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# The effect of the flavonoid diosmin, grape seed extract and red wine on the pulmonary metastatic B16F10 melanoma

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**Summary.** Objective: To study the effect of different phenolic compounds and red wine on pulmonary metastatic melanoma. Methods: Swiss mice were inoculated with $5x10^5$ melanocytes B16F10 and given oral doses of diosmin, grape seed extract (GSE) and red wine. A macroscopic count was made of the metastatic nodules on the lung surface and a microscopic study by image analysis of five sections, calculating the implantation percentage and tumoral growth and invasion indices. Results: Macroscopically, the group treated with diosmin showed the greatest reduction (52%) in the number of metastatic nodules compared with the control group, which was treated with ethanol, while GSE and red wine caused decreases of 26.07 and 28.81%, respectively. Microscopically, there was a decrease in the implantation percentage after the administration of diosmin (79.4%) and red wine (20.19%), and an increase of 2.12% after the administration of GSE, all relative to the ethanol-treated control. As regards the growth index, diosmin produced a reduction of 67.44% and red wine a reduction of 20.62%, while GSE again produced an increase (25.33%). The reductions in the invasion index were 45.23, 31.65 and 17.57% with diosmin, GSE and red wine, respectively. Conclusions: Diosmin originated the greatest reduction in pulmonary metastases, both at the macroscopic and microscopic levels.

**Key words:** B16F10, Pulmonary metastasis, Procyanidins, Flavonoids, Diosmin

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## Introduction

Melanoma is a serious challenge in oncology because of the ineffectiveness of known treatments and the progressive increase in mortality recorded in fair skinned people from all over the world (Holme et al., 2001). Despite representing only 4% of skin cancers, it is responsible for 80% of skin cancer deaths (Horn-Ross, 2003), most as a consequence of metastasis. It is one of the neoplasias that metastasise most frequently, especially in the lymphatic glands or the lung. In the latter organ, it occurs with a frequency of between 12.2% (Harpone et al., 1992) and 20% (Majeski, 1999). Pulmonary metastasis constitutes one of the most important causes of death in oncological patients (Kumar et al., 2004). The difficulty of treating metastases lies in the interactions between tumoral cells and the homeostatic mechanisms that replace them (Fidler, 2002). They show one of the worst response rates to chemotherapy, basically due to the resistance of cells to antineoplastic agents (Helmbach et al., 2001) and also because of the secondary problems, which are common. Hence, the interest in finding new antimetastatic agents; in this sense, some polyphenolic compounds have been described as potentially chemoprotective dietary agents against cancer (Miller et al., 1994). Numerous epidemiological studies have suggested that the consumption of fruit and vegetables, besides having other benefits, reduces the risk of cancer due to the polyphenolic compounds they contain (O'Brien, 2001). For its part, diosmin is a flavonoid widely used in medicine as a antivaricose agent and vasoprotector.

Epidemiological studies have suggested that the low incidence of coronary heart disease in France is due to the protective effect of red wine ("the French paradox")

(Donovan et al., 1999). Red wine is rich in polyphenols, including quercetin, rutin, gallic acid, catechin procyanidins, resveratrol, etc. The actual concentration and number of polyphenols in red wine depends on many factors, including vine variety, climate, soil, pressing and fermentation techniques, etc (Lopez-Velez et al., 2003). Several assays have shown that it is possible to inhibit pulmonary metastases induced by intravenous injection of melanoma cells in mice using polyphenolic compounds such as: curcumin, epicatechin, rutin (Menon et al., 1995), isoflavones (Li et al., 1999), apigenin and quercetin (Caltagirone et al., 2000), etc.

The objective of this work was to study the possible antimetastatic effects of the flavonoid diosmin, GSE and red wine and to compare the results with the effect of ethanol, which was used as and administration vehicle.

