Antioxidant and Anti-Inflammatory Effects of Delphinidin on Glial Cells and Lack of Effect on Secretase Enzyme

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Abstract. Beneficial health effects of anthocyanin extracts such as antioxidation and anti-inflammatory effects have been demonstrated in many studies. However, studies focusing on these effects exerted specifically by delphinidin (anthocyanin extract) are generally poor. The purpose of this study was to explore the in vitro protective functional role of anthocyanin extract, delphinidin, on oxidation- and inflammation-induced injury in C6 neuroglial cells, and to examine its effect on suppression of secretase enzyme, a key factor in Alzheimer's disease development. Cytotoxicity assay results revealed that delphinidin at high concentrations enhanced cell viability of spiked cells. Moreover, in a dose-dependent manner, delphinidin caused glutathione levels to increase in cells, a critical indicator of the cellular redox state, versus cells treated with hydrogen peroxide. Delphinidin decreased the expression of MCP-1 and CINC-1, two major cytokines markers in inflammatory situations versus tumor necrosis factor-α-induced damage. However, delphinidin, even at higher concentrations, did not suppress the secretase enzyme activity, indicating its indirect action in ameliorating the underlying cause of the disease. Our findings suggested that delphinidin could be a promising candidate for the development of therapeutics for the management of diseases associated with oxidative stress and inflammatory expressions such as neurodegenerative diseases.

Keywords: Anthocyanin; Delphinidin; Antioxidants; Anti-inflammatory; Neurodegenerative diseases

1. Introduction
In the last decade, the incidence of neurodegenerative diseases (NDs) like Alzheimer disease (AD), Parkinson disease (PD) and multiple sclerosis (MS) has exponentially increased. NDs are generally characterized by progressive loss of neuron cells, neuroinflammation and mitochondrial dysfunction. Mounting evidence suggests that the overproduction of reactive oxygen species (ROS) play substantial roles in the development of these diseases [1, 2]. In fact, neuron cells are specifically vulnerable to oxidative damage due to their high content of polyunsaturated fatty acids in membranes and increased oxygen consumption with poor antioxidant defense [3]. The pathogenesis of several NDs may be associated with the aggregation of proteins which induce inflammatory response in the brain and trigger ROS release in turn, leading to oxidative stress (OS) [4]. Numerous studies have shown that antioxidants, including pigmented plants, possess great potential in mediating NDs and could be an area of interest for future research [2].

Pigmented fruits and flowers are an economically potential resource of functional foods and nutraceuticals due to their antioxidant capacity and nutritional quality. Thus, they play an important
role in human nutrition and health [5, 6]. These plant parts possess diverse polyphenolic compounds (such as flavonoids (anthocyanins, flavanols, and catechins) and tannins), vitamin C and E and carotenoids, which are favorable dietary antioxidants for the endogenous defense strategies [7, 8]. Anthocyanins are blue, red, or purple pigments found in plant parts, especially flowers, fruits and tubers, and they have been traditionally used as a natural food colorant. The color variation and stability of these pigments are influenced by pH, light, temperature and structure [9]. In alkaline condition, anthocyanin appears as a blue pigment while red pigment anthocyanin is found in acidic conditions. Cyanidin, delphinidin, pelargonidin, peonidin, malvidin and petunidin are the most common anthocyanin extracts distributed in plants. Among the anthocyanin pigments, cyanidin and delphinidin are the most abundant anthocyanins (reaching up to 65%) found in most of the plants [10]. Recently, anthocyanins have gained an increased attention due to their beneficial health effects. They are known to have antioxidant, anti-inflammatory, anti-allergic, antimicrobial, antiviral, anticarcinogenic, antimutagenic, and anti-proliferative effects and thus play an essential role in preventing and modulating various inflammatory and degenerative diseases including NDs [8, 11-13].

