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Supercritical rosemary extracts, their antioxidant activity and effect on hepatic tumor progression

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Abstract

The use of supercritical fluid technology as an innovative technology to extract bioactive compounds has grown considerably in recent decades. Particularly, the recovery of antioxidants from different herbs is a matter of continuous research and development. Antioxidants can protect cells against the effects of free radicals and thus, play an important role in heart illness, cancer and other diseases.

Rosemary (*Rosmarinus officinalis* L.) has been recognized as one of the Lamiaceae plant with many important biological activities. Particularly, large antioxidant power has been recognized in rosemary and main substances related with this activity were the phenolic diterpenes such as carnosol, rosmanol, carnosic acid, methyl carnosate, and phenolic acids such as the rosmarinic and caffeic acids. Moreover, carnosic acid and carnosol are recognized as the most abundant antioxidants present in rosemary.

In this work, supercritical fluid technology was applied to produce rosemary extracts with different composition and thus, with different bioactivity properties. 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. These extracts showed a dose-dependent effect on inhibiting the proliferation of human hepatoma cells. Moreover, observed citoestaticity appeared to be significantly influenced by their different composition, suggesting a relevant role of the technology to produce the extracts and the consequently obtained compositions on the potential antitumoral activity of rosemary.

Keywords: *Rosmarinus officinalis*; Antioxidant; Supercritical extraction; Hepatic cancer.

1. 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, and many scientific studies are currently under development to prove that these substances possess specific functional activities. For example, the catechins of green tea [3], resveratrol present in grapes, berries and peanuts [4], lycopene of tomato [5], ellagic acid which is a natural phenol antioxidant found in numerous fruits and vegetables [6], have been reported to show the capability to prevent cancer development.

Particularly, rosemary (*Rosmarinus officinalis* L.) is a perennial herb from Lamiaceae family, typical of the Mediterranean region, which has been recognized to have numerous and important biological properties, such as hepatoprotective [7], antidiabetic [8], antioxidant [9], antiproliferative [10], antiviral [9], antimicrobial [12], antinociceptive [13] and antidepressant [14], 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 isolated components show inhibitory effects on the growth of breast, liver, prostate, lung and leukemia cancer cells [14] and represses the initiation and promotion of tumorogenesis of melanoma and glioma in animal models [15-17]. However, the potential synergism among components, as well as the putative mechanism of action by which it exerts this biological activity has not been clearly addressed to date.

One of the most appreciated properties 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 [18-20]. Moreover, carnosic acid and carnosol are the most abundant antioxidant of rosemary. Some *in vitro* investigations have shown that carnosic acid has an antioxidant activity three times higher than that of

carnosol [21]. Nevertheless, also the contrary conclusion was reported, depending on the method employed to evaluate the antioxidant activity [22].

On the other hand, different authors [23, 24] 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 extracts has been extensively investigated and reported; the reader is referred to some of the abundant literature available in this respect [23-31]. The main advantage of SFE is related to the possibility of fractionation of the extract to separate the essential oil substances from the phenolic compounds. In general, fractionation was accomplished by applying different conditions in two sequential extractions (multi-step fractionation) or by producing a cascade decompression of the extract in two or more separator vessels (on-line fractionation). Further, to increase the concentration of phenolic compounds in the extract and get more antioxidant power, the supercritical CO_2 extraction using small amounts of a polar cosolvent (ethanol) was applied.

Multi-step fractionation arrangement consist in performing a first extraction step at low CO_2 density to extract the most soluble compounds (e.g. the volatile oil) followed by a second extraction step at high CO_2 density to remove the less soluble substances (e.g. antioxidants). Ibañez et al. [29] employed this fractionation scheme and a low-antioxidant but essential oil rich fraction was obtained in the first step (10 MPa and 40°C, CO_2 density = 630 kg/m^3) and a high-antioxidant fraction was produced in the second step (40 MPa and 60°C, CO_2 density = 891 kg/m^3). Ibáñez et al. [30] and Ivanovic et al., [31] employed similar multi-step fractionation scheme to isolate an antioxidant fraction from rosemary.

