JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Functional Fatty Fish Supplemented with Grape Procyanidins. Antioxidant and Proapoptotic Properties on Colon Cell Lines

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This work shows the properties of grape procyanidins with additional anticarcinogenic properties for increasing the shelf life of functional seafood preparations. Galloylated procyanidins (100 ppm, 2.7 mean degree of polymerization, 25% galloylation) extended the shelf life of minced horse mackerel muscle stored at 4 °C more than 8 days compared to controls without addition of polyphenols. The levels of endogenous α-tocopherol, EPA, and DHA of fish muscle were also preserved after 10 days at 4 °C. Therefore, the presence of procyanidins increased the stability of a product based on minced fish muscle during cold storage and maintained its functionality associated with the presence of polyunsaturated fatty acids and α-tocopherol. In addition, grape procyanidins showed a significant capacity to induce apoptosis in colon cancer cells (HT29 cell line) while being inactive in noncancer control cells (IEC-6). Thus, the product based on fatty fish muscle supplemented with grape procyanidins appears to be a stable functional food offering the combined action of ω-3 fatty acids and natural polyphenols.

KEYWORDS: Fish lipids; procyanidins; functional seafood; anticarcinogenic

INTRODUCTION

Functional foods is a term used to refer to foods and isolated food ingredients that provide an additional physiological benefit beyond that of meeting basic nutritional needs (*1*). Within the past decade, there has been an increment of consumer and industrial interest in the health-enhancing role of specific foods or physiologically active food components (*2*). Marine bioactive lipids are a main goal of food companies which already have products in the market claiming to be stable ω-3 oils from marine sources. This fact has a special relevance for fatty and semifatty fish species such as horse mackerel, mackerel, herring, etc. Their muscles contain high amounts of ω-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), which have shown potential benefits to human health, particularly in preventing cardiovascular diseases (*3*, *4*). Fish is also an excellent source of other compounds with potential activities for human health such as ascorbic acid and α-tocopherol (*1*), involved in the stabilization of the high content of PUFA and rapidly degraded during the oxidative postmortem processes (*5*).

However, the oxidative development of off-flavors and rancidity continues to be the main objection in the exploitation and commercialization of products based on fish muscle. Moreover, the biological functionality of their lipids and other compounds is lost even at low storage temperatures (−18 °C) (*5*, *6*). Grape seeds and skin are a rich source of oligomeric catechins (procyanidins, **Figure 1**) which have been recently employed as natural antioxidants for stabilizing marine lipids (*7*). Such polyphenols are potent free radical scavengers (*8*) increasingly appreciated as chemopreventive agents against health conditions such as cancer and cardiovascular diseases (*9*, *10*). Apart from their scavenging activity, catechins, particularly those including a gallate ester moiety, appear to interact with the replicating machinery of tumor cells, probably through enzyme−ligand interactions with some key protein domains (*11*, *12*). They also trigger apoptosis (programmed cell death) by mechanisms that are currently under investigation (*13*, *14*).

This work is aimed at studying the functional properties of grape procyanidins with additional anticarcinogenic properties, as an effective additive against oxidation of fish functional compounds. For such a purpose, a seafood product based on minced horse mackerel muscle was supplemented with galloylated procyanidins and the oxidation of molecules associated with fish functionality such as PUFA and α-tocopherol was studied. The polyphenolic mixture will be, at least in part, bioavailable in the colon after ingestion of the supplemented seafood (*15*). Since procyanidins are potential cancer cell inhibitors reported to exert actions on cancer cells (*16*), their effect on colon cancer cells has been evaluated.

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10.1021/jf0527145 CCC: $33.50
Published on Web 04/22/2006