## Material and methods

### *Cell line*

We used the highly metastatic sub-line B16F10, of the B16 murine melanoma (European Collection of Cell Cultures, UK), cultivated with Eagle minimum essential medium (EMEM, Gibco, EEUU), buffered at pH 7.2-7.4 and supplemented (10%) with foetal bovine serum (FBS, Gibco, EEUU), streptomycin and penicillin (100 µg/ml and 100u/ml respectively). The absence of microplasm spp. was checked by direct fluorescence with specific colorant for DNA (H33233, Hoecht, Alemania).

### *Animals*

65 female white Swiss mice were used, 10-12 weeks old and weighing 28-36 g at the beginning of the experiment. These were obtained from the Murcia University Laboratory Animal Service (licence 30030-2AB, Spanish Ministry of Agriculture, Fisheries and Food, 13-10-88). Food and water was administered "ad libitum" during the study.

### *Flavonoids*

Grape seed extract corresponded to a compound with a flavan-3-ol structure, principally composed of procyanidins (99.45%) with only small quantities of (+)-catechin (0.32%) and (-)-epicatechin (0.23%), obtained by physical techniques from grape seeds by Furfural Español S.A. (Murcia, Spain), which also provided the diosmin used. The GSE and Diosmin, were dissolved at 0.2% (w/v) in a solution of water and ethanol (Merck, Madrid) in a proportion of 98.8: 1.2 (w/v), and provided “ad libitum” as food and drink (see below). The wine Hécula was provided by Castaño Yecla (Murcia). It had an alcohol content of 13.5%, which was reduced to 1.2% by adding water (91.2 water: 8.8 wine, v/v), and was also administered “ad libitum” in the food and drink. The feed, A04 from Panlab (Barcelona), was triturated and the GSE, diosmin or wine solutions were added in the proportion of 1L of solution per kg of feed. The control group received the same feed made up with the same volume of water. The individual mixtures were homogenised and water was added to obtain pellets, which were dried at 80°C.

### *Experimental procedure*

Each animal was inoculated with $5x10^5$ cells/200 µl of culture medium in the lateral vein of the tail. The specially prepared drink and feeds were provided 11 days prior to inoculation and 21 days afterwards. The following groups, each of 13 animals, were established: I, control (only inoculation of $0.5x10^5$ cells); II, ethanol solution (98.8 water: 1.2 ethanol); III, wine; IV, diosmin; V, GSE.

Twenty-one days after inoculation, the animals were sacrificed by cervical dislocation, after which the autopsy and macroscopic study of the lungs were carried out according to Model l. All the organs were fixed in 10% buffered neutral formol. The lungs were processed by the normal method for inclusion in paraffin, placing the five lobules of both lungs in a cassette and making serial 3 µm sections, before selecting one in every fourteen for staining with hematoxylin and eosin.

A quantitative evaluation of the metastatic nodules was made by two observers using the following models:

Model I

Macroscopic study by stereoscopic magnifying glass (Olympus), counting the metastatic nodules of the pleural surface of the five lobules.

Model II

Microscopic image analysis of the five lobules of each lung, using an Olympus SZ11 magnifying glass connected to a Sony DXC 151-Ap video camera and System MIP-4 image analyser (Digital Image System, Barcelona), with which the zones for study were chosen interactively. In accordance with Lentini et al. (2000), the initial parameters evaluated were: total area of lobule calculated at 21x magnification; total area of the metastases and mean area of metastasis per lobule calculated at 55x magnification. The areas were calculated from the maximum and minimum diameters and the mean areas by dividing the total area of metastasis by the number of metastatic nodules.

With these parameters, we calculated: 1. The implantation percentage of = (area of metastasis per lobule / total area) x 100. 2. Growth index = mean area of metastasis / total area. 3. Invasion index = area of metastasis per lobule / mean area of metastasis.

### *Statistical analysis*

A descriptive statistical analysis was made by calculating the distribution frequencies, mean, error of

the mean and standard, maximum and minimum deviation. Comparison between groups was made with a one way analysis of variance on a logarithmic scale. This analysis was complemented by comparison between pairwise t-test of means group, using least significant difference (LSD). P-values of ≤ 0.05 were considered significant.