On-line fractionation is another fractionation alternative which allows operation of the extraction vessel at the same conditions during the whole extraction time, while several

separators in series (normally, no more than two or three) are set at different temperatures and decreasing pressures. The scope of this operation is to induce the selective precipitation of different compound families as a function of their different saturation conditions in the supercritical solvent. This procedure has been applied with success in the SFE of essential oils as it was well established by Reverchon and coworkers in the 1990s [32-34].

Supercritical rosemary extraction and on-line fractionation in a two-step depressurization system was studied by Cavero et al. [25] using pure CO_2 and CO_2 with ethanol cosolvent; the antioxidant fraction was isolated in the first separator, while the volatile oil was recovered in the second separator. Nevertheless, the authors concluded that for increased CO_2 densities a decrease of carnosic acid recovery was obtained. Further, when using ethanol as cosolvent, the differences in the distribution of carnosic acid between fractions recovered in the first and second separators were smaller, showing a decrease in selectivity.

A different on-line fractionation alternative to improve the isolation and yield of the rosemary antioxidants has been recently presented by the authors [35]. The temperature and pressure of the extractor vessel were kept constant (30 MPa and 40°C) during the whole extraction time, but the depressurization procedure was varied with time. At the beginning (first period) on-line fractionation of the extract was accomplished; owing to the lower solubility of the antioxidant compounds in comparison to the essential oil, antioxidants would precipitated in the first separator (S1) while the essential oil would be recovered in the second separator (S2). Nevertheless, after some time, the amount of volatile oil in the plant matrix would be significantly reduced but large amounts of antioxidants would still remain in plant matrix [28]. Then, during the rest of the extraction (second period) the pressure of the first separator is reduced and all substances extracted were recovered in S1 (and mixed with the material recovered there during the first period). The authors [35] varied the time of the first period and determined the optimum in order to maximize antioxidant activity and yield in the

fraction collected in S1. In this way, a product was obtained with a 2-fold increase of antioxidants in comparison with a scheme with no fractionation, and with a yield almost five times higher than that obtained when on-line fractionation is accomplished during the whole extraction time.

In this work, rosemary supercritical extracts with different concentration of antioxidant and volatile oil compounds were produced, using diverse extraction conditions such as pressure, amount of co-solvent (ethanol) and taking advance of the different fractionation procedures reported in the literature and concisely explained above. 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 potential antitumor activity of the extracts when added to liver cancer cells.

2. Materials and methods

2.1 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). CO_2 (N38) was supplied from Air Liquid.

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.

2.2 Supercritical extraction and fractionation schemes

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, where CO_2 is condensed, pumped up to the desired extraction pressure and heated up to the selected extraction temperature.

The extraction conditions were planned on the basis of previous studies reported in the literature [23-31, 35] with respect to the SFE of rosemary leaves to produce antioxidant fractions. Different fractionation alternatives (described in the Introduction) to improve the concentration of antioxidants were scheduled also according to prior studies.

The differences between the SFE assays carried out in this study are described in detail in Table 1. The temperature of the extraction cell and separators was maintained at 40°C and CO_2 flow rate was 60 g/min in all experimental assays (Extractions 1 to 5 in Table 1). For each experimental assay 0.55 kg of rosemary leaves (ground and sieved to 200-600 μm) were employed. In selected assays (see Table 1) 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 and the other from S2. 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.

The solid fractions obtained in S1 and S2 were recuperated and placed in vials. In order to ensure an accurate determination of extraction yield with time, separators were washed with ethanol and the residual material recovered in each case was mixed with the corresponding

solid fraction. Ethanol was eliminated by evaporation (35°C) and then, homogeneous solid samples were obtained and kept under N2 at -20°C in the dark until analysis.

2.3 GC-MS 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 60ºC isothermal for 4 min then increased to 106 ºC at 2.5 ºC/min and from 106ºC to 130ºC 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 volatiles were identified by comparison with standard mass spectra, obtained in the same conditions and compared with the mass spectra from library Wiley 229. The rest of compounds were identified by comparison with mass spectra from Wiley 229 library. A calibration curve was employed to quantify each of the key volatiles. GC-MS analyses were carried out by duplicate and the average standard deviation obtained was ± 0.08%.

2.4 HPLC analysis

Carnosic acid and carnosol content 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 [36]. 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, 280 and 350 nm. Samples were analyzed by HPLC in duplicate and the obtained average standard deviation was ± 0.13%.

