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Radioprotective−Antimutagenic Effects of Rosemary Phenolics against Chromosomal Damage Induced in Human Lymphocytes by γ-rays

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The radioprotective effects of carnosic acid (CA), carnosol (COL), and rosmarinic acid (RO) against chromosomal damage induced by γ-rays, compared with those of L-ascorbic acid (AA) and the S-containing compound dimethyl sulfoxide (DMSO), were determined by use of the micronucleus test for antimutagenic activity, evaluating the reduction in the frequency of micronuclei (MN) in cytokinesis-blocked cells of human lymphocytes before and after γ-ray irradiation. With treatment before γ-irradiation, the most effective compounds were, in order, CA > RO ≥ COL > AA > DMSO. The radioprotective effects (antimutagenic) with treatment after γ-irradiation were lower, and the most effective compounds were CA and COL. RO and AA presented small radioprotective activity, and the sulfur-containing compound DMSO lacked γ-ray radioprotection capacity. Therefore, CA and COL are the only compounds that showed a significant antimutagenic activity both before and after γ-irradiation treatments. These results are closely related to those reported by other authors on the antioxidant activity of the same compounds, and the degree of effectiveness depends on their structure. Furthermore, the results for treatments before and after γ-ray irradiation suggest the existence of different radioprotective mechanisms in each case.

KEYWORDS: Rosemary; polyphenols; antioxidant; radical scavenger; micronuclei; antimutagenic; radioprotection; radiation effects; carnosic acid; carnosol; rosmarinic acid

INTRODUCTION

It is widely accepted that fruits and vegetables have many healthful properties. It is generally assumed that the active dietary constituents contributing to these protective effects are the antioxidant nutrients (*1*). A large number of polyphenolic compounds with antioxidant activity have been identified in the Labiatae plant *Rosmarinus officinalis*, including phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epirosmanol, 7-methylepirosmanol, and methyl carnosate (*2*−*9*). In addition, several flavonoids, such as genkwanin, hispidulin 7-*O*-glucoside, cirsimaritin, luteolin, and isoscutellarein 7-*O*-glucoside, are found in Labiatae plants (*8*, *10*); the phenolic compounds rosmarinic and caffeic acids are also present (*5*, *12*, *13*).

The antioxidant activity of rosemary extracts depends on their phenolic composition and has been determined by various methods in different lipid and aqueous systems. In lipid systems, the extracts with a high content of carnosic acid and carnosol are more effective (*2*−*4*, *14*, *15*), whereas in aqueous systems, rosmarinic acid shows the highest antioxidant activity (*5*, *13*, *15*).

Recent papers have shown specifically the antioxidant properties of the main polyphenols present in *R. officinalis* in different cell lines. Carnosic acid enhances the anticancer activity of vitamin D_3 and its analogues (*16*, *17*), inhibits proliferation of HL-60 and U937 human myeloid leukemia cells (*18*), promotes the synthesis of nerve growth factor in T98G human gioblastoma cells (*19*), and inhibits endothelial cell functions as a novel potential cancer chemoprotective agent (*20*). On the other hand, rosmarinic acid suppressed synovitis in a murine collagen-induced inflammatory arthritis model (*21*).

It is known that ionizing radiations such X- and γ-rays generate reactive oxygen species (ROS) in organisms and induce cellular DNA damage, which leads to mutations and chromosomal aberrations (*22*−*24*). Recently, the scavenging ability of certain plant extracts containing several polyphenols, flavones, catechins, and procyanidins against ROS and their inhibitory effects against X- and γ-ray-induced cell transformation were reported, both in vivo and in vitro (*22*, *24*−*28*).

The micronucleus assay, originally developed in vivo by Schmidt (*29*), provides a simple and rapid method for detecting chromosomal damage (*30*−*33*). At present, the micronucleus assay in human lymphocytes irradiated and treated with cytokinesis-block, as described by Fenech and Morley, is the most

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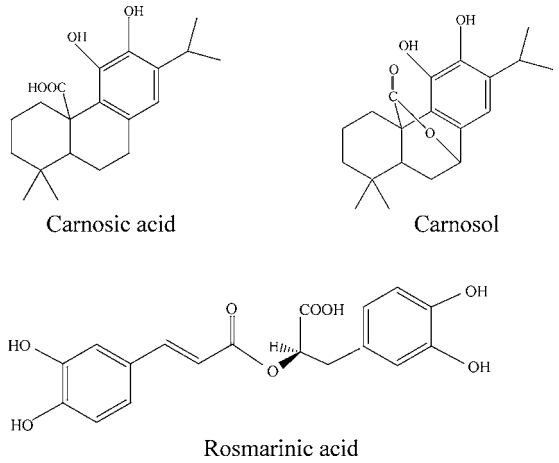

Figure 1. Chemical structures of the rosemary phenolics studied: carnosic acid (CA), carnosol (COL), and rosmarinic acid (RO).

widely used test for analyzing the mutagenic capacity of chemical substances and physical agents (*32*, *33*).

