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Role of Galloylation and Polymerization in Cytoprotective Effects of Polyphenolic Fractions against Hydrogen Peroxide Insult

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ABSTRACT: Byproducts and wastes generated by agricultural, food, and forestry industries contain large amounts of polyphenols, which can be potentially used as sources of natural or semisynthetic antioxidants. This study examined and compared the protection against peroxidative damage induced in erythrocytes and 3T3 cell line of polyphenolic fractions from white grape pomace, pine bark, and witch hazel bark. The work pays special attention to the different degrees of polymerization and galloylation of the extracts to contribute to the understanding of their mechanisms of action. Fractions demonstrated different protections against erythrocyte lipid peroxidation, hemolysis, and 3T3 cytotoxicity caused by H_2O_2. Galloylation is claimed to be related to antioxidant protective capacity, and it is also responsible for the pro-oxidant effect observed at high doses. The results show that not only the percentage of galloylation but also the degree of polymerization are important modulators of their antioxidant capacity. In this sense, it is crucial that novel polyphenolic fractions were prepared attending a value of 3 for the mean degree of polymerization and did not exceed a 30% of galloylation to reach the highest antioxidant capacity with the lowest cytotoxic effects. For this reason, the grape extracts appear to be the best strategy to fight against hydrogen peroxide cell damage.

KEYWORDS: antioxidant, chemoprevention, toxicity, grape pomace, pine bark, witch hazel bark

INTRODUCTION

Increasing evidence suggests that excessive concentrations of reactive oxygen species (ROS) in the human body are involved in a number of pathological events and that oxidative damage to cell components may play an important pathophysiological role in many types of human diseases. ROS induce molecular alterations in cellular components, such as lipid peroxidation and DNA damage,[1,2] which are considered to be key phenomena in the development of chronic illnesses such as inflammatory and heart disease, hypertension, and some forms of cancer.[3,4] Natural foods and food-derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention because they are known to function as chemopreventive agents against oxidative damage.[5,6] Important sources of polyphenols include fresh fruit and raw vegetables, tea, cacao, grains, and seeds.[7,8] However, many byproducts and wastes generated by the agricultural, food, and forestry industries contain still large amounts of polyphenols, which can be potentially used as sources of natural or semisynthetic antioxidants.[9] In fact, numerous members of this group of natural compounds have been demonstrated to possess radical scavenging/antioxidative activity, especially when studied in cell-free systems; however, this does not necessarily indicate a protective activity against the deleterious effects of oxidative stress in intact cells in vitro, or even in vivo.[10]

On the basis of previous studies performed by our group with several polyphenolic fractions and compounds extracted from several natural sources,[11−14] we focused our attention on the potential antioxidant activity of polyphenolic fractions from different natural sources such as white grape (*Vitis vinifera*) pomace, pine (*Pinus pinaster*) bark, and witch hazel (*Hamamelis virginiana*) bark. The work pays special attention to the different degrees of polymerization and galloylation of the extracts in contributing to the understanding of their mechanisms of action. Grape and pine extracts contain essentially procyanidins with low gallate content or no galloylation (pyrogallol content) at all, respectively. In contrast, the homologous fractions from witch hazel bark are composed of gallocatechins and prodelphinidins (monomeric and oligomeric catechins with three hydroxyls on ring B) with a high proportion of gallates and thus with high pyrogallol content.[11,12,14]

The study was designed to evaluate whether polyphenolic fractions prevent H_2O_2-induced lipid peroxidation and hemolysis of human erythrocytes and the extent to which these compounds can protect the 3T3 cell line from an oxidative insult, that is, against H_2O_2-induced cell damage, on the basis of their sensitivity. However, we evaluated the impact of different degrees of galloylation and polymerization.

