Protective Effect of Anthocyanidins on Astrocytes and Apoptosis Induced by Oxidative Damage

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Abstract

As a result of the longer life expectancy in the human population, neurodegenerative diseases are increasing, so finding effective treatments together with prevention strategies are urgently needed. Anthocyanins are polyphenolic compounds from vegetal species that have shown strong antioxidant and anti-inflammatory activities in various experimental models. The effect of the anthocyanidins cyanidin, malvidin, and pelargonidin at different concentrations (5 to 100 µM) on experimental models of cell oxidation and apoptosis was studied and compared with the positive control Trolox. The human astrocyte glioblastoma, cellular line U373, was used for a cellular injury model using Fenton's reagent (0.5 mM $FeSO_4$/1 mM H_2O_2) and to measure the lactate dehydrogenase enzyme activities and lipid peroxidation level (thiobarbituric acid reactive substances) by fluorimetric analysis and malondialdehyde release by HPLC. Also, caspase 3, 8, and 9 activities were measured by fluorimetric analysis. The results show that the studied anthocyanidins are able to revert the induced apoptosis related to caspase activation and lipid peroxidation. All the assayed anthocyanidins significantly decreased the activation of caspases 8 and 3, although malvidin and cyanidin increased the activity of caspase 9 when compared to non-treated cells.

Key words
antioxidant · neuroprotection · anthocyanidins · cyanidin · pelargonidin · malvidin · oxidative stress

Neurodegenerative diseases are known to be related to oxidative stress which is due to an imbalance between the production of reactive oxygen species (ROS) and the ability of intrinsic antioxidant systems, both enzymatic and non-enzymatic, to scavenge these radicals [1]. Oxidation of macromolecules such as proteins, lipids and DNA may lead to cell degeneration and death due to an increase in the release of apoptotic-inducing factors. Thus, different strategies have been proposed for the prevention and treatment of ROS-mediated diseases, with special emphasis on antioxidant therapy [2–4].

Anthocyanidins are polyphenols with a strong scavenging activity, which is related to their phenolic structure characterized by B ring hydroxyl groups and a conjugated double bond system. Orally administered anthocyanins have been proven to suppress stress-induced cerebral oxidative damage [5], reduce lipid peroxidation, increase glutathione levels and antioxidant enzymes activity, and improve spatial memory in the hippocampus of the adult rat [6].

In this study, we analyzed the antioxidant activity of the anthocyanidins cyanidin, malvidin, and pelargonidin at different concentrations on the human astrocyte glioblastoma, which is known as a useful model for the study of astrocyte functions under both physiological and pathological conditions. Then, the potential protective effect of these compounds against an oxidative injury chemically induced by Fenton's reagent, which induces a double-strand DNA breaking, was assessed [7]. For this purpose, lactate dehydrogenase (LDH) activity and the lipid peroxidation level through thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) levels were evaluated. Finally, apoptotic caspases 3, 8, and 9 were tested to determine whether anthocyanidins may influence cell apoptosis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analogue of vitamin E with strong antioxidant capacity that is widely used as a positive control in several tests conducted to assay the antioxidant ability of new compounds. It is able to diminish ROS production, block the loss of MMP, prevent cytotoxicity in human cancer cell lines, and rescue cells from apoptotic death [8,9]. Trolox was chosen as a positive control in all the assays conducted in this work.

Results and Discussion

The direct effect of the anthocyanidins on astrocyte viability (LDH) showed a statistically significant lower LDH release than the toxic control (Fenton's reagent), similar to the positive control (0.5 mM Trolox) but higher than untreated cells, except for 25 µM cyanidin (**Fig. 1A**). Their protective effect was assessed by a decrease in LDH release, although with a different profile. For cyanidin, the highest tested concentration showed the highest LDH value, while the mean concentration (25 µM) showed the lowest LDH value. Pelargonidin induced a dose-dependent production of LDH, while the inverse profile was observed for malvidin, as lower LDH levels were detected for the higher concentrations. These results were similar than those observed for the positive control (**Fig. 1B**).

