5-2023

Polyphenolic prevention of deoxyribose damage using spectroscopic methods

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April 21, 2023

Departmental Honors Thesis

ABSTRACT

Hydroxyl radical formation via Fe^{2+}/Cu^{+} metal leads to oxidative DNA damage that is implicated in a number of neurodegenerative and cardiovascular diseases such as Parkinson's diseases and atherosclerosis. Specifically, oxidative stress can lead to strand breaks and other DNA damage. Antioxidants can prevent this damage through metal binding and radical scavenging mechanisms. Polyphenols are one class of abundant antioxidants shown to provide protection against radical species and are found in foods such as fruits, nuts, and berries. Therefore, polyphenol compounds were tested for their ability to prevent deoxyribose degradation by iron and hydrogen peroxide using the deoxyribose assay, a low-cost, screening method that measures the formation of malondialdehyde (MDA), a byproduct of degraded deoxyribose, using ultraviolet-visible (UV-vis) spectroscopy. Previous studies showed low reproducibility when reporting deoxyribose damage prevention by antioxidants. Using a newly modified deoxyribose assay in the Brumaghim lab that improves reproducibility, quinolinic acid and four polyphenols (MEGA, MEPCA, PrEGA, and PCA) were tested for their antioxidant abilities. These results were compared to polyphenol prevention of plasmid DNA damage by iron and hydrogen peroxide using gel electrophoresis, a method that uses DNA as the substrate but is lower-throughput. Compared to plasmid DNA damage prevention, MEGA, MEPCA, PrEGA, and PCA, show greater deoxyribose damage prevention using the deoxyribose assay. Relating antioxidant activity measurements between these two methods enables accurate use of the deoxyribose assay as a higher-throughput method for determining DNA damage or its inhibition compared to DNA gel electrophoresis methods.

INTRODUCTION

Damage of biomolecules and oxidative stress *in vivo* can be caused by hydroxyl radical.[1] Oxidative stress can disrupt normal redox conditions in cells, leading to protein, lipid, and DNA damage. Iron-mediated oxidative DNA damage by hydroxyl radical is the primary cause of cell death under oxidative stress conditions in both prokaryotes and eukaryotes, including humans.[1] Radical-induced DNA damage occurs at both the negatively charged phosphate backbone and the electron-rich nucleotide bases. Metal ions, such as Fe^{2+} and Cu^{+} bind to the nucleotide bases of DNA, specifically at the N7 position of the guanine base. When iron-binding happens sequence specifically at RTGR sites, the iron preferentially localizes at the N7 of guanine occurring immediately 3′ to the nicked thymidine nucleotide.[2] This localization of metal ions is likely due to N7 having the most negative electrostatic potential among the DNA base moieties.[3]

Hydrogen peroxide is present in cells as a byproduct of respiration, and the Fe^{2+} or Cu^{+} metal ions localized on DNA react with hydrogen peroxide to form highly reactive $^{\bullet}OH$ in immediate proximity to DNA. In turn, the hydroxyl radical abstracts a 5' hydrogen atom from the deoxyribose sugar backbone, initiating DNA strand breaks.[4] Reaction 1 depicts this phenomenon, also known as the Fenton reaction. Fe^{2+} or Cu^{+} interacts with hydrogen peroxide, forming the respective oxidized metal, Fe^{3+} or Cu^{2+}, and hydroxyl radical. Hydroxyl radical can participate in oxidative reactions that differ according to each DNA base and can form various sugar degradation products.[5] Oxidative stress involves excessive hydroxyl radical generation in cells, leading to damage in biomolecules, tissues, and organs. This type of metal-mediated oxidative damage has been implicated in the pathology of numerous neurodegenerative diseases, cardiovascular diseases, and cancers.[6]

$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + {}^{\bullet}OH$ (Reaction 1)

If left unchecked, the radical reactions can continue unless stopped by a defense mechanism against oxidative stress.[6] Antioxidants can act as radical scavengers by neutralizing the radical and reducing its ability to harm biomolecules such as DNA. Antioxidant balance can be managed through intracellular enzymes or through diet.[6] Polyphenols are abundant antioxidants in the diet, typically found in green and black teas, olive oils, and chocolate.[7] During absorption of polyphenols in the gut, they often undergo extensive modifications, such as methylation, sulfation, and glucuronidation, which can hinder or enhance their role as antioxidant.[8] These metabolic processes increase the difficulty of elucidating and comparing the antioxidant ability of polyphenols *in vitro* and *in vivo*. However, there is indirect evidence that absorption of polyphenols is related to the increase in antioxidant capacity of the plasma after consumption of polyphenol-rich foods.[9,10] These results suggest that polyphenol absorption and antioxidant ability is dependent on chemical structure and not on overall dietary concentration, revealing a need to classify antioxidant ability according to structure.

Polyphenols are a class of well-studied, strong antioxidants that act through various mechanisms, including iron binding and radical scavenging.[7] Among the different classes of polyphenols, such as flavonoids, phenolic acids, stilbenes, and lignans, their antioxidant mechanisms can differ. Flavonoids for example, participate in radical scavenging,[11] exhibit metal chelating activity,[12,13] and may stabilize radicals through forming complexes with them.[14] Many stilbene derivatives are involved in radical scavenging and in activating cellular-enzymatic antioxidant defenses.[15]

Catechol and gallol groups in polyphenols are known for their iron binding and metal chelating activities that lead to antioxidant activity.[16] Octahedral coordination geometry is expected of general Fe^{2+}-polyphenol complexes, where Fe^{2+} then autoxidizes to Fe^{3+} in the

presence of O_2.[17] The Fe^{2+} can coordinate up to three catecholate or gallate groups from the polyphenol, and studies have found that Fe^{2+} binding compounds with a gallol group results in significantly faster iron oxidation rates than for the analogous catechol compound.[17] However, polyphenols widely vary in their structures, resulting in a variety of combinations of gallol and catechol groups where metal ions can coordinate. Through binding and coordinating with the Fe^{2+} metal, the polyphenol can inhibit the Fenton reaction from occurring, thereby decreasing hydroxyl radical formation and subsequent DNA damage.

