Antioxidant Effects of Elderberry Anthocyanins in Human Colon Carcinoma Cells: A Study on Structure–Activity Relationships

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Scope: Glycosylation is a way to increase structure-stability of anthocyanins, yet compromises their bioactivity. The study investigates the antioxidant activity of purified cyanidin (Cy)-based anthocyanins and respective degradation products in Caco-2 clone C2BBe1 aiming to identify structure–activity relationships.

Results and Methods: Cyanidin 3-O-glucoside (Cy-3-glc) and cyanidin 3-O-sambubioside (Cy-3-sam) proved to be most potent regarding antioxidant properties and protection against hydrogen peroxide (H₂O₂)-induced reactive oxygen species (ROS)-levels measured with the dichloro-fluorescein (DCF) assay. Cyanidin 3-O-sambubioside-5-O-glucoside (Cy-3-sam-5-glc) and cyanidin 3-O-rutinoside (Cy-3-rut) were less efficient and not protective, reflecting potential differences in uptake and/or degradation. Following ranking in antioxidant efficiency is suggested: (concentrations <10 × 10⁻⁶ M) Cy-3-glc > Cy-3-sam > Cy-3-sam-5-glc ~ Cy-3-rut ~ Cy; (concentrations ≥50 × 10⁻⁶ M) Cy-3-glc ≈ Cy-3-sam ≥ Cy > Cy-3-sam-5-glc ~ Cy-3-rut. Cy and protocatechuic acid (PCA) reduced ROS-levels as potent as the mono- and di-glycoside, whereas phloroglucinol aldehyde (PGA) displayed pro-oxidant properties. None of the degradation products protected from oxidative stress.

Gene transcription analysis of catalase (CAT), superoxide dismutase (SOD), glutathione-peroxidase (GPx), heme oxygenase-1 (HO-1), and glutamate-cysteine ligase (γGCL) suggest no activation of nuclear factor erythroid 2-related factor 2 (Nrf2).

Conclusion: More complex residues and numbers of sugar moieties appear to be counterproductive for antioxidant activity. Other mechanisms than Nrf2-activation should be considered for protective effects.

1. Introduction

Anthocyanins are plant pigments present in high amounts particularly in intensively colored berries. They gain great interest regarding potential protective effects against several chronic and degenerative diseases such as cancer,[1] cardiovascular disease,[2,3] diabetes,[4] and diseases associated with oxidative stress.[5] Anthocyanins can scavenge reactive oxygen species (ROS) due to their hydrogen (electron) donation ability.[6,7] Another effective approach to protect cells from oxidative stress is the induction of antioxidant defense systems such as the redox-sensitive nuclear factor erythroid 2-related factor 2 (Nrf2)-pathway. Many antioxidative enzymes, amongst them catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), and glutamate-cysteine ligase (γGCL) are targets of the Nrf2 transcription factor.[8–11] A low absorption rate of anthocyanins was reported even though major effects can be observed with nanomolar serum-concentrations.[12] For instance in an intervention study with healthy male volunteers we found a reduction of oxidative DNA damage and an increase in glutathione (GSH) status already after a daily...
intake of 700 mL of red multi-fruit juice (total anthocyanin content 197.9 mg L⁻¹ Cy-3-glc equivalents) over a 4-week period. A recent in vivo study revealed DNA-protective and modulating effects of Nrf2-signaling by consumption of consumer-relevant amounts of anthocyanin-rich beverages including a red-fruit beverage comprising 332.0 ± 48.8 mg L⁻¹ of total anthocyanins. In a human pilot study with healthy individuals and ileostomy probands transcription of Nrf2-dependent genes in peripheral blood mononuclear cells was affected after consumption of an anthocyanin-rich bilberry (Vaccinium myrtillus L.) pomace extract only in healthy subjects, suggesting a role of colonic processes for bioactivity. The results were supported by Nrf2-activating properties of the intestinal anthocyanin degradation product phloroglucinol aldehyde (PGA), indicating that digestive processes regulated by the intestinal microbiota are crucial for bioactivity of anthocyanins.

Generally, glycosylation can substantially influence the chemical reactivity of anthocyanidins and enhance the stability of the otherwise unstable aglycones. Glycosylation can also enhance the hypochromic effect and bluing of the color, thus being attractive for the development of natural blue food colorants. On the other hand, bioavailability is negatively affected by glycosylation. Regarding the impact of glycosylation site, number and type of sugar on the antioxidant activity of anthocyanins the literature is quite heterogeneous.

Within the scope of the European project AnthoPLUS (http://www.anthoplus.com/), anthocyanins of different complexity in their side chain decoration and sugar moieties were produced and assessed for their quality and properties as potential colorants and food additives. Furthermore, as a novel concept, we have developed test kits for research from different fruit juice sources, comprising varying fractions of purified anthocyanins, which we called AnthoKits. In the present study, the first time, an AnthoKit from elderberry (Sambucus nigra) juice was produced and tested. The kit comprised cyanidin 3-O-sambubioside (Cy-3-sam), cyanidin 3-O-sambubioside-5-glucoside (Cy-3-sam-5-glc) (Figure 1), and a mixture of both anthocyanin fractions based on their natural composition in the elderberry juice. To enlarge the list of Cy-based anthocyanins for systematic structure–activity analysis, cyanidin 3-O-glucoside (Cy-3-glc) and cyanidin 3-O-rutinoside (Cy-3-rut) were added to the study.

Our aim was to elucidate the structure–activity relationship of Cy-based anthocyanins with respect to their antioxidant potential and protective effects against H₂O₂-induced oxidative stress in human colon carcinoma cells. Furthermore, the question was addressed whether the antioxidant potency of anthocyanins is founded in the anthocyanin itself and/or in degradation products, such as cyanidin (Cy), protocatechuic acid (PCA), and the intestinal degradation product PGA (Figure 1). Kropat et al. observed an impact of PGA on the Nrf2-pathway therefore we hypothesized that this redox-sensitive pathway might play a role in the protection against H₂O₂-induced ROS production by Cy-based anthocyanins.

