JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 3785–3792 3785
DOI:10.1021/jf903714z

Influence of Anthocyanins, Derivative Pigments and Other Catechol and Pyrogallol-Type Phenolics on Breast Cancer Cell Proliferation

Iva Fernandes,[†] Ana Faria,[†,‡] Joana Azevedo,[†] Susana Soares,[†] Conceição Calhau,[‡] Victor De Freitas,[†] and Nuno Mateus*[,†]

[†]Chemistry Investigation Centre (CIQ), Department of Chemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal, and [‡]Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal

Anthocyanins (cyanidin-3-glucoside (Cy-3-gluc) and delphinidin-3-glucoside (Dp-3-gluc)) and their respective vinylpyranoanthocyanin-catechins (portisins) were studied in order to evaluate the cytotoxicity effect on the estrogen responsive human breast cancer cell line (ER+) MCF-7 and their effect on estrogen receptor (ER-α and ER-β) expression. Other flavonoid classes and phenolic molecules were also tested, aiming to study possible structural features related with these effects. Also, the antiproliferative effect of Cy-3-gluc and Dp-3-gluc was studied by an immunofluorescence assay. Generally, all the anthocyanin pigments studied inhibited, in a dose-dependent manner, the growth of the (ER+) MCF-7. The cytotoxicity effect was higher when cells were treated with Dp-3-gluc and its respective portisin. Altogether, the results point to the *ortho* trihydroxylated moiety in the phenolic ring as an important structural feature for more potent cytotoxicity effect on MCF-7 cells comparatively to the effect observed with the similar dihydroxylated compounds. In order to elucidate the molecular mechanism involved, expression of estrogen receptor was assayed by RT-PCR and real time RT-PCR. The higher antiproliferative effect observed after cell treatment with Dp-3-gluc was not followed by modification on ER expression. However, the anthocyanin Cy-3-gluc was able to induce a downregulation of ER levels although with no significant effect on MCF-7 proliferation.

KEYWORDS: Anthocyanins; breast cancer; portisins; pyranoanthocyanins

INTRODUCTION

Over the past decades the public interest in questions of health and life quality has progressively increased. Products promising wellness and longevity have developed into a huge and still growing market. One class of preparations available as food supplements are anthocyanin-rich fruit extracts. Anthocyanins are widely found in food of plant origin, namely, berries, dark grapes, cabbages and other pigmented fruits and vegetables (*1*, *2*). Depending on pH and the presence of chelating metal ions, anthocyanins are intensely colored blue, violet or red, contributing substantially to the natural coloring of a multitude of foods. They belong to the widespread class of phenolic compounds collectively known as flavonoids. The structural differences between individual anthocyanins are related to the number of hydroxyl and methoxyl groups, the nature and number of sugars and the position of these attachments in the aglycon and the degree of sugar acylation (*3*).

Depending on the nutrition habits, the daily intake of anthocyanins in humans has been estimated to be from 3−15 (*4*, *5*) up to 150 mg/day (*6*). Since the moderate consumption of such compounds through the intake of products such as red wine (*7*) or bilberry extract (*8*) is associated with a lower risk of coronary heart disease, it has been proposed that anthocyanins may exert therapeutic activities on human diseases associated with oxidative stress, e.g. coronary heart disease and cancer (*9*). The antioxidant properties of anthocyanins have been demonstrated by both *in vitro* and *in vivo* experiments (*3*, *10*−*15*). It has also been suggested that anthocyanins play an important role in the prevention against mutagenesis and carcinogenesis mediating some physiological functions related to cancer suppression (*16*). Anthocyanins show inhibitory effects on the growth of some cancer cells (*17*−*20*) and also inhibit cell transformation (*21*).

More recently, new stable anthocyanin-derived pigments, namely, anthocyanin−pyruvic acid adducts and vinylpyranoanthocyanin-catechins (portisins), have been detected in red wine (*22*−*24*). These compounds exhibit unusual colors (orange and blue) more resistant to color bleaching by sulfur dioxide, and more color capacity at higher pH values than do their anthocyanin precursors (*25*, *26*). The appealing chromatic properties of these anthocyanin derivatives make them interesting ingredients for the development of putative food colorants.

The increasing popularity of food supplements and the developing market for functional foods may contribute substantially to a rise in daily intake levels of new naturally occurring food colorants. However, despite the possible intake by humans,

*Author to whom correspondence should be addressed. E-mail: nbmateus@fc.up.pt. Fax: +351.220402659. Tel: +351.220402562.

Published on Web 02/19/2010

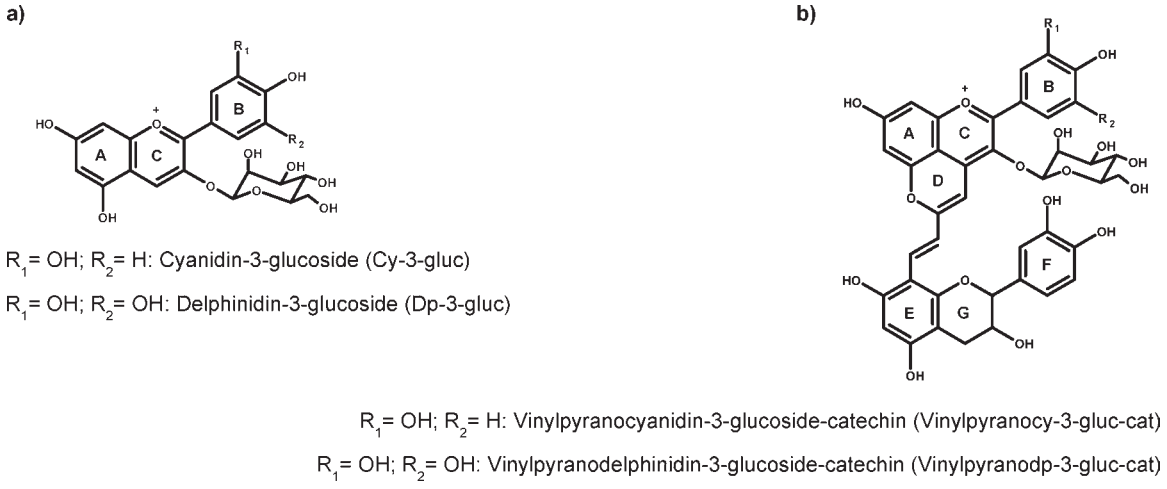

Figure 1. Structure of the anthocyanins (**a**) and the vinylpyranoanthocyanin-catechins (portisins) (**b**) tested.

information on potential cellular mechanisms of these compounds is rather limited. Therefore, it is worthwhile to evaluate bioactivities and mechanisms of anthocyanins that can support their health benefits. Little is known about the biological activities of anthocyanin derivative pigments (*27*). The antioxidant properties of anthocyanin-derived extracts (portisins) previously studied showed that these pigments have a higher antioxidant/antiradical capacity than the original anthocyanin extract (*27*).

