Antioxidant Activity and Radioprotective Effects against Chromosomal Damage Induced in Vivo by X-rays of Flavan-3-ols (Procyanidins) from Grape Seeds (*Vitis vinifera*): Comparative Study versus Other Phenolic and Organic Compounds

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The quantitative distribution of several flavan-3-ols was determined using HPLC in a grape (*Vitis vinifera)* seed extract (GSE) of four cultivars grown in the region of Murcia. Polymer ≥ C_4 units made up the largest group of procyanidins in the GSE (90.92%, expressed as HPLC % area). The antioxidant activity of GSE and other reference compounds was investigated by measuring their ability to scavenge the $ABTS^{\bullet+}$ radical cation (TEAC). The most effective compounds were, in order: GSE > rutin > (+)-catechin > diosmin ≥ ascorbic acid. The radioprotective effects of GSE and other reference compounds were determined by using the micronucleus test for anticlastogenic activity, any reduction of the frequency of micronucleated polychromatic erythrocytes (MnPCEs) being evaluated in the bone marrow of mouse exposed to X-rays. The most effective compounds were, in order: GSE > rutin > dimethyl sulfoxide (DMSO) > ascorbic acid > 6-*n*-propyl-2-thiouracil-6c (PTU) > diosmin. The higher $ABTS^{\bullet+}$ scavenging capacity and anticlastogenic activity of GSE can be explained, structurally, by the high number of conjugated structures between the catechol groups in the B-rings and the 3-OH free groups of the polymeric polyphenolic skeleton and, in addition, by the stability of the aroxyl flavonoid radical generated in the above processes.

Keywords: *Vitis vinifera; flavan-3-ols; procyanidins; radical scavenger; antioxidant; ABTS radical; X-irradiation; radioprotection; anticlastogen*

INTRODUCTION

The mature seeds of *Vitis vinifera* are an important natural source of oligomers and polymers of catechin and epicatechin, which are also denominated procyanidins. Several reports describe the catechin, epicatechin, and general procyanidin content of mature grape seeds from several countries: Spain (Escribano-Bailón et al., 1992a,b; Santos-Buelga et al., 1995), North America (Fuleki and Ricardo da Silva, 1997), France (Bourzeix et al., 1986a,b; Romeyer et al., 1986; Dumon et al., 1991; Ricardo da Silva et al., 1991a, 1992a), Portugal (Ricardo da Silva et al., 1992b), and Morocco (Hmamouchi et al., 1994).

Procyanidins are constituted by a variable number of flavan units regularly linked by C_4-C_6 or C_4-C_8 bonds and may be present in the grape seed extracts in a mixture formed by dimers, trimers, tetramers, polymers up to 15−16 units (Prieur et al., 1994) and small amounts of catechin and epicatechin (Ricardo da Silva et al., 1990, 1991c; Bombardelli and Morazzoni, 1995). The distribution and the absolute content of procyanidins in grape seed extracts principally depend on the raw material used, but they can be changed and/or modified by the procedures used to extract these polyphenols, as well as in subsequent purification and/or crystallization operations. It is important to note the relation that exists between the molecular profile of procyanidin compounds and their physical properties and their biological and medical activities. Furthermore, it is not clearly agreed upon what size oligomer or polymer is responsible for which degree of biological activity. Some sources agree that only the dimers and trimers provide any benefit to health, while others maintain that larger polymers may also be linked to the healthful properties of *V. vinifera* seed extracts (Masquelier, 1988; Facino et al., 1994; Vennat et al., 1994; Gali et al., 1994; Jimenez-Ramsey et al., 1994; Amoroux et al., 1998; Saito et al., 1998).

Procyanidins from *V. vinifera* seeds are valued as therapeutic agents in the treatment of vascular disorders, such as collagen instability in the arterial wall, arterially localized histamine formation and cholesterol oxidation (Masquelier, 1988; Zafirov et al., 1990; Jimenez-Ramsey et al., 1994; Maffei Facino et al., 1996). They show antiinflammatory (Gábor, 1986), antihypertensive (Terencio et al., 1991), antiviral (Takechi et al., 1985; Amoroux et al., 1998), spare vitamin C and E (Masquelier, 1988; Maffei Facino et al., 1998), and antimutagenic (Liviero et al., 1994; Gali et al., 1994)

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activities and inhibit some undesirable enzymatic activities (Maffei Facino et al., 1994; Wang et al., 1996).