2. Results and Discussion

2.1. Phytochemical Characterization of Elderberry Raw Juice

The total anthocyanin content of freshly squeezed elderberry juice has been determined to be 1.19 µg mL⁻¹. Based on 100 mL elderberry juice a degreased extract was prepared to reach 100 mg total anthocyanins for subsequent purifications. After lipid removal and filtration, the raw juice sample was analyzed by LC-UV/MS (Figure 2). The UV chromatogram at 280 nm showed a number of chromatographic peaks, likely related to polyphenols naturally present in the elderberry juice (Figure 2A, top). In contrast, only two major chromatographic peaks were observed at 515 nm, the wavelength specific for anthocyanins (Figure 2A, bottom). The sum of mass spectra across these two chromatographic peaks revealed m/z 743.20 as the main molecular ion present in peak 1 and m/z 581.15 in peak 2 (Figure 2B). Based on MS/MS fragmentation pattern (Figure S2, Supporting Information) and comparison with literature data the related compounds were tentatively annotated as Cy-3-sam-5-glc (further named anthocyanin 1) and Cy3-sam (further named anthocyanin 2). The additional molecular ions detected in the chromatographic peaks 1 and 2 were tentatively annotated as cyanidin 3,5-O-di-glucoside (m/z 611.16) and Cy3-glc (m/z 449.10), and likely present co-eluting anthocyanins. The molecular ion with m/z 338.33 detected in the sum mass spectrum of peak 1, did not produce molecular fragment ions during MS/MS analysis and no extracted ion chromatogram could be generated for this molecular mass. We thus concluded it representing a molecular ion produced by in-source decay during LC-UV/MS analysis.

2.2. Phytochemical Characterization of Elderberry Anthocyanin Fractions and AnthoKit Preparation

We aimed to distinguish biomedical effects of individual anthocyanins from those of anthocyanin combinations (AnthoKit mix). Thus, fractions of individual anthocyanins have been isolated and an elderberry AnthoKit was prepared thereof as described in materials and methods (concept schematically presented in Figure S3, Supporting Information).

Phytochemical compositions of the isolated fractions were analyzed by LC-UV/MS (Figure 3) and compound annotations based on MS/MS fragmentation pattern (Figure S4, Supporting Information). The fraction anthocyanin 1 showed one prominent chromatographic peak at a retention time of 3.30 min, which has been proven to be enriched for Cy-3-sam-5-glc (m/z 743.20) with minor co-elution of cyanidin 3,5-O-di-glucoside (m/z 611.16) as presented in Figure 3A.D. Similarly, one major peak was enriched in fraction anthocyanin 2 eluting at a retention time of 3.82 min and corresponding to Cy3-sam (m/z 581.15) with co-elution of Cy3-glc (m/z 449.10); see Figure 3B.E. Both fractions contained minor remaining amounts of other polyphenols as observed from the UV-chromatograms at 280 nm.
By means of plate photometric assays anthocyanin and sugar amounts were determined (Table 1). The sugars (glucose, fructose, and sucrose) were nearly completely removed by the chromatographic separations. Glucose could not be detected in fractions anthocyanin 1, anthocyanin 2, and polyphenols. The content of fructose was markedly reduced to levels ≤0.1 mM. Sucrose was not detected in raw juice fraction and was found with low amounts (≤0.2 mM) in fractions anthocyanin 1, anthocyanin 2, and polyphenols, likely released from anthocyanins and other polyphenols due to mild acidic hydrolysis during purification procedures. The final fractions (anthocyanin 1, anthocyanin 2, and polyphenols) contain 10.36 mg, 43.05 mg, and 14.78 mg of anthocyanins, respectively.

To obtain an AnthoKit mix we combined the individual purified anthocyanin fractions based on their natural composition in the elderberry juice (Figure S3, Supporting Information), namely 12% w/w of fraction anthocyanin 1 and 88% w/w of fraction anthocyanin 2. All samples were dried under liquid nitrogen and the weight was measured. We calculated a multiplication factor for the utilization in the biomedical assays according to the anthocyanin concentration estimated by plate photometric assay. All fractions were covered with argon and stored at −20 °C until their use.

The main anthocyanins reported for elderberry are Cy-3-glc, Cy-3-sam, Cy-3-sam-5-glc, and cyanidin 3,5-O-di-glucoside; with 65.7%, 32.4%, 1.1%, and 0.8% in this order.16,17 Thus, we resume our tentative identifications as plausible, although different proportions have been observed in our samples (Figure 2). Under our chromatographic conditions we have observed co-elution of the main Cy-3-sam (anthocyanin 2) with Cy-3-glc and of Cy-3-sam-5-glc (anthocyanin 1) with cyanidin 3,5-O-di-glucoside, which is consistent with other reports on elderberry anthocyanins.18,19 (Figure S4, Supporting Information).

Figure 1. Structures of elderberry anthocyanins, and respective degradation products Cy: cyanidin, Cy-3-glc: cyanidin 3-O-glucoside, Cy-3-sam: cyanidin 3-O-sambubioside, Cy-3-sam-5-glc: cyanidin 3-O-sambubioside-5-glucoside, Cy-3-rut: cyanidin 3-O-rutinoside, PCA: protocatechuic acid, PGA: phloroglucinol aldehyde.
Figure 2. LC-UV/MS analysis of elderberry raw juice. A) UV-chromatograms at 280 nm (top) and 515 nm (bottom) from 2 to 12 min. B) Sum of mass spectra from retention time 3.2–3.4 min (top, anthocyanin 1) and 3.8–3.9 min (bottom, anthocyanin 2). Tentative annotation based on MS/MS fragmentation pattern (Figure S2, Supporting Information) and comparison with literature data.

The main molecular ions detected in the polyphenols fraction in our study (Figures S5 and S6, Supporting Information) have not been described in the literature and thus could not be annotated in our study.

2.3. Impact on Cell Viability

In order to rule out cytotoxic effects of the test compounds on C2BBe1 colon cells, causing potential artefacts in the interpretation of data, the sulforhodamine B (SRB) assay was performed. H2O2 was used as a positive control, a substance known to have potent cytotoxic effects in C2BBe1 cells.

Neither the different Cy-based anthocyanins nor the respective degradation products Cy, PCA and PGA (up to 200 × 10^{-6} M) caused statistically significant changes in C2BBe1 cell growth after 24 h incubation compared to the solvent control (Table S1, Supporting Information). H2O2 potently reduced viability of C2BBe1 cells to about 39 ± 13%. Forster et al. reported a growth inhibitory effect for PGA with an IC_{50} of 76.7 × 10^{-6} M after 72 h and a reduction of cell viability already at 100 × 10^{-6} M yet incubated in the absence of catalase. The tendency of a minor proliferative effect of Cy in C2BBe1 at 200 × 10^{-6} M fits with the heterogeneous literature. Renis et al. described a growth inhibition of around 20% for 200 × 10^{-6} M Cy (24 h), whereas at 100 × 10^{-6} M, with a longer incubation time (68 h), no toxicity could be detected. For HT-29 colon carcinoma cells, the range spans from 57 × 10^{-6} M IC_{50} to no inhibition up to 300 × 10^{-6} M which may be attributed to the use of catalase in the latter case. HCT-116 cells on the other hand displayed a considerable growth inhibition of 82% when incubated with 200 × 10^{-6} M Cy, a value dropping to about 40% at 100 × 10^{-6} M, concurring with the IC_{50} of 85 × 10^{-6} M of another working group. In conclusion, our results regarding cytotoxicity of Cy-based anthocyanins and respective degradation products are reliable based on literature.