## Results

### *Macroscopic study*

Quantifiable metastatic nodules were taken to be those structures of a blackish colour on the lung surface that were sufficiently separated to be individually countable (Fig. 1a,b).

Figure 2 shows the count of metastatic nodules made in all the groups studied. The control group (group I) showed between 120 and 340 metastatic nodules randomly distributed over the lung surface, with a mean of 176.30±19.26 metastatic nodules, while group II, which was given ethanol alone, showed a mean of 330±23.18 , which represented an 87% increase in these numbers, compared with the control. In actual fact, group II acted as the real control group because ethanol was the vehicle used for administering the diosmin and GSE, and because of the final ethanol content of the wine, which was diluted until it reached the same alcoholic degree as group II.

In decreasing order of the number of metastatic nodules observed, the next group was group III (261.42±32.87), corresponding to the wine treatment, which showed a reduction of 20.81% with respect to the ethanol-treated group, with a statistical significance of $p<0.05$. This was followed by the GSE group (V) (186.64 ±18.71) with a reduction of 26.07% with respect to the ethanol-treated group ($p<0.05$). The group that showed the greatest reduction in the number of metastatic nodules was that given diosmin, which showed between 98 and 228 metastatic nodules, with a mean of 160±18.20,representing a 52% decrease compared with group II (with a very high degree of statistical significance, $p<0.00005$). This was the only group to show a reduction (9.09%) in the number of metastatic nodules compared with group l, although the decrease was not statistically significant.

In decreasing order of the number of nodules observed on the lung surface we observed: ethanol>red wine>GSE>control>diosmin.

### *Microscopic study*

The localisation of the metastases varied widely, although they were a constant feature at subpleural level, where they took on two basic patterns: linear and solid. At the intraparenchymal level, they appeared mainly around the capillary vessels or veins and bronchioles or bronchi, where they were usually larger than at the subpleural level (Fig. 3a,b). Morphologically, the nodules were composed of solid accumulations of neoplastic melanocytes, which, in the largest cases, usually showed small, generally multiple, areas of necrosis in the central parts with frequent, usually peripheral, inflammatory infiltrates. Cytologically, they showed a moderate degree of cellular and nuclear polymorphism. The mytotic index varied from 6 to 10 mytoses per ten fields of high magnification (x500). Melanic pigment was variable and usually arranged in

