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Antioxidant and antimicrobial properties of dried Portuguese apple variety (*Malus domestica* Borkh. cv Bravo de Esmolfe)

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ABSTRACT

Malus domestica Borkh apples are one of the most consumed fruits in the world, due to their sweetness and flavour. Herein, 'Bravo de Esmolfe' apple fruits were characterized regarding their nutritional value, chemical composition and bioactive properties. Besides nutrients, flavan-3-ols (i.e., epicatechin and B-type procyanidins) as also hydroxycinnamoyl-quinic acids and phloretin derivatives were identified in the samples. Extracts prepared from 'Bravo de Esmolfe' also proved to have antioxidant activity and antibacterial effects against Gram-positive bacteria, namely methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes* and *Enterococcus faecalis*, and against the Gram-negative bacteria *Escherichia coli*, *Escherichia coli* (ESBL) (producing extended spectrum β-lactamases) and *Morganella morganii*. There is very little information about 'Bravo de Esmolfe' apple, so this study is important to inform consumers about an alternative source of nutritional and bioactive compounds.

1. Introduction

'Bravo de Esmolfe' is a Portuguese apple variety with an intense aroma, highly appreciated by consumers. This apple was recognised as a product with Protected Designation of Origin (PDO), being therefore a high added value product with impact in the local and national economy (N°1107/96, 2001; Reis, Rocha, Barros, Delgadillo, & Coimbra, 2009). In the last few years the 'Bravo de Esmolfe' apple has doubled its price compared to exotic varieties, such as Golden and Starking (Feliciano et al., 2010). Its production is carried out in a restricted and small inland region in northern Portugal, corresponding to a production of 200,000 kg per year, but commercial demand is now increasing, due to its appealing sensory properties, namely sweetness and flavour (Bhatti & Jha, 2010). The regular consumption of fruits and vegetables has been associated with reduced risk of developing chronic diseases. These benefits are often attributed to their high phytochemical content and antioxidant power (Serra et al., 2010). Apple fruits have a wide variety and well-balanced composition, being moderately energetic and well-proportioned in sugar and acid contents, giving it a pleasant taste. The chemical composition of apples varies depending on the cultivar, production region and horticultural practices (Róth et al., 2007). They are mostly constituted by water (84%), minerals, complex B vitamins (Feliciano et al., 2010), monosaccharides, dietary fibre, and various biologically active compounds, such as vitamin C, and certain phenolic compounds (Róth et al., 2007; Wu et al., 2007) Feliciano et al., (Feliciano et al., 2010) studied several nutritional parameters in apple varieties, including the "Bravo de Esmolfe" apple. Wu et al. (Wu et al., 2007) reported the sugars and organic acids composition as also the phenolic profile of different apple cultivars. Various authors (Malec et al., 2014; Mayr, Treutter, Santos-Buelga, Bauer, & Feucht, 1995; Scafuri et al., 2016; Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006; Shoji et al., 2003; Verdu et al., 2013; Wojdyło, Oszmiański, & Laskowski, 2008) also presented the phenolic profile of different apple cultivars, but none of the previously mentioned authors have studied the bioactive properties and compounds from the cultivar 'Bravo de Esmolfe'. Therefore, to the best of the author's knowledge, there is still scarce information about this apple variety.

The aim of the present work, was to characterize the nutritional and chemical composition of *Malus domestica* Borkh cv 'Bravo de Esmolfe', as also its bioactive properties in terms of phenolic compounds, antioxidant and antibacterial properties.

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2. Materials and methods

2.1. Standards and reagents

HPLC grade acetonitrile (99.9%), *n*-hexane (95%) and ethyl acetate (99.8%) were purchased from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (standard 47885-U), formic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), L-ascorbic acid, tocopherol, sugars and organic acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenolic standards were acquired from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). *p*-Iodonitrotetrazolium chloride (INT) from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB) and Mueller-Hinton (MH) from Biolab® (Hungary). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

Apple samples (*Malus domestica* Borkh. cv 'Bravo de Esmolfe'), were kindly supplied by the RBR foods company from Castro Daire (Portugal), in the dry form (without skin), since the company's objective is to market this as a snack. After reception, the material was reduced to a fine dried powder (20 mesh), mixed to obtain a homogenate sample and stored in a desiccator, protected from light, until further analysis. All the assays were performed in triplicate.

