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ORIGINAL CONTRIBUTION

Anti-angiogenic properties of carnosol and carnosic acid, two major dietary compounds from rosemary

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Abstract
Background The use of rosemary (*Rosmarinus officinalis*) leaves and their constituents as a source of dietary antioxidants and flavoring agents is continuously growing. Carnosol and carnosic acid, two major components of rosemary extracts, have shown activity for cancer prevention and therapy.
Aim of the study In this study, we investigate the cytotoxic and anti-angiogenic activities of carnosol and carnosic acid, in order to get further insight into their mechanism of action.
Results Our results demonstrate that the mentioned diterpenes inhibit certain functions of endothelial cells, namely, differentiation, proliferation, migration and proteolytic capability. Our data indicate that their growth inhibitory effect, exerted on proliferative endothelial and tumor cells, could be due to, at least in part, an induction of apoptosis. Inhibition of the mentioned essential steps of in vitro angiogenesis agrees with the observed inhibition of the in vivo angiogenesis, substantiated by using the chick chorioallantoic membrane assay.
Conclusions The anti-angiogenic activity of carnosol and carnosic acid could contribute to the chemopreventive, antitumoral and antimetastatic activities of rosemary extracts and suggests their potential in the treatment of other angiogenesis-related malignancies.

Keywords Angiogenesis · Carnosic acid · Carnosol · Rosemary · Chemoprevention

Auxiliadora López-Jiménez and Melissa García-Caballero contributed equally to this work.

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Introduction

Rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world. Rosemary leaves are often used as spices and flavoring agents. Because of the desirable flavor and antioxidant activity of the dried leaves of the plant, they are widely used in food processing, including the preparations of meats, sausages, soups, salads or potato chips, among others. Recently, rosemary extracts have been adopted into European Union legislation, allowing food companies to use the label, "antioxidant: rosemary extract". In all probability, directives 2010/67/EU and 2010/69/EU will impulse the use of rosemary extracts in the market as a safe, effective and natural alternative to synthetic antioxidants, in response to consumers' demand for "natural" or "organic" foods [1, 2].

There is also an increasing interest in the pharmaceutical properties of rosemary, being used in traditional medicine to improve memory and relieve pain, or for its antimicrobial, hepatoprotective, anti-inflammatory, antitumorigenic or chemopreventive activity [3–13]. However, the literature concerning the mechanisms of the rosemary constituents in preventing cancer is still limited.

Phenolic compounds are responsible for many of the biological activities of *R. officinalis*. Carnosic acid (CA)

and carnosol (CS) (Fig. 1) are the major phenolic constituents in fresh, fresh dried and dried leaves from the plant, and they are believed to be the main responsible of their antioxidant, anti-inflammatory and cytotoxic properties [10, 14–17]. CA is the major phenolic diterpene derived from rosemary and has been reported to have chemopreventive, antioxidant, antimicrobial, antiobesity, antiplatelet and antitumor activities, as well as inhibitory effects on the anticancer drug efflux transporter P-glycoprotein [4, 12, 18–21]. CA inhibits cytokine-induced adhesion molecules expression and monocyte adhesion to endothelial cells through a mechanism that involves NFκB, what could be involved in the anti-inflammatory properties of this compound [22]. CA may undergo an oxidative degradation and rearrangement cascade, giving rise to other rosemary antioxidant compounds such as CS, rosmanol, galdosol and rosmariquinone. CS biological activities resemble those of CA, having been described as an antioxidant, anti-inflammatory and anticancer agent (recently reviewed in [23]).

Angiogenesis, the process of formation of new blood vessels from other pre-existing ones, is a highly regulated process, limited in the adult to some processes related to reproductive cycles, wound healing and bone repair. Nevertheless, a deregulated and persistent activation of angiogenesis is related to diseases such as proliferative retinopathies, psoriasis and rheumatoid arthritis and is essential for tumor growth and metastasis [24]. Angiogenesis is considered to be a hallmark of cancer, playing a pivotal role in the progression and malignization of tumors [25]. For these reasons, the search of new inhibitors of angiogenesis is a hot topic in the field of pharmacological research, with hundreds of thousands of patients benefited from the clinical use of inhibitors of angiogenesis, and a continuously increasing number of anti-angiogenic therapies gaining approval, not only for cancer, but also for a number of non-neoplasic angiogenesis-related diseases [26]. Yet, the clinical results have shown up several limitations of these therapeutic strategies, reinforcing the need of new multitargeted anti-angiogenic therapies [27, 28].

The main responsible for the new vessels formation in angiogenesis are endothelial cells that, in response to the angiogenic stimulus, become activated and undergo a series of phenotypic changes leading them to release proteases that will allow them to degrade the extracellular matrix, migrate, proliferate, avoid apoptosis that could be triggered by the loss of survival signals and, finally, differentiate to form new vessels. Any of these steps could be a target for the pharmacological inhibition of angiogenesis [29]. Our group is actively involved in the identification of angiogenesis modulators from natural sources and the characterization of their effect on the different steps of angiogenesis [30–34]. In this report, we describe for the first time that CA and CS are potent inhibitors of in vitro and in vivo angiogenesis. Our data indicate that they both inhibit different relevant steps of the angiogenic process, suggest their potential application in the treatment of cancer and other angiogenesis-related diseases, and reinforce the chemopreventive potential of those compounds and that of rosemary preparations.

