Anthocyanin-rich extracts derived from wine byproducts exert neuroprotective and anti-inflammatory properties

Nikoletta Christoudia [1], Eirini Kanata [2], Athanasia Chatziefsthathiou [1], Mathias Schmitz [3], Inga Zerr [3], Ioannis Tsamesidis [4], Konstantinos Xanthopoulos [2], Dimitra Dafou [1] and Theodoros Sklaviadis [2]

[1] Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece

[2] Neurodegenerative Diseases Research Group, Department of Pharmacy, School of Health Sciences, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece

[3] Department of Neurology, German Center for Neurodegenerative Diseases (DZNE), University Medicine Göttingen, 37075, Göttingen, Germany

[4] Department of Prosthodontics, School of Dentistry, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

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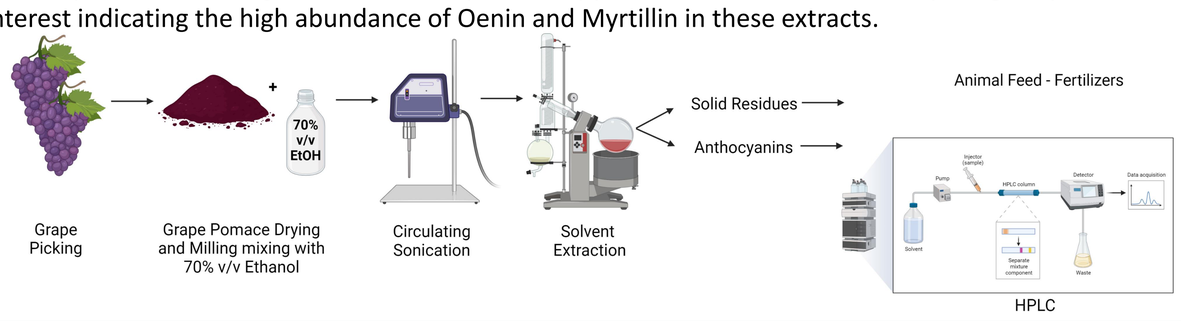
Introduction

Anthocyanins (ACNs) are water soluble vacuolar pigments found mostly in fruits, vegetables and in vegetative organs. They are glucosides of the anthocyanidins, flavonoid derivatives produced via the phenylpropanoid pathway. These compounds are safe, and can be easily supplemented through dietary intake on the brain and nervous system. ACNs possess antioxidant, anti-inflammatory and anti-apoptotic properties, which may protect neurons from oxidative stress, inflammation and finally cell death. This is particularly relevant in the context of Prion diseases, which are fatal neurodegenerative disorders, characterized by the conversion of the normal cellular prion protein (PrP^{C}), to its disease associated isoform (PrP^{Sc}). Currently, no treatments are available for these diseases.

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 different grape varieties (Mavrotragano, Syrah and Merlot), using 70% v/v Ethanol (EtOH) followed by sonication and ACN concentration analysis and HPLC for characterization has been established. Our protocol is based only on Generally Recognized as Safe (GRAS) material, which is ready for consumption. Combining HPLC and UV spectroscopy of these extracts and comparing to pure ACNs of interest indicating the high abundance of Oenin and Myrtillin in these extracts.