2.5 Antioxidant activity by the DPPH test

The method consists in the neutralization of free radicals of DPPH by an antioxidant sample [37]. 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 ($y = 0.0265 \cdot x$; $R^2 = 0.9998$). 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 EC_{50}, the higher the antioxidant power.

2.6 Cell culture

Human hepatoma HepG2 cells, 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 penicillin 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%).

2.7 Cell viability assay

The antiproliferative activity of supercritical rosemary extracts was measured by MTT assay. Cells in the exponential growth phase were seeded in 96-well plates using 200 µL of cell suspension at a density of 6000 cells per well, and incubated overnight. Then, the number of viable cells in the control wells was determined by colorimetric assay (described below); immediately afterwards, medium was replaced with new culture medium (blank wells) or supplemented with increasing concentrations of the corresponding rosemary extract. Cell viability was determined after 48. In order to determine the number of viable cells, 20 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.

2.8 Statistical analysis

Experimental supercritical extractions were carried out by duplicate in the SFE system. Standard deviations of extraction yields obtained were calculated as follows:

$$StD = \sqrt{\frac{1}{2} \times \left[(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 \right]} \quad (1)$$

Being x_1 and x_2 the values obtained in each of the experiments and $\bar{x}$ the corresponding average value.

Quantification of carnosic acid and main volatile oil compounds together with the antioxidant activity tests were also carried out by duplicate, employing the mixture of extracts obtained in the duplicate extraction assays. Equation (1) was applied in order to test the reproducibility of the data obtained.

Cell viability assays were carried out in quadruplicate, and two independent experiments were performed with each selected rosemary extract. Concentration values corresponding to cell sensitivity (IC50), growth inhibition (GI50) and cytostaticity (TGI) were calculated according to the NIH definitions using a logistic regression. These parameters, as well as the ratio of viable cells, were expressed as mean ± s.e.m., which was calculated as follows:

$s.e.m = \frac{StD}{\sqrt{n}}$ being n the number of independent experiments performed. Comparisons between groups were done using the non-parametric Man-Whitney test. Two side p-values less than 0.05 were considered statistically significant. All calculations were performed using SPSS software, version 19.0 (SPSS Inc, Chicago, Illinois).

3. Results and discussion

3.1. Supercritical rosemary extracts

The different conditions applied in the rosemary supercritical extractions (Table 1) were target to produce samples with different content of antioxidant substances and volatile oil compounds, with the intention of detecting a relation between the composition of the extract and its effect on liver cancer cells.

Table 2 shows the extraction yield, the carnosic acid content and the total content (% w/w) of the most abundant volatile compounds (borneol, bornyl acetate, camphor, 1,8-cineol and verbenone) of the supercritical rosemary extracts produced in Extractions 1 to 5 defined in Table 1. Low amounts of carnosol (< 3 % w/w) were obtained in all samples collected. Additionally, the normalized composition (% peak area) of the main volatile oil compounds was determined and is given in Table 3.

As can be observed from Table 2, the higher carnosic acid contents were obtained when ethanol was employed as CO_2 cosolvent (M1, M3-2 and M4-2 samples). Additionally, a low content of essential oil compounds were determined in samples M3-2 and M4-2, what could be attributed to the fact that, in both experiments, the plant matrix was previously extracted with pure CO_2 and thus, essential oil substances were almost exhausted.

Lower % w/w of carnosic acid was obtained in M2 than in M1 demonstrating a decrease of selectivity of the process when high amounts of a polar cosolvent is employed. That is, the high yield obtained in Extraction 2 (10% w/w cosolvent) supposes a high co-extraction of substances other than antioxidants or essential oil. Thus, the concentrations of both carnosic acid and volatile oil compounds obtained in M2 sample (10% w/w ethanol) were considerably reduced with respect to M1 sample, which was produced at identical extraction conditions but using lower percentages of cosolvent (5% w/w cosolvent).

As expected, due to the fractionation procedure accomplished in Extraction 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). Nevertheless, lower extraction yield was obtained for M5-1 fraction in comparison to the samples obtained using ethanol as cosolvent.

