Production of Supercritical Rosemary Extracts and their Effect on Tumor Progression

Gonzalo Vicente1*, Susana Molina2, Margarita González-Vallinas2, Mónica R. García-Risco1, Tiziana Fornari1, Guillermo Reglero1,2 and Ana Ramírez de Molina2

1 Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM). C/Nicolás Cabrera 9, Universidad Autónoma de Madrid, 28049 Madrid, Spain.
2 IMDEA-Food Institute, C/Faraday 7, 28049, Madrid, Spain.
Corresponding author: gonzalo.vicente@uam.es Phone: (+34) 910 017 972 ; Fax: (+34) 910 017 905

ABSTRACT

Supercritical fluid technology is the most innovative method to recover bioactive compounds for use as supplements for functional foods. Particularly, the recovery of antioxidant compounds from different herbs is being a matter of continuous research and development. Besides their role as food stabilizers, antioxidants can protect cells against the effects of free radicals and thus, play an important role in heart disease, cancer and other diseases.

Rosemary (Rosmarinus officinalis L.) has been recognized as one of the Lamiaceae plant with large antioxidant activity. Main substances associated with the antioxidant activity are the phenolic diterpenes such as carnosol, rosmarinol, carnosic acid, methyl carnosate, and phenolic acids such as the rosmarinic and caffeic acids. Particularly, carnosic acid is accepted as the most abundant antioxidant present in rosemary.

In this work, supercritical fluid technology was applied to produce rosemary extracts with different composition (phenolic compounds and volatile oil content) and thus, with different antioxidant power. For this purpose, pure CO2 and CO2 modified with ethanol were utilized as supercritical solvents, and diverse extraction conditions (temperature, pressure, amount of cosolvent and fractionation procedure) were applied.

Selected extracts, from the variety of samples obtained, were used to study the capability of rosemary supercritical extracts to inhibit the proliferation of human liver carcinoma cells. Moreover, the cytostatic effect of the different selected extracts were determined, revealing a dose-dependent effect of the different compositions of the extracts on the response of human hepatocarcinoma cells to the potential antitumoral effect of rosemary.

INTRODUCTION

Recent studies reveal that the extracts of many plants and herbs are potential anticancer drugs owing to their capacity to prevent, reverse and/or inhibit certain processes of carcinogenesis before the development of invasive cancer [1, 2]. This effect has been attributed to certain substances present in the vegetal matter; scientific studies are currently under development to prove that these substances possess specific functional activities.

Rosemary (Rosmarinus officinalis L.) extracts have been reported to have several and important biological properties, such as hepatoprotective [3], antidiabetic [4], antioxidant [5], antiproliferative [6], antiviral [5], antimicrobial [7], antinociceptive [8] and antidepressant [9], among others. Some of these activities point to a promising beneficial effect of rosemary in controlling cancer development. Accordingly, it has been previously reported that rosemary extracts and their components show inhibitory effects on the growth of breast, liver, prostate, lung and leukemia cancer cells [10] and represses the initiation and promotion of tumorigenesis of melanoma and glioma in animal models [11,12,13].

One of the most appreciated property of rosemary extract is its antioxidant capacity, which is related to the presence of antioxidant phenolic substances, such as carnosol, rosmanol, carnosic acid, methyl carnosate, rosmarinic and caffeic acids [14, 15, 16].

Different authors [17, 18] compared rosemary extracts produced by supercritical fluid extraction (SFE) with those obtained using liquid solvents (ethanol and hexane) or hydro-distillation, and demonstrated the superior antioxidant activity of the supercritical extracts.
The SFE of rosemary leaves to produce natural antioxidant products has been extensively studied and reported; the reader is referred to some of the abundant literature available at this respect [19, 9, 20, 21, 22]. To increase the concentration of phenolic compounds and get more antioxidant power, fractionation of the supercritical extract has been proposed. In general, fractionation was accomplished by applying different conditions in two time sequential extractions (multi-step fractionation) or by producing a cascade decompression of the extract in two or more separator vessels (on-line fractionation).

