

The Antioxidant and DNA Repair Activities of Resveratrol, Piceatannol, and Pterostilbene

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Abstract

Lifestyle diseases represent a large burden on developed societies and account for much morbidity worldwide. Research has shown that eating a diet rich in fruit and vegetables helps to ameliorate and prevent some of these diseases. Antioxidants found in fruits and vegetables may provide a substantial benefit in reducing disease incidence. This study examines the antioxidant properties of resveratrol, piceatannol, and pterostilbene, and the ability of Burkitt's Lymphoma (Raji) cells to uptake these three antioxidants. It also studies the effect of the antioxidants in protecting against DNA damage, and their role in DNA repair following oxygen radical exposure in Raji cells. The Oxygen Radical Absorbance Capacity (ORAC) assay was used to measure overall antioxidant contribution as well as the ability of Raji cells to uptake antioxidant following exposure to 2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH). The single cell gel electrophoresis (Comet) assay was used to assess DNA damage and DNA repair rates of cells. Results showed that Raji cells, following oxygen radical exposure, significantly uptake pterostilbene ($p < 0.0001$), but not piceatannol or resveratrol. Piceatannol provided protection against hydrogen peroxide induced DNA damage, but pterostilbene and resveratrol increased DNA damage following hydrogen peroxide treatment. None of the compounds showed any effect on DNA repair. Overall, this study indicates there is merit for further research into the bioactive roles, including antioxidant capacity, of all three compounds. Such research may provide evidence for the more widespread use of these and other food based compounds for preventing lifestyle diseases.

Keywords: antioxidant, comet assay, DNA repair, orac, piceatannol, pterostilbene, resveratrol

1. Introduction

It has been estimated that an unhealthy diet coupled with physical inactivity account for 10% of the global burden of disease (England, Andrews, Jago, & Thompson, 2015). Unhealthy dietary habits include a low consumption of fruits and vegetables. Cardiovascular disease, ischemic stroke, diabetes, and cancer are some diseases that may develop due to poor lifestyle choices such as diets with low consumption of fruits and vegetables. Recent research has shown that a diet with high fruit and vegetable consumption may reduce the risk of these and other diseases (Bussel et al., 2015; Carter, Gray, Troughton, Khunti, & Davies, 2010; Epps, 2013; Joshipura et al., 1999).

Lifestyle diseases are a large area of concern and are the subject of intensive research globally (Edwards et al., 2014). Oxidative stress is believed to be one of the major causes of these diseases (Mahalingaiah & Singh, 2014; Singh, Vrishni, Singh, Rahman, & Kakkar, 2010). Our bodies naturally produce oxygen radicals as part of our cellular metabolism, but our cells also possess mechanisms to deal with those stresses (A. R. Garrett et al., 2014). These natural mechanisms provide considerable protection; however, excess oxygen radicals and reduced antioxidant availability may lead to imbalance and disease (Koppenhofer et al., 2015). Oxidative imbalance due to lifestyle factors can be partially prevented by consuming antioxidants. Antioxidants from diet, such as those found in fruits and vegetables, reduce oxidative stress and prevent oxygen radical DNA damage (Kuate, 2013; Sagrillo et al., 2015). Many fruits and vegetables have been shown to exhibit robust antioxidant activity (Gupta-Elera et al., 2012; Gupta-Elera, Garrett, Martinez, Robison, & O'Neill, 2011). One antioxidant, resveratrol, has previously been reported to have high antioxidant activity and was believed to be a central factor

in what is known as the French Paradox (Aschemann-Witzel & Grunert, 2015; Yamagata, Tagami, & Yamori, 2015). The French Paradox is the observation that even though the French diet includes large amounts of saturated fats, they have a low incidence of coronary heart disease. Their high intake of red wine was believed, among other things, to be responsible for the lower disease incidence rates because it contains the powerful antioxidant resveratrol. Resveratrol can be found naturally in many food sources such as grapes, peanuts, and berries (Calamini et al., 2010; Rimando, Kalt, Magee, Dewey, & Ballington, 2004). There has been evidence suggesting that the *in vivo* effect of resveratrol may be limited due to its low bioavailability (Davidov-Pardo & McClements, 2015; Semba et al., 2014). Bioavailability is the ability of a drug to enter the circulation and cause an effect once it is consumed.