Anthocyanidins have already been reported as having the potential to induce breast and ovarian cancer cell proliferation (*28*). Moreover, anthocyanin structures are similar to other flavonoids such as flavanones and isoflavones that have been described to possess estrogenic and antiestrogenic activities (*29*). Therefore the anthocyanins or derivative pigments might also exhibit estrogenic or antiestrogenic potential.

In the present study, the effect of anthocyanins and derived pigments on breast adenocarcinoma MCF-7 cell viability was assayed (**Figure 1**). The structural requirements of anthocyanins that effectively were responsible for that effect were also investigated, using several structurally related molecules, in order to give more insights on a potential structure−activity relationship. Furthermore, the effect of two anthocyanins, Dp-3-gluc and Cy-3-gluc, in MCF-7 (ER+) proliferation and on the expression of estrogen receptor ER-α and ER-β isoforms was evaluated intending to better understand the effects observed.

MATERIALS AND METHODS

Reagents. Toyopearl gel was purchased from Tosoh (Tokyo, Japan); catechol, gallic acid, phloroglucinol, minimum essential medium (MEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, trypan blue, PBS, pyruvic acid, sulforhodamine B (SRB), tris-HCl, pyrogallol, quercetin, myricetin and antibiotic/antimycotic solution (100×) were supplied from Sigma-Aldrich (Madrid, Spain).

Acetic glacial acid and dimethyl sulfoxide (DMSO) were purchased from Fluka (Madrid, Spain).

Trichloacetic acid (TCA) was purchased from Merck (Darmstadt, Germany). Tissue culture supports were supplied by TPP (Trasadingen, Switzerland).

Anthocyanin Isolation. Grape skin anthocyanins (*Vitis vinifera*) were extracted with a solution of 50% aqueous methanol (pH 1.5, acidified with HCl), for 2 days at room temperature. The grape anthocyanin extract was filtered in a 50 μm nylon membrane, and then nonacylated anthocyanins were purified by TSK Toyopearl gel column (250 × 16 mm i.d.) chromatography eluted with water/ethanol 10% (v/v) according to the procedure described previously (*30*).

Synthesis of Portisins. Vinylpyranoanthocyanin-catechins (portisins) were prepared through reaction of anthocyanin−pyruvic acid adducts with catechin and acetaldehyde according to the procedure described elsewhere (*31*). Briefly, anthocyanin−pyruvic acid adducts were prepared through reaction of the genuine anthocyanin extract with pyruvic acid in water (pH 2.6, 35 °C) at an approximate molar ratio, pyruvic acid/anthocyanin, of 50:1 during 5 days. The resulting extract was purified by Toyopearl gel column chromatography, and the anthocyanin pyruvic acid adduct fraction was eluted with water/ethanol 20% (v/v). Vinylpyranoanthocyanin-catechins (portisins) were prepared through reaction of anthocyanin−pyruvic acid adducts with catechin and acetaldehyde at 35 °C in 20% aqueous ethanol (pH 1.5) at an approximate molar ratio of catechin/acetaldehyde/anthocyanin−pyruvate of 50:25:1. After 10 days of reaction the portisin extract was isolated by Toyopearl gel column chromatography by elution with water/ethanol 85% (v/v), following the procedure previously described (*32*). All the extracts were freeze-dried and stored at −18 °C until used.

HPLC-DAD Analysis. HPLC analysis of the anthocyanin and vinylpyranoanthocyanin-catechins was performed on an Elite Lachrom system (L-2130) equipped with a 250 × 4.6 mm i.d. reversed phase C18 column (Merck, Darmstadt); detection was carried out at 520 and 570 nm, respectively, using a diode array detector (L-2455).

The solvents were A, H_2O/HCOOH (9:1), and B, H_2O/CH_3CN/HCOOH (6:3:1). The gradient consisted of 20−80% B for 70 min at a flow rate of 1.0 mL min^{-1}.

The column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min. Detected peaks were scanned between 200 and 600 nm. Compounds were first identified according to retention time and UV−vis spectra.

Portisins were also analyzed by HPLC using the same conditions with a different solvent B $CH_3CN/H_2O/CH_3COOH$ (8:1.95:0.05).

LC−MS Analysis. HPLC analysis of the anthocyanin and vinylpyranoanthocyanin-catechins was performed on a liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUA (Phenomenex, Torance, CA) reversed-phase column (150 × 4.6 mm, 5 μm, C18), thermostated at 35 °C. Solvents were A, H_2O/HCOOH (9.9:0.1), and B, H_2O/CH_3CN/HCOOH (6.9:3:0.1). The HPLC gradient used was the same as reported above for the HPLC analysis. Double online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corporation, San Jose, CA) equipped with an API source, using an electrospray ionization (ESI) interface. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature 190 °C. Spectra were recorded in positive ion mode between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS−MS of the most intense ion using relative collision energies of 30 and 60.

Portisins were also analyzed by LC−MS using the same conditions with a different solvent B $CH_3CN/H_2O/CH_3COOH$ (8:1.95:0.05).

Purification of Individual Pigments by Preparative HPLC. The anthocyanins were isolated by preparative HPLC (Knauer K-1001) on a reversed-phase column (250 × 25 mm, 10 μm, C18) (Merck, Darmstadt),

detection was carried out at 520 nm using a UV−vis detector (Hitachi, L-2420) (Merck, Darmstadt). The solvents were A, H_2O/HCOOH (9:1), and B, H_2O/MeOH/HCOOH (4:5:1). The gradient consisted of 65−15% B for 70 min at a flow rate of 10 mL min^{-1}. The column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min.

Portisins were also isolated by preparative HPLC using slightly different conditions. The gradient consisted of 40−5% B for 50 min at a flow rate of 10 mL min^{-1}, with a different solvent, B, $CH_3CN/H_2O/CH_3COOH$ (8:1.95:0.05).

The anthocyanins cyanidin-3-glucoside (Cy-3-gluc) and delphinidin-3-glucoside (Dp-3-gluc) and respective portisins, vinylpyranocyanidin-3-glucoside-catechin (vinylpyranocy-3-gluc-cat) and vinylpyranodelphinidin-3-glucoside-catechin (vinylpyranodp-3-gluc-cat), were isolated. The purity of the isolated compounds was assayed by HPLC-DAD−MS and by NMR.