Based on the SFE assays carried out in this work, it can be concluded that high amounts of antioxidants (e.g. carnosic acid) might be obtained only when a polar co-solvent (ethanol) is

employed in the supercritical CO_2 extraction procedure. Further, is more convenient the use of low percentages of ethanol cosolvent (≈ 5% w/w) to produce a supercritical rosemary extract with high concentration of antioxidants. At this respect, if no ethanol is utilized, fractionation of the extract can improve the antioxidant activity of one of the fractions collected, but process yield might be noticeably reduced.

Analysis of the essential oil composition (Table 3) show that despite the concentration of essential oil obtained in the extracts, the composition of the essential oil recovered is quite similar, being 1,8 Cineole and Camphor the more abundant key volatiles present in rosemary essential oil.

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 (around 12 % w/w), except M4-2 which contains a significant reduced amount of volatile oil compounds (2 % w/w). Figure 1 show a comparison between the GC chromatogram obtained for samples M5-1 and M4-2. 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 the commercial production of the extract, since evaporation of cosolvent is an expensive task to be accomplished.

Table 4 shows the EC_{50} value determined for the selected samples using the DPPH test. As expected, the EC_{50} value decreased (and the antioxidant power of the samples increased) as the content of carnosic acid antioxidant increased. Also given in Table 4 is the carnosic acid / key volatiles ratio; as mentioned before while M4-1, M5-1 and M1 contain ratios close to 1-2, while sample M4-2 was almost completely deodorized (carnosic acid / key volatiles ratio = 15).

3.3. Differential effect of supercritical rosemary extracts on the inhibition of the proliferation of human hepatoma cells.

Despite the reported hepatoprotective activity of rosemary [7], its potential activity against liver tumor progression has not been described yet. Thus, in order to address this issue, and to examine the potential effect of the different selected rosemary supercritical extracts (M4-1, M5-1, M1 and M4-2 samples), cell proliferation was analyzed by MTT assay in human hepatoma cancer cells after treatment with increasing concentrations (from 0 to 120 µg/mL) of the different compositions of extracts for 48 h. As it can be observed in Figure 2, each supercritical rosemary extract exhibited a significant dose-dependent effect on cell proliferation. Furthermore, those extracts with the highest content of carnosic acid, M1 and M4-2, are significantly more active against human hepatoma cells than those with the lowest content of this compound, M4-2 and M5-1 (Figure 2).

In addition, values representing cell sensitivity to the extracts (IC50), growth inhibition (GI50) and cytostaticity (TGI) were determined (Table 5). The variation of these parameters with the % w/w of carnosic acid of the sample is depicted in Figure 3. As it can be observed 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, in accordance with the conclusion attained by Yesil-Celiktas et al. [38]. These authors recently compare the anticarcinogenic activity of soxhlet and supercritical CO_2 extracts of rosemary, as well as their main antioxidant components, carnosic and rosmarinic acid, on the growth of various human cancer cell lines, and including liver carcinoma cells. They concluded that the findings confirm the superiority of supercritical CO_2 extraction over solvent extraction yielding higher amounts of active compounds, particularly carnosic acid, which was in turn reflected by the high antiproliferative effects.

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. Accordingly, percentage of human hepatoma viable cells after treatment with the different extracts is comparable and significantly higher for M1 and M4-2 (Figure 2), though M4-2 shows a 50% increased antioxidant activity. Furthermore, though to a lower extent, the biological activity found for M4-1 and M5-1 is also comparable (Figure 2), whereas antioxidant activity is two-fold higher for M5-1, reaching levels even close to that of M1 (Table 4). Thus, these results suggest that the potential antitumoral activity of rosemary extracts against human hepatoma cells is not related to their antioxidant activity, but it is to the extract composition which is determined by the extraction procedure employed.

In this sense, though comparable range of antiproliferative effect is observed for M1 and M4-2, the most active extract in abrogating liver tumoral cell growth is M1 (Table 5), containing around 12% w/w of volatile oil compounds whereas M4-2 contain only ca. 2%w/w (Table 2). Thus, these results suggest that reaching a significant content of carnosic acid, the presence of volatile oil compounds do not interfere with its antitumoral activity, but by contrast, might synergize in this effect.