The greater part of these health-related properties of polyphenolic compounds is based on their antioxidant activity (antioxygen, antiradical, antilipoperoxidant, and metal-chelating activities) (Bombardelli and Morazzoni, 1993), which is principally related to their structural characteristics (Benavente-García et al., 1997). The procyanidins of *V. vinifera* show high antioxidant activity (Uchida et al., 1987; Ozaki et al., 1990; Ariga and Hamano, 1990; Elstner and Kleber, 1990; Ricardo da Silva et al., 1991b; Arpentine et al., 1992; Frankel et al., 1993; Maffei Facino et al., 1994; Tanahashi et al., 1995; Shimoi et al., 1996; Liu et al., 1998).

The main objectives of the present work were as follows: (i) to obtain a global grape seed extract representative of the characteristic *V. vinifera* varieties of the region of Murcia and to characterize and quantify of the different flavan-3-ols therein, (ii) to describe the antioxidant activities of this procyanidin extract compared with those of several flavonoids widely used as pharmaceuticals and other reference compounds through the extent of their abilities to scavenge the $ABTS^{\bullet+}$ radical cation and clarifying the structural elements that confer this antioxidant capacity in an aqueous system, and (iii) to study the radioprotective effects of this extract against to chromosomal damage induced by X-ray. The reactive oxygen species (ROS) are formed by a sequential electron reduction mechanism, which may arise from causes that are endogenous or exogenous to the medium under consideration, among the exogenous causes being ionizing radiation. It is known that X- and γ-rays generate hydroxyl radicals in organisms and induce cellular DNA damage which may lead to mutations and chromosomal aberrations (Kasai et al., 1986; Riley, 1994; Komatsu et al., 1994; Shimoi et al., 1996). Recently, reports have been published on the scavenging ability of certain tea extracts containing several polyphenols and catechins against active oxygen species and the inhibitory effects they have on X-ray induced cell transformations (Yoshikawa et al., 1990; Komatsu et al., 1994). To further this line of investigation, in vivo radioprotective activity of the grape seed extract obtained and other reference compounds was investigated using X-rays as oxidative DNA damaging agent and evaluating any reduction in the frequency of micronucleated erythrocytes (polychromatic erythrocytes of mouse bone marrow) in mouse exposed to X-ray**.** The micronucleus assay on mouse bone marrow polychromatic erythrocytes, originally developed by Schmid (1975), is probably the most frequently used in vivo short-term genotoxicity test. Bone marrow micronucleated erythrocytes provide a simple and rapid method for the detection of chromosomal damage by chemical and physical agents (Heddle and Salamone, 1981; Almassy et al., 1987; Mavournin et al., 1990; Krishna et al., 1992; Mazur, 1995). The relationship between antioxidant and anticlastogenic activities is discussed.

MATERIALS AND METHODS

Plant Material. Four different varieties of *V. vinifera* grapes were selected during the 1998 harvest in different areas of the community of Murcia (Spain): "Macabeo" and "Airen" are white grapes, and "Tempranillo" and "Monastrel" are red grapes. The grapes were picked at their optimum commercial maturity when enologically ripe. The seeds were removed manually until 1000 g of seeds from each cultivar had been collected. The seeds were air-dried at 30 °C for 24 h and stored at −30 °C until use and analysis. The frozen seeds of the four varieties were mixed and pulverized in a Waring blender.

Chemicals. (+)-Catechin, (−)-epicatechin, gallic acid, rutin, and diosmin were obtained from Extrasynthèse S. A. (Genay, France). Ascorbic acid (AA), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt), Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid), manganese dioxide, fetal calf serum, and 6-*n*-propyl-2-thiouracil-6c (PTU) were obtained from Sigma Chemical Co. (Madrid, Spain). DMSO was obtained from Merck (Darmstadt, Germany).

