Title: Tannase enhances the anti-inflammatory effect of grape pomace in Caco-2 cells treated with IL-1 β

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Abstract

Grape pomace (GP) is rich in polymeric polyphenolics and glycosides which have lower bioefficacy than monomeric and aglycone counterparts. The aim of this study was to determine whether tannin acyl hydrolase [tannase (TNS)] can improve the antioxidant and anti-inflammatory actions of GP in Caco-2 cells treated with IL-1 β . TNS increased quercetin content by 45% and decreased quercetin-3-O-rutinoside by 71% as compared to untreated GP. Further, TNS increased total phenols and DPPH, ORAC, and FRAP values by 39, 57, 215, and 12%, respectively. However, GP and GPTNS at 100 and 200 μ g/mL (dry extract wt/v) displayed comparable efficacy in the reduction of ROS in Caco-2 cells. After 24 h pre-treatment, GPTNS (200 μ g/mL) decreased IL-1 β -induced PGE₂ and IL-8 secretion by 107 and 83% respectively, and down-regulated NF- κ B activation by 63%. Thus, TNS appears to enhance antioxidant and anti-inflammatory activities of GP polyphenolics and suggests their use as a value-added agroindustrial residue.

Keywords: grape pomace; tannase; phenolic compounds; Caco-2 cells; gut inflammation.

46 **Abbreviations**

47 GP – grape pomace

48 TNS – tannase (tannin acyl hydrolase)

49 GPTNS – tannase treated grape pomace

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1. Introduction

Food residues derived from production of processed foods, e.g., wines, orange and grape juices, vegetable oils, dairy, and cereals, are generally underutilized. Recently, using these residues for the extraction and production of value-added products in biotechnological applications, e.g., enzymes, proteins, organic acids, and bioactive compounds, has been recognized among scientific communities and agrofood industries (Roselló-Soto et al., 2015; Wong et al., 2015). Further, these applications can reduce detrimental impacts on environments caused by disposal of these residues in land or water, e.g., inhibiting plant germination, increasing biochemical and oxygen demands, and shifting fauna and flora discharge zones (Galanakis, 2012).

Grape pomace (GP) produced during wine and grape juice production contains fiber and phenolic compounds (Mildner-Szkudlarz, Bajerska, Zawirska-Wojtasiak, & Górecka, 2013). Phenolics in GP have been reported to display antioxidant (Mildner-Szkudlarz et al., 2013), anti-inflammatory (Wang et al., 2011), anti-proliferative (Jara-Palacios et al., 2015), and antimicrobial activities (Friedman, 2014). These putative beneficial attributes provide a basis for formulating GP into food products and dietary supplements for health promotion and prevention (Jara-Palacios et al., 2015; Mildner-Szkudlarz et al., 2013; Wang et al., 2011).

Chronic inflammation in the intestine stimulates excessive production of cytokines and reactive oxygen species (ROS) by activated leukocytes via the NF- κ B pathway (Biasi, Leonarduzzi, Oteiza, & Poli, 2013a). Phytochemicals have been found to have a potential for mitigating inflammation (Russell & Duthie, 2011). For example, proanthocyanidins in grape seed ameliorated colitis in rats via the modulation of the NF-

κB pathway (Wang et al., 2011), grape seed and red wine extracts decreased TNF-α or IL-1β-induced IL-8 secretion in Caco-2 cells (Biasi et al., 2013b; Romier-Crouzet et al., 2009), and raisins and white grape pomace inhibited proliferation of Caco-2 cells (Jara-Palacios et al., 2015; Kountouri et al., 2012). Thus, polyphenols from grape pomace may be protective against inflammatory conditions in the gastrointestinal tract.

Polyphenols are generally present in food in polymeric, glycosidic or ester forms, which impact their bioavailability and ultimate bioefficacy at target tissues (Scalbert & Williamson, 2000). A growing number of new technologies and approaches have been developed to optimize the extraction efficiency of beneficial compounds from foods or wastes, e.g. use of half factorial design for the extraction of phenolic compounds in palm kernel and the application of high voltage electrical discharges, pulsed electric field or ultrasound to enhance the extraction of protein and phenolic compounds from olive kernel and blackberries (Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015; Roselló-Soto et al., 2015; Wong et al., 2015). Furthermore, means to transform polyphenols via fermentation or enzymatic hydrolysis to aglycones and/or monomers may enhance their bioavailability and bioactivity in the gut or whole body. Tannin acyl hydrolase [tannase (TNS)] can cleave the ester or depside bonds of tannin, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (Lekha & Lonsane, 1997; Macedo et al., 2012). Biotransformation of polyphenols in tea infusions and orange juice mediated by tannase display higher antioxidant capacity and chemopreventive potential (Ferreira, Macedo, Ribeiro, & Macedo, 2013; Macedo et al., 2012).

