RADIOPROTECTIVE EFFECTS OF FLAVONOIDs AGAINST TO CHROMOSOMAL DAMAGE: RELATION BETWEEN THE STRUCTURE AND ACTIVITY.

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Abstract. The polyphenolic distribution of Vitis vinifera seeds (GSE), Citrus spp. fruits (CE) and Olea europaea L. leaves (OL) extracts were characterized and quantified by HPLC methods. Radioprotective effects of these extracts and the reference flavonoids diosmin and rutin, widely used as pharmaceuticals, were determined by using the micronucleus test for anticlastogenic activity, evaluating the reduction of the frequency of micronucleated polychromatic erythrocytes (MnPCEs) in bone marrow of mouse exposed to X-rays. The most effective compounds were, in order: GSE > CE > rutin > dimethylsulphoxide (DMSO) > OL > diosmin. These results show that the free radicals scavenging capacity, mainly vs hydroxyl radical, and consequently, the anticlastogenic activity of these polyphenolic compounds are based, structurally, on the presence of specific functional groups in their structures, mainly the B-ring catechol, the 3-hydroxyl group and the 2,3-double bond conjugated with 4-oxo function and, in addition, by the stability of the aroxyl radical generated in the processes.

1. Introduction
It is widely accepted that fruits and vegetables have many healthful properties. There is a considerable amount of epidemiological evidence revealing an association between those who have a diet rich in fresh fruit and vegetables and a decrease risk of cardiovascular disease and certain forms of cancer [1]. It is generally assumed that the active dietary constituents contributing to these protective effects are the antioxidant nutrients, although more recent work has highlighted the additional role of the polyphenolic components of the higher plants [2], which may act as antioxidants or agents of other mechanisms that contribute to their anticarcinogenic or cardioprotective actions. These compounds have applications in food stabilization due to their ability to protect against peroxidation of oxygen sensitive foods.

The Mediterranean diet, rich in fresh fruits and vegetables, has been associated with a lower incidence of cardiovascular disease and cancer, partly because of its high proportion of bioactive compounds such as vitamins, flavonoids and polyphenols. Flavonoids are a widely distributed group of polyphenolic compounds characterized by a common benzo-γ-pyrone structure, that have been reported to act as antioxidants in various biological systems [1,3,4]. Flavonoids are present in a wide variety of edible Mediterranean plants, especially Vitis vinifera, Olea europaea and Citrus species.

The Vitis vinifera mature seeds are an important natural source of oligomers and polymers of catechin and epicatechin, also denominated procyanidins. The procyanidins are constituted by a variable number of flavan units regularly linked by C₄-C₆ or C₄-C₈ bonds and may be present in the grape seed extracts in a mixture formed by dimers, trimers, tetramers, polymers up to 7-8 units and small amounts of catechin and epicatechin [5-7]. Procyanidins from V. vinifera seeds are considered as agents with high value in the treatment of vascular disorders as collagen unstabilization in the arterial wall, arterial localized histamine formation and cholesterol oxidation [8-11] and show anti-inflammatory [12], antihypertensive [13], antiviral [14, 15], spare vitamin C and E [8, 16], antimutagenic [17, 18] activities, and inhibit some undesirable enzymatic activities [19, 20]. The procyanidins of V. vinifera show a high antioxidant activity [19, 21-30].

Four types of flavonoids, (flavanones, flavones, flavonols and anthocyanins, the last only in blood oranges) occur in Citrus [31] and more than 60 individual flavonoids have been identified. Recent studies on the quantitative distribution of these flavonoids in Citrus have shown that the 7-O-glycosylflavanones are the most abundant flavonoids in all species of the genus, whose aglycones are intermediates in the biosynthetic pathway [32-36]. Although flavones and flavonols have been found
in low concentrations in *Citrus* tissues, these types of flavonoids have been show to be powerful antioxidants and free radical scavengers [37]. Recently, these flavonoids have attracted attention as potentially important dietary cancer chemoprotective agents [2]. In addition, the possible antitumor action of certain flavonoids has also generated interest [38-40]. *Citrus* flavonoids, due to their antioxidant properties and their ability to absorb UV light, may act in all stages of the carcinogenic process [37]: damage to the DNA (or initiation step) [41, 42], tumor growth (or promotion step) [38, 43, 44] and invasion (or proliferative step) [45, 46].