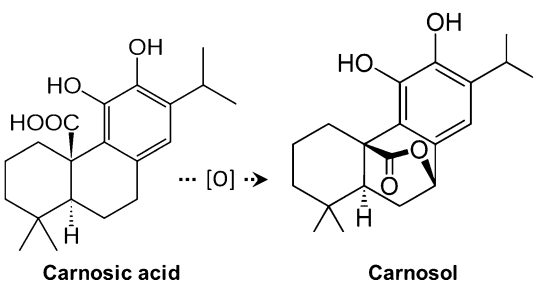

Fig. 1 Chemical structure of carnosic acid and its oxidation product carnosol

Materials and methods

Materials

Cell culture media were purchased from Biowhittaker (Walkersville, MD, USA). Fetal bovine serum was a product of Harlan-Seralab (Belton, UK). Matrigel was purchased from Becton–Dickinson (Bedford, MA, USA). CS and CA were purchased from ChromaDex (Irvine, CA, USA). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark).

Cell culture

BAECs were isolated from bovine aortic arches as previously described [33] and maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/L) and amphotericin (1.25 mg/L) supplemented with 10% fetal bovine serum. HUVECs were isolated from human umbilical cords by collagenase digestion [35] and maintained in 199 medium containing glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/L) and amphotericin (1.25 mg/L) supplemented with 20% fetal bovine serum. Human fibrosarcoma cells HT-1080 were obtained from the American Tissue Cell Collection (ATCC) and maintained in DMEM containing glucose (4.5 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/L) and amphotericin (1.25 mg/L) supplemented with 10% fetal bovine serum. Human promyelocytic leukemia HL60 cells were maintained in

RPMI1640 medium containing glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/L) and amphotericin (1.25 mg/L) supplemented with 20% fetal bovine serum.

Cell growth assay

The MTT dye reduction assay in 96-well microplates was used, as previously described by us [30, 36 and Online Resource 1]. 3×10^3 cells in a total volume of 100 µl of complete medium were incubated for 3 days with serial dilutions of the compound, and IC50 values were calculated as those concentrations of compounds yielding 50% cell survival, taking the values obtained for control as 100%.

Apoptosis assays

Apoptosis induction was assessed after treatments for 14 h with the indicated concentrations of CS or CA in complete medium by Hoechst staining, cell cycle analysis, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) assay and caspase-3/7 activity experiments, as described by us elsewhere [34 and Online Resource 1].

Tube formation on Matrigel by endothelial cells

Matrigel (50 µl of about 10.5 mg/mL) at 4 °C was used to coat each well of a 96-well plate and allowed to polymerize at 37 °C for a minimum of 30 min. 5×10^4 cells were added with 200 µl of complete medium. Finally, different amounts of CS or CA were added and incubated at 37 °C in a humidified chamber with 5% CO_2. After 7 h of incubation, cultures were observed and photographed with a NIKON inverted microscope DIAPHOT-TMD (NIKON Corp., Tokyo, Japan). Each concentration was tested in duplicate, and two different observers evaluated the inhibition of tube formation (Online Resource 1).

Endothelial cell migration assay

The migratory activity of BAEC was assessed using a wounding migration assay as previously described [34 and Online Resource 1]. The amount of migration at 4 h was determined by image analysis in both controls and treated wells and normalized with respect to their respective values at zero time, using the NIH Image 1.6 software.

Gelatinolytic assay

BAE cells were grown at 75% subconfluency in 6-well plates, medium was aspirated, cells were washed twice with phosphate-buffered saline (PBS) and each well received 1.5 ml of DMEM/0.1% BSA containing 200 KIU of Aprotinin/mL. Additionally, some wells received the indicated concentration of CS or CA. After 24 h of incubation, conditioned media were collected. The cells were washed twice with PBS and harvested by scrapping into 0.5 mL of 0.2% Triton X-100 in 0.1 M Tris/HCl containing 200 KIU of Trasylol/mL. Media and cell lysates were centrifuged at $1{,}000 \times g$ and 4 °C for 20 min. Afterward, the supernatants were collected and used for gelatin zymography as previously described by us [30 and Online Resource 1]. Duplicates were used to determine cell number by using a Coulter counter.

In vivo chorioallantoic membrane assay

Fertilized chick eggs were incubated horizontally at 38 °C in a humidified incubator, windowed by day 3 of incubation and processed by day 8. The indicated amount of CS or CA was added to a 1.2% solution of methylcellulose in water, and 10 µl drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood. Then, the methylcellulose disks were implanted on the CAM, and the eggs were sealed with adhesive tape and returned to the incubator for 48 h. Negative controls were always made with DMSO mixed with the methylcellulose. After the re-incubation, the CAM was examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area (Online Resource 1).

In vitro VEGFR2 kinase inhibition assay

VEGFR2 inhibition assay was performed using an HTScan® VEGFR2 kinase kit (Cell Signaling Technology, USA) combined with colorimetric ELISA detection [34 and Online Resource 1]. The kinase assay was repeated twice.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Comparisons of two groups were performed with the two-sided Student's t test. Values of $p < 0.05$ were considered to be statistically significant.