For example, on-line fractionation in a two-step depressurization system was studied by Cavero et al. [18] using pure CO₂ and CO₂ with ethanol cosolvent to increase the solubility of polar substances; the antioxidant fraction was isolated in the first separator, while the volatile oil was recovered in the second separator. Multi-step fractionation approach was employed by Ibañez et al. [23] to extract the volatile oil in a first step (10 MPa and 313 K) while the antioxidant fraction was obtained in a second step (40 MPa and 323 K) without using a cosolvent. Ivanovic et al., [24] employed similar multi-step fractionation scheme to isolate an antioxidant fraction from rosemary.

In this work, rosemary supercritical extracts with different concentration of antioxidant compounds were produced, by using diverse extraction conditions such as temperature, pressure, amount of co-solvent (ethanol) and fractionation procedures. The antioxidant power of the different samples produced was evaluated by the DPPH test, and some selected supercritical rosemary extracts were employed to study the antitumor activity of the extracts when added to liver cancer cells.

**MATERIALS AND METHODS**

**Chemicals and samples**

2, 2- Diphenil-1-pycril hydrazyl hydrate (DPPH, 95% purity), Camphor (>97%), Bornyl acetate (95%) and Linalool (>97%) were purchased from Sigma-Aldrich. Carnosic acid (≥96%) and Carnosol was purchased from Alexis Biochemical. 1,8 cineole (98%) and Borneol (>99%) were purchased from Fluka. Ethanol and phosphoric acid (85%) were HPLC grade from Panreac. Acetonitrile was HPLC grade from Lab Scan (Dublin, Ireland). CO₂ (N38) was supplied from Air Liquid. LO, GO and SO were purchased from a local market.

The rosemary (Rosmarinus officinalis L.) raw material consisted of dried leaves (water content < 5 % wt) obtained from an herbalist’s producer (Murcia, Spain). The sample was ground in a cooled mill. Sample particle size was in the range of 200 and 600 µm.

**Supercritical extraction**

Extractions were carried out using a supercritical fluid pilot-plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity with independent control of temperature (± 2°C) and pressure (± 1 bar). The extraction equipment also includes a recirculation system, were CO₂ is condensed, pumped up to the desired extraction pressure and heated up to the selected extraction temperature.

The temperature of the extraction cell and separators was maintained at 40°C and CO₂ flow rate was 60 g/min in all experimental assays. In selected assays, fractionation of the extracted material was accomplished by setting the pressure of the first separator (S1) to 100 bar, while the second separator (S2) was maintained at the recirculation system pressure (50 bar). In this case, two different samples were collected: one sample from S1 cell and the other from S2 cell. When no fractionation of the extract was accomplished, S1 was set to the recirculation system pressure and thus, only one sample was recovered from S1. Extraction conditions were selected on the basis of previous studies reported in the literature [17, 19, 10, 21, 22, 25, 23]. The SFE assays are explained in detail as follows.

In the first extraction (Ext. 1 in Table 1) the extraction pressure was set to 15 MPa and 5% (w/w) of ethanol was employed as cosolvent. The time of extraction was 180 minutes; no fractionation was accomplished and then only one sample was collected in S1 (M1 sample). Ext. 2 is identical to Ext. 1, but 10 % (w/w) of ethanol cosolvent was employed. In Ext. 3 no cosolvent was employed during a first step of extraction (15MPa, 60 min) and then 10 % w/w ethanol was used during the second step (15 MPa, 120 min). Thus, in Ext. 3 two samples were collected from S1 separator, corresponding to the first (M3-1 sample) and second (M3-2 sample) extraction steps. Ext. 4 was also accomplished in two sequential steps: first, extractor pressure was set to 300 bar during 360 min and no fractionation of the extracted material was accomplished (M4-1 sample); then, extractor pressure was 150 bar and 10 % w/w ethanol as cosolvent was employed during 180 min (M4-2 sample). Finally, Ext. 5 was carried out without ethanol, at 300 bar and fractionation of the extracted material was accomplished during the first 60 min. Then, extraction continued for 300 min without fractionation. Two samples were collected: one from S1 (M5-1 sample) and the other from S2 (M5-2).
Chemical analysis
The essential oil compounds of samples were determined by GC-MS-FID using 7890A System (Agilent Technologies, U.S.A.), comprising a split/splitless injector, electronic pressure control, G4513A auto injector, a 5975C triple-Axis mass spectrometer detector, and GC-MS Solution software. The column used was an Agilent 19091S-433 capillary column, 30 m x 0.25 mm I.D. and 0.25 µm phase thickness. Helium, 99.996% was used as a carrier gas at a flow of 29.4 ml/min and inlet pressure of 28.823 Psi. Oven temperature programming was 60°C isothermal for 4 min then increased to 106 ºC at 2.5 ºC/min and from 106ºC to 130°C at 1ºC/min and finally from 130ºC to 250 ºC at 20ºC/min, this temperature was kept constant for 10 min. Sample injections (1 µl) were performed in split mode (1:10). Injector temperature was of 250ºC and MS ion source and interface temperatures were 230 and 280ºC, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. Key volatile oil compounds (1,8 cineole, camphor, borneol, verbenone and linalool) were identified by comparison with standard mass spectra, obtained in the same conditions and compared with the mass spectra from library Wiley 229. A calibration curve was employed to quantify each of the key volatile oil compounds. GC-MS analyses were carried out by duplicate and the average standard deviation obtained was ± 0.08%.