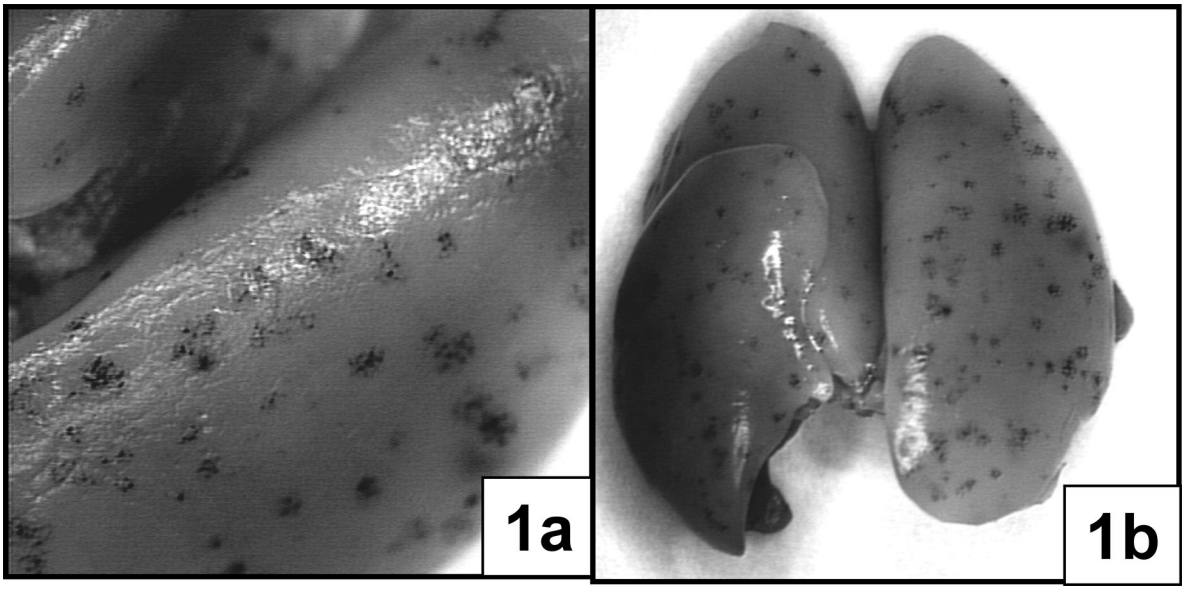


**Fig. 1. a, b.** Macroscopic characteristics of the pulmonary metastatic nodules.

small blackish-brown deposits.

Percentage of implantation (Fig. 4)

The control group (group I) showed a mean invasion of the lung parenchyma of 2.64±0.54%, while the ethanol-treated group (II), the real comparative group, with a mean of 9.4 ±2.88, showed a 254% increase over this level of invasion ($p<0.05$). The GSE group showed a higher implantation percentage (9.614±3.318) with a 2.12% increase over group II (ethanol), while the red wine-treated group (III) showed a mean 7.5±3.65 which represented a 20.19% reduction compared with group II, although this was not a statistically significant difference.

The diosmin-treated group (IV), with a mean of 1.94±0.88, showed the greatest reduction in invasion compared with the ethanol group, the 79.4% reduction being statistically significant ($p<0.05$). Furthermore, it was the only group which showed a reduction with respect to the control (I) (26.8%), although not to a statistically significant degree. The implantation percentage gave the following decreasing order: GSE ≈ > ethanol > red wine >> control > diosmin.

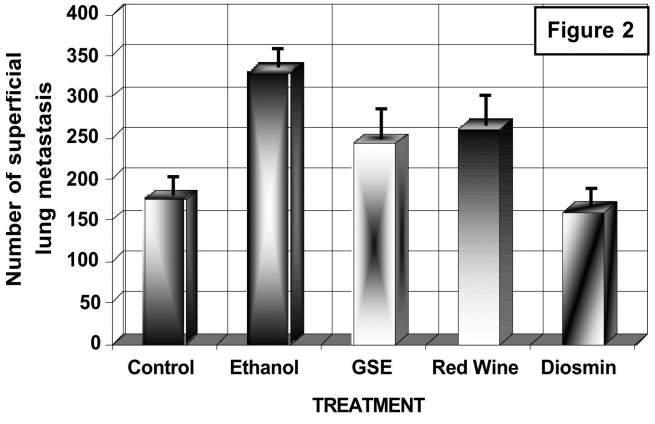


**Fig. 2.** Frequency of the pulmonary metastatic nodules in the control group and groups treated with ethanol, GSE, red wine and diosmin (mean ± error of the mean).

Growth Index (Fig. 5)

The control group showed a mean growth index of 0.0011±0.0004, while the ethanol group (II) was 254% higher (0.0038±0.0012). Of the other groups, the GSE group (V), with a mean of 0.0048±0.0014, showed a 25.33% increase over the ethanol group, while the red wine-treated group (0.0031±0.0009) showed a 20.26% decrease. The greatest reduction (67.44%) was seen in the diosmin-treated group (IV) which showed a mean of 0.0013±0.0004. In decreasing order, then, the growth index was: GSE > ethanol > red wine >> diosmin > control.