The major lipid component of the Mediterranean diet is the drupe-derived olive oil that can be distinguished from other seed oils for the peculiar composition of its non-triglyceride fraction. In fact, several minor components, including polyphenols, grant the oil its particular taste and aroma. Historically, olive leaf has been used as a folk remedy for combating fevers and other diseases as malaria. Several reports showed that olive leaf extract had the capacity to lower blood pressure in animals [47] and increased blood flow in the coronary arteries [48], relieved arrhythmia and prevented intestinal muscle spasms. The bitter compound oleuropein, the major constituent of the secoiridoid family in the olive (*Olea europaea* L.) trees, has been shown to be a potent antioxidant endowed with antiinflammatory properties. Has been reported that oleuropein had antimicrobial activity against viruses, retroviruses, bacteria, yeasts, fungi, molds and other parasites [49-52]. Other clinical effects of oleuropein were the potentiation of cellular and organismal protection through the macrophage-mediated response [53] and the inhibition of platelet aggregation and eicosanoid production [54]. Finally, to consider that the oxygen reactive species are formed by a sequential electron reduction mechanism, that may arise from causes that are endogenous or exogenous to the medium under consideration, among the exogenous causes are ionizing radiations. It is known that X and γ-rays generate hydroxyl radicals in organisms and induced cellular DNA damage which leads to mutations and chromosomal aberrations [29, 55-57]. Recently, scavenging ability of certain tea extracts containing several polyphenols and catechins against active oxygen species and inhibitory effects in X-ray-induced cell transformation were reported [57, 58]. Therefore, *in vivo*, radioprotective activity of the *Vitis vinifera* seeds (GSE), *Citrus* spp. fruits (CE) and *Olea europaea* leaves (OL) extracts and the reference flavonoids diosmin and rutin, widely used as pharmaceuticals, was investigated, using X-rays as oxidative DNA damaging agent, and evaluating the reduction of the frequency of micronucleated erythrocytes (polychromatic erythrocytes of mouse bone marrow) in mouse exposed to X-ray. The relationship between the antioxidant and anticlastogenic activities is discussed.

2. Materials and Methods

2.1. Plant Material.

Grape Seeds Extract (GSE) was obtained from four different varieties of *V. vinifera* grapes, selected in different areas of the community of Murcia (Spain): “Macabeo” and “Airen” as white grapes and “Tempranillo” and “Monastrel” as red grapes. The grapes were picked at their optimum commercial maturity when enologically ripe.

Citrus Fruit Extract (CE) was obtained from immature fruits of several characteristic cultivars from the region of Murcia from three *Citrus* species: *Citrus limonia*, *Citrus sinensis* and *Citrus aurantium*. The fruits were harvested from the trees by natural abscission during the initial phase of the fruit growth.

Olive Leaf Extract (OL) was obtained from *Olea europaea* L. leaves of five cultivars: Villalonga, Alfaarenca, Picual, Cornicabra & Blanketa from the regions of Andalucia and Murcia. The leaves were collected when the olive fruits were picked at their usual commercial time.

2.2. Chemicals Reagent.

Diosmin and Rutin were obtained from Extrasynthese S.A. (Genay, France). DMSO was obtained from Merck (Darmstadt, Germany). All flavonoids and substituted phenols used as standards were obtained from Extrasynthese S.A. (Genay, France). Fetal calf serum was obtained from Sigma Chemical Co. (Madrid, Spain).

2.3. Extraction of polyphenolic compounds from plant material.

Grape Seeds Extract (GSE), Citrus Extract (CE) and Olive Leaf Extract (OL) were manufactured and supplied by Furfural Español S.A. (Alcantarilla, Murcia, Spain).