MATERIALS AND METHODS

Polyphenolic Fractions. The polyphenolic total extract (OW) was obtained from *P. pinaster* bark (OWP), from Parellada grape (*V. vinifera*) pomace (OWG), and from chopped witch hazel bark (OWH), following the procedure previously described.[9,14] From these total extracts our group obtained mixtures of variable phenolic composition using a combination of chromatographic techniques, and we then generated a set of homologous fractions of each type, which differed in composition and procyanidin structure. We labeled these homologous fractions OWP, IVP, and XIP for pine; OWG, IVG, and XIG for grape; and OWH, IVH,

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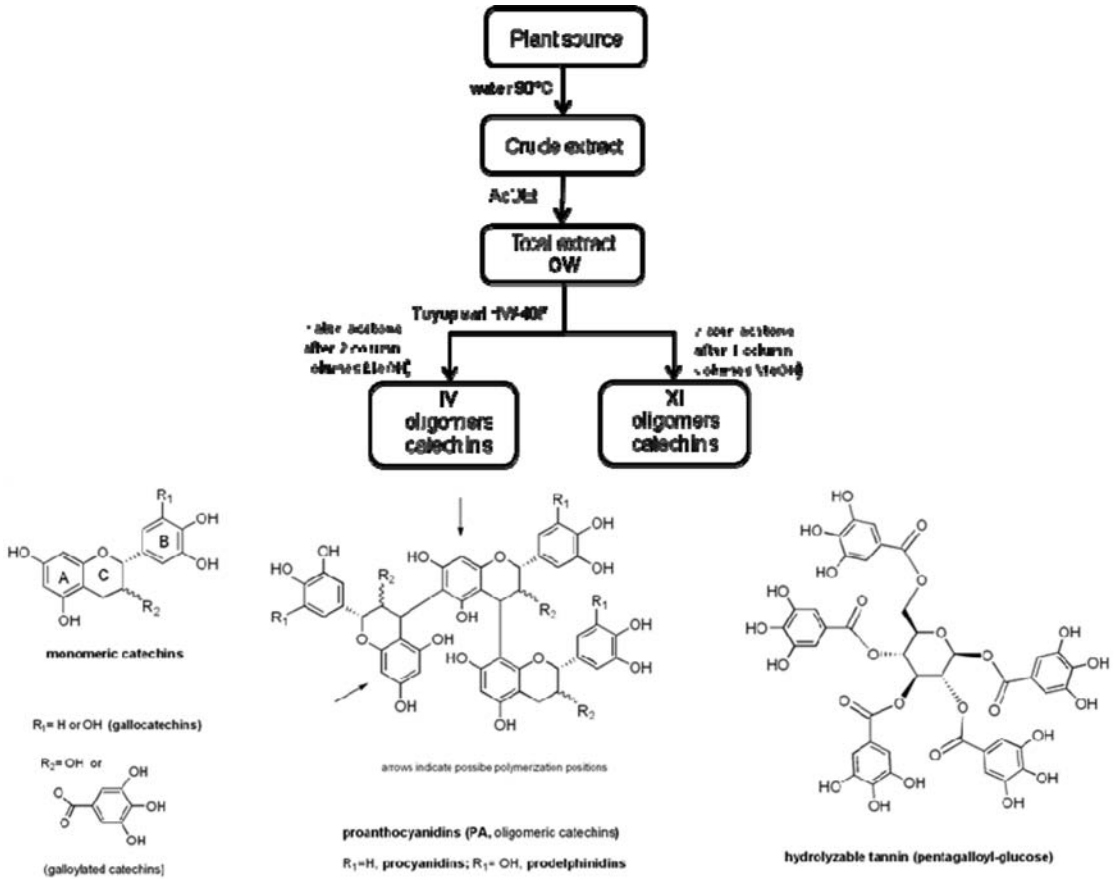

Figure 1. Scheme of the preparation of the different fractions and the structures of the polyphenolic compounds studied.

and XIH for *Hamamelis*. The fractions from the three sources were mainly differentiated by their degree of galloylation (presence of gallate esters). We compare homologous fractions from pine and grape with different degrees of polymerization and galloylation and witch hazel, which is a material differing in molecular structure but with a high degree of galloylation. Figure 1 shows the scheme of preparation of the different fractions and the structures of the polyphenolic compounds present in them. Table 1 summarizes the degree of polymerization (mDP) and the percentage of galloylation previously described by our group.[10−12,14]

General Chemicals. 2-Thiobarbituric acid, hydrogen peroxide 30% (w/w) solution, and sodium azide were purchased from Sigma (St. Louis, MO); trichloroacetic acid solution 20% w/v, extra pure, was purchased from Scharlau (Sentmenat, Spain).