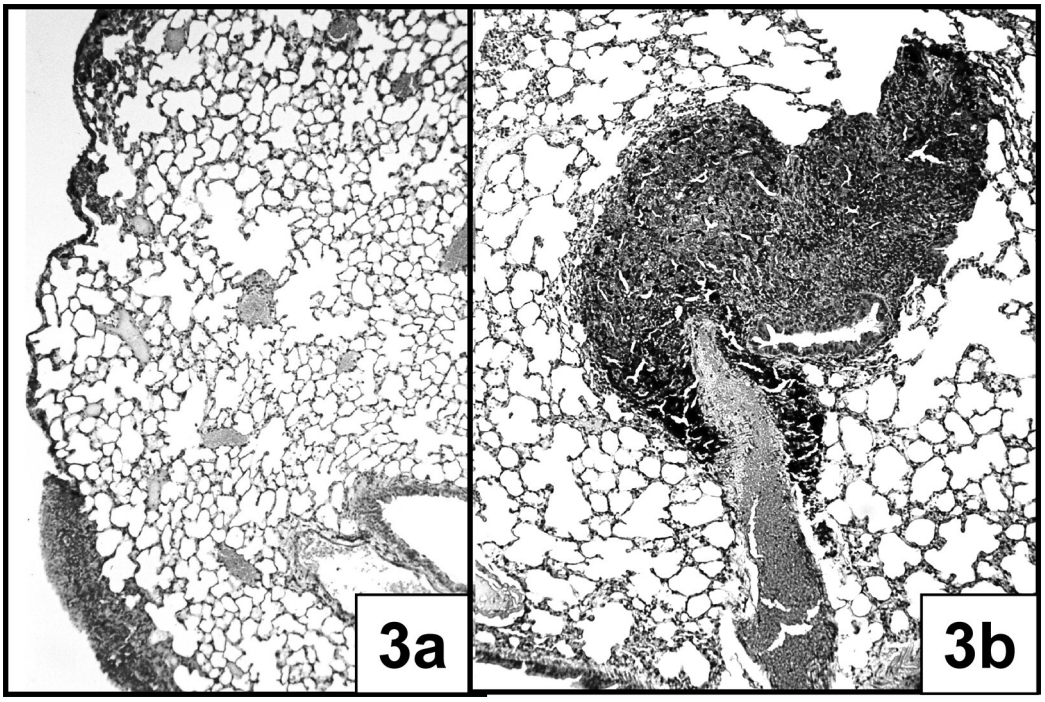


**Fig. 3.** Microscopic characteristic linear pattern at pleural level **(a)** and metastatic nodules around vessels and bronchi **(b)**. x 125

Invasion index (Fig. 6)

The control group showed a mean invasion index of 29.46±2.6, while the ethanol (II) group, with a mean of 36.34±3.41, showed a 23.31% increase (but not statistically significant). Of the other groups, that receiving the red wine showed the highest index (30.04±4.65), which represented a 17.57% reduction with respect to group II ($p<0.05$), followed by the group treated with GSE (V) (24.91±3.66) with a 31.65% reduction compared with group II ($p<0.05$). The group showing the greatest reduction in the invasion index was the one treated with diosmin (VI) (19.96±2.6), which showed a 45.23% reduction ($p<0.005$) compared with group II and a 32.28% reduction compared with the control group I. The following order was obtained for the invasion index: ethanol > control >> red wine > GSE > diosmin.

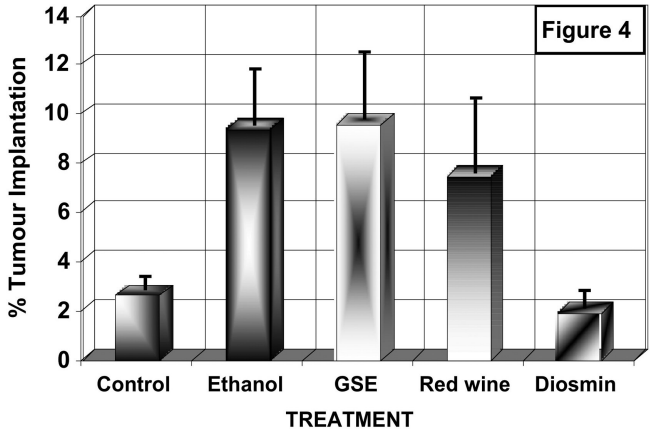


**Fig. 4.** Implantation percentage of control groups and groups treated with ethanol, GSE, red wine and diosmin (mean ± error of the mean).

