Inhibitory Effects of Rosemary Extracts, Carnosic Acid and Rosmarinic Acid on the Growth of Various Human Cancer Cell Lines

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Abstract The leaves of Rosmarinus officinalis harvested from three different locations of Turkey were extracted by both methanolic and supercritical CO₂ extraction. Subsequently, six extracts and the active compounds, carnosic acid, and rosmarinic acid were applied to various human cancer cell lines including NCI-H82 (human, small cell lung, carcinoma), DU-145 (human, prostate, carcinoma), Hep-3B (human, black, liver, carcinoma, hepatocellular), K-562 (human chronic myeloid leukemia), MCF-7 (human, breast, adenocarcinoma), PC-3 (human, prostate, adenocarcinoma) and MDA-MB-231 (human, breast, adenocarcinoma) by MTT assay. Supercritical CO₂ extracts had superior antiproliferative effect compared to the soxhlet extracts. Although the extracts exhibited various cytotoxic effects against different cell lines, comparatively low IC₅₀ values ranging between 12.50 and 47.55 μg/ml were attained against K-562, being the most sensitive cell line. Moreover, carnosic acid caused the lowest cell viability with values ranging from 13 to 30 % at a concentration of 19 μM after 48 h of treatments, resulting in superior antiproliferative effect. Rosemary extract is a potential candidate to be included in the anti-cancer diet with pre-determined doses avoiding toxicity.

Keywords Rosmarinus officinalis · Carnosic acid · Rosmarinic acid · Anticarcinogen · Cytotoxicity · Supercritical CO₂ extraction

Introduction

Chemoprevention is the long term pharmacological management of disease risk. Several plants have been investigated for their potential anticarcinogenic properties. Rosemary (Rosmarinus officinalis L.) possesses phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, epi- and iso-rosmanol and the phenolic constituent, rosmarinic acid. Additionally, rosmanol, epi- and iso-rosmanol are considered to be minor components resulting from degradation of carnosic acid [1, 2].

Although, rosemary extracts have been widely used as a preserving agent in the food industry due to inherent high antioxidant activity, several studies indicate that rosemary extracts and their components inhibit both the initiation and tumor promotion stages of carcinogenesis in mice and rat models [3–6]. Singletary and Nelshoppen [6] showed that dietary supplementation with 0.5 % and 1.0 % rosemary extract inhibited total in vivo binding of 7,12-dimethylbenz[a]anthracene (DMBA) to mammary epithelial cell DNA by an average of 42 %. Huang et al. [5] evaluated the effects of a methanol extract of rosemary on tumor initiation and promotion in mouse skin, and showed that the application inhibited the covalent binding of benzo(a)pyrene (B(a)P) to epidermal DNA and inhibited tumor initiation by B(a)P and DMBA. Additionally, the effect of rosemary extract on cell-mediated immunity of young rats was investigated [7]. Offord and co-workers [8] demonstrated that rosemary extract inhibited the genotoxic effects of the lung procarcinogen B(a)P in human bronchial epithelial cells, BEAS-2B,
and that strong antioxidant components, carnosol and carnosic acid, were responsible for this effect. The study carried out by Singletary et al. [9] showed that carnosol can prevent DMBA-induced DNA damage and tumor formation in the rat mammary gland and thus, has potential to be used as a breast cancer chemopreventive agent. Offord et al. [10] studied also the chemoprotective effects of rosemary extract in human liver and bronchial cells and pointed out two mechanisms; one of which was inhibition of the metabolic activation pathway catalyzed by the phase I cytochrome P450 enzymes and the other was induction of the detoxification pathway catalyzed by the phase II enzymes, such as glutathione S-transferase. Another in-depth study was by Huang and co-workers [4], in which carnosol is shown to inhibit the invasion of highly metastatic mouse melanoma B16/F10 cells in vitro and concluded that carnosol targets MMP-mediated cellular events in cancer cells and provides a new mechanism for its anticancer activity. Additionally, Visanji et al. [11] presented that induction of G2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels.

The purpose of our study was to screen and compare anticarcinogenic activity of soxhlet and supercritical CO2 extracts of *Rosmarinus officinalis* (rosemary) harvested from different locations of Turkey, as well as their components, carnosic and rosmarinic acid versus NCI-H82 (human, small cell lung, carcinoma), K-562 (human chronic myeloid leukemia), MCF-7 (human, breast, adenocarcinoma), Hep-3B (human, black, liver, carcinoma, hepatocellular) PC-3 (human, prostate, adenocarcinoma), DU-145 (human, prostate, carcinoma) and MDA-MB-231 (human, breast, adenocarcinoma) cell lines.