Resveratrol has two relatively lesser studied analogs, pterostilbene and piceatannol, which may provide valuable alternatives to resveratrol for protection against chronic disease. Piceatannol structurally differs from resveratrol by an additional 3' hydroxyl group (Figure 1), which may contribute to better antioxidant and bioavailability properties than resveratrol (A. R. Garrett et al., 2014; Tang & Chan, 2014). Piceatannol can be naturally found in berries, grapes, and passion fruit (Maruki-Uchida et al., 2013; Rimando et al., 2004). Pterostilbene is a dimethyl ether form of resveratrol (Figure 1), and is also found naturally in grapes and blueberries (Sato et al., 2014). Pterostilbene has also been shown to have greater bioavailability than resveratrol and may serve as a more attractive alternative to resveratrol for antioxidant and other functional properties (Kapetanovic, Muzzio, Huang, Thompson, & McCormick, 2011). Overall these three compounds have been reported to exhibit a variety of potential biological effects such as anticancer properties (Xia, Deng, Guo, & Li, 2010), cardiovascular disease prevention (McCormack & McFadden, 2013), and apoptosis induction (Jancinova, Perecko, Nosal, Svitkova, & Drabikova, 2013).

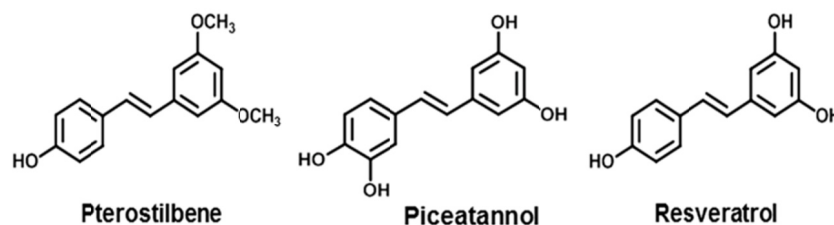


Figure 1. Structures of pterostilbene, piceatannol, and resveratrol

The purpose of this study was: (1) to compare the antioxidant properties of resveratrol, pterostilbene, and piceatannol, (2) to assess the ability of Burkitt's Lymphoma (Raji) cells to uptake these three compounds following challenge with an oxidizing stimulus, (3) to determine the ability of these compounds to protect against DNA damage, and (4) to identify the effect of resveratrol, piceatannol, and pterostilbene on DNA repair rates in Raji cells. We used the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the overall antioxidant properties as well as the ability of cells to uptake antioxidant following exposure to AAPH and the single cell gel electrophoresis (Comet) assay to measure DNA protection and DNA repair. Due to the structural differences discussed earlier, we hypothesized that pterostilbene and piceatannol would exhibit higher utilization and protective effect compared to resveratrol following oxidative challenge. We also hypothesized that these compounds would provide protection against DNA damage and possibly modulate DNA repair when compared to a placebo phosphate buffered saline (PBS) treatment.

2. Materials and Methods

2.1 Chemicals

2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH), Fluorescein sodium salt, Propidium Iodide, Resveratrol, and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). Piceatannol and Pterostilbene were purchased from Cayman Chemical (Ann Arbor, MI). Hydrogen Peroxide was purchased from Fisher Scientific (Pittsburg, PA).

2.2 Materials

Costar 3694 96 well plates were obtained from Corning Inc. Cellstar 12 well cell culture plates were obtained from Greiner Bio-One International.

2.3 Equipment

A BMG FLUOstar Optima plate reader (S/N 413-0225) was used to measure fluorescence readings for ORAC assays. A Zeiss AxioScope fluorescence microscope was used to image all Comet experiments. A Misonix Sonicator 3000 was used for cell lysis.

2.4 Cell Culture

Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations at 37 °C and 5% CO₂.