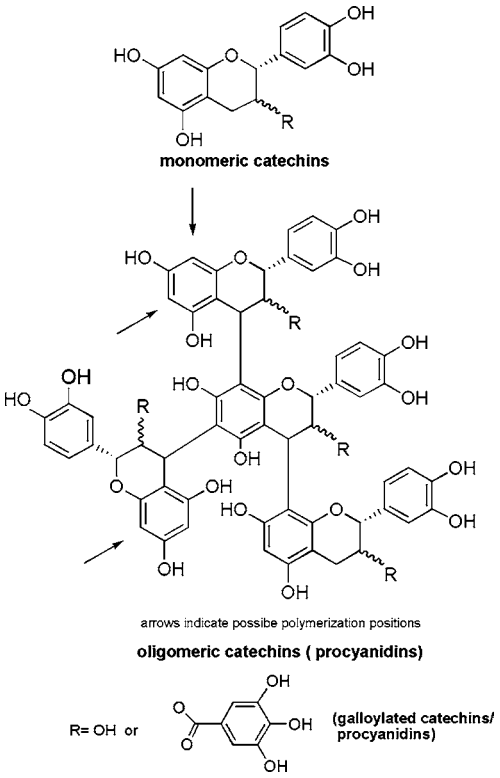

Figure 1. Structures of monomeric and oligomeric catechins.

MATERIALS AND METHODS

Materials. Fresh Atlantic horse mackerel (*Trachurus trachurus*) was supplied by a local market. Grape procyanidins were isolated from a total phenolic fraction extracted from grape pomace (*Vitis vinifera*) essentially as described by Torres et al. (*17*). The fraction employed in this work was a mixture of procyanidins (**Figure 1**), labeled as procyanidins **IVB**. They were selected after previous experiments on fish lipids and minced muscle (*7*) and consisted of oligomers with a mean degree of polymerization of 2.7 and 25% galloylation, both estimated from HPLC analysis after depolymerization with cysteamine as described by Torres and Selga (*18*). All chemicals and solvents used for procyanidin and fish analysis were either analytical or HPLC grade. Dulbecco's Modified Eagle's Medium (DMEM) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Sigma Chemical. Co (St. Louis, MO), antibiotic (10 000 U/mL penicillin, 10 000 μg mL streptomycin) from Gibco-BRL (Eggenstein, Germany), Fetal calf serum (FCS) was obtained from Nalgen. Trypsin EDTA solution C (0.05% trypsin−0.02% EDTA) was purchased from Biological Industries (Kibbutz Beit Haemet, Israel). Trypan Blue solution 0.2%, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI), and Igepal CA-630 were from Sigma Chemical Co (St. Louis, MO); α,α,α-tris(hydroxymethyl)-aminomethane was from Aldrich-Chemie (Steinheim, Germany), and Annexin V/FITC kit was from Bender Med Systems (Med Systems Diagnostics GmbH, Vienna, Austria).

Preparation and Storage of Horse Mackerel Muscle. Fifty different fish, 16 kg, of fresh Atlantic horse mackerel (*Trauchurus trauchurus*) were deboned and eviscerated, and the white muscle was separated and minced to obtain a muscle homogenate. Streptomycin sulfate (200 ppm) was added for inhibiting microbial growth. Procyanidins **IVB** were added at concentrations of 50 and 100 ppm (w/w). Portions of 10 g of minced muscle were placed into plastic air bags and then sealed. Controls and samples with procyanidins **IVB** were kept refrigerated at 4 °C for 13 days. After that, samples showed microbial growth. Duplicate samples were taken every day. Ninety samples were prepared and analyzed. The experiment was performed in duplicate.

Sensory Analysis. A total of four panelists trained in descriptive analysis of fishy off-flavors sniffed the same raw samples that were used for chemical determinations. Approximately 10 g of muscle was placed in separate sterile polystyrene Petri dishes and put on a tray of ice. Panelists concentrated on detecting rancidity/painty odors using a hedonic scale from 7 to ≤1; 7 showed absolutely fresh and ≤1 was putrid (*19*).

Lipid Extraction. Lipids were extracted from fish muscle according to Bligh and Dyer (*20*) and quantified gravimetrically (*21*).

Peroxide Value and Thiobarbituric Acid Reactive Substances (TBARS) Analyses. The peroxide value of fish muscle was determined by the ferric thiocyanate method (*22*) and was expressed as milliequivalents of oxygen molecule per kilogram of oil (mequiv of O_2/kg of oil). Analyses were performed in duplicate. TBARS, expressed as millimoles of malonaldialdehyde per kilogram of muscle (mmol of MDA/kg of muscle), was determined according to Vyncke (*23*). Analyses were performed in duplicate.

Inhibition of formation of peroxides and TBARS was calculated during the propagation period of controls according to Frankel (*5*). Induction periods were calculated as the time (in days) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve (*24*).

