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Rosmarinic acid, a photo-protective agent against UV and other ionizing radiations

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ABSTRACT

Solar UV and other ionizing radiations cause a generation of reactive oxygen species, induce cellular DNA damage and alter skin homeostasis. The use of exogenous antioxidants is increasingly frequents, we attempt to demonstrate that a rosmarinic acid extract acts as photo-protector; both free radical scavenger as an inducer of the body's own endogenous defence mechanisms by regulating tyrosinase activity and stimulating melanin production. Malonyldialdehyde formation (TBARS) was delayed when RA was used. The protection factor was 3.24 times *vs* AA. TEAC value for RA was 1.6 times *vs* AA. The radioprotective-antimutagenic effects of RA were measure using the micronucleus test. The level of micronucleous for treatments before irradiation was: RA [14] < AA [22] < DMSO [28] < Control [32], and after irradiation was: RA [23] < AA [25] < DMSO [31] < Control [32]. RA increased the Tyr activity and its expression level in B16 melanoma cells after stimulation lasting 48 h compared with the negative control. In *vivo* experiments show the capacity of RA orally administered to inhibit cutaneous alterations caused by UVA exposure (skin photocarcinogenesis). Therefore, according all these experiences, RA can be proposed as a proper photo-protective agent.

1. Introduction

Solar ultraviolet radiation is a major environmental factor that dramatically alters skin homeostasis. In recent years, the CFC and other chemical emissions have sharply increased this damage because these substances destroy the ozone layer, the natural radiation filter in the atmosphere. In countries where sunlight exposition is high or people are mainly fair-haired with a low pigmented skin, the risk is increased. Ionizing radiations, included UV ray, cause a massive generation of cytotoxic reactive oxygen species (ROS) and induce cellular DNA damage (Riley, 1994). Their cytotoxicity increases by interaction with membrane phospholipids, thus inducing peroxidative processes and the generation of lipoperoxy-radicals (Diplock et al., 1998). Under these oxidative conditions, the endogenous antioxidant systems might be insufficient, and exogenous agents with a strong-radical scavenging or antioxidant capacity could be beneficial. The use of exogenous antioxidants to fight against oxidative stress is increasingly frequent.

However, it is also important to look for new substances that can induce the human body to increase the activity of its endogenous mechanisms. Melanin pigment in human skin is a major defence mechanism against ultraviolet sun light. Melanin biosynthesis is regulated by melanogenic enzymes such as tyrosinase (Tyr) that presents two functions (tyrosin hidroxilase and dopa oxidase), 5Styrosinase-related protein1 (Trp1) (Aroca et al., 1990; Jimenez-Cervantes et al., 1994; Kobayashi et al., 1994; Cooksey et al., 1997). Thus, a proper photo-protective agent must not inhibit melanogenesis, and new chemopreventive methods are necessary to protect the skin from photodamaging effects of solar UV radiation.

The plant kingdom is a large source of natural active ingredients among which it is possible to find some plant extract with UV

Abbreviations: AA, ascorbic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt; BSA, bovine serum albumin; CB, cytokinesis blocked; CFC, chlorofluorocarbonated; Cyt, cytochalasin; DMSO, dimethylsulphoxide; FCS, foetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; MBTH, 3-methyl-2-benzothiazoline hydrazone; MDA, malonyldialdehyde; MEM, minimum essential medium; MN, micronuclei; PBS, phosphate buffered saline; αPEP7, tyrosinase antibody targeted to the C-terminal peptide of mouse tyrosinase; PMSF, phenylmethylsulphonyl fluoride; RA, rosmarinic acid extract; RO, rosmarinic acid; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TEAC, trolox equivalent antioxidant capacity; Trp, tyrosinase related protein; Tyr, tyrosinase.

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absorption properties that can be used in photo-protective products as enhancers of physical or chemical sunscreens. Dietary polyphenols, commonly present in edible plants, are known to have beneficial properties, including antioxidant, anti-inflammatory and photo-protective effects (Hertog et al., 1993; Benavente-García et al., 1997). The plant extracts suitable for such applications are those that contain compounds with similar chemical structures to synthetic sunscreens.

Rosmarinic acid (RO) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is commonly found in species of the *Boraginaceae* and the subfamily *Nepetoideae* of the *Lamiaceae*, mainly, *Rosmarinus officinalis* L. (Del Baño et al., 2003). RO has a number of interesting biological activities, including antiviral, antibacterial, anti-inflammatory and antioxidant effects. The presence of RO in medicinal plants, herbs and spices has beneficial and health promoting effects. In plants, RO is supposed to act as a preformed constitutively accumulated defence compound. Plant extracts containing RO also have excellent potential as antioxidants for food preservation (Frankel et al., 1996). The pharmacological effect of RO has been demonstrated to act through the inhibition of several complement-dependent inflammatory processes (Peake et al., 1991). Therefore, it has tremendous potential as a therapeutic agent for control of complement activation diseases (Peake et al., 1991; Engleberger et al., 1988). It is also known to have complement-independent effects such as scavenging of oxygen free radicals (Nuytinck et al., 1985) and inhibition of elastase. The relative safety of RO in relation to other methods of complement depletion is well documented (Nuytinck et al., 1985).