2.5 Compound Preparation

Resveratrol, pterostilbene, and piceatannol were each dissolved initially at 40 mM concentrations in DMSO. These samples were then diluted in PBS to 400 µM aliquots. These aliquots were further diluted, in PBS, as needed to working concentrations for their respective assays. Aliquots were stored in fluorescence protection bags at -20 °C until needed. Aliquots were tested before freezing and after being frozen to ensure effectiveness; no difference in effectiveness was observed.

2.6 Procedure

2.6.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were diluted to a 24 µM concentration and then analyzed according to the method published in Gupta-Elera et al. (2012) with the following modifications: 20 µL of AAPH was used instead of 25 µL AAPH, and assays were run for 120 minutes instead of 90 minutes. The final concentration of all samples tested was 2 µM. The ORAC assay was used because it is a well-established method of measuring antioxidant activity *in vitro* (Andrew R. Garrett, Murray, Robison, & O'Neill, 2010). The sample to be tested was mixed with a fluorescent molecule (Fluorescein) and an oxygen radical generator (AAPH). The level of fluorescence was measured every 2 minutes over a 120 minute period to create fluorescence decay curves. Experimental samples were run in the same plate as a standard (Trolox) and control samples (Fluorescein alone and Fluorescein plus AAPH). Samples were run in a BMG FLUOstar Optima plate reader with 485 nm emission and 590 nm excitation filters. Samples were run in six different plates with twelve replicates per plate for each compound.

2.6.2 Standard Curve

The area under the curve (AUC) was measured for all experimental and standard samples from their respective fluorescence decay curves. The AUC for each sample and standard was calculated by subtracting the AUC of Fluorescein plus AAPH from the AUC of the sample/standard. The AUC for the standard samples were then plotted to create a standard curve. The AUC of each sample was compared to the standard curve to determine its antioxidant activity in Trolox Equivalents per mg sample (TE/mg).

2.6.3 Cellular Uptake of Antioxidant following Oxygen Radical Generator Exposure

Cellular uptake of antioxidant by Raji cells after oxygen radical generator exposure was measured by following the method published in Gupta-Elera et al. (2011) with some modifications. In short, Raji cells were incubated with AAPH for 10 minutes to simulate oxidative stress. Following incubation, they were washed and then treated with 10 µM antioxidant for 10, 20, 45, or 60 minutes. Following treatment, the samples were washed 2 times and sonicated to lyse. Following sonication, the samples were centrifuged for 30 minutes at 3000 g. The supernatant was then removed as the lysate fraction. The pellet was washed and used as the membrane fraction. Three different plates per compound were run for both membrane and lysate fractions with twenty-four replicates per plate.

2.6.4 Protection Against DNA Damage

Raji cells were incubated for 90 minutes at a 10 µM concentration of the compounds. Following incubation, cells were washed twice and then exposed to 10 µM H₂O₂ for 10 minutes. The cells were then washed and re-suspended in warm RPMI 1640 and prepared for comet assay as described below.

2.6.5 DNA Repair

Raji cells were incubated in 500 µL of 10 µM H₂O₂ for 10 minutes to induce DNA damage. Following incubation, 1 mL of cold RPMI 1640 media was added to the cells. The cells were then centrifuged at 450 g for 5 minutes at 4 °C, washed with cold PBS, and re-suspended in fresh warm media. Cells were then treated with the compounds and allowed to repair for a predetermined amount of time (0, 5, 15, 30, 60, and 90 minutes). Samples were then prepared for comet assay as described below.

2.6.6 Comet Assay

Following both protection and repair protocols, samples were prepared for comet analysis by following the methods described by Xiao et al. (2014) with slight modifications. Briefly, samples were mixed with low melting point agarose and layered on double frosted microscope slides (Xiao et al., 2014). The slides were placed in alkaline lysis buffer for 1 hour, rinsed with ddH₂O and then placed in alkaline electrophoresis buffer for 20 minutes. They were then electrophoresed for 30 minutes. Following electrophoresis, slides were allowed to rest in ddH₂O for 15 minutes, then fixed in -20°C 100% ethanol for 5 minutes and allowed to dry prior to being stained with Propidium Iodide and imaged. All comets were scored using TriTek CometScore Freeware v1.5. Samples were run in three different trials with fifty comets imaged per sample, totaling 150 comets per sample.