Results

Carnosol and carnosic acid inhibit the growth of endothelial and tumor cells

We investigated the ability of CS or CA to inhibit the proliferation of actively growing endothelial and tumor

cells. IC50 values of this growth inhibitory effect were 50.0 ± 4.9 and 36.3 ± 2.5 μM for CS and CA, respectively, in BAEC, and IC50 = 35.2 ± 5.0 and 36.0 ± 1.1 μM for CS and CA, respectively, in HUVEC (all expressed as means of 3 different experiments ± SD). Data obtained with HL60 leukemia cell line were IC50 = 5.3 ± 0.6 and 5.7 ± 0.7 μM for CS and CA, respectively, and for HT1080 fibrosarcoma cell line, values were obtained of IC50 = 6.6 ± 2.3 μM for CS and 9.0 ± 4.8 μM for CA (means of 3 different experiments ± SD). According to these data, both rosemary diterpenes showed a higher inhibitory activity of on tumor cell growth in comparison with that exerted on non-transformed endothelial cells (Fig. 2a, b).

Carnosol and carnosic acid induce apoptosis in endothelial and tumor cells in vitro

As a first approach to determine whether the growth inhibitory activity of CS or CA could be, at least in part, due to the induction of apoptosis, nuclear morphology was investigated after treatment of endothelial and tumor cells with different concentrations of these compound. Figure 3a shows that concentrations above 10 μM of CS or CA induced chromatin condensation in endothelial (BAE and HUVE) and tumor (HL60 and HT1080) cells, suggesting that both compounds could induce cellular apoptosis. Similar results were obtained by using TUNEL assay to detect the DNA fragmentation induced by the compounds (Fig. 3b).

Flow cytometric analysis of cell cycle after incubation with different doses of the compounds was performed after propidium iodide staining. Results showed that CS or CA significantly increased apoptotic sub-G1 cells in a concentration-dependent manner (Fig. 3c).

To determine whether caspases were activated as a result of the treatment with the rosemary diterpenes, we used a caspase-3/7 substrate DEVD-AMC, which is cleaved to a fluorescent product by caspase-3 and other caspases with similar substrate cleavage sequences. As shown in Fig. 4, the "effector caspase-3" was significantly activated in a dose-dependent pattern in HL60 leukemia cells after treatment with CS or CA. This effect was not so clearly observed in the other cell types. As a positive control of caspase activation, 10 μM 2-methoxyestradiol (2-ME) was used.

Carnosol and carnosic acid inhibit the capillary tube formation by endothelial cells

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. As shown in Fig. 5, a complete inhibition of this endothelial alignment on Matrigel was observed after incubation with 10 μM CS. CA completely inhibited endothelial morphogenesis on Matrigel of BAEC and HUVEC at 10 and 25 μM, respectively (with partial inhibition in HUVEC at 10 μM). The concentrations of compounds required to inhibit the differentiation of BAEC and HUVEC did not affect their viability after 7 h (results not shown).

Carnosol and carnosic acid inhibit the migratory capability of endothelial cells

Angiogenesis involves the acquisition by endothelial cells of the capability to migrate through extracellular matrix. To investigate the effect of CS or CA on endothelial cell migration, a wound-healing assay with BAE and HUVE cells was used. As shown in Fig. 6, both compounds produced a dose-dependent inhibition of the migratory capability of endothelial cells.

Carnosol and carnosic acid induce a decrease in the endothelial cells MMP-2 activity

The capability to degrade the basement membrane and, in general, to remodel the extracellular matrix is essential in

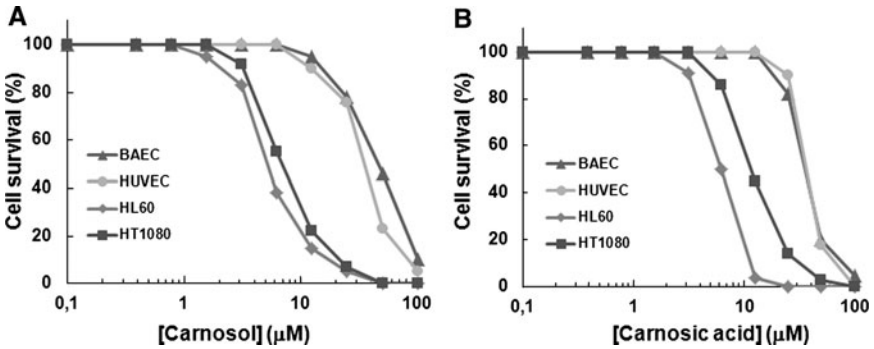

Fig. 2 Carnosol and carnosic acid inhibit endothelial and tumor cell growth. Dose-dependent effect of CS (**a**) or CA (**b**) on the in vitro growth of BAE (*filled triangle*), HUVE (*filled circle*), HL60 (*filled diamond*) and HT1080 (*filled square*) cells. Cell survival is represented as a percentage of control-cell growth in cultures containing no drug. *Each point* represents the mean of quadruplicates; SD values were typically lower than 10% of the mean values and are omitted for clarity