Blood Samples and Preparation of Red Blood Cells. Human blood was obtained from the Blood Bank of the Hospital Vall d'Hebrón (Barcelona, Spain) following the ethical guidelines of this institution. The experiments were performed the same day of arrival to avoid alterations in enzyme activity. The erythrocytes were washed three times in a phosphate-buffered solution (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4, and 5.6 mM KH_2PO_4 in distilled water (pH 7.4; 300 mOsmol/L) to remove plasma, platelets, and leucocytes. Cells were then suspended in PBS solution at a cell density of 8×10^9 cells/mL. The final packet cell volume was adjusted to 12.5% under the assay conditions. Sodium azide at 2 mM in PBS was added to the cell suspension and was preincubated for 15 min in continuous rotation to enable inactivation of erythrocyte catalase by the sodium azide.

In Vitro Assay for the Inhibition of Human Erythrocyte Hemolysis. We measured the hemolysis of red blood cells (RBCs) mediated by H_2O_2 using a modification of the method described previously.[15] The erythrocyte suspension (250 μL) was incubated in the presence of H_2O_2 at a final concentration of 20 mM for 90 min in a shaker at 37 °C.[16]

The protective activity of fractions was tested by adding several concentrations of the compounds dissolved in PBS, ranging from 25 to 75 μg/mL, to the RBC suspension in the presence of 20 mM H_2O_2.

A blood sample incubated at the same conditions but without H_2O_2 or fractions was included as a control for the spontaneous hemolysis.

Table 1. Degree of Polymerization (mDP) and Percentage of Gallate Groups of Fractions from Pine Bark (P Fractions), Grape Pomace (G Fractions), and Witch Hazel Bark (H Fractions)

fraction	mDP[a]	galloylation[b] (%)
OWP	2.1	
IVP	2.9	
XIP	3.4	
OWG	1.7	15
IVG	2.7	25
XIG	3.7	31
OWH	1.2	44
IVH	1.6	52
XIH	2.7	64

[a] mDP corresponding to the proanthocyanidins within the fractions. [b] Total galloylation from both proanthocyanidins and hydrolyzable tannins from refs 9, 10, and 12.