Methyl 3,4,5-trihydroxybenzoate (MEGA; Figure 1) is a gallate ester that is often extracted from the leaves of various plants. MEGA has been shown both *in vitro* and *in vivo* to exhibit anti-tumor properties, anti-inflammatory, anti-HIV, and antioxidant activity.[18,19,20,21] Methyl 3,4-dihydroxybenzoate (MEPCA) is a methyl ester with a catechol group and is a major metabolite found in green teas. MEPCA has been shown *in vivo* and *in vitro* to have antioxidant and

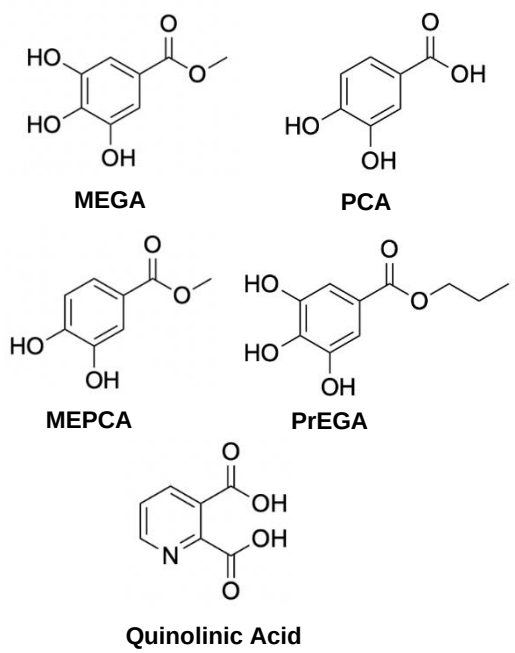


Figure 1. Polyphenols in this study: methyl 3,4,5-trihydroxybenzoate (MEGA), methyl 3,4-dihydroxybenzoate (MEPCA), *n*-propyl gallate (PrEGA), protocatechuic acid (PCA, and 2,3-pyridinedicarboxylicacid (quinolinic acid).

neuroprotective effects and to mitigate oxidative stress.[22,23,24,25] *n*-Propyl gallate (PrEGA) is an ester form of gallic acid used to protect oils and fats from oxidation. PrEGA is known for its high antioxidant capacity in radical scavenging[26] and anticancer effects.[27] Protocatechuic acid (PCA) is a phenolic acid plant metabolite with a catechol group. PCA is reported to have strong antioxidant effects in the prevention and therapy of many neurodegenerative and hepatic diseases, but the mechanisms behind these effects are not well understood.[28] PCA is also shown to have potent antibacterial, anticancer, antihyperlipidemic, antidiabetic, and anti-inflammatory effects.[28]

2,3-Pyridinedicarboxylic acid, also known as quinolinic acid, is an intermediate of the kynurenine metabolic pathway of tryptophan that produces $NADP^+$.[29] At high concentrations, quinolinic acid can act as a neurotoxin and induce oxidative stress in brain tissue *in vitro* and *in vivo*.[30] However, the antioxidant abilities of quinolinic acid have been difficult to measure using the deoxyribose assay, making its role as a prooxidant or antioxidant, or both, unclear.[30] The deoxyribose assay used to test quinolinic acid involving $FeCl_3$, H_2O_2, and ascorbate provided results with substantial errors at high quinolinic acid concentrations that shed little light on its antioxidant or prooxidant activity.[31]

MEGA, MEPCA, PrEGA, and PCA were identified from plasmid DNA gel electrophoresis studies as having strong antioxidant abilities.[32] Gel electrophoresis methods more closely simulate biologically relevant conditions compared to the deoxyribose assay, likely allowing for more accurate determinations of DNA damage or prevention. On the other hand, the simpler deoxyribose assay uses UV-vis spectroscopy to yield a pink chromogen (Figure 2).[33] When 2-deoxyribose is treated with Fe^{2+} and hydrogen peroxide to form hydroxyl radical (Reaction 1), it degrades malonaldehyde (MDA).[33] MDA reacts with thiobarbituric acid (TBA) to form a pink chromogen

that absorbs at 532 nm.[33] Although the deoxyribose assay does not use DNA, the major benefit of the deoxyribose assay is that it allows for a faster screening of DNA damage or prevention by a variety of compounds, including polyphenols.[32]

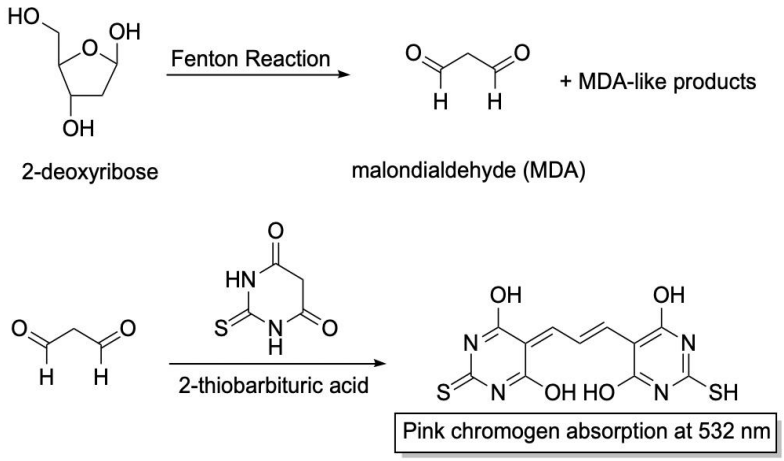


Figure 2. Reaction mechanism from 2-deoxyribose to its TBA-MDA degradation product that can be measured as a pink chromogen with an absorption at 532 nm.

Previous studies using the standard deoxyribose assay have noted or demonstrated low reproducibility when reporting deoxyribose damage prevention by antioxidants.[46,47,48] Therefore, one goal of this work is to modify the deoxyribose assay to increase its reproducibility by using this modified method to test the antioxidant abilities of quinolinic acid and four polyphenols (MEGA, MEPCA, PrEGA, and PCA). These results obtained from this modified deoxyribose assay will be compared to polyphenol prevention of plasmid DNA damage by Fe and H_2O_2 using gel electrophoresis, a lower-throughput method that uses DNA as the substrate. Through the comparison of these two methods, the modified deoxyribose assay will be assessed as a higher-throughput method that could determine or represent DNA damage inhibition accurately.

EXPERIMENTAL METHODS

Materials. 2-Deoxy-D-ribose (TCI), 2-(*N*-morpholino)ethanesulfonic acid (Cabiochem), iron (II) sulfate heptahydrate (Thermo Scientific), quinolinic acid (AmBeed) methyl 3,4-dihydroxybenzoate (Alfa Aesar), methyl 3,4,5-trihydroxybenzoate (Alfa Aesar), propyl gallate (Acros Organics), protocatechuic acid (Frontier Scientific), quinolinic acid (AmBeed), 30% H_2O_2 solution (Fisher), 2-thiobarbituric acid (MP Biomedicals), and trichloroacetic acid (VWR) were used as received.