Most recent studies confirm that caffeoyl esters as RO show the high specific antioxidant activity, delay vitamin E depletion, decrease pro-inflammatory lysophosphatidylcholine production and prevent the oxidation of LDL, which is compatible with its anti-inflammatory and anti-atheroesclerotic role in pathophysiological conditions (Cartron et al., 2001). RO also inhibits cytokine-induced mesagial cell proliferation (Makino et al., 2000) and suppress synovitis in a murine collagen-induced inflammatory arthritis model (Youn et al., 2003).

In the present work, we attempt to demonstrate by a series of *in vitro* and orally applied *in vivo* experiments that RO acts as exogenous and endogenous photo-protector, in the first case acting as free radical scavenger and in the second as an inducer of the body's own endogenous defence mechanisms by regulating tyrosinase activity and stimulating melanin production. In this way, we hope to obtain an overall view of how RO acts as photo-protector.

2. Materials and methods

2.1. Plant material

Rosemary leaves were obtained from three month old *Rosmarinus officinalis* L. plants grown in greenhouses at the University of Murcia. The plant materials were collected immediately dried at 40 °C and ground for extraction.

2.2. Extraction and purification of rosmarinic acid extract from dried rosemary leaves

Rosmarinic acid was extracted from ground dried rosemary leaves (5000 g) with a mixture of water–ethanol (80:20) in a ratio of 100 g/l under N_2 for 1 h. After filtration, the extraction solvent was evaporated under vacuum at 50 °C until the ethanol was removed. The aqueous medium was washed with n-hexane to remove liposoluble substances and the remaining solvent was removed by evaporation. This crude extract was purified using an Amberlite XAD 16 column, and eluted with ethanol for desorption of polyphenols. The ethanol was removed under vacuum at 40 °C. In this way, 50.6 g of crystalline and hygroscopic powder of rosmarinic acid extract (RA) was obtained.

2.3. HPLC analysis of purified rosmarinic acid extract (RA)

The solid obtained was dissolved in water for analytical chromatography; this solution was filtered through a 0.45 μm nylon membrane. The HPLC equipment used was a Hewlett–Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C_{18} LiChrospher 100 analytical column (250 × 4 mm i.d.) with a particle size of 5 μm (Merck, Darmstadt, Germany) thermostated at 30 °C. The flow rate was 1 ml/min. The absorbance changes were monitored at 340 nm.

The HPLC method used was previously published by us (Del Baño et al., 2003). Rosmarinic acid standard (98% HPLC assay, CAS no 20283-92-5) was obtained from Extrasynthèse (Genay, France). The quantitative HPLC analysis of the obtained rosmarinic acid extract (RA) results in an absolute concentration of 80.62% of pure rosmarinic acid (*vs* standard). Fig. 1 shows the molecular structure of rosmarinic acid.

2.4. Materials and reagents

Linoleic acid, α-tocopherol, lard, ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid diamonium salt), Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid), manganese dioxide, Fetal bovine serum, 1,6% phytohaemaglutinin, 1% penicillin/streptomycin and cytochalasin B (Cyt. B), 3-Isobutyl-1-methylxanthine (IBMX), l-3,4-Dihydroxyphenylalanine (l-dopa), d-dopa, Nonidet P40, 3-methyl-2-benzothiazoline hydrazone (MBTH), EDTA, o-phenanthroline, 2,2-dipyridyl, dithizone, PMSF and hydroxyapatite (type I suspension in 1 mM phosphate buffer, pH 6.8) and eosin were obtained from Sigma Chemical Co. (Madrid, Spain).

HPLC grade ethanol, methanol, water, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), dimethylsulphoxyde (DMSO), acetic acid, Brij 35, SDS, Tris and BSA were obtained from Merck (Darmstadt, Germany). Minimum essential medium (MEM) and foetal calf serum (FCS) were obtained from Gibco–Invitrogen (Carlsbad, CA, USA).

L-Ascorbic acid (AA) and formaldehyde was obtained from Panreac (Madrid, Spain). SDS/PAGE reagents were from Bio-Rad (Richmond, CA, USA). Sodium periodate, potassium cyanide and metal ions (always used as the sulphate salt) were supplied by Probus (Barcelona, Spain). The bicinchonic acid kit for protein determination was purchased from Pierce (Rockford, IL, USA). Triturated maintenance feed for A04 mice was obtained from Panlab (Cornellá, Spain); haematoxylin was obtained from Analema (Vigo, Spain). All solutions were prepared in double-distilled water passed through a Milli-Q system (Millipore) (resistance higher than 18 M × cm).

