

Rosmarinus officinalis Extract Inhibits Human Melanoma Cell Growth

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Rosmarinus officinalis L. is receiving increasing attention due to its anti-inflammatory and antioxidative constituents. Our recent studies showed that *R. officinalis* extract, containing 31.7 % of carnosic acid, was able to counteract the deleterious effects of UV-R, by protecting plasmid DNA from hydroxyl radicals generated by UV-A. In this work, we evaluated the effects of this extract on pBR322 DNA cleavage induced by nitric oxide, and the growth inhibitory activity against two human melanoma cell lines, M14 and A375. The extract showed a protective effect on plasmid DNA damage, and at concentrations of 10-80 µg/mL was able to reduce significantly ($p < 0.001$) the growth (MTT assay) of both melanoma cell lines. In addition, our results indicate that apoptotic cell demise is induced in M14 and A375 cells. No statistically significant increase in LDH release was observed in melanoma cells, correlated to a fragmentation of genomic DNA, determined by COMET assay.

Keywords: *Rosmarinus officinalis* L., nitric oxide, melanoma, cancer cells, DNA damage.

UV-R is considered to be the major etiological factor in skin cancer. In humans, both UV-B (280–320 nm) and UV-A (320–400 nm) can cause gene mutations and suppress immunity. These biological events can lead to skin cancer, including melanoma. Melanoma is an aggressive, therapy-resistant malignancy of melanocytes [1]. Of the major forms of skin cancer, it carries the highest risk of mortality from metastasis. It is, therefore, of primary interest to search for new therapeutic agents that are able to prevent and contrast this aggressive tumor. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by both UV-A and UV-B contribute to inflammation, immunosuppression, gene mutation and carcinogenesis. Therefore, substances able to inhibit these reactive species could be used in the prevention of skin cancer, including melanoma [1].

Rosmarinus officinalis L. (rosemary) is a typical Mediterranean species, but is now cultivated all over the world. Usually the plant is clonally propagated because of the poor germinability of its seeds and the genetic diversity of the seedlings [2a]. *R. officinalis* is

used as a folk medicine around the world, as well as in cosmetics. In medicine, the extract is receiving increasing attention due to its anti-inflammatory and antioxidative constituents [2b]. Our recent studies evidenced that *R. officinalis* extract, containing 31.7% of carnosic acid, [2b], 0.4% of rosmarinic acid [2b], and 5.9% of carnosol (unpublished data), was able to counteract the deleterious effects of UV-R, protecting plasmid DNA from hydroxyl radicals generated by UV-A [2b]. In this work, we evaluated the effect of this extract on pBR322 DNA cleavage induced by nitric oxide, and its growth inhibitory activity against two human melanoma cell lines (M14 and A375).

It has been reported that NO liberated following UV-R irradiation plays a significant role in initiating erythema and inflammation [3]. NO can combine with UV-induced superoxide to form peroxynitrite, which exists in equilibrium with peroxynitrous acid. These reactive nitrogen species are very toxic, and can cause DNA damage, nitrosylation of tyrosine residues in proteins, and initiate lipid peroxidation, all of which interfere with cellular function [4a].

Table 1: Effect of *R. officinalis* methanolic extract on Angeli's salt-mediated DNA damage.

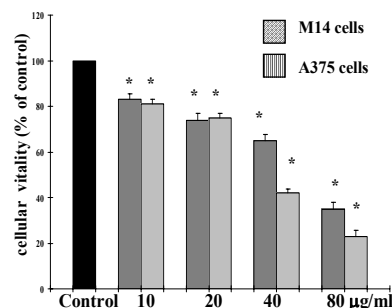
Treatments	UD of supercoiled DNA (% of native DNA)
scDNA	100
Angeli's salt 0.2 mM	12 ± 15*
extract	
200 µg/mL	56 ± 4.7*°
400 µg/mL	91 ± 2.6*°
800 µg/mL	94 ± 3.6*°

The values are expressed as densitometric units (D.U.) obtained by scanning the agarose gel electrophoresis photos.

Each value represents the mean ± SD of three experiments, performed in duplicate. *Significant vs. supercoiled DNA ($p < 0.001$); °significant vs. Angeli's salt treated DNA ($p < 0.001$).