Aqueous Deoxyribose Assay. In 15 mL conical tubes, water (0.8 mL), 2-deoxyribose (0.1 mL of a 33.6 mM stock solution dissolved in MES buffer (1.5 mL of a 96.2 mM stock solution at pH 6 for a final buffer concentration 8.02 mM in the samples) for a final 2-deoxyribose concentration of 2.8 mM), $FeSO_4$ (0.1 mL of a 1200 mM stock solution dissolved in water for a final sample concentration of 100 µM), compound to be tested (0.1 mL of 60, 120, 300, 600, 1200, 2400 µM stock solutions prepared in 1.5 mL microcentrifuge tubes were transferred to the conical tubes for a final sample concentration of 5, 10, 25, 50, 100 or 200 µM, respectively; water was used for Control 1), and H_2O_2 (0.1 mL of a 30 mM stock solution for a final sample concentration of 2.5mM) to reach a final sample volume of 1.2 mL) were added sequentially. Between each addition, the sample were mixed briefly using a vortex mixer. After H_2O_2 addition, the conical tubes were then centrifuged for 1 min at 3,200 rpm and then allowed to stand at room temperature for 30 min to damage the deoxyribose. To develop the assay samples, TBA (1 mL, 1% w/v in 50 mM NaOH) and TCA (1 mL, 2.8% w/v in water) were added to each sample. The samples then were placed in a water bath at 100° C for 20 min and cooled for an additional 20 min. Using a UV-vis spectrometer (Thermo Scientific GENESYS^{TM} 40/50 Vis/UV-Vis Spectrophotometers), sample absorbances were obtained at 532 nm.

Control 1: A positive control with 0.1 mL water added instead of 0.1 mL polyphenol compound was run for every trial to determine the maximum deoxyribose damage.

Control 2: Because the deoxyribose-Fe^{3+} complex also has an absorbance at 532 nm,[34] a control with water, MES buffer, deoxyribose, $FeCl_3$, and H_2O_2 was also performed to determine this interfering absorbance.

Data analysis. All samples were run in triplicate and values reported are means with standard deviations. To find the percent deoxyribose damage inhibition, the following formula was used: (1 - (Abs of test sample - Control 2) / Control 1)) * 100. Kubicova *et al.*[30] used thiobarbituric acid reactive species (TBARS) to measure deoxyribose damage, so in our assay, the percent damage inhibition calculated was subtracted from 100, since the opposite of inhibition is damage. Data tables for all deoxyribose assay results are shown in the supporting information (Tables S1-S5). To compare *p*-values in the deoxyribose assay, a t-test was used to determine if the triplicate percent deoxyribose damage inhibition values at the respective compound concentrations were statistically different than maximum deoxyribose damage. To determine *p*-value comparisons between deoxyribose assay data and data from plasmid DNA electrophoresis studies, a comparison of means test was used.

RESULTS AND DISCUSSION

Deoxyribose Assay Modifications to Increase Reproducibility. The deoxyribose assay with Fe^{3+}, ascorbate, and hydrogen peroxide is reported to have difficulties with reproducibility and large errors.[35,36,37] To improve assay reproducibility, we made several modifications to the assay reagents and procedures. First, ascorbate is commonly added in the deoxyribose assay to reduce Fe^{3+} (from $FeCl_3$) to Fe^{2+}, which is oxidation state needed to react with hydrogen peroxide to form

hydroxyl radical (Reaction 1). However, ascorbate is also a known antioxidant,[38] making it difficult to determine whether observed damage inhibition is due to the ascorbate or the added antioxidant compound. Specifically, ascorbate acts as a donor of single reducing equivalents (H or $H^+ + e-$) and cycles between the fully reduced ascorbate and its radical anion, monodehydroascorbate.[39] Monodehydroascorbate reacts preferentially with other radicals, making it not just a radical scavenger but also a terminator of radical chain reactions.[40] In addition, the ascorbate concentration is high compared to $FeCl_3$, (1000 μM vs. 50 μM, respectively, as exemplified in one study[35]), which can reduce Fe^{3+} to Fe^{2+}, allowing redox cycling of the iron and to the formation additional hydroxyl radical (Reaction 1). To address this issue, we used an Fe^{2+} salt ($FeSO_4$) that does not require iron reduction to generate hydroxyl radical and eliminated ascorbate from the deoxyribose assay method.

Second, Phosphate-buffered saline (PBS) Buffer is also commonly used in the deoxyribose assay;[34,41,42] however, Fe^{3+} from $FeCl_3$ can precipitate in PBS buffer at near-neutral pH, and these assays are commonly conducted around a pH of 7.4. Additionally, the phosphates in PBS buffer coordinate iron, requiring more iron addition to see deoxyribose damage, worsening potential precipitation issues. To prevent iron precipitation and lower the iron concentrations required in this assay, the non-metal-binding 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer was used at pH 6, which reduces precipitation and has been shown to have no complex formation between MES and Fe^{2+}.[43, 44]

Third, in typical deoxyribose assay protocols, H_2O_2 is added before the antioxidant compound to be tested. However, this promotes deoxyribose damage before the antioxidant is added. In our modified protocol, H_2O_2 was added last to ensure that hydroxyl radical is not generated until all the components are added, reducing systematic errors in this method.

A caveat in the literature for the deoxyribose assay is that oxidized Fe^{3+} binds to deoxyribose and creates an interfering absorbance at 532 nm.[34] Despite this report, several papers describing the deoxyribose assay do not account for this Fe^{3+}-interference and therefore underestimate the deoxyribose damage inhibition properties of the tested compounds. To account for this interference, we performed a control reaction with water, MES buffer, deoxyribose, $FeCl_3$, and H_2O_2, measured its absorbance at 532 nm, and subtracted out this absorbance to account for formation of the Fe^{3+}-deoxyribose complex.

Using our modified assay, we tested the ability of quinolinic acid, a neurotoxin that can both induce oxidative stress in brain tissue and act as an antioxidant depending on concentration.[30] Previous studies have discussed the difficulty of accurately measuring the deoxyribose damage inhibition at high quinolinic acid concentrations.[30] In Kubicova *et al*., the deoxyribose assay of quinolinic acid using $FeCl_3$, ascorbate, and H_2O_2 calculated the deoxyribose damage using percentage of thiobarbituric acid reactive species (TBARS) formed.[31] For the modified deoxyribose assay, in contrast, we calculated its inverse, as percentage of deoxyribose damage inhibition and converted this value to percent TBARS formed to compare these methods.