Thus, the primary aim of this study was to examine whether constituents in the GP treated with *Paecilomyces variotii* tannase (GPTNS) (Battestin & Macedo, 2007) would be more potent to diminish IL-1 β -induced oxidative stress and inflammation in Caco-2 cells than the untreated GP.

2. Material and Methods

2.1. Reagents

Gallic acid, catechin, epicatechin, caffeic acid, rutin (quercetin-3-O-rutinoside), quercetin, myricetin, ferulic acid, vanillic acid, p-coumaric acid, 3,4-dihydroxyphenylacetic acid, protocatechuic acid, interleukin 1-beta (IL-1 β), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), bovine serum albumin (BSA), arachidonic acid (AA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 4,6-tripyridyl-s-triazine (TPTZ), methanol and acetonitrile (ACN) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium sulfate, sodium acetate, and fluorescein were purchased from Ecibra (São Paulo, Brazil). Tannic acid was purchased from Ajinomoto OmniChem Division (Wetteren, Belgium). Monobasic and dibasic sodium phosphate were obtained from LabSynth (Diadema, Brazil). Fetal bovine serum (FBS) was purchased from ATCC (Rockville, MD, USA). Advanced Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, L-glutamine, Hank's balanced salt solution (HBSS), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) probe were

purchased from Life Technologies (Grand Island, NY, USA). 2,2-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was obtained from Wako Chemicals (Richmond, USA). Bicinchoninic acid (BCA) kit and sandwich human interleukin-8 (IL-8) ELISA Kit were purchased from Pierce Biotechnology (Rockford, IL, USA). Cyclooxygenase 2 (COX-2) ELISA kit was purchased from EMD Millipore (Bedford, MA, USA). Nucleic acid extraction kit, NF- κ B p65 ELISA kit and prostaglandin E₂ (PGE₂) enzyme immunoassay kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Phosphate buffer saline (PBS) and radioimmunoprecipitation assay (RIPA) buffer were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Grape pomace

GP was obtained from a winery in Valinhos, São Paulo State, Brazil during the wine production period between January and March 2013. GP comprised skins, seeds, and stems, as well as microbiota derived from the fermentation during the wine production. The grape variety was “Maximo IAC 138-22,” a hybrid of Syrah (75%) and Seibel (25%) varieties. The GP was frozen at -20°C, lyophilized, ground with a portable laboratory grinder, and then stored at -80°C.

2.3. Production of tannase extract

Tannase was produced by *Paecilomyces variotii* using our previously published procedure with minor modifications in the scale and formulation of the culture medium (Battestin & Macedo, 2007). Briefly, the culture medium was prepared by mixing 20 g wheat bran, 20 mL distilled water, and 10% tannic acid (w/w) in a 250-mL conical flask,

and a *Paecilomyces variotii* suspension (5×10^7 spores/mL) was inoculated into the sterilized medium. After fermentation for 120 h at 30°C, 160 mL of 20 mmol/L acetate buffer (pH 5.0) was added, followed by shaking for 1 h (Fermentation Design Incorporated, Allentown, USA). The resulting supernatant generated after filtration and centrifugation at 9,650 x g for 30 min at 4°C (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA) was treated with solid ammonium sulfate (80% saturation) at 4°C for overnight. After centrifugation (9,650 x g for 30 min), resuspension, dialysis with distilled water for 48 h, and lyophilization, semi-purified tannase extract was produced for the study.

2.4. Tannase treatment of grape pomace

GP (10 g) was incubated with 500 mg (1.2 U/mg) tannase extract in 100 mL of 0.02 M sodium acetate buffer (pH 5.0) for 5 h at 40°C according to Battestin & Macedo (2007). At the end of the hydrolysis, the mixture was chilled in an ice bath for 15 min to inhibit enzyme activity and then mixed with 240 mL methanol. After 60 min at room temperature, the mixture was sonicated for 10 min in a water-bath sonicator and spun at 9,000 x g for 15 min (Daigger model SD, Illinois, USA). The supernatant was collected, dried under N₂ air, lyophilized, and stored at -80°C until use. The dry extract was reconstituted in PBS for tests.