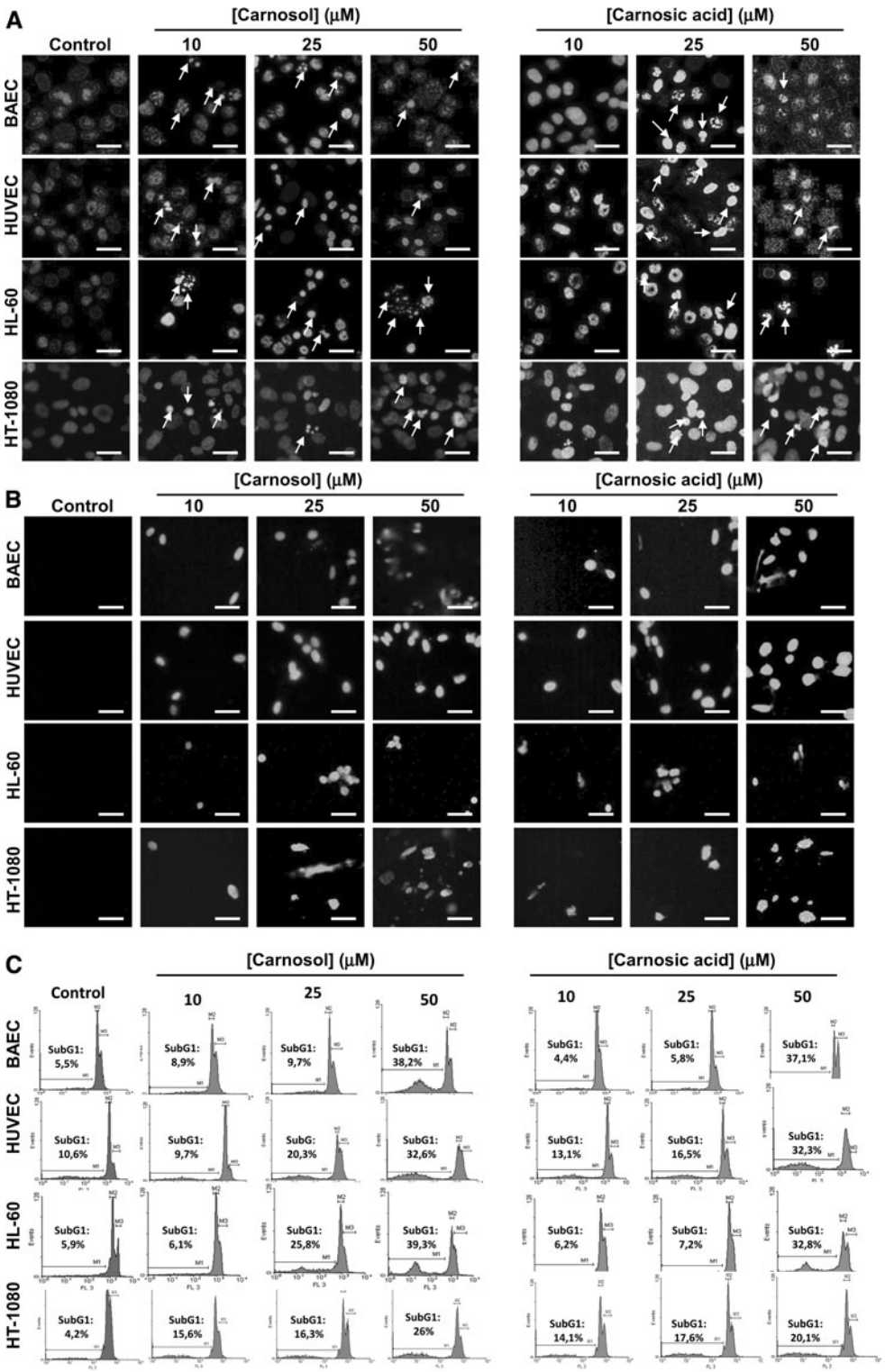

Fig. 3 Induction of apoptosis by carnosol and carnosic acid in endothelial and tumor cells. **a** Effect of CS and CA on cell morphology (Hoechst staining) (*bar* 50 μm). **b** Effect of CS and CA on DNA fragmentation (TUNEL assay) (*bar* 50 μm). **c** Effect of CS and CA on endothelial cell cycle distribution. Percentages of sub-G1 cell populations, determined using a MoFlo Dako Cytomation cytometer, are indicated. Representative of one of two experiments done with superimposable results

angiogenesis. Gelatin zymographies of conditioned media and cell extracts of BAEC, untreated and treated for 24 h with the indicated concentrations of CS or CA (Fig. 7a), show that the concentrations of MMP-2 in the cell lysates or in the medium conditioned by the rosemary-compound-treated cells are clearly lower than those of untreated cells. Quantitative analyses show a dose-dependent significant inhibition of the MMP-2 activity in both, the cell lysates and in the medium conditioned by CS- or CA-treated cells (Fig. 7b).

