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Procyanidins as Antioxidants and Tumor Cell Growth Modulators

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Five procyanidin fractions with different structural complexities were obtained after fractionation of a grape seed extract. The procyanidin fraction's abilities to inhibit lipid peroxidation induced by 2,2′-azobis-2-methyl-propanimidamide dihydrochloride in a liposomal membrane system were examined. The antioxidant capacities of all fractions were evaluated through monitoring oxygen consumption and by measuring the formation of conjugated dienes. All tested fractions provided protection of membranes against peroxyl radicals by increasing the induction time of oxidation. This effect increased up to fraction II but decreased with the increase of the structural complexity of further procyanidin fractions, possibly due to steric hindrance effects exhibited by the more complex fractions. In addition, the antiradical properties and the reducing power of these fractions were determined by using 2,2-diphenyl-1-picrylhydrazyl and ferric reducing/antioxidant power methods, respectively. Moreover, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide reduction and DNA synthesis were measured in Michigan Cancer Foundation 7 (MCF-7), a human breast cancer cell line, treated with catechin or procyanidin fractions in order to evaluate the effect of these compounds on cell viability and proliferation. The results obtained showed that at 30 μg/mL, fractions I and II decreased cell viability and proliferation, which was not observed with 60 μg/mL of the same fractions. Catechin was also able to decrease cell viability and proliferation at 30 and 60 μg/mL. It is interesting to notice that the procyanidin fractions that exhibited higher antioxidant activity were the same to affect cell viability and proliferation.

KEYWORDS: Antioxidant; antiproliferative agents; breast cancer; grape seed extract; MCF-7 cells; procyanidins

INTRODUCTION

Procyanidins, or condensed tannins, are a complex family of polyphenol polymers widespread in nature, which occur in products such as red wine, apples, tea, and cocoa or chocolate (*1*). These polyphenols are composed of monomeric flavan-3-ol units [(+)-catechin and/or (−)-epicatechin]. The size and composition of oligomeric procyanidins appear to be related to their antioxidant activity, through differences in both the intrinsic scavenging capacity and the physicochemical properties governing their partition behavior within biological environments (*2*, *3*).

Several biological effects are attributed to procyanidins, namely, antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and antiallergic activity (*4*, *5*). Moreover, a special interest has been devoted to their antioxidant activity since procyanidins can exert a protective action in the organism against protein and lipid oxidation, reducing the risk of chronic diseases such as coronary heart disease (*6*−*8*) and certain types of cancer (*9*, *10*).

Apart from their direct antioxidant properties, flavonoids exert other activities that may not be related to their radical scavenging capacity. These activities, mostly mediated by receptor−ligand interactions, include antiproliferation, cell cycle regulation, and induction of apoptosis (*11*). It is becoming evident that the activity of plant flavonoids as preventive agents must be evaluated from different angles to cover not only their antioxidant capacity but also the influence of the physicochemical environment on the antioxidant effectiveness and the occurrence of other biological activities.

The aim of this work was to study the ability of procyanidin fractions from grape seed as antioxidants or preventive agents against cell aging and cancer. For this purpose, the antiradical properties and the reducing power of these fractions were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assays, respectively. The

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Table 1. Average Molecular Weights of Procyanidin from Grape Seed Fractions Determined by LSIMS

fraction	recovery time (h)	average MW
I	2:30–4	600
II	4–4:35	800
III	4:35–6	900
IV	6–7	1000
V	7–8	1200

activity against lipid peroxidation, induced by AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride), was determined using soybean phosphatidylcholine (PC) liposomes as a membrane model system. Artificial phospholipid vesicles, so-called liposomes, have been frequently used as models of cellular and subcellular membranes. In particular, liposomes prepared from PC containing polyunsaturated fatty acids are available for investigating the mechanism of lipid peroxidation and the antioxidative action of components in phospholipids bilayers. Unilamellar liposomes seem to be a more suitable model for free radical oxidation in vivo because biomembranes, in general, consist of unilamellar structures (*12*).