2.5. Total phenols and antioxidant capacity

Total phenol content was determined according to Singleton & Rossi (1965) with results expressed as mg gallic acid equivalents (GAE)/mg dried extract (DE).

Antioxidant capacity was assessed using the DPPH radical scavenging activity (DPPH), oxygen radical absorbance capacity (ORAC), and ferric-reducing antioxidant power (FRAP) assays (Benzie & Strain, 1996; Brand-Williams, Cuvelier, & Berset, 1995; Macedo et al., 2012; Prior et al., 2003). All values are expressed as $\mu\text{mol Trolox equivalents (TE)/mg DE}$.

2.6. Detection and quantification of polyphenolic compounds (HPLC-ECD)

Polyphenols were profiled using a high performance liquid chromatography (HPLC) system equipped with an electrochemical detector (ECD) (ESA Inc., Chelmsford, MA) and an Agilent ZORBAX SB-C18 column (5 μm , 4.6 mm $\times$ 250 mm, Santa Clara, CA), according to the method of Chen, Milbury, Lapsley, & Blumberg (2005). The identification of individual compounds (gallic acid, catechin, epicatechin, caffeic acid, rutin, quercetin, myricetin, ferulic acid, vanillic acid, *p*-coumaric acid, 3,4-dihydroxyphenylacetic acid, protocatechuic acid) was achieved by comparing retention time and electrochemical response with authenticated standards. The concentrations were calculated using curves constructed with the authenticated standards.

2.7. Cell Culture

2.7.1. Caco-2 cells maintenance

Human colon adenocarcinoma-derived Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell passages between 25 and 40 were used in the experiments. The cells were grown in 75 cm^2 flasks in DMEM supplemented with 10% FBS, 1% antibiotics (penicillin and streptomycin) (v/v), 1% L-

glutamine, and 10 mmol/L of HEPES buffer at 37°C in a humidified atmosphere with 5% CO₂. Culture medium was changed 3 times each week and cells were passaged at 75-85% confluence.

2.7.2. Cell viability

Cell viability was evaluated using the MTT assay, according to the method of Mosmann (1983) with minor modifications. Briefly, Caco-2 cells (1×10^5 /well) were seeded in 24-well plates for 24 h. After the removal of the medium, the cells were treated with GP extracts in DMEM without FBS at the selected concentrations (50-1000 µg/mL) for 48 h. At the end of the treatment, the medium was removed and replaced with 0.5 mg/mL MTT in DMEM without FBS. After 3 h incubation at 37°C, formazan was dissolved in 200 µL DMSO and measured at 570 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). Data are expressed as the percent of control.

2.7.3. Intracellular reactive oxygen species (ROS)

Intracellular ROS content was measured using an oxidant-sensitive fluorescent probe, CM-H₂DCFDA (Wang & Joseph 1999). Briefly, Caco-2 cells (3×10^4 /well) were seeded in 96-well black microplates. Once cells reached 80% confluence, the culture medium was replaced with HBSS containing 10 µmol/L CM-H₂DCFDA and incubated for 30 min at 37°C. After washed with PBS to remove unabsorbed probe, the cells were treated with GP or GPTNS in HBSS at 100 or 200 µg/mL with and without (as control) 50 µmol/L AAPH as a ROS generator for 5 h. Fluorescence at 490ex and 520em were

monitored hourly. Results are expressed as the % increase in fluorescence from the respective baseline value.

2.7.4. IL-1 β -induced inflammation

Inflammation in Caco-2 cells was induced by treating the cells with 1 or 20 ng/mL IL-1 β in DMEM without FBS for 24 h. The IL-1 β doses and duration were selected based on preliminary data (data was not shown).

The anti-inflammatory effect of GP extracts at 100 or 200 μ g/mL was evaluated under 2 conditions: pre-treatment (prior to IL-1 β exposure) and pre-treatment plus co-treatment, to reflect a potential for prevention and treatment, respectively (**Fig. 1**). A post-treatment only condition was also performed, but these results are not shown as preliminary experiments indicated no significant changes between control and treatment groups. The test GP concentrations were selected based upon average fasting and postprandial liquid volumes in the small and large intestine at ~100 mL (Mudie et al., 2014); thus, a 200 μ g/mL could be achievable when 20 mg of the extract is consumed, an amount approximately equivalent to 100 mg dried GP.