The objective of the present work was to study the radioprotective effects (antimutagenic activity) of carnosic acid, carnosol, and rosmarinic acid against chromosomal damage induced in human lymphocytes by γ-rays, compared with the protective effects of L-ascorbic acid and the S-containing compound dimethyl sulfoxide, using the micronucleus test. The relationship between the molecular structure and the antioxidant, radioprotective, and antimutagenic activities is discussed.

MATERIALS AND METHODS

Chemicals. Carnosic acid. 82% (CA), and carnosol, 86% (COL), were supplied by Nutrafur-Furfural Español S.A. (Murcia, Spain). Rosmarinic acid, 95% (RO), was obtained from Extrasynthèse (Genay, France). **Figure 1** shows the molecular structure of these compounds. L-Ascorbic acid, 99% (AA), was obtained from Sigma Co. (Madrid, Spain). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany).

Blood Samples and Irradiation Procedure. Heparinized samples of human peripheral blood were obtained from two healthy young nonsmoking female donors. The rosemary phenolics used in this study, CA, COL, and RO, were dissolved in 5% aqueous DMSO at the ratio of 1 mg/mL, and AA was dissolved in 5% aqueous DMSO at the ratio of 2.5 mg/mL. For the before-γ-irradiation treatments, 20 μL of these solutions was added to 2 mL of human blood to obtain a 25 μM concentration, and the samples were homogenized just before γ-irradiation. For the after-γ-irradiation treatments, also 20 μL of these solutions was added to 2 mL of irradiated human blood (25 μM) and homogenized for 15 min after γ-irradiation. The DMSO group was included in this study not only because it was added as solvent but also because it is generally considered to be a classic radical scavenger (*34*, *35*) and a radioprotective agent according to structural and experimental data (*36*).

The blood samples were exposed to ^{137}Cs γ-rays from an Irradiator IBL 437 C (CIS, France) at a dose of 2 Gy $\pm$ 3%. The irradiation was performed at room temperature for 40 s with a dose rate of 5 cGy/s at the moment of the study. The γ-ray exposure was established by means of thermoluminescent dosimeters (TLDs) (GR-200, Conqueror Electronics Technology Co. Ltd., China). The TLDs were supplied and measured by CIEMAT (Ministry of Industry and Energy, Spain).

Culture Technique. After γ-irradiation (with addition of phenolics before and after γ-irradiation), the micronucleus assay was carried out on human lymphocyte culture according to the method of Fenech and Morley (*32*, *33*). Whole blood (1 mL) was cultured at 37 °C for 72 h in 9 mL of F-10 medium (Sigma Co.), containing 15% fetal bovine serum (Sigma Co.), 1.6% phytohemagglutinin (Sigma Co.), and 1% penicillin/streptomycin (Sigma Co.). Forty-four hours after initiation of the lymphocyte culture, cytochalasin B (Cyt B) (Sigma Co.) was added at a concentration of 3 μg/mL. At 72 h the lymphocytes were

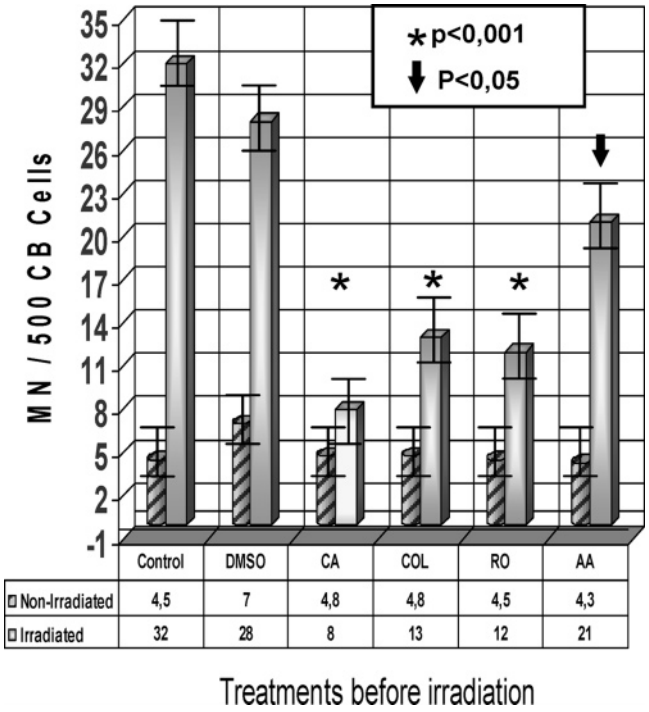

Figure 2. Influence of treatments administered before γ-ray irradiation on the frequency of micronuclei in human lymphocytes.