2.4. Antioxidant Effects: Comparison of Anthocyanins and Degradation Products

Effects of Cy-based anthocyanins and potential degradation products on cellular ROS levels of C2BBe1 cells were investigated with the DCF assay. Cells were incubated with solvent and the respective test compounds in the presence of catalase for 1 h (measurement every 15 min). ROS levels significantly lower than the levels of solvent-treated cells were considered as antioxidant effect. Since the data of time point t_{60} showed the most significant differences from solvent control, this time point was chosen for data presentation (Figure 4).

Cy-3-glc as well as its degradation products Cy and PCA potently reduced ROS production in C2BBe1 cells. (Figure 4). Cy-3-glc and PCA were equipotent, while Cy appeared to be less potent, as significantly reduced ROS levels were observed only starting at a concentration of 1 × 10^{-6} M compared to 0.1 × 10^{-6} M for Cy-3-glc. At concentrations ≥50 × 10^{-6} M Cy-3-glc, Cy, and PCA were equipotent, reducing ROS levels at 200 × 10^{-6} M to 11 ± 4%, 18 ± 6%, and 14 ± 3% respectively compared to the solvent control (p < 0.001). Other anthocyanin degradation products have been shown to decrease ROS production as well, such as the grape phenolic compounds gallic acid (1 and 10 × 10^{-6} M) and syringic acid (10 × 10^{-6} M) that were measured in Caco-2 cells with the DCF assay. To our knowledge no data are available so far for...
PCA in Caco-2 cells. Yet, DCF assays using different cell lines showed also antioxidant properties of PCA. Guttenplan et al. reported that incubation with $4 \times 10^{-6}$ M PCA of the human oral cell line MSK-Leuk 1 leads to a 10% reduction of fluorescence after 90 min. This effect appears to be relatively weak compared to the 56% fluorescence reduction caused by $5 \times 10^{-6}$ M PCA in C2BBe1 cells. However, it shows that PCA has the capability to lower intracellular ROS in different cell lines.

The results clearly show no significant difference between the antioxidant effect of Cy-3-glc and its degradation products in C2BBe1 cells, except for PGA. Despite being a main degradation product PGA did not lower basal ROS levels in C2BBe1 cells. Instead, an increase in ROS with a peak at $25 \times 10^{-6}$ M at all measured time points (Figure 4, $t_{60}$ T/C: 155.7 ± 29.6%, $p < 0.001$) was detected, suggesting a potential pro-oxidant effect. Kropat et al. reported PGA to activate the main regulator of antioxidant defense, Nrf2, in HT-29 colon tumor cells. As a degradation product of the gut microbiota PGA indeed might have pro-oxidant effects in the gut, tickling the Nrf2-mediated defense system. However, it must be pointed out that the concentration...
Table 1. Anthocyanin and sugar concentration before and after chromatographic separation of elderberry juice.

| Fraction     | Anthocyanin concentration [μg mL⁻¹] | Volume [mL] | Total anthocyanin amount [mg] | Sugar concentration [mM] |
|--------------|-------------------------------------|-------------|------------------------------|--------------------------|
| Raw juice    | 1.19                                | 84          | 99.96                        | Glucose: 5.962           |
| Anthocyanin 1| 2.59                                | 4           | 10.36                        | Fructose: 4.872          |
| Anthocyanin 2| 2.05                                | 21          | 43.05                        | Sucrose: 0.131           |
| Polyphenols  | 1.64                                | 9           | 14.78                        |                          |

n.d. – not detected.

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of PGA appears to be critical as the pro-oxidant effect turns up only in a narrow range of low PGA concentrations.

2.5. Antioxidant Effects: Comparison of Different Cy-Based Anthocyanins

Whether type and size of the sugar moiety of the flavylium structure impact the antioxidant properties of Cy-based anthocyanins was addressed by comparing their ability to diminish the fluorescence signal in the DCF assay.

All tested anthocyanins lead to significant lower ROS levels in C2BBe1 cells after 1 h incubation as observed in DMSO-treated cells. Since data of time point 60 showed the most significant differences from solvent control, this time point was chosen for comparison between the different anthocyanins (Figure 5A) with respect to antioxidant efficiency. The aglycone Cy was included for comparison as well.