Extraction and Purification of Polyphenolic Compounds (Flavan-3-ols). Polyphenolic compounds (flavan-3-ols) were extracted from ground grape seeds (4000 g) with 75% aqueous methanol in a ratio of 100 g/L, under N_2 for 1 h. After filtration, the extraction solvent was evaporated under vacuum at 40 °C until the methanol was removed. The aqueous medium was washed with *n*-hexane to remove liposoluble substances and the remaining solvent was removed by evaporation. The flavan-3-ols were fixed on an Amberlite XAD 2 column and eluated with methanol for desorption of polyphenols. The methanol was removed under vacuum at 40 °C. In this way, 24 g of crystalline grape seeds extract powder (GSE) was obtained (0.6% w/w).

HPLC Chromatographic Analysis of *V. vinifera* Seed Extract (GSE). The solid obtained was dissolved in methanol in the ratio 3 mg/mL 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 280 nm.

A modification of the method described by Ricardo da Silva et al. (1990) was used for the HPLC separation of different flavan-3-ols present in the polyphenolic extract (GSE). The mobile phases for chromatographic analysis were (A) water, (B) acetic acid/water (10:90) and (C) methanol/acetonitrile (50:50). A linear gradient was run from 90% (A), 10% (B) to 30% (A), 70% (B) for 45 min, changed to 22% (A), 78% (B) in 15 min (total 60 min); changed to 100% (B) in 10 min (total 70 min) and changed to 100% (C) in 5 min (total 75 min), this isocratic composition was maintained for 10 min (total 85 min); and then reequilibrated in 10 min (total 95 min) to the initial composition.

Antioxidant Capacity ($ABTS^{\bullet+}$). The antioxidant capacity was measured using the method of Miller et al. (1996), which is based on the ability of different substances to scavenge the $ABTS^{\bullet+}$ radical cation, compared with a standard antioxidant (Trolox) in a dose−response curve.

$ABTS^{\bullet+}$ 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 preincubated at 30 °C prior to use. Fresh $ABTS^{\bullet+}$ 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.

All compounds were dissolved in DMSO to a concentration of 50 μM. After addition of 1 mL $ABTS^{\bullet+}$ solution to aliquots of Trolox or of the phenolic and reference compounds (1 to100 μL, depending on the activity of the particular compound), 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 and DMSO blanks were run in each assay. 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 activities of several compounds were assayed at four different concentrations, which had been

determined to be within the range of the dose−response curve. Each compound was analyzed 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 each compound.

The molecular mass of GSE was determined using the ponderated mean of molecular mass of different compounds (monomers, dimers, trimers, and polymers longer than trimers) present in GSE. Polymers longer than trimers are considered globaly as a hexamer, in accordance with Bombardelli and Morazzoni (1995).

Animals. The adult male Swiss mice used in the experiments weighed between 27 and 30 g. All mice were acclimatized for at least 1 week prior to dosing in constant environmental conditions with a 12/12 h light/dark cycle. They were fed standard granulated chow and given drinking water ad libitum. Each group consisted of five to seven mice.

Chemicals and Treatment. The substances were administered orally. All the solutions were freshly prepared immediately before treatment of the animals. *V. vinifera* extract (GSE), PTU, and ascorbic acid were dissolved in 0.2% drinking water and administered for 5 days before the X irradiation. DMSO was dissolved in water (50 g/100 mL). Rutin and diosmin were dissolved in DMSO (300 mg/mL). DMSO, rutin and diosmin were injected in a single dose of 0.6 mL directly into the lumen gastric 6 h before the X-irradiation.

Exposure to X-rays. The mice were whole-body X-irradiated using CGR apparatus with radioscopy (General Electric, Spain). During exposure to X-rays, the animals were placed in a well-ventilated acrylic box. Irradiation conditions: 120 kV, 1.4 mA, filter 2.5 mm Al, exposure rate of 2 cGy/min, target distance 100 cm. The mice were exposed to a single dose of 48 cGy, and X-ray exposure was measured by means of a thermoluminiscence dosimeter GR-200 (TLD) (China).