Cell Culture Conditions. One human breast cancer tumor cell line, the ER (+) MCF-7, was grown as monolayer, from passage number 29 to 46. For routine maintenance, cells were cultured in 75 cm^2 flasks as monolayer and maintained in MEM (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution (100 units mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 0.25 μg mL^{-1} of amphotericin B) at 37 °C in a humidified atmosphere with 5% CO_2.

Cells were harverested by trypsinization (0.25% (w/v) trypsin-$EDTA_4$-Na) twice a week. The exponentially growing MCF-7 cells were obtained by plating 1.5×10^5 cells mL^{-1} followed by 24 h incubation. One day later, the seeding medium was replaced by fresh MEM containing the tested compounds for 24 h. To avoid compound oxidation the cell medium was replaced by fresh MEM containing the tested compounds for more 24 h.

The effect of the vehicle solvent (DMSO) was evaluated in all experiments by exposing untreated control cells to the maximum concentration (0.1%) of DMSO used in each assay. A stock solution of each compound was prepared in DMSO and kept at −20 °C. Appropriate dilutions of each compound were freshly prepared just prior to every assay.

Sulforhodamine B Assay. The effect of the compounds on the growth of the human breast adenocarcinoma cell line was evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) that uses the protein-binding dye sulforhodamine B (SRB) (Sigma Chemical Co, Saint Louis, MO) to assess cell growth (*33*, *34*).

Briefly, MCF-7 cells (1.5×10^5 cells mL^{-1}) were spread into 96-well plates and allowed to grow for 24 h before treatment. Thereafter, cells were incubated with the respective drug for 2 days, with a maximal solvent concentration of 0.1% DMSO. The medium was replenished after 24 h of incubation to avoid compounds oxidation. Incubation was stopped by addition of TCA (50% solution). After 1 h at 4 °C, plates were washed four times with water. The plates were dried at room temperature overnight or for 1 h at 37 °C and were stained with a 0.4% solution of SRB for 30 min in the dark. The excess of staining solution was washed out with 1% acetic acid. The dye was eluted with tris-buffer (10 mM, pH 10.5) and quantified photometrically at 492 nm. Cytotoxicity was determined as percent survival, determined by the number of treated (*T*) over control (*C*) cells × 100 (% *T*/*C*).

BrdU Proliferation Assay. MCF-7 (1.5×10^5 cells/mL) were cultured following standard conditions or the treatment procedures for 48 h. Cells were also incubated with a 5′-bromodeoxyuridine (BrdU) solution at a final concentration of 10 μM for 1 h, and then the in situ detection was performed using 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche, Indianapolis, IN), according to the manufacturer's instructions. DAPI was used to counterstain DNA (blue). The results are given as mean ± SEM and are expressed as percentage of proliferating control cells. This percentage was evaluated as a 200× magnification field. One thousand nuclei were examined, and three independent experiments were performed.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Briefly, MCF-7 cells (1.5×10^5 cells mL^{-1}) were spread into 25 cm^2 flasks and allowed to grow for 24 h before treatment. Thereafter, cells were incubated with 100 μM of Dp-3-gluc and Cy-3-gluc or 0.1% DMSO (vehicle) for 2 days. The medium was renewed after 24 h of incubation to avoid compound oxidation. RNA was extracted from the MCF-7 cells using Tripure Isolation Reagent (Roche, Indianapolis, IN), according to the producer's instructions. RNA was dissolved in water (diethylpyrocarbonate treated) and stored at −80 °C. Five micrograms of RNA was used as template for cDNA production through the incubation with reverse transcriptase (Finnzymes, Espoo, Finland) for 1 h at 45 °C, in 10 μM random hexamers, 0.375 mM each deoxynucleotide triphosphate, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, and 40 units of RNase inhibitor (RNaseOUT, Gibco BRL, Barcelona, Spain), followed by 10 min at 95 °C to inactivate the enzyme. Samples were incubated for 30 min at 37 °C with 0.1 mg/mL RNase (Sigma, St. Louis, MO). PCR amplification was performed in the presence of 2 mM $MgCl_2$, 0.5 mM each primer, 0.2 mM deoxynucleotide triphosphates, 2 U of Taq DNA polymerase (DFSTaq DNA polymerase, Bioron GmbH), and 4 μL of RT product, in a final volume of 50 μL.

For ER-α the following primers were used: 5′-CCACCAACCAGTGCACCATT-3′ (forward primer) and 5′-GGTCTTTTCGTATCCCACCTTTC-3′ (reverse primer) (*35*). Thermocycling consisted of 5 min at 94 °C followed by 30 cycles of 1 min 30 s at 94 °C, 1 min 30 s at 56 °C and 1 min 30 s at 72 °C and stopped with 10 min at 72 °C. For ER-β the following primer was used: 5′-AAAAGAATCATTCAATGACA-3′ (forward primer), 5′-ATTAACACCTCCATCCAACA-3′ (reverse primer) (*36*). Thermocycling consisted of 5 min at 94 °C followed by 30 cycles of 1 min 30 s at 94 °C, 1 min 30 s at 58 °C and 1 min 30 s at 72 °C and stopped with 10 min at 72 °C. Simultaneous amplification of the invariant housekeeping gene glyceraldehyde- 3-phospathe dehydrogenase (GAPDH) was performed. GAPDH primer was the following: 5′-ACT GGC GTC TTC ACC ACC AT-3′ (forward primer), 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse primer) (Primers from Metabion International, Martinsried, Germany). The predicted size of the PCR product was 682 bp.

PCR products were visualized on a 1.6% agarose gel with ethidium bromide staining. The expression of all tested enzymes was normalized to the expression of GAPDH of each sample and compared using Gel Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

Quantitative Real-Time PCR. MCF-7 cells were treated as previously described for RT-PCR. RNA was extracted from the MCF-7 cells using Tripure Isolation Reagent (Roche, Indianapolis, IN), according to the producer's instructions.

Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen Corporation, Carlsbad, CA) and 0.5 μg of resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 20 μL of final reaction volume, according to the manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade unreacted RNA. For the real-time quantitative PCR, 2 μL of the 20 μL reverse transcription reaction mixture was used. For the calibration curve, MCF-7 standard cDNA was diluted in five different concentrations.

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ). Twenty microliter reactions were set up in microcapillary tubes using 0.5 μM of each primer and 4 μL of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95 °C for 5 min), amplification and quantification [95 °C for 10 s, annealing temperature (AT) for 5 s, and 72 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for 10 s segment] repeated 45 times, a melting curve program [(AT + 10)°C for 15 s and 95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement], and a cooling step to 40 °C. Data were analyzed using LightCycler analysis software. The following human-specific primers: ER-α (AT-64 °C) forward primer 5′-GAAGAGGGTGCCAGGCTTTGT-3′ and reverse primer 5′-CGCCAGACGAGACCAATCATC-3′; ER-β (AT 65 °C) forward primer 5′-GTTCGACCAAGTGCGGCTCTT-3′ and reverse primer 5′-TCCCCTCATCCCTGTCCAGAA-3′.