Comet assay results are reported in terms of Tail Moment. Tail moment is defined as the product of the tail length and the % of DNA in the tail. These values are given as part of the output by the CometScore software and are widely reported for Comet analysis (Olive, Banath, & Durand, 2012).

2.7 Statistics

All statistics were performed using JMP Pro 11. ANOVA and the Tukey-Kramer HSD tests were used to compare compounds and PBS for all ORAC and cellular uptake data. Welch's T test was used to compare treatment and non-treatment groups for the Comet Repair data. A Dunnett's Procedure was used to compare all treatment groups with the PBS treatment for the DNA protection data. All statistics were calculated at the family-wise $\alpha = 0.05$ level. All error bars represent the 95% confidence interval of the mean.

3. Results

3.1 ORAC Antioxidant Values

The raw antioxidant values of pterostilbene, piceatannol, and resveratrol as measured by ORAC are given in Figure 2. Resveratrol exhibited the highest antioxidant activity with a mean value 311.403 TE/mg (CI $302.63 \leq \mu \leq 320.18$, N=72) followed by piceatannol and pterostilbene with a mean of 283.520 TE/mg (CI $274.75 \leq \mu \leq 292.29$, N=72) and 126.875 TE/mg (CI $118.10 \leq \mu \leq 135.65$, N=72) respectively. There was a significant difference ($p < 0.0001$) in the mean TE/mg values among the tested compounds.

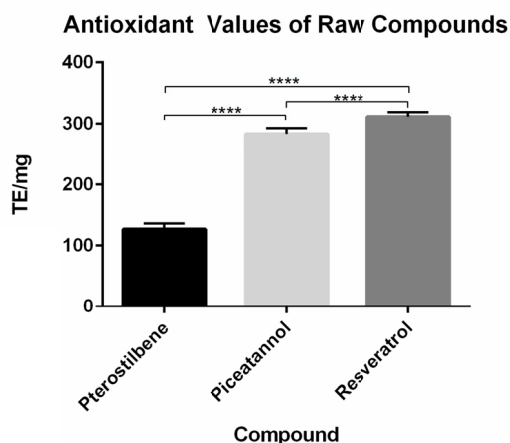


Figure 2. Antioxidant values of raw compounds as measured by ORAC assay reported as mean Trolox Equivalents per milligram (TE/mg). Each mean was significantly different than those of the other two compounds ($p < 0.0001$, $n = 72$ respectively).

3.2 Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure

3.2.1 Lysate Fraction

Figure 3 shows the results from our cellular uptake model for the lysate fraction of Raji cells. There was a significant difference in the mean antioxidant capacity between the lysates of cells treated with the different compounds. Treatment of Raji cells with pterostilbene had the highest antioxidant activity with a mean of 22,407 TE/L/ 10^6 cells followed by piceatannol and resveratrol with mean values of 16,319 and 12,424 TE/L/ 10^6 cells,

respectively.

Treatment of Raji cells with pterostilbene produced significantly higher antioxidant values ($p < 0.0001$), compared to piceatannol, resveratrol and the control. We also found significantly higher antioxidant values in Raji cells treated with piceatannol compared to those treated with resveratrol ($p = 0.0283$).

3.2.2 Membrane Fraction

Contrary to the lysate, the mean antioxidant values for the membranes of cells treated with any of the three compounds were less than raw Trolox (standard), (Figure 4). The treatment with the highest antioxidant value was pterostilbene, (mean = -4614 TE/L/ 10^6 cells). This treatment was the only treatment that was significantly different from the other groups ($p < 0.0001$). Piceatannol, resveratrol, and PBS had mean antioxidant values of -11024, -11390, and -10612 TE/L/ 10^6 cells, respectively. Antioxidant values are reported as negative because they are being compared to a standard curve of raw Trolox, as opposed to cells treated with Trolox. Thus, the amount of antioxidant in the membrane fraction is not as high as raw Trolox. There is no precedent for a Trolox standard curve from cells incubated with Trolox so results were compared to a raw Trolox standard curve.

