Neuroprotective effects of Anthocyanins with anti-Prion activity

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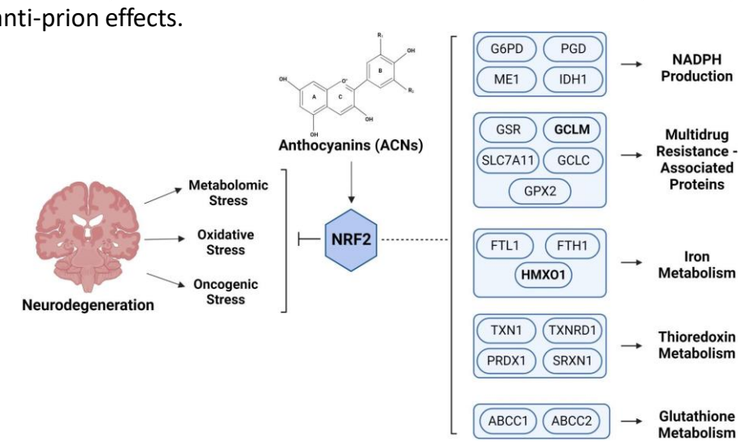
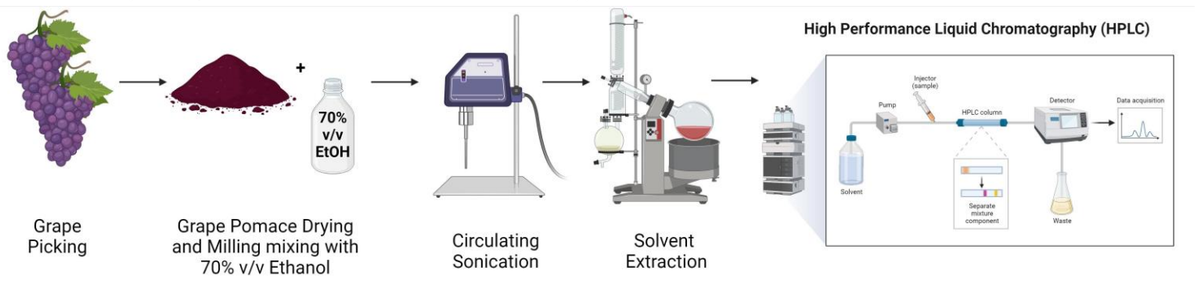
Introduction

Prion diseases refer to a group of rare, progressive neurodegenerative disorders (NDs), that affect both humans and animals. The key event in pathogenesis is a translational process, during which a conformational change of the normal structural prion protein, PrP^{C}, to the disease-associated pathogenic isoform, PrP^{Sc}, takes place. Oxidative stress has been found to be associated with the onset or worsening of neurodegenerative diseases. Anthocyanins (ACNs) are polyphenolic chemicals, and function as secondary plant metabolites. They are characterized as water-soluble bioactive compounds and have the ability to act as inhibitors of oxidative stress, by promoting the nuclear translocation of nuclear factor erythroid 2 (Nrf2)-related factor 2 or by stimulating the activity of antioxidant enzymes, such as SOD, CAT and GPx. Therefore, they can exploit as antioxidants and were tested for their anti-prion effects.



Materials and Methods

Following the principles of circular economy, the exploitation of vinification byproducts is targeted for the extraction of natural bioactive compounds with antioxidant and neuroprotective properties. An ACN extraction protocol from winery byproducts, using 70% v/v Ethanol followed by sonication and ACN concentration analysis and HPLC for characterization has established. Our protocol is based only on Generally Recognized as Safe (GRAS) material, which is ready for consumption. Combining HPLC and UV spectroscopy of such extracts from vinification residues compared to pure ACNs of interest indicate the high abundance of these two compounds (Oenin and Myrtillin) in the extracts from vinification byproducts.