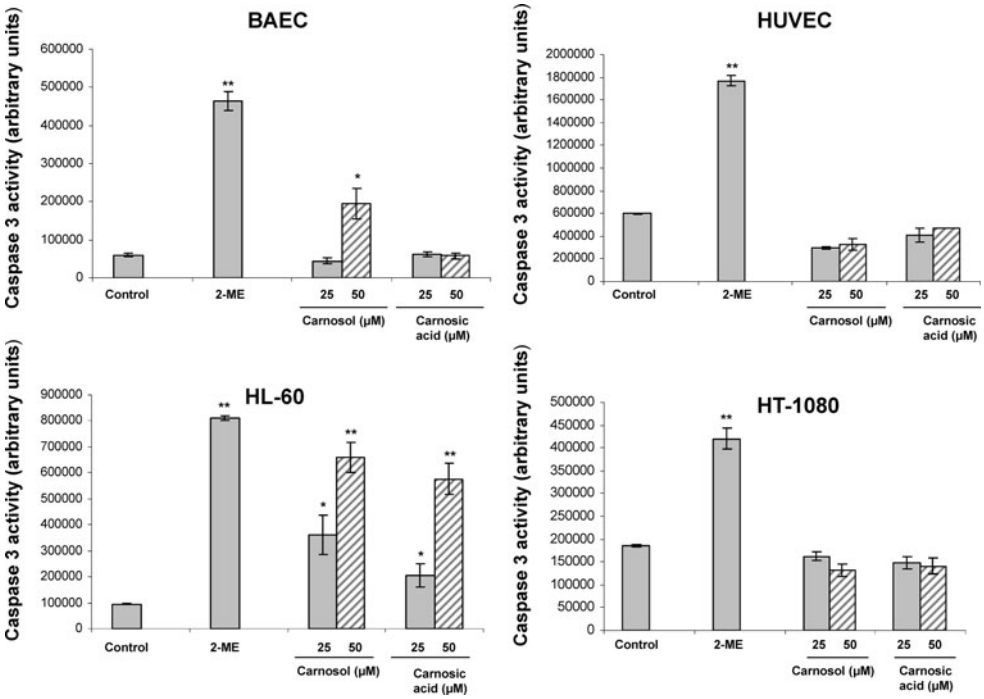

Fig. 4 Effect of carnosol and carnosic acid on the endothelial and tumor cells caspase-3-like activity. Cells were plated in 96-well plates and treated with the indicated concentrations of CS or CA or 2-methoxyestradiol (2-ME) for 14 h. Then, caspase-3/7 reagent was added to wells according to the manufacturer's instructions, and the luminescence was recorded at 30 min with a microplate luminometer. Results are expressed as mean ± SD, $*p < 0.05$;$**p < 0.001$ *versus* untreated control ($n = 3$)

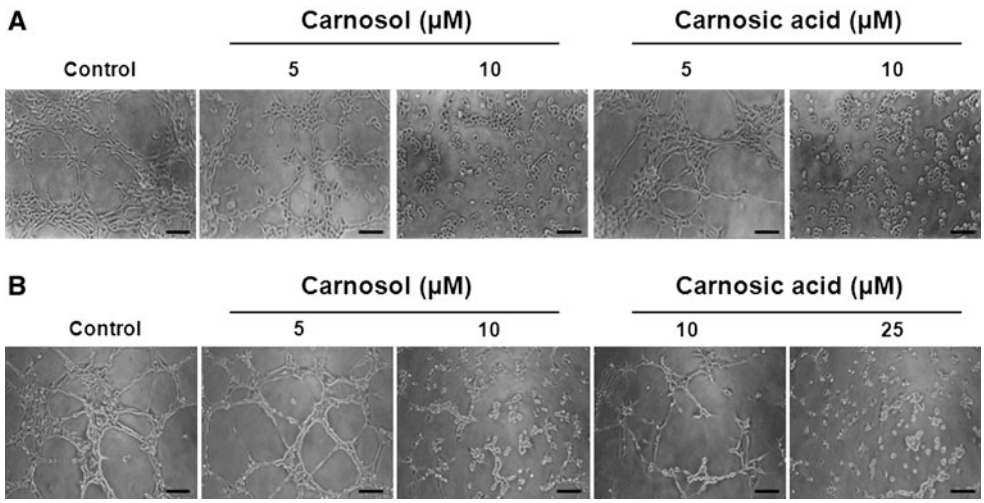

Fig. 5 Carnosol and carnosic acid inhibit endothelial cell tube formation. BAEC (**a**) and HUVEC (**b**) seeded on Matrigel formed tubes (*left panels*). CS and CA inhibited endothelial cell tubulogenesis in vitro in a dose-dependent manner at non-toxic doses. Cells were photographed 7 h after seeding under an inverted microscope (*bar* 100 μm)

Carnosol and carnosic acid do not inhibit the kinase activity of VEGFR2

Vascular endothelial growth factor receptor 2 (VEGFR2, KDR, Flk-1) is a major receptor for VEGF-induced signaling in endothelial cells. Upon ligand binding, VEGFR2 undergoes autophosphorylation and becomes activated. Since VEGFR2 kinase is the molecular target of a number of clinically successful angiogenesis inhibitors [26], we explored the effect of the rosemary diterpenes on this enzymatic activity by means of an in vitro assay that measures directly the enzymatic activity of the human recombinant VEGFR2 kinase on a biotinylated substrate. Our results showed that incubation with either CS or CA at a concentration of 100 μM did not exert any effect on the in vitro kinase activity of a human recombinant VEGFR2 (results not shown).