The content of carnosic acid in the samples were determined using an HPLC (Varian Pro-star) equipped with a Microsorb-100 C 18 column (Varian) of 25 cm × 4.6 mm and 5 µm particle size. The analysis is based on the work of Almela et al. [26]. The mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23% A, 8-25 min, 75% A, 25-40 min 75% A and the 40-45 min 23% A . Initial conditions were gained in 5 min. The flow rate was constant at 0.7 ml/min. Injection volume was 20 µl and the detection was accomplished by using a diode array detection system (Varian) storing the signal at a wavelength of 230 and 280 nm. Samples were analyzed by HPLC in duplicate and the obtained average standard deviation was ± 0.13%.

Antioxidant activity by the DPPH test
The method consists in the neutralization of free radicals of DPPH by an antioxidant sample [27]. An aliquot (50 µl) of ethanol solution containing 5-30 µg/ml of rosemary extract, was added to 1.950 µl of DPPH in ethanol (23.5 µg/ml) prepared daily. Reaction was completed after 3 h at room temperature and absorbance was measured at 517 nm in a Nanovette Du 730 UV spectrophotometer (Beckman Coulter, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression (y = 0.0265·x; R² = 0.9998). Ethanol was used to adjust zero and DPPH-ethanol solution as a reference sample. The amount of extract necessary to decrease the initial DPPH concentration by 50% or EC 50 (µg/ml) was determined and employed to value the antioxidant power of the sample; the lower the EC50, the higher the antioxidant power

Cell culture
Human hepatoma cancer cells HepG2, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% of antibiotic-antimycotic solution (containing 10 000 units/mL of penicilllin base, 10 000 µg/mL of streptomycin base, and 25 000 ng/mL of amphotericin B; Gibco). The cells were maintained under standard conditions of temperature (37ºC), humidity (95%), and carbon dioxide (5%).

Cell viability assay
The antiproliferative activity of SFRE was measured by MTT assay. Cells in the exponential growth phase were placed in 24 well plates using 500 µL of cell suspension per well at a density between 1.5 x 104 and 6.0 x 104 cells, and incubated overnight. Then, the number of viable cells in the control wells was determined by colorimetric assay (described below); immediately afterwards, medium from the corresponding wells was replaced with new culture medium (blank wells) or supplemented with different concentrations of 5-FU (Sigma), SFRE, or a combination of both, according to the experiment. Cell viability was determined after 48 or 72 h. In order to determine the number of viable cells, 50 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h; subsequently, the medium was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to lyse the cells and resuspend the formazan (the metabolic product of MTT). Quantities of formazan product, which are directly related to the number of viable cells, were measured at 560 nm using a scanning spectrophotometer microplate reader (UVM 340 Biochrom, Cambridge, UK). At least three independent experiments were performed in triplicate. Concentration values corresponding to cell sensitivity (IC50), growth inhibition (GI50) and cytostaticity (TGI) were calculated according to the NIH definitions using a logistic regression.
RESULTS AND DISCUSSION

Supercritical rosemary extracts
The different conditions applied in the supercritical rosemary extraction were targeted to produce a sample with high content of antioxidant substances. Table 1 shows the extraction yield, the carnosic acid content and the total content (% w/w) of main volatile compounds (borneol, bornyl acetate, camphor, 1,8-cineol and verbenone) of the supercritical rosemary extracts produced in the Ext. 1 to 5 defined before. Low amounts of carnosol (< 3 % w/w) were obtained in all samples.