In general, anthocyanins are able to reduce the risk of these diseases which can be shown by direct and indirect pathways. These colored extracts can directly reduce the risk of several chronic diseases through scavenging free radicals and thus lowering OS status usually associated with such diseases. While, the indirect actions may involve in downregulation of enzymatic activities, cell proliferation and apoptosis caused by OS and lipid peroxidation [14]. It is theorized that their antioxidants activity is owing to free radical scavenging activity by reducing agents in the electron-transfer reaction pathway via donating electrons to the free radicals and detoxifying them [15]. Studies have shown that anthocyanins serve as antiperoxidative by inhibition the lipid peroxidation of cell membrane [16, 17]. It has also been stated that anthocyanins can prevent oxidation of LDL catalyzed by metal or peroxyl radical through quick chelation of metal ions forming stable anthocyanin-metal complexes [18].

Anti-inflammatory action is another main pharmacologic effect of anthocyanin. In this context, lipid inflammatory mediators such as prostaglandins produced via cyclooxygenase and leukotrienes via lipoxygenases are attractive targets of anthocyanins [19]. It has been reported that delphinidin and some other anthocyanin extracts are potent inhibitors of phospholipaseA2, an enzyme catalyzes hydrolysis of membrane glycerophospholipids to generate prostaglandins and leukotrienes and other free fatty acids [20]. Their possible anti-inflammatory mechanisms include the attenuation of oxidative damage, inhibition of inflammation, stimulation of the immune system, reduction of platelet aggregation, and modulation of cholesterol synthesis and hormone metabolism [21]. Previous studies have demonstrated the role of anthocyanins in reducing the risk of cardiovascular disorders, NDs, and some cancers via inhibiting the production of pro-inflammatory modulators. It has been found that several inflammatory and anti-inflammatory cytokines were significantly upregulated in NDs [22, 23].

The antioxidant and anti-inflammatory effect of cyanidin, the most common anthocyanin, is extensively investigated, however, the effects of other anthocyanins including delphinidin (the second most abundant anthocyanin extract) is rarely reported. Delphinidin has a chemical characteristic similar to most of the anthocyanins and it appears as a blue-reddish or purple pigment in the plant. The blue hue of flowers is related to the delphinidin pigment. As compared to cyanidin, delphinidin has higher solubility in water and methanol with better stability at acidic pH and bioavailability [24, 25].

In this study, we investigated the effects of anthocyanin extract, delphinidin (Figure 1) on the OS status and inflammatory markers in an in vitro neurodegenerative model using C6 glial cells. Hydrogen peroxide (H$_2$O$_2$) was used to induce OS status in the C6 glial cell culture while TNF-$\alpha$ was used to initiate inflammatory situation. Then, the cell culture model was treated with a range of concentrations of delphinidin. Cell viability of stressed- and inflammatory-spiked cells with and without treatment with delphinidin was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Furthermore, the extent of alleviation of OS status and attenuation of inflammatory situation caused by the antioxidant and anti-inflammatory effects of the anthocyanin derivative (delphinidin) were investigated. Glutathione (GSH) (as a marker for the OS levels) were quantified in the cell culture model and compared with untreated cells. The downregulation of pro-inflammatory cytokines expression by delphinidin effect was measured by comparing the suppression of the secretion of monocyte chemoattractant protein-1 (MCP-1) and
cytokine-induced neutrophil chemoattractant (1) (CINC-1) cytokines levels in treatment-free and treated cells. Finally, the effect of delphinidin on lowering γ-secretase levels (an enzyme behind the pathological accumulation of beta-amyloid plaques in AD) was investigated using enzyme-linked immunosorbent assay (ELISA)-based analysis. This is the first study that demonstrate the effect of anthocyanin extract, delphinidin, on the neuroglial cells exploiting its benefits against OS and inflammation associated with NDs.

![Chemical structure of delphinidin chloride](image)

**Figure 1:** Chemical structure of delphinidin chloride (Chemical formula C_{13}H_{11}ClO_7 and molecular weight: 338.70 g/mol).