Determination of α-Tocopherol. α-Tocopherol was extracted by adaptation of the Burton et al. (*25*) procedure as described by Pazos et al. (*26*). The analysis of α-tocopherol was performed by HPLC according to Cabrini et al. (*27*).

Fatty Acid Analysis. Fatty acid composition of lipids extracted from horse mackerel muscle was determined by gas chromatography (*28*).

Cell Culture. Human colorectal adenocarcinoma HT29 cells (ATCC HTB-38) and two noncancer rat intestinal cell lines IEC-6 (ECCAC No. 88071401) and IEC-18 (ECCAC No. 88011801) were used. The cells have epithelial morphology and adherent growth properties. HT29, IEC-6, and IEC-18 cells were maintained as monolayer culture at 95% humidity, 5% CO_2, and 37 °C. Cells were passaged at preconfluent densities by the use of trypsin EDTA solution C. Cells were cultured and passaged in DMEM supplemented with 10% heat-inactivated fetal calf serum and 0.01% streptomycin/penicillin.

Cell Growth Inhibition. HT29, IEC-6, and IEC-18 were seeded into 96-well flat-bottomed microtiter plates; the samples contained 200 μL of cell suspension at 15×10^3, 25×10^3, and 5×10^3 cells/mL, respectively. After the adherence of cells for 24 h of incubation at 37 °C, the procyanidins **IVB** were dissolved in fresh medium, aliquoted, and added to the cells to obtain final concentrations from 1 to 300 μg/mL. The cultures were incubated for 72 h in a incubator with 95% humidity, 5% CO_2 at 37 °C. After incubation, the medium was removed, 50 μL of MTT (5 mg/mL in phosphate-buffered saline (PBS)) and 50 μL of medium were added to each well, and the mixtures were incubated for 1 h. The blue MTT formazan precipitate was dissolved in 100 μL of DMSO, and the absorbance values at 550 nm were recorded on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN Australia). Absorbance was proportional to the number of living cells. The growth inhibition curves and the concentrations that caused 50% of inhibition cell growth (IC_{50}) were calculated using Grafit 3.0 software. The assay was performed by a variation of the MTT assay (*29*).

Cell Cycle Analysis. The assay was carried out by flow cytometry by using a fluorescence-activated cell sorter (FACS). HT29, IEC-6, and IEC-18 cells were plated in six-well flat-bottomed microtiter plates, at a density of 87.3×10^3, 146×10^3, and 29.1×10^3 cells/well, respectively. The number of cells was estimated using Trypan Blue and a Neubauer cell counter chamber (Brand, Wertheim, Germany). The cultures were incubated for 72 h, in the absence or presence of the polyphenolic mixture at their respective IC_{50} values. Thereafter, the cells were trypsinized, pelleted by centrifugation, and stained in Tris-buffered saline (TBS) containing 50 μg/mL PI, 10 μg/mL RNase free of DNase, and 0.1% Igepal CA-630 for 1 h at 4 °C in the dark. Cell cycle analysis was performed by FACS (Epics XL flow cytometer, Coulter Corporation, Hialeah, FL) at 488 nm. All experiments were performed in triplicate.

Apoptosis. The occurrence of apoptosis (programmed cell death) was determined by measuring Annexin V/FITC and propidium iodide (PI) staining by FACS. Cells were seeded, treated, and collected as described above. Following centrifugation, cells were washed in binding

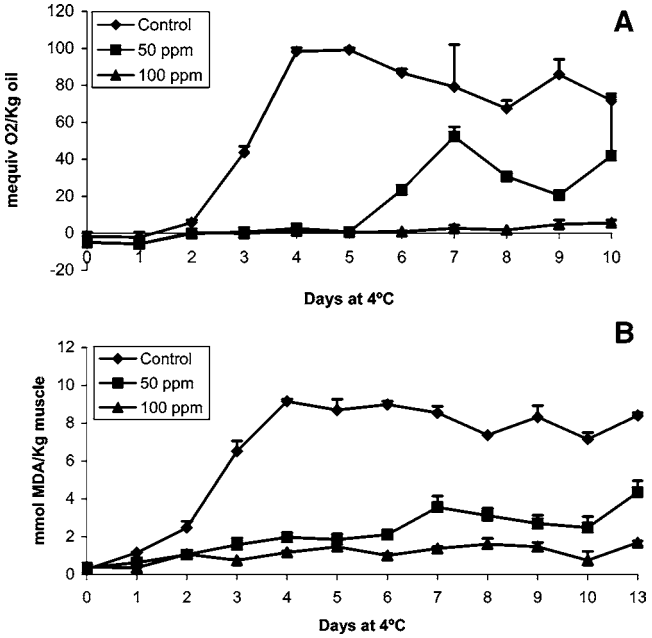

Figure 2. Hydroperoxide **(A)** and TBARS **(B)** formation during chilled storage of minced fish muscle at 4 °C (mean ± standard deviation of experiments performed in duplicate).