MDA, the main compound resulting from lipid peroxidation, was evaluated in the presence or absence of oxidative stress conditions. In the absence of an oxidative stimulus, no statistically significant differences were found between anthocyanidin concentrations, Trolox treatment, and control cells on TBARS levels, except for 5 µM cyanidin and 25 µM malvidin (**Fig. 2A**). Under oxidative stress injury, a protective effect was shown through a reduction in these values (**Fig. 2B**).

Under normal conditions, every anthocyanidin induced statistically significant lower MDA levels than the toxic alone, and similar to that of the control and Trolox groups (**Fig. 3A**), except for pelargonidin at the highest concentration. When the cells were previously treated with anthocyanidins, the lipid peroxidation damage induced by Fenton's reagent was significantly decreased, even more than the positive control, Trolox (**Fig. 3B**).

A direct effect of cyanidin, malvidin, and pelargonidin on caspase activities showed no significant differences for caspases 3 and 8. Significant differences were found between the control and malvidin-treated cells for caspase 9 activity; also, 25 µM cyanidin showed a higher caspase 9 activity than untreated cells (**Fig. 4A**). When testing the protective effect on a cellular injury model induced by 500 µM H_2O_2 (**Fig. 4B**), every tested compound reduced caspase 3 and 8 activities, while caspase 9 was not activated by the toxic treatment.

This study demonstrates that cyanidin, malvidin, and pelargonidin are capable of protecting human astrocytes against oxidative injury mainly influencing caspase activity. The potential preventive effect of the anthocyanidins when an injured cell model was