2.5. Antioxidant activity: auto-oxidation of linoleic acid (TBARS method)

Two grams of linoleic acid was solubilized in 50 ml ethanol. Then, 100 ml of 0.1 M phosphate buffer, pH 7.0 and 50 ml of water were added. Finally, the solution of the RA or AA in 50 ml of ethanol was added for a final concentration of 200 mg/kg. The reference solution was prepared by final addition 50 ml of ethanol. Oxidation was performed by letting in atmospheric air at 25 °C. To detect the malondialdehyde (MDA) formed, 5 ml of TCA were added to 2.5 ml of oxidated solution for stopping the oxidation and then 2.5 ml of TBA (0.67% in water) was added. The reaction mixture was shaked and incubated in a boiling water-bath for 15 min. The mix was cooled and centrifuged at 1200 rpm for 20 min. The final colour developed was investigated at 532 nm using a Perkin Elmer UV/VIS spectrophotometer. The protection factor, also called the activity index, of the antioxidant is defined as the ratio of the induction times measured with and without antioxidant and can be taken as a measure of the antioxidant efficacy.

2.6. Antioxidant activity: $ABTS^{\cdot+}$ scavenging capacity (TEAC method)

$ABTS^{\cdot+}$ radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS through manganese dioxide (MnO_2) on a Whatman no 5 filter paper. Excess MnO_2 was removed from the filtrate by passing through a 0.4 μm nylon syringe filter. This solution was then diluted in 5 mM phosphate buffered saline (PBS) pH 7.4 to an absorbance of 0.70 (±0.02) at 734 nm and pre-incubated at 30° C prior to use (Rice-Evans and Miller, 1996; Kuskoski et al., 2004). Fresh $ABTS^{\cdot+}$ radical cation solution was prepared each day. 2.5 mM Trolox was prepared in PBS for use as stock standard. Fresh working standards were prepared daily by diluting 2.5 mM Trolox with PBS. RA was dissolved in water to a concentration of 50 μM. After addition of 1 ml $ABTS^{\cdot+}$ solution to aliquots of Trolox or of RA, the solutions were vortexed for exactly 30 s and, exactly 1 min after initiation of mixing, the absorbance at 734 nm was read in a Unicam UV-2 spectrophotometer (Cambridge, UK) at 30° C. PBS blanks were run in each assay.

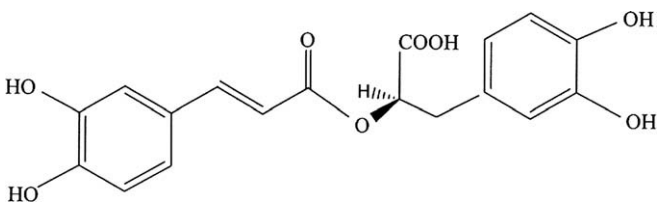

Fig. 1. Chemical structure of the rosmarinic acid.

The dose-response curve for Trolox consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation (blank) and was based on triplicate determinations. The activity of RA was assayed at four different concentrations, which had been determined to be within the range of the dose-response curve, and was analysed in triplicate at these four concentrations. By reference to the Trolox dose-response curve, the mean Trolox equivalent antioxidant capacity (TEAC) value was derived for RA.

2.7. Radioprotective-Antimutagenic effects of RA against chromosomal damage induced in human lymphocytes by γ-rays

2.7.1. Blood samples and irradiation procedure

Heparinized samples of human peripheral blood were obtained from two healthy young non-smoking female donors. RA was dissolved in 5% aqueous DMSO at the ratio of 1 mg/ml, and AA, as reference compound, was dissolved in 5% aqueous DMSO at the ratio of 2.5 mg/ml. For the pre γ-irradiation treatments, 20 μl of these solutions were added to 2 ml of human blood to obtain a 25 μM concentration and the samples were homogenized just before γ-irradiation.

For the post γ-irradiation treatments, also 20 μl of these solutions were added to 2 ml of irradiated human blood (25 μM) and homogenized 15 min after γ-irradiation. The DMSO group was included in this study, not only because was added as solvent, but also because it is generally considered to be a classic radical scavenger (Cillard and Cillard, 1988; Darmon et al., 1990) and radioprotective agent according to structural and experimental data (Brown et al., 1982).

The blood samples were exposed to ^{137}Cs γ-rays from an Irradiator IBL 437 C (CIS, France) at a dose of 2 Gy ± 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).

2.7.2. Culture technique

After γ-irradiation (with addition of compounds (RA, AA and DMSO) pre and post γ-irradiation), the micronucleus assay was carried out on human lymphocyte culture according the methods of Fenech and Morley (Fenech and Morley, 1985; Fenech, 1993). Whole blood (1 ml) was cultured at 37 °C for 72 h in 9 ml of F-10 medium containing 15% fetal bovine serum, 1.6% phytohaemaglutinin and 1% penicillin/streptomycin. Forty-four hours after initiation of the lymphocyte culture, Cyt. B was added in a concentration of 3 μg/ml. At 72 h the lymphocytes were 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.