Bone Marrow Preparation and Staining. The animals were killed by cervical dislocation about 24 h after X-irradiation, and bone marrow samples were taken. Two femurs were removed from each mouse. Both the proximal and distal ends of the femur were cut off and the bone marrow cells were gently flushed out with fetal calf serum. These cells were dispersed by gently pipetting and collected by centrifugation at 1000 rpm for 5 min at 4 °C. Cell pellet was resuspended in a small volume of fetal calf serum and bone marrow smears (two slides per mouse) were prepared. The slides were coded to avoid observed bias. After 24 h air-drying, the smears were stained with May-Grünwald/Giemsa (Schmidt, 1975; Pascoe and Gatehouse, 1986). With this method polychromatic erythrocytes (PCEs) stain reddish-blue and normochromatic erythrocytes (NCEs) stain orange, while nuclear material is dark purple.

Slide Analysis. The number of micronucleated polychromatic erythrocytes (MnPCEs) in 1000 PCEs per mouse (500 PCEs per slide) was determined. The slides were examinated at 1000 × magnification using a Zeiss light microscope (Oberkochen, Germany).

Statistical Evaluation. Differences in the incidence per animal of MnPCEs and PCEs per 100 erythrocytes (PCEs + NCEs) were compared by analysis of variance

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

where F_{control} = frequency of MnPCEs in irradiated animals and F_{treated} = frequency of MnPCEs in animals treated before the X-ray irradiation (diosmin, rutin, ascorbic acid, GSE, DMSO, and PTU groups) (Sarma and Kesavan, 1993).

RESULTS AND DISCUSSION

HPLC Polyphenolic Distribution in *V. vinifera* Seed Extract (GSE). Spectral Characteristics of the Main Flavan-3-ols. Figure 1 shows a characteristic HPLC chromatogram of GSE in which different flavan-3-ols and gallic acid were identified. The relative distribution (normalized area values) of the main compounds numbered (1−11) are summarized in Table 1. Figure 2 shows the normalized absorption spectra of several of these compounds (diode array spectra in the elution solvent of Figure 1). They show a characteristic maximum at 280 nm, with different extinction coefficients as a function of their molecular weight. This spectral distribution is consistent with compounds that have a flavan-3-ol skeleton. In this grape seed extract, polymer ≥ C_4 units formed the largest group of (90.92%). As regards the other procyanidins, monomers were found to be the most abundant flavan-3-ol, with (+)-catechin (2.11%) being more abundant than (−)- epicatechin (1.06%). The other procyanidins present in relatively large quantities in this extract were dimers, in which the elemental units were bound principally by a type C_4−C_8 interflavan bond: these were B4 (0.96%), B3 (0.71%), B1 (0.52%), B2 (0.48%), and the 3-O-

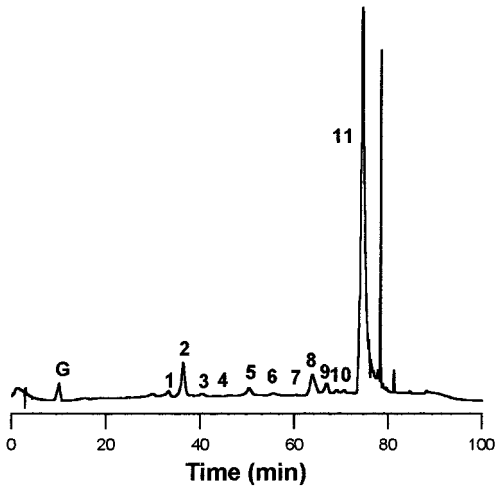

Figure 1. HPLC chromatogram of *V. vinifera* seed extract (GSE), using a C_{18} Lichrospher 100 analytical column (250 × 4 mm i.d.), with a particle size of 5 μm, thermostated at 30 °C. The flow rate was 1 mL/min, and the absorbance changes were monitored at 280 nm. Peak identification (t_R, min) reference data (Ricardo da Silva et al., 1990; Santos-Buelga et al., 1995; Fuleki and Ricardo da Silva, 1997): G, gallic acid; 1, B3 (34.3); 2, (+)-catechin (37.6); 3, B1 (40.6); 4, T2 (45.4); 5, B4 (49.9); 6, B2 (54.0); 7, B2-3′-*O*-gallate (59.1); 8, (−)-epicatechin (62.9); 9, B1-3-*O*-gallate (64.4); 10, C1 (67.4); 11, procyanidins ≥ C_4 units (74.4).