Carnosol and carnosic acid inhibit in vivo angiogenesis in the chick chorioallantoic membrane assay

The CAM assay was used to determine the ability of the compounds to inhibit angiogenesis in vivo. In controls, blood vessels formed a dense and spatially oriented,

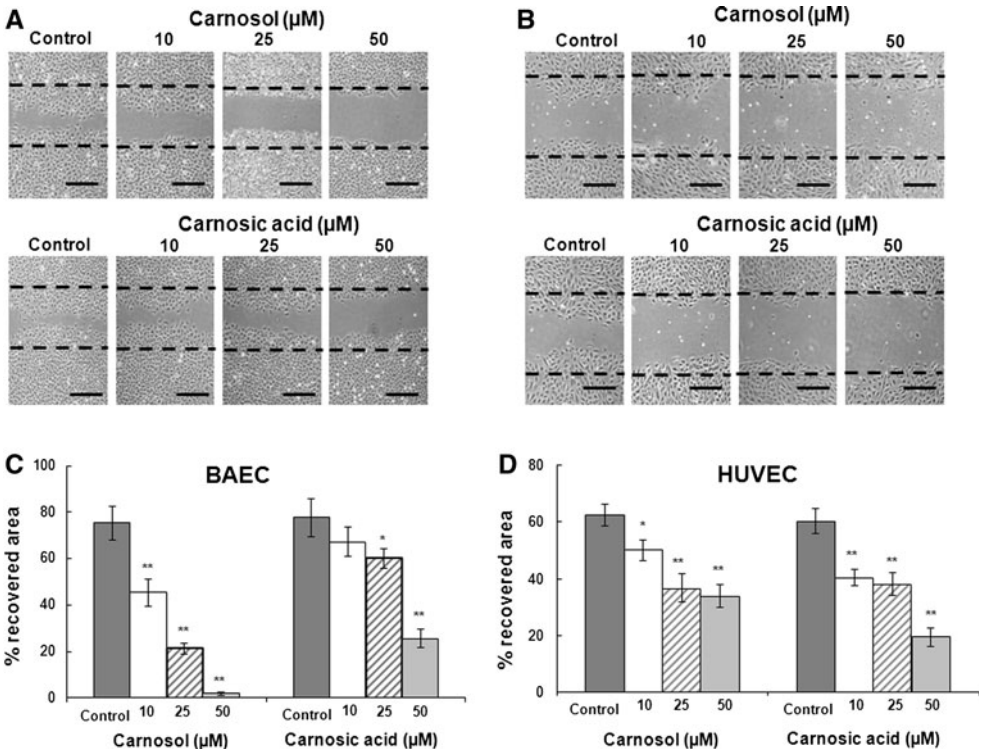

Fig. 6 Effect of carnosol and carnosic acid on endothelial cell migration in vitro. **a** Confluent BAEC or **b** HUVEC monolayers were wounded, and fresh culture medium was added either in the absence or in the presence of the indicated concentrations of CS or CA (*bar* 200 μm). Photographs were taken at the beginning of the assay and after 4 h of incubation. *Broken lines* indicate the wound edges. The regrowth of **c** BAEC or **d** HUVEC into the cell-free area was measured after 4 h, and percentages of recovered area are expressed as mean ± SD, $*p < 0.05$;$**p < 0.001$ *versus* control ($n = 3$)