Results

Pure ACNs reduce Reactive Oxygen Species (ROS) Levels in neuronal cell lines

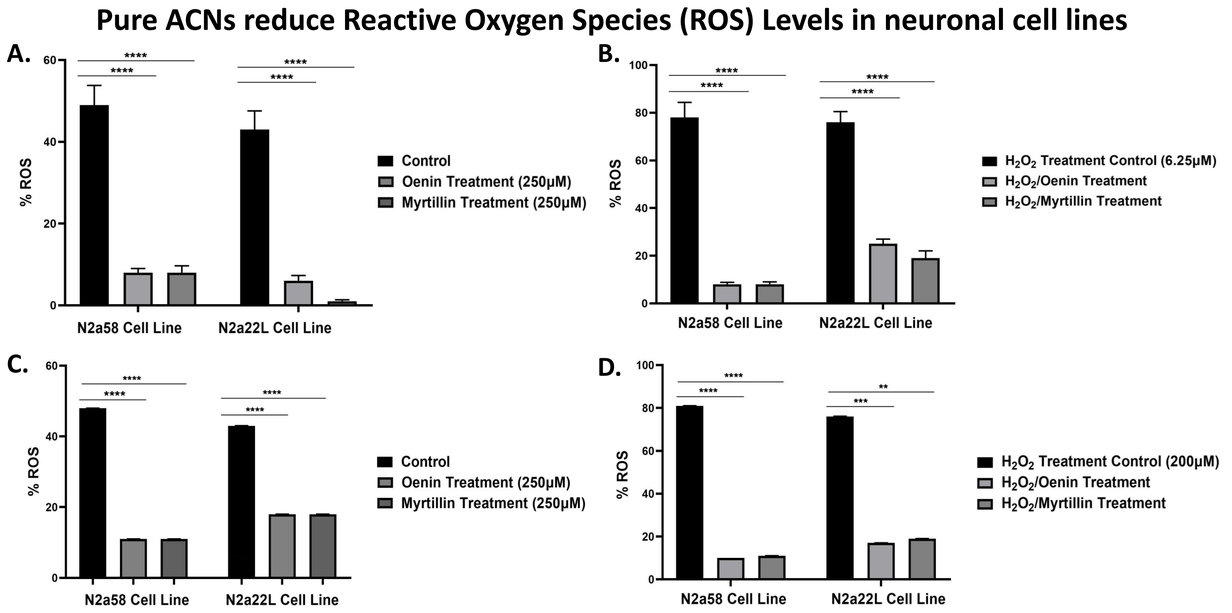


Figure 1. Reactive Oxygen Species (ROS) levels measured using H_2DCFDA in the murine neuroblastoma cell line, N2a58, and the scrapie infected cell line, N2a22L (ScN2a, prion disease model) after treatment with the indicated concentrations of Oenin and Myrtillin without (**A.** and **C.**) or following (**B.** and **D.**) pre-treatment with different concentrations of H_2O_2 (6.25 and 200 μM), to induce oxidative stress. Controls received DMSO at concentrations matching those delivered with the compounds. 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.

