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Evaluation of pH and concentration effects on antioxidant and pro-oxidant activities of lysine- and lysine methyl ester-based antioxidant tetramers derivatized with syringaldehyde and vanillin

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Abstract

Previous studies indicated that antioxidant (AO)/pro-oxidant (PO) activities could depend on pH/concentrations. To see if antioxidants carrying multiple hindered phenolic units are also affected by the pH/concentrations, we synthesized tetrameric antioxidants with different solubilities: lysine-based hydrophilic antioxidants and lysine methyl ester-based hydrophobic antioxidants, each carrying four hindered phenolic units (*ortho*-methoxy containing syringaldehyde or vanillin).

The AO activities of the synthesized antioxidants were measured using AAPH-derived radicals and pBR322 plasmid DNA. Hydrophilic sodium ascorbate and hydrophobic quercetin were used as comparison standards. Based on the study, all tetrameric antioxidants and the standards in PBS showed increasing AO activities as concentrations increased. In the study of pH effects on AO activities using pH 4, pH 7, and pH 9 buffers, the AO activities of all hydrophilic and hydrophobic antioxidants increased with increasing pH (pH 9 > pH 7 > pH 4), indicating that their AO activities are pH-dependent.

In the PO assay employing Cu(II) ions and pBR322, sodium ascorbate and quercetin in PBS produced strong PO effects, which increased as concentrations increased. In the buffers at pH 4, 7, and 9, sodium ascorbate and quercetin showed PO effects in the order of pH 9 > pH 7 > pH 4. Within the same pH level, both sodium ascorbate and quercetin produced significantly stronger PO effects at high concentrations than at low concentrations, suggesting that PO effects are concentration-dependent as well as pH-dependent."

According to the results obtained from sodium ascorbate and quercetin, both AO and PO activities increase as pH/concentrations increase, indicating that a powerful antioxidant can also function as a powerful pro-oxidant. However, the tetrameric antioxidants synthesized with hindered phenolic building blocks did not show any significant PO effects regardless of the pH/concentrations, although they showed strong AO activities. It suggests that sterically crowded phenolic units are beneficial in building potent antioxidants without PO effects. None of our tetrameric antioxidants showed the concentration- and pH-dependent activity crossover from AO to PO and vice versa.

1. Introduction

It is well-known that oxidative stress caused by free radical damage is associated with the pathogenesis of various human diseases, indicating that antioxidants can contribute to preventing diseases in which oxidative stress is the underlying problem. There have been many attempts to

investigate the therapeutic potential of antioxidants for human disease treatment using individual antioxidants, a mixture of antioxidants, or antioxidants along with drug molecules (combination therapy).[1,2,3,4,5,6]

Antioxidant (AO) potency, in terms of free radical scavenging, is known to be related to the number of phenolic hydroxyl (OH) groups because antioxidants donate a hydrogen atom (or electron) of the OH group to quench radicals.[7,8,9] Electron-donating groups that are *ortho*/*para* to the OH group increase hydrogen atom donating potential by stabilizing the phenoxyl radical that is formed after the hydrogen atom donation, meaning the electron-donating group contributes to enhancing antioxidant efficacy.[10,11,12,13] Extended π-bonds on the AO also contribute to increasing antioxidant potency by stabilizing the resulting AO radical via resonance stabilization.[14] A majority of natural antioxidants with these features produce potent antioxidant efficacy but unfortunately, they are also known to produce pro-oxidant (PO) side effects, limiting their use in bio-applications.

According to previous reports, the AO/PO activities of antioxidants are affected by certain factors. Concentration is one of the factors, producing confounding AO/PO effects. For example, Trolox produces AO effects at low concentrations (2.5-15 μM) in HeLa cells while it exhibits PO effects at high concentrations.[15] Catecholestrogens show PO activities at low concentrations but AO activities at high concentrations in human low-density lipoprotein incubated with cupric sulfate.[16] On the other hand, curcumin at low concentrations (<10 μM) prevents glutathione depletion (AO action) but decreases glutathione levels (PO action) at high concentrations.[17] Vitamin C supplementation at 500 mg/day exhibited an increased level of 8-oxoadenine, a biomarker for DNA damage caused by oxygen radicals, in DNA isolated from lymphocytes.[18] However, doses below 500 mg/day produced AO effects. Seo et al.[19] also reported that the AO effects predominated at low doses (30-100 mg/kg body weight) of vitamin C in ischemia-induced oxidative stress in rats, whereas the PO effects prevailed at high doses (1000 mg/kg body weight). Lycopene and β-carotene were also stated to have concentration-dependent AO/PO effects.[20] Concentration-dependent activity changes observed in complex biological systems have been reported to depend on the prevailing molecular conditions in the tissues, including the concentrations of transition metal ions, oxygen tension, and interactions with biological membranes or other molecules,[21,22,23] as well as dose, time, and locations.[24]

The AO/PO properties are also reportedly affected by pH conditions. For example, caffeic acid and other phenolic acids (or esters) in unilamellar vesicles displayed weak AO activities at pH 4, but their AO activities were significantly enhanced with increasing pH (pH 4–8).[25] Food extracts also exhibited increasing radical scavenging effects with increasing pH values.[26] Jodko-Piórecka et. al.[27] reported that increasing the pH from 5.5 to 7.4 resulted in a significant increase in the rate constants for the reaction between catechol amines and DPPH radicals. In micellar and liposomal systems, bilirubin shows a change in activity at pH 6. Below this pH, it functions as a retardant, while at pH levels between 6 and 9, it acts as an antioxidant.[28] Based on these reports, the AO properties appear to be accelerated at higher pH levels. This increased activity at elevated pH is likely due to the deprotonation of the phenolic hydroxyl group, resulting in the formation of the phenoxide anion, which facilitates electron donation and thereby enhances the antioxidant effect.[29]

The studies reporting the combined effects of pH and concentration on antioxidant activity indicate that antioxidants generally produce dose-dependent pro-oxidant effects at pH levels of 2 to 4, and antioxidant effects at slightly higher pH levels of 5 to 7.[30,31,32] As per these studies, both concentration and pH are responsible for exerting net AO or PO effects. For antioxidants intended for bio-applications, it is important to ascertain the pH and concentration at which they exhibit strong AO efficacy while minimizing PO effects.

Despite many studies on pH effects, only a limited number have examined pH levels above 8. The pKa values of phenolic OH groups in antioxidants typically range from 8 to 10. This means that at pH levels below this range, the phenolic OH groups are predominantly in their neutral forms, while at pH levels above this range, the phenoxide forms become the more dominant species. Therefore, evaluating antioxidants for their AO and PO properties at acidic and neutral pH levels, as well as at basic pH levels where the deprotonation of the phenolic OH groups is likely to occur, will provide a more comprehensive understanding of their activities.

Our research focuses on developing various syringaldehyde or vanillin-derivatized dendritic antioxidants for potential use in oxidative stress-related human disease treatment for combination therapy so that we can tackle the diseases via two different mechanisms, antioxidation by antioxidant and pharmacological action by drug molecules. Both syringaldehyde and vanillin are small *ortho*-methoxy group-containing (hindered) phenolic aldehydes and occur naturally. They have been reported to produce antioxidant properties as well as various beneficial pharmacological effects.[33,34,35,36,37,38,39,40] In our previous studies using them as building blocks for dendrimer synthesis, we observed that their antioxidant activities significantly increased when incorporated into dendritic structures.[41,42,43] For instance, the IC50 values for syringaldehyde and vanillin were measured at 1.8 mM and 7.4 mM, respectively, in the DPPH assay. In contrast, their dendritic derivatives containing eight units exhibited IC50 values of 3.1 μM and 5.4 μM.[41] Additionally, these dendrimers did not produce pro-oxidant effects, which is a significant benefit. In the present study, we synthesized lysine-based hydrophilic antioxidants and lysine methyl ester-based hydrophobic antioxidants by derivatizing them with syringaldehyde or vanillin. We then assessed the effects of concentration and pH (at pH 4, pH 7, and pH 9) on their AO and PO properties. The results will establish a foundation for estimating the properties of future macromolecular dendritic antioxidants with the same or similar surface antioxidant units.

2. Materials and Methods

2.1 Chemicals

Syringaldehyde, vanillin, lysine, lysine methyl ester, sodium ascorbate (BioXtra), quercetin, sodium triacetoxyborohydride ($NaBH(OAc)_3$), 2-picoline borane, copper(II) chloride ($CuCl_2$), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), N,N-diisopropylethylamine (DIPEA), triethylamine (TEA), phosphate-buffered saline (PBS), and ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, tris-acetate-EDTA (TAE) were purchased from Sigma Aldrich (Milwaukee, WI). Sodium phosphate dibasic and monobasic were obtained from Fisher Scientific (Fair Lawn, NJ). All chemicals were used as received without further purification. All reaction solvents, as well as deuterated NMR solvents, were purchased from VWR (Detroit, MI). LC-MS grade acetonitrile and formic acid were sourced from HoneyWell in Germany and Thermo-Scientific, respectively. Ultrapure water was obtained from our Milli-Q UVPlus unit (Millipore, France). Agarose (electrophoresis grade) was obtained from MP Biomedicals (Solon, OH). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Cayman Chemical (Ann Arbor, MI). The plasmid DNA pBR322 was obtained from New England BioLabs (Ipswich, MA) and supplied in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, with a DNA concentration of 1,000 μg/mL. The GelRed DNA stain was purchased from Biotium (Fremont, CA).