Pre-confluent Caco-2 cells (2×10^5 cells/well) were grown in 24-well plates for 24 h and then treated with GP extracts for 6 or 24 h. Subsequently, the cells were exposed to 1 ng/mL IL-1 β for 24 h. At the end of the treatment, medium was collected for IL-8 quantification and determined according to the manufacturer's instructions. Values were calculated using a standard curve constructed with a recombinant human IL-8 standard. After the adjustment for the protein content of cell lysates, results are expressed as the percent change from control, obtained using the following **formula (1)**:

$$\% = [(sample - negative\ control) - (positive\ control - negative\ control) / (positive\ control - negative\ control)] \times 100 \quad (1)$$

where the positive control is the mean value obtained from IL-1 β treated wells and negative control is the value obtained from wells receiving no treatment.

For the determination of PGE₂, Caco-2 cells (2 x 10⁵ cells/well) were grown in 24-well plates as described above for IL-8. At the end of the treatment, the cells were rinsed twice with HBSS, pH 7.4, followed by incubation in HBSS containing 0.1% (w/v) BSA and 10 μ mol/L arachidonic acid for 30 min at 37°C. PGE₂ in HBSS was determined using an ELISA kit according to the manufacturer's instructions. PGE₂ concentrations were calculated using a standard curve constructed with PGE₂ standard with adjustment for the cell lysate protein. Results are expressed as the percent change from control using the IL-8 formula.

To assess COX-2 protein expression, Caco-2 cells (5 x 10⁵/well) were cultivated in 6-well plates for 24 h, followed by the GP extracts and IL-1 β treatments as the above-mentioned protocol. GP extracts concentrations were the same but IL-1 β was increased from 1 to 20 ng/mL, as our preliminary results showed the higher dose could potentiate the production of COX-2 protein. At the end of the treatment, cells were lysed using RIPA buffer in ice for 5 min in an orbital shaking platform. COX-2 protein in the cell lysate was determined using an ELISA kit. COX-2 concentrations were calculated using a standard curve constructed with a COX-2 standard with adjustment for the cell lysate

protein. Results are expressed as the percent change from control using the IL-8 formula.

To evaluate NF- κ B activation, Caco-2 cells were grown and treated in the same manner as for COX-2 determination. At the end of the treatment, cells were scraped from the wells, followed by nucleic acids extraction using a nucleic acid extraction kit. The resulting nuclear fractions were used for the quantification of NF- κ B p65 protein using an ELISA kit. After adjustment for cell lysate protein, the results were expressed as the percent change from control.

Protein concentration in cell lysates was determined using a BCA protein assay kit.

2.8. Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). One-way ANOVA was performed to assess statistical significance for the data on total phenols, antioxidant capacity, polyphenol quantification, cell viability, and cellular ROS production. If statistical significance was obtained, Tukey-Kramer HSD multi-comparison or Student's t-test was performed. For the inflammatory biomarkers, a 3-way ANOVA with enzyme, time, and dose as the independent variables was performed to analyze statistical significance, followed by the Tukey-Kramer HSD multi-comparison test. A P-value ≤ 0.05 was considered statistically significant. The Pearson's correlation test was used to analyze the correlation between variables.

3. Results

3.1. Tannase effect on GP total phenols, antioxidant capacity, and polyphenolic profile

Tannase treatment increased total phenols, DPPH, ORAC, and FRAP by 39, 57, 215 and 12%, respectively, as compared to GP (**Table 1**). Using HPLC-ECD, we quantified 6 phenolic acids and 5 flavonoids (**Table 2**). Among the quantified flavonoids, catechin and epicatechin were predominant with their concentrations in GP being at least 1-fold larger than the other 3 compounds. The content of 2 flavanols were not altered by tannase treatment. In general, the content of the analyzed phenolic acids in GP was increased after tannase treatment. The tannase treatment increased gallic, protocatechuic, 3,4-dihydroxyphenylacetic, and vanillic acids, myricetin, and quercetin by 124, 51, 221, 100, 84, and 45%, respectively, as compared to GP. Caffeic and *p*-coumaric acids became detectable after the treatment, but rutin was decreased by 71%.

3.2. Cell viability after GP and GPTNS treatments

Toxicity of GP and GPTNS toward Caco-2 cells was tested at the concentrations ranging from 50 to 1000 µg/mL for 48 h (**Fig. 2**). GP and GPTNS at ≤200 µg/mL did not induce changes in cell viability ($P > 0.05$). At 250 µg/mL, GP significantly reduced cell viability, but not GPTNS. At the highest concentrations, both GP and GPTNS significantly reduced Caco-2 cell viability by 65%. Thus, GP and GPTNS at 100 and 200 µg/mL, which induced less than 5% toxicity, were administered to evaluate their anti-inflammatory and antioxidant effects in Caco-2 cells.