2.4. HPLC chromatographic analysis of *Vitis vinifera* seeds extract (GSE).

GSE 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 C18 LiChroSphere 100
analytical column (250 x 4 mm i.d.) with a particle size of 5 µm (Merck, Darmstadt, Germany) thermostated at 30ºC. The flow rate was 1 mL/min. The absorbance changes were monitored at 280 nm. A modification of the method described earlier [5] 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) during 45 min, changed to 22% (A), 78% (B) in 15 min (total 60 min); in 10 min (total 70 min) changed to 100% (B); in 5 min (total 75 min) changed to 100% (C), maintaining this isocratic composition during 10 min (total 85 min); after, reequilibrate in 10 min (total 95 min) to initial composition.

2.5. HPLC chromatographic analysis of Citrus spp. fruits extract (CE)
The methods used for the HPLC elucidation and quantification of flavonoids present in CE have been described in several previous papers [33-35, 59].

2.6. HPLC chromatographic analysis of Olea europaea L. leaves extract (OL)
For the quantification of phenolics in OL, the extract was dissolved in DMSO, in the ratio of 5 mg/ml, 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 C18 LiChrospher 100 analytical column (250 x 4 mm i.d.) with a particle size of 5 µm (Merck, Darmstadt, Germany) thermostated at 30ºC. The flow rate was 1 ml/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95 % (A) and 5 % (B) to 75 % (A) and 25 % (B) during 20 min; changed to 50 % (A) and (B) in 20 min (40 min, total time); in 10 min changed to 20 % (A) and 80 % (B) (50 min, total time), after reequilibrate in 10 min (60 min, total time) to initial composition.

2.7. Animals.
The adult male Swiss mice employed in the experiments weighed between 25 and 30 g and 9-12 weeks old. All mice were acclimatized for at least one week prior to dosing. They were maintained under constant environmental conditions with 12/12 h light/dark cycle. They were fed standard granulated chow (Rodent toxicology diet®, BYK Universal beekay feeds, France) and given drinking water ad libitum. Each group consisted of 5-7 mice.

2.8. Chemicals and Treatment.
The polyphenolic extracts were administered orally. All solutions were freshly prepared directly before treatment of the animals. GSE, CE, and OL were dissolved in 0.2 % drinking water and administered during 5 days before the X-irradiation. DMSO was dissolved in water (50 g/100 ml). Diosmin and rutin were dissolved in DMSO (300 mg/ml). DMSO, diosmin and rutin were injected at a single dose of 0.6 ml directly into the lumen gastric 6 h before the X-irradiation.

2.9. 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. The X-rays exposure was stablished by means of thermoluminiscent dosimeters (TLDs) (GR-200®, Conqueror Electronics Technology Co.Ltd, China). The TLDs were supplied and measured by CIEMAT (Ministry of Industry and Energy, Spain)

2.10. Bone Marrow Preparation and Staining.
The animals were killed by cervical dislocation at 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. The bone marrow cells were dispersed by gently pipetting and collected by centrifugation at 1000 rpm for 5 min at 4ºC. 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 [60, 61]. With this method polychromatic erythrocytes (PCEs) stain reddish-blue and normochromatic erythrocytes (NCEs) stain orangey while nuclear material is a dark purple colour.

2.11. Slide Analysis.
The number of micronucleated polychromatic erythrocytes (MnPCEs) among 2000 PCEs per mouse (1000 PCEs per slide) were determined. The slides were examine at 1000 x magnification using a Zeiss light microscope (Oberkochen, Germany).

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

The magnitude of protection (%) was calculated using the formula:

\[
\text{Magnitude of protection} \, (\%) = \left( \frac{F_{\text{control}} - F_{\text{treated}}}{F_{\text{control}}} \right) \times 100.
\]

Where

- \( F_{\text{control}} \) = frequency of MnPCEs in irradiated animals
- \( F_{\text{treated}} \) = frequency of MnPCEs in animals treated before the X-ray irradiation (diosmin, rutin, GSE, CE, OL and DMSO) [62].