Statistical Analysis. Values are expressed as the arithmetic means ± SEM. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni test. For the RT-PCR assays values are expressed as the arithmetic means from three independent experiments with identical observations. Statistical significance of the difference between samples was evaluated by Student's *t* test. Differences were considered to be statistically significant when $*p < 0.05$ vs control.

RESULTS

After a continuous exposure to a serial range of concentrations from 12.5 to 100.0 μM of the two classes of pigments (anthocyanins and the respective vinylpyranoanthocyanin-catechins

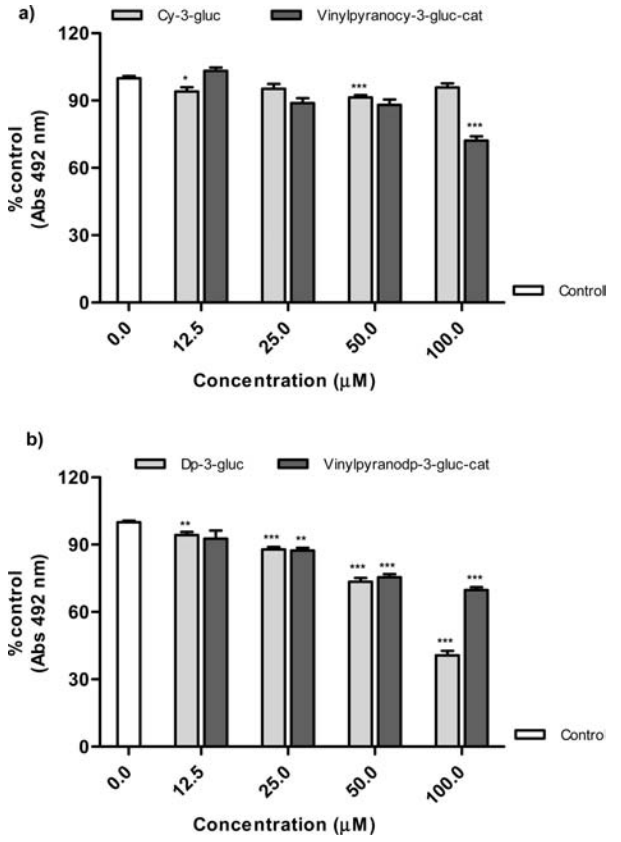

Figure 2. Effect of Cy-3-gluc and Dp-3-gluc and its corresponding vinylpyranoanthocyanin-catechins (portisins) on MCF-7 cells proliferation evaluated by SRB assay. MCF-7 cells, seeded in 96 well plates, were treated with a broad concentration range (12.5−100.0 μM) of each compound for 48 h. Each value represents the mean ± SEM (n = 6−30). *p < 0.05, **p < 0.001, ***p < 0.0001 (significant decrease vs control).

(portisins)) for 48 h, the dose−response profiles were obtained (**Figure 2**). Vinylpyranocy-3-gluc-cat revealed a higher cytotoxic activity than Cy-3-gluc for the highest concentration tested (100.0 μM). At the same concentration the inverse trend was observed when MCF-7 cells were treated with Dp-3-gluc and vinylpyranodp-3-gluc-cat. Indeed, the results obtained with Dp-3-gluc showed a potent growth inhibitory effect reaching 40.60% ± 2.08 for the highest concentration tested and was higher than the value of its respective portisin 69.76% ± 1.33 at the same concentration.

The striking difference observed between the effect of Cy-3-gluc and Dp-3-gluc has directed this study toward the evaluation of the only structural feature which differs in their structures: the hydroxylation pattern of ring B. In order to understand the influence of the substitution pattern in the anthocyanin ring B, other compounds belonging to flavonoid classes (quercetin and myricetin), phenolic acids (protocatechuic and gallic acid) and phenols (catechol and pyrogallol) with similar structural features, namely, dihydroxyl and trihydroxyl phenolic rings, were also tested. The results obtained are shown in **Figure 3**. All phenolic classes revealed a similar trend with the compounds bearing a trihydroxyl groups being more effective than the ones bearing dihydroxyl groups in inhibiting the growth of MCF-7 cells. This effect was more pronounced at higher concentrations.

Generally, and despite common structural features, the phenolic acids and phenols (smaller compounds) were the most active in inhibiting MCF-7 cell growth comparatively to the flavonoids tested.

The effect of the anthocyanins in cell proliferation was also tested by BrdU incorporation assay. 5-Bromo-2′-deoxy-uridine (BrdU) can be incorporated into DNA in place of thymidine. Cells which have incorporated BrdU into DNA can be quickly detected using a monoclonal antibody against BrdU and an FITC-labeled anti-BrdU antibody (green). DAPI was used to counterstain DNA (blue). Subsequently, the sample is evaluated using an immunofluorescence microscope.

The number of MCF-7 in S phase was quantified after incubation with 0,1% DMSO, 100 μM of Dp-3-gluc and 100.0 μM de Cy-3-gluc during 48 h.

The number of cells in S phase was significantly reduced after the treatment with Dp-3-gluc comparatively to control cells. In the case of Cy-3-gluc no antiproliferative effect was observed (**Figure 4**).

Furthermore, the effect of Dp-3-gluc and Cy-3-gluc on the expression of estrogen receptor ER-α and ER-β isoforms in MCF-7 cells was assayed aiming to better understand the opposite antiproliferative effect observed with these two anthocyanins.

The expression of estrogen receptor ER-α and ER-β isoforms in MCF-7 cells was first determined by RT-PCR. MCF-7 cells expressed both isoforms after incubation with Dp-3-gluc and Cy-3-gluc (**Figure 5**). A significant decrease in the ER-α isoform was found after incubation with Cy-3-gluc. In the case of ER-β no significant effect was observed although this result presents a higher error deviation than all the others. Slightly reduced ER-β expression was observed after incubation with Dp-3-gluc, although not reaching statistical significance.

Evaluation of ER mRNA levels after treatment with the two anthocyanins was also performed by quantitative real time RT-PCR. The mRNA levels of the ER-α and ER-β transcripts from Dp-3-gluc treated cells were similar to that in the corresponding controls (**Figure 6**). The mRNA levels of ER-β in MCF-7 cells treated with Cy-3-gluc were significantly reduced compared to that in control samples (**Figure 6**).

DISCUSSION

In the present study, the effect of anthocyanins and portisins on the growth of MCF-7 cells and a putative structure activity relationship were investigated.