2.2 Synthesis Methods

All target compounds were prepared by following the previously reported method with some modifications.[44]

2.2.1 Compound **1** (Lysine methyl ester-syringaldehyde derivative): (S)-N,N-bis(4-hydroxy-3,5-dimethoxybenzyl)-5-(bis(4-hydroxy-3,5-dimethoxybenzyl)amino)-6-oxohexan-1-aminium.

To a 250 mL round-bottom flask, lysine methyl ester dihydrochloride (1.002 g, 4.298 mmol, 233.14 g/mol) was added, followed by the addition of 100 mL of 1,2-dichloroethane. After stirring the reaction mixture for 5 min, DBU (1.28 mL, 8.576 mmol, 152.24 g/mol, d = 1.02 g/mL) was added. Then, two equivalents of syringaldehyde (1.563 g, 8.578 mmol, 182.2 g/mol) were added and stirred for 1 hour. After that, 2 equivalents of $NaBH(OAc)_3$ (1.818 g, 8.58 mmol, 211.9 g/mol) were added, and the reaction was run for 14 h. Another two equivalents of syringaldehyde were added, and the reaction mixture was stirred for 1 h. Subsequently, two equivalents of $NaBH(OAc)_3$ were added. The reaction was run for 48 h.

After confirming that the target compound had formed as the major component, the reaction mixture was filtered, and the filtrate was dried on a rotary evaporator. The resulting residue was re-dissolved in acetone (5 mL) and mixed with silica gel (10 g) to make a silica slurry, which was then allowed to dry. The dried silica gel mixture was loaded onto a flash chromatography column containing 40 g of silica gel and purified using a gradient hexane-ethyl acetate (5:1 to 1:1) solvent system (details are provided in section 2.3, Purification Methods). The purified target compound was analyzed using an NMR spectrometer and LC/MS(ESI) mass spectrometer, as outlined below (section 2.4 Analysis).

Yield = 32% (1.13 g); Appearance: yellowish-brown flaky solid; R_f = 0.34 (hexane-ethyl acetate = 1:4); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.56 (s, 8H), 3.84 – 3.823 (s + s, 24H), 3.72 (s, 3H), 3.43 – 3.34 (s + s, 8H), 3.30 (t, J = 15.0 Hz, 1H), 2.36 (m, 2H), 1.69 (m, 2H), 1.43 (m, 2H), 1.25 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 147.1, 133.6, 130.8, 105.3, 61.1, 58.4, 56.1, 54.7, 53.4, 51.1, 29.4, 26.8, 24.4; HPLC: one peak at 9.32 min; HR-MS (LC/MS(ESI)-TOF) m/z: $[M + H]^+$ calculated for $C_{43}H_{57}N_2O_{14}$, 825.3804; found, 825.3810.

2.2.2 Compound **2** (Lysine methyl ester-vanillin derivative): (S)-N,N-bis(4-hydroxy-3-methoxybenzyl)-5-(bis(4-hydroxy-3-methoxybenzyl)amino)-6-methoxy-6-oxohexan-1-aminium

In a 250 mL round-bottom flask, lysine methyl ester dihydrochloride (0.4565 g, 1.958 mmol, 233.14 g/mol) and two equivalents of vanillin (0.5958 g, 3.917 mmol, 152.1 g/mol) were mixed, followed by the addition of 100 mL of 1,2-dichloroethane. After stirring the mixture for 0.5 hours, DBU (0.6 mL, 4.02 mmol, 152.24 g/mol, d = 1.02 g/mL) was introduced. The mixture was stirred for 5 hours, after which two equivalents of $NaBH(OAc)_3$ (1.270 g, 5.993 mmol, 211.9 g/mol) were added. The mixture was allowed to react for 14 h. Two additional equivalents of vanillin were added and stirred for 1 hour, followed by the addition of two equivalents of $NaBH(OAc)_3$. The reaction was run for 48 h.

The reaction was monitored with Thin Layer Chromatography (TLC) using a hexane-ethyl acetate (1:2) solvent system. After confirming that the target compound was formed as the major product via LC/MS, the reaction mixture was filtered, and the filtrate was dried using a rotary evaporator. The resulting residue was redissolved in 5 mL of acetone and mixed with 10 g of silica gel to make a slurry, which was then allowed to dry. The dried slurry was loaded onto a commercially available 40 g silica gel flash column and purified with a gradient hexane-ethyl acetate solvent system (5:1 to 1:1).

Yield = 59% (0.81 g); Appearance: flaky orange colored solid material; R_f = 0.36 (hexane-ethyl acetate = 1:2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.82 (m, 12H), 5.60 (br.s, 3H), 3.82–3.80 (s + s, 12H), 3.73 (s, 3H), 3.48–3.34 (s + s, 8H), 3.30 (t, J = 6.0 Hz, 1H), 2.34 (m, 2H) 1.65 (m, 2H), 1.40 (m, 2H), 1.25 (m

2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.7, 146.6, 144.7, 131.6, 121.6, 114.0, 111.1, 60.8, 57.9, 56.0, 54.4, 53.2, 51.1, 29.4, 26.8, 24.4; HPLC: one major peak at 9.49 mins; HR-MS (LC/MS(ESI)-TOF) m/z: $[M + H]^+$ calculated for $C_{39}H_{49}N_2O_{10}$, 705.3382; found, 705.3387.

2.2.3 Compound **3** (Lysine-syringaldehyde derivative): (S)-2,6-bis(bis(4-hydroxy-3,5-dimethoxybenzyl)ammonio)hexanoate

In a 250 mL round-bottom flask, lysine (1.002 g, 6.854 mmol, 146.19 g/mol) was added, followed by 100 mL of anhydrous methanol. The vessel was flushed with argon, and the mixture was stirred for 10 min. Then, DIPEA (2.38 mL, 13.66 mmol, 129.25 g/mol, d = 0.742 g/mL) was added to the mixture, which was stirred for 1 h. Next, two equivalents of syringaldehyde (2.500 g, 13.723 mmol, 182.17 g/mol) were added and stirred for 1 h. Molecular sieves were added to absorb the water by-product. Subsequently, two equivalents of 2-picoline borane (1.463 g, 13.685 mmol, 106.9 g/mol) were added to the mixture, which was stirred for 14 h. Another two equivalents of syringaldehyde were added, and the mixture was stirred for 1 h. Then, two equivalents of 2-picoline borane were added, and the reaction was allowed to run for 72 h.

TLC was run to detect new compounds using a methylene chloride–methanol (7:3) mixture containing 1% TEA. LC/MS was used to confirm the formation of the target compound. Upon completion, the reaction mixture was filtered, and the filtrate was dried using a rotary evaporator. The crude mixture was dissolved in methanol (10 mL) and mixed with silica gel (10 g) to make a slurry, which was then dried using a rotary evaporator. The dried silica gel mixture was loaded onto a 40 g prepacked silica gel column and purified using a gradient solvent system of ethyl acetate and methanol (9:1 to 7:1). Alternatively, the crude mixture was dissolved in 10 mL of THF, directly loaded onto a silica gel column, and purified with the same solvent system.

Yield = 40% (2.22 g); Appearance: deep reddish orange solid; R_f = 0.62 (methylene chloride–methanol = 7:3 in 1% TEA); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.57 (s, 4H) 6.51 (s, 4H), 3.77 (s, 12H), 3.72 (s, 12H), 3.36 (s, 8H), 3.16 (t, J = 7.5 Hz, 1H), 2.32 (m, 2H), 1.67 (m, 2H), 1.42 (m, 2H), 1.25 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.9, 147.0, 146.9, 133.5, 133.3, 132.3, 130.9, 105.2, 105.1, 63.5, 58.1, 56.2, 56.0, 54.9, 53.9, 30.8, 27.0, 25.2; HPLC: one major peak at 8.19 mins; HR-MS (LC/MS(ESI)-TOF) m/z: $[M + H]^+$ calculated for $C_{42}H_{55}N_2O_{14}$, 811.3648; found, 811.3654.

2.2.4 Compound **4** (Lysine-vanillin derivative): (S)-2,6-bis(bis(4-hydroxy-3-methoxybenzyl) ammonio)hexanoate

In a 250 mL round-bottom flask, lysine (1.001 g, 6.847 mmol, 146.19 g/mol) and two equivalents of vanillin (2.0822 g, 13.685 mmol, 152.15 g/mol) were mixed, followed by the addition of 100 mL of dry methanol. The vessel was flushed with argon, and the mixture was stirred for 0.5 h. Afterward, DIPEA (2.39 mL, 13.72 mmol, 129.25 g/mol, d = 0.742 g/mL) was added to the mixture, which was stirred for 14 h, which resulted in complete dissolution. Molecular sieves were added to absorb the water byproduct. Then, two equivalents of 2-picoline borane (1.4643 g, 13.70 mmol, 106.9 g/mol) were added to the reaction mixture, which was allowed to run overnight. An additional two equivalents of vanillin were subsequently added and stirred overnight, followed by the addition of another two equivalents of 2-picoline borane. The reaction continued for 24 hours. TLC (methylene chloride–methanol = 7:3 with 1% TEA) and LC/MS were employed to assess the formation of the target compound. An additional equivalent of vanillin was added to the reaction mixture if the target

compound remained the minor component. The mixture was stirred for 14 h, after which an additional equivalent of 2-picoline borane was added. The reaction was allowed to proceed for 48 h.