3. Results and Discussion

3.1. HPLC polyphenolic distribution in *Vitis vinifera* seeds extract (GSE)

Figure 1 shows a characteristic HPLC chromatogram of GSE and their distribution was characterized for the presence of different flavan-3-ols, and also detected gallic acid. The relative distribution (normalized area values) of the main compounds are summarized in Table 1. They show a characteristic maximum at 280 nm, with different extinction coefficients in function of their molecular weight.

In GSE, polymer \( \geq C_4 \) units was the group of procyanidins present in the highest concentration (90.92%). With respect to the other compounds, monomers were found to be the most abundant flavan-3-ols, (+)-catechin (2.11%) is more abundant than (-)-epicatechin (1.06%). The other procyanidins present in relatively large quantities in this extract were dimers in which the elemental units are bound principally by type \( C_4-C_8 \) interflavan bond, the main are B4 (0.96%), B3 (0.71%), B1 (0.52%), B2 (0.48%) and the 3-O-galloylated derivative of dimer B1 (0.52%). The compounds included in the peak with \( t_R \) 74.4 min are tetramers and polymers longer than tetramers; new findings are in progress for to obtain the isolation and identification of each of the polymeric polyphenolic structures present in this procyanidin fraction.

3.2. HPLC polyphenolic distribution in *Citrus* spp. fruit extract (CE)

Figure 2 shows a HPLC chromatographic profile of CE. The retention time and abundance of the main flavonoids in CE are summarized in Table 2. The flavonones naringenin and hesperetin are widely found in *Citrus* species in their 7-O-glycoside forms: isonaringin and hesperidin as rutinosides, and naringin and neohesperidin as neohesperidosides. Also, there are significative concentrations of 7-O-glycosides of eriodictyol: eriocitrin and neoeiocitrin, rutinoside and neohesperidoside, respectively. Flavanones are the most abundant flavonoids, but the flavones exhibit in vitro higher biological activity even through they occur in lower concentrations. It is important to note the presence of the glycosides of luteolin, diasmetin (diosmin and neodiosmin), as well the neohesperidoside of apigenin, rhoifolin.

3.3. HPLC polyphenolic distribution in *Olea europaea* L. leaves extract (OL)

The HPLC profile of phenolic compounds present in Olive Leaf Extract is shows in the Figure 3. The retention times and abundance of the main compounds in OL are show in Table 3. Five group of compounds are present principally: oleuropeosides (oleuropein and verbascoside); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diasmetin-7-glucoside, luteolin and diasmetin); flavonols (rutin); flavan-3-ols (catechin) and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid).

The most abundant compound in OL is oleuropein, followed by hydroxytyrosol, the flavone-7-glucosides of luteolin and apigenin and verbascoside. Hydroxytyrosol is a precursor of oleuropein and verbascoside is a conjugated glucoside of hydroxytyrosol and caffeic acid.

3.4. X-ray radioprotective effects. Anticlastogenic activity

It is know that the different radicals responsible for the cell oxidation processes are the following: singlet oxygen (\( ^1O_2 \)), superoxide anion (\( O_2^- \)), hydroxyl radical (OH) and peroxyl radical (R-OO-). Except the first, formed by photosensitization [63], the remaining oxygenic oxidative species are formed by a sequential electron mechanism, by means of which molecular oxygen gives rise to superoxide radical, hydrogen peroxide and hydroxyl radical successively [37]. The electron supply mechanism may arise from causes that are endogenous or exogenous to the medium under consideration. Among the exogenous causes are solar radiation, photosensitization by visible light, termic shocks, environmental contaminants, drugs biotransformations, the induction of redox cycles and the cause studied in the present work, ionizing radiations, specifically X-irradiation.

X-rays caused *in vivo* the high generation of hydroxyl radical, by homolytic cleavage of body water or from 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 about $10^{-9}$ s [64]. The high reactivity of this radical implies immediate reaction at the place where it is generated. When the hydroxyl radical generation is massive, like X-irradiation, the citotoxic effect isn’t only located, but that can to propagate intracellular and extracellularly, increasing the interaction of these radicals with phospholipid structures, inducing peroxidation processes that increase the hydroxyl radical activity in DNA oxidative damage [37, 65].