2.7.3. 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.

2.7.4. 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 analyse.

$$\text{Magnitude of protection}(\%) = ((F_{\text{control}} - F_{\text{treated}})/F_{\text{control}}) \times 100.$$

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 (RA, AA and DMSO) (Sarma and Kesavan, 1993).

2.8. Effects of RA on melanogenic enzymes of mouse B16 melanoma cells

2.8.1. Cell culture and treatments

B16 mouse melanoma cells were cultured in MEM with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomicyn. The cultures were performed in a water-saturated atmosphere with 5% CO2. For RA treatment, semi confluent cells were stimulated by 48 h incubation to 100 μM of RA dissolved in the appropriated cell culture medium and filtered through Millipore filters.

2.8.2. Enzyme activity determinations

Cells were washed with PBS, trypsin harvested and counted before solubilization in 1% Igepal CA-630, 0.1 mM PMSF in 10 mM phosphate buffer (pH 6.8). Tyrosine hydroxylase activity was determined by a radiometric method (Jara et al., 1988). Dopa oxidase activity was measured spectrophotometrically in the presence of 3-methyl-2-benzothiazolinone (MBTH) (Nordlund and Abdel-Malek, 1988). Activity units are defined as the amount of enzyme transforming 1 μmol of substrate/minute.

2.8.3. Immunochemical techniques

Western blotting was performed as described (Winder and Harris, 1991; Martinez-Esparza et al., 1997), using the specific antisera αPEP7 (anti-Tyr), a gift from Prof. V. Hearing (NIH, Bethesda, MD).

2.8.4. Other procedures

Protein concentration was measured with the bicinchonic acid method using BSA as a standard (Martinez-Esparza et al., 1998).

2.9. Cutaneous alterations resulting from UVA exposure (in vivo)

2.9.1. Animals

Twenty female albino Swiss mice aged 10–12 weeks and weighing a mean 33.83 ± 3.72 g at the beginning of the study were obtained from the laboratory Animal Service of the University of Murcia (SAI, no REGA ES300305440012), following EU guidelines (86/609/EEC). The animals were distributed into two groups: I, exposed control (UVA) and II, group treated daily with RA at 2% in food and drink (UVA+RA). The feed was prepared weekly, grinding and mixing it with the RA dissolved in water, before forming it into pellets and drying in a stove at 80° C. The mice were fed "*ad libitum*".

2.9.2. Method

The mice from groups I and II were shaved 48 h before being exposed to UVA ray using a Philips Typl HB 171/A facial sun lamp (composed of Philips Cleo 15 w tubes with an emission spectrum of 300–425 nm (maximum 354 nm)). The animals were exposed for 120 min per session (33 weeks, 3 sessions by week, 100 sessions) at a distance of 20 cm from the UVA source. At the end of the experiment the animals were sacrificed and skin samples taken from the back and ears, and viscera. All the tissues were fixed in 10% neutral buffered formaldehyde, included in paraffin and sectioned at 5 μm before being stained with haematoxylin–eosin. The sections were studied by two observers, who evaluated several dermal and epidermal parameters.

3. Results

3.1. Antioxidant activity in aqueous systems: TBARS and TEAC assays

First, the antioxidant activity was studied, especially as RA free radical scavenging activity, using two contrasting systems: the TBARS test, evaluating the linoleic acid peroxidation by measuring of MDA production, and the TEAC method, which is based on the ability of different substances to scavenge the $ABTS^{\cdot+}$ radical cation, compared with a standard antioxidant (Trolox) in a dose-response curve.

The linoleic acid peroxidation was indexed by measuring of MDA production using the TBARS test. Fig. 2 shows the oxidation of linoleic acid with the time, measured by the MDA formation at 532 nm, for different linoleic acid solutions when RA or AA was added in a concentration of 200 mg/kg at 25 °C. MDA formation was delayed when RA was used. A theoretical protection factor for RA was quantified at 21 days (induction time of AA and control), according to its corresponding absorbance at the same time, results in a value higher than 3.92 (3.24 times *vs* AA).

The abilities of RA and AA, as reference compound to scavenge the $ABTS^{\cdot+}$ radical cation in comparison with Trolox under given conditions, are determined. The antioxidant capacity measured as TEAC value (Trolox equivalent antioxidant capacity) for RA in this aqueous system is 1.79 ± 0.08 mM (1.6 times *vs* AA).

3.2. Radioprotective-antimutagenic effects of RA against chromosomal damage induced in human lymphocytes by γ-rays

To determine if the qualified "antioxidant" compound by its antioxidant capacity according to the above *in vitro* chemical models, are really effective against oxidative stress induced by ionizing radiations, we used γ-ray as oxidant agent and measure the genotoxic damage with the micronucleus test.