2.3. Nutritional composition

The proximate composition was determined according to AOAC procedures (AOAC, 2016), including protein by the macro-Kjeldahl method (991.02); crude fat using a Soxhlet apparatus and extracting the powdered sample with petroleum ether (989.05) and ash contents (935.42) by incineration at 550 ± 15 °C. The total carbohydrates (including fiber) were calculated by difference ([Total carbohydrates (g/100 g) = 100 − (g fat + g protein + g ash)]) and total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat).

2.4. Fatty acids

Fatty acids were determined after Soxhlet extraction using the powdered sample and after a *trans*-esterification process. The analysis was performed by GC-FID (DANI model GC 1000 instrument, Contone, Switzerland) and separation was achieved using a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df). The results were expressed in relative percentage of each fatty acid (Barros, Pereira, & Ferreira, 2013; Dias et al., 2015).

2.5. Tocopherols

Tocopherols (four isoforms) were analysed in the powdered sample and analysed by HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) and separation was achieved using a Polyamide II (5 μm, 250 × 4.6 mm) normal-phase column from YMCWaters (YMC America, Inc., Allentown, PA, USA). Tocol was used as an internal standard and the quantification was based on the fluorescence signal response of each standard, The results were expressed in mg per 100 g of dry plant weight. (Barros et al., 2013; Dias et al., 2015).

2.6. Soluble sugars

Soluble sugars were determined in the powdered sample by HPLC coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described by Barros et al. (2013). Separation was achieved using a Eurospher 100-5 NH2 column (5 μm, 4.6 × 250 mm, Knauer) and quantification was performed using internal standard method (IS, melezitose). The results were expressed in g per 100 g of plant dry weight.

2.7. Organic acids

Organic acids were determined in the powdered samples and analysed by HPLC coupled to photodiode array detector (UFLC-PDA; Shimadzu Corporation, Kyoto, Japan) and separation was performed with a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 × 4.6 mm i.d.). The quantification was performed by comparison of the peak area recorded at 215 nm as preferred wavelength. The results were expressed in g per 100 g of plant dry weight (Barros et al., 2013; Dias et al., 2015).

2.8. Hydromethanolic extracts preparation

The hydromethanolic extracts were prepared by mixing 1 g of the powered dried apple sample with a methanol: water mixture (80:20, *v*/*v*) at 25 °C and 30*g* during 1 h, followed by filtration through a Whatman filter paper No. 4. The remain residue was re-extracted with an additional portion of methanol:water mixture and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and stored at −20 °C for further analysis.

2.9. Phenolic compounds analysis

The phenolic compounds were determined in the hydromethanolic extract solution (5 mg/ml) by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), following a procedure previously described by the authors (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016). Chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 × 150 mm). For the double online detection, a DAD (280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer performed in negative mode (Linear Ion Trap LTQ XL mass spectrometer equipped with an ESI source, ThermoFinnigan, San Jose, CA, USA) were used and connected to the HPLC system. The identification was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra. If no standard compound was available, phenolic compounds were identified by comparing the obtained information with available data reported in the literature, giving a tentative identification. Quantification was made from the areas of the peaks recorded at 280 nm by comparison with calibration curves obtained from standards. The results were expressed as mg/100 g dry weight (dw).

2.10. Antioxidant and antibacterial activity of the hydromethanolic extracts

The antioxidant activity was evaluated in the extracts re-dissolved in methanol:water mixture (10 to 0.3125 mg/ml) through DPPH radical-scavenging, reducing power, inhibition of β-carotene bleaching and TBARS inhibition assays. Trolox was used as positive control and the results were expressed in EC_{50} values Barros et al. (2013). The antibacterial activity was determined in the extracts re-dissolved in water (stock solution 20 mg/ml). The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. The antibiotic susceptibility profile was screened previously (Dias et al., 2016) for all the tested bacteria. Microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay were used to determine