Results

High abundance of Oenin and Myrtillin in the extracts from vinification byproducts

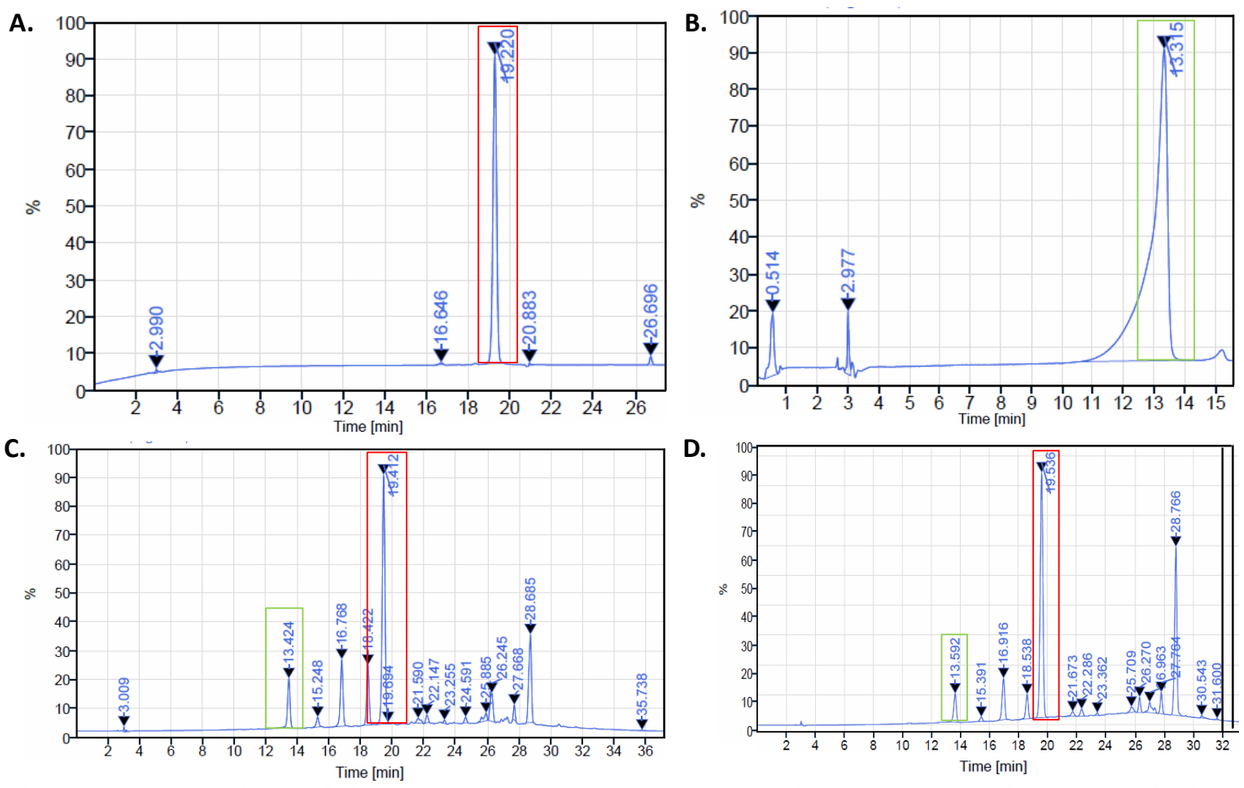


Figure 1. Representative HPLC Chromatographs acquired from the analysis of **A.** Reference Sample of Oenin, **B.** Reference Sample of Myrtillin, **C.** Extracts from winery byproducts of Variety Mavrotragano, **D.** Extracts from winery byproducts of Variety Merlot. Combining HPLC and UV spectroscopy of such extracts from vinification residues compared to pure Anthocyanins of interest indicate the high abundance of Oenin and Myrtillin in the extracts from vinification byproducts.

In RT-QuIC assays Anthocyanins prevented the conversion of recPrP^{C} to PrP^{Sc}

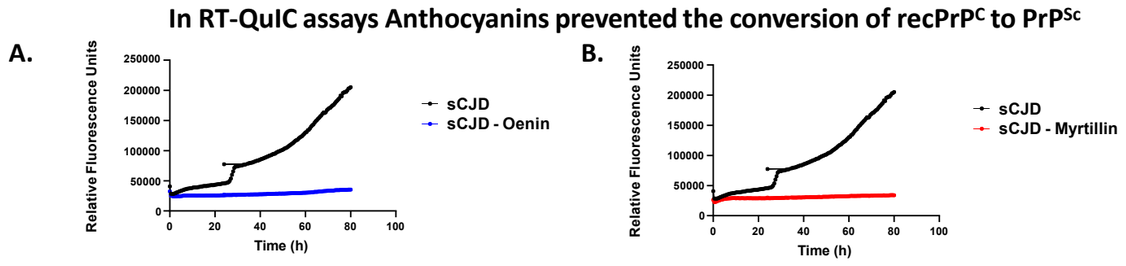


Figure 2. RT-QuIC assays. Aggregation of recPrP^{C} in RT-QuIC assays using CSF from three different sCJD patients as a seed was evaluated in the presence or absence of **A.** Oenin and **B.** Myrtillin (5 μM final concentration). Anthocyanins were added to the reactions from the beginning, and the Th-T fluorescence was recorded every 30 min.