treated with hypotonic solution (KCl, 0.075 M) for 3 min and fixed using methanol/acetic acid (3:1). Air-dried preparations were made, and the slides were stained with May-Grunwald Giemsa.

Scoring of Micronuclei. The number of micronuclei (MN) in at least 500 cytokinesis-blocked cells (CB cells) was scored using a Zeiss light microscope (Oberkochem, Germany) with 400× magnification for surveying the slides and 1000× magnification to confirm the presence or absence of MN in the cells.

Statistical Analysis. The degree of dependence and correlation between variables was assessed using analysis of variance, complemented by a contrast of means using p value ($p < 0.05$). Quantitative means were compared by regression and lineal correlation analysis

$$\text{magnitude of protection (\%)} = [(F_{\text{control}} - F_{\text{treated}})/F_{\text{control}}] \times 100$$

where F_{control} = frequency of MN in irradiated blood lymphocytes and F_{treated} = frequency of MN in blood lymphocytes treated before and after the γ-ray irradiation (CA, COL, RO, AA, and DMSO) (*37*).

RESULTS

γ-Ray Radioprotective Effects: Antimutagenic Activity. **Figure 2** shows the influence of treatments before γ-ray irradiation on the frequency of MN in non-irradiated and irradiated human lymphocytes, which permits a comparison of the potential genotoxicity (non-irradiated) of each compound versus its antimutagenic capacity (irradiated). In non-irradiated human lymphocytes, all phenolic compounds show the same level of MN as the control, whereas the sulfur compound DMSO shows higher toxicity than the others. In irradiated human lymphocytes, the order of treatments, from lowest to highest level of MN induced by radiation, was CA < RO ≤ COL < AA < DMSO.

Figure 3 shows the influence of treatments after γ-ray irradiation on the frequencies of MN. The frequencies are higher than observed in the treatments before γ-ray irradiation. It is clear that only CA and COL show significant antimutagenic activity. RO and AA present a low degree of radioprotective activity, and the sulfur-containing compound DMSO lacks a γ-ray radioprotection capacity. The order of treatments, from

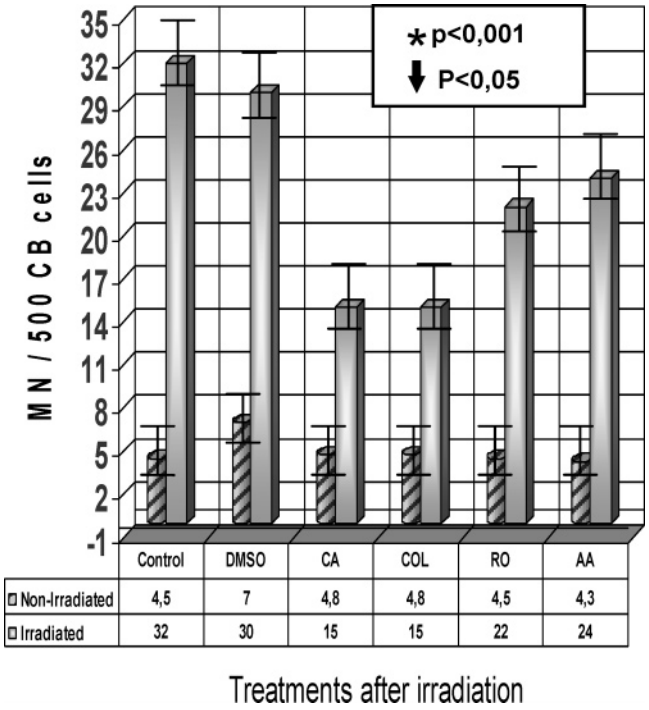

Figure 3. Influence of treatments administered after γ-ray irradiation on the frequency of micronuclei in human lymphocytes.