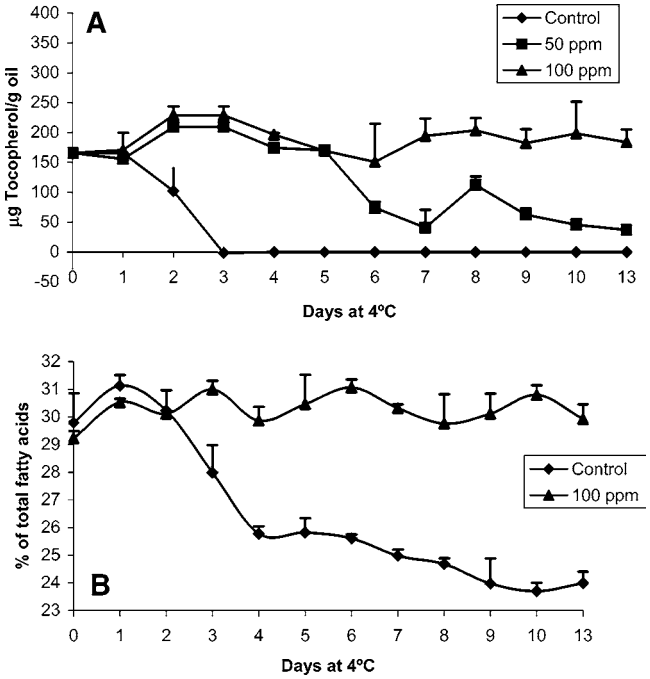

Figure 3. Concentration of α-tocopherol **(A)** and DHA **(B)** during chilled storage of minced fish muscle at 4 °C (mean ± standard deviation of experiments performed in duplicate).

buffer (10 mM Hepes/sodium hydroxide pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer, Annexin V/FITC was added according to the Annexin V/FITC kit. After 30 min of incubation at room temperature and darkness, propidium iodide was added 1 min before FACS analysis at 20 μg/mL. Experiments were performed in triplicate. Assessment of apoptosis for HT29, IEC-6, and IEC-18 cells was done after 72 h of treatment with procyanidins **IVB** at their respective IC_{50} concentrations.

Statistical Analysis. The data from fish experiments were compared by one-way analysis of variance (ANOVA) (*30*), and the means were compared by a least squares difference method (*31*). Significance was declared at $p < 0.01$. Cell growth, cell cycle, and apoptosis assays were analyzed by the Student's *t*-test and were considered statistically significant at $p < 0.05$ or $p < 0.001$. Data given are representative of three independent experiments.

RESULTS AND DISCUSSION

Preservation of Fish Lipids from Oxidation. Procyanidins **IVB** supplemented to minced fish muscle promoted a significant preservation of fish lipids from oxidation (**Figure 2** and **Table 1**). As the sensory and chemical data demonstrate, the overall quality of the minced fish product was maintained longer in the samples containing procyanidins **IVB** than in controls. Sensory analysis revealed that control samples lost sensory quality by the third day in which panelists first detected a clear rancid odor. Minced fish muscle supplemented with procyanidins **IVB** maintained a very fresh odor for 7 days. By the seventh day, a slightly rancid odor was detected in samples supplemented with 50 ppm. After 10 days at 4 °C, rancid odors were not detected in fish muscle supplemented with 100 ppm. Additionally, the panelists were not able to appreciate significant changes in the taste of the fish homogenate supplemented with 100 ppm of procyanidins **IVB**.

In agreement with the sensory analysis, the formation of peroxides and TBARS in controls was significant by the third day and was retarded in samples containing procyanidins **IVB** (**Figure 2**). The induction periods of peroxide and aldehyde formation were 1.8 and 1.9 days for controls, 4.9 and 5.8 days for samples with 50 ppm procyanidins **IVB**, and longer than 10 days in samples with 100 ppm procyanidins **IVB**. The amount of oxidation products, peroxides, and MDA equivalents formed was significantly lower in fish muscle supplemented with procyanidins **IVB** than in controls.