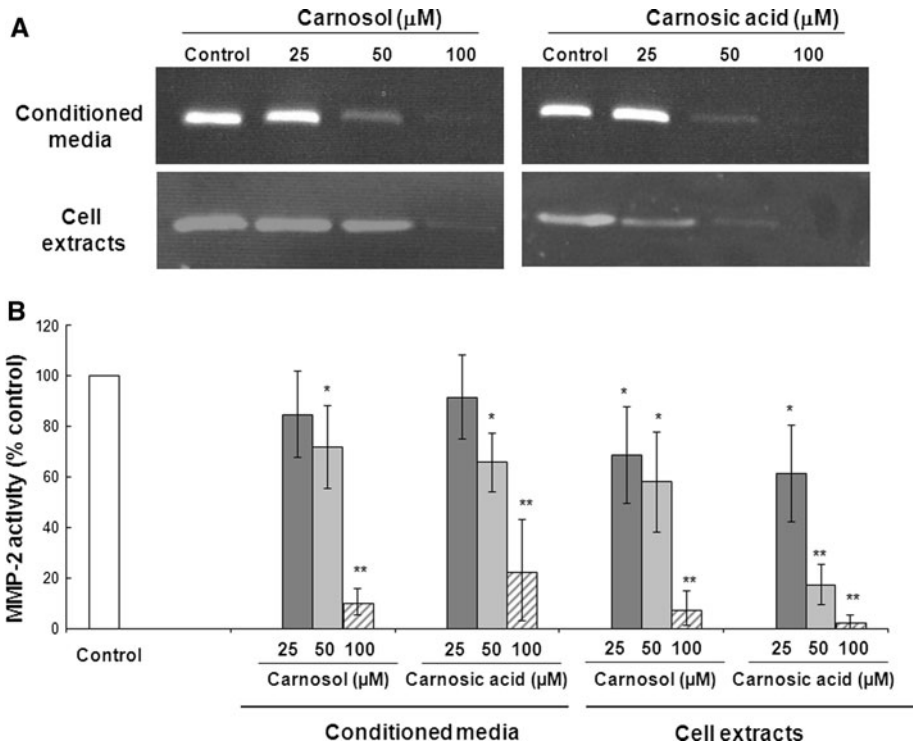

Fig. 7 Carnosol and carnosic acid inhibit endothelial cell MMP-2 activity. **a** Conditioned media and cell extracts from BAEC treated during 24 h with the indicated concentrations of CS or CA were normalized for equal cell density and used for gelatin zymography as indicated in "Materials and methods". **b** Quantification of the normalized relative inhibitory effect. *Data* are given as percentage of the untreated control, and they are means ± SD of three experimental values. $*p < 0.05$;$**p < 0.001$ *versus* control

leaf-like branching network composed by vascular structures of progressively smaller diameter as they branch (Fig. 8a). Table 1 summarizes the evaluation of the in vivo inhibition of angiogenesis in the CAM assay by CS or CA. As shown in that table, treatment with CS caused a dose-dependent anti-angiogenic effect, which is maintained as low as 5 nmol per CAM, where 40% of the eggs scored positive. CA exhibited a higher activity in the CAM assay, with 100% total inhibition at 5 nmol/CAM. Anti-angiogenic effects of CS or CA were observed as an inhibition of

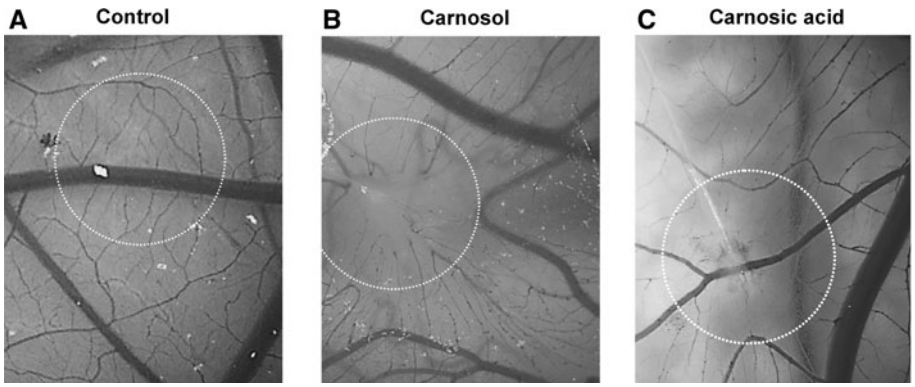

Fig. 8 Inhibition of in vivo angiogenesis by carnosol and carnosic acid. Chorioallantoic membrane assay of the rosemary compounds was performed as described in the "Materials and methods" section. **a** Methylcellulose disk containing the substance vehicle alone. **b** Methylcellulose disk containing 5 nmol of CS. **c** Methylcellulose disk containing 5 nmol of CA. *Circles* show the locations of the methyl cellulose disks

Table 1 Inhibition of in vivo angiogenesis by carnosol and carnosic acid

	Dose (nmol/CAM)	Positive/total	% inhibition
Carnosol	0	0/20	0
	1.25	0/4	0
	2.5	1/4	25
	5	2/5	40
	10	4/7	63
	30	4/6	77
	50	5/6	95
Carnosic acid	0	0/20	0
	1.25	1/4	25
	2.5	4/6	77
	5	6/6	100
	10	5/5	100

In vivo chorioallantoic membrane assay was carried out with different doses of CS or CA, as described in the "Materials and methods" section. Data are given as percentage of eggs with inhibited angiogenesis in their CAMs per total number of treated egg CAMs

the ingrowth of new vessels in the area covered by the methylcellulose disks and a centrifugal growth of the peripheral vessels (relative to the position of the disk), which seemed to avoid the treated area, where a decreased vascular density could be observed (Fig. 8b, c).

Discussion

Preventive therapy of cancer is the long-term pharmacological management of the disease. Several plants and their constituents have been investigated for their chemopreventive potential, in many cases due to the regulation of signaling pathways such as nuclear factor-*k*B (NF-*k*B), activator protein-1 (AP-1) or mitogen-activated protein kinases (MAPK) [37–40]. Chemoprevention of cancer by phytochemicals aims to block one or more steps in the process of carcinogenesis: initiation, promotion and progression. Early experimental evidences suggested that rosemary extracts or their constituents might exert their antitumorogenic activity by a multistage inhibition of those steps [9]. Our results reinforce this hypothesis, adding evidences of the tumor growth inhibitory activity of CS or CA, and demonstrating for the first time that they are potent inhibitor of angiogenesis in vitro and in vivo.