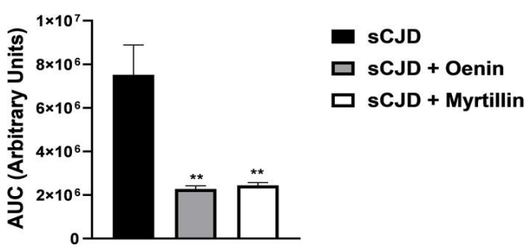
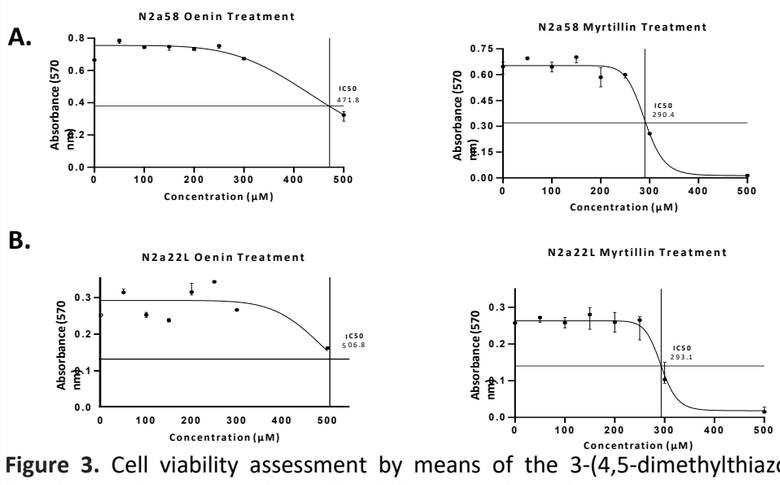


Figure 2C. Quantification of Oenin and Myrtillin effects on PrP conversion and aggregation inhibition. Bar graphs represent the mean ± SD of the area under the curve (AUC) calculated for the individual fluorescence curves of each replicate reaction. AUC values were used as a measure of protein conversion and aggregation Stars indicate statistical significance (unpaired, one-tailed, T-test). **: p value < 0.01, ***: p value < 0.001.

Toxicity levels of Myrtillin is particularly higher than Oenin



Oenin:
- N2a58 IC_{50}: 471.80μM
- N2a22L IC_{50}: 506.80μM

Myrtillin:
- N2a58 IC_{50}: 290.4μM
- N2a22L IC_{50}: 293.1μM

Figure 3. Cell viability assessment by means of the 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for Oenin and Myrtillin, following 48 h incubation in **A.** N2a58 and **B.** N2a22L cell lines.
Graphs depict the absorbance at 570 nm determined for each cell line following treatment with the indicated compounds at different concentrations. The background absorbance of the plates at 630 nm was also measured and subtracted from 570 nm measurement. LD_{50} was estimated for each compound and cell line based on non-linear regression analysis for curve fitting using the GraphPad software (v 8.0). The black dots in graphs for Anthocyanins represent the fitted curve in each case; determined LD_{50} values are depicted in each graph.

Production of ROS was significantly decreased in the presence of Anthocyanins

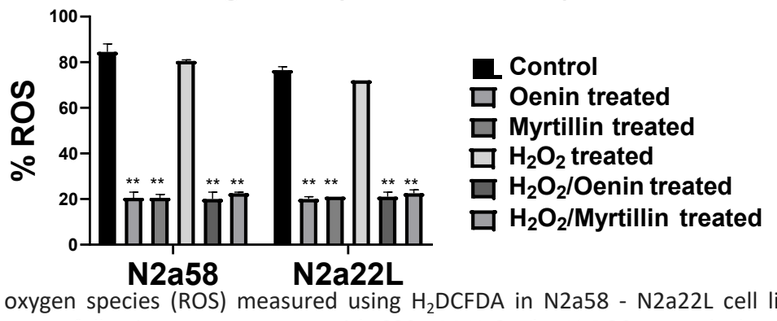


Figure 4. Reactive oxygen species (ROS) measured using H_2DCFDA in N2a58 - N2a22L cell lines after treatment with Anthocyanins, without or following pre-treatment with 600 μM H_2O_2.The % ROS was calculated based on the maximum ROS production value (3 mM). Data represent means ± SD; stars denote statistical significance (unpaired, one-tailed, *T*-test); *: *p*-value < 0.05, **: *p*-value < 0.01, ***: *p*-value < 0.001.