These data are in agreement with the contents of α-tocopherol (**Figure 3A**). The oxidation of α-tocopherol can be significantly related to the oxidation produced in postmortem fish (*6*). The content of α-tocopherol in controls dropped significantly by the second day, whereas such a decrease was observed only by the sixth day in samples with 50 ppm **IVB** and was not observed in samples with 100 ppm after 10 days. Recent studies have described that α-tocopherol is the last defense of fish muscle to inhibit oxidation and its reduction below a critical level leads lipid oxidation (*6*). These data demonstrated that 100 ppm

Table 1. Development of Rancid Odors, Percentage Inhibition[a] of the Formation of Peroxides and TBARS, and Proportion of Original α-Tocopherol Remaining in Minced Fish Muscle during Storage at 4 °C by the Supplementation of 50 and 100 ppm Procyanidins (Mean ± SD)[b]

	sensory analysis		hydroperoxides		TBARS		α-tocopherol	
procyanidins IV	day 3	day 7	day 3	day 6	day 3	day 7	day 3	day 6
control	rancid odor	rancid odor	0.0 ± 0.2 a	0.7 ± 0.5 a	0.1 ± 0.1 a	0.1 ± 0.1 a	0.0 ± 0.5 a	0.1 ± 0.1 a
50 ppm	fresh odor	rancid odor	98.6 ± 2.8 b	72.9 ± 2.7 b	75.8 ± 5.5 b	58.4 ± 7.0 b	100.2 ± 1.5 b	55.3 ± 2.2 b
100 ppm	fresh odor	fresh odor	100.1 ± 0.2 b	99.0 ± 0.8 c	88.4 ± 0.7 c	83.9 ± 1.0 c	105.3 ± 3.4 b	99.8 ± 2.0 c

[a] % inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed and S = oxidation product formed in sample. [b] Values in each column with the same letter were not significantly different ($p < 0.01$). Number of samples analyzed in the experiment: 90.

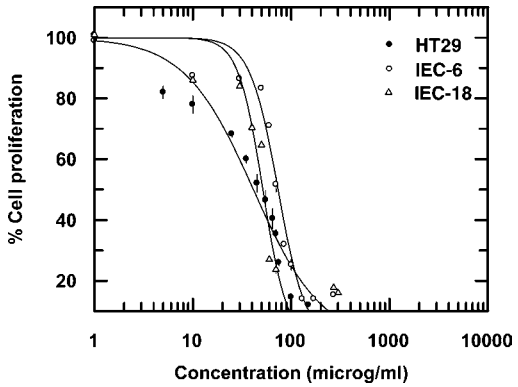

Figure 4. Percentage of proliferation of HT29 (●), IEC-6 (○), and IEC-18 (△) cells as a function of procyanidin concentration. Cells were treated for 72 h. Data are shown as the mean ± standard deviation of experiments performed in triplicate.

procyanidins **IVB** maintained the levels of endogenous α-tocopherol in minced horse mackerel muscle for 13 days at 4 °C.

Regarding the stability of fish bioactive PUFA, a significant reduction of the amount of DHA in control samples during chilling storage was recorded (**Figure 3B**). The DHA content of control samples decreased from an initial proportion of 29.8% total fatty acids to 25.8% by the fourth day and to 23.7% by the tenth day. Fish minced muscle supplemented with 100 ppm procyanidins **IVB** conserved its original levels of DHA for 10 days at 4 °C.

The data of this study revealed that the supplementation of procyanidins **IVB** with 25% galloylation stabilized the fish product based on minced muscle and maintained its functionality associated with the presence of PUFA and α-tocopherol. The supplementation of 100 ppm procyanidins **IVB** extended the shelf life of minced horse mackerel muscle for more than 8 days comparative to controls. Other polyphenolic antioxidants of natural origin such as olive oil phenolics or tea catechins have provided evidence to inhibit lipid oxidation of fish muscle (*32*, *33*). However, the effectiveness reported seems to be lower than that achieved by the supplementation of procyanidins **IVB**. Mackerel muscle containing 300 ppm tea catechins showed 60% inhibition of the formation of TBARS during the first 4 days at 4 °C and 20% after 6 days (*32*). The effectiveness of procyanidins **IVB** was also higher than that of hydroxytyrosol for preserving frozen fish fillets from oxidation (*33*). In other fish products such as sterilized tuna, 100 ppm olive oil phenolics slightly inhibited thermal oxidation of muscle after 4 days (*34*).