At high quinolinic acid concentrations, our deoxyribose assay showed a % TBARS formation of 52.9 ± 4.76 % at 200 µM, which decreased to a low of 25.8 ± 1.40 % at 100 µM before increasing to a high of 96.1 ± 1.71 % at 5 µM (Figure 3A). This U-shaped effect is seen in both Kubicova *et al*. and our deoxyribose assay (Figure 3A and 3B), which indicates that at low concentrations, quinolinic acid shows antioxidant abilities through Fe^{2+} coordination, but at high concentrations, the toxicity of quinolinic acid[30,31] seems to act in a prooxidant manner by inducing furthermore production of the hydroxyl radical.

Furthermore, the deoxyribose assay data from Kubicova *et al*. with $FeCl_3$, ascorbate, and H_2O_2 for quinolinic acid deoxyribose damage (TBARS) was compared to that seen in our deoxyribose assay. TBARS formation at the corresponding quinolinic acid concentrations is lower for our modified deoxyribose results. This systematic difference indicates that deoxyribose damage inhibition by quinolinic acid is greater in our modified deoxyribose assay. This difference is unsurprising, since Kubicova *et al*.[31] did not report subtracting the deoxyribose-Fe^{3+} complex absorbance interference at 532 nm,[34] likely leading to an artificial decrease in the reported deoxyribose damage inhibition values.

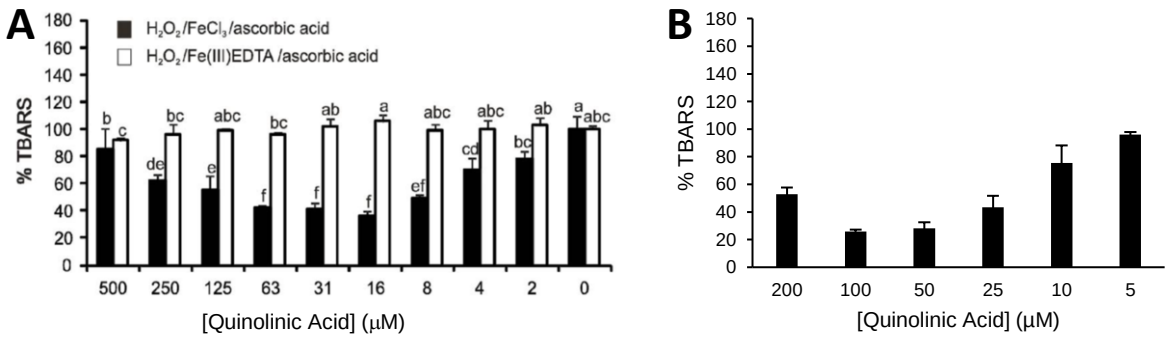


Figure 3. A) Percent TBARS formation vs. quinolinic acid concentration reported by Kubicova *et al*. (Kubicova, L.; Hadacek, F.; Chobot, V. *Int. J. Mol. Sci.* **2013**, *14*, 21328–21338). Black bars represent the deoxyribose assay using the $H_2O_2/FeCl_3$/ascorbate system and indicates formation of quinolinic acid-Fe coordination complexes, and the white bars represent a control that confirms noncomplexed quinolinic acid. B) Percent TBARS formation vs. quinolinic acid concentration in our modified deoxyribose assay.

The calculated standard deviations in the modified deoxyribose assay data appear similar to or less than those reported by Kubicova *et al* (Figure 3).[31] Since Kubicova *et al*. did not report subtracting out the additional absorbance from the deoxyribose-Fe^{3+} interactions (~20-30% of the maximum deoxyribose damage signal in our modified assay). If this correction were made, the error bars in the graph in Figure 3A would be significantly larger. Results from our modified deoxyribose assay also follow the U-shaped trend of increased TBARS formation, similar to the trend reported by Kubicova *et al*.[31] In our studies, greater than 200 μM quinolinic acid stock solutions were found

to start precipitating, which is why higher concentrations from >200 µM to 500 µM, could not be tested to definitively establish the U-shaped trend. However, in our modified deoxyribose assay, the trend of leveling off to sharp increase at 50 µM to 200 µM, respectively, is also seen in Kubicova *et al.* from approximately 31 µM to 500 µM (Figure 3B), giving evidence for the U-shaped trend. From this preliminary U-shaped trend observed in our assay, this could show that our modified deoxyribose assay follows trends seen in *in vivo*, such as quinolinic acid acting as a neurotoxin at high concentrations. Furthermore, this could implicate our modified deoxyribose assay as a method to test other classes of antioxidants as well for larger-scale use in the future.

Comparing Deoxyribose and Plasmid DNA Damage Inhibition. To determine how damage inhibition measured using the modified deoxyribose assay compares to damage inhibition measured by plasmid DNA electrophoresis assays, four polyphenol compounds (Figure 1) were tested using both methods. In the modified deoxyribose assay, MEPCA showed a dose-dependent response for deoxyribose damage inhibition with 3 $\pm$ 5 % inhibition at 5 µM MEPCA rising to 87 $\pm$ 2 % inhibition at 200 µM MEPCA (Figure 4A). The standard deviations for the values shown in Figure 3A were between 2 and 11%, with an average standard deviation of 5%.