As calculated with the relative fluorescence data and ANOVA (Bonferroni), Cy-3-glc and Cy-3-sam-5-glc significantly (p < 0.001) reduced cellular ROS production to 69 ± 17% and 71 ± 19% respectively, starting at a concentration of 0.1 × 10⁻⁶ M to a maximum of 11 ± 4% and 31 ± 13%, respectively at 200 × 10⁻⁶ M (Figure S7A,C, Supporting Information). Cy-3-sam displayed significant antioxidant effects already at 0.01 × 10⁻⁶ M (p < 0.01) and reduced basal ROS levels to 79 ± 6% up to a maximum of 8 ± 11% at 200 × 10⁻⁶ M (p < 0.001) (Figure S7B, Supporting Information). Cy-3-rut was significantly effective starting at 1 × 10⁻⁶ M (p < 0.01) reducing fluorescence to 71 ± 13% and up to a maximum of 26 ± 10% at 200 × 10⁻⁶ M (p < 0.001) (Figure S7D, Supporting Information). The degradation product Cy (p < 0.001) reduced ROS levels significantly to 75 ± 11% starting with 1 × 10⁻⁶ M and to 18 ± 6% at 200 × 10⁻⁶ M (Figure S7E, Supporting Information). The positive control, H₂O₂, potently increased ROS production in C2BBe1 cells up to about 4-fold in comparison to the solvent control (Figure S7, Supporting Information). Cy-3-glc and Cy-3-sam seem rather similar and the most potent in their antioxidant properties in C2BBe1 cells after 1 h of incubation. Based on the graphical plot (Figure 5A) Cy-3-glc decreases ROS levels significantly better than Cy-3-sam-5-glc, Cy-3-rut, and Cy at the concentration of 1 and 10 × 10⁻⁶ M, whereas Cy-3-sam appears less potent yet with no statistical significance. Cy-3-glc and also Cy-3-sam at concentrations ≥50 × 10⁻⁶ M reach significant differences to Cy-3-sam-5-glc and Cy-3-rut whereas statistical significance is lost to Cy. Thus, at higher concentrations both glycosides act equipotent in reducing ROS. With increasing concentration Cy-3-sam-5-glc slowly reaches the same and even higher fluorescence than Cy with statistical significance at 100 × 10⁻⁶ M, suggesting less potency compared to Cy. The most potent fluorescence-reducing effect is observed with Cy-3-sam at 200 × 10⁻⁶ M with 8 ± 11% (p < 0.001) compared to the solvent control. In contrast, the aglycon Cy possesses lower antioxidant potency than Cy-3-glc at low concentrations (≤10 × 10⁻⁶ M), potentially due to its low stability. At higher concentrations (≥50 × 10⁻⁶ M) the antioxidant activity of Cy is found to be improving compared to the di-glycoside and rutinoside. Based on these results we suggest following ranking in antioxidant efficiency of Cy-based anthocyanins with different sugar moieties: at low concentrations (1–10 × 10⁻⁶ M) Cy-3-glc ≥ Cy-3-sam > Cy-3-sam-5-glc ≈ Cy-3-rut ≈ Cy, at high concentrations (≥50 × 10⁻⁶ M) Cy-3-glc ≈ Cy-3-sam ≥ Cy > Cy-3-sam-5-glc ≈ Cy-3-rut. As the most potent antioxidant effect in C2BBe1 colon cells is observed for Cy-3-glc and Cy-3-sam, more bulky residues and numbers of sugar moieties appear to be counterproductive for antioxidant effects in colon tumor cells. Of note, at low concentrations (1, 10 × 10⁻⁶ M) Cy-3-glc showed the most potent antioxidant effect, whereas at concentrations ≥50 × 10⁻⁶ M Cy-3-sam is equipotent suggesting potential differences in the uptake and/or stability of the compounds.
Mixture of Anthocyanins

2.6. Antioxidant Effects: Comparison of Single Anthocyanins and Mixture of Anthocyanins

Two highly purified fractions (Cy-3-sam and Cy-3-sam-5-glc) from the elderberry AnthoKit were tested regarding antioxidant properties as well as the reconstituted mix (AnthoKit mix) comprising both fractions. To compare the effects of the different fractions, the AnthoKit mix was used as Cy-3-sam equivalent in the DCF assay. Cy-3-sam equivalent (μM) represents the concentration of Cy-3-sam calculated based on its portion (88% w/w) present in the AnthoKit mix.

Already the lowest concentration of 0.1 × 10⁻⁶ M AnthoKit mix showed a significant decrease of about 38 ± 19% (Figure 5B and Figure S7F, Supporting Information). At the highest concentration (200 × 10⁻⁶ M) the cellular ROS level was decreased to 0 ± 6% after 1 h incubation. Thus, increasing concentrations of AnthoKit mix up to 200 × 10⁻⁶ M Cy-3-sam equivalents appeared to yield lower cellular ROS levels as observed for the single fractions. For example, a concentration of 10 × 10⁻⁶ M AnthoKit mix decreased the cellular ROS content to 37 ± 10% after 1 h compared to solvent control. Cy-3-sam induced a reduction of ROS to about 47 ± 17% at the same concentration and time point, Cy-3-sam-5-glc to 64 ± 14%. Thus, the mix of the two fractions appears to be more potent regarding antioxidative effects than the single fractions. However, statistical analysis (one-way ANOVA and Bonferroni) reveals no difference in potency between Cy-3-sam and the AnthoKit mix. Cy-3-sam-5-glc clearly reduced ROS levels less effectively than the AnthoKit mix at concentrations ≥1 × 10⁻⁶ M and at ≥50 × 10⁻⁶ M also than Cy-3-sam. In summary, the AnthoKit mix is more potent than Cy-3-sam-5-glc and equipotent to Cy-3-sam. Thus, the presence of a less efficient anthocyanin in the mixture did not affect the overall effect of the more potent Cy-3-sam, presumably as the percentage is rather low.

2.7. Protective Effects Against H₂O₂-Induced Stress

A modified version of the DCF assay was used to assess whether and to which extent a 24 h pre-incubation with anthocyanins and respective degradation products can modify the cells’ response to oxidative stress induced by H₂O₂. We hypothesized that potentially strengthening cellular mechanisms such as the Nrf2/Keap1-pathway are involved to protect from H₂O₂-stress. The effect was detected with the DCF assay as a reduction in fluorescence and thus in ROS levels.

Pre-incubation with Cy-3-sam-5-glc, the AnthoKit mix, Cy-3-rut, and the degradation products Cy, PCA and PGA up to 200 × 10⁻⁶ M did not reduce the fluorescence generated by H₂O₂ over a time period of 1 h with statistical significance (data not shown), suggesting no protective effect against H₂O₂-induced ROS production by these compounds. In contrast, for Cy-3-glc a significant difference to stressed control cells was evident at the highest concentration of 200 × 10⁻⁶ M (t₀/₂: %, p < 0.001) based on one-way ANOVA and Bonferroni post hoc test (Figure 6A). The fluorescence signal was reduced to 61 ± 11% in comparison to stressed control cells.

Similar activity can be assumed for Cy-3-sam. However due to significantly different variance homogeneity (as calculated with the Brown–Forsythe test) a non-parametric Kruskal–Wallis ANOVA was performed, which shows significant differences in median values (t₀/₂: p < 0.001) at 200 × 10⁻⁶ M, with the ranking shown in the inserted table of Figure 6B. Cy-3-glc and Cy-3-sam both showed an apparent trend to a dose dependent reduction in fluorescence and thus in H₂O₂-induced ROS, yet with statistical...
Figure 6. Effect of A) Cy-3-glc and B) Cy-3-sam on H₂O₂-induced ROS production in C₂BBe1 cells after pre-incubated for 24 h. Data plotted as mean ± SD of test over control (T/C) in %. Cells were stressed for 1 h with 1 mM H₂O₂ while fluorescence intensity was quantified with a Cytation3 plate reader. The mean of fluorescence change from t₀ was calculated in at least triplicates from at least five independent experiments and is presented as mean ± SD normalized to DMSO solvent control (T/C in %). DMSO = acidified 0.8% DMSO with 1 mM H₂O₂ in colorless medium. Non-normalized data outliers were eliminated with Nalimov. Statistical analysis with non-normalized data is used 1) for (A) and 2) for (B). 1) Significant differences to DMSO solvent control were calculated with one-way ANOVA (Bonferroni) (α = 0.05: *) and Student’s t-test (α = 0.001: ###). Kolmogorov–Smirnov test for normal distribution and Brown–Forsythe test for variance homogeneity was performed. 2) Significant differences to solvent control as calculated with Kruskal–Wallis ANOVA (α = 0.05: *), Mann–Whitney U test (α = 0.001: ###). Variance homogeneity was calculated with the Brown–Forsythe test and found significant different, hence non-parametric tests for significance were chosen. Inserted table presents mean ranking of Kruskal–Wallis ANOVA for Cy-3-sam.