In addition, the effect of these procyanidin fractions was evaluated on cell viability and proliferation. For this purpose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) reduction and DNA synthesis were measured in Michigan Cancer Foundation 7 cells (MCF-7), a human breast cancer cell line, treated with the studied compounds.

MATERIALS AND METHODS

Reagents. Toyopearl gel was purchased from Tosoh (Tokyo, Japan), and AAPH, DPPH, $FeCl_3$, trolox, Hepes, NaCl, and soybean L-α-PC were purchased from Sigma-Aldrich (Madrid, Spain), TPTZ (2,4,6-tripyridy-s-triazine) was purchased from Fluka (Madrid, Spain), and [methyl-^{3}H]thymidine (88.0 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Grape Seed Extract. Condensed tannins were extracted from *Vitis vinifera* grape seed tissues with an ethanol/water/chloroform solution (1:1:2, v/v/v) using a blender (Ultra-Turrax) as described elsewhere (*13*). The 50% aqueous ethanol upper layer, containing polyphenols, was separated from the chloroform layer containing chlorophylls, lipids, and other undesirable compounds. Ethanol was removed using a rotary evaporator, and the resulting aqueous solution, containing the polyphenolic compounds, was extracted with ethyl acetate, followed by precipitation with hexane, to obtain the procyanidin oligomers, according to the procedure described in the literature (*14*).

Fractionation of Grape Seed Procyanidins. The grape seed extract was fractionated through a TSK Toyopearl HW-40(s) gel column (250 mm × 16 mm i.d., with 0.8 mL min^{-1} methanol as the eluent) according to the procedure described in the literature with some modifications (*15*). Fractions were all obtained after elution with 99.8% (v/v) methanol; the first 120 mL, corresponding to the elution of catechin monomers, was eliminated, and elution was followed over 5 h in order to elute the procyanidin oligomers; all of the fractions were mixed with deionized water; the solvent was eliminated using a rotary evaporator under reduced pressure at 30 °C and then freeze-dried. One part of procyanidin fractions was dissolved in water and maintained at −20 °C until use for antioxidant activity experiments. The other part was dissolved in ethanol and maintained at −80 °C until use in the proliferation assay. The resulting solids were analyzed by laser secondary ionization mass spectrometry (LSIMS) (**Table 1**).

Radical DPPH Scavenging Activity. Following the method described in the literature (*16*) with some modifications, radical activities were determined by using DPPH as a free radical. The reaction for scavenging DPPH radicals was performed in polypropylene tubes at room temperature (22−23 °C). For each tube, an aliquot of procyanidin fractions (2.5 μg/mL final concentration) was added to 3 mL of DPPH solution (60 μM in methanol). The decrease in absorbance was measured at 515 nm, at $t = 0$, and every 10 min for 30 min. Methanol was used as a blank solution, and DPPH solution without any sample served as the control. The antiradical activity was calculated from the equation determined from linear regression after plotting known solutions of Trolox with different concentrations (2.5−50 μM) (*16*, *17*). For the final results, the 0−20 min reaction time window was used. The antiradical activity was expressed as μM Trolox equivalents.

FRAP. The FRAP assay developed by Benzie and Strain (*18*) was performed with some modifications. In short, FRAP reagent [10 vol of 300 mM acetate buffer, pH 3.6 + 1 vol of 10 mM TPTZ (2,4,6-tripyridyl-1,3,5-triazine) in 40 mM HCl + 1 vol of 20 mM $FeCl_3$] was diluted to one-third with acetate buffer and prewarmed at 37 °C. Three milliliters of this reagent was mixed with an aliquot of procyanidin fraction (2.5 μg/mL). This mixture was shaken, and the absorbance was read at 593 nm. The test was performed at 37 °C, and the 0−4 min reaction time window was used. Results are expressed as Trolox equivalents determined using a calibration curve.