3.3. GP and GPTNS present cellular ROS scavenging activity

After 5 h of incubation with H₂DCFDA, the cumulative ROS level inside Caco-2 cells was increased by 49% as compared to the baseline. Both GP and GPTNS at 100 and 200 µg/mL displayed similar efficacy in the reduction of endogenous ROS production by 17%, as compared to negative control (**Fig. 3A**). Because IL-1β treatment did not result in a significant increase in ROS production (data not shown), AAPH (50 µmol/L) was employed to test radical scavenging activity of GP constituents. AAPH alone enhanced ROS production by 142% compared to negative control at 5 h (**Fig. 3B**). When Caco-2 cells were treated with AAPH and GP extracts simultaneously, the AAPH-induced ROS production was completely abolished. Similar to the result noted in **Fig. 3A**, a dose-dependent effect was not evident for GP and GPTNS.

3.4. Effects on inflammation biomarkers in IL-1β-treated Caco-2 cells

A preliminary experiment was performed to optimize treatment duration and dose of IL-1β in the induction of IL-8 production (data not shown). We found that the treatment at 1 ng/mL for 24 h stimulated Caco-2 cells to produce 3.1 ng/mg protein IL-8 as compared to an undetectable level in the negative control. In the pre-treatment experiment, all dose, time, and tannase factors were significant in the IL-1β-induced IL-8 production (**Fig. 4A**). The largest inhibition was achieved with GPTNS at 200 µg/mL for 24 h, decreasing IL-8 by 83%, followed by GP at 200 µg/mL for 24 h by 59% and pre-treatment with GPTNS at 200 µg/mL for 6 h by 51%. The pre-treatment with GP/GPTNS at 100 µg/mL did not yield a significant inhibition on the IL-1β-induced IL-8 production. In the pre/co-treatment experiment, the inhibitory effect was independent of the dose. The largest inhibition occurred with GPTNS for 24 h, decreasing by 70%, and

the other test conditions inhibited IL-8 production by ~44% (**Fig. 4B**). NF- κ B activation (i.e., NF- κ B p65 translocation to nucleus) was correlated with IL-8 production ($r = 0.36$, $P = 0.012$).

IL-1 β at 20 ng/mL enhanced NF- κ B activation by 223% compared to negative control. In the pre-treatment experiment, either tannase or treatment duration significantly effected the activation of NF- κ B (**Fig. 4C**). On average, GPTNS diminished 48% NF- κ B activation compared to GP at 27%. The 24-h pre-treatment decreased activation 49% compared to 26% after 6 h pre-treatment. Overall, the greatest inhibition at 63% was noted after the pre-treatment with GPTNS for 24 h, independent of the dose. In the pre/co-treatment experiments, dose, duration, and tannase treatment had no effect on the NF- κ B activation as all test conditions resulted in ~33% decrease in the translocation (**Fig. 4D**), which was lower than those of the pre-treatments.

The basal secreted PGE₂ was 652.73 pg/mg protein in the unstimulated cells. In cells treated with IL-1 β at 1 ng/mL for 24 h (positive control), secreted PGE₂ was increased by 214%. In the pre-treatment experiment, the dose and the tannase-dose interaction significantly reduced PGE₂ (**Fig. 5A**) with the average reduction at 200 μ g/mL by 95% and at 100 μ g/mL by 40%. The largest inhibition was 107% when the cells were pre-treated with GPTNS at 200 μ g/mL, followed by 79% when pre-treated with GP 200 μ g/mL, independent of time (not statistically significant). In the pre/co-treatment experiment, all treatment conditions resulted in a comparable inhibition in PGE₂ production (~88%) (**Fig. 5B**) with IL-8 and PGE₂ being strongly correlated ($r = 0.85$, $P \leq 0.0001$).

In order to assess COX-2 protein expression, IL-1 β at 20 ng/mL for 24 h was administered to simulate a detectable increase in Caco-2 cells. The baseline COX-2 protein was 3.77 ng/mg protein and after IL-1 β challenge, this value was increased by 228%. In contrast to our expectation, an inhibitory effect of GP and GPTNS on COX-2 protein expression was not observed. In both pre-treatment and pre/co-treatment experiments, dose, time, and tannase-dose-time interactions contributed to the increase in COX-2 protein expression (**Fig. 5CD**). In both experiments, the high dose and 6-h duration stimulated COX-2 protein expression by ~64 and 47%, respectively. Both GP and GPTNS at 100 μ g/mL maintained COX-2 protein levels as positive control.