Figure 7. Effect of Cy, Cy-3-glc, Cy-3-sam and Cy-3-sam-5-glc on H₂O₂-induced ROS-levels in C₂BBe1 cells after pre-incubated for 24 h. Data plotted as mean ± SD of test over control (T/C) in %. Cells were stressed for 1 h with 1 mM H₂O₂ while fluorescence intensity is quantified with a Cytation3 plate reader. Mean and SD of fluorescence change from t₀ was calculated in at least triplicates from at least five independent experiments and was normalized to DMSO solvent control (T/C in %). DMSO = acidified 0.8% DMSO with 1 mM H₂O₂ in colorless medium. Non-normalized data outliers were eliminated with Nalimov. Data is plotted at t₁ = 60 min. ANOVA and post hoc Bonferroni (or *) or Kruskal Wallis ANOVA was performed for every concentration level with normalized data. Significantly different change of fluorescence between the substances (α = 0.05) noted with a) Cy, b) Cy-3-glc, c) Cy-3-sam, d) Cy-3-sam-5-glc. Significant differences to solvent control was calculated with ANOVA (Bonferroni) (α = 0.05: *; 0.01: **, 0.001: ***) and Student’s t-test (α = 0.001: ###) for each substance separately. Significance only at the highest concentration of 200 × 10⁻⁶ M (T/C at t₆₀ of Cy-3-glc: 61 ± 11%; Cy-3-sam: 62 ± 17%).

Since the aglycon Cy is reported to have low stability in cell culture media while the glycosides remain stable with a degradation of 57% (Cy-3-glc) compared with 96% for Cy after 4 h incubation[33,34] this may, together with a potential uptake via glucose transporters,[35] be an explanation for the lacking protective effect of Cy and the notable effect of the glycosides. One-way ANOVA and Bonferroni post hoc test for comparison between anthocyanins and the aglycon at each separate concentration with normalized data show that Cy-3-sam lowered the fluorescence intensity induced by H₂O₂ significantly better than Cy, first at 1 × 10⁻⁶ M (p < 0.05) and at 200 × 10⁻⁶ M (p < 0.001) (Figure 7). Cy-3-glc lowered the fluorescence significantly better than Cy at 100 × 10⁻⁶ M (p < 0.05) and 200 × 10⁻⁶ M (p < 0.001). The two mono-glucosides, Cy-3-glc and Cy-3-sam, seem rather similar in their protective effect against H₂O₂-induced ROS. Notably, the AnthoKit mix in Cy-3-sam equivalent concentrations displayed no protective effect. This might point to a potential weaker protective activity due to combinatorial effects.

Our results regarding the protective effect of Cy contradict the results of Cvoric et al.[24] who found a dose-dependent inhibition of fluorescence increase while incubating Cy with...
Caco-2 cells and stressing with the hydroxyl radical-generator 2,2′-azobis(2-aminopropane). It should be noted that both delphinidin and Cy were even inducing the formation of free radicals in LoVo/ADR cells, although the incubation time was only 1 h. A slightly longer incubation (3 h) with Cy-3-glc in Caco-2 cells on the other hand showed a significant protection against 2,2′-azobis(2-aminopropane) dihydrochloride-induced ROS-formation. The results from non-tumorigenic colonic NCM460 cells with elderberry extract however are in line with the present results, as an inhibition of fluorescence intensity of 13%, 20%, and 40% at 0.01, 0.1, and 1 mg mL$^{-1}$ respectively was apparent. Although, it has to be considered that other compounds with potential antioxidant activity are present in the fruit extract as well. So far to our knowledge no experiments have been performed regarding protective effects of elderberry anthocyanins in C2BBe1 colon cells.

Despite many in vitro studies regarding the chemical antioxidant activity of the most common anthocyanins and of anthocyanin-rich extracts, the relevance in vivo is often questioned, since most test systems do not consider uptake, metabolism and bioavailability of the compounds. Nevertheless, cell culture experiments are of great value as model systems to screen for effects and mechanisms in a biological system where it is possible both to test high concentrations in order to screen for toxicity and to use biologically relevant concentrations that may in vivo reach the cell of interest. The concentrations we used in our study span both points of interest, as Esselen et al. calculated, that based on an ileostomy-study where up to 85% of ingested blueberry extract could reach the colon, 200 g blackberries (50 mg total anthocyanins 100 g$^{-1}$ FW) would supply comparable concentrations in colon cells as proven bioactive in their study (max. test concentration: 100 × 10$^{-6}$ M Cy and Cy-3-glc). Analogue, considering 134.94 mg Cy-3-sam 100 g$^{-1}$ fresh weight of elderberries, a concentration of 197.4 × 10$^{-6}$ M may be reached in the colon; both concentrations which showed a protective effect against ROS-production in the protective DCF assay with Cy-3-glc and Cy-3-sam. When calculated with the lower percentage of Cy-3-glc (28.3%) arriving in the stoma-bags it would still be 65.7 × 10$^{-6}$ M, which was yet potent in lowering the intracellular ROS-production. Considering intestinal degradation products of Cy-based anthocyanins, only PGA might be a good candidate for induction of protective mechanisms against H$_2$O$_2$-induced oxidative stress as has been hypothesized by Kropat et al. As the antioxidant properties of Cy-3-glc in vitro appeared to be not affected by this rather pro-oxidant effect of PGA, it might be speculated that PGA is not been formed under the respective cell culture conditions. Though Kay et al. detected PGA as a degradation product of Cy-3-glc in cell culture medium, thus letting assume that the concentrations of PGA might be too low to catch up the antioxidant effect of the anthocyanin.