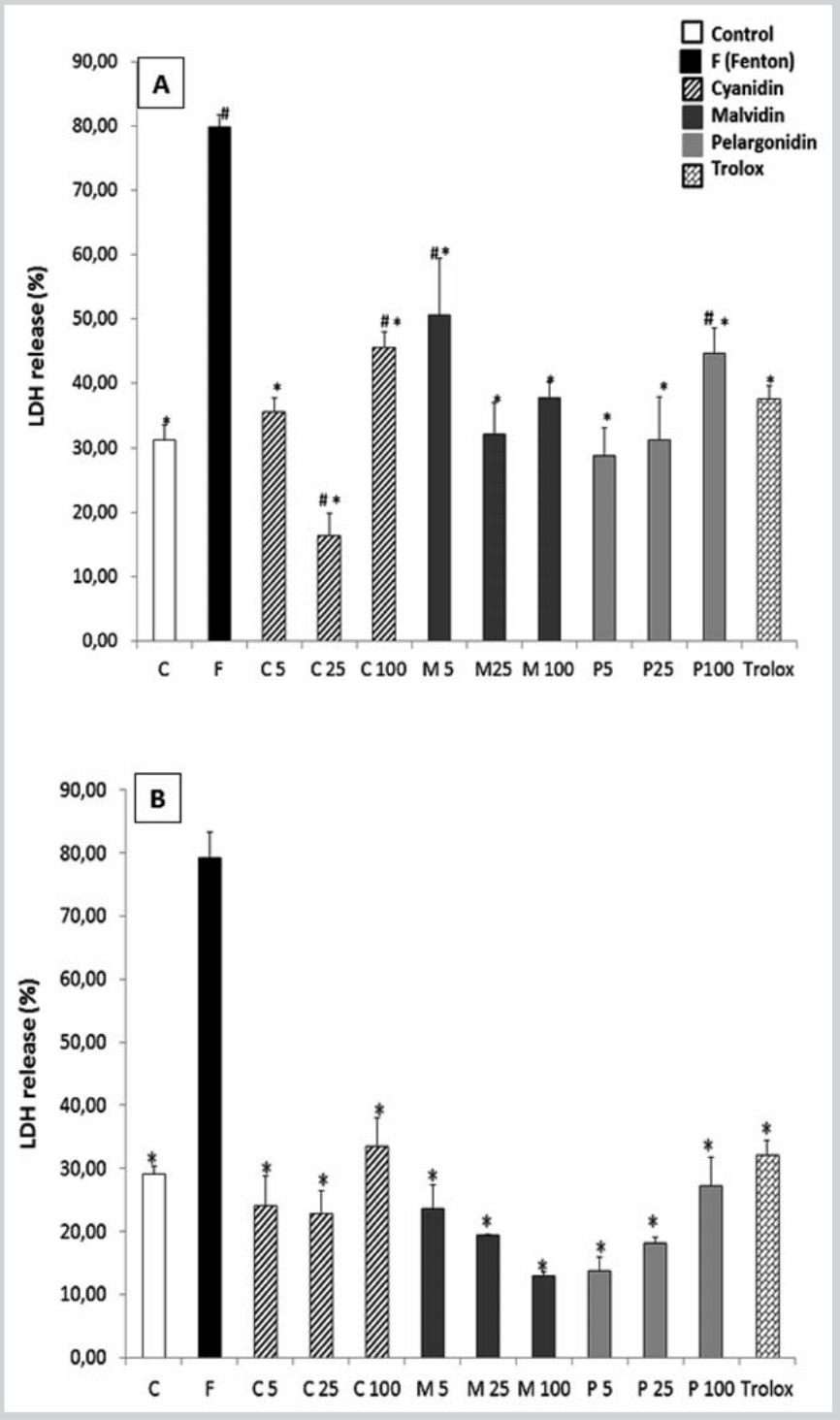

Fig. 1 Effect of anthocyanidins (cyanidin, malvidin, and pelargonidin – 5, 25, and 100 µM) on cell viability compared to the positive control (0.5 mM Trolox). **A** Direct effect: U-373MG cells were treated with different concentrations of the isolated compounds for 24 h. **B** Protective effect: U-373MG cells were treated with different concentrations of the isolated compounds for 24 h. Then cells were washed and Fenton's reagent was added to all the cultures except for the controls for 3 h. LDH leakage is expressed as the percent of LDH activity. Values are means ± SD (n = 3, four replicates). *P < 0.05 vs. F; #p < 0.05 vs. control.

implemented proved their ability to reduce LDH release to culture media up to values close to untreated cells, but with a different behavior, probably due to the different chemical structure of these compounds. Under oxidative stress conditions, polyunsaturated fatty acids of cell membranes may break down to different secondary products such as aldehydes with MDA being one of the main products derived from lipid peroxidation, which is widely used as an index of peroxidation in biological sciences [10, 11]. In this study, an HPLC method was applied to quantify the peroxidation metabolite (data not shown). Results proved the protective effect from anthocyanidins, but with a different profile. These differences may be related to the structure-activity relationship within anthocyanidins, as anthocyanidin trihydroxilated derivates are more effective in neutralizing ROS than monohydroxilated ones (pelargonidin), which in turn are more efficient in inhibiting proteasome activity (which could also inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells-NF-κB pathway) [12].

These results are in accordance with previous studies that proved a similar effect of cyanidin-3-glucoside over endothelial cells