In the first part of this work, the two classes of pigments were generally shown to induce a dose-dependent decrease in the growth of the breast cancer cells. Two anthocyanin precursors were studied: Cy-3-gluc and Dp-3-gluc. These compounds differ only in the hydroxylation pattern of ring B (**Figure 1a**). Among the anthocyanins and derivative compounds studied, the antiproliferative effect was higher when cells were treated with Dp-3-gluc and vinylpyranodp-3-gluc-cat (**Figure 2b**).

The results obtained seem to indicate a direct relationship between increased inhibitory effect on cell proliferation and the number of hydroxyl groups in the phenolic ring B.

This trend was also observed using flavonols (quercetin and myricetin) and other nonflavonoid compounds such as phenols (catechol and pyrogallol) and benzoic acids (protocatechuic and gallic acid). Indeed, all compounds bearing trihydroxyl groups were found to be more effective in inhibiting cell growth than the similar dihydroxyl compounds (**Figure 3**).

Also, the position of the three hydroxyl groups in the aromatic ring seems to be important attending to the results obtained with phloroglucinol (1,3,5-trihydroxybenzene). This latter did not inhibit the MCF-7 cell growth oppositely to what was observed with pyrogallol (**Figure 3c**).

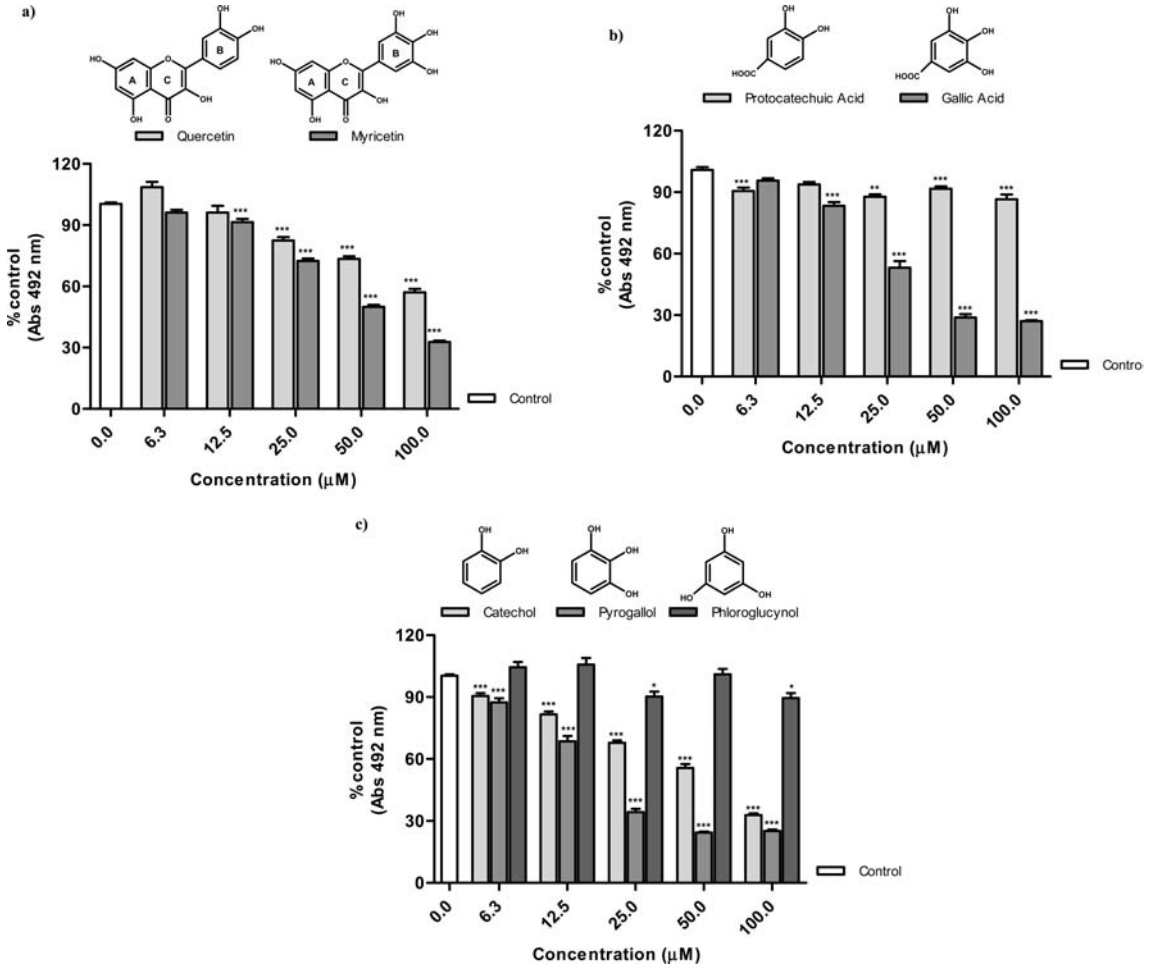

Figure 3. Effect of (**a**) flavonols, (**b**) hydroxybenzoic acids and (**c**) phenols on MCF-7 cells proliferation evaluated by SRB assay. MCF-7 cells, seeded in 96 well plates, were treated with a broad concentration range (6.3−100.0 μM) of each compound for 48 h. Each value represents the mean ± SEM (n = 6−30). *p < 0.05, **p < 0.001, ***p < 0.0001 (significant decrease vs control).

Previous studies on structure−activity relationship in flavonoids have already shown that both antioxidant and pro-oxidant activities of the flavonoids depend on the number of hydroxyl groups in the aglycon moiety (*37*).

Moreover, pyrogallol itself has been reported to undergo auto-oxidation (*38*, *39*).

The reduction in cell viability observed when cells were treated with Dp-3-gluc observed for the SRB assay was associated with a reduction in the proliferation of MCF-7 evaluated for the BrdU assay. Bearing this, a possible mechanism of action of Dp-3-gluc could be the result of antiproliferative effect associated with the production of toxic oxygen species and/or reactive intermediates by auto-oxidation that can be related with apoptosis induction (*40*).

Since the growth inhibitory effect of Dp-3-gluc was observed in ER (+) MCF-7 cells, this could suggest that this effect could be dependent on the presence of estrogen receptors. In ER (+) MCF-7 cells, phytoestrogens normally exhibit a biphasic effect, which consists on a stimulatory effect on cell growth at low concentrations and an inhibitory effect at high concentrations (*41*). While the stimulatory effect of phytoestrogens seems to be mediated via estrogen receptors, the antiproliferative effect appears to involve ER-independent cellular mechanisms (*42*). Indeed, a recent work has reported the decrease in cell viability of MDA-MB-231 (ER−) treated with high concentrations of Dp-3-gluc (*43*). Since the potential to stimulate breast and ovarian cancer cell proliferation for lower concentrations has already been reported for anthocyanidins (*28*), this could suggest that these compounds could exhibit a biphasic effect in the ER (+) MCF-7 cells.