Conclusions

Supercritical rosemary extracts were produced employing different extraction and fractionation conditions. Fractionation of the extract improved the antioxidant activity of one of the fractions collected, although process yield was reduced. Moreover, the higher amounts of antioxidants were obtained only when ethanol was employed as cosolvent.

Supercritical rosemary extracts with different content of antioxidants (carnosic acid) and essential oil compounds were investigated on their effect to inhibit the proliferation of human liver carcinoma cells. Rosemary abrogates the growth of human hepatoma cells. In addition, a considerably reduction of the proliferative activity of the cells is observed for increasing amounts of carnosic acid in the samples. However, although the concentration of carnosic acid demonstrated to have a crucial effect on growth inhibition and cytostaticity, the putative antitumoral activity of supercritical rosemary extracts might not be exclusively attributed to carnosic acid antioxidant content. Thus, substances comprising the volatile oil fraction might synergize with rosemary in its antitumoral action. These results suggest that M1 might constitute an efficient composition to further analyze its effects as an antitumoral agent against liver cancer, and additional studies will be developed on this direction.

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Figure captions

Figure 1. Comparison between the GC chromatogram obtained for (a) M4-2 and (b) M5-1 supercritical rosemary extracts.

Figure 2. Supercritical rosemary extracts inhibit the proliferation of human hepatoma cells in a dose-dependent manner.

Dose-dependent effect of selected rosemary extracts on inhibiting the proliferation of human hepatoma cells. Values represent the mean ± s.e.m. of two independent experiments each performed in quadruplicate. Asterisks indicate statistically different values in treated cells respect to control.

Figure 3. IC50 (a), GI50 (b) and TGI (c) as a function of the carnosic acid content (% w/w) of the different extracts tested.

Cell sensitivity and cytoestaticity determined as IC50 (a), GI50 (b) and TGI (c) of the different supercritical rosemary extracts on human hepatoma cells is represented as a function of their carnosic acid content (% w/w). Results are shown as the mean ± s.e.m. of two independent experiments each performed in quadruplicate.

Table 1. Production of supercritical rosemary extracts applying different process conditions. P: extraction pressure; C: % weight cosolvent (ethanol); t: extraction time.

Extraction number	Extraction and fractionation conditions	Samples obtained
1	P = 150 bar, C = 5 % w/w, t = 180 min. No fractionation of the extract.	One sample was collected from S1 separator (M1).
2	P = 150 bar, C = 10 % w/w, t = 180 min. No fractionation of the extract.	One sample was collected from S1 separator (M2).
3	P = 150 bar. First step (t = 60 min): C = 0. Second step (t = 120 min): C = 10 % w/w.	Two samples from the first (M3-1) and second (M3-2) steps.
4	First step: P = 300 bar, t = 360 min. Second step: P = 150 bar; C = 10 % w/w, t = 180 min.	Two samples from the first (M4-1) and second (M4-2) steps.
5	P = 300 bar, fractionation of the extract was accomplished during t = 60 min. Then, extraction continued for t = 300 min without fractionation.	Two samples: one from S1 (M5-1) and the other from S2 (M5-2).

Table 2. Extraction yield, carnosic acid and main volatile oil compounds content (% w/w) in the supercritical rosemary samples produced.

Ext.	Sample	Yield (g extract / g rosemary leaves x 100) [a]	Carnosic acid [b] (% w/w)	Main volatiles compounds [c] (% w/w)
1	M1	7.26	25.66	10.42
2	M2	13.44	14.18	4.69
3	M3-1	1.42	2.00	36.92
	M3-2	3.02	28.49	4.81
4	M4-1	4.52	10.89	12.79
	M4-2	4.93	30.69	2.04
5	M5-1	2.83	16.90	13.59
	M5-2	1.53	3.12	21.70

[a] mean standard deviation < 0.24

[b] values reported correspond to average value between duplicates; mean standard deviation < 0.53

[c] values reported correspond to average value between duplicates; mean standard deviation < 0.41

Table 3. Normalized (% peak area) composition[a] of main volatile oil compounds identified in rosemary supercritical extracts.