Pure ACNs inhibit *de novo* formation of PrP^{Sc} aggregates

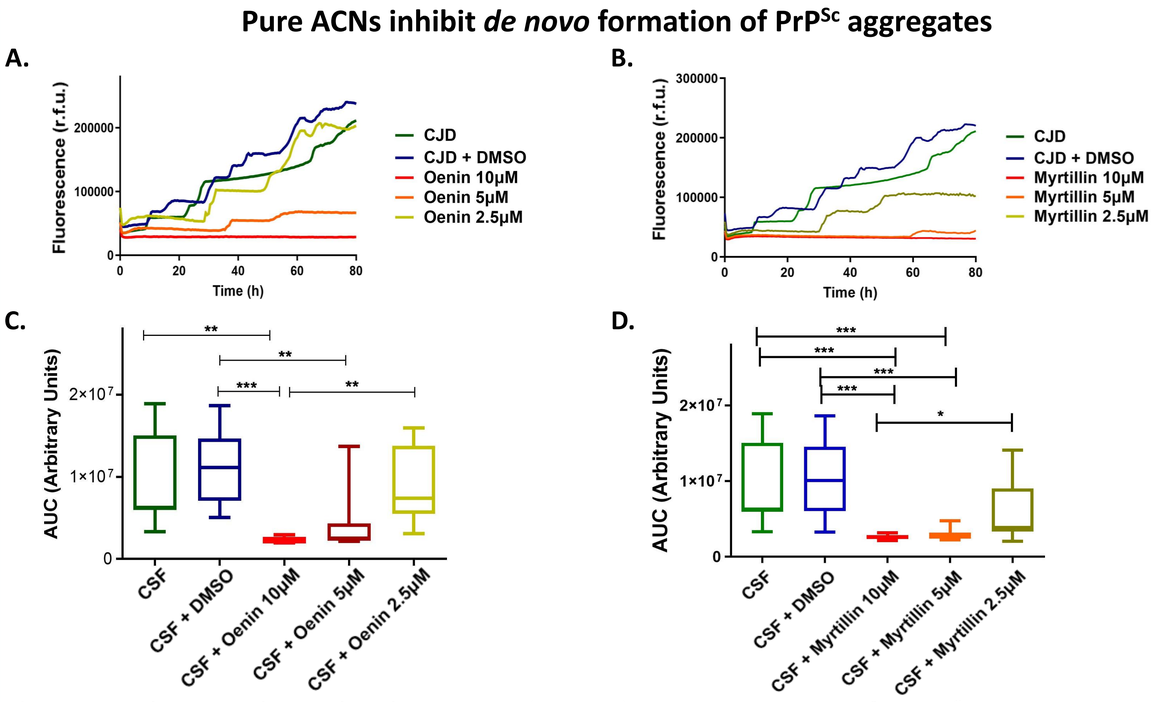


Figure 2. Real-time quaking-induced conversion (RT-QuIC) assays. Aggregation of $recPrP^{C}$ in RT-QuIC assays using Cerebrospinal Fluid (CSF) from twelve different Creutzfeldt–Jakob disease (CJD) patients, as a seed was evaluated in the presence of different concentrations of these two compounds. **A.** Oenin and **B.** Myrtillin were added in the reaction mixture in three different concentrations (2.5, 5 and 10 μM) from the beginning of the reactions, and the results were compared with that from CSF only and CSF with DMSO. Thioflavin-T (Th-T) fluorescence, as a measure of protein aggregation, was recorded every 30 min. Quantification of **C.** Oenin and **D.** Myrtillin effects on PrP conversion and aggregation inhibition. Box plots represent the Standard Error of Mean (SEM) of the Area Under 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.

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Co-funded by the European Union

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Winery byproducts extracts contain high levels of polyphenols

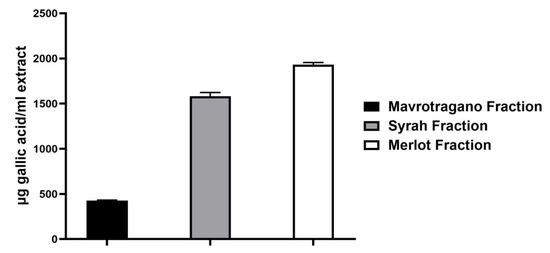

Figure 3. Measurement of Total Polyphenol Content (TPC) for Mavrotragano, Syrah and Merlot Fractions, respectively, using the Folin-Ciocalteu assay. Graphs depict the absorbance at 765 nm determined for each winery byproducts extract.

Winery byproducts extracts have antioxidant capacity

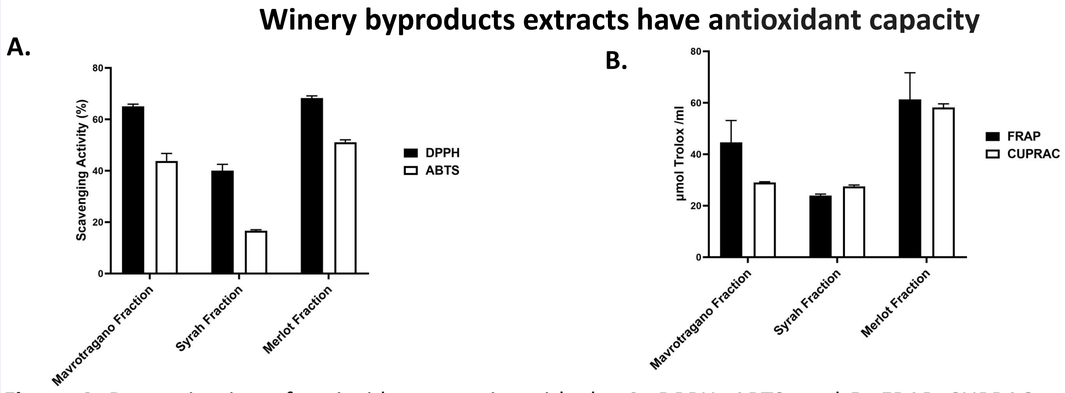

Figure 4. Determination of antioxidant capacity with the **A.** DPPH, ABTS, and **B.** FRAP, CUPRAC methods. **A.** The DPPH and ABTS methods determine the scavenging activity of the different winery byproducts extracts, in terms of DPPH and ABTS free radicals, respectively. The DPPH uses a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$). The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with a Trolox standard. **B.** The FRAP method measures the ferric reducing power of antioxidants, while the CUPRAC method is based on Cu^{2+} into Cu^{+} by the action of non-enzymatic antioxidants present in the sample.