Liposome Preparation. Liposomes were prepared by evaporation to dryness of L-α-PC from soybean solution in chloroform with a stream of argon; the film was then left under vacuum over 3 h to remove all traces of the organic solvent. The resultant dried lipid film was dispersed with Hepes buffer (10 mM Hepes; 0.1 M NaCl; pH 7.4), and then, the mixture was shaken above the phase transition temperature to produce multilamellar liposomes (MLV). Frozen and thawed MLVs were obtained by repeating the cycle five times: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 °C. Lipid suspensions were equilibrated at 37 °C for 30 min and extruded 10 times through polycarbonate filters of 100 nm pore size in a 10 mL stainless steel extruder in order to form large unilamellar vesicles (LUV) (*19*).

Lipid Peroxidation Procedures. Lipid peroxidation of soybean LUVs was induced by peroxyl radicals, generated at a constant rate, by thermal degradation of the azocompound, AAPH, in the presence or absence of antioxidants and followed by measuring the oxygen consumption and conjugated diene production.

Oxygen Consumption. The rate of oxygen consumption was measured continuously with a Clark type oxygen electrode (Hansatech) provided with an automatic recording apparatus. The reaction mixture containing 1.3 mL of Hepes buffer, 200 μL of LUV (340 μM final concentration), and the antioxidant tested (313 ng/mL for procyanidin fractions solved in water) was left in a 37 °C thermostated bath for 1 h. This mixture was introduced in a closed glass vessel, protected from light, thermostated at 37 °C, and provided with a stirrer, and the reaction was started by the addition of AAPH (10 mM final concentration) (*20*). The induction periods in the presence of procyanidin fractions were determined graphically from the profiles of oxygen consumption by the coordinates of the interception of the tangents to the inhibited and uninhibited rates of oxidation. Results were expressed relative to the ones obtained with Trolox.

Conjugated Diene Formation. Soybean liposomes (500 μM final concentration) were incubated in a water bath at 37° with the procyanidin fractions (313 ng/mL) to test. The reaction was started with the addition of 10 μL of the azo initiator (5 mM final concentration). Aliquots of liposomes (60 μL) were taken at 15 min intervals and dissolved with 940 μL of absolute ethanol directly in a 1 cm quartz cell (*21*). The formation of conjugated dienes was followed by recording the absorbance at 233 nm. The induction periods in the presence of procyanidin fractions were determined graphically from the profiles of conjugated dienes formation by the coordinates of the interception of the tangents to the inhibited and uninhibited rates of oxidation. Results were expressed relative to the ones obtained with Trolox.

Cells and Culture Conditions. MCF-7 cells, from an estrogen receptor-positive human breast cancer cell line, were used between passage numbers 48−62. MCF-7 cells were maintained in a humidified atmosphere of 5% CO_2−95% air and were grown in minimum essential medium (MEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B (all from Sigma). The culture medium was changed every 2−3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically

(0.25% trypsin−EDTA, 5 min, 37 °C), split 1:4, and subcultured in plastic culture dishes (21 cm^2; ∅ 60 mm; Corning Costar, Corning, NY).

Effects on Viability of MCF-7 Cells Determined by the MTT Reduction Method. For the experiments, the MCF-7 cells were seeded into 24 well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar). After 24 h in culture, the cells were treated with compounds and control cells were incubated in culture medium containing 0.1% ethanol. After 48 h of treatment, the cell viability was assessed by the MTT assay (*22*). This is a colorimetric assay dependent on the cellular reduction of MTT to a blue formazan product by the mitochondrial dehydrogenase of viable cells. The intensity of the blue color is a measure of cell viability (A_{540}). Briefly, culture medium was removed and cells were preincubated with the tested compounds in culture medium at 37° for 48 h. This treatment was removed, and the cells were incubated for 3 h with 500 μL of culture medium and 50 μL/well of MTT (5 mg/mL of PBS). This solution was carefully aspirated, and the formazan product of mitochondrial dehydrogenase activity was dissolved in DMSO. Absorbance at 660 nm corresponds to unspecific reduction of MTT. The $A_{540} - A_{660}$ value was calculated and registered. Results were expressed in % of control.