Ext	Sample	1,8 cineole	Camphor	Borneol	Verbenone	Bornyl acetate
1	M1	54.82	28.12	8.62	6.20	2.25
2	M2	56.23	27.95	9.44	6.38	n.d.
3	M3-1	58.40	19.62	6.75	9.20	1.15
	M3-2	59.98	24.56	9.54	5.92	n.d.
4	M4-1	66.75	22.83	8.45	n.d.[b]	1.97
	M4-2	61.23	24.01	14.76	n.d.	n.d.
5	M5-1	64.43	23.96	5.78	4.14	1.69
	M5-2	48.28	32.29	10.44	7.27	1.71

[a] deviations between two injections < 0.08%

[b] n.d. = not detected

Table 4. EC_{50} values and content of carnosic acid (% w/w) of selected supercritical rosemary samples produced in this work.

Rosemary extract	EC_{50} value [a] (µg/ml)	carnosic acid (% w/w)	carnosic acid / volatile oil ratio
M4-1	32.97	10.89	0.85
M5-1	15.91	16.90	1.24
M1	14.77	25.66	2.46
M4-2	9.8	30.69	15.04

[a] values reported correspond to average value between duplicates; mean standard deviation < 1.1

Table 5. Cell sensitivity (IC50), growth inhibition 50 (GI50), and tumor growth inhibition (TGI), indicative of the required concentration to induce a cytostatic effect of HepG2 cells after 48 h treatment with the different extracts (μg/mL).