As can be observed from Table 1, higher carnosic acid contents were obtained when ethanol was employed as CO₂ cosolvent (M1, M3-2 and M4-2 samples). In the case of samples M3-2 and M4-2, the low content of essential oil compounds determined could be attributed to the fact that, in both experiments, the plant matrix was previously extracted with pure CO₂ and thus, essential oil substances were almost exhausted. On the other side, the high yield obtained in Ext. 2 (10% w/w cosolvent) supposes a high co-extraction of other substances and thus, the concentration of both carnosic acid and volatile oil compounds obtained in M2 sample was considerably reduced with respect to M1 sample, which was produced at identical extraction conditions but using 5% w/w cosolvent.

As expected, due to the fractionation procedure accomplished in Ext. 5 (no cosolvent was employed), the extract collected in S1 (M5-1) contains higher amounts of carnosic acid and lower amounts of volatile oil compounds than the sample collected in S2 (M5-2).

Table 1. Extraction yield, carnosic acid and main volatile oil compounds (% w/w) in the supercritical rosemary samples produced.

<table>
<thead>
<tr>
<th>Ext.</th>
<th>Sample</th>
<th>Yield (g extract / g rosemary leaves x 100)</th>
<th>Carnosic acid content (% w/w)</th>
<th>Main volatiles compounds (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M1</td>
<td>7.26</td>
<td>25.66</td>
<td>10.42</td>
</tr>
<tr>
<td>1</td>
<td>M2</td>
<td>13.44</td>
<td>14.18</td>
<td>4.69</td>
</tr>
<tr>
<td>1</td>
<td>M3-1</td>
<td>1.42</td>
<td>2.00</td>
<td>36.92</td>
</tr>
<tr>
<td>1</td>
<td>M3-2</td>
<td>3.02</td>
<td>28.49</td>
<td>4.81</td>
</tr>
<tr>
<td>2</td>
<td>M4-1</td>
<td>4.52</td>
<td>10.89</td>
<td>12.79</td>
</tr>
<tr>
<td>2</td>
<td>M4-2</td>
<td>4.93</td>
<td>30.69</td>
<td>2.04</td>
</tr>
<tr>
<td>5</td>
<td>M5-1</td>
<td>2.83</td>
<td>16.90</td>
<td>13.59</td>
</tr>
<tr>
<td>5</td>
<td>M5-2</td>
<td>1.53</td>
<td>3.12</td>
<td>21.70</td>
</tr>
</tbody>
</table>

The rosemary supercritical samples selected to carry out the studies about their antitumor effect on liver cancer cells were M4-1, M5-1, M1 and M4-2. Moreover, all samples contain similar amounts of key volatile oil compounds (≈ 12 % w/w), except M4-2 which contains a significant reduced amount of volatile oil compounds (≈ 2 % w/w). Particularly, M4-1 and M5-1 were selected since both samples were produced without using ethanol as cosolvent. This is an important factor to be considered to evaluate a commercial rosemary supercritical extract production, since evaporation of cosolvent is an expensive task to be accomplished.

Table 2 shows the EC₅₀ value determined for samples M4-1, M5-1, M1 and M4-2, using the DPPH test. As expected, the EC₅₀ value decreased (and the antioxidant power of the samples increased) as the content of carnosic acid antioxidant increased.

Table 2. EC₅₀ values and content of carnosic acid (% w/w) of selected supercritical rosemary samples produced in this work.