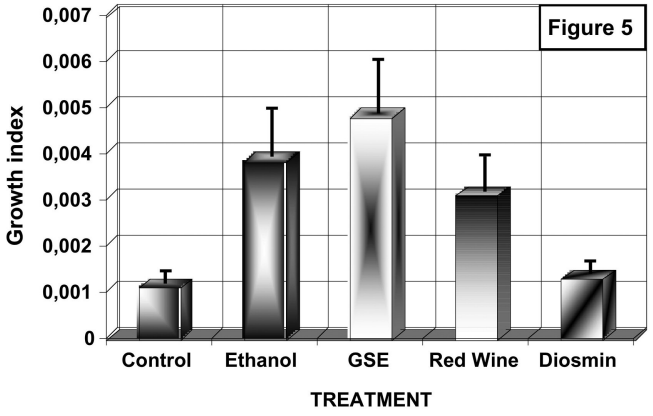


**Fig. 5.** Growth index of control groups and groups treated with ethanol, GSE, red wine and diosmin (mean ± error of the mean).

To summarise, diosmin produced the greatest reduction in the number of metastatic nodules (52%), implantation percentage (79.4%), growth index (67.44%) and invasion index (45.23%), all compared with the group receiving ethanol. The diosmin group also showed a reduction in the number of metastatic nodules, implantation percentage and invasion index with respect to the control group (l) with reductions of 9.09, 26.8 and 32.28%, respectively.

## Discussion

Several studies have shown that the different sublines of the B16 melanoma, with their different chemical and immunological characteristics and ability to adhere, have a predilection for certain organs. The B16F10 and B16BL6 sublines show the greatest metastatic capacity in the lung (Gath et al., 1986) when tumor cells are injected into the tail vein, while the same cells generate liver metastases when injected intrasplnically (Vidal-Vanaclocla et al., 2000; Mendoza et al., 2001). Again, mice inoculated in the left cardiac ventricle develop metastasis in several organs according to an IL-1 dependent or independent pattern (Anasagasti et al., 1997). In our study, the first of the above sublines originated pulmonary metastases in practically all the mice inoculated. As regards the number of cells necessary to cause such metastases, although Kikkawa et al. (2000) mentions a million as being suitable, our assays using different concentrations (Vicente Ortega et al., 2003) have shown that, in the case of B16F10, a million cells produces such an extensive dissemination of metastatic nodules that they are impossible to quantify. For this reason, in the present study we used an inoculation of 500,000 cells, which produces independent metastases that are possible to count.

To evaluate the effect of the different treatments, we used a macroscopic quantitative method (Bossserhoff et

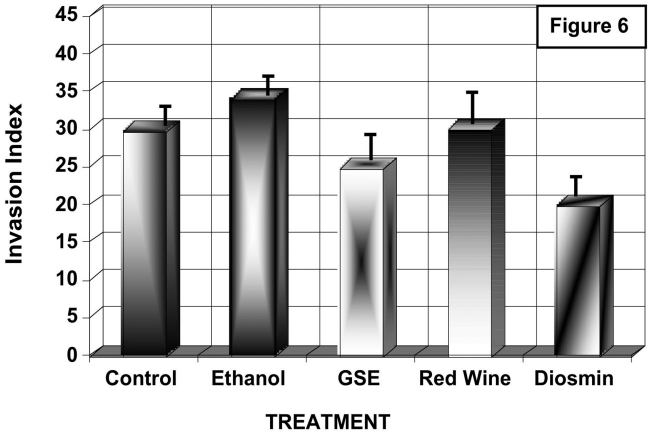


**Fig. 6.** Invasion index of control groups and groups treated with ethanol, GSE, red wine and diosmin (mean ± error of the mean).

al., 2001; Fuzii and Travassos, 2002), which enables the operator to count the nodules on the lung surface by means of a stereoscopic magnifying glass, while for the microscopic study we followed the method described by Lentini et al. in 1998, which correlates the number of metastaic nodules with the invasion phase of the melanoma cells, and the mean area of metastasis with the degree of cell proliferation. We considered the three indices described by Lentini et al. in 2000.