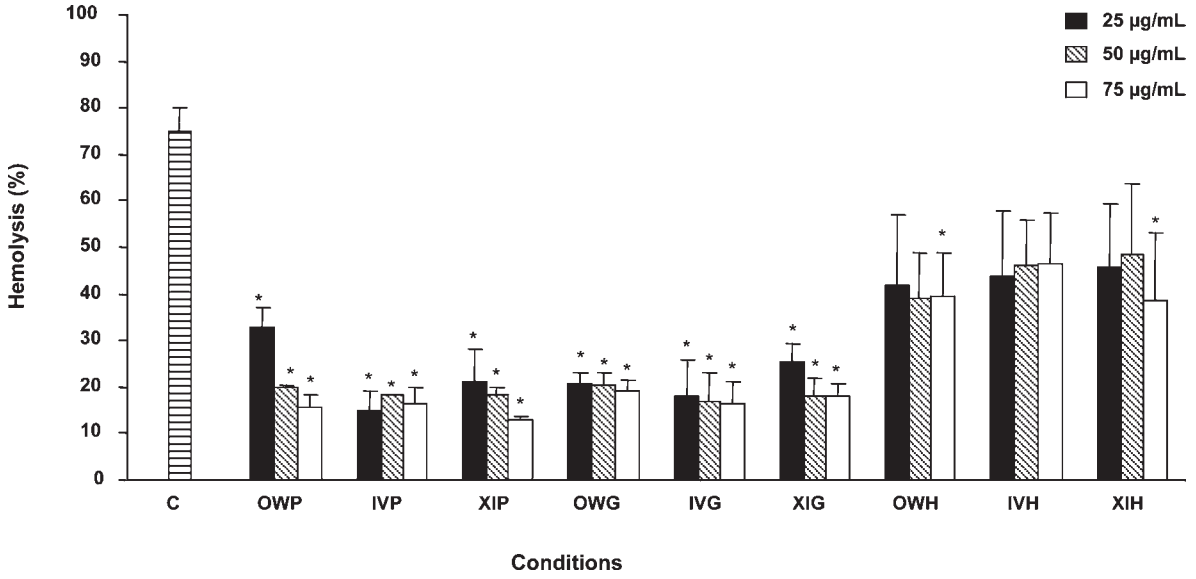

Figure 2. Protective effects of polyphenolic fractions against hemolysis induced by H_2O_2. Erythrocytes were incubated in the presence of 20 mM H_2O_2 (C) or H_2O_2 plus polyphenolic fractions (OWP, IVP, XIP, OWG, IVG, XIG, OWH, IVH, and XIH) at different concentrations. Results are expressed as percentage of hemolysis with respect to totally lysed cells. Statistical differences with respect to control treated cells (20 mM H_2O_2, C) were considered to be significant for *, $p < 0.05$.

After the incubation period, cells were centrifuged and the percentage of hemolysis was determined by a spectrophotometer at 540 nm (release of hemoglobin). The ratio of hemolysis was calculated by using the equation

$$H\,(\%) = \frac{A_p}{A_{water}} \times 100$$

where H (%) is the hemolysis of erythrocytes incubated with the different compounds, A_p is the absorbance of sample supernatant, and A_{water} is the absorbance after complete hemolysis with distilled water.[17]

In Vitro Assay for the Inhibition of Lipid Peroxidation on Erythrocytes. Lipid peroxidation mediated by H_2O_2 led to malondialdehyde (MDA) production, which was indirectly measured by a spectrophotometric method determining the thiobarbituric acid reactive (TBAR) substances. The principle of this method depends on the extraction of MDA from erythrocyte suspension by trichloroacetic acid (TCA) solution and the subsequent reaction of this MDA with thiobarbituric acid (TBA), which yields a pink complex (maximum absorption at 532 nm).[18] To induce lipid peroxidation, RBCs were incubated under the same conditions as the hemolysis assay (i.e., with 20 mM H_2O_2 alone or with different concentrations of test compound at 37 °C for 90 min). Following incubation, the RBC suspension was mixed with 1 mL of 20% w/v TCA solution to remove potentially interfering substances.[19] Samples were then centrifuged, and 1 mL of supernatant was mixed with 1 mL of 1% TBA. Finally, samples were heated at 90 °C for 50 min, cooled, and centrifuged before the absorbance of the supernatant was measured at 532 and 600 nm to discard possible interferences. The appropriate blanks and controls were run alongside the test samples. The degree of lipid peroxidation was expressed in arbitrary absorbance units after subtraction of the absorbance of controls. The percentage of lipid peroxidation inhibition was calculated on the basis of the absorbance of treated samples related to controls.

3T3 Cell Line Culture. The mouse embryonic fibroblast cell line 3T3 was obtained from the Banco de Células Eucariotas, Barcelona (Spain). Cells were grown in DMEM (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and a 1% penicillin (10000 U/mL)−streptomycin (10000 µg/mL) mixture and maintained in a humidified incubator at 37 °C and 5% CO_2. Cells were routinely cultured in 75 cm^2 culture flasks.

When the cells were approximately 80% confluent, they were harvested with trypsin/EDTA and seeded at a density of 8.5×10^4 cells/mL into the central 60 wells of 96-well plates and then incubated for 24 h at 37 °C and 5% CO_2.