Regarding the stability and biological activity of grape procyanidins during chilled storage, some studies have found that grape procyanidins and their antioxidant activity were stable for more than 1 year at 4 °C (*35*). Additionally, the fish homogenate supplemented with 100 ppm procyanidins **IVB** did not show significant oxidation for more than 15 days (data not shown), meaning that the procyanidins were still active even when the fish product was not proper for consumption (microbiological growth).

Functional Properties of Grape Procyanidins. The procyanidins **IVB** described so far as food antioxidants may also exert a beneficial action on the humans consuming the supplemented fish product. Because procyanidins appear to be poorly absorbed in the gut and reach the end of the intestinal tract mostly intact (*15*), their putative preventive effect is likely to be more evident in the colon. The effect of 25% galloylated procyanidins **IVB** on the cell viability, cell cycle, and apoptosis

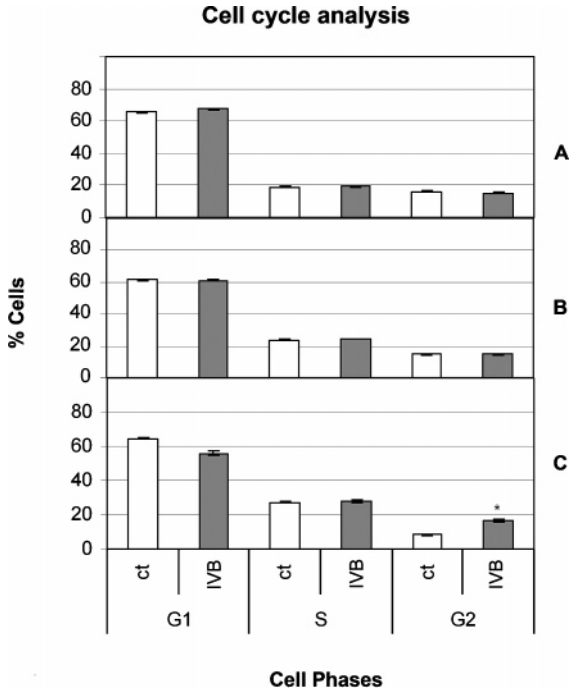

Figure 5. Cell cycle analysis by PI staining. Cells treated with procyanidins **IVB** at their IC_{50} values: **(A)** 75 μg/mL, IEC-6; **(B)** 56 μg/mL, IEC-18; and **(C)** 59 μg/mL, HT29. Percentages of cells at different cell stages (G1, S, and G2) are represented. % cells ± standard error of the mean (SEM) (*, $p < 0.05$; **, $p < 0.001$). ct, untreated control. Experiments were performed in triplicate.

in colon cancer cells was investigated using the HT29 epithelial colon carcinoma immortalized cell line. Two other cell lines (IEC-6 and IEC-18) were used as control models of noncancer colon cells.

Cell Viability. Cells were treated with different concentrations of procyanidins **IVB**, and the results showed that the decrease in cell number was dose-dependent (**Figure 4**). The calculated mean IC_{50} values were for HT29, 59 ± 3 μg/mL; for IEC-6, 74 ± 4 μg/mL; and for IEC-18, 52 ± 8 μg/mL. The procyanidins affected very little the cell viability in all three cell lines.

Cell Cycle. There was a moderate arrest of the cell cycle in the G2 phase in cancer HT29 cells (**Figure 5C**). The cell cycle distribution in noncancer IEC-6 and IEC-18 rat intestinal cell lines was not affected by procyanidins (**Figure 5A**,**B**). An arrest in G2 has also been described for other galloylated and nongalloylated polyphenols possibly by a mechanism involving enzyme inhibition (*36*).