Fig. 3 shows the influence of treatments before and after γ-ray irradiation on the frequency of MN in irradiated human lymphocytes, together with the data for non-irradiated human lymphocytes, which permits a comparison of the potential genotoxicity

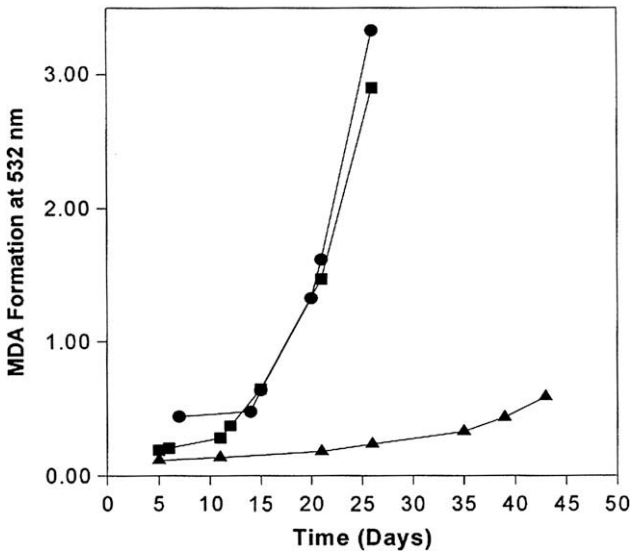

Fig. 2. MDA formation quantified by the absorbance at 532 nm versus time for linoleic aqueous system: (●) Control; (■) added 200 mg/kg of L-ascorbic acid; (▲) added 200 mg/kg of RA.

(non irradiated) of each compound *vs* its antimutagenic capacity (irradiated). In non irradiated human lymphocytes, RA and AA show the same level of MN than control, while the sulphur-compound DMSO shows higher toxicity than others.

In irradiated human lymphocytes, the order of treatments before γ-ray irradiation, from lowest to highest level of MN induced by radiation was: RA [14] < AA [22] < DMSO [28] < Control [32]. In the case of treatments after γ-ray irradiation, RA and AA present a lower degree of radioprotective activity than observed in the treatments before γ-ray irradiation, while the sulphur-containing compound DMSO lacked γ-ray radioprotection capacity; thus, the order of treatments, from lowest to highest level of MN induced by radiation was: RA [23] < AA [25] < DMSO [31] < Control [32].

The radioprotective effects and, consequently, the antimutagenic (or antigenotoxic) activity of the different compounds assayed (before and after γ-ray irradiation), were established by the decrease in MN numbers according to the above equation (Sarma and Kesavan, 1993) (see Material and Methods), obtaining a percentage value that determines the degree of protection of each compound. Fig. 4 shows the values of these protection capacities for treatments before and after γ-ray irradiation.

In addition, we measure the results (level of MN numbers) of antimutagenic (or antigenotoxic) activity, before and after γ-ray irradiation, using different concentrations of RA, 25 and 50 μM. The obtained results were 14 and 23, respectively, for 25 μM, and 13 and 20, for 50 μM.

3.3. Effects of RA on melanogenic enzymes, tyrosinase activities of mouse B16 melanoma cells. Effect of RA on tyrosinase expression

In order to study the mechanism by which the RA induced tyrosinase activity, the expression of this protein was examined in mouse B16 melanoma cells; the tyrosinase activity was studied as the step-limiting rate of the melanogenic pathway of cells treated with this polyphenol. Cells were incubated for 48 h under the following conditions: RA at 100 μM, in the absence of any stimulus as negative control, and with IBMX (high concentration at) 500 μM. Proteins were extracted and analyzed as both tyrosinase (tyrosin hydroxylase and dopa oxidase). As we can see from Fig. 5, RA increased the tyrosinase activity in B16 cells after stimulation lasting 48 h in all of the assays, compared with the negative control values in a 20% in both tyrosinase activities. Statisitical studies shows that RA increases the tyrosinase activitie in a ratio of 1,2. The experiment was realized in six independent assays. The degree of dependence and correlation between variables was assessed using Analysis of Variance.

Our next goal was to study the mechanism by which the RA increased tyrosinase activity. For this, the tyrosinase expression of cells stimulated with RA was investigated. B16 mouse melanoma cells were incubated for 48 hours under the following conditions: RA at 100 μM, and in the absence of any stimulus as negative control and the positive control of the experiment was IBMX (500 μM).