Cytoprotection against H_2O_2-Induced Damage. To assay the ability of the compounds to protect the 3T3 fibroblast cell line against ROS-mediated oxidative stress, cells were preincubated overnight (18−20 h) with polyphenolic fractions at different concentrations (12.5, 25, 50, and 75 µg/mL) prepared in DMEM supplemented with 5% FBS previously sterilized by filtration. The stock antioxidant solutions were prepared immediately before each set of experiments. After the preincubation period, the excess of polyphenols was completely removed and the medium was changed before the addition of H_2O_2 to avoid a direct reaction between the polyphenol and the oxidant source in the medium.[1] H_2O_2 was added to cell culture dissolved in DMEM at a final concentration of 2 mM[16] and incubated for 2 h. After incubation, the medium was eliminated and cells were washed with PBS. Cell viability as end point was used to measure cytoprotective effects of the compounds against H_2O_2-induced cell damage.

Cell Viability Assay. Cell viability was determined by the neutral red uptake (NRU) assay, performed as previously described [20] and modified to remove the use of formaldehyde.[21]

Following treatments, the medium was removed and neutral red solution was added (100 µL per well). After 3 h of incubation at 37 °C and 5% CO_2, the medium was aspirated, cells were washed twice with PBS, and a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the dye absorbed into the viable cells. After 10 min on a microtiter-plate shaker, the absorbance of neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader. Results are given as the percentage of viability compared with control cells (the mean optical density of untreated cells was set to 100% viability).

Statistical Analysis. Results are expressed as the mean ± SE of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons between fractions with respect to the H_2O_2 controls or the Scheffé post hoc test to compare results between extracts, all using SPSS software (SPSS Inc., Chicago, IL). Statistical differences at $p < 0.05$ were considered to be significant.

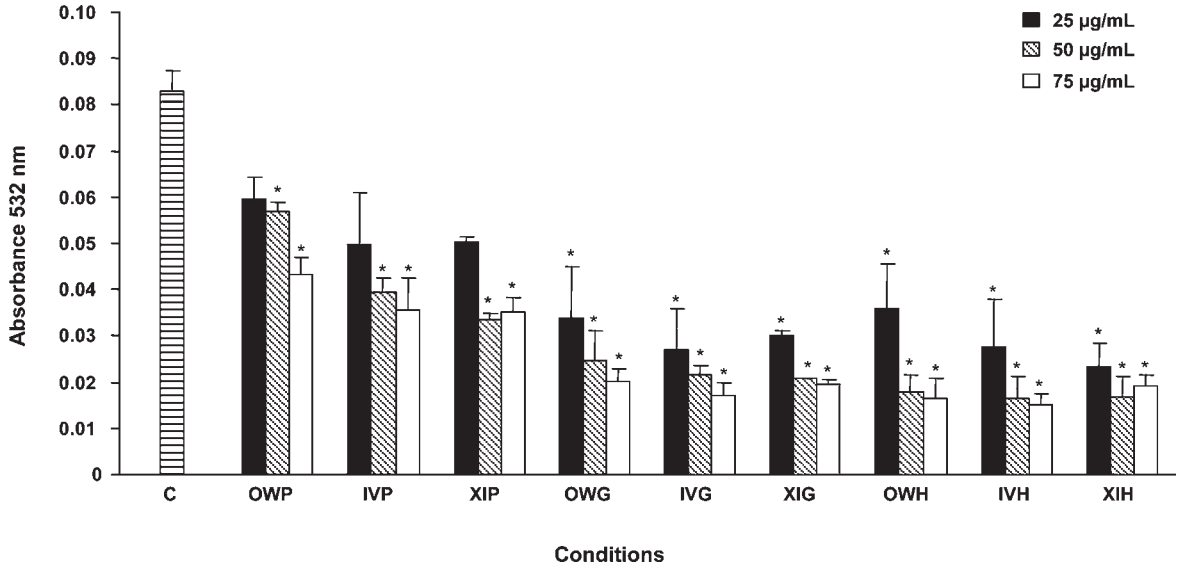

Figure 3. Effects of polyphenolic fractions against lipid peroxidation induced by H_2O_2. Erythrocytes were treated with 20 mM H_2O_2 (C) and 20 mM H_2O_2 plus the polyphenolic fractions (OWP, IVP, XIP, OWG, IVG, XIG, OWH, IVH, and XIH) at different concentrations. Results are expressed as absorbance at 532 nm produced by the presence of the MDA−TBA complex. Statistical differences with respect to control treated cells (20 mM H_2O_2, C) were considered to be significant for *, $p < 0.05$.