The pure chemical antioxidant activity of anthocyanidins often has a higher ORAC-value than their respective glycosides, which is attributed to the reactivity of the aglycon, while for Cy and Cy-3-glc this was however the opposite case. Our data regarding the impact on cellular ROS levels show a similar picture for Cy and Cy-3-glc. Generally, glycosylation is considered to increase the stability of anthocyanidins potentially leading to different kinetic behavior and delayed or no biological effects, as has been observed with some of the tested Cy-based anthocyanins in our study. On the level of chemical structure the addition of a third sugar moiety may reduce the antioxidant impact, as shown for cyanidin 3-xylosyl-galactoside and cyanidin 3-xylosyl-glucoside-galactoside and here for Cy-3-sam-5-glc.

An uptake study with elderberry anthocyanins in aortic endothelial cells reported all four anthocyanins (Cy-3-glc, Cy 3,5-O-di-glucoside, Cy-3-sam, and Cy-3-sam-5-glc) to be found in the cell membrane, while only Cy-3-glc and Cy-3-sam were detected in the cytosol. Significantly more Cy-3-glc than Cy-3-sam was detected in the cytosol, which the authors attribute to the more complex structure of Cy-3-sam and that it may be degraded to Cy-3-glc in the cell. A lack of protection by Cy-3-sam-5-glc may thus be attributed to its poor cellular uptake due to the complex glycoside moiety, although uptake studies in C2BBe1 cells would be needed for confirmation. However, Cy-3-sam-5-glc displayed antioxidant properties by reducing ROS production in C2BBe1 cells after 1 h of incubation, albeit to a weaker extent, implying more likely potential degradation products being responsible for this effect. Conversely, Cy-3-rut bearing the disaccharide formed by glucose and rhamnose acted not protective against oxidative stress and displayed weaker antioxidant properties, thus suggesting that the structure of substituted sugar may be as well important for bioactivity.

2.8. Impact on Transcription of Protective Key Enzymes of Oxidative Stress

To investigate a potential role of Nrf2 in the defense against H$_2$O$_2$-induced ROS production, we performed transcription analysis of HO-1, γGCL, CAT, SOD, and GPx after 2, 6, and 24 h of incubation with Cy-3-glc and Cy-3-sam up to 200 × 10$^{-6}$ M. The results revealed no inducing effects by both anthocyanins on the transcription of tested genes at any time point (Figure S8, Supporting Information). The results in C2BBe1 cells are not in line with data on Cy-3-glc activity in other cell lines. For instance, Speciale et al. report the activation of the Nrf2/ARE pathway in endothelial cells and Sukprasansap et al. show the induction of CAT, SOD, and GPx in neuronal cells, however at much lower Cy-3-glc concentrations as used in the present study. PCA was described to intensify the antioxidant capacity of cells potentially by increasing the activity of antioxidant enzymes like CAT in hypertensive rats, and GPx in macrophages.

Albeit a potential involvement of Nrf2 in the protective effects of Cy-3-glc and Cy-3-sam in C2BBe1 cells could not be corroborated by qRT-PCR results, considering the pro-oxidant effect of the microbial degradation product PGA and the induction of Nrf2-regulated genes in HT-29 cells a potential role of Nrf2 in protection against oxidative stress is conceivable in the gut. Nevertheless, PGA was ineffective in C2BBe1 cells with respect to protection against H$_2$O$_2$-induced stress, suggesting either only a short time pro-oxidant effect of PGA or in case of Cy-3-glc and Cy-3-sam involvement of other protective mechanisms in C2BBe1 cells than modulation of Nrf2-signaling. Furthermore, it cannot be excluded, that other Nrf2-responsive genes play a role for the observed effect on the ROS production. A broad spectrum of genes has already been described to possess an antioxidant response element in their promoter region, thus being potentially responsive to Nrf2-activation. Also, the possibility of pathway
The hypothesized role of Nrf2 in the protection against oxidative stress could not be corroborated by transcription analysis of the selected genes, suggesting potentially other alternative defense mechanisms.

4. Experimental Section

**Chemicals:** Freshly squeezed elderberry juice was obtained from Bayernald Frechheitsverwertung KG (Hengersberg, Germany) and stored at −80 °C until further use. Cyanidin chloride ≥98% was bought from PlantMetaChem (Gießen, Germany). Sulforhodamine B sodium salt (SRB), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) ≥98%, catalase from bovine liver (3356 U mg⁻¹), PGA (2,4,6-trihydroxybenzaldehyde), and PCA, purity ≥97% were purchased from Sigma-Aldrich (München, Germany). Cy-3-glc was isolated from blackberry juice. Structure and purity (>95%) were analyzed by LC-UV/MS using methodologies described in [31]. Cy-3-rut was from inhouse stocks (IPK-Gatersleben) with a purity of 66.3% based on Q-TOF MS analysis (Figure S1, Supporting Information).

**Anthocyanin Isolation and Preparation of Elderberry AnthoKit:** Insoluble material was removed from 100 mL elderberry juice, with an anthocyanin content of 1.19 μg mL⁻¹, by centrifugation for 30 min at 13000 x g. The supernatant was sequentially filtered through 100 and 20 μm nylon net filters. Degreasing was realized by thoroughly mixing the extract with two volumes (v/v) of n-heptane. The lower phase (degreased extract) was carefully collected after phase separation. The remaining organic solvent was removed under vacuum by means of a rotation evaporator and then the degreased extract was adjusted to a final concentration of 2% methanol and 0.5% formic acid (FA). Prior to chromatography the degreased extract was sequentially filtered down to 0.2 μm through nylon net filters. This sample was named raw juice and was analyzed by LC-UV/MS and LC-MS/MS.

For anthocyanin isolation the degreased extract, containing a maximum of 100 mg total anthocyanins, was first loaded on a solid phase extraction column (RediSep RF Gold C18aq column, 15.5 g, 20–40 μm, Teledyne Isco, Lincoln, NE, USA) equilibrated with 2% methanol, 0.5% FA. Compound elution was realized by step gradients at 2%, 10%, 25%, 40%, and 98% of methanol with 0.5% FA using 7.4 column volumes each, at a flow rate of about 20 mL min⁻¹ utilizing a Chromabond vacuum chamber (Machery-Nagel, Düren, Germany). Resulting fractions were analyzed by LC-UV/MS. Fractions containing anthocyanins were then used for further anthocyanin isolation; those were the two crude fractions from elution with 10% and 25% methanol, 0.5% FA. Other fractions containing polyphenols but no anthocyanins were combined and named polyphenols fraction. The volume and the content of organic solvent of these fractions were reduced under vacuum by means of a rotation evaporator.