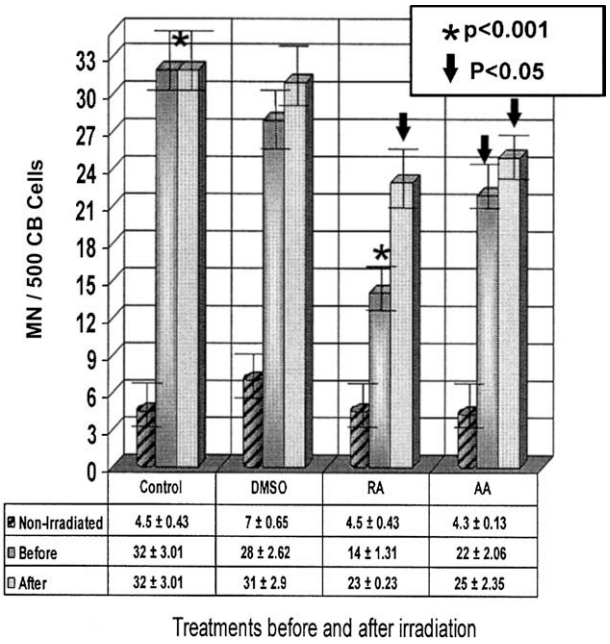

Fig. 3. Influence of treatments administered before and after γ-ray irradiation on the frequency of micronuclei in human lymphocytes.

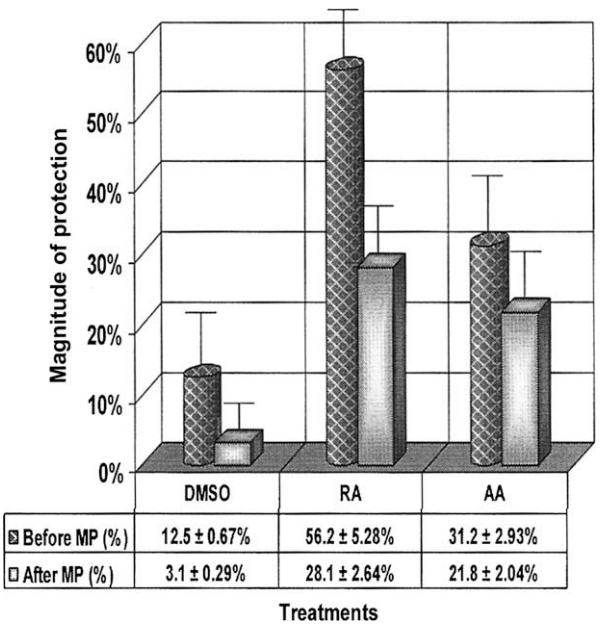

Fig. 4. Magnitude of protection (%) of different treatments administered before and after γ-ray irradiation.

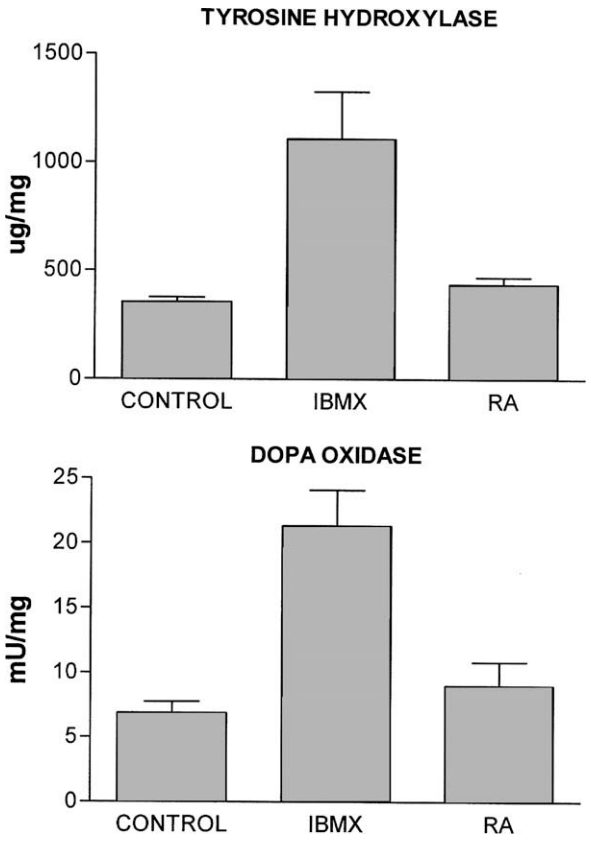

Fig. 5. Increase of tyrosine hydroxylase activity and DOPA oxidase (μU/mg protein) of mouse B16 melanoma cells by RA. B16 mouse melanoma cells were exposed to 100 μM RA for 48 h, and the tyrosine hydroxylase, DOPA oxidase and protein content was analyzed. Results expressed as histograms are the mean of six independent experiments.

Then tyrosinase expression was evaluated by Western-blot analysis. As can be seen in Fig. 6 the level of tyrosinase protein increased when incubated for 48 h with 100 μM RA, so that the increase in tyrosinase activity was related with the tyrosinase expression level in cells stimulated with RA.

3.4. Cutaneous alterations caused by UVA exposure (in vivo)

Since UVA radiation is mainly responsible for photoageing and skin photocarcinogenesis, *in vivo* experiments were carried out in which the capacity of RA orally administered to inhibit such alterations was studied. All the animals of group I exposed to UVA showed severe or moderate dysplasia, while the animals of group II (orally treated with RA) only presented slight dysplasia in 30% of cases (Table 1).