RESULTS AND DISCUSSION

Protective Effect of Polyphenolic Fractions against RBC Oxidative Damage. In previous studies, our group characterized the antioxidant and cytotoxic properties of a polyphenolic group of fractions from different natural sources (pine bark, grape pomace, and witch hazel bark).[11−14] In these studies, antioxidant characterization was performed both by cell-free systems and chemical methods (DPPH, HNTTM, ABTS, etc.) and by AAPH assay in red blood cells. However, because flavonoids display antioxidant activity against several injurious agents, we considered it appropriate to determine the potential cytoprotective activity of these fractions against peroxide insult and to evaluate the impact of different degrees of galloylation and polymerization.

The protective effect of polyphenolic fractions was evaluated by oxidative hemolysis experimentally induced with H_2O_2 in RBC. Under the given conditions, H_2O_2 caused considerable RBC hemolysis (75%) that was significantly inhibited by polyphenolic fractions except in the case of *Hamamelis* fractions (Figure 2). The inhibitory effect observed in the case of pine and grape fractions was dose-dependent in almost all cases, reaching values up to 80%.

Free radicals attack erythrocyte membrane components, such as proteins and lipids, causing changes in the structure and function of membranes, which may result in hemolysis. In this sense, antioxidant products appear to be an excellent strategy to exert protective effects and thus prevent disruption of erythrocyte membrane. The polyphenolic fractions studied in the present work showed good antihemolytic activity, being higher when there is no galloylation or it does not exceed a percentage of 30%, which is the case for pine and grape polyphenolic fractions, respectively. By contrast, the *Hamamelis* extracts with the highest degree of galloylation exhibited very poor beneficial effect, protecting erythrocyte hemolysis against peroxidative insult.

Moreover, the protection of peroxidative damage was also evaluated by measuring the level of MDA expressed as absorbance at 532 nm (Figure 3). Erythrocyte controls without treatments revealed an absorbance of 0.0317 ± 0.001 (mean ± SEM), which was subtracted for each treatment. The addition of H_2O_2 induced an increase of MDA level, thus indicating relevant oxidative damage of cell membranes that can be prevented by antioxidants. In this sense, the presence of our polyphenolic fractions inhibited the formation of H_2O_2−TBAR in a dose-dependent manner as shown by the decrease in absorbance at 532 nm. This inhibitory effect was statistically significant ($p < 0.05$) in all cases, except for pine fractions at the lowest concentration tested. When homologous fractions are compared, grape and *Hamamelis* extracts presented similar inhibitory effects; that is, they hold the best protective effects against peroxidative insult.

The mechanism of free radical induced hemolysis is not completely understood, although a competitive model between lipid and protein oxidation occurring simultaneously is usually proposed as a hypothesis.[22] However, among the potential mechanisms by which natural proanthocyanidins and other phenolics may protect erythrocytes from hemolysis, some authors have proposed to include the reduction of lipid peroxidation.[23] Nevertheless, on the basis of our results, it seems that there is no correlation between the reduction of lipid peroxidation and the hemolysis protection induced by oxidative insult.

Some authors have attributed a direct relationship between protection against lipid peroxidation and the degree of galloylation of compounds.[24] Witch hazel barks have been recently described as a source of hydrolyzable tannins with a central nucleus of glucose having several gallate groups that are present in much higher concentrations than procyanidins.[25] In this regard, the *Hamamelis* fractions have the highest content in gallate moieties (from 44 to 64%), and they showed good protection against lipid peroxidation over erythrocyte membranes, but not better than the one recorded by the grape fractions with a percentage of galloylation between 15 and 31%. Moreover, the more modest protection measured for pine fractions in this assay correlates well with their lack of galloylation.