The reaction mixture was filtered, and the filtrate was dried using a rotary evaporator. The crude mixture was dissolved in 10 mL of methanol and combined with 10 g of silica gel to form a slurry, which was then dried with a rotary evaporator. The dried slurry was subsequently loaded onto a prepacked 40 g silica gel column and purified using a gradient ethyl acetate-methanol (10:0 to 9:1) solvent system containing 0.5% TEA.

Yield = 57% (2.69 g); Appearance: reddish brown solid material; R_f = 0.73 (methylene chloride–methanol = 7: 3 in 1% TEA); ^{1}H NMR (500 MHz, DMSO) δ 8.81 (br.s, 4H), 6.83 (s, 4H), 6.71 (s, 8H), 3.72 (s, 6H), 3.68 (s, 6H), 3.43 (s, 4H), 3.41 (s, 4H), 3.13 (t, J = 7.5 Hz, 1H), 2.31 (m, 2H), 1.60 (m, 2H), 1.37 (m, 2H), 1.23 (m, 2H); ^{13}C NMR (125 MHz, DMSO) δ 174.5, 147.8, 145.8, 131.0, 115.6, 112.7, 60.6, 55.8, 55.7, 53.9, 49.1, 29.1, 24.3, 7.9; HPLC: one major peak at 8.96 mins; HR-MS (LC/MS(ESI)-TOF) m/z: $[M + H]^+$ calculated for $C_{38}H_{47}N_2O_{10}$, 691.3225; found, 691.3231.

2.3 Purification Methods

All target compounds were purified using commercially available silica gel-based flash chromatography columns (40 g, 230-400 mesh, Luknova, Mansfield, MA, USA) and a solvent delivery module (AnaLogix Bottomless Solvent Reservoir 0962-1). Compounds **1** and **2** were purified using a gradient hexane-ethyl acetate solvent system (5:1→ 1:1). Compounds **3** and **4** were purified with a gradient ethyl acetate-methanol solvent system (9:1 → 7:1). The flow rate for the purification was 20 mL/min. The fractions were analyzed via TLC using the TLC solvent systems indicated in section 2.2 Synthesis Methods. A Spectroline UV lamp (254 nm) was used to visualize the spots.

LC/MS was employed to identify the target compound in every fifth-column fraction. The neighboring fractions were then analyzed with a standalone HPLC system (Hitachi, Japan) to combine similar fractions. This HPLC system includes an autosampler (L-7200), a pump (L-7100), a UV detector (L-7400), and an interface (D-7000). The mobile phase consisted of a gradient of acetonitrile and H_2O, varying from 5% to 95% acetonitrile, with the incorporation of 0.1% trifluoroacetic acid. The flow rate was maintained at 1 mL/min, and the analytes were detected at a wavelength of 214 nm.

2.4 Analysis

^{1}H NMR spectra were recorded on a 500 MHz spectrometer (Bruker, MA, USA). Chemical shifts (δ) are reported in ppm using tetramethylsilane as the internal standard in the deuterated solvent. $CDCl_3$ was used for compounds **1**, **2**, and **3**, while DMSO was used for compound **4**. The concentration of the NMR samples was 20 mg/mL. Coupling constants (J) are given in Hz. The multiplicities of signals are reported as follows: s = singlet, br.s = broad singlet, t = triplet, m = multiplet. ^{13}C NMR spectra were recorded on a 125 MHz spectrometer (Bruker, MA, USA). ^{1}H NMR and ^{13}C NMR spectra for compounds **1**-**4** are shown in S7 of the Supporting Information.

Mass spectra were obtained using either an Ultra-Performance Liquid Chromatography (UPLC)-Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometer (AdvanceBio 6545XT, Agilent, USA) or a High-Performance Liquid Chromatography (HPLC)-Time-of-Flight (TOF) mass spectrometer (G6230B, Agilent, USA). Both UPLC and HPLC systems are equipped with a multi-wavelength Diode Array Detector (Agilent, USA).

The LC/MS samples were prepared at a concentration of 1 ng/mL. In the case of fractions from column chromatography, samples were prepared by three serial dilutions with the matrix (water/acetonitrile = 50/50 containing 0.1 % formic acid). Before injection, all samples were filtered using a syringe filter (Minisart RC4 cellulose membrane with 0.2 μM pore, obtained from Sartorius,

UK). The sample injection volume was 0.3 µL. LC separations were performed on an Agilent C18 column (InfinityLab Poroshell 120 EC-C18, mean particle size: 1.9 µm, inner diameter: 2.1 mm, and column length: 50 mm) using water-acetonitrile gradient systems (5 → 95% acetonitrile) containing 0.1% formic acid at a flow rate of 0.4 mL/min over 10 minutes, with a column oven temperature set at 35 ℃. Mass analysis for all samples was conducted using Dual Agilent Jet Stream Electrospray Ionization (AJS ESI) as the ionizing source under the following conditions: gas temperature at 320 ℃, drying gas flow rate at 8 L/min, nebulizer gas at 35 psi, sheath gas temperature at 350 ℃, sheath gas flow rate at 11 L/min, capillary voltage at 3500 V, nozzle voltage at 1000 V, fragmentor voltage at 120 V, skimmer voltage at 65 V, MS range of 100 – 3000 m/z, and an acquisition rate of 1 spectrum/s. Tuning and calibration of the instrument were performed using an ESI-L low-concentration tuning mix and hexamethoxyphosphazine (0.1 mM HP-0321) purchased from Agilent.

2.5 Software

ACD/ChemSketch and/or ChemDraw programs were used to draw the chemical structures of the synthesized compounds and to determine their IUPAC names. $^{1}H/^{13}C$ NMR signal estimation and NMR data processing were carried out using MestreNova NMRPredict and Mnova NMR, respectively.

2.6 Antioxidant Activity Assay

Antioxidant activity assays were conducted according to previously published methods with modifications.[45] A 1% w/v agarose gel was prepared by adding 50 mL of 1x Tris-Acetate-EDTA (TAE) buffer to 0.5 g of agarose and microwaving it for 2-3 min until completely dissolved. GelRed (5 µL) was mixed into the molten agarose, allowed to cool to room temperature, and then transferred into an Owl EasyCast B1A gel electrophoresis kit (Thermo Fisher Scientific, Detroit, MI, USA). The gels were set for 30-45 min. Hydrophilic compounds were dissolved in ultrapure water to prepare stock solutions (1 mM). The same water was used for serial dilutions (0.5 – 0.00195 mM). AAPH was dissolved in PBS to achieve a concentration of 221 mM. A working DNA (pBR322) solution of 10 ng/µL was prepared by diluting the stock DNA solution (1000 ng/µL) with PBS (e.g., 5 µL of DNA stock solution + 495 µL of PBS). PBS buffer was utilized as blanks for both positive and negative controls.

Hydrophobic antioxidants were initially dissolved in DMSO and subsequently diluted with water to make stock sample solutions (1 mM) containing 1% DMSO. For serial dilutions, water with 1% DMSO was used to prepare 0.5 – 0.00195 mM antioxidant solutions. The 1% DMSO in water served as blanks for both positive and negative controls.

Solution X was prepared by combining the working DNA solution with the AAPH solution in a 20:1 ratio (v/v, e.g., 300 µL working DNA solution + 15 µL AAPH solution).

The negative control contained 52.25 µL of the working DNA solution and either 7.75 µL of PBS (for hydrophilic compounds) or 1% DMSO (for hydrophobic compounds), placed in Eppendorf tube 1. The positive controls included 55 µL of solution X and 5 µL of PBS buffer or 1% DMSO (tube 2). Test samples were prepared by mixing 55 µL of solution X with 5 µL of antioxidants at concentrations ranging from 1 to 0.00195 mM (tubes 3-12).

The final concentrations of antioxidants, AAPH, and DNA were 83.3 µM–0.16 µM, 10 mM, and 9 ng/µL, respectively. The controls and samples were incubated for 1 h at 37 ℃. Following this, 10 µL of gel loading buffer was added to each tube, and 20 µL of the solution was loaded into the gel. The 1x TAE buffer was used as the electrode buffer to conduct gel electrophoresis with the Bio-Rad PowerPac 300 (Hampton, NH, USA) at 100 V for 70 min. Gel imaging was performed using a 312 nm variable intensity transilluminator (Fisher Scientific, Pittsburgh, USA). Gel electrophoresis was repeated three times for each compound.

Antioxidant activity protecting DNA was assessed by comparing test samples with the positive control (AAPH-treated DNA) and the negative control (native Supercoiled (SC) DNA).

Increased intensities of SC bands in test samples relative to the positive control indicate antioxidant effects, whereas increased DNA damage suggests pro-oxidant effects. A comparison with the negative control shows the preservation of native SC DNA.

2.7 Pro-oxidant Effect Assay

The pro-oxidant activity assay was conducted by following previously published methods with modifications.[46] The protocols for the pro-oxidant effect test are identical to the aforementioned antioxidant effect tests, except that $CuCl_2$ is used instead of AAPH.