To further elucidate the molecular mechanism involved in the antiproliferative effect observed in this study, the potential implication of an ER-independent mechanism was investigated.

The antiproliferative effect observed after treatment of MCF-7 cells with Dp-3-gluc was not followed by modification of ER expression. Corroborating these findings, a previous work has shown that a 48 h treatment with tamoxifen, a clinically important selective estrogen receptor modulator (SERM), caused no change in ER-α mRNA levels in MCF-7 cells growing in tissue culture. In fact, antiestrogen tamoxifen stabilized the nuclear form of ER-α (*44*).

Other possible pathways not involving direct association with the receptor may be concerned. Previous work has shown that the treatment with anthocyanidin Dp results in the effective shutting off of the MAP kinase pathway downstream of the epidermal growth-factor receptor (EGFR), a crucial signaling event in the regulation of cell proliferation (*19*). Dp has been reported to contribute to the inhibition of carcinogenesis by blocking activation of the MAPK signaling pathways which are required for AP-1 activation (*9*). The induction of AP-1 activity results in neoplastic transformation which can be blocked by chemopreventive agents.

Dp-3-gluc can thus act as a chemopreventive agent by mechanisms that may involve binding to EGFR and subsequent blocking the activation of the MAPK.

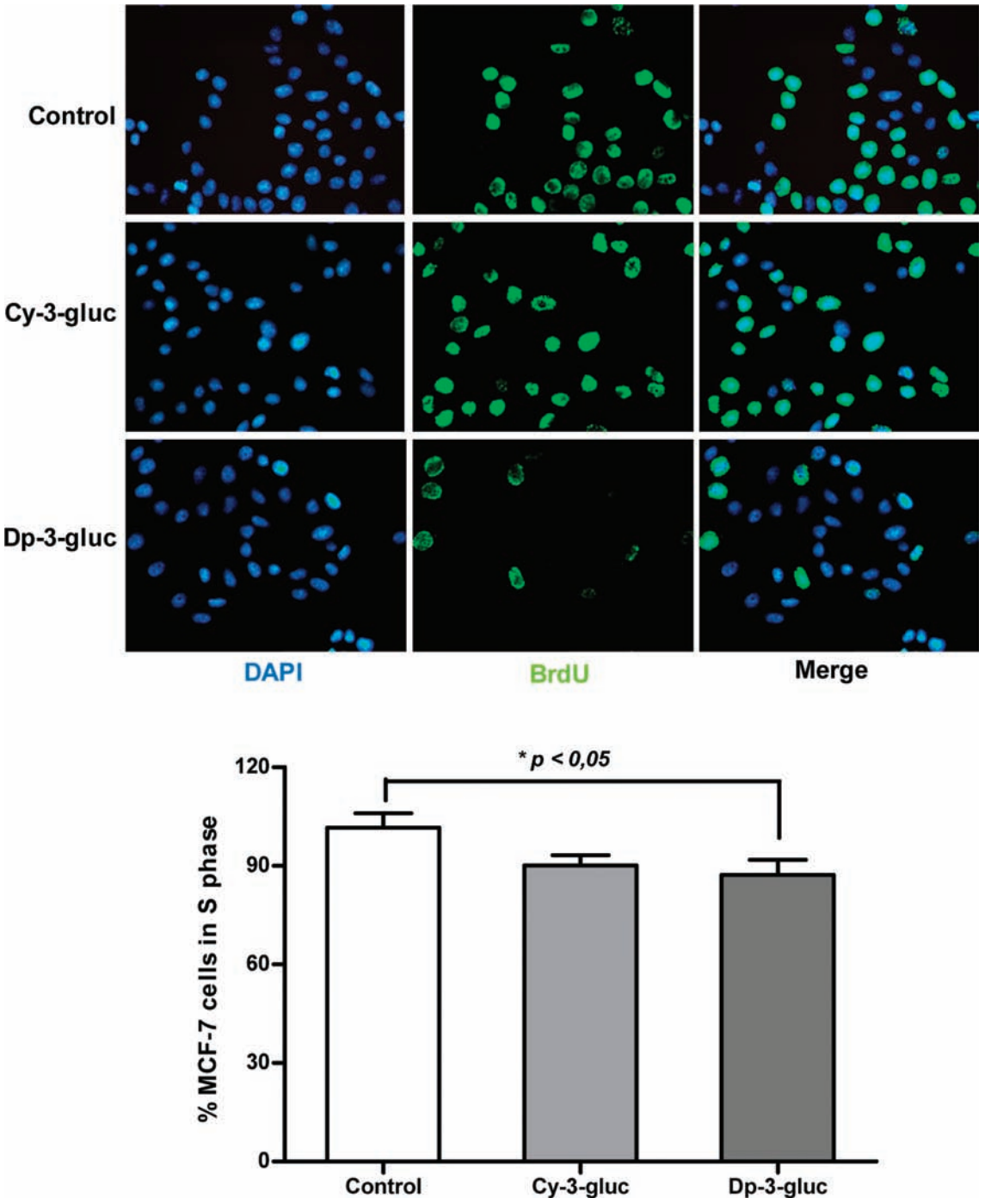

Figure 4. S phase arrest detected by BrdU incorporation analysis. MCF-7 cells were incubated in the presence of Cy-3-gluc or Dp-3-gluc (100 μM) for 48 h. After removing the BrdU, the cells were washed, and fixed for measurement of BrdU incorporation using FITC-labeled anti-BrdU antibody (green) and DAPI to counterstained DNA (blue). A representative experiment is shown from three independent experiments with identical observations. Each value represents the mean ± SEM (n = 3). *p < 0.05.

Oppositely, Cy-3-gluc showed no significant antiproliferative effect on MCF-7 proliferation which is in accordance with previous studies (*45*). However, this anthocyanin was able to induce a downregulation of ER-α level in RT-PCR assay.

Although both real-time quantitative RT-PCR and RT-PCR showed a decrease in ER expression, in the first one Cy-3-gluc selectively reduced expression of the ER-β gene with no apparent effect on expression of ER-α.

It appears that ER-β is antiproliferative, in many ways antagonizing the function of ER-α (*46*). In MCF-7 cells, which are known to have a predominance of ER-α over ER-β, Cy-3-gluc induced a decrease in ER-β expression resulting in a high ER-α: ER-β ratio, which parallels the physiological state linked to the absence of antiproliferative effect.

Overall, the anthocyanins and derivatives tested bearing a pyrogallol type structure were the most active against MCF-7 cell proliferation. This effect was also perceived with other flavonoid classes bearing similar structural features. Further studies will be helpful for clarifying a putative structure−activity relationship and to explore possible synergies or antagonisms between them.