Table 1. HPLC Relative Distribution (Normalized Area Values) of the Main Flavan-3-ols Present in *V. vinifera* Extract (GSE)[a]

peak	t_R (min)	flavan-3-ols	% rel value
G	9.7	gallic acid	0.83
1	34.3	B3 (dimer)	0.71
2	37.6	(+)-catechin	2.11
3	40.6	B1 (dimer)	0.52
4	45.4	T2 (trimer)	0.11
5	49.9	B4 (dimer)	0.96
6	54.0	B2 (dimer)	0.48
7	59.1	B2-3′-*O*-gallate	0.16
8	62.9	(−)-epicatechin	1.06
9	64.4	B1-3-*O*-gallate	0.52
10	67.4	C1 (trimer)	0.20
11	74.4	polymer procyanidins ≥ C_4 units	90.92
others	[20−80]	other dimer and trimer flavan-3-ols	1.40

[a] Peak identification according to reference data (Ricardo da Silva et al., 1990; Santos-Buelga et al., 1995; Fuleki and Ricardo da Silva, 1997)

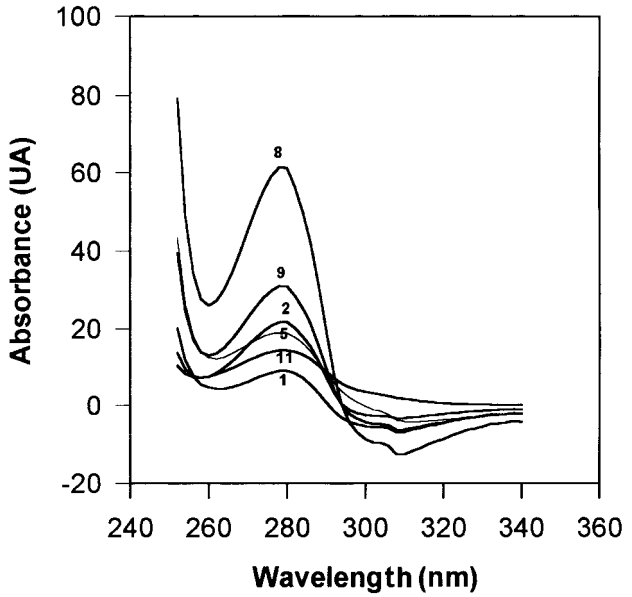

Figure 2. Normalized UV spectra (in the elution solvent of Figure 1, on the diode array detector) of the main procyanidins present in the *Vitis vinifera* seed extract (GSE): 1, B3; 2, (+)-catechin; 5, B4; 8, (−)-epicatechin; 9, B1-3-*O*-gallate; 11, procyanidins ≥ C_4 units.

Table 2. Antioxidant Activity: ABTS$^{\bullet+}$ Radical Cation Scavenging Ability

compound	TEAC (mM)
rutin	2.75 ± 0.05
diosmin	1.14 ± 0.09
ascorbic acid	1.12 ± 0.06
(+)-catechin	1.37 ± 0.07
GSE	8.21 ± 0.16

galloylated derivative of dimer B1 (0.52%). All compounds were identified by comparison with the respective references (Ricardo da Silva et al., 1990; Santos-Buelga et al., 1995; Fuleki and Ricardo da Silva, 1997) and their quantitative values are expressed as HPLC % area.

The compounds included in the peak at t_R 74.4 min (number 11) are tetramers and polymers longer than tetramers; new findings are in progress in an attempt to isolate and identify each of the polymeric polyphenolic structures present in this procyanidin fraction.