Among pine fractions, the strongest lipid peroxidation inhibitory capacity corresponded to the mixture with the highest

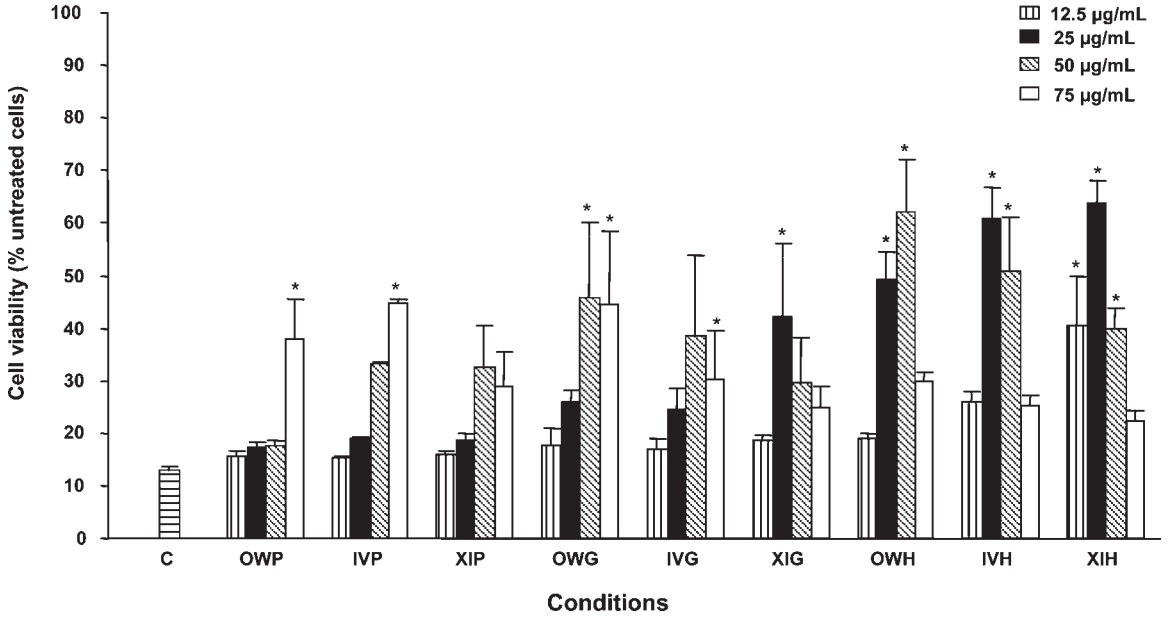

Figure 4. Comparative protective effect of polyphenolic fractions toward H_2O_2-induced cytotoxicity on 3T3 cells. Cells were preincubated overnight with polyphenolic fractions (OWP, IVP, XIP, OWG, IVG, XIG, OWH, IVH, and XIH) at different concentrations, washed, and incubated with 2 mM H_2O_2 for 2 h. Cell viability was expressed as percentage of the untreated cells (100%). Statistical differences with respect to control treated cells (20 mM H_2O_2, C) were considered to be significant for $*$, $p < 0.05$.

degree of polymerization (XIP, mDP of 3.4). In grape and *Hamamelis* mixtures the protection against lipid peroxidation was proportional to mDP and galloylation. Thus, as previously pointed out,[13,26] we conclude that both mDP and galloylation modulate the antioxidant capacity of polyphenolic substances.

Therefore, we postulate that in the case of H_2O_2-induced hemolysis, a degree of polymerization of about 3 and a percentage of galloylation around 30% determine the best protective capacity of the polyphenolic fractions studied in the present work. Moreover, some degree of galloylation enhances the inhibitory effects against lipid peroxidation. For this reason, grape extracts appear to be the best option to fight against hydrogen peroxide erythrocyte damage.