Effects on DNA Synthesis in MCF-7 Cells. MCF-7 cells were seeded into 24 well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar) in a final volume of 0.5 mL culture medium containing 10% FBS. After 24 h in culture, the cells were treated with different concentrations of procyanidin fractions in culture medium containing 5% FBS. Control cells were incubated in culture medium containing 0.1% ethanol. After 48 h, the cells were incubated with 0.2 mL of methyl[^{3}H]thymidine (0.5 μCi/well) for 4 h. The medium was removed, and the cells were fixed by incubation in 10% TCA for 1 h at 4 °C. The cells were then washed twice with 10% TCA to remove unbound radioactivity. The plates were air-dried, and the cells were lysed with 1 M NaOH (0.28 mL/well). A 0.25 mL aliquot of the lysate was neutralized with HCl prior to the addition of scintillation fluid. The radioactivity of the samples was quantified by a liquid scintillation counter. The counts (disintegrations per min) of each treatment were averaged and expressed as percent of controls (adapted from ref *23*).

Statistical Analysis. Values are expressed as the arithmetic mean ± standard error of the mean. Statistical significance of the difference between various groups was evaluated by one-way analysis variance followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

The procyanidin fractions were obtained after fractionation of a grape seed extract by low-pressure column chromatography. Five fractions were yielded, containing procyanidins with different structural complexities (**Table 1**) and consequently different molecular weights (MWs). The general structure of the compounds present in these fractions is illustrated in **Figure 1**. All of the fractions were freeze-dried and used for the antioxidant, antiradical, and antiproliferative assays.

DPPH and FRAP. The free radical scavenging activity of the procyanidin fractions was tested using the DPPH method. The tested substances react with DPPH, which is a stable free radical, and induce a decrease of the absorbance measured at 515 nm, which indicates the scavenging potential of the extracts. As it can be seen from **Table 2**, the antiradicalar activity of the tested procyanidin fractions was not statistically different. Nevertheless, it would be expected that the presence of more hydroxyl groups in the more complex fractions would increase the scavenging capacity, but the larger structure may cause stereochemical hindrances and these groups may no longer be available for donating hydrogens and, thus, scavenging radicals. Moreover, the reducing power of these compounds was assessed with the FRAP assay. The results obtained were in agreement with the possibility that the *ortho*-catechol groups of procyanidins were hardly accessible with the enhancement of the overall structure. It has been described that the reduction of the metals is positively correlated with the number of hydroxyl groups present in the molecules (*24*) and that the points of attachment of transition metal ions to the flavonoid molecule are at the *ortho*-catechol group in the B ring (*25*). As seen in **Table 2**, the more complex fraction (V) presents a lower capacity for reducing metal ions, which is probably related to some stereochemical impedance that avoids metal to reach the *ortho*-catechol group of ring B. This steric problem has already been described in a work of Saint-Cricq (*3*).

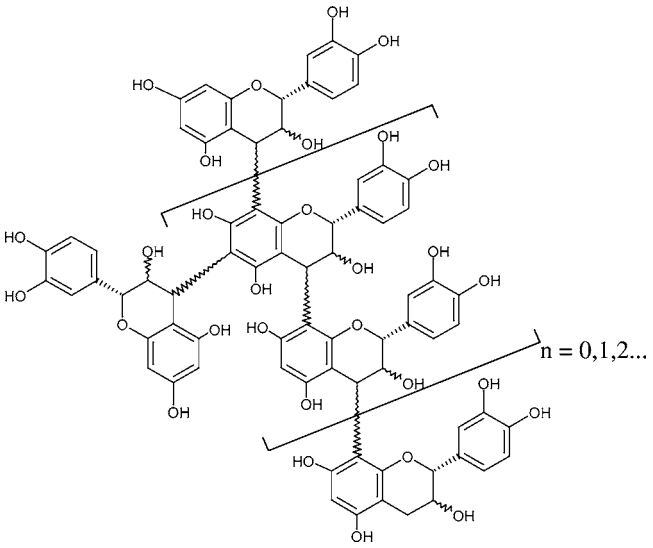

Figure 1. General structure of procyanidins (condensed tannins).