<table>
<thead>
<tr>
<th>Rosemary extract</th>
<th>EC₅₀ value (µg/ml)</th>
<th>Carnosic acid (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4-1</td>
<td>32.97</td>
<td>10.89</td>
</tr>
<tr>
<td>M5-1</td>
<td>15.91</td>
<td>16.90</td>
</tr>
<tr>
<td>M1</td>
<td>14.77</td>
<td>25.66</td>
</tr>
<tr>
<td>M4-2</td>
<td>9.8</td>
<td>30.69</td>
</tr>
</tbody>
</table>
Effect of supercritical rosemary extracts on the proliferation of liver cancer cells

To examine the effect of selected rosemary supercritical extracts (M4-1, M5-1, M1 and M4-2 samples) in human hepatoma cancer cells, cell proliferation was analyzed by MTT assay after treatment with different concentrations (from 0 to 120 µg/mL) of the different compositions of extracts for 48 h. As it can be observed in Table 4, each rosemary supercritical extract exhibited a dose-dependent effect on cell proliferation. Values representing cell sensitivity to the extracts (IC50), growth inhibition (GI50) and cytostaticity (TGI) were determined (Table 4).

The variation of these parameters with the % w/w of carnosic acid of the sample is depicted in Figure 1. As can be seen in the individual graphs, a considerably reduction of the proliferative activity of the cells is observed for increasing amounts of carnosic acid from M4-1 to M1 samples. That is, the higher the concentration of carnosic acid in these samples, the lower the values of IC50, GI50 and TGI.

However, although sample M4-2 contains higher concentration of carnosic acid than sample M1 and consecutively presents higher antioxidant activity, M4-2 anti-proliferative effect is not increased with respect to M1, resulting even lower. On the other hand, while all M4-1, M5-1 and M1 samples contain around 12 % w/w of volatile oil compounds, M4-2 contain only ca. 2 % w/w.

According to the results obtained, the anti-proliferative activity of rosemary supercritical extracts on liver cancer cells could not be attributed exclusively to carnosic acid antioxidant content but other substances, probably substances comprising the volatile oil fraction, may act synergically. These results suggest that M1 might constitute an efficient composition to further analyze its effects as an antitumoral agent against liver cancer.

**Table 4.** Cell sensitivity (IC50), growth inhibition (GI50) and cytostaticity (TGI) of HepG2 cells after 48 h treatment with the different extracts (µg/mL).

<table>
<thead>
<tr>
<th></th>
<th>M4-1</th>
<th>M5-1</th>
<th>M1</th>
<th>M4-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% carnosic acid</td>
<td>10.89</td>
<td>16.90</td>
<td>25.66</td>
<td>30.69</td>
</tr>
<tr>
<td>IC50</td>
<td>110.71 ± 18.7</td>
<td>93.26 ± 22.1</td>
<td>42.16 ± 5.9</td>
<td>48.01 ± 3.2</td>
</tr>
<tr>
<td>GI50</td>
<td>78.98 ± 15.7</td>
<td>55.00 ± 10.0</td>
<td>20.00 ± 5.0</td>
<td>26.50 ± 6.5</td>
</tr>
<tr>
<td>TGI</td>
<td>99.18 ± 19.2</td>
<td>67.47 ± 12.3</td>
<td>28.40 ± 0.9</td>
<td>44.80 ± 6.0</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Supercritical rosemary extracts produced intentionally with different content of antioxidants (carnosic acid) were investigated on their effect to inhibit the proliferation of human liver carcinoma cells. A considerably reduction of the proliferative activity of the cells is observed for increasing amounts of carnosic acid in the samples. Although the concentration of carnosic acid demonstrated to have a crucial effect on growth inhibition and cytostaticity, certain synergic effect with rosemary essential oil seem to be also important. Additional studies will be developed on this direction.

**ACKNOWLEDGES**

This work has been supported by project AGL2010-21565(subprogram ALI) and project INNSAMED IPT-300000-2010-34 (subprogram INNPACTO) from Ministerio de Ciencia e Innovación (Spain) and Comunidad Autónoma de Madrid (project ALIBIRD-S2009/AGR-1469).
Figure 1. IC50 (a), GI50 (b) and TGI (c) as a function of the carnosic acid content (% w/w) of the different extracts tested.

REFERENCES