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

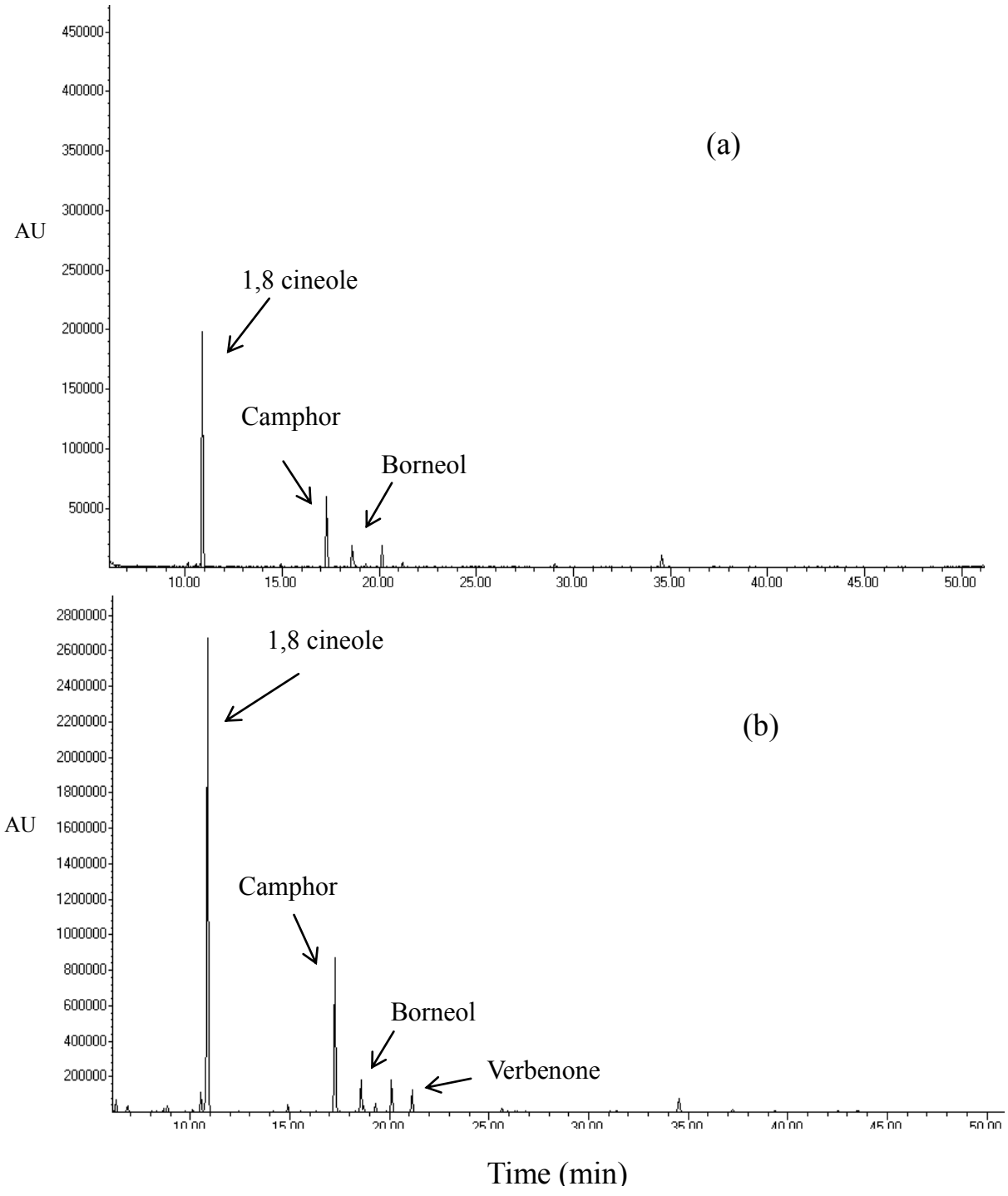

(a)
AU
1,8 cineole
Camphor
Borneol
(b)
AU
1,8 cineole
Camphor
Borneol
Verbenone
Time (min)

Figure 1.

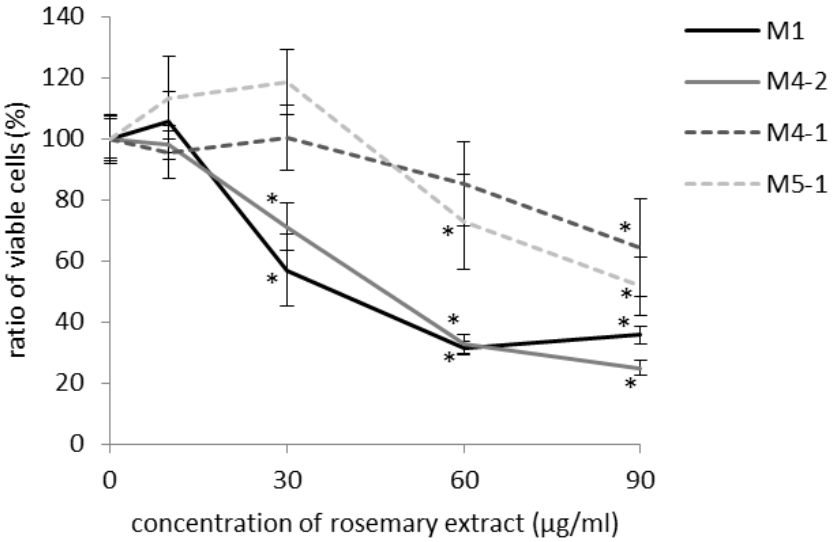

140
120
100
80
60
40
20
0
ratio of viable cells (%)
0
30
60
90
concentration of rosemary extract (μg/ml)
M1
M4-2
M4-1
M5-1

Figure 2.