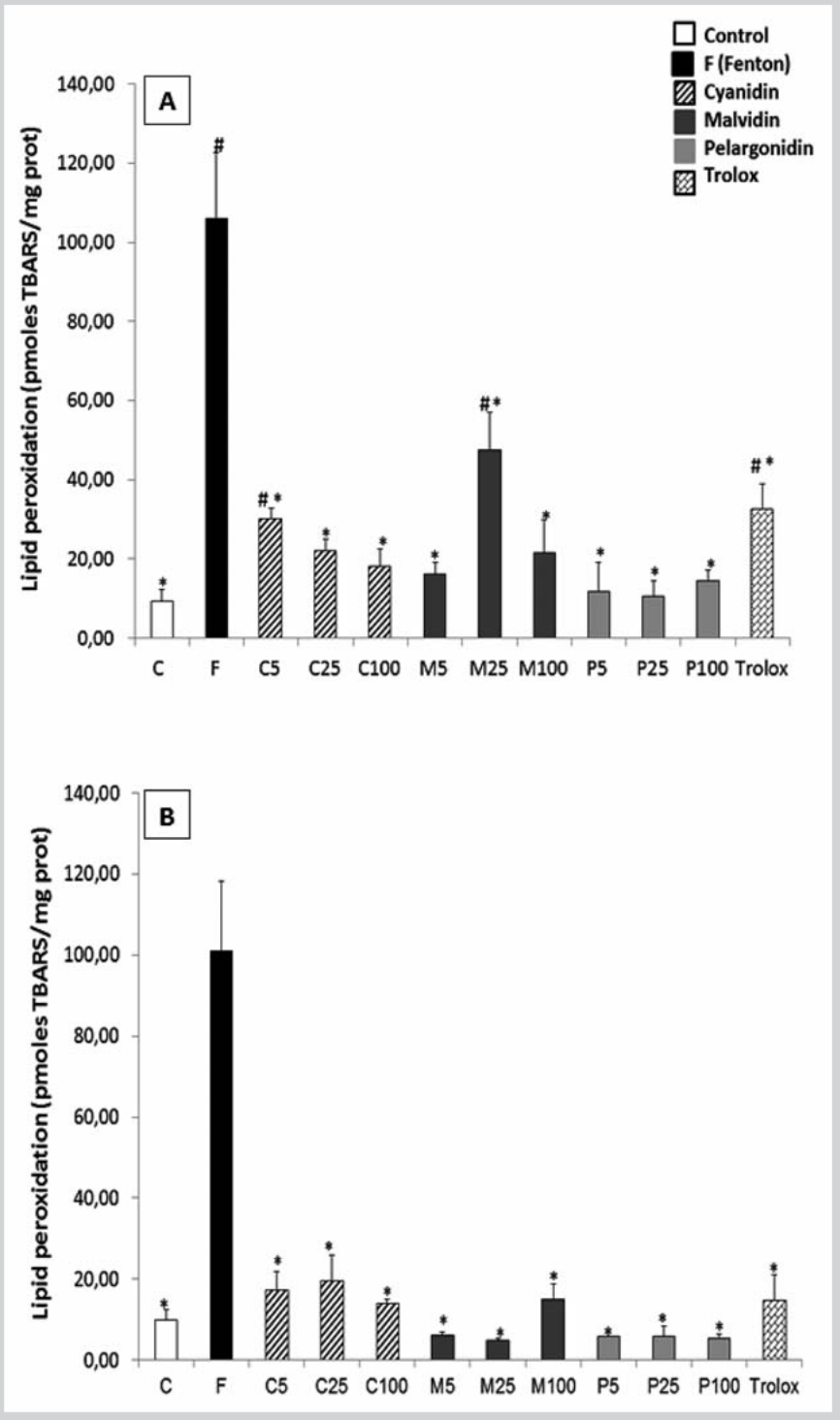

Fig. 2 Effect of anthocyanidins (cyanidin, malvidin, and pelargonidin – 5, 25, and 100 µM) on lipid hydroperoxide concentration (pmol TBARS/mg protein) compared to the positive control (0.5 mM Trolox). **A** Direct effect. **B** Protective effect under stress conditions (Fenton's reagent). Values are means ± SD (n = 3, four replicates) of three different experiments. *P < 0.05 vs. F; #p < 0.05 vs. control.

[13], as well as other anthocyanidin-rich products such as beans, which significantly reduced free radical time-dependent generation and exerted antioxidant activity [14].

In this work, the effect of anthocyanidins showed no significant differences between treatments on caspase 3 (executioner) activity and untreated cells; neither were differences found for caspase 8 (initiator) activity. However, every malvidin concentration and 25 µM cyanidin improved caspase 9 activity (initiator). The study on the protective effect after oxidative injury showed a decrease in caspase 3 and 8 activities, with no statistically significant differences between the compounds and concentrations. In view of the results, and taking into account the previous studies in this field, it is proposed that the protective effect of anthocyanidins should be initiated via an extrinsic pathway and not an intrinsic one [6, 15–18].