Agarose gel (1% w/v) was prepared by adding 50 mL of 1x Tris-Acetate-EDTA (TAE) buffer to 0.5 g of agarose and microwaving for 2-3 min until completely dissolved. GelRed (5 µL) was added to the molten agarose, which was then allowed to cool to room temperature before being transferred into an Owl EasyCast B1A gel electrophoresis kit (Thermo Fischer Scientific, Detroit, MI, USA). Gels were allowed to set for 30-45 min.

Hydrophilic compounds were dissolved in ultrapure water to prepare stock solutions (1 mM). The same water was used for serial dilutions (0.5 - 0.00195 mM). Copper(II) chloride ($CuCl_2$) was dissolved in the water with an initial concentration of 0.22 mM. A working DNA (pBR322) solution of 10 ng/µL was prepared by diluting a stock DNA solution (1000 ng/µL) with PBS (e.g., 5 µL DNA stock solution + 495 µL PBS). The PBS buffer served as blanks for all controls.

Hydrophobic antioxidants were first dissolved in DMSO and then diluted with water to create stock sample solutions (1 mM) containing 1% DMSO. For serial dilutions, water with 1% DMSO was used to prepare 0.5 - 0.00195 mM antioxidant solutions. The 1% DMSO in water served as blanks for all controls.

Solution X was made by combining the working DNA solution with the $CuCl_2$ solution in a 20:1 (v/v) ratio (e.g., 300 µL working DNA solution + 15 µL $CuCl_2$ solution).

The negative control contained 52.25 µL of the working DNA solution and either 7.75 µL of PBS (for hydrophilic compounds) or 1% DMSO (for hydrophobic compounds), placed in Eppendorf tube 1. Another control (the second (2^{nd}) negative control) contained 55 µL of solution X and 5 µL of PBS buffer or 1% DMSO (tube 2). Test samples were prepared by combining 55 µL of solution X with 5 µL of antioxidants at concentrations ranging from 1 to 0.00195 mM (tubes 3-12).

The final concentrations of antioxidants, $CuCl_2$, and DNA were 83.3 µM–0.16 µM, 10 µM, and 9 ng/µL, respectively. The controls and samples were incubated for 1 h at 37 ºC. Following that, 10 µL of gel loading buffer was added to each tube, and 20 µL of the solution was loaded into the gel. A 1x TAE buffer was used as the electrode buffer to run the gel electrophoresis utilizing the Bio-Rad PowerPac 300 (Hampton, NH, USA) at 100 V for 70 min. Gel imaging was conducted using a 312 nm variable intensity transilluminator (Fisher Scientific, Pittsburgh, USA). Gel electrophoresis was performed three times for each compound.

If the test samples exhibit greater DNA damage than the 2^{nd} negative control, which contains only DNA and Cu(II) ions, it indicates a PO effect.

2.8 pH Effect Study

The pH 4 buffer was prepared using sodium dihydrogen phosphate, the pH 9 buffer was prepared using disodium hydrogen phosphate, and the pH 7 buffer was prepared by adding the pH 9 buffer to the pH 4 buffer until the pH reached 7.

Hydrophilic antioxidants were initially dissolved in ultrapure water, and the stock solution (1 mM) was then serially diluted with the respective buffers to prepare sample solutions (0.5 – 0.0625 mM) at three different pH levels. The final concentrations of antioxidants were 41.7 μM, 20.8 μM, 10.4 μM, and 5.2 μM. If intact DNA was still observed at the lowest test concentration (5.2 μM), four additional initial concentrations, ranging from 0.03125 to 0.0039 mM (resulting in final concentrations of 2.6 μM, 1.3 μM, 0.65 μM, and 0.33 μM) were tested. AAPH was dissolved in water to make a stock solution (0.5 M), and a working solution (221 mM) was made from the stock solution using the pH buffers to create AAPH in three different pH levels. The final concentration of AAPH was 10 mM. The DNA (supplied in Tris buffer) was also diluted with the respective buffers to prepare working solutions at pH 4, pH 7, and pH 9.

Hydrophobic compounds were dissolved in DMSO, and then the solution was diluted with water to make a stock solution of 1% DMSO in water. This stock solution was then serially diluted with the pH buffers (pH 4, pH 7, and pH 9), each containing 1% DMSO. Working DNA at different pH levels was prepared by diluting the stock DNA solution with the respective buffers containing 1% DMSO to ensure consistency with the test samples. The AAPH solution was made by following the same methods as the hydrophilic antioxidants. The final concentration of AAPH was 10 mM.

For the pro-oxidant effect assay under the test pH conditions, the same protocols used in the antioxidant activity assay were employed, except that $CuCl_2$ was used instead of AAPH. A stock $CuCl_2$ solution (9.385 mM) was prepared in water and working $CuCl_2$ solutions (0.22 mM) at different pH levels were prepared from the stock solution using the respective buffers.

Gel electrophoresis was performed three times for each compound.

2.9 Solubility Study

The solubility of all synthesized antioxidants was evaluated by dissolving each compound in water at a concentration of 1 mM, and the solutions were stirred for two hours. Compounds that did not dissolve within this timeframe were classified as hydrophobic. These hydrophobic compounds were first dissolved in DMSO and then diluted with water to prepare stock solutions containing 1% DMSO. Sodium ascorbate was used as the hydrophilic standard for comparison, while quercetin served as the hydrophobic standard.

3. Results and Discussion

3.1 Chemistry

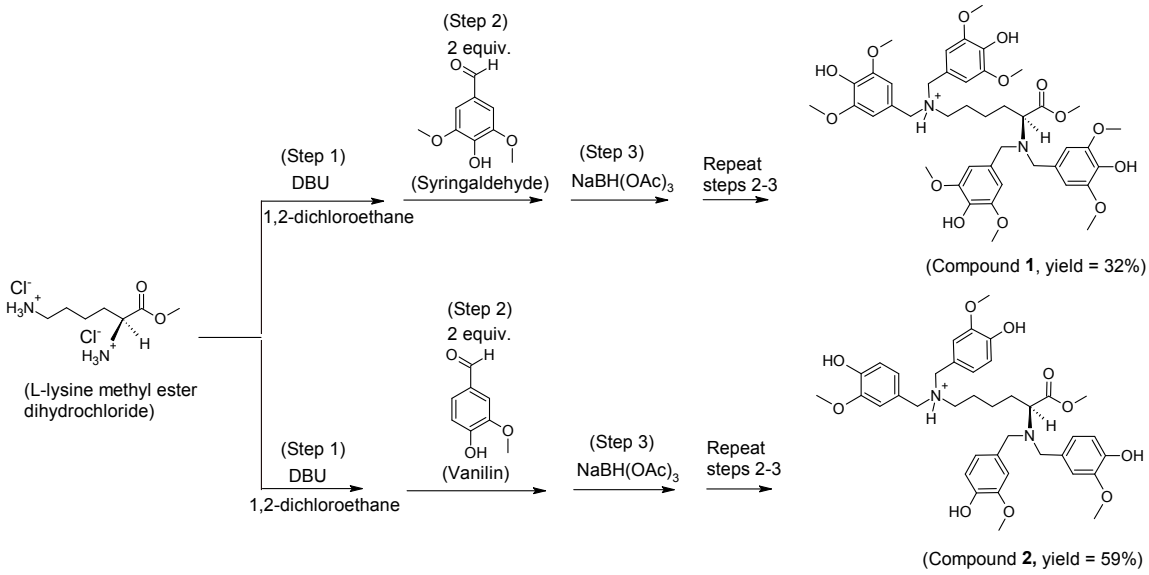
Tetrameric antioxidants carrying four phenolic antioxidant units were synthesized. Lysine methyl ester was used as the scaffold to create hydrophobic antioxidant tetramers (Scheme 1) and lysine, to form hydrophilic antioxidants (Scheme 2). Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) and vanillin (4-hydroxy-3-methoxybenzaldehyde) were used as antioxidant-producing building blocks (BBs).

The target compounds **1** and **2** were synthesized via reductive amination using lysine methyl ester as the core and syringaldehyde or vanillin as a BB (Scheme 1). $NaBH(OAc)_3$ was used as the reducing agent and 1,2-dichloroethane as the solvent.

The core scaffold, lysine methyl ester carrying two NH_3^+ groups, was first treated with DBU to enhance its solubility in the reaction solvent. Although $NaBH(OAc)_3$ is regarded as a mild reducing agent and compatible with aldehyde groups,[44] we observed a reasonable amount of aldehyde reduction when the reagents were added together.[43] Therefore, the reductive amination was run stepwise by adding 2 equivalents of BB and then 2 equivalents of reducing agents with a minimum of 1 h interval between

the additions. The same steps were repeated to form the target compounds with 4 BBs attached. The stepwise reactions took longer (3~4 days) to form the target compounds compared to the one-step reaction, but the overall reaction yields were much higher. The reactions after purification afforded the target compounds **1** and **2** with overall yields of 32% and 59%, respectively.

Similar reactions were run with BBs that were protected with *tert*-butyldimethylsilyl (TBDMS) to increase solubility in the reaction solvent and to make the final target compounds elute faster during column purification. However, the TBDMS protecting group on the BB was deprotected upon the addition of DBU, resulting in the formation of the target compound without the protecting group.