Table 2. Antiradical Activity and Reducing Power of 2.5 μg/mL of Procyanidin Fractions Assessed by the DPPH Method and FRAP Method, Respectively

fraction	DPPH	FRAP[a]
I	12.106 ± 1.550	11.810 ± 0.795 a
II	13.348 ± 1.441	10.459 ± 0.866 b
III	12.071 ± 1.289	9.886 ± 0.628 a,c
IV	11.804 ± 1.425	8.376 ± 0.865 a,b
V	10.803 ± 1.594	7.332 ± 0.479 a,b,c

[a] The mean values followed by the same letters in each column are significantly different at $P < 0.05$.

Oxidation of Soybean PC Liposomes. Liposomes were used in this study as they are usually employed to mimic biological targets (e.g., cellular membranes). The evaluation of the antioxidant capacity of the procyanidin fractions, against oxidation of soybean PC liposomes, was performed using AAPH as a peroxidation initiator. Despite not being a relevant biomolecule, AAPH has been thoroughly used in this kind of study. Indeed, this compound undergoes thermal decomposition at 37 °C and generates peroxyl radicals at a known and constant rate, thereby allowing reproducible and quantitative analysis of the fractions' antioxidant capacity (*26*). The antioxidant/antiradical capacity of the extracts was assessed at the initial and propagation stages of oxidation by monitoring oxygen consumption. Their influence in a further stage of oxidation was evaluated by measuring the conjugated diene formation. The results are expressed relative to the ones obtained with Trolox.

The generation of peroxyl radicals from AAPH induces a significant oxidation of PC, since they are able to subtract hydrogen atoms from polyunsaturated acyl chains, yielding lipid

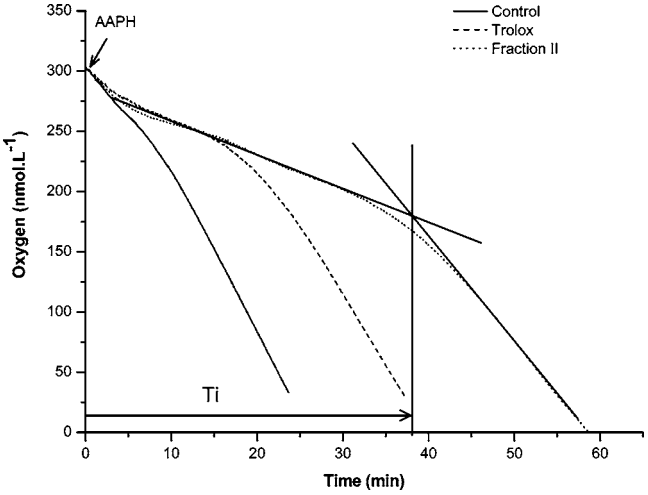

Figure 2. Oxygen consumption during the oxidation of soybean PC liposome membranes (LUV) (340 μM) induced by AAPH (10 mM) in the absence of antioxidants (control assay) and in the presence of 313 ng/mL of trolox and procyanidin fraction II. Ti, induction time.

radicals that lead to the propagation chain reaction (*27*). The data obtained from the oxygen consumption assays showed that all of the procyanidin fractions scavenged efficiently the peroxyl radicals generated in the aqueous phase comparatively to the control (without any procyanidin fraction), as it can be seen through the lag phase produced in the oxygen consumption graph (**Figure 2**). Nevertheless, it should be noted that the induction of the lag phase for the more complex fractions was much lower than the one for simpler fractions. This effect is easily perceived in **Figure 3a**, which displays the relevance of this feature relative to Trolox. There was a decrease in the protection effect with the increase of structural complexity (**Figure 3a**); fraction V presented less protection toward liposome oxidation. This is probably due to difficulties for the larger molecules to reach liposome membrane and scavenging peroxyl radicals.

