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Analytical Methods

Influence of extraction procedures on phenolic content and antioxidant activity of Cretan barberry herb

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ABSTRACT

The main goal of present study was the development, optimization and application of different extraction protocols, especially those employing green technologies, in order to obtain from *Berberis cretica* extracts with high antioxidant capacity. For this purpose, the applied methods: maceration, ASE and SFE coupled with ASE were incorporated. The antioxidant assessment was carried out using DPPH and total phenolic content (Folin–Ciocalteu) assays. Major constituents were elucidated using HPLC-DAD and UHPLC–HRMS/MS (hybrid IT-Orbital trap spectrometer) equipped with an ESI probe.

The chromatographic and spectral data revealed the presence of several simple phenolic acids, derivatives of both caffeic and benzoic acids, and flavonoids in the produced extracts. It was clearly evidenced that the extraction method and solvents used affected both the activity and the chemical content of the results, significantly. The most beneficial conditions were calculated for methanol and water:ethanol (50:50) extracts derived from the combination of SFE and ASE methodologies. Obtained results classify Cretan barberry as a strong antioxidant agent.

1. Introduction

The herb *Berberis cretica* L. of Berberidaceae family was selected to be investigated in the present study. It is a spiny deciduous thorn bush growing in the mountains of Greece and Western Asia (Israel) above 900 m. In the late summer plant produces tiny and dark blue berries (Knight, 1835; Rashmi, Rajasekaran, & Pant, 2008; www.west-crete.com/flowers/berberis_cretica.htm).

Barberry species belong to edible plants and are used as cholagogue, choleretic, anti-inflammatory and antipyretic medicines both in homoeopathy and allelopathy (Das, Singh, Ansari, & Agrawal, 2005; Kozlovskaya et al., 1998; Wang et al., 2007). Fruits of *Berberis* species are known as potent sources of vitamin C and are present to known food supplements (Chalise et al., 2010).

Phenolic substances (simple phenols, phenolic acids, flavonoids or hydroxycinnamic acid derivatives) are phytonutrients characterized of strong antioxidant properties (Loliger, 1991; Velioglu, Mazza, Gao, & Oomah, 1998). The principle function of antioxidants is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals (Namiki, 1990). As a result, antioxidants may reduce oxidizing damage, which remains a confirmed cause of cancer and cardiovascular problems (Lindley, 1998; Papas, 1999). Moreover, several studies show a negative association between the intake of fruits, vegetables and medicinal plants and certain diseases (Papas, 1999).

Advantages of free-radical scavengers' intake lead to much investigation on this field. Numerous papers treat on isoquinoline alkaloids' properties. Isoquinoline alkaloids – main secondary metabolites of *Berberis* genus – are found to exhibit moderate free radical scavenging properties. Phenolic acids and flavonoids highly

Abbreviations: HPLC, high performance liquid chromatography; SFE, Supercritical Fluid Extraction; GAE, gallic acid equivalents; ASE, Accelerated Solvent Extraction; SFE–ASE, Supercritical Fluid Extraction followed by Accelerated Solvent Extraction; LC–MS, liquid chromatography–mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

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represented in barberry's extracts have impact on its antioxidant potential as well (Bezakova, Misik, Malekova, Svajdlenka, & Kostalova, 1996; Chen et al., 1997; Grycova, Dostal, & Marek, 2007; Leitao Da-Cunha, Fechine, & Guedes, 2005; Misik, Bezakova, Malekova, & Kostalova, 1995; Muller, Ziereis, & Gawlik, 1995; Rackova, Majekova, Kostalova, & Stefek, 2004; Schiff, 1996).

Extraction from the source, as the first step in the medicinal plants' analysis, remains crucial. Application of conventional extraction methods is related to small extraction yields, long extraction time, high usage of solvents and significant toxicity. That is why in the course of current study more advanced extraction techniques such as Accelerated Solvent Extraction – ASE and Supercritical Fluid Extraction – SFE were compared with conventional maceration of plant material (Mustafa & Turner, 2011). Higher automation, extraction yields and recovery of active compounds together with lower toxicity of employed solvents and shorter duration are some of the advantages of contemporary extraction techniques. Nontoxic solvents, such as ethanol and water, as well as carbon dioxide were employed in the current study to recover phenolic substances with possibly highest nutritional value.

In the course of the conducted study, novel use of Cretan barberry extracts as strong antioxidant agents is proposed. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Folin–Ciocalteu tests were employed to calculate the radical scavenging properties of extracts obtained in different conditions. Apart from the optimization of extraction methods, detailed study on phenolic content of all obtained extracts has been conducted by means of HPLC, HRMS and HRMSMS.