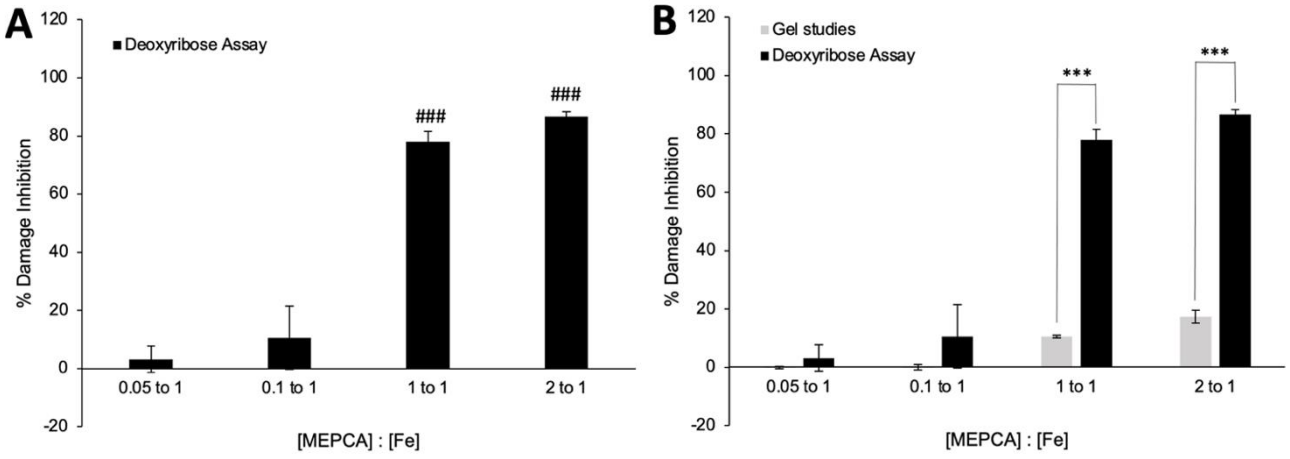


Figure 4. A) Percent Damage Inhibition vs. [MEPCA] : [Fe] in our modified deoxyribose assay. B) Percent Damage Inhibition vs. [MEPCA] : [Fe] in our modified deoxyribose assay and gel studies reported by Perron *et al.* (Perron, N.; Hodges, J.; Jenkins, M.; Brumaghim, J. *Inorg*. *Chem*. **2008**, *47*, 6153–6161). Black bars: percent damage inhibition for MEPCA measured using the modified deoxyribose assay; grey bars: percent DNA damage inhibition

using plasmid DNA gel electrophoresis. All samples were run in triplicate and values reported are means with standard deviations. ###$p < 0.001$ when compared to maximum deoxyribose damage inhibition. ***$p < 0.001$ when compared to plasmid DNA gel electrophoresis data.

Because DNA gel electrophoresis methods use substantially less Fe^{2+} than the modified deoxyribose assays (2 vs. 100 μM, respectively[32]), comparisons between these methods were made by calculating [MEPCA] : [Fe] ratios for the different assays (Figure 4B). In the modified deoxyribose assays, MEPCA showed approximately 4-6-fold more damage inhibition than measured using plasmid DNA electrophoresis at higher MEPCA concentration ratios where the differences were determined to be statistically significant. This large, systematic difference between deoxyribose damage inhibition measured using only deoxyribose or full DNA is likely due to competition between polyphenol vs. DNA for iron binding compared to polyphenol vs. only deoxyribose for iron binding. Deoxyribose has significantly weaker affinity for iron binding compared to the phosphates and nucleobases of DNA, making polyphenol-iron binding the major contributor to deoxyribose damage inhibition. Polyphenol-iron binding is known to inhibit deoxyribose cleavage,[45] so the greater polyphenol-iron interactions in the modified deoxyribose assay likely leads to greater deoxyribose damage inhibition.

Similar to MEPCA, MEGA also showed a dose-dependent response in deoxyribose damage inhibition to a maximum at 73 ± 1 % inhibition at 200 μM (Figure 5A). Under these assay conditions, MEGA concentrations greater than 200 μM showed observable precipitation. Comparing deoxyribose and plasmid DNA assay results, MEGA showed approximately 2-6-fold more damage inhibition in the deoxyribose assay (Figure 5B), similar to comparison results for MEPCA.

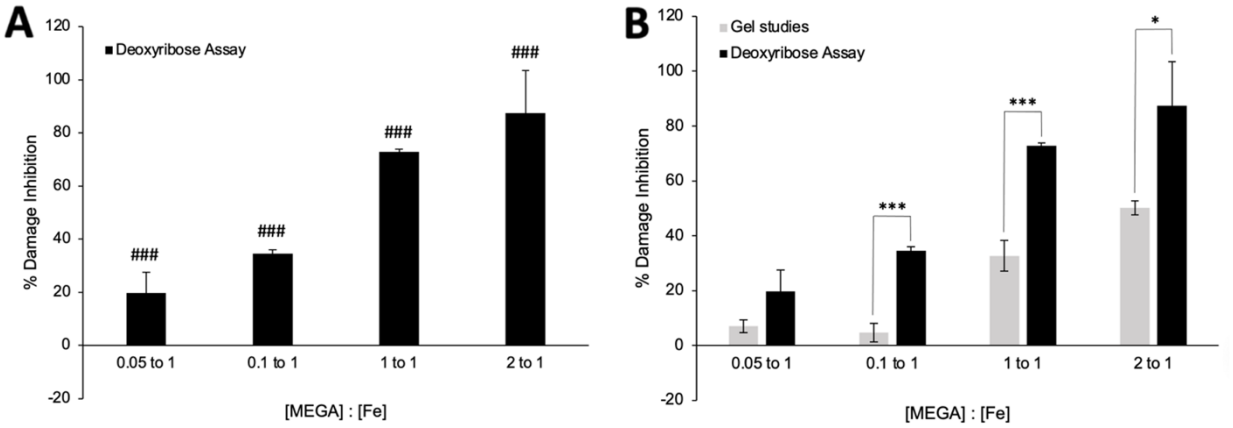


Figure 5. A) Percent Damage Inhibition vs. [MEGA] : [Fe] in our modified deoxyribose assay. B) Percent Damage Inhibition vs. [MEGA] : [Fe] in our modified deoxyribose assay and gel studies reported by Perron *et al.* (Perron, N.; Hodges, J.; Jenkins, M.; Brumaghim, J. *Inorg*. *Chem*. **2008**, *47*, 6153–6161). Black bars: percent damage inhibition for MEGA measured using the modified deoxyribose assay; grey bars: percent DNA damage inhibition using plasmid DNA gel electrophoresis. All samples were run in triplicate and values reported are means with standard deviations. ###$p < 0.001$ when compared to maximum deoxyribose damage inhibition. ***$p < 0.001$ when compared to plasmid DNA gel electrophoresis data. *$p < 0.05$ when compared to plasmid DNA gel electrophoresis data

PrEGA also showed a dose-dependent response for deoxyribose damage inhibition (Figure 6A). At low PrEGA concentrations (5 and 10 µM), deoxyribose inhibition of deoxyribose damage was not statistically different from 0 but rose to a maximum of 96 ± 4 at 10 µM. PrEGA is reported to have high antioxidant abilities,[23] and the high deoxyribose damage inhibition values found for PrEGA are consistent with these results. Similar to MEPCA and MEGA, PrEGA showed approximately 2-3-fold more damage inhibition in the deoxyribose assay than in plasmid DNA electrophoresis studies (Figure 6B).