It is not yet possible to classify anthocyanins and portisins as new classes of phytoestrogens since the possible molecular

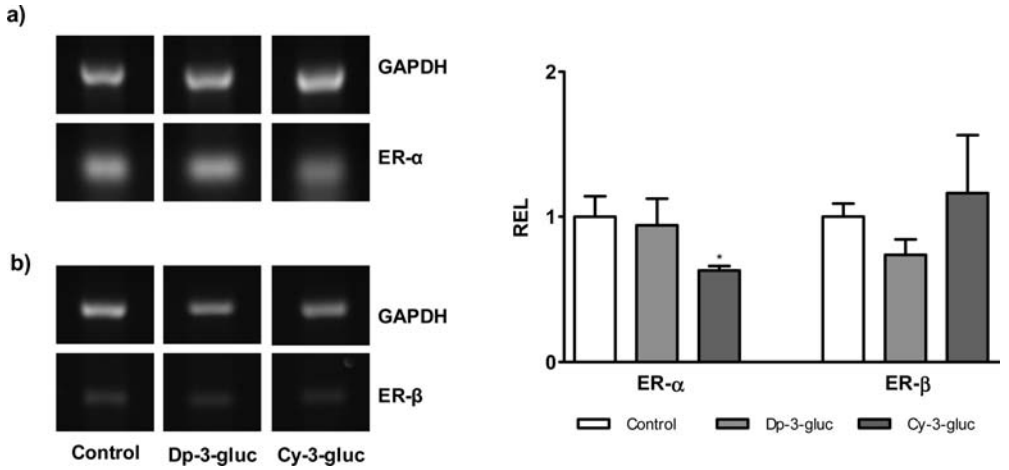

Figure 5. Expression of ER isoforms in MCF-7 cells. Differential RT-PCR for ER-α (**a**), ER-β (**b**) and GAPDH gene products in cultures of MCF-7 cells after incubation with Dp-3-gluc or Cy-3-gluc, 100.0 μM for 48 h. A representative blot is shown from three independent experiments with identical observations. Each value represents the mean ± SEM The relative expression level, REL, of each band was normalized and compared to the expression of GAPDH of each sample. *$p < 0.05$ vs control.

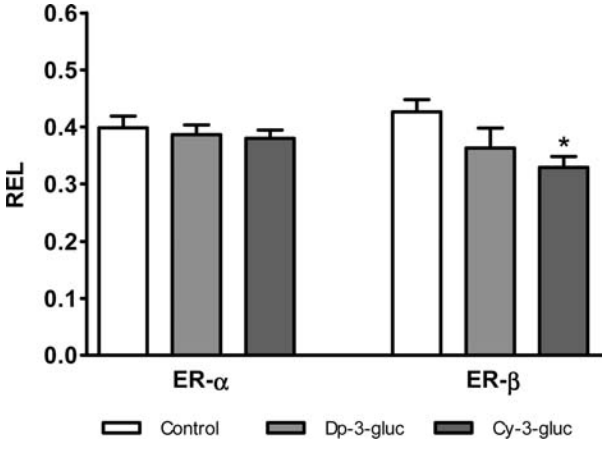

Figure 6. Expression of ER isoforms in MCF-7 cells. Differential RT^2-PCR for ER-α, ER-β and GAPDH gene products in cultures of MCF-7 cells after incubation with Dp-3-gluc or Cy-3-gluc, 100.0 μM for 48 h. Each value represents the mean ± SEM. The relative gene expression level; REL was normalized and compared to the expression of GAPDH. *$p < 0.05$ vs control.

mechanism for the antiproliferative effect observed is not already clarified. The antiproliferative effect exhibited by these compounds could be dependent or independent of estrogen receptors or other molecular pathways may be involved.

The data of this work proposes that individual structural features of anthocyanins and derivatives, namely, the hydroxylation pattern of ring B, differently affect the cellular mechanisms involved in the antiproliferative effects of these natural food compounds.

LITERATURE CITED

(1) Harborne, J. B.; Williams, C. A. Anthocyanins and other flavonoids. *Nat. Prod. Rep.* **2001**, *18*, 310–333.

(2) Bohm, H.; Boeing, H.; Hempel, J.; Raab, B.; Kroke, A. Flavonols, flavone and anthocyanins as natural antioxidants of food and their possible role in the prevention of chronic diseases. *Z. Ernahrungswiss.* **1998**, *37*, 147–163.

(3) Kong, J. M.; Chia, L. S.; Goh, N. K.; Chia, T. F.; Brouillard, R. Analysis and biological activities of anthocyanins. *Phytochemistry* **2003**, *64*, 923–933.

(4) Chun, O. K.; Chung, S. J.; Song, W. O. Estimated dietary flavonoid intake and major food sources of U.S. adults. *J. Nutr.* **2007**, *137*, 1244–1252.

(5) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.

(6) Heinonen, M. Antioxidant activity and antimicrobial effect of berry phenolics - a Finnish perspective. *Mol. Nutr. Food Res.* **2007**, *51*, 684–691.

(7) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523–1526.

(8) Muth, E. R.; Laurent, J. M.; Jasper, P. The effect of bilberry nutritional supplementation on night visual acuity and contrast sensitivity. *Altern. Med. Rev.* **2000**, *5*, 164–173.

(9) Hou, D. X. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.* **2003**, *3*, 149–159.

(10) Lazze, M. C.; Pizzala, R.; Savio, M.; Stivala, L. A.; Prosperi, E.; Bianchi, L. Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells. *Mutat. Res.* **2003**, *535*, 103–115.

(11) Kahkonen, M. P.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **2003**, *51*, 628–633.

(12) Tsuda, T.; Horio, F.; Kitoh, J.; Osawa, T. Protective effects of dietary cyanidin 3-O-β-D-glucoside on liver ischemia-reperfusion injury in rats. *Arch. Biochem. Biophys.* **1999**, *368*, 361–366.

(13) Tsuda, T.; Kato, Y.; Osawa, T. Mechanism for the peroxynitrite scavenging activity by anthocyanins. *FEBS Lett.* **2000**, *484*, 207–210.

(14) Tsuda, T.; Shiga, K.; Ohshima, K.; Kawakishi, S.; Osawa, T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris L. Biochem. Pharmacol.* **1996**, *52*, 1033–1039.

(15) Ramirez-Tortosa, C.; Andersen, O. M.; Gardner, P. T.; Morrice, P. C.; Wood, S. G.; Duthie, S. J.; Collins, A. R.; Duthie, G. G. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biol. Med.* **2001**, *31*, 1033–1037.