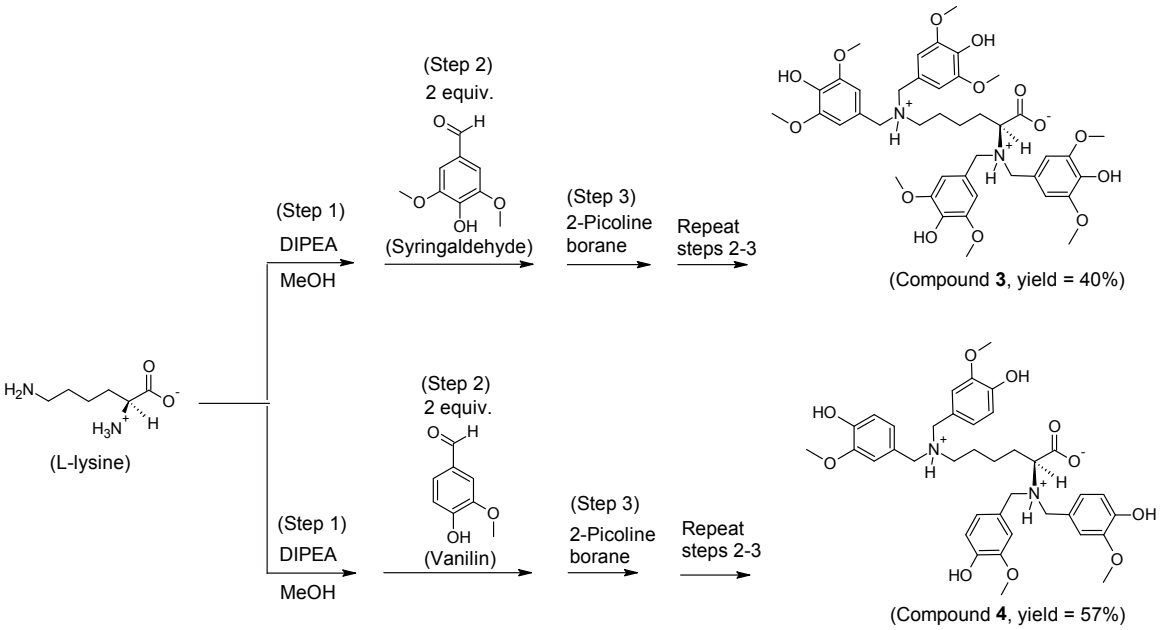


Scheme 1. Synthesis of lysine methyl ester-based antioxidants by reductive amination.

To synthesize compounds **3** and **4**, lysine was used as the scaffold to which syringaldehyde or vanillin was attached using reductive amination (Scheme 2). Compounds **3** and **4**, which lack a protecting group on their carboxyl (COOH) groups, can be obtained by hydrolyzing compounds **1** and **2**. However, they were synthesized using methods similar to those used for compounds **1** and **2**, with some modifications. This approach was preferred because synthesizing without the need for protection and deprotection steps is generally more convenient and often leads to higher reaction yields.

Lysine with zwitterions does not dissolve in 1,2-dichloroethane or THF, which are suitable solvents for reductive amination involving $NaBH(OAc)_3$. However, it is highly soluble in water and dissolves reasonably well in methanol. Since both BBs also dissolve well in methanol, we chose methanol as the reaction solvent. There are a few reducing agents that are compatible with methanol, such as 2-picoline borane and sodium cyanoborohydride. Our choice was 2-picoline borane because its reaction could be heated if needed. The addition of a base like DIPEA, DBU, or TEA increased the solubilities of lysine as well as reaction intermediates in methanol. While DBU performed better than DIPEA and TEA in solubilizing the reaction mixtures, we opted to use either TEA or DIPEA because DBU co-eluted with the target compound, complicating the column chromatography purifications. In our typical synthesis of dendritic compounds, the purification process to obtain the desired products is usually the time-limiting factor, rather than the initial formation of the target compounds. Thus, the base that does not hamper purification is more important than the amount of time needed to dissolve the starting materials and reaction intermediates. As we observed with $NaBH(OAc)_3$ in the reductive amination reactions, the aldehyde groups on BBs were not able to withstand 2-picoline borane well, and

a considerable amount of the BBs were reduced to their respective alcohols. As a result, to lessen aldehyde exposure to the reducing agent, BB, and the reducing agent were added sequentially.



Scheme 2. Synthesis of lysine-based antioxidants by reductive amination.

3.1.1. Solubilities of Synthesized Compounds

The structures of compounds **1-4** shown in Scheme 1 are their predominant molecular species at pH 7, estimated at 85.9%, 84.1%, 88.3%, and 95.4%, respectively (calculated using the Chemaxon pKa calculator). All tetramers carry a net positive charge of 1, based on their structures at pH 7. According to the calculated Log D values (obtained from the Chemaxon Log D calculator, Table 1) for antioxidants at pH 4, pH 7, and pH 9, antioxidants display varying solubilities.

Table 1. Calculated Log D values for antioxidants.

		Compound **1** (LME-SA)	Compound **2** (LME-Van)	Compound **3** (Lysine-SA)	Compound **4** (Lysine-Van)	Sodium ascorbate	Quercetin
Log D	pH 4	0.29	0.5	-0.75	-0.2	-2.03	2.13
	pH 7	4.31	4.53	1.6	1.86	-2.06	0.01
	pH 9	4.75	5.8	1.54	2.77	-2.90	-3.29

We also observed differences in solubility during our tests of antioxidants in pH 4, pH 7, pH 9, and PBS buffers at concentrations of 1 mg/mL and 0.25 mg/mL. If not all antioxidants dissolve in every test buffer, it is advisable to prepare all AO stock solutions in a common medium and then treat them with the respective pH buffer to assess changes in their AO activities under those pH conditions.

Therefore, we evaluated the solubilities of all synthesized antioxidants by dissolving each antioxidant in water at a concentration of 1 mM and stirring for two hours. If a compound did not dissolve during this period, it was categorized as hydrophobic. Additionally, we tested sodium ascorbate and quercetin as comparison standards. Based on the solubility tests, sodium ascorbate and compound 3 were found to be hydrophilic, while quercetin, along with compounds **1**, **2**, and **4**, was identified as hydrophobic. Although compound **4** has log D values comparable to those of compound **3**, its solubility in water is significantly lower than that of compound **3**. It took 20 hours to dissolve 0.25 mg in 5 mL of

water, preventing us from conducting a comparative study with other hydrophilic compounds. As a result, compound **4** was classified as hydrophobic. All hydrophilic antioxidants were dissolved directly in water, while all hydrophobic compounds were first dissolved in DMSO and then diluted with water to make AO stock solutions containing 1% DMSO in water.

3.2 Antioxidant Activity

Syringaldehyde and vanillin are small, hindered phenolic antioxidants that exhibit minimal antioxidant activity individually. However, our previous studies clearly demonstrated that dendrimers featuring four to eight units of syringaldehyde (or vanillin) on their surface display significantly enhanced antioxidant properties, likely due to the synergistic effects inherent in dendritic structures.[41,42,43] In the current study, it became apparent that the individual syringaldehyde and vanillin provided negligible antioxidant effects and were ineffective in protecting DNA at all the tested concentration ranges. As a result, these compounds were excluded from further testing.

AO activity tests were performed for the synthesized compounds and comparative standards using AAPH as the radical source and plasmid pBR322 DNA as the substrate, following a previously reported method.[45] This method is one of the most reliable and reproducible AO assays based on our experience. In this assay, a gel electrophoresis method is utilized to visualize both intact and damaged DNA, enabling us to evaluate the protective effects of antioxidants.

The incubation of native DNA (in supercoiled form) with AAPH results in damage to the DNA caused by AAPH-derived peroxyl and carbon radicals. This process transforms the supercoiled (SC) DNA into the nicked open circular (OC) form and/or the cleaved linear form, as previously reported by Hiramoto et al.[47] In gel electrophoresis, native SC DNA migrates faster than the damaged OC and linear forms of DNA due to its compact structure, which encounters less resistance as it moves through the gel matrix. Damaged DNA with breaks or nicks becomes relaxed and thus migrates more slowly during electrophoresis. The SC form appears as the top band, while the relaxed OC form is seen as the bottom band. If the OC form undergoes further damage and is converted into a cleaved linear form, it migrates faster than the OC form, appearing as the middle band. When DNA is exposed to excessive radicals, linear DNA can break down into smaller fragments of varying sizes, producing a smear on the gel.

Coincubation with antioxidants may help prevent DNA damage. The effectiveness of antioxidants in protecting DNA is assessed by comparing the bands in the test samples to those of the negative control (native SC DNA, the 1st lane in each panel) and the positive control (DNA with AAPH, the 2nd lane in each panel). The negative control serves as a baseline, providing insights into the preservation of the undamaged SC form of DNA, while the positive control helps gauge the level of relative DNA damage. If the SC band is more intense than that of the positive control, it suggests antioxidant effects. Conversely, if there is greater DNA damage compared to the positive control, it indicates a pro-oxidant effect.

3.2.1 Effects of Concentration on Antioxidant Activity

For the initial assessment of the relative strengths of AO activity among the test antioxidants, we incubated pBR322 DNA (final concentration, 9 ng/μL), along with AAPH (final concentration, 10 mM) and antioxidants (final concentrations, 0.16 μM – 83.3 μM), at 37 °C for 1 hour. We employed gel electrophoresis to evaluate the level of DNA protection provided by these antioxidants (the gel images are presented in Supplementary Materials S1).