Current study employs the comparison between classical room temperature maceration, ASE, and SFE. Incorporation of green extraction technologies towards the high recovery of antioxidant agents remains the main target of current study. Fractions rich in free radical scavengers obtained using nontoxic solvents could be perceived as dietary supplements of high value. The efficiency of employed methods, temperature settings and solvents' composition have been evaluated using the antioxidant *in vitro* tests: a DPPH assay and a Folin–Ciocalteu method for the evaluation of free-radical scavenging activity and total phenolic content, respectively. The amount of extracted alkaloids and phenolics is proportional to the scavenging power of the extract against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (IC_{50} values) and Folin–Ciocalteu reagent (gallic acid equivalents (GAE)).

2. Materials and methods

2.1. Chemicals and reagents

Standards of phenolic acids (chlorogenic, ferulic, gallic, p-coumaric, p-hydroxybenzoic, protocatechuic, rosmarinic, quinic, sinapinic, syringic, and vanillic acid) and flavonoids (apigenin, hesperidin, hyperoside, luteolin, quercitrine, rhamnetin, rutin), as well as sodium carbonate (99% purity) and 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95% purity) were purchased from Sigma–Aldrich (Syeinheim, Germany). Folin–Ciocalteu reagent, ethanol reagent grade, dichloromethane reagent grade, DMSO reagent grade, methanol reagent grade and gradient grade, hydrochloric acid (37%), ammonia (40%), acetic acid (99%), and acetonitrile gradient grade were produced by Merck (Darmstadt, Germany).

2.2. Plant material

The herb of Cretan barberry used in the course of current survey was collected by Dr. Nektarios Aligiannis and Dr. Eleftherios Kalpoutzakis on the island of Crete (Greece), at the beginning of plant's flowering (in April 2002) from the rocky locations of Rouvas forest in Nidha Plateau and identified by Dr. Eleftherios Kalpoutzakis. A voucher specimen (no. KL006R) was deposited at the Herbarium of the Division of Pharmacognosy, University of Athens.

2.3. Extraction

2.3.1. Maceration

After the collection 400 g of dried and ground herb was macerated in dark glass bottles – successively with cyclohexane (2 L × 3), ethanol (2 L × 4) and distilled water (2 L × 4), for 2 days each time. The derived extracts were filtered and evaporated each time. Filtrates coming from the same solvent were joined together. This procedure resulted in three extracts from the herb of Cretan barberry: cyclohexane, ethanol and aqueous one.

2.3.2. Accelerated Solvent Extraction (ASE)

An ASE 300 apparatus (Dionex, Sunnyvale, CA, USA) with 100 mL stainless steel vessels was used for the pressurized liquid extraction. 25 g of *B. cretica* L. herb powder was placed in an extraction cell each time. Following the addition of solvents (Table 1), the cell was pressurized, heated, and extracted statically under following conditions: static time – 15 min, purge time – 100 s, preheating time – 5 min while the pressure was set at 103 bar.

The extracts were evaporated to dryness using a rotary evaporator at 40 °C. Aqueous extracts were primarily frozen in a GFL freezer and lyophilized using Martin Christ Alpha 2-4LD plus lyophilizer.

2.3.3. Supercritical Solvent Extraction (SFE)

The extraction was conducted using a SFE-1-2 No 4218 (Separex F 54250 Champigneulles) system. 220 g of dry powdered herb of Cretan barberry was placed in the stainless steel extraction cell. Pure carbon dioxide was delivered on extraction basket with the flow of 5.0 kg/h. The extraction temperature was set at 50 °C as well as the temperature of separators. Different pressure levels were applied in the course of extraction process: 100, 200, and 300 bar (Table 2). Extracts were collected every half an hour to dark glass bottles. Twenty fractions were collected within 17.5 h. Their chemical content was evaluated using thin layer chromatography (TLC) and fractions with similar composition were put together.

After 5 h extraction with carbon dioxide, ethanol was added as co-solvent to modify the polarity of the solvent system. In the course of the study, the flow rate of ethanol was changed from 2.5 mL/min to 20 mL/s according to the protocol which is presented in Table 2, while the carbon dioxide flow was decreased (2.5 kg/h) at the end of the procedure in order to increase the percentage of ethanol in the solvent mixture (Table 2).

2.3.4. Supercritical Solvent Extraction coupled with Accelerated Solvent Extraction

Portions of 25 g of the residue obtained from CO_2 processing of the herb were subsequently transferred to ASE cell and extracted with different solvent systems. All extraction conditions are listed in (Table 1).

2.4. GC–MS profiling of volatile fractions

An amount of each volatile extract (S01 and S02 extracts – see Table 1) was diluted in 0.5 mL dichloromethane R at a concentration of 1 mg/mL and kept at 4 °C in a sealed brown vial. An aliquot of 10 µl of each solution was directly injected into the GC–MS instrument.

GC–MS analysis was conducted using an Hewlett–Packard 6890-5973 instrument (EI mode) provided in a capillary DB-5 col-