The need to use certain quantities of ethanol to dissolve the polyphenols made it necessary to set up a second control, in which the mere presence of ethanol produced surprising results as regards the numbers of metastases produced at both microscopic and macroscopic level. The ethanol group, for example, showed an 86% increase at macroscopic level and an invasion index increase of 23.1% at the microscopic level. Blank and Meandows (1996) observed a similar increase in the number of pulmonary metastases, although the study was confined to macroscopic observations. Increases were also observed in the indices related with the area invaded: a 256% increase in the implantation percentage (which relates the lobule area occupied by metastases with the total lobule area) and a 254% increase in the growth index (mean area of metastases versus total lobule area). These data indicate that there is a significant increase in the extent of invasion and, particularly, in the proliferation of tumoral cells in the lung. However, the action mechanism of ethanol is not totally understood and contradictory effects have even been reported. Some studies have pointed to an effect that increases the number of metastases, certain growth factors (erbB2, erbB3 and erbB4) being stimulated in breast cancer cells (Luo and Miller, 2000), or favouring angiogenesis (Gu et al., 2001). Other "in vitro" studies have described the stimulating effect of ethanol on cell migration, both in the B16F10 melanoma (Silberman et al., 1990) and T47D line of breast cancer (Zhu et al., 2001). An immunosuppressor effect has also been described for methanol since it decreased the cytotoxic activity of NK lymphocytes (Hebert and Pret, 2003).

Low concentrations of ethanol have been seen to activate fibronolysis, which would hinder the development of metastases (Tabengwa et al., 2002). It has also been described as preventing platelet aggregation (Visioli et al., 2000), which would also inhibit pulmonary metastases. In this respect, subcutaneous administration of the anticoagulant Tizaparin for fourteen days reduced pulmonary metastases by 96% compared with a control (Amirkhosravi et al., 2003) and other previous papers also mentioned the antineoplastic and antimetastatic effects of anticoagulants (Nieswandt et al., 1999).

The wide range of effects attributed to flavonoids reflects their chemical structure, which confers on them antioxidant and antiproliferative properties, as well as the ability to regulate different enzymatic activities (Halliwell et al., 1992). In our study, diosmin produced the greatest reduction both in the number and area of metastases. The reduction observed in the number of nodules at macroscopic level (52%) and at microscopic level (reduction in the invasion index of 45.23%) suggests that diosmin acts by inhibiting invasion, which might be related with its action on the vascular system, where it diminishes vein distensibility and, at the microcirculation level, reinforces capillary resistance (Lyseng-Williamson and Perry, 2003). Its capacity to inhibit the release of inflammation mediators, such as prostaglandins (PGE2) (Pickelmann et al., 1999), its action on key enzymes in prostaglandin biosynthesis (Korthui and Gute, 2002) and its modulation of leukocyte adhesion and prevention of endothelial damage (Coleridge Smith, 2003), may also be related to its action mechanism.

Notwithstanding, diosmin seems to possess a marked anti-proliferative capacity since the decrease observed in the implantation percentage (79.4%) and growth index (67.44%) was greater than its effect on the number of metastatic nodules. In other "in vivo" studies diosmin seems to have had an anti-proliferative effect on different tumors produced by carcinogenic substances: oesophagus (Tanaka et al., 1997a), colon (Tanaka et al., 1997b), oral (Tanaka et al., 1997c), urinary-bladder (Yang et al., 1997). However, in other "in vivo" studies, such anti-proliferative effects were scarce (Zheng et al., 2002).