Winery byproducts extracts are not toxic in neuronal and microglial cell lines

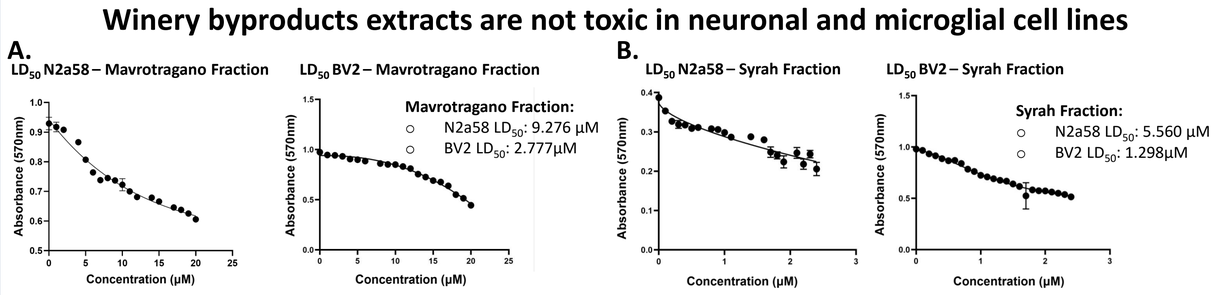

Figure 5. Cell viability assessment by means of the 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for **A.** Mavrotragano and **B.** Syrah Fraction following 48 h incubation in N2a58 and BV2 cells, respectively. Graphs depict the absorbance at 570 nm determined for each cell line following treatment with the indicated fractions. Concentrations refer to Oenin and Myrtillin levels determined for each fraction using HPLC. The background absorbance of the plates at 630 nm was also measured and subtracted from 570 nm measurement. LD_{50} was estimated for each fraction and cell line based on non-linear regression analysis for curve fitting using the GraphPad software (v 8.0.2). The black dots in graphs for different grape fractions represent the fitted curve in each case; determined LD_{50} values are depicted in each graph.

Winery byproducts extracts exert antioxidant properties, by upregulating *Keap1-Nrf2* pathway in neuronal and microglial cell lines

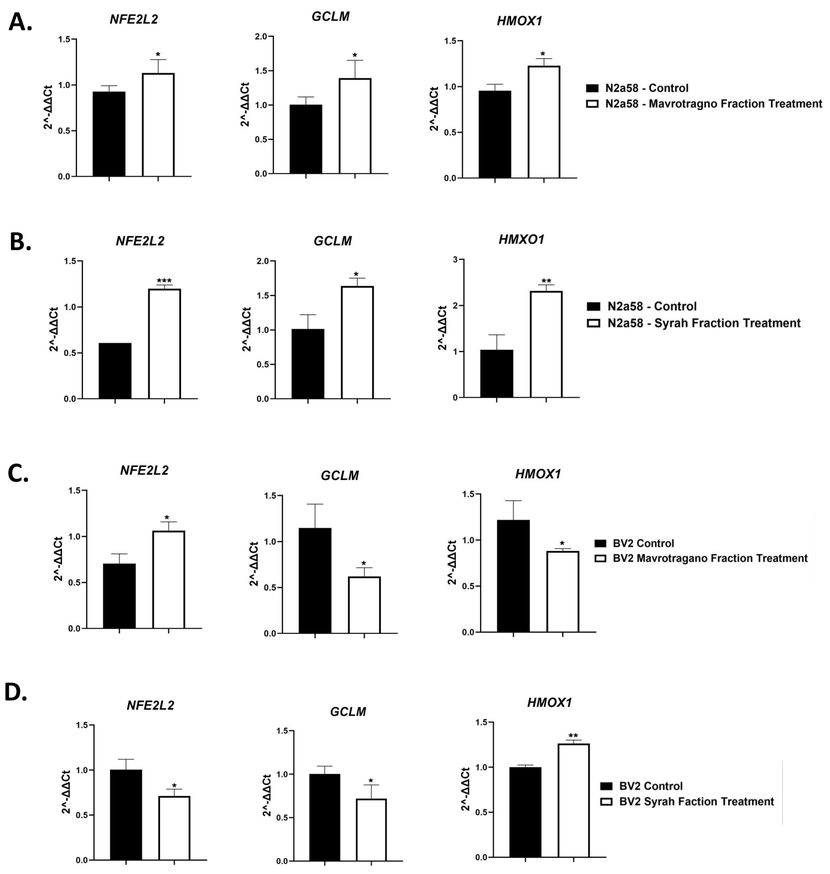

Figure 6. Expression of genes regulated by the antioxidant response *Keap1-Nrf2* pathway was performed using mRNA extracted from (**A.** and **B.**) N2a58 and (**C.** and **D.**) BV2 cells treated with 0.5 × LD_{50} of Mavrotragano and Syrah fractions for 48 h, relative to non-treated control cells (Cntrl). Control cells received EtOH at concentrations matching those delivered with grape fractions. 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.

Conclusions

- Winery byproducts have high abundance of pure ACNs.
- Winery byproducts exert antioxidant effects in neuronal and microglial cell lines.
- Further studies in *in vitro* and *in vivo* models of Prion diseases are in progress, to evaluate the potent beneficial effects of winery byproducts.