2. Materials
C6 glial cell line and F-12K (Kaighn's) medium were purchased from ATCC laboratories, UK. Fetal bovine serum (FBS) was supplied by PAA Laboratories Ltd., UK. Horse serum was obtained from Gibco, UK. CINC-1 ELISA kit and H_2O_2 were obtained from Thermo-Fisher Scientific, UK. delphinidin chloride, MTT, dimethylsulfoxide (DMSO), TNF-α, MCP-1 ELISA kit, GSH assay kit and other chemicals were supplied from Sigma-Aldrich, UK. γ-secretase ELISA Kit was obtained from Abbexa Ltd., UK.

3. Experimental protocols

3.1. Cell culture and treatment
The complete culture media of C6 cells comprised of F-12 medium, supplemented with FBS to a final concentration of 2.5% v/v, and horse serum to a final concentration of 15% v/v. The cells were incubated at 37°C in a humidified, 5% CO_2, 95% air atmosphere (Sanyo-MCO715). Cell stocks were maintained by routinely culturing as monolayers on T-25 flasks at a seeding density of 2×10^5 cells/cm^2. The fourth to fifth passage cells were used for all experiments at 80–90% confluence. Based on the preliminary experiments (data not shown), a dose-response assay regarding viability showed that delphinidin, H_2O_2 and TNF-α were not toxic to the cells at concentrations of less than 100 µg/mL, 300 µM and 1 µg/mL respectively. The cells were seeded in the desired-size plates (based on the type of experiment) and allowed to adhere for 24 h. In a separate set of experiments, the cells were treated with 1, 10, 50, and 100 µg/mL delphinidin for 18 h, followed by H_2O_2 (300 µM) or TNF-α (1 µg/mL) stimulation for 6 h. The absorbance of all samples and standards was adjusted with the use of culture media as background. Cells treated with stimulants only (H_2O_2 or TNF-α) were considered as positive control while treatment-free cultures were used as negative control.

3.2. Cell viability detection by MTT assay
This assay relies on oxidation reduction reaction which accomplished by various mitochondrial dehydrogenase enzymes, and generates nicotinamide adenine dinucleotides (NADH and NADPH) in viable cells supernatants [26]. MTT solution was freshly prepared at a 0.5 mg/mL concentration in culture medium without FBS and incubated at 37°C. Prior to use, the solution was sterilized by using a 0.22 µm pore size filter. MTT solution (100 µL) was added to 96-well culture plates containing the treated cells. The plates were incubated at 37°C for 4 h. The supernatant was removed and DMSO was then added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes standing, the plates were read spectrophotometrically (Mindray, China) at 540 nm.
3.3. Determination of antioxidant activity of delphinidin

GSH was measured using GSH assay kit according to the manufacturer's protocol. Briefly, the measurement uses a kinetic assay in which catalytic amounts of GSH cause a continuous reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) and the formed reduced GSH is recycled by GSH reductase and NADPH. The reaction rate is proportional to the concentration of liberated GSH. The intensity of yellow color due to formation of TNB was measured by spectrophotometer at 412 nm wavelength. Following treatment and incubation, the supernatant was used to quantify the amount of GSH in treated and untreated cell culture samples using a standard curve of reduced GSH established from a series of dilutions of standard solution of GSH (supplied with the kit).

3.4. Determination of anti-inflammatory activity of delphinidin

For the analysis of inflammatory cytokines, MCP-1 and CINC-1, kits were used to quantify the attenuation in these two cytokines levels induced by delphinidin addition in cells spiked with TNF-α. Both ELISAs were conducted according to the manufacturer's instructions. The absorbance for each set of standards, samples, and controls was measured at 450 nm and the mean was calculated after subtracting the average zero standard optical density. The standard curves of MCP-1 and CINC-1 were plotted with standard concentration on the x-axis and absorbance on the y-axis drawing the best-fit curve through the standard points. All reactions were performed in duplicates and experiments were repeated six times for statistical analyses. Levels of cytokines are calculated and expressed in ng/mL.