Various compounds in foods, as well as in medicinal plants, have been widely used for wound-healing, anti-aging, and disease treatments of skin. The biological activity of these compounds has been correlated, in part, to their capacity to counteract the oxidative and nitrosative stress. Our results suggest that the *R. officinalis* extract could also act in this way in skin protection. In fact, sample, at concentrations of 200-800 µg/mL, exhibited protection against plasmid DNA damage induced by Angeli's salt, a NO donor [4b], which in our experimental conditions produced a significant decrease in the scDNA band intensity (Table 1). The treatment of plasmid DNA with extract alone did not change the migration pattern (data not shown).

In this study, we also tested the effect of this natural product on melanoma cells M14 and A375, and the results obtained show that the extract inhibited the growth of cancer cells. In fact, the results, summarized in Figure 1, show that the extract used at non toxic concentration in normal cells (data not shown), exhibited a dose dependent inhibitory effect on both human melanoma cells examined. In particular, in M14 and A375 cells treated with our sample at 40 µg/mL concentration for 72 h, the cell vitality was 65% and 42%, respectively. In cutaneous cells, there was a homeostatic relationship between cell proliferation and apoptosis. Alterations in either cell proliferation or cell death can lead to a loss of growth control, and thus play a major role in the process of tumorigenesis. Defects of apoptotic pathways influence also drug resistance, and because of these defects chemotherapy often fails [5]. Recent studies have suggested that the resistance of human melanoma to apoptosis is an important mechanism underlying this cancer's aggressiveness and its poor response to chemotherapeutic agents [5]. The induction of apoptosis in tumor cells is considered very useful in the management and therapy of cancer, including melanoma [5]. It is thus considered

**Figure 1:** Cell growth, assayed using MTT test, of M14 and A375 cells untreated and treated with the methanolic extract of *R. officinalis* at different concentrations for 72 hours. Stock solution of the extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p < 0.001$).

important to screen apoptotic inducers from plants, either in the form of extracts or as components isolated from them. Necrosis results in a disruption of the cytoplasmic membrane and the necrotic cells release cytoplasmic LDH and other cytotoxic substances into the medium. We therefore examined the membrane permeability of the treated cells and the existence of LDH in their culture medium. No statistically significant increase in LDH release was observed in M14 and A375 cells treated with the extract at 10-40 µg/mL concentrations. Conversely, a significant increase in LDH was observed at 80 µg/mL (Table 2).

Table 2: Lactate dehydrogenase (LDH) release, expressed as percentage of LDH released into the cell medium with respect to total LDH in M14 and A375 cells untreated and treated with the methanolic extract of *R. officinalis* at different concentrations for 72 hours. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone.

Treatments	% LDH released
M14	
control	4.7 ± 0.9
extract	
20 µg/mL	5.8 ± 0.7
40 µg/mL	4.8 ± 0.6
80 µg/mL	40 ± 3.6*
A375	
control	5.8 ± 1.5
extract	
20 µg/mL	6.6 ± 1.2
40 µg/mL	5.1 ± 2.6
80 µg/mL	35 ± 1.6*

Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p < 0.001$).

Nuclear DNA fragmentation was analyzed using the COMET assay, a sensitive method for detecting DNA strand breaks in individual cells and a versatile tool that is highly efficacious in human bio-monitoring of natural compounds [6a]. The COMET assay also allows the differentiation between apoptotic and necrotic cells based on the DNA fragmentation pattern [6b]. The COMET pattern significantly differs between

Table 3: COMET assay of genomic DNA of M14 and A375 cells untreated and treated with extract from *R. officinalis* at different concentrations for 72 h.

Treatments	TDNA	TMOM
M14 cells		
Control extract	17.7±3.0	86±3.1
20 µg/mL	145±5.0*	1113±11*
40 µg/mL	183±2.3*	1667±41*
80 µg/mL	68±5.5*	256±10*
A375 cells		
Control extract	12.1±3.0	69±4.3
20 µg/mL	173±3.0*	1165±5.3*
40 µg/mL	288±5.8*	2519±12*
80 µg/mL	94±5.9*	331±15*

The values were expressed as TDNA (percentage of the fragmented DNA) and TMOM, the product of TD (distance between head and tail) and TDNA. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells (p<0.001).