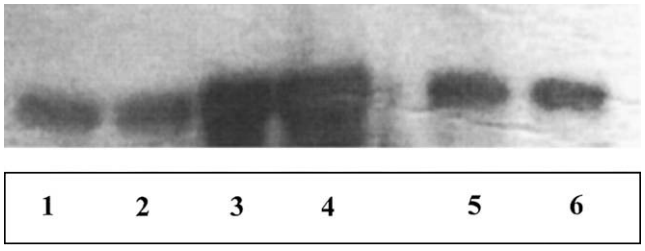

Fig. 6. Western-blot analysis of tyr expression in B16 melanoma cells treated with RA. Tyrosinase was detected with a αpep7 (anti-tyrosinase) as primary antibody. Lanes 1 and 2, Control; 3 and 4, IBMX; 5 and 6, 100 μM RA.

Table 1
Grade of dysplasia (%) of the cutaneous modifications generated by exposition to UVA ray.

Grade	UVA (I)	UVA+RA (II)
Absence	0	70
Light	0	30
Moderate	80	0
Severe	20	0

4. Discussion

It is know that the ROS responsible for cell oxidation processes are the following: singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (**.**OH) and peroxyl radical (R-OO**.**).

The hydroxyl radical is the most cytotoxic of all those so far described, with an estimated half-life of about 10^{-9} s (Diplock et al., 1998). The high reactivity of this radical implies immediate reaction at the place where it is generated. When hydroxyl radical generation is massive, the cytotoxic effect is not only local, but can propagate intra-cellularly and extra-cellularly, increasing the interaction of these radicals with phospholipoid structures and inducing peroxidation processes that increase the hydroxyl radical activity in DNA oxidative damage (Benavente-García et al., 1997; Sáez-Tormo et al., 1994).

Lipid peroxidation may be prevented by free radicals scavengers or by singlet oxygen quenchers. It has been shown that, in TBARS assay, the addition of rosmarinic acid, shows a decrease in oxidation compounds during the autoxidation of linoleic acid. Also, the results obtained on the determination of the ability of RA to scavenge the $ABTS^{\cdot+}$ radical cation, confirm the antioxidant capacity of rosmarinic acid concentrate extract. The presence of two catechol structures, conjugated with a carboxylic acid group is, probably, the most important structural element in the antioxidant activity of this compound (Del Baño et al., 2003).

In vivo, γ-rays cause a massive generation of hydroxyl radicals, by homolytic cleavage of body water or of endogenous hydrogen peroxide (formed by reduction of the superoxide anion) by two mechanisms: the Haber-Weiss and Fenton models. In this study, RA, AA and DMSO were used as radioprotective-antimutagenic 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. DMSO is a classic radical scavenger, with a high capacity as hydroxyl radical scavenger *in vitro*. However, when applied in radioprotective doses, in the absence of any subsequent irradiation, it is highly toxic in animals (Murray et al., 1988; Weiss et al., 1990).

The results obtained concerning radioprotective effects (antimutagenic activity) for RA were close to those reported by other authors on its antioxidant activity (Del Baño et al., 2003; Frankel et al., 1996; Aruoma et al., 1996; Farag et al., 1989). Obviously, the degree of RA effectiveness depends on its structure. It is know that the capacity to scavenge hydroxyl radical is principally based on the combination of conjugated structures in the polyphenolic skeleton, mainly the o-dihydroxy-phenol or catechol structure; thus, the presence in RA of two catechol groups conjugated with a carboxylic acid group, increases its antioxidant activity in aqueous media. In fact, according to the above mentioned structural considerations, the antimutagenic activity of RA given before γ-irradiation is consistent with its antioxidant properties and specific activities as free radical scavenger. These data also confirm the higher antimutagenic activity of RA than those of AA and DMSO.

When the phenolics were added after γ-ray irradiation, the ROS present in the cells, according to the half-life of superoxide anion and hydroxyl radicals (Diplock et al., 1998), were lipoperoxy (R-OO**.**) and other radicals (R**.**, RO**.**,. . .), 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. Also, lipooxygenase and cyclooxigenase are involved in other processes (endoperoxide formation, prostaglandins, leukotrienes, etc). In these complex oxidative stress conditions, is very difficult to make a structural linear evaluation of the experimental data obtained for the antimutagenic activity measured in different post-irradiation treatments, however, some considerations are possible. The results obtained in the post-irradiation treatments show that RA, a watersoluble compound, presents a significant decrease of its radioprotective-antimutagenic activity, showing similar results to AA, while DMSO lacked γ-ray radioprotection capacity.

According these data *in vitro*, and considering recent studies that confirm the presence of free rosmarinic acid in human plasma after oral intake, RA could be use *in vivo* as photo-protective agent, but it is also very important to determine how this antioxidant compound could affect melanogenesis, because a proper photoprotector should not downregulate melanin biosynthesis.