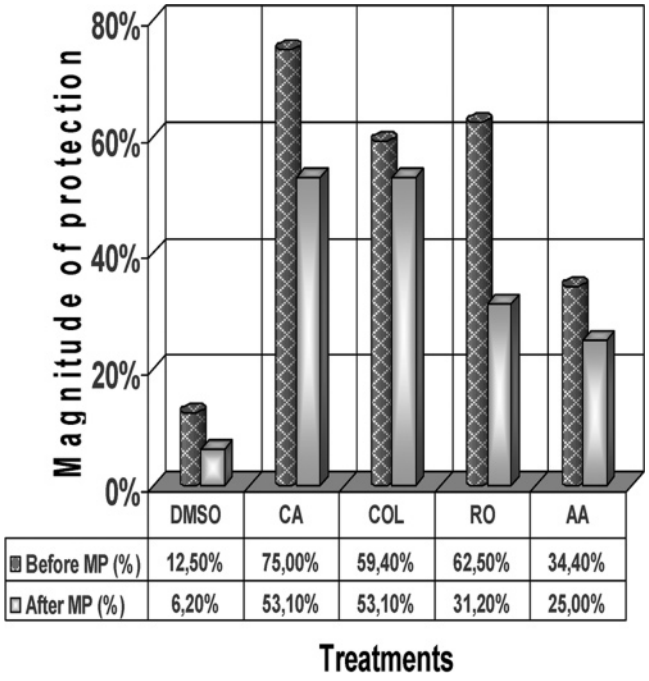

Figure 4. Magnitude of protection (percentage) of different treatments administered before and after γ-ray irradiation.

lowest to highest level of MN induced by radiation, was CA = COL < RO < AA < DMSO.

The radioprotective effects and, consequently, the antimutagenic (or antigenotoxic) activity of the different compounds assayed (before and after γ-ray irradiation), were established according to the decrease in MN numbers according to the above equation (*37*) (see Material and Methods), obtaining a percentage value that determines the degree of protection of each compound. **Figure 4** shows the values of these protection capacities, the orders of efficacy being CA > RO ≥ COL > AA > DMSO for treatments before γ-ray irradiation and CA = COL > RO > AA > DMSO for treatments after γ-ray irradiation.

DISCUSSION

It is know that the ROS responsible for cell oxidation processes are the following: singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($R-OO^{\bullet}$). Except the first, which is formed by photosensitization (*38*), the remaining ROS are formed by a sequential electron mechanism, by means of which molecular oxygen gives rise to superoxide radical, hydrogen peroxide, and hydroxyl radical successively (*39*).

In vivo, γ-rays cause a high generation of hydroxyl radicals, by homolytic cleavage of body water or endogenous hydrogen peroxide (formed by reduction of the superoxide anion) by two mechanisms: the Haber−Weiss and Fenton models. The hydroxyl radical is the most cytotoxic of all those so far described, with an estimated half-life of $\sim 10^{-9}$ s (*40*). The high reactivity of this radical implies immediate reaction at the place where it is generated. When hydroxyl radical generation is massive, as with γ-irradiation, the cytotoxic effect is not only local but can propagate intracellularly and extracellularly, increasing the interaction of these radicals with phospholipoid structures and inducing peroxidation processes that increase the hydroxyl radical activity in DNA oxidative damage (*39*, *41*).

In these oxidative stress conditions, when even the endogenous antioxidant systems are defective or insufficient, exogenous agents with a strong radical scavenging capacity must be used. This capacity depends on high absolute reactivity against different radicals or the high stability of the intermediate aroxyl radical formed (*39*). In this study, CA, COL, RO, AA, and the sulfur-containing compound DMSO were used as radioprotective agents. AA is considered to be one of the most powerful and least toxic of natural antioxidants; it is water-soluble and is found in high concentrations in many tissues. On interaction with ROS, AA is oxidized to dehydroascorbate via the intermediate ascorbyl free radical and recycled back to ascorbic acid by the enzyme dehydroascorbate reductase. DMSO is a classic radical scavenger, with a high capacity for in vitro hydroxyl radical scavenging (*34*, *35*). However, when applied in radioprotective doses, in the absence of any subsequent irradiation, it is highly toxic in animals (*42*−*44*).

The results obtained concerning radioprotective effects (antimutagenic activity) of the different phenolics of *R. officinalis* in our study were closely related to those reported by other authors on the antioxidant activity of the same compounds (*4*, *5*, *14*, *15*, *45*−*48*). Obviously, the degree of effectiveness depends on their structure. It is known that the capacity to inhibit hydroxyl radical is principally based on the combination of conjugated structures in the polyphenolic skeletons, mainly the *o*-dihydroxyphenol or catechol structure, and also the presence of a carboxylic group. The greater activity of these compounds is also due to the stability of the polyphenolic radical generated in the process (*39*, *49*).