4. Discussion

GP derived from wine and juice production has been considered as a potential value-added waste by-product due to the antioxidant and other functional actions of its phytochemical constituents. However, the benefits from GP may not be fully realized because most of its polyphenols are present in glycosidic forms associated with lower bioavailability (Scalbert & Williamson, 2000). We assessed whether tannase could enhance the antioxidant and anti-inflammatory efficacy of GP in cultured Caco-2 cells.

The application of tannase for biotransformation of polymeric or glycosidic polyphenols have been reported to increase polyphenolic content and antioxidant activity. For example, Chamorro, Viveros, Alvarez, Vega, & Brenes (2012) found that tannase treatment increased total phenols by 41% in grape seed extract and 29% in grape pomace extract, as well as augmented antiradical (DPPH) activity by 16.7% in GP extract. Similarly, Arnous & Meyer (2010) observed that pectinolytic and cellulolytic

enzyme treatments yielded similar outcomes in grapes skins and seeds. Roselló-Soto et al. (2015) and Wong et al. (2015) also showed that the pretreatment of palm and olive kernels with high voltage electrical discharges augments total antioxidant capacity and total phenolic amounts of the final products. We report here that tannase treatment enabled the release of caffeic, *p*-coumaric, gallic, and vanillic acids conjugated in oligomeric/polymeric polyphenol complexes. Particularly, caffeic and *p*-coumaric acids were only detected after the tannase treatment. Further, the increase in quercetin content and the decrease in rutin (quercetin rutinoside) confirmed the deglycosidic action of tannase.

Enhanced oxidative stress in the gastrointestinal tract promotes inflammation related diseases/disorders, partially through ROS-mediated induction and amplification of inflammation (Lam, Apostopoulos, Zulli, & Nurgali, 2015). Consistent with the results of antioxidant activity noted in DPPH, ORAC, and FRAP assays, antioxidants in both GP and GPTNS appeared to be capable of scavenging peroxy radicals produced from AAPH and radicals endogenously generated within cells, but independent of dose and tannase treatment. Our results are consistent with findings that the proanthocyanidins of grape seeds and flavanols were found to effectively scavenge ROS in Caco-2 cells (Pinent et al., 2015; Ramos, Rodríguez-Ramiro, Martín, Goya, & Bravo, 2011). The lack of dose-response and tannase-mediated polyphenol hydrolysis effects is not clear. We speculate that the amount of polyphenols being transported through the cell membrane and then reaching sites of intracellular ROS production was not only small but also comparable between GP and GPTNS. In the AAPH-treated condition, we also speculate that polyphenols in GP and GPTNS could effectively scavenge peroxy radicals in the

extracellular compartment where they were produced before they could react with the radical probe in the intracellular compartment (Matias et al., 2014). Under a constant environmental stress, ROS produced for an extended time can elicit a chronic, low-grade inflammation and contribute to the development and progression of chronic diseases (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). The capacity of GP and GPTNS for scavenging ROS may be a possible key factor related to the inactivation of the NF- κ B transcription factor and the downregulation of associated inflammatory responses (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008).

NF- κ B plays a central role in the connection between external pro-inflammatory stimuli and gene expression of inflammatory responses in the nucleus (Karin, 1999). Thus, interfering the NF- κ B activation may particularly be beneficial to diseases related with chronic, low-grade inflammation (Kaulmann & Bohn, 2014). We found that constituents in GP and GPTNS suppressed the IL-1 β -induced NF- κ B activation. These results are consistent with results obtained from wine, grape powder, and grape seed extracts in Caco-2 cells, adipocytes, and in rats with TNBS-induced ulcerative colitis, respectively (Chuang et al., 2011; Guina et al., 2015; Wang et al., 2011). While GP effectively attenuated IL-1 β -induced NF- κ B activation, its treatment with tannase appeared to enhance the anti-inflammatory potency. It is interesting to note that all the results from the pre/co-treatment experiments produced comparable efficacy on the attenuation of the NF- κ B activation, independent of tannase, dose, and duration. Further, the magnitude of the protection conferred in the pre/co-treatment experiment was either similar or lower than the corresponding condition in the pre-treatment experiment. We speculate that the affinity of polyphenols to proteins may decrease the

availability of IL-1 β for the activation of NF- κ B when polyphenols in GP and GPTNS were incubated with IL-1 β in pre/co-treatment experiment (Xiao & Kai, 2012).