The formation of conjugated dienes, comprised of hydroperoxides and respective degradation products, was monitored by measuring their characteristic absorbance at 233 nm. When lipids are protected in the presence of antioxidant, the oxidation induction time varies with the antioxidative capacity of the tested compound. The phenolic compounds present in the extracts are thought to trap AAPH-derived peroxyl radicals and inhibit the initiation of lipid peroxidation, but if located at the surface of the liposome, they may also quench liposome-derived peroxyl radicals, inhibiting the chain propagation (*28*). Consequently, formation of conjugated diene compounds was inhibited until exhaustion of the antioxidant compounds. As can be seen in **Figure 3b**, fractions I, II, and III inhibited significantly the formation of conjugated dienes. Fractions IV and V presented very little protection against conjugated dienes formation, which is in agreement with the hypothesis that larger molecules have difficult accessibility to fatty acyl chains and therefore exert less effect than fractions with a lower polymerization degree. These results agree with the ones yielded from the oxygen consumption assay.

Effects on Viability and on DNA Synthesis in MCF-7 Cells. The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and nowadays are used to treat human tumors. As therapeutic agents, the phytochemicals kill the cancer cells or stop their growth, leading to cancer remission. Considering the procyanidin antioxidant capacity, it is of interest to evaluate

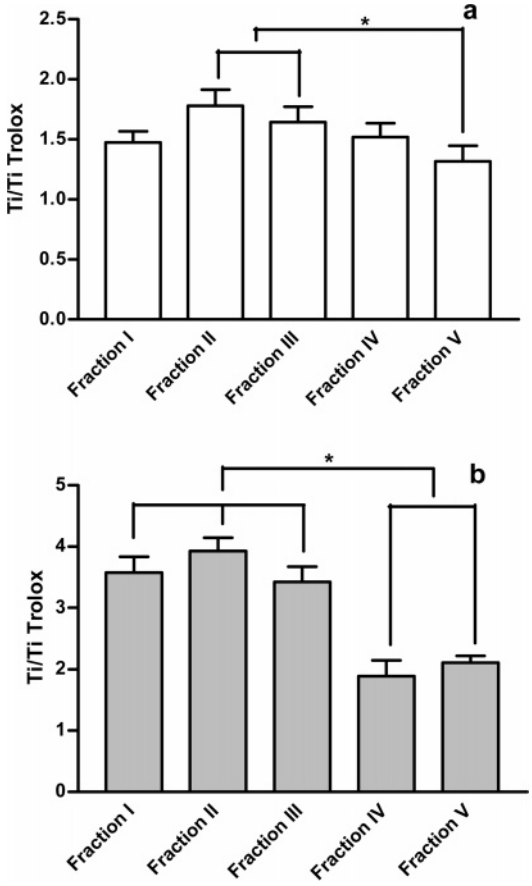

Figure 3. Inhibition of AAPH-initiated oxidation in soybean PC liposome membranes (LUV) by 313 ng/mL of procyanidin fractions measured by oxygen consumption (**a**) and conjugated diene formation (**b**). Columns represent mean values ± standard deviation (SD). *P < 0.05.

the effect of catechin and procyanidin (catechin oligomers) fractions on cell growth. A human breast cancer cell line, MCF-7, was used for this purpose. Treatments for 48 h with catechin and procyanidin fractions were performed using the MTT assay to evaluate cell viability. It was found that catechin, fraction I, and fraction II induced a decrease of cell viability at 30 μg/mL (**Figure 4a**). When a 60 μg/mL concentration was used, only catechin was able to decrease the cell viability of MCF-7 cells.

To analyze if the observed effect on cell viability was due to a decrease in cell proliferation, DNA synthesis in the presence of the same compounds was evaluated. For this purpose, cells were treated with the tested compounds for 48 h and incorporation of labeled thymidine into cells was quantified. Catechin induced inhibition of cell proliferation at concentrations of 30 and 60 μg/mL (**Figure 4b**), and fractions I and II showed the same effect but only when 30 μg/mL was used. Because of these results, lower concentrations of procyanidin fractions and catechin were tested (0.06−30 μg/mL), but no significant effect on thymidine incorporation was observed (data not shown).