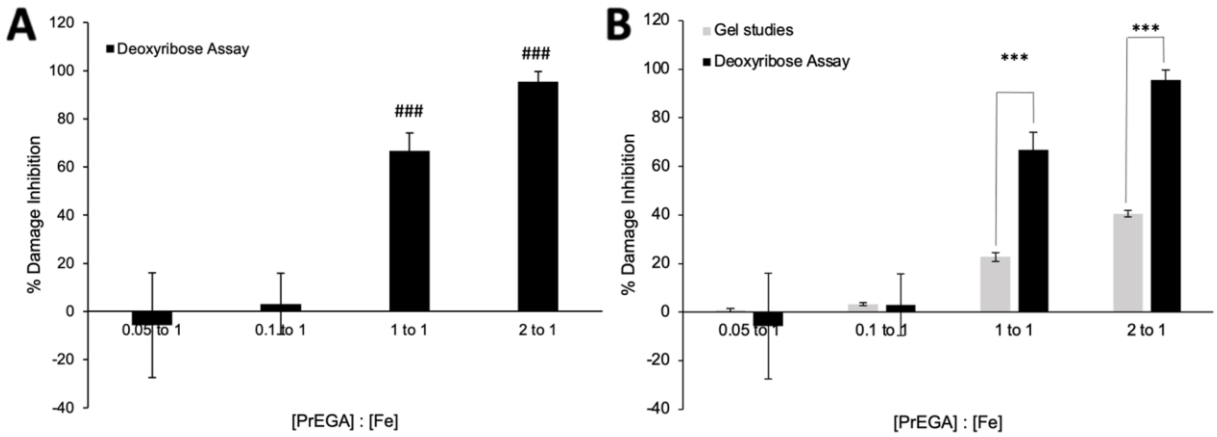
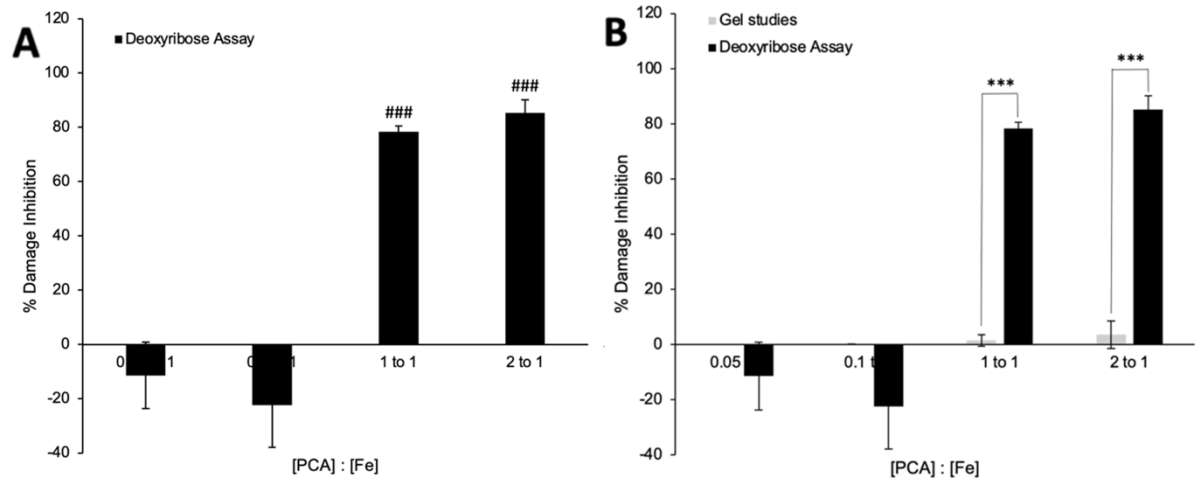


Figure 6. A) Percent Damage Inhibition vs. [PrEGA] : [Fe] in our modified deoxyribose assay. B) Percent Damage Inhibition vs. [PrEGA] : [Fe] in our modified deoxyribose assay and gel studies reported by Perron *et al.* (Perron, N.; Hodges, J.; Jenkins, M.; Brumaghim, J. *Inorg*. *Chem*. **2008**, *47*, 6153–6161). Black bars: percent damage inhibition for PrEGA measured using the modified deoxyribose assay; grey bars: percent DNA damage inhibition using plasmid DNA gel electrophoresis. All samples were run in triplicate and values reported are means with standard deviations. ###$p < 0.001$ when compared to maximum deoxyribose damage inhibition. ***$p < 0.001$ when compared to plasmid DNA gel electrophoresis data.

In deoxyribose assays with PCA, the two lowest concentrations (5 and 10 µM) showed negative deoxyribose damage inhibition with high standard deviations that make these values not statistically different from zero (Figure 7A). From 25 to 200 µM, deoxyribose damage inhibition



increased, reaching a maximum of 85 ± 5 % at 200 µM. In comparison with plasmid DNA electrophoresis studies, PCA showed approximately 19-46-fold more damage inhibition (Figure 7B), having the highest fold damage inhibition in comparison to the other polyphenols tested.

Figure 7. A) Percent Damage Inhibition vs. [PCA] : [Fe] in our modified deoxyribose assay. B) Percent Damage Inhibition vs. [PCA] : [Fe] in our modified deoxyribose assay and gel studies reported by Perron *et al.* (Perron, N.; Hodges, J.; Jenkins, M.; Brumaghim, J. *Inorg. Chem.* **2008**, *47*, 6153–6161). Black bars: percent damage inhibition for PCA measured using the modified deoxyribose assay; grey bars: percent DNA damage inhibition using plasmid DNA gel electrophoresis. All samples were run in triplicate and values reported are means with standard deviations. ###$p < 0.001$ when compared to maximum deoxyribose damage inhibition. ***$p < 0.001$ when compared to plasmid DNA gel electrophoresis data.