![FIG 1 and Table I. HPLC chromatogram of Vitis vinifera Seeds Extract (GSE),](image1)

<table>
<thead>
<tr>
<th>Flavon-3-ols</th>
<th>Retention time (min)</th>
<th>% Relative Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>9.7</td>
<td>0.83</td>
</tr>
<tr>
<td>B3 (dimer)</td>
<td>34.3</td>
<td>0.71</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>37.6</td>
<td>2.11</td>
</tr>
<tr>
<td>B1 (dimer)</td>
<td>40.6</td>
<td>0.52</td>
</tr>
<tr>
<td>T2 (trimer)</td>
<td>45.4</td>
<td>0.11</td>
</tr>
<tr>
<td>B4 (dimer)</td>
<td>49.9</td>
<td>0.96</td>
</tr>
<tr>
<td>B2 (dimer)</td>
<td>54.0</td>
<td>0.48</td>
</tr>
<tr>
<td>B2-3'-O-gallate</td>
<td>59.1</td>
<td>0.16</td>
</tr>
<tr>
<td>(-)-epicatechin</td>
<td>62.9</td>
<td>1.06</td>
</tr>
<tr>
<td>B1-3-O-gallate</td>
<td>64.4</td>
<td>0.52</td>
</tr>
<tr>
<td>C1 (trimer)</td>
<td>67.4</td>
<td>0.20</td>
</tr>
<tr>
<td>Polymer</td>
<td>74.4</td>
<td>90.92</td>
</tr>
<tr>
<td>Procyanidins ≥ C4 units</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>Other dimer and trimer flavan-3-ols</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![FIG 2. and Table 2. HPLC chromatogram of Citrus spp. Extract (CE),](image2)

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>Retention time (min)</th>
<th>% Absolute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriocitrin</td>
<td>5.67</td>
<td>2.24</td>
</tr>
<tr>
<td>Neoorocitrin</td>
<td>8.86</td>
<td>1.25</td>
</tr>
<tr>
<td>Luteolin 7-O-rutinoside</td>
<td>9.55</td>
<td>0.60</td>
</tr>
<tr>
<td>Luteolin 7-O-neohesperidioside</td>
<td>11.47</td>
<td>0.06</td>
</tr>
<tr>
<td>Isoarisingin</td>
<td>13.13</td>
<td>0.20</td>
</tr>
<tr>
<td>Naringin</td>
<td>15.93</td>
<td>10.76</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>18.87</td>
<td>6.68</td>
</tr>
<tr>
<td>Rhodofolin</td>
<td>19.93</td>
<td>7.65</td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>23.10</td>
<td>0.51</td>
</tr>
<tr>
<td>Diosmin</td>
<td>36.96</td>
<td>0.32</td>
</tr>
<tr>
<td>Neodiosmin</td>
<td>50.44</td>
<td>0.20</td>
</tr>
</tbody>
</table>

![FIG..3 and Table 3 HPLC chromatogram of Olea europaea Leaves Extract (OL),](image3)

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Retention time (min)</th>
<th>% Absolute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>4.80</td>
<td>1.46</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>5.83</td>
<td>0.71</td>
</tr>
<tr>
<td>Catechin</td>
<td>8.41</td>
<td>0.04</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>11.56</td>
<td>0.34</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>14.17</td>
<td>0.63</td>
</tr>
<tr>
<td>Vanillin</td>
<td>14.79</td>
<td>0.05</td>
</tr>
<tr>
<td>Rutin</td>
<td>17.22</td>
<td>0.05</td>
</tr>
<tr>
<td>Luteolin-7-glucoside</td>
<td>18.10</td>
<td>1.38</td>
</tr>
<tr>
<td>Verbascoside</td>
<td>20.06</td>
<td>1.11</td>
</tr>
<tr>
<td>Apigenin-7-glucoside</td>
<td>21.28</td>
<td>1.37</td>
</tr>
<tr>
<td>Diosmetin-7-glucoside</td>
<td>21.95</td>
<td>0.54</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>22.76</td>
<td>2.54</td>
</tr>
<tr>
<td>Luteolin</td>
<td>28.61</td>
<td>0.21</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>31.59</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The micronucleus assay on mouse bone marrow polychromatic erythrocytes, originally developed by Schmidt in 1975 [60], is probably the most frequently used in vivo short-term genotoxicity test. Bone marrow micronucleated erythrocytes provide a simple and rapid method for detection of chromosomal damage by chemical and physical agents [66-70].