Antioxidant Capacity Cellular Model Lysate Fraction

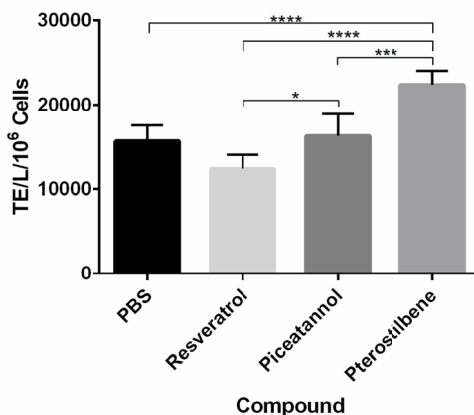


Figure 3. Mean antioxidant values of Raji cell lysates. Antioxidant values of cell lysate following challenge with AAPH and recovery with the specified antioxidant treatments. $n = 72$ for each treatment

Antioxidant Capacity Cellular Model Membrane Fraction

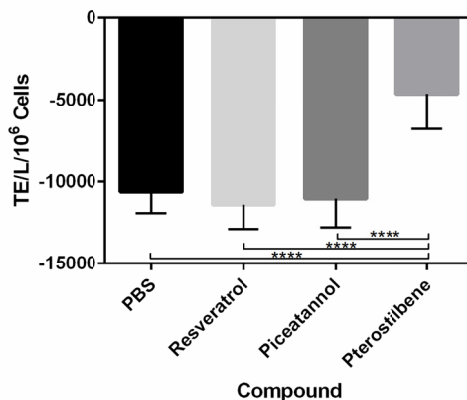


Figure 4. Mean antioxidant values of Raji cell membrane fractions following challenge with AAPH and recovery with the specified antioxidant treatments. $n = 72$ for each treatment

3.3 DNA Protection

The DNA protection assay was performed by pretreating Raji cells with one of the antioxidant compounds or the control (PBS) prior to treatment with hydrogen peroxide (Figure 5). Surprisingly, samples pretreated with pterostilbene and resveratrol exhibited larger tail moments than samples pretreated with PBS alone, ($p < 0.0001$). Samples pretreated with piceatannol had smaller tail moments than samples pretreated with PBS ($p < 0.0001$). No difference was observed between samples pretreated with PBS and samples receiving no pretreatment.

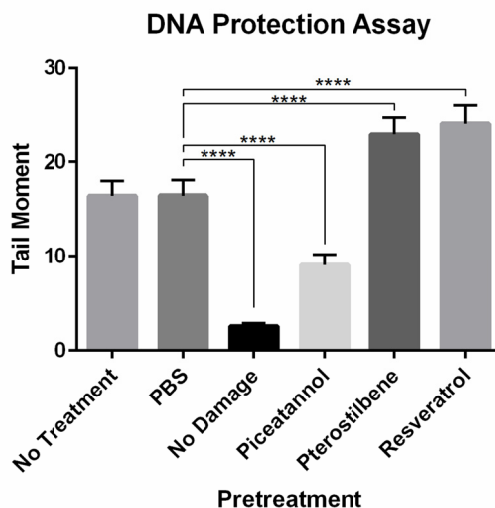


Figure 5. DNA protection. Raji cells were pretreated with antioxidant compounds prior to treating with H₂O₂. Larger tail moments indicate more DNA damage, n = 150 for each treatment

3.4 Comet Repair Assay

The results from our Comet repair assay showed a decrease in the tail moment of cells over time (Figures 6, 7, and 8); however, there was no difference in tail moment for samples treated with or without pterostilbene, piceatannol, or resveratrol after 90 minutes of repair $p=0.9488$, 0.7750 , and 0.6785 respectively.