**Materials and Methods**

**Plant Material**

*Rosmarinus officinalis* specimens were collected from three different locations namely, Canakkale (southern Marmara region, the coolest climate), Izmir (Aegean region, moderately hot) and Mersin (eastern Mediterranean region, the hottest) on September 2004 (I-S, C-S, M-S for soxhlet and C-SC, I-SC, M-SC for supercritical CO2 extracts). The specimens were dried at 30 °C and stored in the cold room (4 °C) of Ege University Science and Technology Center.

**Materials and Reagents**

The six extracts were dissolved in methanol to a final concentration of 100 mg/ml (1000X). Subsequent dilutions were made in culture medium. The same proportion of methanol/culture medium was added to the controls. The final methanol content was never above 0.1 %. MTT [3′-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] and etoposide (Catalog Number: E1383) were purchased from Sigma-Aldrich (St Louis, MO, USA). Rosmarinic acid (97 %) (lot no: 456976/1) was purchased from Fluka and carnosic acid (93 %) (A7781) was from A.G. Scientific. The HPLC grade organic solvents methanol and acetonitrile were purchased from Merck. All other chemicals were of analytical grade purity.

**Preparation of SFE Extracts**

The dried aerial parts were ground and then 100 g of ground rosemary were submitted to water distillation for 4 h using a Clevenger apparatus in order to remove volatile oils. After the hydro-distillation, the air-dried distillate was extracted by using supercritical CO2 extraction. An Isco (Isco Inc., Lincoln, NE, USA) supercritical fluid extractor was used to perform all the experiments. About 0.49 g of sample was placed into a 2.5 ml stainless steel cartridge. CO2 flow rate was kept between 2 and 3 ml/min at an average of 2.5 ml/min controlled using a valve. Finally, the sample was extracted at 350 bar and 100 °C, using 5 % of methanol as co-solvent. Extraction time was 5 min static extraction followed by 35 min dynamic extraction. Supercritical CO2 extracts were collected in vials and kept at −20 °C before use.

**Preparation of Methanolic Extracts**

The same distillates were also extracted with methanol using a Soxhlet apparatus for nine cycles [12], the solvents were evaporated using a rotary vacuum evaporator and the extracts were stored at −20 °C before use.

**Cell Culture**

The human cancer cells NCI-H82 (human, small cell lung, carcinoma), K-562 (human chronic myeloid leukemia), MCF-7 (human, breast, adenocarcinoma), Hep-3B (human, black, liver, carcinoma, hepatocellular), PC-3 (human, prostate, adenocarcinoma), DU-145 (human, prostate, carcinoma) and MDA-MB-231 (human, breast, adenocarcinoma) were obtained from American Cell Culture Collection (ATCC Manassas, VA, USA). The cells were cultured in RPMI 1640 or DMEM-Ham’s F12 supplemented with 10 % fetal bovine serum, L-glutamine, (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37 °C with 5 % CO2 in a humidified atmosphere.

**Cytotoxic Activity Assay**

The cytotoxic activities of the six extracts and etoposide (positive control, 250 μg/ml) on various cancer cells
were measured by MTT assay. Cells in exponential growth phase were placed in 96-wells plates to make 6,000 cells/wells and sample solutions were added at concentrations ranging from 6.25 to 100 μl/ml in each well, subsequently the cells were incubated for 48 h. Negative control was treated with 0.1 % methanol. Cell proliferation was determined by adding 0.5 mg/ml per well, prepared as a sterile stock-solution of 5 mg/ml in Dulbecco’s-phosphate buffered saline (Gibco, USA), diluted 1:10 with medium prior to use. Medium was removed 4 h later and blue formazan crystals dissolved in 200 μl 100 % dimethylsulfoxate (DMSO) per well. Quantities of blue formazan product were measured at 570–690 nm using a microplate reader (Versamax, Tunable Microplate Reader, USA). For the cells, strong correlations between numbers of cells present and amounts of MTT formazan product were observed. The data were obtained from three independent assays, using three wells for each assay. Cytotoxicity was determined according to percent cell viability.