IL-8 is produced by neutrophils and macrophages during intestinal inflammation (Mitsuyama et al., 1994). Epithelial cells, lamina propria and mucosal tissues also synthesize this chemokine in small quantity (Daig et al., 2000; McCormack et al., 2001). Caco-2 cells upon the stimulation of IL-1 β via the NF- κ B pathway can synthesize and secrete IL-8 (Garat, 2003). Grape seed, GP, and wine extracts, as well as individual phenolic compounds, i.e., gallic acid, caffeic acid, catechin, epicatechin, and quercetin have been reported to decrease IL-8 secretion in Caco-2 cells (Biasi et al., 2013b; Gessner et al., 2012; Guina et al., 2015). Consistent with these studies, we found GP and GPTNS decreased the IL-1 β -induced IL-8 secretion in Caco-2 cells in a manner depending on treatment conditions, i.e., pre- and pre/co-treatment, tannase, dose, and duration. The largest reduction was noted with GPTNS at 200 μ g/mL for 24 h in the pre-treatment experiment. The lower effectiveness in the attenuation of the IL-8 secretion in the pre/co-treatment may be ascribed to the affinity of GP and GPTNS polyphenols toward IL-1 β as discussed above. The significant correlation between IL-8 and NF- κ B suggests that constituents in GP and GPTNS mediated the IL-8 attenuation via their inhibition of the NF- κ B pathway (Hoffmann, Dittrich-Breiholz, Holtmann, & Kracht, 2002).

An array of other mediators, besides pro-inflammatory cytokines and chemokines, are synthesized in inflammatory conditions. For example, prostaglandins, generated from arachidonate catalyzed by COX, are involved in the development of signs like inflammation redness, swelling, and pain (Ricciotti & FitzGerald, 2011).

Consistent with the attenuated effect on IL-1 β -induced inflammatory responses noted with IL-8 and NF- κ B, we found GP and GPTNS displayed a comparable inhibition of PGE₂ synthesis and secretion. Similarly, grape seed extract and resveratrol have been found to decrease the production and secretion of PGE₂ in Caco-2 cells (Cianciulli et al., 2012; Romier-Crouzet et al., 2009). In contrast to the PGE₂ results, COX-2 protein, which is inducible during inflammation, was enhanced by the high dose of GP and GPTNS but unchanged by the low dose. While our results do not provide a clear mechanism for this effect, the inhibition of COX-2 protein expression has been reported in adipocytes treated with grape powder extract (Chuang et al., 2011), in HT29 cells with raisin extract (Kountouri et al., 2012) and in rats fed GP (Nishiumi, Mukai, Ichiyanagi, & Ashida, 2012). We speculate that the decrease in PGE₂ may be attributed to increased PGE₂ degradation mediated by 15-hydroxy prostaglandin dehydrogenase and/or down-regulation of prostaglandin E₂ synthase-1 activity which catalyzes the final step of PGE₂ synthesis (Koeberle et al., 2009; Liu et al., 2014). Future studies are warranted to elucidate the discordant results of PGE₂ and COX-2 protein.

5. Conclusions

We found that tannase treatment liberated monomeric polyphenolics and aglycones from corresponding polymers and glycones in GP, an action that may enhance the bioavailability and bioefficacy of these ingredients. We also found that GP and GPTNS attenuated the IL-1 β -induced inflammatory responses in Caco-2 cells, including the secretion of IL-8 and PGE₂, and NF- κ B activation and that tannase treatment augmented these actions. While the mechanisms responsible for the

observed anti-inflammatory effect remain to be elucidated, the antioxidant and anti-inflammation effects of GP and GPTNS constituents may play an important role. Our results support the potential of GP and GPTNS for protection against oxidative stress and inflammation in the gastrointestinal tract, and inform development of agroindustrial residues rich in bioactive compounds as functional ingredients.

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646

Figure captions

Fig. 1. Design of cell culture experiments to evaluate the effect of grape pomace (GP) and grape pomace treated with tannase (GPTNS) on IL-1 β (1 or 20 ng/mL)-induced inflammation in Caco-2 cells. Abbreviation: PBS, Phosphate Buffer Saline.

Fig. 2. Viability of Caco-2 cells treated with grape pomace (GP) or tannase treated grape pomace (GPTNS) extracts. DMEM serves as control and PBS in DMEM as vehicle control. Values are expressed as mean $\pm$ SD. *Means significantly differ from control ($P \leq 0.05$), tested using one-way ANOVA, followed by post-hoc Tukey HSD.