Antioxidant Activity: ABTS$^{\bullet+}$ Scavenging Capacity. The abilities of GSE, (+)-catechin, the most abundant monomer flavan-3-ol in *V. vinifera* extracts, diosmin (flavone) and rutin (flavonol), the two flavonoids most widely used as pharmaceuticals, and ascorbic acid (AA) as reference compound to scavenge the ABTS$^{\bullet+}$ radical cation in comparison with Trolox under given conditions are shown in Table 2. This table shows that the most effective monomeric-flavonoid compound in ABTS$^{\bullet+}$ radical cation scavenging was the flavonol rutin (quercetin-3-*O*-rhamnoglucoside). These results confirm the importance of the 2,3-double bond (rutin, diosmin) in conjugation with a 4-oxo function (rutin, diosmin), the presence of a free or glycosylated 3-hydroxyl group (catechin, rutin) and the catechol structure (*o*-dihydroxy) in the B-ring (rutin, catechin). It is known that the methylation of the 4′-hydroxyl group in the B-ring (diosmin) significantly reduces the antioxidant ability of flavonoids, because their electron donor capacity with respect to free hydroxyl group is reduced (Chen et al., 1996; Benavente-García et al., 1997). The absence of catechol structure (diosmin) had a greater effect on the scavenging of the ABTS$^{\bullet+}$ radical cation than the lack of the 2,3-double bond and 4-oxo function (catechin). Ascorbic acid showed a scavenging capacity similar to that of diosmin.

GSE showed the highest ABTS$^{\bullet+}$ scavenging capacity of all the compounds tested, due to its high content of polymeric flavan-3-ol structures, that permit the existence of a high number of conjugated structures between the 3-OH groups and the catechol groups (*o*-dihydroxy) in the B-ring, despite the absence of 2,3-double bonds conjugated with the 4-oxo function. In addition, the presence of many C_4-C_8 linkages structurally increased the electron dislocation capacity of GSE and, consequently, its free radical scavenging capacity.

These results confirm the importance of the free 3-OH group associated with the catechol structure in the B-ring for maximal ABTS$^{\bullet+}$ radical scavenging capacity and strongest radical absorption (Bors et al., 1990a; Bors et al., 1990b; Salah et al., 1995; Miller et al., 1996; Rice-Evans and Miller, 1996). Such activity is higher in GSE because of the high number of associations between the above two structures, which permit the stabilization of the aroxyl radical through electron dislocation between the A, B and C rings. This generates multiple mesomeric structures after hydrogen donation to the ABTS$^{\bullet+}$ radical cation.

X-ray Radioprotective Effects. Anticlastogenic Activity. X-rays caused a high degree of in vivo hydroxyl radical generation by homolytic cleavage of body water or from the endogenous hydrogen peroxide formed by reduction of the superoxide anion by the Haber−Weiss or Fenton mechanism (Diplock et al., 1998). The hydroxyl radical is the most cytotoxic radical of all those so far described, with an estimated half-life of about 10^{-9}s (Diplock et al., 1998). Its high reactivity leads to an immediate reaction at the place where it is generated. When hydroxyl radical generation is massive, as it is during X-irradiation, the cytotoxic effect is not merely local but may result in intracellular and extracellulary propagation. This increases the interaction of these radicals with phospholipid structures, inducing peroxidation processes that increase hydroxyl radical activity in DNA oxidative damage (Sáez-Tormo et al., 1994; Benavente-García et al., 1997).

In these oxidative stress conditions, when 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 (Benavente-García et al., 1997). In the present study GSE, ascorbic acid (AA), DMSO, PTU, and the flavonoids rutin and diosmin were used as radioprotective agents. All these compounds have been shown to protect small rodents from the effects of total body X-irradiation when they are injected or ingested before X-ray exposure. (+)-Catechin was not used according to bibliographic references respect to its lower hydroxyl radical scavenging activity than rutin and diosmin (Cillard and Cillard, 1988; Darmon et al., 1990).

Vitamin C is considered to be one of the most powerful and least toxic natural antioxidants; it is water-soluble and is found in high concentrations in many tissues. On interaction with ROS, it is oxidized to dehydroascorbate via the intermediate ascorbyl free radical and recycled back to ascorbic acid by the enzyme dehydro-ascorbate

reductase. As a scavenger of oxygen radicals it has been shown to be effective against all the above-mentioned species.