In these oxidative stress conditions, when even the endogenous antioxidant systems are defective or insufficient, is necessary the use of exogenous agents with a strong-radical scavenger capacity, in function of a high absolute reactivity against different radicals and a high stability of the intermediately
formed “antioxidant radical” [37]. In this study were used as radioprotective agents, also GSE, CE, OL, the flavonoids diosmin and rutin, and DMSO. These compounds shown to protect small rodents from the effects of total body X-irradiation when are or ingested before the X-ray exposure.

It is know that sulfur-containing compounds used in toxic doses markedly affect the metabolism and cellular ultrastructure of mammalian tissues. However, the changes caused by the radioprotective and also toxic doses of the thiols given prior to irradiation of animals are connected with the increased efficiency of the repair system and protection of cells from radiation damage. Various mechanisms or combinations of them for radioprotection by the thiols have been proposed, at the molecular level: by free radical scavenging hydrogen donation, binding to critical biological targets and mixed disulphide formation; at the biochemical-physiological level: by hypoxia, biochemical shock and hypothermia, and at the organ level: by stimulation of recovery of cell populations [70].

DMSO is considered according to structural and experimental data as radioprotective agent [71], in addition, is a classic radical scavenger, with a capacity for in vitro hydroxyl radical scavenging higher than many flavonoids: 30 % higher than quercetin (aglycon of rutin), 40 % than diosmin and 50 % than (+)-catechin [72, 73]. However, when applied in radioprotective doses, without subsequent irradiation of animals, this S-containing compound is highly toxic [70, 74, 75].

![FIG.4](image1.png)

**FIG.4.** Influence of treatments and X-rays irradiation on the frequencies of MnPCEs.

![FIG.5](image2.png)

**FIG.5** Magnitude of protection of different treatments in relation to X-irradiation.

Flavonoids are excellent hydroxyl scavengers [37, 64, 72, 73, 76] and, obviously, their degree of effectiveness depends of their structure. It is know that this capacity to inhibit hydroxyl radical is based principally in the binary substitution model in B-ring (o-dihydroxy or catechol structure) and, in smaller grade, in the presence of 3-OH group in the flavonoid skeleton [37]. This greater activity of compounds with catechol structure is due to the stability of the flavonoid radical generated in the process [37, 77, 78].

Our results confirm these previous findings. Figure 4 shows the influence of treatments on the frequencies of MnPCEs in bone marrow of animals non-irradiated and irradiated, permitting thus to compare the potential toxicity of each treatment vs their anticlastogenic activity. Diosmin, rutin, GSE, CE and OL show very low levels of MnPCEs generation, similars respect to non-irradiated control data; the sulfur-containing compound, DMSO, present a toxicity levels higher than the other studied compounds. Also, Figure 4 shows the influence of X-rays irradiation on the frequencies of MnPCEs in mouse bone marrow. The order of treatments with respect to the minor level of MnPCEs generated after irradiation is: GSE < CE < rutin < DMSO < OL < diosmin. The radioprotective effects and
consequently the anticlastogenic activity of the different treatments used, was established according to the increase of MnPCEs level in animals after irradiation and their relation with this level in animals control, obtaining a porcentual value that determine the magnitude of protection of each treatment. Figure 5 shows the values of these protection capacities, being the order of effectivity: GSE > CE > rutin > DMSO > OL > diosmin.