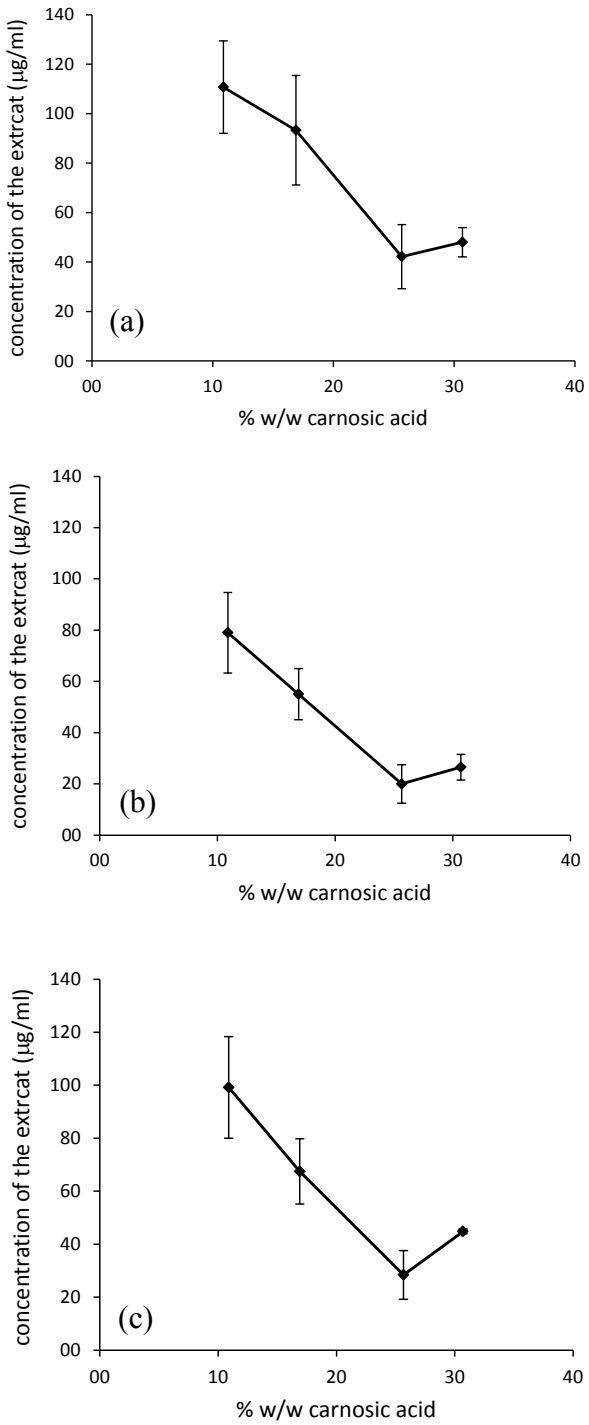
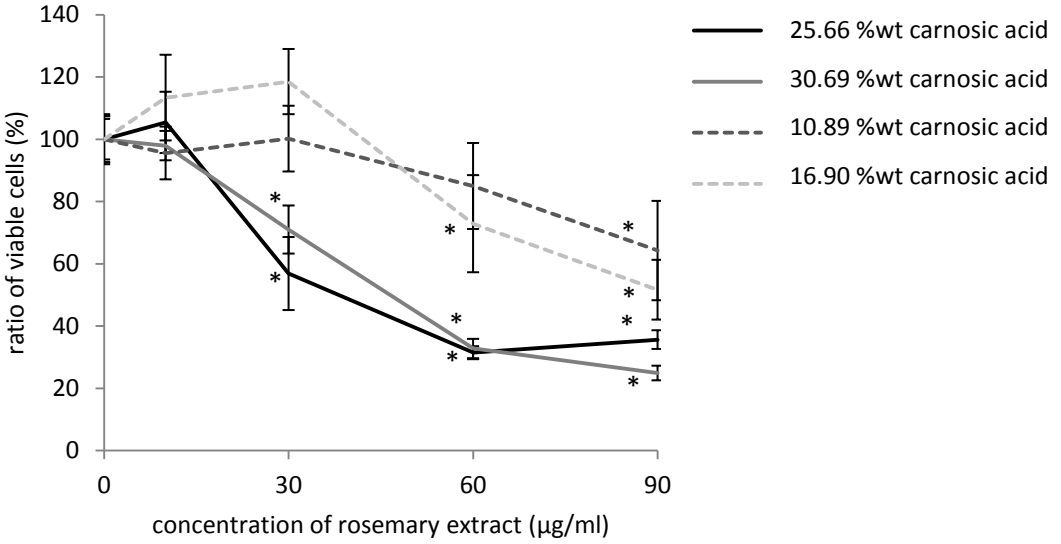

concentration of the extrcat (µg/ml)
140
120
100
80
60
40
20
00
(a)
00
10
20
30
40
% w/w carnosic acid
concentration of the extrcat (µg/ml)
140
120
100
80
60
40
20
00
(b)
00
10
20
30
40
% w/w carnosic acid
concentration of the extrcat (µg/ml)
140
120
100
80
60
40
20
00
(c)
00
10
20
30
40
% w/w carnosic acid

Figure 3.

***Graphical Abstract (for review)**



Highlights

- Production of different antioxidant supercritical rosemary extracts
- Their capability to inhibit the proliferation of human liver carcinoma cells
- Antioxidant content has a crucial effect on growth inhibition and cytostaticity
- Antitumoral activity might not be exclusively attributed to antioxidants content