3.5. Amelioration of γ-secretase enzyme levels by delphinidin

C6 glial cells were cultured in 24-well plates and allowed to reach confluence, then treated with 1, 10, 50, and 100 µg/mL delphinidin. Wells containing untreated C6 glial cells were also included as a control. After 24 h incubation, the cells were washed with phosphate buffer and lysed via repeated freezing to -20°C and thawing to room temperature (3 times). The cells were centrifuged at 1500 rpm for 10 min to remove any cellular debris and the supernatant was collected and analyzed immediately as recommended to avoid any possible degradation. The analysis was conducted using ELISA kit for assaysing the γ-secretase levels (according to the kit instructions). Optical densities obtained from the samples were measured by spectrophotometer and the levels of γ-secretase were calculated using a standard curve drawn from a series of dilutions of standard γ-secretase solution (supplied with the kit).

3.6. Statistical analysis

The data of each group was analyzed using t-tests to determine significant differences among different treatments. Any significant difference in mean between tested groups was reported by carrying out a one-way ANOVA with Tukey’s post-hoc test, using Minitab® 17. The means of six independent experiments ± standard deviation (SD) were compared and differences were considered significant at P<0.05 (two-sided confidence intervals).

4. Results

Effects of delphinidin on cell viability. As shown in Figure 2A, H₂O₂ incubation led to a significantly (P<0.05) reduced cell viability, which was obviously recovered by delphinidin pretreatment, and the protective effect was dose-dependent around the tested range of concentrations. Compared to the control, treatment with H₂O₂ caused reduction in C6 glial cells viability to 58.4%, indicating that OS happened in cells leading to oxidative damage. However, delphinidin significantly (P<0.05) restored the cellular viability to 68.6% and 79.9% at 50 and 100 µg/mL concentrations, respectively. Such reduction was not observed in lower concentrations (1 and 10 µg/mL) with no significant difference from cells treated with H₂O₂ only (P>0.05).
Figure 2: Effect of delphinidin on cytoprotection of C6 glial cells based on cell viability analyzed by MTT assay. (A). In H$_2$O$_2$-induced OS cells. (B). In TNF-α-induced inflamed cells. Data represent means± SD. Values with different letters above are significantly different, P<0.05.

Similar results were seen for cells spiked with TNF-α which showed reduction in cell viability reaching 52.4% (Figure 2B), suggesting cellular damage by induction of inflammatory situation. Nevertheless, the assay showed that 50 and 100 µg/mL delphinidin significantly (P<0.05) increased cell viability, peaking at 75.8% and 92.2% respectively. Same effects were not found in cells incubated with lower concentrations of delphinidin, pointing out its dose-dependent effect.

Effects of delphinidin on OS. In this study, the extent of OS relief was measured in terms of the change in GSH levels caused by the antioxidant capacity of delphinidin introduced into the sample. Basal GSH levels were significantly (P<0.05) decreased by approximately 65% in H$_2$O$_2$-treated C6 glial cells, while pretreatment with delphinidin significantly (P<0.05) elevated GSH levels. As illustrated in Figure 3, we found that a 300 µM H$_2$O$_2$ challenge lowered GSH levels in treated cells (without H$_2$O$_2$: 35.16±4.16; with H$_2$O$_2$: 10.2±3.7). However, the addition of delphinidin, even in low concentrations (10 µg/mL), neutralized the reactive oxygen-generating ability of H$_2$O$_2$ as expressed by elevated GSH levels reaching 38.8±6.64 in the highest tested concentration (100 µg/mL).

Figure 3: Effects of different concentrations of delphinidin on GSH levels in cells treated with H$_2$O$_2$ compared to untreated cells. Data represent means ± SD. Columns with different superscripts are significantly different from each other at P<0.05.
Anti-inflammatory effects of delphinidin. To evaluate the levels of proinflammatory cytokines in TNF-α-spiked C6 glial cells, MCP-1 and CINC-1 concentrations were quantified in the supernatant (with and without delphinidin) using ELISA kits. As shown in Figure 4A and B, TNF-α markedly increased MCP-1 and CINC-1 levels compared to control ($P<0.01$) reaching more than 3 folds of the basal level of MCP-1 (Figure 5A) and more than 2.5 folds the level of CINC-1 (Figure 4B). On the other hand, both cytokine markers levels were significantly ($P<0.01$) and dose-dependently downregulated by delphinidin supplementation returning to the basal levels especially at the higher concentrations.