The results obtained with regard to the magnitude of protection (**Figure 4**) offered by different treatments given before and after γ-ray irradiation suggest the probable existence of different radioprotective mechanisms in each case. In the treatments before irradiation, the radioprotective effects (antimutagenic activity) of phenolics is theoretically based, as mentioned earlier, on the scavenging capacity against superoxide anion ($O_2^{\bullet -}$) and, especially, hydroxyl radical ($^{\bullet}OH$), which is massively generated during γ-ray irradiation. In fact, according to the above-mentioned structural considerations, the antimutagenic activity of the tested compounds given before γ-irradiation are consistent with their antioxidant properties and specific activities as free radical scavengers.

The results for CA, COL, and RO confirm the higher radioprotective effects (antimutagenic activity) of phenolics with

o-dihydroxyphenol substitution, polyphenol skeletons, a catechol structure with conjugated double bonds, and carboxylic groups. These data also confirm the higher antimutagenic activity of this structure than of AA and the sulfur-containing compound DMSO.

The presence of a catechol group in the aromatic ring (C11−C12) of the rosemary phenolic diterpene skeleton is probably the most important structural element in the antioxidant activity of these compounds. The presence of a free carboxylic group on this diterpene skeleton increases the radioprotective activity, according to the data shown by CA and COL, the corresponding γ-lactone of CA. The presence of two catechol structures conjugated with a carboxylic acid group in RO increases its antioxidant activity in aqueous media, showing results similar to those for COL. In addition, the greater activity of CA is probably due to the stability of the diterpenoid radical formed in the irradiation process, which is greater for acid structure than for lactone structure. It is important to note that the radioprotective activity of CA, in the same test conditions, is higher than that of the powerful antioxidant grape seed extract (procyanidins), which shows a protection capacity (*27*) of 61.29% versus the 75.0% of CA.

Recently, Offord et al. (*50*) studied the photoprotective potential of several dietary antioxidants, among them AA and CA, using human dermal fibroblasts exposed to UV-A light. The authors suggested as a possible protection mechanism the influence of these phenolics on the metalloproteinase 1 (MMP-1) mRNA activity. UV-A irradiation of human fibroblasts led to a 10−15-fold rise in MMP-1, which was suppressed in the presence of low concentrations of AA and CA.

When the phenolics were added after γ-ray irradiation, the only ROS present in the cells, according to the half-life of superoxide anion and hydroxyl radicals (*40*), were probably lipoperoxy radicals (R−OO$^{\bullet}$), which are responsible for continuous chromosomal oxidative damage. In addition, ionizing radiations enhance lysosomal enzyme secretion and arachidonic acid release from membranes through lipooxygenase, cycloxygenase, and phospholipase activities, increasing the inflammatory cell response. In these complex oxidative stress conditions, it is very difficult to make a structural linear evaluation of the experimental data obtained for the antimutagenic activity measured in different after-irradiation treatments; however, some considerations are possible.

The distinction between antiradical (versus superoxide anion and hydroxyl radicals) and anti-lipoperoxidant (versus lipoperoxy radicals) activities proposed by Pincemail et al. (*51*) seems to be reasonable, according to the data obtained in the before- and after-γ-ray irradiation models, and could be the cause for the different behaviors with regard to the lipid peroxidative processes of the compounds tested. The anti-lipoperoxidant activity of phenolics and flavonoids depends in a complex way on various factors, among them the nature of the lipid substrate susceptible to oxidation, operational cell conditions, and even the method used to evaluate this potential (*39*).

The results obtained in the post-irradiation treatments show that the diterpenes CA and COL are the only compounds that support a significant radioprotective capacity; this agrees with the above-mentioned considerations, because CA and COL are also the only liposoluble (oil-soluble) compounds. RO, a water-soluble compound, presents a significant activity slope, showing results similar to those for AA. The sulfur-containing compound DMSO lacks γ-ray radioprotection capacity.

New experiments are in progress to study the specific and synergic antioxidant and antimutagenic activities of these rosemary phenolics and, likewise, the role of the intestinal microflora in their catabolism, their disposition in mammalian tissues after oral and parenteral administration, and their dose−response curves, using in vivo systematic treatment models before and after γ-ray and other ionizing radiations. In addition, the apparent null toxicity of these rosemary phenolics, according to verified tests widely used in food products (generally regarded as safe status), gives them a high capacity for use as nutraceutical and pharmaceutical agents.

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