Various mechanisms or combinations thereof have been proposed to explain the radioprotective role of thiols: at the molecular level, by free radical scavenging hydrogen donation, binding to critical biological targets and mixed disulfide formation; at the biochemical-physiological level, by hypoxia, biochemical shock and hypothermia, and at the organ level, by stimulation of the recovery of cell populations (Mazur, 1995). DMSO and PTU can be considered as radioprotective agents according to structural and experimental data (Brown et al., 1982). PTU and other thiouracil compounds, particularly, have been shown to have a chemical radioprotective effect against X-irradiation on the thyroid gland of rat, acting through inhibition of thyroid peroxidase with a consequent diminution of oxygenation and thyroid metabolism (Greig, 1969). DMSO is a classic radical scavenger, with a capacity for in vitro hydroxyl radical scavenging higher than that shown by many flavonoids: 30% higher than quercetin (aglycon of rutin), 40% higher than diosmin and 50% higher than (+)-catechin (Cillard and Cillard, 1988; Darmon et al., 1990). However, when applied in radioprotective doses, in the absence of any subsequent irradiation, these S-containing compounds are highly toxic in animals (Murray et al., 1988; Weiss et al., 1990; Mazur, 1995).

Flavonoids are excellent hydroxyl scavengers (Pincemail et al., 1985; Cillard and Cillard, 1988; Darmon et al., 1990; Benavente et al., 1997; Diplock et al., 1998), their effectiveness obviously depending on their structure. It is known that this capacity to inhibit hydroxyl radical is based principally on the binary substitution model in the B-ring (*o*-dihydroxy or catechol structure) and, to a lesser extent, on the presence of a 3-OH group in the flavonoid skeleton (Benavente-García et al., 1997). This greater activity of compounds with a catechol structure can be attributed to the stability of the flavonoid radical generated in the process. The decay rate constants of flavonoid aroxyl radicals generated by their interrelation with other radicals shows that all the most stable aroxyl radicals, without exception, contain the 3′,4′-catechol B-ring substitution pattern. All other polyphenolic compounds form far less stable aroxyl radicals (Bors et al., 1990b; Benavente-García et al., 1997).

Our results confirm these previous findings. Figure 3 shows the influence of treatment on the frecuencies of MnPCEs in the bone marrow of nonirradiated and irradiated animals, permitting a comparison of the potential toxicity of each treatment vs their anticlastogenic activity. AA, diosmin, rutin and GSE show very low levels of MnPCEs generation with respect to control while the sulfur-containing compounds, DMSO and PTU, present higher toxicity levels than the other compounds studied. Figure 4 shows specifically the influence of X-ray irradiation on the frecuencies of MnPCEs in mouse bone marrow. The order of treatments with respect to MnPCEs generation after irradiation is: GSE < rutin < DMSO < AA < PTU < diosmin. The radioprotective effect and, consequently, the anticlastogenic activity of the different treatments used was established according to the increase in MnPCEs in animals after irradiation and a comparison with the levels observed in control animals. This gave percentile value that reflected the degree of protection offered by

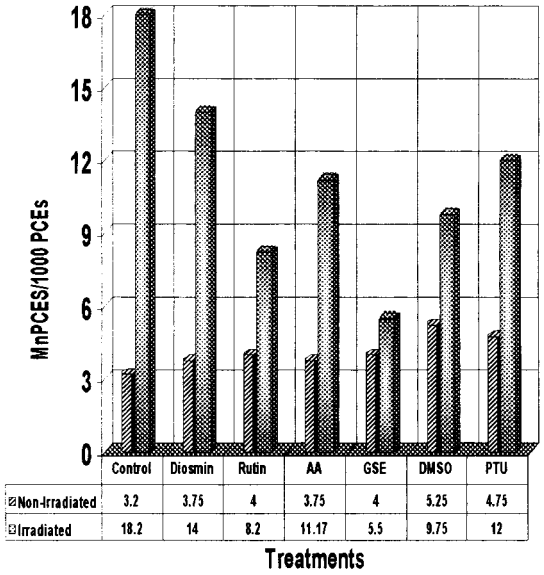

Figure 3. Influence of treatments on the frequency of MnPCEs in mouse bone marrow (irradiated and nonirradiated).