For final purification the crude isolated fractions were loaded on a preparative HPLC column (Gemini 5 µm NX-C18 110 A, LC Column 250×30 mm, AXIA Packed, Phenomenex, Aschaffenburg, Germany) equipped with a pre-column (SecurityGuard PREP Cartridge Gemini-NX C18 15×30mm) and equilibrated with water + 2% of 5% ammonium formate in FA. Compound elution was realized by a linear gradient from 2% to 30% acetonitrile within 170 min at a flow rate of 10 mL min⁻¹ utilizing a Varian ProStar chromatography instrument (Agilent Technologies, Santa Clara, CA, USA) with UV monitoring at the wavelengths of 280 and 515 nm. Resulting fractions, showing anthocyanin specific absorption at 515 nm, were analyzed by LC-UV/MS and properly combined. Finally, two anthocyanin fractions enriched in the main elderberry anthocyanins Cy-3-sam-5-glc (further named anthocyanin 1) and Cy-3-sam (further named anthocyanin 2) were obtained. Again, other fractions containing polyphenols but no anthocyanins were combined and added to the polyphenols fraction. The volume and the content of organic solvent of the final pure isolate fractions were reduced under vacuum.

A final purification step was performed to remove remaining low molecular weight contaminations (e.g., salt and sugar). Each fraction (anthocyanin 1, anthocyanin 2, and polyphenols) was adjusted to a final concentration of 2% methanol, 0.5% FA. In addition, 10 mL of the original elderberry juice were mixed with 10 mL of 2% methanol, 0.5% FA, and then cleared by various filtration steps on nylon membranes down to...
Each fraction (anthocyanin 1, anthocyanin 2 and polyphenols, and the cleared elderberry juice) was loaded on a separate solid phase extraction cartridge (Chromabond C18, 70 mL, 10 g, Macherey-Nagel), which was conditioned with two column volumes of 2% methanol, 0.5% FA. The flow through was observed to be clear and the anthocyanins bound to the C18 column matrix. Remaining sugars and salts were washed off with two column volumes of 2% methanol, 0.5% FA. Elution of the bound anthocyanins and polyphenols was performed with 98% methanol, 0.5% FA. Aliquots were taken from all purification steps and analyzed for anthocyanin and sugar content by plate photometric assays. For preparation of the AnthoKit mix the individual purified anthocyanin fractions based on their natural composition in the elderberry juice; namely 12% of Cy-3-sam-5-gluc (anthocyanin 1) and 88% of Cy-3-sam (anthocyanin 2) was mixed. All resulting fractions were dried under nitrogen stream. The obtained dry powder was covered with argon and stored at −20 °C until further use.

**Anthocyanin Quantification by Spectral Photometric Detection:** Total anthocyanin content was quantified by means of the pH differential method described by Giusti and Wrolstad. In brief, the absorbance of pH 1.0 (25 mM KCl) and pH 4.5 (0.4 M Na-acetate) buffer-diluted samples was measured at 515 and 700 nm with a SPEKTRAMAX Pro UV-visible spectrophotometer ( Molecular devices, Sunnyvale, CA, USA). Measurements were done in duplicates. The differential absorbance was calculated using the following equation: A = (A515 - A700) pH 1.0 – (A515 - A700) pH 4.5. The total anthocyanin content was then calculated based on reference curves for Cy-3-gluc in the range from 0.001 to 0.050 mg mL$^{-1}$.

**Methods for LC-UV/MS and LC/MS/MS Analysis of Anthocyanin Fractions:** Anthocyanins were analyzed by LC-UV/MS as described in Oerter et al. Details for LC-UV/MS and LC/MS/MS fragmentation analyses are provided in Supporting information. LC-UV/MS data were analyzed by means ofBruker Compass DataAnalysis version 4.1 software. The annotation of compounds was based on comparison of the measured retention times and molecular ion masses with reference standards and fragmentation patterns as obtained from LC-MS/MS experiments (Supporting information, more details).

**Determination of Glucose, Fructose, and Sucrose:** Determination of soluble sugars was performed as described in Stitt et al. by sequential enzymatic degradation of glucose, fructose, and sucrose; using one unit each of glucose-6-phosphate dehydrogenase (Hoffmann-La Roche, Basel, Switzerland) for baseline generation, and then hexokinase, phosphoglucoisomerase, and invertase (β-fructofuranosidase, all from Sigma-Aldrich), respectively. Quantities were calculated based on reference curves for authentic standards in the range from 0.1 to 1.0 mM.

**Cell Culture and Treatment:** The clone of Caco-2, C2BBE1 (ATCC CRL-2102), was purchased from LGC Standards GmbH (Wesel, Germany). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) GlutaMAX with 4.5 g L$^{-1}$ glucose and sodium pyruvate (Fisher Scientific, Vienna, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% v/v penicillin (5 000 units mL$^{-1}$)/streptomycin (5 000 μg mL$^{-1}$) (P/S) at 37 °C, 5% CO$_2$, and 95% humidity. Cells were tested regularly for mycoplasma contamination.

Test compounds were dissolved in acidified 80% dimethyl-sulfoxide (DMSO) and added to the incubation media, resulting in a final concentration of 0.8% (v/v) DMSO. Previously, anthocyanins were found to generate H$_2$O$_2$ in cell culture medium, accelerating the chemical degradation of the compounds and possibly causing oxidative cell damage. To prevent these effects, catalase (100 units mL$^{-1}$) was added to the culture medium immediately before incubation with test compounds. C2BBE1 cells were used in a non-differentiated state for all experiments and cultivated for 24 h prior incubation with compounds.

**Cytotoxicity Assay:** The sulforhodamine B assay (SRB assay) was performed according to a modified method of Skhean et al. The assay measures the optical density (absorbance) of cellular protein stained with the dye sulforhodamine B, showing a high linear correlation between protein content and optical density. Briefly, 15000 cells per well were seeded in 96-well plates and allowed to attach for 24 h. Cells were incubated with solvent control (0.8% acidified DMSO) and respective concentrations of Cy-based anthocyanins or Cy, PCA, and PGA in the presence of catalase (100 units mL$^{-1}$) for 24 h in the dark. After incubation, cells were fixed by addition of trichloroacetic acid to the incubation medium and subsequently stained with SRB solution (0.4% w/v in 1% acetic acid). After washing with water and 1% v/v acetic acid, the color was eluted with Tris buffer (10 mM, pH 10). Absorbance was measured at 570 nm with the Cytation 3 microplate reader (BioTek Instruments, Winooski, VT, USA). Effects on cell growth were referred to the solvent control and plotted as test over control (T/C) in percent (%) from at least five biological replicates. H$_2$O$_2$ (1 mM) was used as positive control.

