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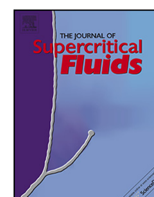

# Supercritical fluid extraction vs conventional extraction of myrtle leaves and berries: Comparison of antioxidant activity and identification of bioactive compounds

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ABSTRACT

In this work, the antioxidant capacity of extracts of Portuguese myrtle (*Myrtus communis* L.) is being studied over a period of three years. The samples were leaves of myrtle collected at the flowering stage and berries sampled at an early ripened stage. Supercritical fluid extraction (SFE) extracts were obtained at 23 MPa, 45 °C and a $CO_2$ flow of 0.3 kg $h^{-1}$ using ethanol as co-solvent with a flow rate of 0.09 kg $h^{-1}$. Hydrodistillation was carried out in a Clevenger type apparatus and the aqueous phase was extracted with diisopropylether having obtained what is hereby designated as liquid phase extract (LPE).

The antioxidant capacity of all the extracts was determined by using three different methods: the Folin–Ciocalteu, the Trolox Equivalent Antioxidant Capacity (TEAC) and the Oxygen Radical Absorbance Capacity (ORAC). The results show that the SFE extracts present a significantly higher antioxidant capacity. The extracts were characterized and quantified by HPLC-DAD-MS/MS methods. The bioactive compounds identified in all the extracts were phenolic acids (only in the LPE extracts), flavonoids and anthocyanins (only in the SFE extracts). The results indicate that the higher antioxidant capacity of the SFE myrtle extracts is mainly correlated with the concentration of flavonol glycosides, the myricetin-*O*-glycosides.

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## 1. Introduction

Myrtle (*Myrtus communis* L.), is a wild shrub, typical of the Mediterranean flora, widespread in Portugal in most of the mainland with the exception only of the far northern interior part of the country. Myrtle is an evergreen plant with very aromatic dark green leaves, white, delicate flowers and small, round, dark blue/purple fruit. Its pleasant scent makes it a valuable ingredient for the perfume and cosmetic industries and its flavour makes it an interesting spice for culinary purposes, although this particular aspect has been lost, over the years, in most countries with only a few exceptions. In Portugal, myrtle is mainly used to make regional liqueurs. As a member of the aromatic plants and being typical of the Mediterranean flora, myrtle has been the object of a number of studies which have shown that it also possesses very interesting medicinal properties being referred to as an antiseptic, antimicrobial, disinfectant and hypoglycaemic agent [1].

Studies have shown high antioxidant capacity in plant extracts obtained both by conventional extraction [2–4] and by supercritical fluid extraction [5–7].

This work looks at the antioxidant capacity of leaves and fruit extracts obtained by two different methods, SFE and hydrodistillation, followed by the extraction of the residual liquid with diisopropylether, over a period of three years. Ghasemi et al. [6] have compared SFE and hydrodistillation in a study with the objective of optimizing the SFE parameters for the extraction of myrtle from Iran, though this comparison was made for the composition of the extracts. The paper reports considerably higher yields for the extracts obtained by SFE as well as better selectivity. Abdelkarim et al. [8] studied the SFE of Tunisian myrtle leaves, having determined yields and solubilities of extracts obtained at pressures below 18 MPa. Zermane et al. [9] optimized the SFE of Algerian myrtle leaves, in terms of yields. As far as the authors know, there are no results in the literature for SFE of myrtle berries and the

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respective antioxidant comparison. Costa et al. [7] report a similar comparison study for a different plant, *Lavandula Viridis L'Her.* Their results show higher yields but lower antioxidant for the SFE extracts. However, it should be noted that the pressures used in the aforementioned study are somewhat low in relation to those usually accepted for the extraction of antioxidant compounds, which are in the order of the 20–30 MPa, sometimes even higher [10]. Therefore, the results obtained for the antioxidant might not be as high as they could be if higher pressures were used. The working pressure used in this work, 23 MPa, is the result of an optimization procedure performed on SFE extracts of myrtle leaves [11].

This study was carried out over a period of three years to investigate whether the yield and antioxidant capacity were affected, and to what extent, by climatic conditions. This influence, if present, would have to be taken into account in possible industrial applications.

## 2. Material and methods

### 2.1. Plant material

Samples of wild myrtle leaves and berries were collected over a period of three years from the Sintra area (Portugal). For each year, the leaves were collected in July and the ripe berries in October. These months were selected having taken into account the study of yields and composition of essential oil over the vegetative cycle of Portuguese myrtle in 2006 [12]. No soil tillage, fertilization and pest treatments were carried out in this area. Collection always took place in the same geographical area to avoid variability. After collection, the plant material was identified and deposited in the Herbarium of the Instituto Superior de Agronomia of the University of Lisbon. The plant material kept for analysis was dried for two months out of the sunlight, and then sealed in black bags and kept at −20 °C.

