JOURNAL OF FUNCTIONAL FOODS 11 (2014) 293–303

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Two-step sequential supercritical fluid extracts from rosemary with enhanced anti-proliferative activity

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ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form 7 October 2014
Accepted 8 October 2014
Available online

Keywords:
Rosemary
SFE
Sequential process
Anti-proliferative
Colon cancer cell
HT-29

ABSTRACT

Previously, carnosic acid and carnosol have demonstrated anti-proliferative activity against different types of cancer. To obtain extracts enriched in these two key phenolic compounds, two different processes have been developed in the present work based on the use of two-step sequential supercritical fluid extraction (SFE). By removing the interfering, less active fractions in a first step (150 or 300 bar, 40°C, neat CO_2, 60 min), suitable enrichment is achieved in the second step (150 bar, 40°C, CO_2 + 7% ethanol, 120 min), and this leads to carnosic acid concentrations in the extract as high as 40% of total dry weight, which are among the highest concentrations that have been described with this type of process. The enriched extracts were tested against the HT-29 human adenocarcinoma cell line, showing enhancement of their antiproliferative activity by approximately 3-fold compared to previously reported SFE rosemary extracts and higher inhibitory effects at lower concentrations (30 μg mL^{-1} of extract). Thus, the proposed two-step SFE process effectively improves the carnosic acid and carnosol recovery in shorter processing times (180 min vs. 300 min). Moreover, the obtained extracts possess higher anti-proliferative activity and consume less solvent.

1. Introduction

The influence of dietary polyphenols on human health is currently widely accepted. In fact, these compounds have been suggested to positively influence the prevention of several different chronic diseases, including obesity (Wang et al., 2014), diabetes (Xiao & Högger, 2014), cardiovascular disease (Wang, Chun, & Song, 2013), Alzheimer's disease (Hu et al., 2013), and cancer (Henning, Wang, Carpenter, & Heber, 2013), among others. Moreover, a positive impact on gut microbiota has been proposed (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013), which could also provide additional benefits for human health. However, the particular mechanisms of action of polyphenols on these diseases have not yet been completely elucidated (Del Rio et al., 2013).

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http://dx.doi.org/10.1016/j.jff.2014.10.014
1756-4646/

One of the dietary sources of polyphenols that has garnered attention is rosemary (*Rosmarinus officinalis*). This Mediterranean species belongs to the *Lamiaceae* family and has traditionally been used as a food ingredient to increase food flavor. However, this plant has also been described as an attractive source of phenolic compounds with important bioactivities (Ben Jemia et al., 2013; Borrás Linares et al., 2011).