apoptotic and control cultures, as well as between apoptotic and necrotic ones. Quantification of the COMET data is reported in Table 3 as TDNA, the percentage of the fragmented DNA, and TMOM that is defined as the product of the percentage of DNA in the tail of the COMET and TD value (distance between head and tail). The results clearly demonstrate DNA damage in cells exposed to extract of *R. officinalis* for 72 h, but this natural product produced a high increase in both TDNA and TMOM at concentrations of 20 and 40 µg/mL. These results seem to confirm a programmed cell death, because data in the literature [6c] indicate that only comets with high values of TMOM and TD can be related to apoptosis. On the other hand, previous studies have shown that carnosic acid, the major polyphenol in *R. officinalis*, inhibits the proliferation of different cancer cells [6d,6e,7a], and induces apoptosis in HL-60 cells [7a].

Taken together, our present results suggest that the methanolic extract of *R. officinalis*, because of its ability to counteract nitric oxide-mediated plasmid DNA damage, could be useful in the prevention of cell damage correlated to UV-R, such as dermatitis, premature aging and skin cancer. In addition, this study provided the first evidence that the extract of *R. officinalis* attenuates the growth of human cancer cells by triggering an apoptotic process. In fact, a high DNA fragmentation (COMET assay), not correlated to LDH release, a marker of membrane breakdown, occurred in melanoma cells exposed to the methanolic extract of *R. officinalis* in concentrations of 20–40 µg/mL. Although the molecular mechanism by which apoptosis is induced by this extract remains to be confirmed, the results reported here suggest its possible use as a novel

therapeutic agent for melanoma cancer treatment in association with classic drugs with the aim to reduce their toxicity.

Experimental

***Rosmarinus officinalis* sample:** This came from the special collection of more than 160 individual plants used by BOTANE Ltd., and was collected at Goodwood, Canada. The accession was clonally propagated in order to maintain genetic uniformity, and was planted in November 2001 at Illapel, Chile [2b]. The fresh rosemary sample was dried at 40°C in a forced air circulation oven (MemmertULM500). The leaves were manually separated and ground in a vertical hammer mill (Peruzzo Milly model 35.010) at 12,000 rpm to 0.8 mm mesh. Samples were mixed with 500 mL methanol and stirred for 15 h at 20°C, in the dark. After stirring and filtering under vacuum, the filtrate was evaporated to dryness in a Rotavapor. The samples were extracted again for 6 days (two times) [2b], and the filtrate was evaporated to dryness in a Rotavapor [yield 55.4g (22.9%)]. The content of carnosic acid, rosmarinic acid and carnosol was 31.7%, 0.41% [2b] and 5.93%, respectively.

Analysis of DNA single-strand breaks induced by Angeli's salt: The experiments were performed [7b] by incubating pBR322 plasmid DNA in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA, 0.15 mM Angeli's salt (prepared in 0.01 N NaOH), an appropriate amount of HCl to neutralize the NaOH present in the solution of Angeli's salt, and the extract at different concentrations at 37°C for 1 h (final volume 10 µL, final pH 7.5). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 µg/mL; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

Effects on human tumor cell lines: M14 human melanoma cells was obtained from the American Type Culture Collection (Rockville, MD, USA) and were grown in RPMI containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL fungizone. A375 human melanoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2.0 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL fungizone. The cells

were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. After 24 h incubation at 37°C in a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of the methanolic extract of *R. officinalis* for 72 h under the same conditions. Stock solution of the natural product was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone.

MTT bioassay: Cellular growth was determined using the MTT assay on 96-well microplates, as previously described [7b]. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm., and the results were reported as % of control.

Lactate dehydrogenase (LDH) release: Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation, as previously reported [7b]. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium.

DNA analysis by COMET assay: The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), as previously reported [7b]. At the end of the electrophoretic run, the “minigels” were neutralized in 0.4 M Tris-HCl, pH 7.5, stained with ethidium bromide and scored using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed the analysis and quantification of DNA damage by measuring: a) tail length (TL), intensity (TI) and area (TA); b) head length (HL), intensity (HI) and area (HA). These parameters are employed by the software to determine the level of DNA damage as: i) the percentage of the fragmented DNA (TDNA), and ii) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

Statistical analysis: Statistical analysis of results was performed by using one-way ANOVA, followed by Dunnett’s post-hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA). Each value represents the mean ± SD of three separate experiments performed in quadruplicate.

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