### 2.2. Standards and reagents

#### 2.2.1. Antioxidant capacity

Folin–Ciocalteu reagent, gallic acid and sodium carbonate were purchased from VWR (Leuven, Belgium). Fluorescein was obtained from Panreac (Barcelona, Spain). 2,2′-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and sodium dodecyl sulphate were purchased from Acros Organics (Geel, Belgium). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulphate were purchased from Sigma–Aldrich (Steinheim, Germany). Monobasic potassium phosphate was obtained from Merck (Darmstadt, Germany) and dibasic sodium phosphate was obtained from Fluka (Steinheim, Germany).

#### 2.2.2. Extraction

The diisopropylether was acquired from Merck (Darmstadt, Germany).

#### 2.2.3. Supercritical fluid extraction

Carbon dioxide (N48-99.998%) for extraction was supplied in cylinders by Air Liquide (Lisbon, Portugal). Absolute ethanol p.a. obtained from Merck (Darmstadt, Germany) was used as co-solvent.

#### 2.2.4. HPLC–MS

Methanol and acetonitrile LC–MS grade from Fisher Scientific (Loughborough, Leicestershire, UK), formic acid Agros Organics (Geel, Belgium) and deionised water (Millipore Simplicity® Simpak 2, $R$ = 18.2 MΩ cm, USA) were used in extract and mobile phase preparations. Standards of quercetin-3-β-D-glucoside, myricetin-3-*O*-rhamnoside, malvidin-3-*O*-glucoside and quercetin were purchased from Extrasynthese (Genay, France). Quinic acid was purchased from Sigma–Aldrich (Steinheim, Germany).

### 2.3. Extraction procedure

The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus, for two hours, using 100 g of ground dried plant material. The oil was recovered and the aqueous phase was extracted with diisopropylether [13,14] obtaining what we designate as Liquid Phase Extracts (LPE), which was afterwards subject to antioxidant determination using the three referred methods: Folin–Ciocalteu, TEAC and ORAC assays.

The SFE apparatus in which the experiments were carried out is already described elsewhere [11]. The experimental conditions used were those reported as optimal, in Pereira et al. [11]: temperature: 45 °C, pressure 23 MPa, $SCCO_2$ flow rate 0.3 kg $h^{-1}$, and co-solvent flow rate (ethanol) 0.09 kg $h^{-1}$.

Each extraction run was carried out at the aforementioned conditions of pressure, temperature, $SCCO_2$ flow rate, and ethanol flow rate using 30 g of the ground myrtle leaves or berries, and samples of the extracts were collected at various time intervals. For each of these intervals, records of the mass of extract recovered and volume of $SCCO_2$ were taken.

### 2.4. HPLC-DAD-MS analysis

The extract analyses were performed on a LC–MS with a ProStar 410 Autosampler, two 212-LC chromatography pumps, a ProStar 335 diode array detector and a 500-MS ion trap mass spectrometer with an electrospray ionization (ESI) ion source (Varian, Palo Alto, CA, USA). Data acquisition and processing were performed using Varian MS Control 6.9 software. The separation of the phenolic compounds was performed in a Polaris column (Varian) RP18-A (150 mm × 2 mm I.D., 5 μm particle size) while the anthocyanins were separated in a (Merck) Lichrocart RP18 column (250 mm × 4.6 mm ID, 5 μm particle size). In both cases, the columns were kept at controlled temperature (35 °C). The samples were injected into the column via a Rheodyne injector with a 20 μL loop. The mobile phase, for the phenolic compounds, consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient adopted, at a flow rate of 0.25 mL $min^{-1}$, was as follows: 0–2 min A:B (93:7) isocratic, 8 min A:B (75:25) isocratic, 35 min A:B (20:80) isocratic, 40 min A:B (0:100) isocratic, 45 min A:B (93:7) linear. For the anthocyanins, the mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B). The following gradient was used: 0 min A:B (100:0) isocratic, 20 min A:B (10:90) isocratic, 25 min A:B (10:90) isocratic, 30 min A:B (100:0) linear. A flow rate of 1 mL $min^{-1}$ was used, and the LC effluent was introduced into the ESI source in a post-column splitting ratio of 3:1. The mass spectra were acquired in negative and positive mode in the range from 100 to 1700 Da; the optimized parameters were as follows: ion spray voltage, ±4.9 kV; capillary voltage, 20 and −60 V; RF loading, 90%. Nitrogen was used as nebulizing and drying gas, at pressure of 35 and 10 psi, respectively; drying gas temperature, 350 °C. The $MS^n$ ($n$ = 1–3) spectra were obtained with an isolation window of 2.0 Da, excitation energy values between 1.0 and 2.5 V and an excitation time of 10 ms. The $MS^2$ experiment was used to characterize both flavonoid aglycones and glycosides; $MS^3$ was used in the fragmentation of flavonoid glycosides to their aglycones.

The nomenclature for flavonoid aglycone fragmentations proposed by Claeys and co-workers [15], and the systematic nomenclature for carbohydrate fragmentations introduced by

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