Firstly, we tested the effect of CS or CA on the growth of tumor and endothelial cells. Our results show that both compounds are more active on tumor cells than in non-transformed endothelial cells, suggesting a preferential effect on highly proliferant tumor cells. This is in agreement with the previously observed anticancer activity of those compounds in other tumor cell lines and reinforces their potential as antitumor agents [12, 38, 41].

Apoptosis is associated with characteristic morphological changes, including chromatin condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing and the formation of apoptotic bodies. Our studies on the nuclear morphology of CS and CA-treated cells revealed that they induce nuclear changes, characterized by chromatin condensation and nuclear fragmentation. Those results were confirmed by TUNEL assay, showing DNA fragmentation and by monitoring of the cell cycle distribution, showing an increase in the percentage of cells with sub-diploid DNA content.

Therefore, results here obtained show that the growth inhibitory effect produced by CS and CA on proliferant cells could be due to, at least in part, an induction of apoptosis and are in agreement with pro-apoptotic activity previously described for CS in acute lymphoblastic leukemia (ALL), prostate cancer lines, and for CA in acute myeloid leukemia (AML) cell lines [38, 41, 42].

Measurement of the activity of the effector caspase-3 showed an activation of the caspase proteolytic cascade after treatment with CS or CA in leukemia (HL60) cells, what is in agreement with previously reported data [42]. Nevertheless, our results did not clearly indicate that caspase-3 activity is induced by CS or CA treatment neither in endothelial cells (BAEC or HUVEC), nor in human fibrosarcoma HT1080 cells, suggesting that caspase-independent mechanisms could play a role in the apoptosis induction within those cells [43, 44]. Further works are required to determine whether this phenomenon occurred and to elucidate the underpinning mechanism(s) responsible for the observed apoptosis in CS- and CA-treated cells.

The induction of endothelial apoptosis is a common mechanism exhibited by a number of endogenous and exogenous angiogenesis inhibitors and has been postulated to contribute to the anti-angiogenic potential of these compounds [30, 45]. This anti-angiogenic activity was confirmed by in vivo CAM assay, showing that CS and CA induced morphological features in the CAM neovascularization (including an overall decrease in the vascular density and centrifugal growth of the peripheral vessels avoiding the treated area), similar to those reported for other inhibitors of angiogenesis, and exerted at similar or much lower concentrations of the compounds [30, 31, 34, 46]. The observed inhibition of angiogenesis in vivo by CS and CA could not be explained by a direct inhibition of the tyrosine kinase activity of the vascular endothelial growth factor receptor VEGFR2, since no effect was observed after in vitro incubation of the recombinant VEGFR2 tyrosine kinase with 100 μM of any of them.

A number of in vitro assays were used to characterize the effect of the compounds on the different steps involved in angiogenesis. Our results show that both compounds inhibit in a dose-dependent fashion certain functions of endothelial cells, namely, differentiation, proliferation and migration. CS and CA inhibited capillary-like tube formation by BAEC or HUVEC at concentrations that are lower than those reported for other inhibitors of angiogenesis, including ursolic acid and rosmarinic acid, two natural products found, although at lower concentrations, in *R. officinalis* [46–48]. Migration of endothelial cells is another key step of the angiogenic process [49]. Our results show that CS and CA produced a significant inhibition of the migratory capability of endothelial cells in a dose-dependent manner.

Inhibition of the endothelial cells' differentiation and migration was exerted at concentrations that were lower than their respective IC50 values in the MTT assay, what suggest that the anti-angiogenic activity of CS and CA was dependent on prevention of capillary-like tube formation or endothelial migration rather than proliferation or cell death.

Several lines of evidence support an important function of matrix metalloproteinases (MMPs) in angiogenic or lymphangiogenic processes [50]. Endothelial cells constitutively secrete MMP-2, which is required to trigger tumor angiogenesis in vitro and in vivo [51, 52]. Our data show that incubation with either CS or CA inhibits MMP-2 secretion by BAEC. Given that proteolytic degradation of extracellular matrix components by MMP-2 is critical for angiogenesis, the inhibition of MMP-2 secretion could contribute to the anti-angiogenic effect of the mentioned compounds. Similar decreases in the secreted MMP-2 activity have been described for endothelial cells treated with other anti-angiogenic natural products, including curcumin, halofuginone or aeroplysinin-1, being suggested to play a role in the inhibition of the endothelial cell tube formation or endothelial cell migration required for angiogenesis [30, 53, 54].

All these data reinforce the potential of the popular and world widely used *R officinalis* and its constituents, in the chemoprevention and treatment of cancer. On the one hand, CS and CA exhibit anticancer properties, derived from the direct inhibition of tumor cell growth. On the other hand, these compounds inhibit angiogenesis in vitro and in vivo, affecting several steps of the angiogenesis process, namely endothelial proliferation, migration, differentiation and proteolytic capability. The anti-angiogenic activity of these diterpenes could contribute to the chemopreventive, antitumoral and antimetastatic activities of rosemary extracts and their constituents and suggests their potential in therapeutic applications for the treatment of angiogenesis-related malignancies. Further experiments examining the effects of CS and CA on tumor growth and angiogenesis in vivo are warranted.

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Conflict of interest The authors state no conflict of interest.

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