![Figure 4](image1.png)

**Figure 4:** Effects of different concentrations of delphinidin on TNF-α induced inflammatory markers. (A). MCP-1 marker levels. (B). CINC-1 marker levels. Data represent means ± SD. Peaks with different letters above are significantly ($P<0.05$) different.

γ-secretase activity suppression by delphinidin. To evaluate any potential direct neuroprotective effects of delphinidin, besides its ROS scavenging capacity and anti-inflammatory effects, inhibition of γ-secretase activity was investigated using ELISA assay. As shown in Figure 5, the anthocyanin extract did not promote any significant reduction ($P>0.05$) in the enzyme levels when compared to the control cells. The values fluctuated between 14.51±8.22 pg/mL at the lowest concentration and 18.3±4.6 pg/mL.

![Figure 5](image2.png)

**Figure 5:** Effect of different concentration of delphinidin treatment on λ-secretase enzyme levels in C6 glial cells. No significant ($P>0.05$) inhibitory effects on λ-secretase levels was seen.
5. Discussion

Anthocyanins are key components in the human diet due to their frequent presence in plants, particularly dark-colored fruits, vegetables, and pigmented grains. In the last decade, there has been a growing interest both from consumers and researchers in the role that anthocyanin may have in diseases prophylaxis and prevention. Its dietary supplement is being increasingly recognized as beneficial for modern human health [11, 27]. Anthocyanins possess anti-diabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects, as well as prevention of cardiovascular diseases and neuroprotective effects [14, 20]. Such effects for the most abundant anthocyanin, cyanidin, are extensively studied, however, similar effects related to delphinidin, another major anthocyanin extract, is less frequently investigated.

In this work, C6 glial cell line was chosen to mimic an in vitro neuro model to investigate potential neuroprotective effects of the anthocyanin extract. Cytotoxic activities of delphinidin against H2O2- and TNF-α-induced toxicity in these cells were evaluated by assessing effects on metabolic activity by the MTT assay. Results shown in Figure 2A and B demonstrated that high concentrations of delphinidin reduced the mortality of C6 glial cells in both cases, suggesting its ability to reverse the cellular-induced damage. This finding is supported by previous studies that have reported the effects of pre-treatment with different concentrations of anthocyanins successfully improved the cell viability after spiking with different activators using different cell lines [28-30]. Thus, addition of delphinidin could protect cells against activators and promote viability. Apart from direct antioxidative reactivity, compounds such as certain polyphenols and flavonoids (including anthocyanins) may also activate some intracellular signaling pathways to prolong the cellular defense response [29].

To confirm the antioxidant effects imposed by delphinidin, further investigation was conducted to measure GSH levels as an OS marker. The results demonstrated that administration of delphinidin significantly and dose-dependently increased the activity of GSH (Figure 3). GSH is one of the major antioxidant defense enzyme that is responsible for scavenging free radicals, it is a tripeptide, capable of preventing damage to essential cellular components caused by ROS, such as free radicals, peroxides, lipid peroxides and heavy metals. Our results showed that GSH levels were decreased in C6 glial cells by H2O2 treatment, however, delphinidin administration had a counteractive effect on the H2O2-induced decrease-GSH levels in a dose dependent manner. These results are in agreement with other studies which highlighted the antioxidant abilities of other anthocyanins (rather than delphinidin) [11, 31-33]. OS contributes to the tissue injury and mainly occurs due an imbalance between the production of ROS and the antioxidant defense systems. These ROS attack biomolecules including lipids, proteins and DNA, accelerating the established tissue damage and initiating cell death pathways [34]. ROS are generated as by-products from mechanisms such as "the electron transport chain", NADPH oxidase, metabolism of the cytochrome P-450 and arachidonic acid and xanthine oxidase. These ROS cause cell damage either directly or indirectly by acting as intermediates in numerous cell signaling pathways [35, 36]. Although anthocyanins are extensively used as antioxidants and anti-inflammatory, their mechanisms of action are still not entirely clear. The potential of anthocyanins as antioxidant is greatly regulated by their chemical structure, in particular the number of hydroxyl groups and the availability of donor electrons in the ring [20]. Typically, the antioxidant action of the anthocyanins includes the suppression of ROS formation, via enzyme inhibition or blocking the trace elements involved in the formation of free radicals [15]. It has been proposed that flavonoids, including delphinidin, can interrupt the free radicals chain reaction by hydrogen donating to the peroxyl radical, to form a flavonoid radical ending propagation chain reaction [20].