Fig. 3. Intracellular reactive oxygen species (ROS) production in Caco-2 cells treated with grape pomace (GP) or tannase treated grape pomace (GPTNS) extracts at 100 and 200 μ g dry extract/mL in HBSS and without **(A)** and with 50 μ M AAPH **(B)**. ROS are quantified using the 2',7'-dichlorofluorescein diacetate probe. ^{a,b}Values (mean $\pm$ SD) at the same time point without sharing the same letters significantly differ ($P \leq 0.05$), tested using one-way ANOVA, followed by post-hoc Tukey HSD.

Fig. 4. Percentage change from control (1 and 20 ng/mL IL-1 β) in IL-8 and nuclear NF- κ B p65 respectively, in Caco-2 cells pre-treated or pre- and co-treated with grape pomace (GP) or tannase treated grape pomace (GPTNS) extracts at 100 or 200 μ g/mL. Values are expressed as mean $\pm$ SD. Statistical significance between treatments were tested using three-way ANOVA (enzyme, dose, time), followed by post-hoc Tukey HSD

670 (P ≤0.05): **(A)** pre-treatment: enzyme, P = 0.0083; dose, P ≤0.0001; time, P ≤0.0001;
671 **(B)** pre/co-treatment: enzyme, P = 0.0029; time, P = 0.02; enzyme x time, P = 0.02; **(C)**
672 pre-treatment: enzyme, P = 0.01; time, P = 0.0048; **(D)** pre/co-treatment: model, P =
673 0.80.

674

675 **Fig. 5.** Percentage change from control (1 and 20 ng/mL IL-1β) in PGE₂ and COX-2
676 protein expressions in Caco-2 cells pre-treated or pre- and co-treated with grape
677 pomace (GP) or tannase treated grape pomace (GPTNS) extracts at 100 or 200 µg/mL.
678 Values are expressed as mean ± SD. Statistical significance between treatments were
679 tested using three-way ANOVA (enzyme, dose, time), followed by post-hoc Tukey HSD
680 (P ≤0.05): **(A)** pre-treatment: dose, P = 0.0083; enzyme x dose, P <0.0001; **(B)** pre/co-
681 treatment: model, P = 0.63; **(C)** pre-treatment: dose, P <0.0001; time, P = 0.01; enzyme
682 x dose x time, P = 0.02; **(D)** pre/co-treatment: dose, P <0.0001; time, P = 0.0039;
683 enzyme x dose x time, P = 0.0005.

Table 1. Tannase (TNS) treatment increases total phenols [mg gallic acid equivalent/g dry extract] and antioxidant capacity (μmol Trolox equivalent/g dry extract) in grape pomace (GP)

	GP	GPTNS
Total Phenols	281.70 ± 5.64^a	391.52 ± 9.25^b
DPPH	3382.76 ± 12.83^a	5309.11 ± 47.18^b
ORAC	1333.50 ± 66.08^a	4194.74 ± 71.88^b
FRAP	1069.89 ± 12.77^a	1194.26 ± 11.50^b

^{a,b}Values (mean $\pm$ SD) within a row without sharing the same letters significantly differ ($P \leq 0.05$), tested using Student's t-test.

Table 2. Tannase (TNS) changes flavonoid and phenolic acid contents (mg/g dry extract) in grape pomace (GP)

Phenolic compounds	GP	GPTNS
Gallic acid	3.6 ± 0.21 ^a	8.07 ± 0.22 ^b
Protocatechuic acid	0.67 ± 0.00 ^a	1.01 ± 0.00 ^b
3,4-dihydroxyphenylacetic acid	0.24 ± 0.02 ^a	0.77 ± 0.05 ^b
Catechin	13.55 ± 0.9 ^a	14.32 ± 0.46 ^a
Vanillic acid	0.09 ± 0.00 ^a	0.18 ± 0.01 ^b
Caffeic acid	not detected	0.2 ± 0.02
Epicatechin	7.14 ± 0.56 ^a	7.76 ± 0.49 ^a
<i>p</i> -coumaric acid	not detected	1.65 ± 0.04
Rutin (quercetin-3-O-rutinoside)	1.27 ± 0.11 ^a	0.36 ± 0.03 ^b
Myricetin	0.33 ± 0.03 ^a	0.61 ± 0.03 ^b
Quercetin	0.58 ± 0.04 ^a	0.84 ± 0.06 ^b

^{a,b}Values (mean ± SD) within a row without sharing the same letters significantly differ ($P \leq 0.05$), tested using Student's t-test.