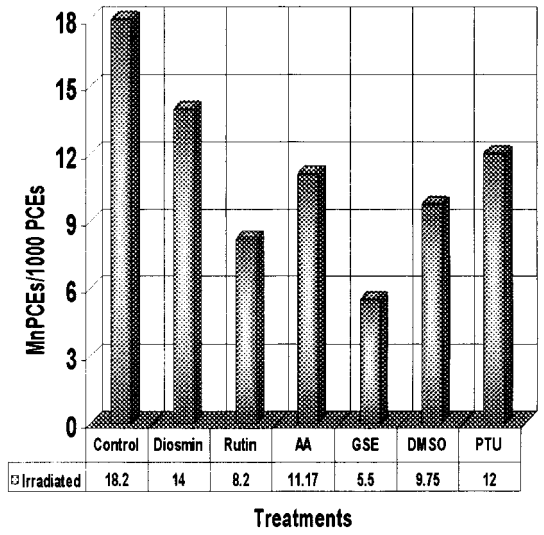

Figure 4. Influence of X-ray irradiation on the frequency of MnPCEs in mouse bone marrow.

each treatment. Figure 5 shows the values of these protection capacities, the order being: GSE > rutin > DMSO > AA > PTU > diosmin.

Relation between Antioxidant and Anticlastogenic Mechanisms. According to the structural considerations mentioned above, the results obtained in the measurement of the anticlastogenic activity of the tested compounds are consistent with their specific activities as hydroxyl radical scavengers, and are in line with their respective capacities to scavenge the $ABTS^{\bullet+}$ radical cation. It is clear that the high degree of polymerization of GSE arising from linkages between (+)-catechin and (−)-epicatechin monomers, whether free or esterified with gallic acid, allows the existence of a conjugate structure spatially very close, with a high number of catechol groups. As a result, that, when GSE operates as free radical scavenger, the aroxyl radical formed is more stable than that generated by the other polyphenolic compounds. In addition, these B-ring catechol structures in conjugation with 3-OH free groups contribute to superoxide anion scavenging and metal chelating activities, and increase the antioxidant capac-

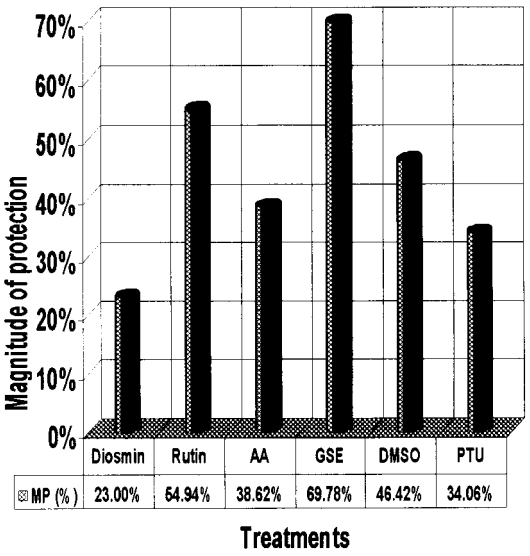

Figure 5. Magnitude of protection of different treatments in relation to X irradiation.

ity of GSE. Consequently, their antioxidant capacity is higher than that of rutin, the most active of the monomers assayed, and much higher than that of diosmin, which has no B-ring catechol configuration (the 4′ hydroxyl group of the B-ring is methylated). Ascorbic acid shows a similar capacity to diosmin for scavenging $ABTS^{\bullet+}$ radical cation, but a greater radioprotective activity. The data for GSE, DMSO, and PTU confirm the higher antioxidant and anticlastogenic activities of flavonoid skeletons with a B-ring 3′,4′-catechol structure associated with the 3-hydroxyl group than those of sulfur-containing compounds, thiol and aminothiol functional groups. In addition, their apparent lack of toxicity, according to verified tests, increases the potential interest of these polyphenolic polymers as nutraceutical and pharmaceutical agents.

New findings are in progress to isolate and identify the different polymers present in the fraction $\geq C_4$ units. Their antioxidant and anticlastogenic activities are being studied, as are systematic treatment models, involving anti- and post-X-ray irradiation treatment and the dose−response curves of several GSE compounds.

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