The effect of some botanical extracts, such as the medicinal extract of *Ephedra herba,* on melanogenesis has been studied (Kim et al., 2006). It was observed that ephedra extract decreases tyrosinase activity and melanin content in a dose-dependent manner in B16 murine melanocytes. Other studies showed that tyrosinase, the rate-limiting enzyme in melanin biosynthesis, is inhibited by green tea components (No et al., 1999). On the other hand, the citrus flavonoid naringenin stimulates melanogenesis in mouse B16 melanoma cells (Ohguchi et al., 2006).

In this paper, it was demonstrated that tyrosinase activity and expression increased in mouse B16 melanoma cells after 48 h stimulation with RA compared with the level of the negative control. In fact, B16 melanoma cells pellets from samples treated with RA, upon visual inspection, have darkness colour than the negative control by the increased melanin content.

On the other hand, the generation of ROS and/or the resulting increase in lipid peroxidation products have been proposed as etiological factors for de-pigmentary natural processes such as hair greying (Nordlund and Abdel-Malek, 1988). Moreover, in mammalian skin, H_2O_2 is formed as a by-product of melanin synthesis, and following UV radiation during the oxidation of melanogenic precursors DHICA and DHI (Nappi and Vass, 1996), and H_2O_2 production by human melanoma cells in culture has also been observed (Bittinger et al., 1998). Also it was demonstrated that the activity of the rate-limiting melanogenic enzyme, tyrosinase, decreases in melanoma cells, treated with H_2O_2 (Jiménez-Cervantes et al., 2001). This inhibition was concentration and time-dependent in the B16 melanoma. As mentioned above, RA is able to scavenger free radicals. So, RA may neutralize the ROS generated in melanogenesis and its downregulatory effects on tyrosinase in mouse B16 melanoma cells, producing a stimulatory effect on melanogenesis. Therefore, RA has a certain stimulatory effect on melanogenesis and can be proposed as a proper photo-protective.

As mentioned above, exposure to UVA is known to generate ROS in the skin of animals, which in turn induces skin photodamage and photoageing, leading to extensive cellular damage and cell death either by apoptosis or necrosis. One approach to protecting human skin against the harmful effects of UVA radiation is by using herbal compounds as photoprotectants. Our study has evaluated the photo-protective effects of RA against UVA-induced skin damage. Exposure to UVA provoked moderate to severe dysplasia. RA acted as protector against UVA-caused lesions. These results suggest that skin damage by UVA-induced ROS generation is reduced by oral supplementation of rosmarinic acid extract, which has a scavenging and quenching activity against ROS. This result agrees with the studies of Yamada et al. (2006) concerning the oral supplementation of caffeic acid.

In other studies (Psotova et al., 2006), when human keratinocytes exposed to UVA (10–30 J/cm^2) were treated with pure rosmarinic acid (0.9–18 mg/l) or a rosmarinic acid extract (1–75 mg/l) for 4 h, both exhibited the ability to partially mitigate the UVA-caused decrease in a cell viability, as monitored by neutral red retention and by LDH released into the medium. Also, significantly suppressed UVA-induced ROS production, which manifests itself as a decrease in intracellular lipid peroxidation, increased ATP and reduced glutathione. In addition, the UVA-induced activation of caspase-3 was inhibited by treatment with rosmarinic acid. Some *Lamiaceae* plant extracts produced a marked reduction in tumorigenesis in a murine, two-stage skin carcinogenesis model (Osakabe et al., 2004). These authors conclude that part of the anticarcinogenic effects of these extracts is due to rosmarinic acid acting via two independent mechanisms: the inhibition of the inflammatory response and the scavenging of reactive oxygen radicals.

In relation with other possible mechanisms, Offord et al. (2002) recently studied the photo-protective potential of several dietary antioxidants, using human dermal fibroblasts exposed to UVA light. The authors suggested as a possible protection mechanism the influence that these phenolics have on the metalloproteinase 1 (MMP-1) mRNA activity. UVA irradiation of human fibroblasts led to a 10–15-fold increase in MMP-1, which was suppressed in the presence of low concentrations of different phenolics.

Obviously, the mechanism by which the RA extract studied acted as photo-protector in this experiment would be due to its antioxidant and, probably, anti-inflammatory properties, which are not only related with its free radical scavenging capacity, but also with the capacity to regulate certain enzymatic activities involved in these processes.

Our results suggest that concentrated rosmarinic acid extracts, from rosemary leaves, could favourably supplement protection against skin disorders caused by solar radiation by offering protection against UV-induced oxidative stress. They may be beneficially included in orally administered and dermatological photo-protective preparations. New experiments are in progress to study the specific and synergic antioxidant, antimutagenic and photo-protective activities of rosmarinic acid extracts. 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 are being studied using in vivo systematic treatment models. In addition, the apparent null toxicity of this concentrate rosmarinic acid extract, according to verified tests widely used in the food product industry (status GRAS), demonstrates their potential as functional, nutraceutical and pharmaceutical agent.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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