The results shown in this work support previous data on the strong antioxidant effect of anthocyanidins in several experimental models. The inhibitory effect on lipid peroxidation, together with direct antioxidant and antiapoptotic effects under oxidative stress injury on astrocytes was assessed. These results may contribute to the knowledge of the molecular mechanism involved in such an antioxidant effect, although more in-depth

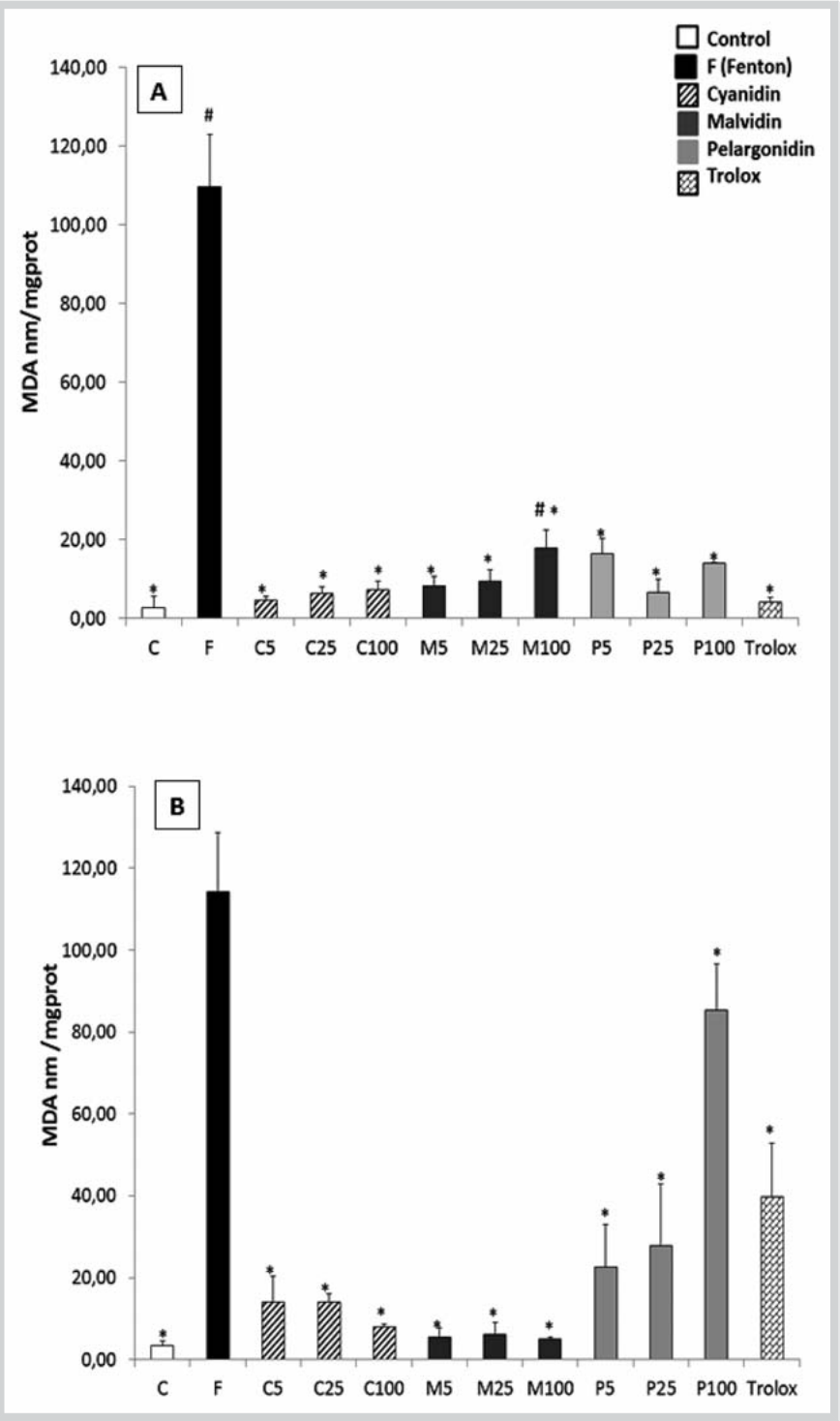

Fig. 3 Effect of anthocyanidins (cyanidin, malvidin, and pelargonidin – 5, 25, and 100 µM) in MDA levels compared to the positive control (0.5 mM Trolox). **A** Direct effect. **B** Protective effect under stress conditions (Fenton's reagent). MDA concentration is expressed as nmol/mg of protein. Results are expressed as means ± SD (n = 3, four replicates). *P < 0.05 vs. F; #p < 0.05 vs. control.

research is needed to evaluate their possible usefulness as natural protection against those diseases in which oxidative stress plays a crucial role.