According to gel electrophoresis, the lowest concentration at which sodium ascorbate provided DNA protection was 41.7 μM (Table 2). Under the same conditions, hydrophilic compound **3** exhibited

protective effects at 5.2 μM and provided slight protection at 2.6 μM. This indicates that hydrophilic compound **3** is approximately eight times more effective as an antioxidant than sodium ascorbate.

The hydrophobic standard quercetin provided DNA protection at ≥10.4 μM, with some protection also observed at 5.2 μM. Compound **1** also showed protective effects at ≥10.4 μM, while compound **2** provided protection at ≥20.8 μM. Compound **4** exhibited protection comparable to that of quercetin, providing protection at ≥10.4 μM and some protection at 5.2 μM. It is important to note that the 1% DMSO used to dissolve the hydrophobic compounds does not disrupt or damage the supercoiled form of DNA; No differences were observed between the controls containing water and PBS, which were used for testing hydrophilic compounds, and those containing 1% DMSO in water, which were used for testing hydrophobic antioxidants (S1).

Table 2. The lowest concentration at which DNA protection occurs (μM).

Solubility	Antioxidant	AO activities (μM)
Hydrophilic AO	Sodium ascorbate	41.7
	Compound **3**	5.2 (some protection at 2.6)
Hydrophobic AO	Quercetin	10.4 (some protection at 5.2)
	Compound **1**	10.4
	Compound **2**	20.8
	Compound **4**	10.4 (some protection at 5.2)

If antioxidants shift their protective activities to pro-oxidant effects as their concentrations increase, we define this as an activity crossover from AO to PO. To determine if a concentration-dependent activity crossover occurs, we tested hydrophilic and hydrophobic antioxidants at higher concentration ranges of 0.33 μM to 667 μM and 0.33 μM to 166.7 μM, respectively, in PBS buffer (S2).

During the study, we observed that sodium ascorbate at high concentrations displayed fluctuating antioxidant activities in one out of every three gel data sets when using sodium ascorbate with a purity of 98%. The highest antioxidant (AO) activities were noted within the concentration range of 40 μM to 400 μM (as shown in S2A), while antioxidant activities decreased at concentrations outside this range. To investigate whether this variation indicated an activity crossover from AO to PO at high concentrations, we repeated the experiment using two newly purchased premium-grade sodium ascorbates under the same concentration ranges and conditions. However, we did not observe this behavior, suggesting that contaminants in the antioxidant may be responsible for the fluctuations in the data and the skewed AO results, as noted by Carr et al.[48]

The hydrophobic antioxidants were tested at lower concentrations (ranging from 0.33 μM to 166.7 μM) compared to the hydrophilic antioxidants due to their limited solubility in the testing solvent, which consisted of PBS with 1% DMSO. The addition of more DMSO was avoided because it possesses antioxidant properties and could potentially produce false positive results for the test antioxidants. Moreover, at higher concentrations, DMSO, commonly used as a solvent for hydrophobic compounds in cell studies, can induce cellular toxicity. DMSO was chosen as a cosolvent because it effectively dissolves the hydrophobic antioxidants and remains stable throughout the incubation period, being less volatile than other solvents like methanol.

According to our data, all antioxidants tested in this study showed increasing AO activities as concentrations increased but did not exhibit concentration-dependent activity crossover from AO to PO.

3.2.2 Effects of pH on Antioxidant Activity

To investigate the effects of pH on AO activities, we tested three different pH conditions: acidic pH 4, neutral pH 7, and basic pH 9. Most antioxidants evaluated in this study lost their protective effects at concentrations between 41.7 μM and 5.2 μM in PBS buffer. Therefore, we used this concentration range to assess how different pH conditions influenced their activities. However, if an antioxidant continued to provide significant protection to DNA within this range, we conducted further tests at four additional concentrations (2.6 μM, 1.3 μM, 0.65 μM, and 0.33 μM) to identify the concentration at which protective effects end and to compare the efficacy among the tested antioxidants.

The effect of pH on AO activities was evaluated by comparing the bands in the test samples with those in the negative control (the 1st lane in each panel) and the positive control (the 2nd lane in each panel) at each pH level. The negative control served as a baseline to estimate the amount of preserved, undamaged DNA in the test samples, while the positive control was used to assess the relative DNA damage.

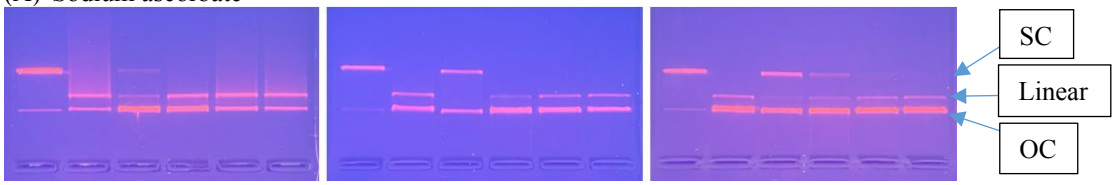
As shown in Fig. 1A, the overall protective effect of sodium ascorbate on DNA improved as the pH increased. At acidic pH 4, only 41.7 μM exhibited very slight protection. However, at pH 7, the top band at 41.7 μM is much brighter, and no visible linear forms are present, indicating stronger protection. In the case of pH 9, not only was a significant amount of DNA protection observed at 41.7 μM, but slight protection was also noted at 20.8 μM. Compound **3** (Fig. 1B) exhibited a trend similar to that of sodium ascorbate; a small amount of DNA protection was observed only at 41.7 μM at pH 4. At pH 7, the top bands across all concentrations appeared brighter compared to those observed at pH 4. In contrast, pH 9 exhibited significantly stronger DNA protection, particularly between concentrations of 41.7 μM and 2.6 μM. Overall, compound **3** showed overall much stronger AO activities at pH 9, followed by pH 7, and then pH 4. According to the study results, hydrophilic antioxidants at a higher pH demonstrate stronger protective effects on DNA against AAPH-derived radicals, suggesting that an increase in pH is beneficial for antioxidant activities.

The pH studies for hydrophobic antioxidants were conducted under the same pH conditions and concentration ranges as those used for hydrophilic antioxidants. The only difference was that a buffer containing 1% DMSO was used at each pH level, instead of using the buffer alone. To ensure consistency, both the negative and positive controls were treated with the same amounts of the buffer containing 1% DMSO as the test samples.

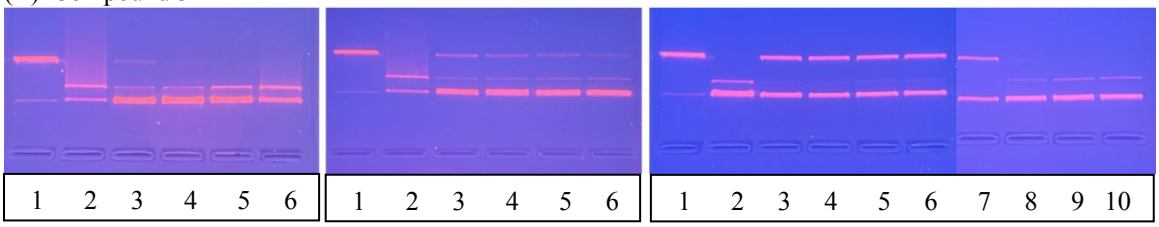
In the assay, quercetin demonstrated no protective effect at acidic pH 4 across all tested concentrations (Fig. 1C). Even when the concentration was increased to 83.3 μM, no protection was observed (data not shown). At pH 7, good DNA protection was noted at concentrations of 41.7 μM to 10.4 μM, with slight protection at 5.2 μM. In comparison, significantly stronger protection was observed at pH 9, particularly within the concentrations of 41.7 μM to 5.2 μM. Compound **1** also exhibited no protective effect at pH 4 across all concentrations (Fig. 1D). However, it provided good protection at concentrations ranging from 41.7 μM to 20.8 μM at pH 7, and from 41.7 μM to 10.4 μM at pH 9. At pH 9, a slight degree of DNA protection was noted even at a concentration of 5.2 μM. Based on the relative thickness of the top band to the bottom band, the level of DNA protection at pH 9 was significantly higher than at pH 7, particularly at high concentrations (41.7 μM to 20.8 μM). Its protective effect at 10.4 μM at pH 9 was comparable to that observed at 41.7 μM at pH 7. Compound **2** also showed no protective effect at acidic pH (Fig. 1E). However, at neutral pH, it provided good DNA protection at 41.7 μM and slight protection at 20.8 μM. In contrast, at pH 9, good DNA protection was observed in the range of 41.7 μM to 20.8 μM, with minor protection at 10.4 μM to 5.2 μM. Compound **4** did not exhibit any protective effect at pH 4, but it demonstrated DNA protection at concentrations as low as 10.4 μM at neutral pH 7 (Fig. 1F). At basic pH 9, its protective capability increased dramatically, showing substantial DNA protection between 41.7 μM and 5.2 μM, with some protection observed even at 2.6 μM. A small amount of intact DNA was visible even at 1.3 μM.