The GSE used in our study was mainly constituted by procyanidins and small quantities of (+)-catequin and (-)-epicatequin, substances that show a powerful antioxidant activity, inhibiting lipid peroxidation (Zhao et al., 1999), dehydrogenase lactate (LDH) (Rong et al., 1995) and the oxidation of LDL-cholesterol (Weisburger, 2001) or protecting against the genotoxic damage caused by X rays (Castillo et al., 2000) and gamma rays Castillo et al., 2001). Several authors have proposed that the action of procyanadins is due to their capacity to inhibit growth, detaining the cell in the G1 stage, and to their ability to cause death by apoptosis (Agarwal et al., 2002) or by inhibiting aromatose, an enzyme involved in oestrogen biosynthesis (Eng et al., 2003). In our study, the number of metastatic nodules was reduced in the macroscopic midel (26.07%) and at the microscopic level (invasion index decresase of 31.65%). However, the area of metastasis (implantation percentage and growth index) increased significantly, which suggests that procyanidins only influence the number of cells that reach the lung or that are implanted there. Previous studies have described its effect in reducing carrageenin-induced oedema in rats (Blazso and Gabor, 1980). It has also been observed that catequin inhibit the binding of B16 cells to extracellular proteins (Suzuki and Isemura, 2001).

Fifteen minutes after inoculating mice with B16 cells, Amirkhosravi et al. (2003) observed a 50% reduction in circulating platelets. In this sense, it has been described that the consumption of procyanidins may inhibit the stimulation of the platelet receptors

(GPIIb-IIIa) by ADP and epinephrin, thus reducing platelet activity (Rein et al., 2000) and increasing the time needed for ADP or epinephrin to bind with the collagen (Pearson et al., 2002). In B16BL6 melanoma model, flavonoids have been found to inhibit invasion (Zhang et al., 2004), to induce apoptosis (Zhang et al., 2005), and to inhibit the in vivo growth of tumor cells (Catagirone et al., 2000). However, an early inhibitory effect of flavonoidson tumor cell adhesion to endothelium, prior to the angiogenesis-dependent vascular proliferative stage, seems even more important in reducing melanoma cell colonies (Mendoza et al., 2001, 2004; Ferrer et al., 2005). Also of interest is the finding that a micronized purified flavonoid fraction (a semisynthetic flavonoid preparation from the diosmin group) has been found to significantly decrease the plasma levels of VCAM-1 in human (Ramelet, 2000).

Most studies on the beneficial effects of red wine were carried out with dealcoholised wine extracts or with some of the polyphenols present in red wine, such as quercetin, rutin, gallic acid, tannic acid, antocyanins, catequin, procyanidins, resveratrol, etc (Damianaki et al., 2000). In our study, on the other hand, we used wine diluted in water to obtain an alcoholic degree equal to that used in the other groups. The wine treatment reduced the number of nodules both at macroscopic (20.18%) and microscopic level (invasion index reduction of 17.57%), although this effect was less pronounced than that recorded in the GSE group, probably because of the lower concentration of polyphenols (procyanidins). On the other hand, it seems to show a higher anti-proliferative and anti-angiogenesis capacity as it induces a reduction in the implantation percentage (20.19%), growth rate (20. 26%) and mean area of metastasis (14.62%). This effect may well be due to the presence of other flavonoids in the wine with a greater anti-proliferative effect, such as quercetin, which has been described as blocking the G1-phase in muscular cells of blood vessels and inhibiting metalloproteinase-9 (Moon et al., 2003); gallic acid, which has been shown to have an inhibitory effect on highly metastatic cell lines such as P815 and to reduce hepatic metastases through a cytotoxic action (Ohno et al., 2001); resveratrol, which acts on different enzymes, such as Map Kinase (Niles et al., 2003) and on the expression of proteins involved in phase G1, such as cdk2, cdk4 and cdk6 (Ahmad et al., 2001), although it also inhibits aromatose (Eng et al., 2003). Certain polyphenols in wine (quercetin, rutin, gallic acid and tannic acid) induce the apoptosis of prostate cancer cells (LNCaP) (Romero et al., 2002).

It is clear is that the effects of these flavonoids in the study carried out are inversely proportional to their antioxidant activity, so that we cannot relate such effects with this capacity but, rather, with their capacity to regulate processes associated with cell proliferation or angiogenesis.

In conclusion, diosmin was the flavonoid that caused the greatest reduction in the number of pulmonary metastases and in their extension (implantation percentage and growth index), which seems to indicate a capacity to impede the proliferation of neoplastic cells and their binding with endolethial cells, thus reducing their invasion capacity.

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