Materials and Methods

Reagents

Cyanidin, malvidin, and pelargonidin were purchased from Extrasynthese (France) with a purity ≥ 96%. Gentamicin, penicillin, and streptomycin were purchased from Sigma Chemical (Spain). Trolox (97% purity) was purchased from Sigma-Aldrich (Spain). Fenton reagent ($FeSO_4/H_2O_2$) and other reagents were of analytical or chromatographic quality.

Cell culture

The human astrocytoma U373 MG line was obtained from the Cell Culture and Biological Resources Unit at Alcala de Henares University (Madrid, Spain). Cells were grown in a humidified incubator at 5% CO_2 and 95% air at 37 °C in Dulbecco's modified Eagle's medium (DMEM) piruvate free, from Invitrogen (Madrid,

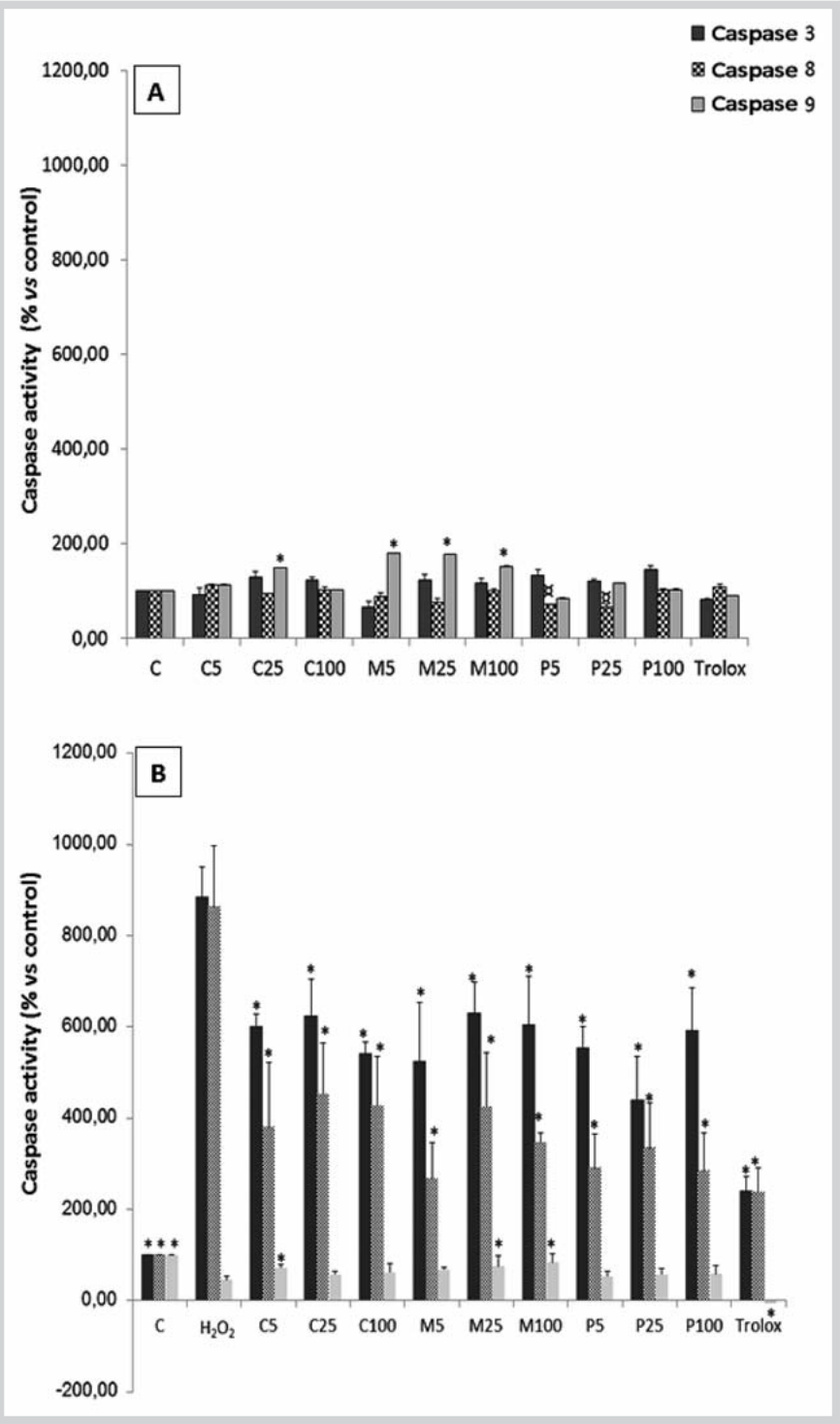

Fig. 4 Effect of anthocyanidins (cyanidin, malvidin, and pelargonidin – 5, 25, and 100 µM) on caspase 3, 8, and 9 activities compared to the positive control (0.5 mM Trolox). **A** Direct effect. **B** Protective effect under oxidative stress conditions (500 µM H_2O_2). Results were expressed as % versus control. Results are expressed as mean ± SD of at least three independent experiments (n = 3, four replicates). *P < 0.05 vs. corresponding control value; *p < 0.05 vs. corresponding exposure to toxic values.

Spain), supplemented with 10% fetal bovine serum (FBS) (Biowhitaker) and 50 mg/l of each one of the following antibiotics: gentamicin, penicillin, and streptomycin.

Cell treatment

Three concentrations of cyanidin, malvidin, and pelargonidin (5, 25, and 100 µM) were dissolved in DMEM and added to the cell plates for 24 h. In order to evaluate the protective effect against an oxidative insult, the culture medium was removed and the cells were treated with Fenton reagent (0.5 mM $FeSO_4$/1 mM H_2O_2) for 45 min.

For all of the experiments, every sample was analyzed in triplicate, with four plates for each condition.

Lactate dehydrogenase activity assay