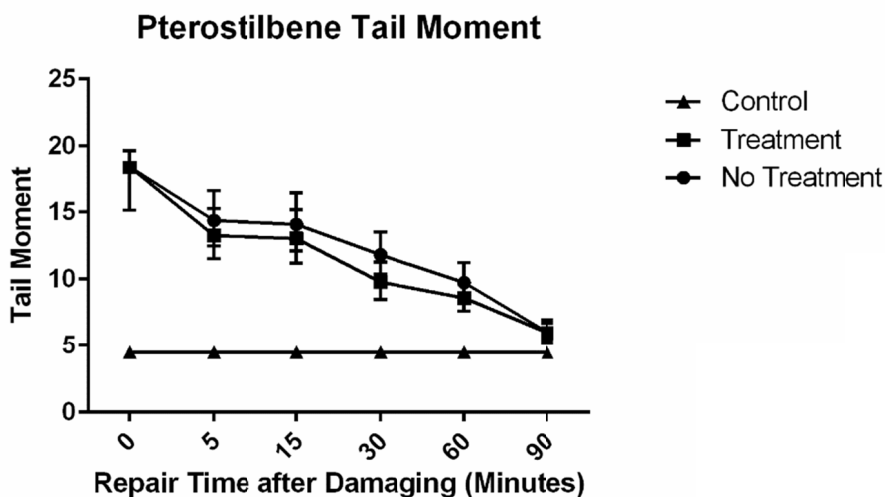


Figure 6. Tail moments for pterostilbene. Tail moments were measured for samples treated either with or without pterostilbene over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.9488$), n = 150 for each treatment at each time point

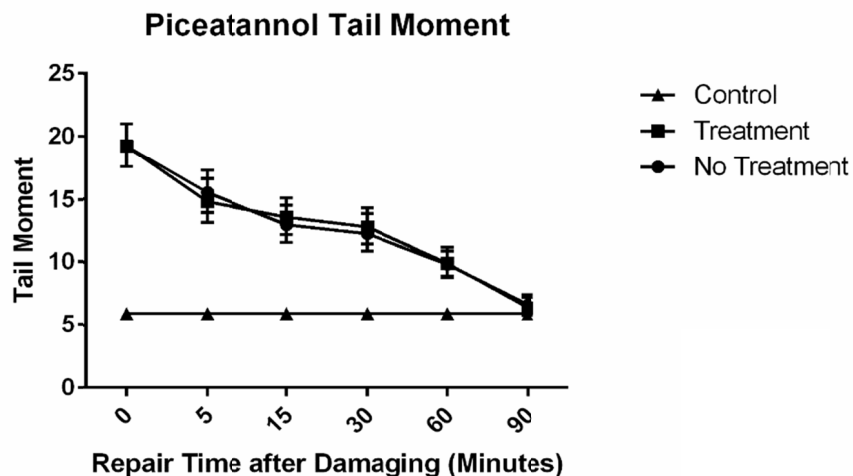


Figure 7. Tail moments for piceatannol. Tail moments were measured for samples treated either with or without piceatannol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.7750$). $n = 150$ for each treatment at each time point

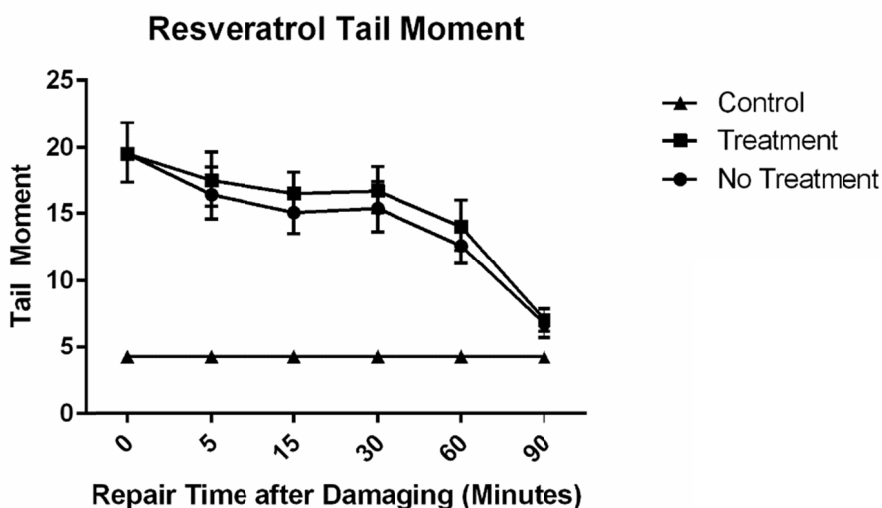


Figure 8. Tail moments for resveratrol. Tail moments were measured for samples treated either with or without resveratrol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.0612$). $n = 150$ for each treatment at each time point

4. Discussion

The results of this study provide interesting information regarding resveratrol and its analogs: piceatannol and pterostilbene.