Antioxidant Protection against H_2O_2-Induced Cytotoxicity. Although erythrocytes are widely used for studying mechanisms of oxidative damage in biomembranes because they lack cytoplasmatic organelles, more complex cell-based systems should be considered to evaluate the potential antioxidant properties of novel compounds. Several studies support the use of the mouse embryo fibroblast 3T3 cell line as a sensitive cellular model for the evaluation of oxidative stress induced by H_2O_2.[27]

Preincubation with polyphenolic fractions for 18 h prior to the H_2O_2 insult resulted in an antioxidant-specific modulation of 3T3 cell viability. Previous papers have shown that preincubation with polyphenolic compounds was required to achieve protective effects in other cell models and also that if flavonoids and H_2O_2 were added and incubated simultaneously, the protective effect was lower than when cells were preincubated.[1,28] The fact that recovery from damage was more marked in cells treated with the tested flavonoids before H_2O_2 incubation suggests that simple scavenging of ROS by flavonoids was not the only mechanism involved in cell protection by these compounds. Moreover, it has been reported that grape seed procyanidin extracts activate antioxidative enzymes and the glutathione cycle,[29] so these compounds could enhance the antioxidant potential of cells during preincubation.

The 3T3 cell line was pretreated with polyphenolic fractions at concentrations between 0 and 75 μg/mL to study their capacity to protect cells when exposed to 2 mM H_2O_2 for 2 h. As shown in Figure 4 the addition of H_2O_2 to cell cultures resulted in a loss of approximately 85% of 3T3 cell viability. Pretreatment with the polyphenolic fractions exerted a dose-dependent protective effect for the pine fractions with the lowest polymerization degree (mDP), OWP and IVP, whereas the remaining fractions lost effectiveness at the highest concentrations tested. It should be emphasized that some of the highly galloylated fractions (XIG, OWH, IVH, and XIH) showed significant biological antioxidant activity against strong ROS-induced oxidative stress at 25 μg/mL, increasing viability up to 5-fold in the case of the XIH fraction compared to H_2O_2 alone.

Polyphenolic structure is essential to determine the activity of antioxidant compounds. For example, galloylation appears to be more influential than polymerization with regard to biological apoptosis and with respect to the hydroxyl and superoxide anion radical scavenging capacity of fractions. Recently, it has been demonstrated that galloylation seems to play an important role in the antioxidant efficacy of the polyphenolic fractions, whereas an intermediate polymerization degree showed the highest activity in fish oil-in-water emulsions.[30] Proanthocyanidins with medium size (two to three monomeric units) and low galloylation degree (0.15−0.25 gallate group/molecule) seem to better inhibit lipid oxidation in pelagic fish muscle.[31] This ability to prevent the imbalance between high-level oxidant exposure and antioxidant capacity, which leads to several pathological processes, may contribute to the chemopreventive effect of the gallic acid derivatives.[32] However, some of these compounds can behave as both anti- and pro-oxidants and their action is dependent on the concentration used. It should be noted that the protective effect of each compound does not always increase in parallel with its concentration. This behavior may be explained by considering the pro-oxidant effect of phenolic compounds.[33,34]

In conclusion, the present study has demonstrated the protective effect of the polyphenolic fractions obtained from pine

bark, grape pomace, and witch hazel bark against the oxidative stress induced by H_2O_2 in different cell models. The compounds protected against erythrocyte lipid peroxidation and hemolysis and also prevented H_2O_2-induced cytotoxicity in 3T3 cells. Galloylation is claimed to be related to antioxidant protective capacity, and it is also responsible for the pro-oxidant effect observed at high doses. However, our results show that not only the percentage of galloylation but, to some extent, also the degree of polymerization can modulate the antioxidant capacity of polyphenolic fractions. In this sense, it is crucial that novel polyphenolic fractions should be synthesized attending a value of 3 for the mean degree of polymerization and not exceed a 30% galloylation to reach the highest antioxidant capacity with the lowest cytotoxic effects. For this reason, the grape extracts appear to be the best strategy to fight against hydrogen peroxide cell damage.

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