(16) Omenn, G. S. What accounts for the association of vegetables and fruits with lower incidence of cancers and coronary heart disease? *Ann. Epidemiol.* **1995**, *5*, 333–335.

(17) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins *in vitro*. *Cancer Invest.* **1995**, *13*, 590–594.

(18) Koide, T.; Hashimoto, Y.; Kamei, H.; Kojima, T.; Hasegawa, M.; Terabe, K. Antitumor effect of anthocyanin fractions extracted from red soybeans and red beans *in vitro* and *in vivo*. *Cancer Biother. Radiopharm.* **1997**, *12*, 277–280.

(19) Meiers, S.; Kemeny, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958–962.

(20) Nagase, H.; Sasaki, K.; Kito, H.; Haga, A.; Sato, T. Inhibitory effect of delphinidin from Solanum melongena on human fibrosarcoma HT-1080 invasiveness in vitro. *Planta Med.* **1998**, *64*, 216–219.
(21) Hou, D. X.; Kai, K.; Li, J. J.; Lin, S.; Terahara, N.; Wakamatsu, M.; Fujii, M.; Young, M. R.; Colburn, N. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. *Carcinogenesis* **2004**, *25*, 29–36.
(22) Mateus, N.; Silva, A. M.; Rivas-Gonzalo, J. C.; Santos-Buelga, C.; de Freitas, V. A new class of blue anthocyanin-derived pigments isolated from red wines. *J. Agric. Food Chem.* **2003**, *51*, 1919–1923.
(23) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401–1407.
(24) Bakker, J.; Timberlake, C. F. Isolation, Identification, and Characterization of New Color-Stable Anthocyanins Occurring in Some Red Wines. *J. Agric. Food Chem.* **1997**, *45*, 35–43.
(25) Vivar-Quintana, A. M.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Anthocyanin-derived pigments and colour of red wines. *Anal. Chim. Acta* **2002**, *458*, 147–155.
(26) Oliveira, J.; Fernandes, V.; Miranda, C.; Santos-Buelga, C.; Silva, A.; de Freitas, V.; Mateus, N. Color properties of four cyanidin-pyruvic acid adducts. *J. Agric. Food Chem.* **2006**, *54*, 6894–6903.
(27) Faria, A.; Oliveira, J.; Neves, P.; Gameiro, P.; Santos-Buelga, C.; de Freitas, V.; Mateus, N. Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *J. Agric. Food Chem.* **2005**, *53*, 6896–6902.
(28) Schmitt, E.; Stopper, H. Estrogenic activity of naturally occurring anthocyanidins. *Nutr. Cancer* **2001**, *41*, 145–149.
(29) Damianaki, A.; Bakogeorgou, E.; Kampa, M.; Notas, G.; Hatzoglou, A.; Panagiotou, S.; Gemetzi, C.; Kouroumalis, E.; Martin, P. M.; Castanas, E. Potent inhibitory action of red wine polyphenols on human breast cancer cells. *J. Cell Biochem.* **2000**, *78*, 429–441.
(30) Pissarra, J.; Mateus, N.; Rivas-Gonzalo, J.; Santos Buelga, C.; Freitas, V. Reaction Between Malvidin 3-Glucoside and (+)-Catechin in Model Solutions Containing Different Aldehydes. *J. Food Sci.* **2003**, *68*, 476–481.
(31) Mateus, N.; Oliveira, J.; Santos-Buelga, C.; Silva, A. M. S.; de Freitas, V. NMR structure characterization of a new vinylpyrano-anthocyanin-catechin pigment (a portisin). *Tetrahedron Lett.* **2004**, *45*, 3455–3457.
(32) Mateus, N.; Oliveira, J.; Haettich-Motta, M.; de Freitas, V. New Family of Bluish Pyranoanthocyanins. *J. Biomed. Biotechnol.* **2004**, *2004*, 299–305.
(33) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
(34) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* **1991**, *83*, 757–766.
(35) Bieche, I.; Parfait, B.; Laurendeau, I.; Girault, I.; Vidaud, M.; Lidereau, R. Quantification of estrogen receptor α and β expression in sporadic breast cancer. *Oncogene* **2001**, *20*, 8109–8115.
(36) Jiang, W. G.; Davies, G.; Fodstad, O. Com-1/P8 in oestrogen regulated growth of breast cancer cells, the ER-β connection. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 253–262.
(37) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biol. Med.* **1997**, *22*, 749–760.
(38) Cos, P.; Ying, L.; Calomme, M.; Hu, J. P.; Cimanga, K.; Van Poel, B.; Pieters, L.; Vlietinck, A. J.; Vanden Berghe, D. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **1998**, *61*, 71–76.
(39) Hodnick, W. F.; Ahmad, S.; Pardini, R. S. Induction of oxidative stress by redox active flavonoids. *Adv. Exp. Med. Biol.* **1998**, *439*, 131–150.
(40) Saeki, K.; Hayakawa, S.; Isemura, M.; Miyase, T. Importance of a pyrogallol-type structure in catechin compounds for apoptosis-inducing activity. *Phytochemistry* **2000**, *53*, 391–394.
(41) Wang, C.; Kurzer, M. S. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr. Cancer* **1997**, *28*, 236–247.
(42) Collins-Burow, B. M.; Burow, M. E.; Duong, B. N.; McLachlan, J. A. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. *Nutr. Cancer* **2000**, *38*, 229–244.
(43) Syed, D. N.; Afaq, F.; Sarfaraz, S.; Khan, N.; Kedlaya, R.; Setaluri, V.; Mukhtar, H. Delphinidin inhibits cell proliferation and invasion via modulation of Met receptor phosphorylation. *Toxicol. Appl. Pharmacol.* **2008**, *231*, 52–60.
(44) Wijayaratne, A. L.; McDonnell, D. P. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J. Biol. Chem.* **2001**, *276*, 35684–35692.
(45) Zhang, Y.; Vareed, S. K.; Nair, M. G. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sci.* **2005**, *76*, 1465–1472.
(46) Hartman, J.; Ström, A.; Gustafsson, J.-Å. Estrogen receptor beta in breast cancer--Diagnostic and therapeutic implications. *Steroids* **2009**, *74*, 635–641.

Received for review October 23, 2009. Revised manuscript received January 15, 2010. Accepted February 4, 2010. This work was supported by FCT (Fundação para a Ciência e Tecnologia) (POCI, FEDER, Programa Comunitário de Apoio) by two Ph.D. student grants (SFRH/BD/28160/2006 and SFRH/BD/38883/2007) and one project grant (PTDC/QUI/65501/2006). This work was support by a student grant from Instituto de Bebidas e Saúde (IBeSa). The authors would like to thank Doctor Eduardo Marques for the use of fluorescence microscopy.