Treatment upregulates Keap1-Nrf2 pathway

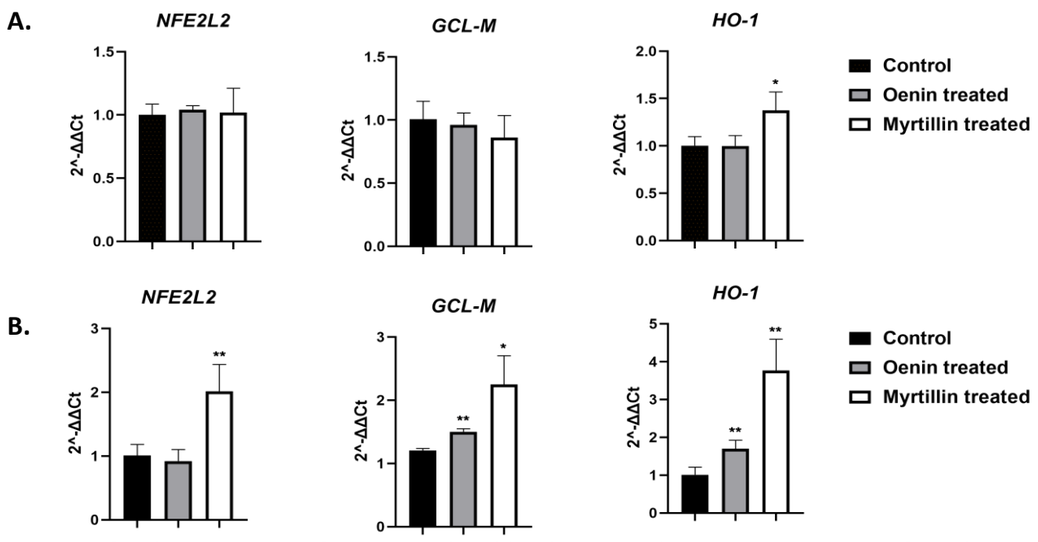


Figure 5. qPCR for the expression of genes regulated by the antioxidant response Keap1-Nrf2 pathway was performed using mRNA extracted from **A.** N2a58 and **B.** N2a22L cell lines with Anthocyanins for 48 h and compared to non-treated control cells (Cntrl). Data represent means ± SD of three independent experiments. Stars denote statistical significance (unpaired, one-tailed, *T*-test); *: *p*-value < 0.05, **: *p*-value <0.01, ***: *p*-value < 0.001, ****: *p*-value < 0.0001.

Western-blot of Anthocyanins treated N2a22L cells validated the prevention of PrP^{Sc} formation

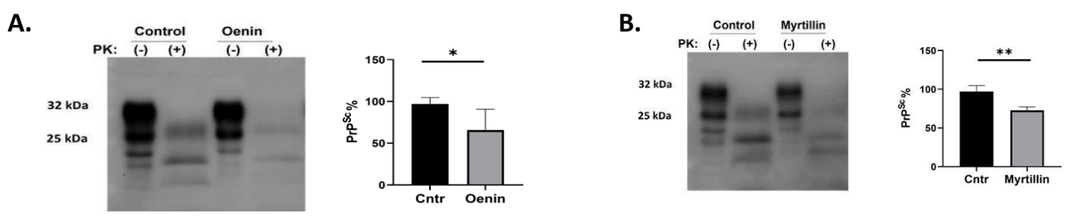


Figure 6. Effect of **A.** Oenin and **B.** Myrtillin treatment on PrP^{Sc} levels. N2a22L cells were incubated for 48 h or left untreated (Control), lysed, and then divided into two different fractions. Fraction lysates were either treated (+) or not (-) with PK, and 50μg of PK-untreated or 150μg of PK-treated fraction was resolved by SDS-PAGE. Densitometric analysis was performed, and bar graphs show the conversion rate of each ACN treated sample relative to the control conversion rate. Data represent means ± SD; stars denote statistical significance (unpaired, one-tailed, T-test); *: p value < 0.05, **: p value < 0.01.

Conclusions

The pleiotropic beneficiary effects of anthocyanins including inhibition of PrP aggregation potentiate their use against not only Prion diseases but also other neurodegenerative proteinopathies, suggesting that they could become preventative and/or therapeutic agents.

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