In the TNF-α-induced cytokines formation experiment (Figure 4), at high concentrations (50-100 µg/mL), delphinidin showed the most potent anti-inflammatory effects, as indicated by abolishing the upregulation in MCP-1 and CINC-1 levels caused by cell spiking with TNF-α. Inflammation is a complex process, including the accumulation and activation of immune suppressor cells, pro-inflammatory cytokines, chemokines, growth and angiogenic factors and activation of several inflammatory signaling pathways mediated predominantly by transcription nuclear factor (NF-κB) [37]. Exposed to external stimuli such as TNF-α, NF-κB transcription inhibitor protein (IkBα) phosphorylation occur, which lead to ubiquitination-dependent degradation of IkBα resulting in an increased release of inflammatory markers such as MCP1 and CINC1 [38]. Anthocyanins are capable
to inhibit NF-κB and NF-κB-dependent mediators. Activation of these mediators, including TNF-α, trigger chronic inflammation which is functionally dependent on the OS in the cell [20, 39]. Previous data has shown that anthocyanins could be the potential alternatives to prevent and treat the inflammation situation in many diseases [37]. Recent study reported action mechanisms of protecting vascular endothelium including modulation of crucial signaling pathways and gene regulation [39]. Further study has shown that malvidin-3-glucoside, another anthocyanin extract, could suppress pro-inflammatory mediators through NF-κB inhibition in bovine arterial endothelial cell [40].

The available evidence from previous studies has suggested that anthocyanins have an effective role in mitigation of AD, however, the exact mechanism of such action has not been yet explained. AD is characterized by accumulation of beta amyloids plaques which resulted from action of γ-secretase enzyme on amyloid precursor protein. Previous experiments performed by Joseph et al. (2003) have shown no effect on beta amyloid peptide production, deposition or amyloid load on transgenic PS1-APP mice brains, after supplementation with anthocyanin extracts (2% of diet) [41]. Moreover, the study of Magdalini et al (2012) has indicated that anthocyanin had no effect on amyloid precursor protein when assessed by western blot in CHO APP/770 cells [42]. In this work, γ-secretase enzymatic activity experiment was performed to investigate whether anthocyanin has any direct effect on modulation of this enzyme and to predict its mechanism of action. C6 glial cells were treated with different concentrations of delphinidin for 24 h; no alterations were observed on λ-secretase levels secreted versus untreated cells (Figure 5). These observations suggest that the neuroprotective activity of anthocyanin in AD may be attributed to its antioxidant and anti-inflammatory aptitude that result in lowering the "redox-sensitive nuclear factor-κB signaling pathway" rather than direct inhibition of enzymatic activities. In this regard, further research may be required to provide full explanation [43].

6. Conclusion
In the present study, anthocyanin extract, delphinidin, provided a protection to C6 glial cells against OS- and inflammatory-induced injury in a dose-dependent manner. Delphinidin enhanced cell viability of C6 glial cells treated with H$_2$O$_2$ and TNF-α. It offered great antioxidant effects by increasing GSH levels. Moreover, downregulation of inflammatory markers levels, MCP-1 and CINC-1 suggesting its potent anti-inflammatory effect. However, lack of activity in the modulation of λ-secretase levels might propose indirect neuroprotective effects of delphinidin. Considering the pivotal roles of OS in NDs, the manipulation of ROS levels by using delphinidin as antioxidants and anti-inflammatory may represent a promising therapeutic option to slow down neurodegeneration process and alleviate associated symptons.

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8. Conflicts of interest
Authors do not have any conflict of interest.

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