The anti-proliferative activity of dietary polyphenols is one of the most-studied bioactivities, and rosemary phenolic compounds are not an exception. Several studies have demonstrated the positive influence of rosemary extracts against different types of cancer (Kontogianni et al., 2013). This activity has been associated with their antioxidant capacity, mainly due to the presence of several phenolic compounds, such as rosmarinic and carnosic acids, carnosol, rosmanol, epirosmanol and methyl carnosate (Herrero, Plaza, Cifuentes, & Ibañez, 2010b), among others. In previous studies, we reported that different advanced, environmentally-friendly extraction techniques, such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE), are able to selectively produce bioactive rosemary extracts with anti-proliferative effects on different human cancer cell lines, specifically colon cancer (Valdés et al., 2013) and leukemia (Valdés et al., 2012) cell lines. Through different foodomics-based approaches, it has been possible to explain some of the molecular mechanisms of action of these phenolic compounds (Ibáñez et al., 2012a and Ibáñez et al., 2012b). SFE is a valuable tool to extract bioactive compounds from natural sources (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013). The use of supercritical CO_2 has many advantages in these applications, allowing the extraction of labile or easily oxidizable compounds. However, because of its very low polarity, CO_2 is unable to extract some of the most interesting natural bioactive components, which possess higher polarity. For this reason, the use of a co-solvent during extraction, often ethanol, is widely employed (Herrero, Mendiola, Cifuentes, & Ibáñez, 2010a). By using a co-solvent, the properties of the CO_2 as a solvent are modified and more polar compounds can be extracted. Among the different extraction techniques previously studied in our laboratory to obtain bioactive rosemary extracts, SFE has shown the most promise, because it was able to produce active extracts when ethanol was employed as cosolvent together with supercritical CO_2 (Valdés et al., 2013). These rosemary extracts were comparatively richer in medium to low polarity phenolic compounds (i.e., carnosic acid and carnosol), whereas other techniques such as PLE were more suited for the enrichment of the more polar phenolics, mainly rosmarinic acid, which were less active (Valdés et al., 2013). Consequently, these less polar phenolic compounds present in rosemary are more interesting as functional ingredients to prevent cell proliferation. In fact, carnosic acid has been the focus of research that has concluded that this compound may have not only anti-proliferative activities (Visanji, Thompson, & Padfield, 2006) but also anti-inflammatory properties (Kuo et al., 2011; Xiang et al., 2013) and neuroprotective activities (Satoh et al., 2008) in addition to its well-known antioxidant capacity (Jordan, Lax, Rota, Loran, & Sotomayor, 2012). The other related phenolic diterpene, carnosol, has also possesses anti-proliferative effects (Lopez-Jimenez, Garcia-Caballero, Medina, & Quesada, 2013 and Johnson, 2011). Thus, strategies directed at the enrichment of carnosic acid and carnosol in rosemary extracts are important for the functional food industry to achieve more active natural fractions.

Therefore, the main goal of the present work was to develop new strategies based on the use of sequential SFE processes to produce supercritical rosemary extracts enriched in carnosic acid and carnosol. Additionally, their anti-proliferative activities against human colon cancer cells were compared to a previously reported SFE extract (Herrero et al., 2010b and Valdés et al., 2013). Moreover, a complete chemical characterization together with *in vitro* activity measurements (total phenol content and antioxidant activity) were performed to create as broad of a picture as possible of their observed bioactivity.

2. Materials and methods

2.1. Samples and chemicals

The rosemary (*Rosmarinus officinalis*) samples consisted of dried rosemary leaves obtained from Herboristeria Murciana (Murcia, Spain). The dried rosemary leaves were ground using a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at low temperature for 30 s. The ground samples were then vacuum-packed and stored at 4 °C until further use.

The solvents used in the supercritical extraction process were 99% pure carbon dioxide purchased from Carburos Metálicos (X50S, Barcelona, Spain) and ethanol (99.5%) provided by VWR Chemicals (Fontenay-sous-Bois, France).

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 99%), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, ≥99%), carnosic acid (≥97%) and carnosol (≥ 98%) were purchased from Sigma-Aldrich. The Folin-Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). For the inhibition of cell proliferation experiments, the dry extracts were dissolved in DMSO (Sigma-Aldrich) at the appropriate concentrations and stored as aliquots at –80 °C until future use.

2.2. Supercritical fluid extraction (SFE)

The extractions were carried out using a pilot scale SFE instrument (Thar Technologies, model SF2000, Pittsburg, PA) equipped with a 2 L extraction cell and two 0.5 L separators with independent pressure and temperature controls. For each extraction, 500 g of rosemary was used with a CO_2 flow rate of 60 g min^{-1}. For each extraction condition studied, a two-step extraction protocol was employed, utilizing a 60 min extraction with neat supercritical CO_2 first and then a second extraction with supercritical CO_2 and 7% ethanol as the cosolvent at 150 bar. Duplicate extraction procedures were carried out, and two different extraction pressures for the first step (150 and 300 bar) were used.

After extraction, the extracts were collected in vials, the residual ethanol was evaporated under vacuum and the dried extracts were stored at –20 °C and protected from light until analysis.

2.3. Total phenol content (Folin-Ciocalteu method)

The total phenol contents of the rosemary extracts were measured using the Folin–Ciocalteu assay with some modifications,