Statistics

Statistical analyses of the data were performed by Student’s t-test. A probability value of $P \leq 0.05$ was considered to denote a statistically significant difference, and $P \leq 0.01$ was also used to show the power of the significance. Data are presented as mean values ± S.E.M. (standard error of the mean).

Results and Discussion

Rosemary extracts have been widely investigated for their ability to exert antioxidant activity. Several groups have studied the antiproliferative properties of the extracts and their active compounds [11, 13, 14] and protection against DNA damage [15]. Rosemary extracts contain several polyphenolic components, including carnosic acid, carnosol, and rosmarinic acid. In this study, we examined the inhibitory effects of soxhlet and supercritical CO$_2$ extracts of Rosmarinus officinalis harvested from different locations of Turkey and

Fig. 1 Dose-dependent cytotoxic activity of carnosic acid (a) and rosmarinic acid (b) on various tumor cells. Tumor cells were treated with 6.25, 12.5, 25 and 50 μg/ml doses of the compounds. “N.C” [cell + medium + 0.1 % MeOH] denotes negative control. Each cell type was incubated with the extracts for 48 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.
the active components, carnosic and rosmarinic acid in human leukemia, breast, prostate, lung and liver cells.

Inhibitory Effects of Carnosic and Rosmarinic Acid

Considering standard compounds, cytotoxic activities of carnosic acid (Fig. 1a) and rosmarinic acid (Fig. 1b) were analyzed by MTT assay. The cytotoxic effects in terms of cell death were determined by treating DU-145, PC-3, Hep-3B, K-562, MCF-7 and MDA-MB-231 cell lines with doses ranging between 6.25 and 100 μg/ml for 48 h.

Carnosic acid caused the lowest cell viability with values ranging from 13 to 30 % at a concentration of 6.25 μg/ml after 48 h of treatments, whereas rosmarinic acid showed

![Fig. 2 Dose-dependent cytotoxic activity of six extracts obtained from R. officinalis on various tumor cells (a- NCI-H82, b- DU-145, c- Hep-B, d- K-562, f- MCF-7). Tumor cells were treated with 6.25, 12.5, 25 and 50 μg/ml doses of the extracts. “N.C” [cell+medium+0.1 % MeOH] denotes negative control. Each cell type was incubated with the extracts for 48 h at 37 °C, and subjected to MTT assays to measure % cell viability](image)
almost no inhibitory effect at a concentration of 50 μg/ml with
values ranging from 98 to 205 %. Gigante and coworkers [16]
investigated the effect of carnosic acid on five human cancer
cell lines, including MCF-7, where the compound showed
50 % inhibition at 24.2 μM concentration, also in our study
70 % inhibition was attained at 19 μM. Carnosic acid
exhibited a 2-fold higher inhibition in estrogen independent
breast cancer cell line (MDA-MB-231) compared to the
estrogen dependent cell line (MCF-7) presenting a different
response in similar tissues. The mechanism of carnosic acid is
reported to be based on the induction of G2/M phase cell cycle
arrest during different stages of this phase, corresponding to
various effects of carnosic acid on cyclin levels [11]. However,
antiestrogens drugs work by competing with the hormone
estrogen to bind to the receptors in estrogen dependent cancer
cells. The drug slows the growth and reproduction of breast
cancer cells by blocking estrogen in the breast. Administration
of estrogen suppressing injections is becoming a common
practice. Comparatively, less non-steroidal treatments are also
available for estrogen independent aggressive cancer cell lines,
where carnosic acid can be a potential candidate for further
clinical trials. Steiner and coworkers [17] reported that
carnosic acid exhibited IC50 values of 6–7 μM in human
myeloid leukemia cells and concluded that carnosic acid was
capable of antiproliferative action in leukemic cells, which
supports our findings where a cell viability of 19 % was
observed at a concentration of 19 μM in K-562 cells.

Rosmarinic acid showed proliferative effects rather than
cytotoxic activity in almost all cell lines tested with the
highest effect in K-562 cells, exhibiting a cell viability of
205 % at 139 μM (50 μg/ml). Moon and coworkers [18]
also reported that RA alone exhibited little effect on the cell
viability in human leukemia cells. However, a combination
of TNF-α and RA induced apoptosis concluded that RA
significantly stimulates TNF-α induced apoptosis in non-
cell type specific manner.