Cells (2×10^5) were seeded in 24-well plates and treated as described in the previous section. Then, 100 µL of each plate were transferred into 96-multiwell plates, while the remaining cells were treated with the lysis buffer (Triton X-100) and transferred into Eppendorf tubes. Sodium pyruvate 80 mM and NADH (2 mg/ml) were added just before reading both samples (culture medium and lysate) [19].

The LDH leakage was measured using a Fluostar optima fluorimeter (λ 340 nm, 37 °C for 10 min) and expressed as a percentage from the ratio between the LDH activity in the culture medium and that of the whole cell content [9].

Lipid peroxidation

Quantification of MDA as the major end product of lipid peroxidation was carried on by chromatographic evaluation and the TBARS fluorimetric assay.

HPLC [20]: Briefly, samples were filtered through one cellulose acetate filter (0.22 µm) and injected on an Agilent 1100 series HPLC-DAD chromatograph with an isocratic elution of methanol : water (50 : 50 V/V) for 20 min. The flow rate was 0.5 mL/min and detection was set at 268 and 532 nm within a photodiode array detector. MDA values are expressed as nanomoles of MDA per milligram of protein.

TBARS Fluorometric Assay [21]: Samples were kept at 90 °C for 45 min at a low pH. Then, thiobarbituric acid was added to yield a fluorescent red chromogen that was read at 530 nm. The results are expressed as a percentage of TBARS, as referred to the toxic value (100%).

Caspase 3, 8, and 9 determinations

The fluorogenic caspases 3, 8, and 9 were used for a fluorimetric assay. After the hydrolytic cleavage of the coumarin derivative by the cytosolic caspases, the fluorescent product was determined using a multiwell fluorescence reader (Bio-Tek Instruments). Fluorescence was measured at 360 nm excitation and 480 nm emission for caspase 3; emission was set at 528 nm for caspases 8 and 9.

Statistical analysis

Statgraphics Centurion 16.1.15 (XV) was used. One-way analysis of variance (ANOVA) followed by Fishers least significant difference (LSD) test was applied to obtain the differences between samples. $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors have no conflicts of interest to declare.

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