Hydrophilic Antioxidants

(A) Sodium ascorbate

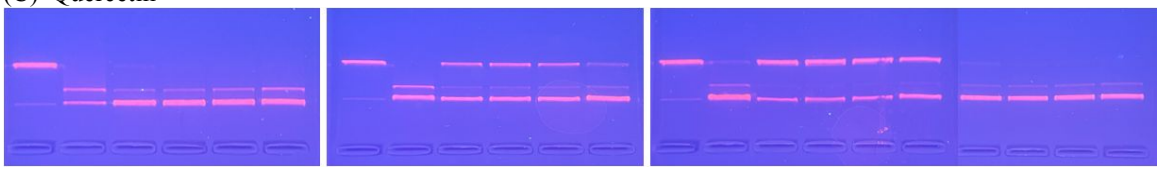


(B) Compound **3**

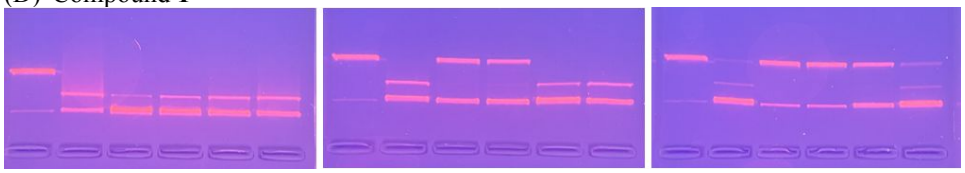


Hydrophobic Antioxidants

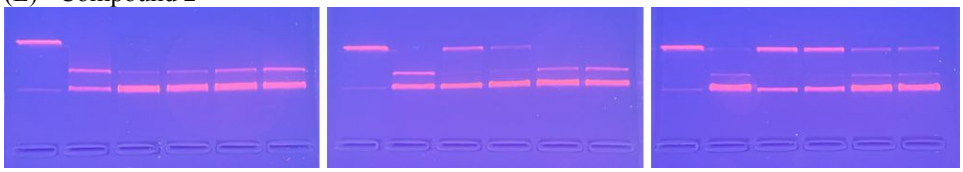
(C) Quercetin



(D) Compound **1**



(E) Compound **2**



(F) Compound **4**

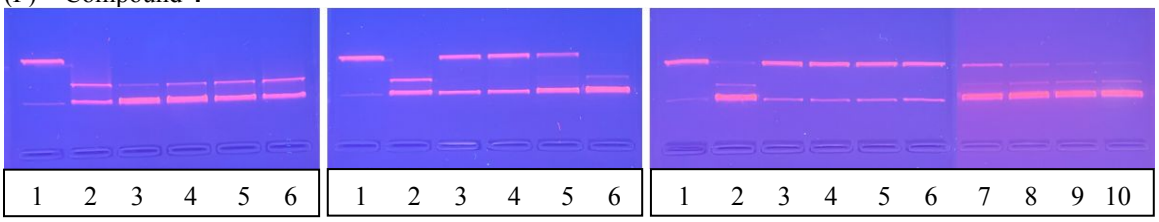


Figure 1. The effects of pH on antioxidant activities. The left panels show samples at pH 4, the middle panels at pH 7, and the right panels at pH 9. In each panel, lane 1 contains native DNA, and lane 2 consists of DNA + AAPH. Lanes 3-6 are DNA + AAPH + antioxidant at various concentrations: 41.7 µM, 20.8 µM, 10.4 µM, and 5.2 µM, respectively. If protection did not stop, four additional concentrations (Lanes 7-10: 2.6 µM, 1.3 µM, 0.65 µM, and 0.33 µM) were tested. Hydrophilic antioxidants: (A) Sodium ascorbate; (B) Compound **3**. Hydrophobic antioxidants: All controls and antioxidants contain 1% DMSO. (C) Quercetin; (D) Compound **1**; (E) Compound **2**; (F) Compound **4**.
*Note: Supercoiled (SC) DNA refers to native, intact DNA. In contrast, the nicked open circular (OC) form and the cleaved linear form represent damaged DNA molecules.

Our results show that both hydrophilic and hydrophobic antioxidants enhance their protective effects on DNA against peroxyl and carbon radicals derived from AAPH as the pH increases across the tested pH conditions. A basic pH of 9 is more favorable for AO activity than a neutral pH of 7, followed by an acidic pH of 4 (a summary of the results is provided in S3). Additionally, none of the test compounds, including the standard comparison controls, exhibited AO to PO crossover activities in the tested pH buffers.

The results of the study indicate that all the tested antioxidants exhibited antioxidant activities in the following order: pH 9 > pH 7 > pH 4. This trend suggests that their enhanced antioxidant activities at higher pH levels are significantly influenced by their pH-dependent ionization states. Each synthesized compound contains four phenolic OH groups, with theoretically calculated pKa values ranging from 8.78 to 10.55 (calculated using the Chemaxon pKa calculator). The pKa values of the COOH groups in the lysine derivatives are less than 2.0. Under varying pH conditions, the amino groups present in both lysine and lysine methyl ester can exist as ammonium groups due to their basic nature. As a result, the pKa values for their protonated amino groups were also determined, with these values ranging from 7.9 to 8.5.

Based on the pKa values, the COOH groups deprotonate at much lower pH levels, indicating that they will not impede the deprotonation of the OH groups at high pH. The ammonium groups overall have lower pKa values compared to the phenolic OH groups, but the differences are small. This suggests that the ammonium groups may influence the deprotonation of phenolic OH groups through proton transfer. We theoretically estimated the total amounts of the phenoxide form at pH 9 for selected compounds **1** and **3**, which were found to be 75% and 80.2%, respectively. This indicates that these compounds have a high [phenoxide]/[phenol] ratio at pH 9. Since a basic pH facilitates the deprotonation of phenolic OH groups, leading to the formation of phenoxide, which can readily transfer an electron to a radical, it is reasonable to conclude that a basic pH contributes to a faster reaction.

Most prior studies report that higher pH correlates with stronger AO activities.[49,50] However, some studies suggest that high pH does not always lead to increased AO activities. The optimal pH for maximum antioxidant activity varies depending on specific antioxidants, as pH influences their structural stability and ionization. For instance, anthocyanins change their structures with pH and degrade beyond pH 7, indicating that the AO activities at each pH level are determined by their predominant forms at that pH or by degradation products.[51] In the case of resveratrol in heterogeneous systems, such as micellar and liposomal environments, resveratrol reaches its optimal ionization state at a pH of 6 within the pH range of 4 to 10. In this optimal state, it enhances its reactivity, promotes the formation of effective resveratrol-derived dimers, and facilitates favorable localization at the lipid-water interface, all of which collectively contribute to the AO activities of resveratrol.[52] The AO activities of plant extracts, saponins,[53] palm juice,[54] and other medicinal herbs[55] have also been reported to show pH dependence; the saponin extract showed the highest AO activities at pH 9, compared to lower (pH 3-6) and higher pH levels (pH 10-12).[53] In palm juice, AO activity increases as the pH rises from 3.5 to 5.5 but decreases at pH 6.5.[54] *Rhodiola rosea*, *Gentiana lutea, Rosa canina*, and *Hyperycum perforatum* showed AO effects under acidic conditions (pH 6.8) but PO effects at alkaline conditions (pH 7.8).[55] According to these studies, the optimal pH for the AO activity of complex AO samples, such as plant extracts and fermentation, or in heterogeneous test protocols, is influenced by other factors involved in these processes and microenvironments.

Our study, which utilizes purified samples and homogeneous testing systems, minimizes the influence of external factors. While it remains uncertain whether our findings with AAPH as the radical source can be extrapolated to other radicals until further research is conducted, our tetrameric antioxidants show greater radical scavenging activities at basic pH conditions. This lays a foundation for

estimating the antioxidant properties of larger dendrimers with the same building blocks under identical testing conditions.

3.3. Pro-oxidant Effect

Pro-oxidant activities can occur under various conditions. One of the better-known situations in which antioxidants behave as pro-oxidants is when they interact with redox-active transition metal ions, such as Fe(III) and Cu(II) ions. Previous studies have reported that antioxidants couple with the redox-active metal ions and then reduce these ions to their lower oxidation states, Fe(II) and Cu(I), respectively.[56,57] The Fe(II) ions catalyze the formation of hydroxyl radicals (OH•) from hydrogen peroxide (H_2O_2) through the Fenton reaction, while Cu(I) ions, in the presence of O_2, contribute to the formation of H_2O_2 via superoxide, which decomposes into OH•.[58,59,60] These reactions result in the regeneration of Fe(III) and Cu(II) ions, which become reduced again by antioxidants in proximity, resuming the redox cycle and contributing to the continuous production of new radicals.[61] This pro-oxidant phenomenon is well known with phenolic (or enolic) antioxidants containing 1,2-dihydroxy[62,63,64,65] 1,2,3-trihydroxy,[65,66] or thiol groups[67] due to their ability to effectively complex with transition metal ions.