3.5. Relation between antioxidant and anticlastogenic mechanisms

According to the structural considerations, above mentioned, the results obtained in the measure of anticlastogenic activity of the tested compounds are consistent with their antioxidant properties and specific activities as hydroxyl radical scavengers. It results clear that the high grade of polymerization of GSE from linkages between (+)-catechin and (-)-epicatechin monomers, free or esterified with gallic acid, allows the existence of a conjugate structure with a high number of catechol groups, very close spatially, this structure is capable of an extensive electron delocation, which is a prerequisite for radical stabilization, generating multiple mesomeric structures. The decay rate constants of flavonoid arxoyl radicals generated by interrelation with other radicals shows that all the most stable arxoyl radicals, without exception, contain the 3',4'-catechol B-ring substitution pattern. All other polyphenolic compounds form far less stable arxoyl radicals [37, 77, 78]. 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 GSE antioxidant capacity.

Citrus flavonoids of CE are excellent hydroxyl scavengers and they too seen to posses a degree of effectiveness which depends on their structure [37]. This degree, which is based on their capacity to inhibit hydroxyl radical, suggest the importance of the binary substitution model in B-ring even when the hydroxyl are sterified with methyl groups. The negative influence of the hydroxyl group in position 3 in monosubstituted B-ring compounds is significant [37]. Similarly to GSE, the greater activity of CE flavonoids with B-ring catechol structure is due to the stability of the flavonoid radical generated in the process, as shall be seen below [37, 77, 78]. The inhibition of hydroxyl radical generation is markedly inhibited by increasing flavonoid concentration above a threshold level, such increases depending on the individual flavonoid and reaction medium [37]. Therefore, the results of anticlastogenic activity suggest that the presence in CE of a heteregeneous mixture of glycosylated flavanones and flavones shows, probably, a synergetic behaviour in their radical scavenging capacity when act in mixed form as occurs in CE.

Consequently, the potentiality of GSE and CE is higher than rutin, the most active of the two pharmaceutical flavonoids assayed, and very higher than diosmin, that hasn’t got B-ring catechol configuration (4’ hydroxyl group of B-ring is methylated).

The anticlastogenic activity showed by OL confirm the importance of the catechol structure in the flavonoid B-ring (luteolin and luteolin-7-glucoside) and substituted phenols (hydroxytyrosol, verbascoside, caffeic acid, etc). Other important structural element described for the antioxidant activity of OL polyphenols is the number and location of their aromatic hydroxyl groups. Antioxidant activity of oleuropein is due mainly to the hydroxytyrosol moiety in its structure. It is very important to note that OL shows a higher anticlastogenic activity than diosmin (flavone with absence of B-ring catechol structure).

New findings are in progress for to isolate and identified the different polymers present in the fraction ≥ C₄ units of GSE, studying their antioxidant and anticlastogenic activities and the synergetic behaviour combined with CE and/or OL extracts, and likewise on the systematic treatment models, dosage before and after of X-irradiation, and dose-response curves of these compounds. In addition, the present study, conventional in vivo MN assay was used and MN were scored only in polychromatic erythrocytes. In accordance with Vijayalaxmi and Venu [79], the drawback with this method is that, it does not discriminate between dividing and nondividing cells. Hence, in recent years MN test is being performed by using cytokinesis block (CB) method [80,81]. This method applied to anticlastogenecity studies is a supplement to conventional MN test and we cant autenticate our observations in the next studies above mentioned.

In conclusion, the data show by GSE, CE and OL confirm the higher antioxidant and radioprotective/anticlastogenic activities of flavonoid skeletons with B-ring 3’,4’-catechol structure associated with 3-hydroxyl group and/or 2,3 double-bond conjugated with 4-oxo function. In addition, their apparent null toxicity, according to verified test, give these polyphenolic compounds a high capacity as nutraceutical and pharmaceutical agents.
4. References


47. G. Samuelsson. The blood pressure lowering factor in leaves of Olea Europaea, Farmaceutisk Revy, 15 (1951) 229-239.


