 \times \text{phenolics} + 0.02 \times \text{tannins} - 0.14 \times \text{flavonoids} - 0.002 \times \text{DPPH} + 0.02 \times \text{FRAP}$$

Regarding classification function coefficients, the following functions were obtained:

$$\text{water} = -5.08 + 0.14 \times \text{phenolics} + 0.04 \times \text{tannins} - 0.32 \times \text{flavonoids} + 0.42 \times \text{DPPH} - 0.04 \times \text{FRAP}$$

$$\text{water; ethanol} = -9.76 + 0.06 \times \text{phenolics} + 0.10 \times \text{tannins} - 0.52 \times \text{flavonoids} + 0.34 \times \text{DPPH} - 0.02 \times \text{FRAP}$$

$$\text{ethanol} = -4.70 - 0.01 \times \text{phenolics} + 0.04 \times \text{tannins} - 0.05 \times \text{flavonoids} + 0.31 \times \text{DPPH} - 0.04 \times \text{FRAP}$$

For PT, the discriminant model selected also 2 significant functions (Fig. 4), which included 100.0% of the observed variance (function 1: 75.1%, function 2: 24.9%). In the obtained model, all samples were correctly classified (sensitivity and specificity were obviously 100.0%). All variables were included in the final model, with DPPH• scavenging activity and total phenolics (both higher in lyophilized samples) as the variables with the highest correlation with function 1 and 2, respectively.

The canonical discriminant function coefficients allowed obtaining the following model:

$$D_1 = -6.63 - 0.14 \times \text{phenolics} + 0.11 \times \text{tannins} - 0.12 \times \text{flavonoids} + 0.36 \times \text{DPPH} - 0.03 \times \text{FRAP}$$

Regarding classification function coefficients, the following functions were obtained:

$$\text{fresh} = -10.59 - 0.40 \times \text{phenolics} + 0.44 \times \text{tannins} + 0.27 \times \text{flavonoids} + 1.66 \times \text{DPPH} - 0.16 \times \text{FRAP}$$

4. Conclusions

The statistical interaction among the type of solvent and type of processing was significant in all cases, showing that the effects caused by each selected solvent might depend on the form in which the sample was processed. In addition, the results obtained with LDA indicate that type of processing had a higher influence on the antioxidant activity and bioactive compounds content of the extracts than the extraction solvent. Phenols and tannins concentrations were maximized in hydroalcoholic extracts of lyophilized samples (which presented also the highest FRAP values), while flavonoids reach the highest yields in ethanolic extracts of the lyophilized samples (which also showed the highest DPPH scavenging activity). These findings might have practical applications to define the best processing methodology regarding the enhancement of elephant foot yam, either for prompt consumption as well as to develop food supplements or pharmaceuticals related products.

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