Apoptosis. The procyanidins **IVB** induced the appearance of 16% apoptotic HT29 cells with respect to the untreated control (**Figure 5**). Interestingly the proapoptotic effect was selective for cancer cells. No induction of apoptosis was recorded for any of the noncancer cell lines. In addition, these two control cell lines presented a small percentage of necrotic cells (6% in IEC-6 and 5% in IEC-18) after treatment with the procyanidins (**Figure 6**). The proapoptotic effect of catechins, particularly tea (−)-epigallocatechin gallate, has also been described for different cell lines (*13*, *37*). Since the galloyl moiety of some catechins appears to be crucial for their proapoptotic effect (*13*, *14*), the presence of galloylated procyanidins **IVB** ($G = 25\%$) may explain, at least in part, the apoptosis elicited on HT29 cells. The influence of procyanidins **IVB** on the cell cycle (arrest in G2) may be attributed to other components of the mixture because the galloylated catechins appear to arrest the cell cycle in the G0/G1 phase in most of the cells tested so far (*12*, *14*).

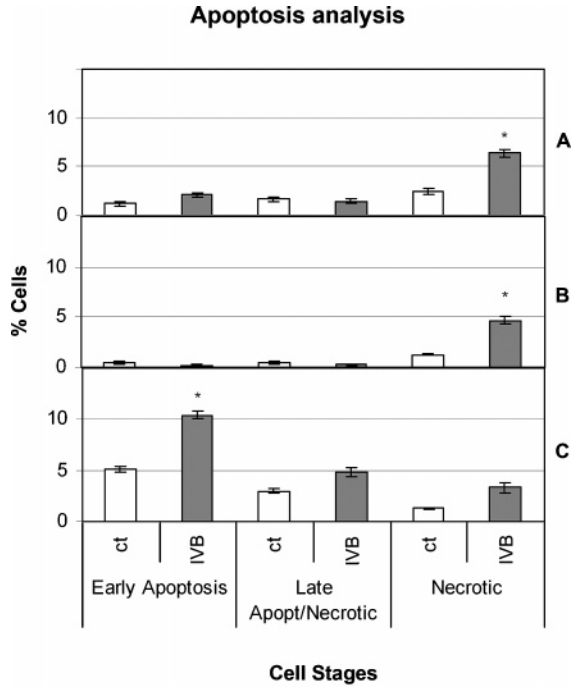

Figure 6. Analysis of apoptosis by annexin V/FITC and PI staining. Cells treated with procyanidins **IVB** at their IC_{50} values: **(A)** 75 μg/mL, IEC-6; **(B)** 56 μg/mL, IEC-18; and **(C)** 59 μg/mL, HT29. Percentages of cells at different cell stages are represented. Cell stages: early apoptosis (Annexin V^+PI^-); late apoptosis/necrotic (Annexin V^+PI^-); necrotic (Annexin V^-PI^+). % cells ± SEM (*, $p < 0.05$; **, $p < 0.001$). Experiments were performed in triplicate.

In any case, and most interestingly, the effects recorded for the antioxidant mixture on cell cycle and apoptosis was selective for cancer cells.

Briefly, data from the cellular studies show that the procyanidins **IVB** exerted little effect on the cell viability and cell cycle integrity of epithelial colon cells of both cancer and noncancer phenotype. The procyanidins exert a mild and selective proapoptotic effect on HT29 colon carcinoma cells. The healthy noncancer cells are not significantly affected by the procyanidins.

Conclusions. Functional ω-3 unsaturated fatty acids as well as α-tocopherol in minced horse mackerel muscle were stabilized by addition of an antioxidant extract composed mainly of procyanidins. The results with cell lines suggest that grape procyanidins **IVB** may also exert a direct preventive effect on colon epithelial cells by acting as an antioxidant and a selective proapoptotic agent. Thus, the product based on fatty fish muscle supplemented with grape procyanidins appears to be an interesting and stable functional food offering the combined action of ω-3 fatty acids and natural polyphenols.

ACKNOWLEDGMENT

M. Jesús Gonzalez and Elsa Silva are gratefully acknowledged for their technical assistance.

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Received for review November 2, 2005. Revised manuscript received March 22, 2006. Accepted March 23, 2006. This work was performed within Research Projects PPQ2003-06602-C04-01, -03, and -04 and AGL2004-07579-C04-02 and -03 financed by the Spanish Ministry of Education and Science. We thank Xunta de Galicia for a postgraduate grant to S.L. and the Spanish Ministry of Education and Science for postgraduate grants to D.L. and S.T.

JF0527145