Inhibitory Effects of *R. officinalis* Extracts

Cytotoxic activities of the rosemary extracts, together with
etoposide, were analyzed by MTT assay. The cytotoxic
effects in terms of cell death were determined by treating
H-82, DU-145, Hep-3B, K-562 and MCF-7 cell lines with
doses ranging between 6.25 and 100 μg/ml for 48 h.

Extracts showed a similar dose dependent inhibition
profile in terms of cell viability in NCI-H82 cells (Fig. 2a).
However, a significant difference was observed in terms of
extraction methods, where SFE extracts showed better
inhibitory activities compared to the soxhlet extracts. While
the most promising samples were C-SC and M-SC with
IC50 values of 24.08 μg/ml and 37.22 μg/ml in NCI-H82
cell line, the soxhlet counterparts had values of 47.34 μg/ml
and 94.92 μg/ml (Table 1).

All SFE extracts displayed better dose dependent activity
compared to the soxhlet extracts in DU-145 cell line as was
observed in NCI-H82 cell line. I-SC had the best cytotoxic
activity with 42 % cell viability at a concentration of
12.5 μg/ml (Fig. 2b) giving an IC50 value of 8.82 μg/ml,
which is 8.4 fold lower than the value of I-S (Table 1).
Referring to the contents of the extracts, I-SC sample
had a carnosic acid (CA) content of 50.04 mg/g extract
and no detectable amounts of rosmarinic acid (RA),
whereas, I-S sample had a CA content of 27.81 mg/g and
RA content of 20.39 mg/g [19, 20]. The 8.4 fold
difference can be attributable to the high amounts of CA
in I-SC and high amounts of RA in I-S, which might
exhibit the same proliferative effect as it demonstrated as a
pure agent.

The effects of extraction methods were not very
significant in Hep-3B cell line except for the Izmir harvest.
Canakkale and Mersin samples exhibited 13–16 % cell
viabilities at a concentration of 100 μg/ml (Fig. 2c). On the
other hand, I-S had an IC50 value of 96.86 μg/ml, whereas
I-SC showed increased cytotoxic activity, which lowered
the IC50 value to 30.27 μg/ml (Table 1).

The variations among the different harvesting loca-
tions were more prominent compared to the methods in
K-562 and MCF-7 cell lines with few exceptions
(Fig. 2d–e). Supercritical and soxhlet treated Mersin
harvests showed the highest cytotoxicity with IC50 values
of 12.50 and 17.63 μg/ml, respectively in K-562. Mersin
harvests had the highest carnosic acid and carnosol contents,

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC50 (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>C-SC</td>
</tr>
<tr>
<td>NCI-H82</td>
<td>24.08 (±0.01)</td>
</tr>
<tr>
<td>DU-145</td>
<td>80.82 (±4.29)</td>
</tr>
<tr>
<td>Hep-3B</td>
<td>22.88 (±0.70)</td>
</tr>
<tr>
<td>K-562</td>
<td>22.88 (±0.68)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>31.87 (±2.99)</td>
</tr>
</tbody>
</table>

Table 1 IC50 values of supercritical CO2 and soxhlet extracts
(M-SC, 111.08 mg/g, 18.43 mg/g; M-S, 115.76 mg/g, 25.47 mg/g, respectively) [19, 20], which are possibly responsible for the high cytotoxic activity in leukemia cell line. Dorrie and coworkers [21] reported carnosol responsible for the high cytotoxic activity in leukemia cell line. The findings confirm the superiority of supercritical CO2 extraction over solvent extraction yielding higher amounts of active compounds, particularly carnosic acid, which was 2-fold lower IC50 value than C-S (Table 1).

### Conclusion

The findings confirm the superiority of supercritical CO2 extraction over solvent extraction yielding higher amounts of active compounds, particularly carnosic acid, which was in turn reflected by the high antiproliferative effects. With a holistic approach, the most sensitive cell line was K-562. The samples showed comparatively low IC50 values ranging between 12.50 and 47.55 μg/ml against K-562. Although the extracts exhibited various cytotoxic effects against different cell lines, no correlation could be found between suspension (NCI-H82, K-562) and solid (DU-145, Hep-3B, MCF-7) tumor lines.

Considering our results along with the preliminary studies, we suggest that carnosic acid alone or in combination with the anticancer drugs may offer a good strategy for the treatment of a variety of human cancers that are resistant to chemotherapy.

### Acknowledgements

This project was supported by the Scientific and Technical Research Council of Turkey (TBAG-2479 (104T078)).

### References