4.1 Analysis of Antioxidant Abilities

Even though resveratrol demonstrated significantly higher raw antioxidant activity than either piceatannol or pterostilbene, its ability to be absorbed by Raji cells for future antioxidant protection was weak. As suspected, pterostilbene did show a significantly increased ability to be absorbed compared to piceatannol, resveratrol, and control. Piceatannol was also shown to have more absorption ability than resveratrol in Raji cells. We tested this absorptive capacity at different time points, 10, 20, 45, and 60 minutes, as well as at 1 μM and 10 μM , concentrations. ANOVA revealed no significant differences between the antioxidant capacity between the two concentrations or the four time points for each individual compound, so the data from all time points and

concentrations was combined into 1 group for each compound. These compound groups were then compared to each other for the results reported here. This evidence helps to support our hypothesis that the structures of pterostilbene and piceatannol affect their ability to protect cells from internal oxidizing damage. These results suggest a need for further studies into the biological roles of both pterostilbene and piceatannol.

4.2 DNA Protection

We investigated the ability of these compounds to protect against DNA damage induced by treatment with hydrogen peroxide. We first wanted to ensure that these compounds were not exerting any cytotoxic effects on the Raji cells, so cells were exposed to each of these compounds. We did not see any loss of viability compared to cells incubated with PBS, as measured by Trypan blue exclusion after 24, 48, or 72 hours indicating that the results we obtained were not due to loss of cell viability (data not shown). We also wanted to ensure that no DNA damage was induced by these compounds so we performed comet analyses on Raji cells treated with each compound. We observed no difference in tail moments of Raji cells treated with these compounds when compared to cells treated with PBS, indicating that these compounds themselves had not caused DNA damage directly to the cells (data not shown).

To assess the ability of these compounds to protect Raji cells from DNA damage, we pretreated Raji cells with each compound and then exposed them to hydrogen peroxide. While piceatannol pretreatment demonstrated the ability to protect from DNA damage ($p < 0.0001$), resveratrol and pterostilbene pretreatment resulted in increased DNA damage following hydrogen peroxide exposure, ($p < 0.0001$). Reasons for this are unclear at this point. Increase in tail moment after pretreatment with resveratrol and pterostilbene may be due to anticancer effects or cell death inducing effects of these agents. We did not observe any loss of viability or increase in DNA damage after treatment with these compounds alone, so pretreatment may predispose Raji cells to undergo cell death following oxidative stress. Xia et al. (2010) reported that resveratrol has anticancer effects for prostate, breast and epithelial cancers. These results suggest that this effect may extend to lymphocytic cancers as well. Further work will help to elucidate and characterize these mechanisms.

4.3 DNA Repair Analysis

In an effort to investigate whether or not these compounds could help to repair previously damaged DNA, we tested both a 1 μM (data not shown) and a 10 μM concentration. No positive effects on DNA repair were observed at either concentration. We also looked at several time points to see if there were differences over time. We saw no significant effect on DNA repair rates of Raji cells that received antioxidant treatment versus those that did not. This result was observed for all time points and concentrations tested.

These results indicate that among the many biologically relevant roles of resveratrol, piceatannol, and pterostilbene, DNA repair modulation does not appear to be one of them. This study provided no additional evidence of resveratrol's usefulness as an intracellular antioxidant, but has shown the potential usefulness of pterostilbene in protecting cells from intracellular oxidizing damage. This work also provided information about the possible anticancer activities of resveratrol and pterostilbene. This preliminary study demonstrates the need to further investigate the biologically active roles of all three compounds. Such work may prove to establish mechanisms which may increase longevity and reduce aging. Foods containing antioxidants should be studied further to provide greater insight into the role of a healthy balanced diet in improving health. Such studies may be valuable in helping to alleviate many lifestyle diseases worldwide.

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