More complex procyanidin fractions (FIII−FV) were tested at similar concentrations (30 and 60 μg/mL). An increase in cell viability was observed, albeit with no relation with cell proliferation, except for FIII at 60 μg/mL (**Figure 4**). Because these fractions (FIII−FV) have a higher polymerization degree, it is expected that they do not pass the plasmatic membrane. Therefore, this effect displayed in cell viability could result, for example, from the protection exerted by these compounds against membrane lipid peroxidation, a well-known mechanism that interferes with cell integrity. On the other hand, we cannot exclude the hypothesis that these compounds directly reduce

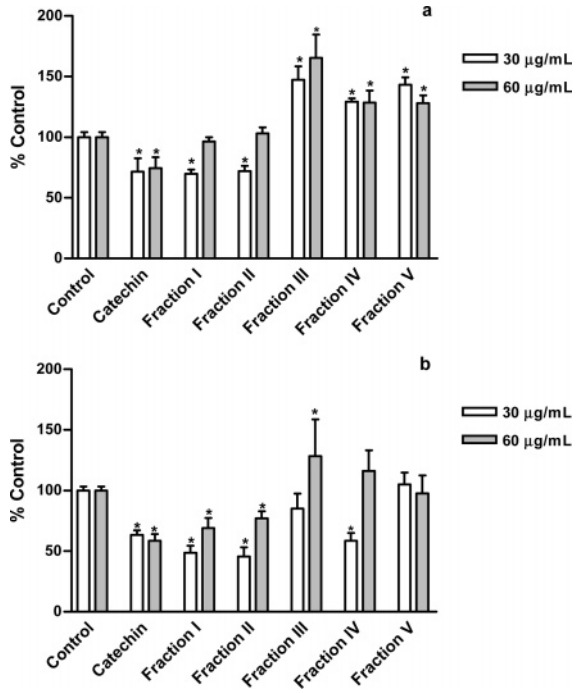

Figure 4. Effects of procyanidin fractions and catechin on cell viability evaluated by MTT assay (**a**) and cell proliferation measured by DNA synthesis (**b**) in MCF-7 cells. MCF-7 cells, seeded in 24 well plates, were treated with 30 and 60 μg/mL of each compound for 48 h in the presence of 5% FBS. *Significantly different from control, $P < 0.05$.

MTT, as described (*29*). These authors showed MTT reduction by flavonoids in the absence of cells. To overcome this flaw, in relation to these fractions, we have the results obtained on DNA synthesis through ^{3}H-thymidine incorporation.

The effects of procyanidin fractions over ^{3}H-thymidine incorporation were different at 60 and 30 μg/mL. We could speculate that at the higher concentration they exert a pro-oxidant effect, capable of contradicting the antiproliferative effect induced by 30 μg/mL.

Curiously, the procyanidin fractions that exhibited higher antioxidant activity were the ones capable of inhibiting cell proliferation. This suggests that antioxidant activity may be, in part, responsible for the effect on cell proliferation.

The antioxidant/antiradical activity of these compounds, together with their antiproliferative effect, observed in this work, is in line with previous studies (*30*−*32*). In agreement with these studies, we observed an antiproliferative effect of several grape polyphenolic compounds. The mode of action of polyphenols on cell growth is not well-established. However, it is now well-accepted that some of these compounds may interact with steroid receptors, such as phytoestrogens (*30*). Nevertheless, this kind of interaction might not be the sole mechanism of the polyphenol inhibitory effect on cell proliferation. Steroid receptor-independent pathways, such as those involved in cell arrest at the G2/M phase (*33*) or at the G1 phase of the cell cycle (*31*), have been described, among others (*34*, *35*).

In conclusion, oligomeric procyanidin fractions from grape seed appear to be efficient antioxidant agents. They exhibited scavenging and reducing activity in the DPPH and FRAP assays and antioxidant capacity against oxidation of soybean PC liposomes induced by an azo-compound. Catechin and procyanidin fractions I and II showed an antiproliferative effect in MCF-7 human breast cancer cells, probably through different mechanisms. The simpler procyanidin fractions displayed higher antioxidant activity when compared to more complex fractions; they were also the only ones with an antiproliferative effect. This is possibly due to steric hindrance effects exhibited by the more complex fractions. Procyanidins thus appear potentially interesting for the prevention and/or treatment of breast cancer. This potential deserves further characterization.

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