We have compared two methods to assess the inhibition of iron-mediated damage by polyphenols: DNA gel electrophoresis and the modified deoxyribose assay. The gel electrophoresis methods have plasmid DNA as its substrate, which more closely resembles biological conditions, while the substrate of the deoxyribose assay is not DNA, but rather 2-deoxyribose, which is the primary site of DNA damage as measured in plasmid DNA electrophoresis methods. These methods are significantly different in their protocols, since plasmid DNA damage is quantified using gel electrophoresis methods, whereas damage in the deoxyribose assay is quantified using UV-vis spectroscopy. The deoxyribose assay serves as a lower-cost, faster screening method to compare antioxidant or prooxidant activity among compounds, so the ability to correlate deoxyribose assay results with more biologically relevant plasmid DNA damage results would be a significant advancement.

From the results of our comparisons with four polyphenol compounds, plasmid DNA electrophoresis methods show less relative DNA damage prevention in comparison to the spectroscopic deoxyribose assay. The difference observed between these methods illustrates the differences between DNA and deoxyribose substrates in these assays. Iron binds to the bases and phosphates in DNA, leading to competition with polyphenol compounds for iron binding. Specifically, Fe^{2+}/H_2O_2-mediated cleavage in DNA relies upon the ability of Fe^{2+} to selectively interact with the N7 position of guanine bases to cause deoxyribose damage and cleavage,[2] similar to that measured in the deoxyribose assay. This increased binding of Fe^{2+} to N7 sites in guanine, coupled with more iron localization on other DNA bases and phosphates, likely leads to less iron-

polyphenol coordination in the plasmid DNA electrophoresis assays and thus less damage prevention. In contrast, the deoxyribose substrate does not have DNA base or phosphate sites for iron localization, and therefore, polyphenol-Fe^{2+} interactions dominate in this system. As a result of less competition, more polyphenol-Fe^{2+} interactions cause greater deoxyribose damage prevention ability as seen in our results. Although the deoxyribose assay overestimates the deoxyribose damage prevention abilities of polyphenols, this trend was relatively consistent for MEPCA, MEGA, and PrEGA, with a consistent 2-6-fold increase in deoxyribose damage inhibition relative to DNA damage inhibition. In contrast, PCA showed 19-46-fold more damage inhibition, indicating that factors in addition to polyphenol-iron binding competition with DNA binding sites at play in this system.

CONCLUSIONS

Polyphenol antioxidant properties have been previously measured through lower-throughput, time-consuming plasmid DNA electrophoresis methods, and we have developed a more robust deoxyribose assay to measure these antioxidant abilities in a more economical and higher-throughput manner. Our modified deoxyribose assay protocols remove factors that could affect reproducibility, such as ascorbate antioxidant activity, metal-coordinating buffers, and deoxyribose-Fe^{3+} complex absorbance interference. These conditions are frequently not accounted for in reports of deoxyribose assay results, calling into question the reliability of results obtained from these assays.

A comparison of polyphenolic antioxidant abilities shows that the deoxyribose assay tends to overestimate the percent deoxyribose damage inhibition when compared to plasmid DNA electrophoresis studies, likely due to the respective iron-binding affinities between deoxyribose

and DNA. However, the deoxyribose assay results generally follow trends reported from the plasmid DNA electrophoresis studies, showing promise in using this revised assay as a more efficient and faster means to test antioxidant activity.

FUTURE WORK

For future work, we will test eight additional polyphenol compounds for deoxyribose damage inhibition that were tested using plasmid gel electrophoresis studies[32] as well as four additional compounds that were tested using previous deoxyribose assay methods[46,47,48] to establish the modified deoxyribose assay as a reproducible screening method (Figure 8).

One of these is Vanillic Acid (VA), which is known as a less metal-binding compound due to its single phenol group. The deoxyribose damage inhibition abilities of VA can serve as a control and reference point for the inhibition abilities of the other polyphenols in Figure 8 that may have multiple phenol groups, such as gallol or catechol groups.

By testing additional compounds, a structure-activity comparison can discern trends between various polyphenol structure components such as gallol and catechol groups to their deoxyribose damage inhibition abilities. These trends will then be compared to the DNA damage prevention trends to further establish the role of our modified deoxyribose assay. Additionally, future studies will use the deoxyribose assay to test Cu^{+} instead of Fe^{2+} under similar assay conditions, since Cu^{+} also generates damaging hydroxyl radical (Reaction 1). Currently, there is little literature on Cu^{+} in measuring deoxyribose damage inhibition by polyphenols, and testing Cu^{+} vs. Fe^{2+} will be useful in distinguishing the role of metals in their damage prevention abilities. We are also focusing on development of a higher-throughput method for this assay using 96-well plates. Overall, this work will establish a new deoxyribose assay method as a low-cost, higher-

throughput screening method for future classes of antioxidants. In conjunction with gel electrophoresis methods, this new deoxyribose assay can act as a higher-throughput method for determining DNA damage inhibition from antioxidants.