**Dichlorofluorescein (DCF) Assay:** The formation of ROS was quantified fluorometrically by using DCFH-DA following the method described by Wang and Joseph. Briefly, 15000 cells well$^{-1}$ were seeded in 96-well plates and allowed to attach for 24 h. Cells were incubated with DCFH-DA for 20 min and washed two times with PBS prior incubation with solvent control (0.8% acidified DMSO) and respective concentrations of anthocyanins or Cy, PCA, and PGA in the presence of catalase (100 units mL$^{-1}$) for 3 h. After incubation at $\lambda = 485$ nm DCF emits light at $\lambda = 528$ nm, which was detected with a Cytation 3 microplate reader (BioTek Instruments, Winooski, VT, USA). The emitted light directly reflects the ROS production in the cells. Fluorescence was detected every 15 min over a period of 1 h. Data analysis was performed according to Wang and Joseph. Data are plotted as test over control (T/C) in percent (%) from at least five biological replicates.

**Protective Dichlorofluorescein (pDCF) Assay:** A modified version of the DCF assay was used to assess the ability of anthocyanins to strengthen the antioxidant defense of cells and thus protection from H$_2$O$_2$-induced ROS production. Cells were incubated with anthocyanins or respective degradation products for 24 h prior to oxidative stress induction by 1 mM H$_2$O$_2$ for up to 1 h. ROS levels were determined as described above with the DCF assay. In case of protective effects less DCFH-DA is oxidized to fluorescent DCF, hence showing a lower signal than solvent control containing the stressor. To show protective effects more clearly, the fluorescence of H$_2$O$_2$-treated cells is set to 100% and the fluorescence of anthocyanin pre-treated cells is plotted as test versus control (T/C) in percent (%) from at least five biological replicates.

**Quantitative Real-time PCR (qRT-PCR):** For transcription analysis of the antioxidant enzymes HO-1, γGCL, CAT, GPX, and SOD in C2BBE1 cells qRT-PCR was applied.

Total RNA was extracted using the RNeasy Mini Prep Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer’s protocol. RNA purity and quantity were determined with the NanoDrop 2000 (Thermo Fisher Scientific, Vienna, Austria). According to the manufacturer’s protocol total RNA was reverse transcribed into complementary DNA (cDNA) using the Quantitect reverse transcription Kit (Qiagen). Quantitect primer assays (Qiagen): Hs_GCLC_1_SG, QT00200039; Hs_SOD1_1_SG, QT01008651 and Quantitect SybrGreen master mix (Qiagen) were used for gene-specific amplification with the StepOne Plus PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). The amplification protocol consisted of an initial activation step of the Taq polymerase for 15 min at 95 °C, 40 cycles comprising denaturation: 15 s, 94 °C, annealing: 30 s, 55 °C, and extension: 30 s, 70 °C followed by a melting curve analysis. Relative gene transcript levels were calculated by applying the ΔΔCT-method as amplification efficiency was comparable. Ct-values of the target genes were normalized to the Ct-values of the control gene β-actin and then compared to the calibrator (respective solvent control sample). Data are presented as the mean of relative transcription (RQ) ± SD of at least three biological replicates.

**Dosage Information/Dosage Regimen:** C2BBE1 cells were incubated with 0.1, 1, 10, 50, 100 and 200 μM anthocyanins or Cy, PCA, and PGA for 1 h (DCF assay) and 24 h (protective DCF assay, SRB assay) in the presence of catalase (100 units mL$^{-1}$) and 0.8% DMSO (final concentration). Effects of anthocyanins have been reported in vitro within this concentration range and timeframe. For qRT-PCR experiments only anthocyanins and concentrations were tested that yielded...
protective effects against H$_2$O$_2$-induced stress. Cells were incubated for 2, 6 and 24 h to catch potential short- as well as long-term effects on the transcriptional level.

Following the low bioavailability of anthocyanins, the use of even lower concentrations might have been justified. However, the situation in the colon is different. Even concentrations beyond 100 $\times$ 10$^{-6}$ M might be reached in the colon due to locally higher bolus-like distribution [10, 39]. The applied concentrations cover approximately a physiological range that can be reached by an anthocyanin-rich meal as well as high concentrations (200 $\times$ 10$^{-6}$ M), reflecting a pharmaceutical rather than a nutritional dosage. Yet, they might be accomplished by the intake of anthocyanin-rich extracts or supplements. The growth of C2BBe1 cells was not impaired by test compounds up to 200 $\times$ 10$^{-6}$ M after 24 h incubation.

**Statistical Analysis:** Statistical tests such as the analysis of variance (ANOVA) and the Student’s t-test assume a normal distribution of data, verified with the Kolmogorov–Smirnov test. Significances were analyzed by one-way ANOVA when normality was confirmed and Brown–Forsythe test was chosen to control for variance of homogeneity. One-way ANOVA was applied to solvent control and test-groups with the addition of the Bonferroni post hoc test for multiple comparison of the means of the groups, which controls the overall type I error. If the variances were not homogeneously distributed the non-parametrical test of Kruskal–Wallis ANOVA was performed, which is a method that tests for differences of the samples’ medians instead of means.

Each experiment had either a positive (1 $\times$ 10$^{-6}$ M H$_2$O$_2$) or a negative control (no stressor) to determine the success of the assay. When normal distribution and variance-homogeneity was assured Student’s two-sample t-test for independent groups was performed between positive/negative control and its respective control. The non-parametrical Mann–Whitney U test was performed to observe a significant difference between the two distributions on the basis of ranked data when samples were either not normally distributed or variances of homogeneity were significantly different.

Outliers were eliminated from raw data of experiments with more than five replicates by using the Nalimov test. Statistical analysis was performed with OriginPro 9.1 (OriginLab Corporation, Northampton, USA).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

Conceptualization, A.M., D.M., HP.M.; methodology, K.A., A.O., T.J-S., S.G., G.P.; software, K.A., A.O., S.G., G.P.; validation and formal analysis, K.A., A.O., S.G., T.J-S., G.P.; resources, A.M.; HP.M. and D.M.; data curation, A.M., D.M.; writing - original draft preparation, G.P.; writing - review and editing, A.M., D.M., HP.M., A.O., K.A., T.J-S., G.P.; visualization, A.M., A.O., K.A., S.G., G.P.; supervision, A.M.; HP.M. and D.M.; project administration, A.M., D.M.; funding acquisition, A.M., D.M., HP.M. All authors have read and agreed to the published version of the manuscript.

**Data Availability Statement**

Data available on request from the authors.

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