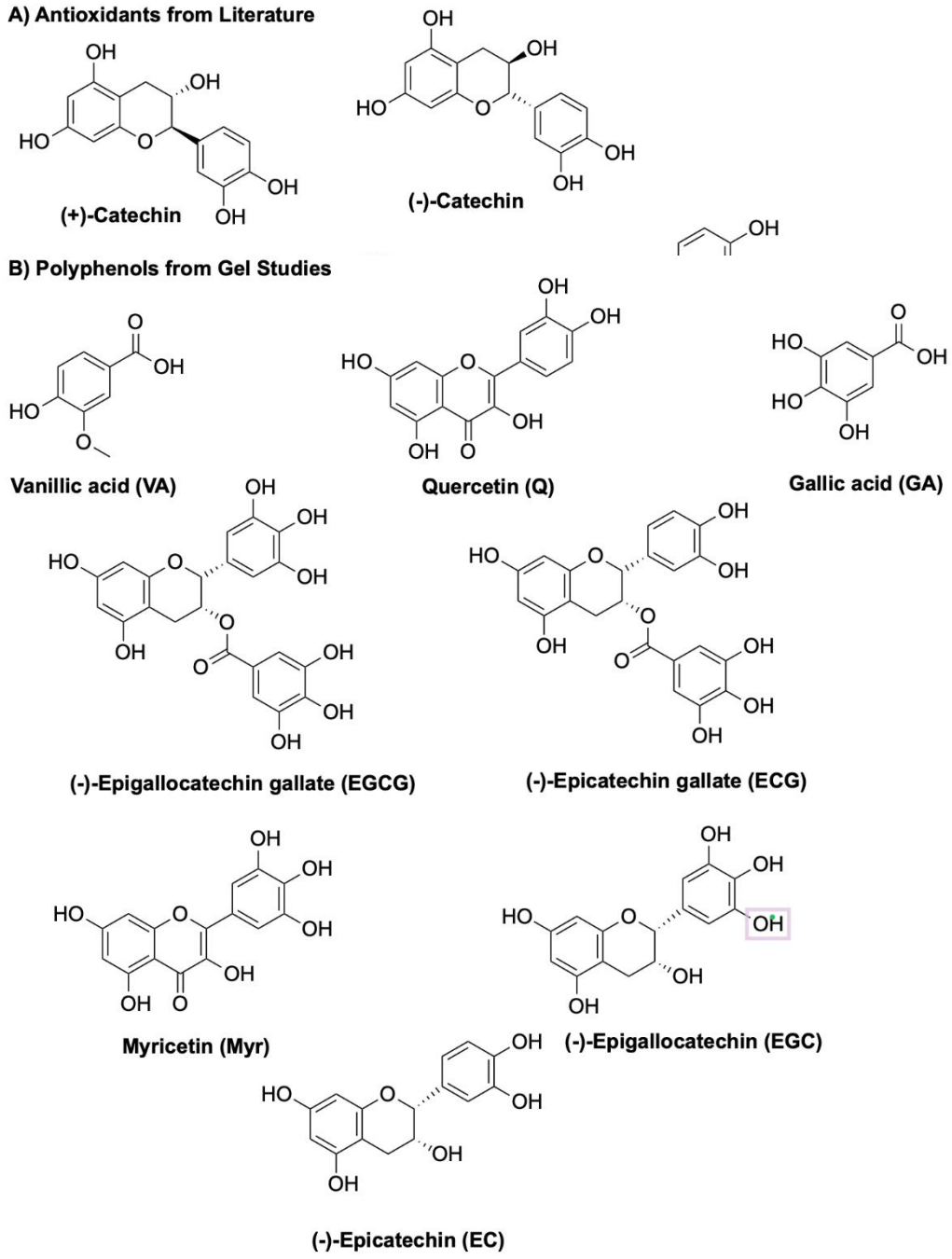


Figure 8. A) Panel of polyphenols identified from literature that previously displayed high error bars and low reproducibility that will be tested in future work.[46,47,48] B) Panel of polyphenols identified from previous plasmid DNA gel studies that will be tested in future work.[32]

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Funding Sources

NSF Grant CHE-1807709, Clemson University Honors College, and the Clemson University Creative Inquiry program.

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SUPPLEMENTAL INFORMATION

Table S1. Percent deoxyribose damage inhibition for quinolinic acid in the modified deoxyribose assay.

[Quinolinic Acid], μM	% Deoxyribose Damage Inhibition	*p*-Value Comparison with Maximum Inhibition
5 μM	3.91 ± 1.71	< 0.05
10 μM	24.4 ± 12.6	< 0.01
25 μM	56.5 ± 8.25	< 0.001
50 μM	71.8 ± 4.33	< 0.001
100 μM	74.2 ± 1.40	< 0.001
200 μM	47.1 ± 4.76	< 0.001

All data are reported as the average of three trials with calculated standard deviations.

Table S2. Percent deoxyribose damage inhibition for MEPCA in the modified deoxyribose assay.

[MEPCA], μM	% Deoxyribose Damage Inhibition	*p*-Value Comparison with Maximum Inhibition	*p*-Value Comparison to Plasmid DNA Studies[32]
5 μM	3.18 ± 4.57	0.294	0.271
10 μM	10.6 ± 10.9	0.169	0.167
25 μM	20.4 ± 5.32	< 0.01	N.D.
50 μM	57.9 ± 4.30	< 0.001	N.D.
100 μM	77.9 ± 3.61	< 0.001	< 0.001
200 μM	86.6 ± 1.64	< 0.001	< 0.001

All data are reported as the average of three trials; the calculated standard deviations are shown. N.D. indicates that this polyphenol concentration was not done in the plasmid DNA electrophoresis assays.

Table S3: % Deoxyribose Damage Inhibition for MEGA in our deoxyribose assay

[MEGA], in μM	% Deoxyribose Damage Inhibition	*p*-Value Comparison with Maximum Inhibition	*p*-Value Comparison to Plasmid DNA Studies[32]
5 μM	19.7 ± 7.84	< 0.001	0.0549
10 μM	34.4 ± 1.45	< 0.001	< 0.001

25 μM	61.4 ± 1.43	< 0.001	N.D.
50 μM	71.3 ± 1.08	< 0.001	N.D.
100 μM	72.9 ± 0.966	< 0.001	< 0.001
200 μM	87.4 ± 16.2	< 0.001	< 0.05

All data are reported as the average of three trials; the calculated standard deviations are shown. N.D. indicates that this polyphenol concentration was not done in the plasmid DNA electrophoresis assays.

Table S4: % Deoxyribose Damage Inhibition for PrEGA in our deoxyribose assay

[PrEGA], in μM	**% Deoxyribose Damage Inhibition**	***p*-Value Comparison with Maximum Inhibition**	***p*-Value Comparison to Plasmid DNA Studies[32]**
5 μM	-5.74 ± 21.7	0.616	0.623
10 μM	3.07 ± 12.7	0.645	0.977
25 μM	22.0 ± 22.1	0.0940	N.D.
50 μM	62.6 ± 3.15	< 0.001	N.D.
100 μM	66.7 ± 7.43	< 0.001	< 0.001
200 μM	95.5 ± 4.13	< 0.001	< 0.001

All data are reported as the average of three trials; the calculated standard deviations are shown. N.D. indicates that this polyphenol concentration was not done in the plasmid DNA electrophoresis assays.

Table S5: % Deoxyribose Damage Inhibition for PCA in our deoxyribose assay

[PCA], in μM	**% Deoxyribose Damage Inhibition**	***p*-Value Comparison with Maximum Inhibition**	***p*-Value Comparison to Plasmid DNA Studies[32]**
5 μM	-11.4 ± 12.3	0.183	N.D.
10 μM	-22.4 ± 15.6	0.0676	0.0668
25 μM	27.0 ± 5.03	< 0.001	N.D.
50 μM	61.8 ± 2.50	< 0.001	N.D.
100 μM	78.4 ± 2.16	< 0.001	< 0.001
200 μM	85.3 ± 4.84	< 0.001	< 0.001

All data are reported as the average of three trials; the calculated standard deviations are shown. N.D. indicates that this polyphenol concentration was not done in the plasmid DNA electrophoresis assays.