3.3.1. Effects of Concentration on Pro-oxidant Activity

The pro-oxidant potentials of all antioxidants were evaluated by incubating each antioxidant in the presence of Cu(II) ions, following a previously published method.[46]

In our PO assay, we used two controls: the negative control containing native DNA(lane 1) and the 2nd negative control containing DNA and Cu(II) ions (lane 2). In Fig. 2, lane 1 appears the same as lane 2, which indicates that Cu(II) ions do not cause DNA damage. Both of our comparison standards, sodium ascorbate and quercetin, are well known for having severe PO effects in the presence of transition metal ions. We also observed strong PO effects from both sodium ascorbate and quercetin (Fig. 2; the results are summarized in Table 3). Sodium ascorbate exhibited severe PO effects between 83.3 μM and 41.7 μM (Fig. 2A), while quercetin displayed effects between 83.3 μM and 10.4 μM (Fig. 2B). Both exhibited greater PO effects at higher concentrations than at lower concentrations. For instance, the PO effects at 83.3 μM were significantly more damaging than those at 10.4 μM, suggesting that they would produce even worse PO effects at concentrations greater than 83.3 μM. In fact, the DNA bands completely vanished when the concentration was increased to 833 μM (data not shown), indicating that even more severe PO effects are anticipated at antioxidant concentrations above 833 μM. This means that the PO effects are concentration-dependent and directly related to the number of available antioxidants that can sustain redox cycling between antioxidant and transition metal ions.

In the copper ion-mediated PO assay, a PO effect is identified when the level of DNA damage in the test samples exceeds that of the 2nd negative control, which contains only DNA and Cu(II) ions. Conversely, if the DNA damage in the test samples is less than or equal to that in the 2nd negative control, it is interpreted as an AO effect. If the PO effect shifts to an AO effect with changes in concentration, it signifies a crossover from PO to AO activity. It is important to note that in the PO assay, the amount of supercoiled (SC) DNA in the test samples cannot exceed the level found in the 2nd negative control (lane 2), as copper ions alone do not cause DNA damage. If the SC band in the test samples is equivalent to that of the 2nd negative control, it may be interpreted as an AO effect. Therefore, in the PO assay, any observed DNA damage is classified as a PO effect. Consequently, the milder DNA damage observed from sodium ascorbate and quercetin at low concentrations can be attributed to less pronounced PO effects rather than AO effects. This is due to an insufficient number of antioxidant molecules available to facilitate the redox cycling of copper ions. Consequently, this should

not be interpreted as a crossover from PO to AO activity. Should a co-antioxidant capable of regenerating these antioxidants be introduced, it may be possible to sustain their PO actions even at low concentrations.

Previous studies reporting concentration-dependent antioxidant/pro-oxidant effects stated that AO effects predominate at low doses, while PO effects prevail at high doses.[68,15,17,18,19] Most of these studies involve biological systems. The AO-PO crossover effects observed in biological systems are complex and may vary depending on the molecular conditions present in tissues, such as the concentrations of transition metal ions.[21,22, 23,24]

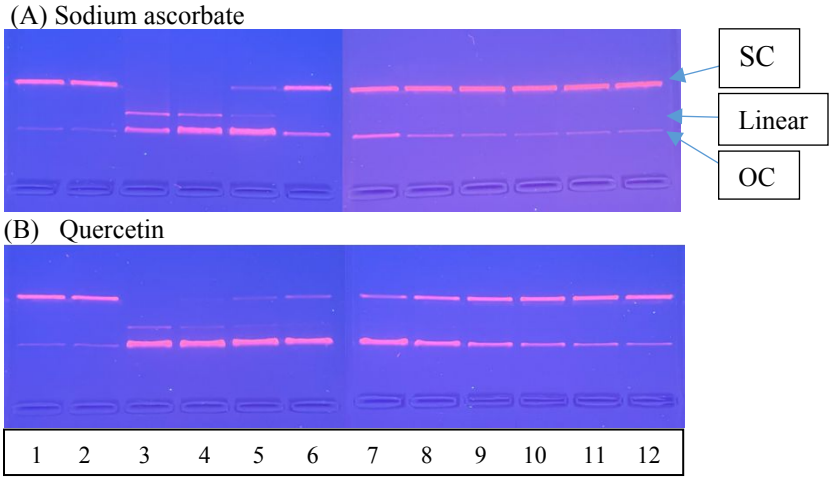


Figure 2. The pro-oxidant effects of antioxidant standards in PBS.
Lane 1 (native DNA), lane 2 (native DNA + Cu(II) ions, without antioxidant), and lanes 3–12 contain native DNA + Cu(II) ions + antioxidant at various concentrations: 83.3 μM, 41.7 μM, 20.8 μM, 10.4 μM, 5.2 μM, 2.6 μM, 1.3 μM, 0.65 μM, 0.33 μM, and 0.16 μM, respectively. (A) Sodium ascorbate; (B) Quercetin.
*Note: Supercoiled (SC) DNA refers to native, intact DNA. In contrast, the nicked open circular (OC) form and the cleaved linear form represent damaged DNA molecules.

Table 3. The concentration range where the PO effect occurs (μM).

Solubility	Antioxidant	Severe PO effects (μM)	Mild PO effects (μM)	Activity crossover
Hydrophilic AO	Sodium ascorbate	83.3 – 41.7	20.8 – 5.2	Sodium ascorbate and quercetin have PO effects at high concentrations and AO effects at low concentrations.
	Compound **3**	No PO		
Hydrophobic AO	Quercetin	83.3 – 10.4	5.2 – 2.6	
	Compound **1**	No PO		
	Compound **2**	No PO		
	Compound **4**	No PO		

All in-house prepared tetrameric antioxidants **1**– **4** did not show any PO effects (S4). As for radical scavenging AO, strong antioxidants are generally strong pro-oxidants,[69,70] because both antioxidant and pro-oxidant actions stem from the reducing properties of antioxidants; electron donation to transition metal ions results in PO effects and an electron transfer to free radicals leads to AO activities. However, our tetrameric antioxidants do not support this notion as our tetrameric antioxidants **1**– **4**, which produce stronger AO effects than sodium ascorbate, did not show any PO effects at all

tested concentrations (Table 3 and S4). Although the concentration was increased to 166.7 μM, they still did not show any pro-oxidant effects under our experimental conditions (S5). The difference between sodium ascorbate/quercetin and our tetrameric antioxidants lies in the fact that both quercetin and sodium ascorbate contain 1,2-dihydroxy groups, while all tetramers consist of hindered phenolic groups. The 1,2-dihydroxy groups are known to effectively chelate copper ions, thereby contributing to the initiation of PO effects.[62,63,64,65] The observation that the tetramers did not exhibit pro-oxidant effects may indicate that their interactions with copper ions are inefficient. As a result, strong antioxidants that do not effectively interact with transition metal ions are not likely to act as strong pro-oxidants. This leads us to conclude that phenolic units substituted with electron-donating groups (EDGs) at the *ortho* position offer dual benefits by enhancing antioxidant effectiveness while minimizing pro-oxidant side effects.

3.3.2 Effects of pH on Pro-oxidant Activity

The evaluation of pH effects on PO activities for both hydrophilic and hydrophobic antioxidants was performed under the same pH conditions (pH 4, 7, and 9) as the AO activity tests described above.

In Figure 3, the 1st lane in each panel contains the native DNA in the respective buffer, while the 2nd lane contains the DNA along with 10 μM Cu(II) ions in their respective buffer. The 2nd lane is the same as the 1st lane in all three panels (Fig 3A), which means that the DNA is not affected by the copper ions in the tested buffers of pH 4, pH 7, and pH 9. The addition of sodium ascorbate clearly induced DNA damage, transforming SC DNA into both OC and linear forms across all three pH conditions (Fig. 3A). In terms of the smear level and band intensity of damaged DNA under the tested pH conditions, the PO effects of sodium ascorbate are most pronounced at pH 9, followed by pH 7, and then pH 4. At a concentration of 41.7 μM in the pH 9 buffer, the DNA completely disintegrated into smaller fragments of varying sizes, resulting in a smear. Even at concentrations of 20.8 μM and 10.4 μM, the linear form band began to streak. At lower concentrations of sodium ascorbate, specifically between 10.4 μM and 5.2 μM, the intensity of the middle band serves as a reliable indicator of the extent of PO effects, as the middle band (cleaved linear DNA) represents more severely damaged DNA than the bottom band (nicked open circular DNA). The middle bands of sodium ascorbate samples become thicker with increasing pH, indicating that its PO effects intensify as the pH increases.

The hydrophobic antioxidant standard quercetin demonstrated significantly greater PO effects at pH 9 compared to those observed at a neutral pH of 7 (Fig. 3B). Under acidic pH conditions, quercetin showed no detectable PO activities. Although the extent of DNA damage caused by PO effects varies with each pH level, the overall trend regarding pH impacts on the PO activities of hydrophobic quercetin aligns well with that of the hydrophilic sodium ascorbate. This indicates that PO effects tend to increase as pH rises, irrespective of the solubility of the antioxidant. At the same pH level, the pro-oxidant effects of sodium ascorbate and quercetin increased progressively with higher concentrations. For example, at pH 9, the PO effect at 41.7 μM was significantly greater than that at 5.2 μM (Fig 3A). Furthermore, the extent of DNA damage at the same concentrations intensified as pH increased. Therefore, the PO effects observed with sodium ascorbate and quercetin depend on both concentration and pH.

In transition metal ion-mediated PO events, the stability of metal ions at different pH levels can influence test results. Previous reports have indicated that reduced transition metals, such as Fe(II) and Cu(I), are more soluble and stable under acidic pH conditions.[71,72] In this context, their enhanced stability should contribute to their continuous availability for participation in redox processes, which would increase the production of $OH^{\bullet}$ and consequently lead to stronger PO effects. Given that copper ions are more stable in acidic pH, we would, therefore, expect more severe PO effects at pH 4 than at pH 9. However, our study showed otherwise.

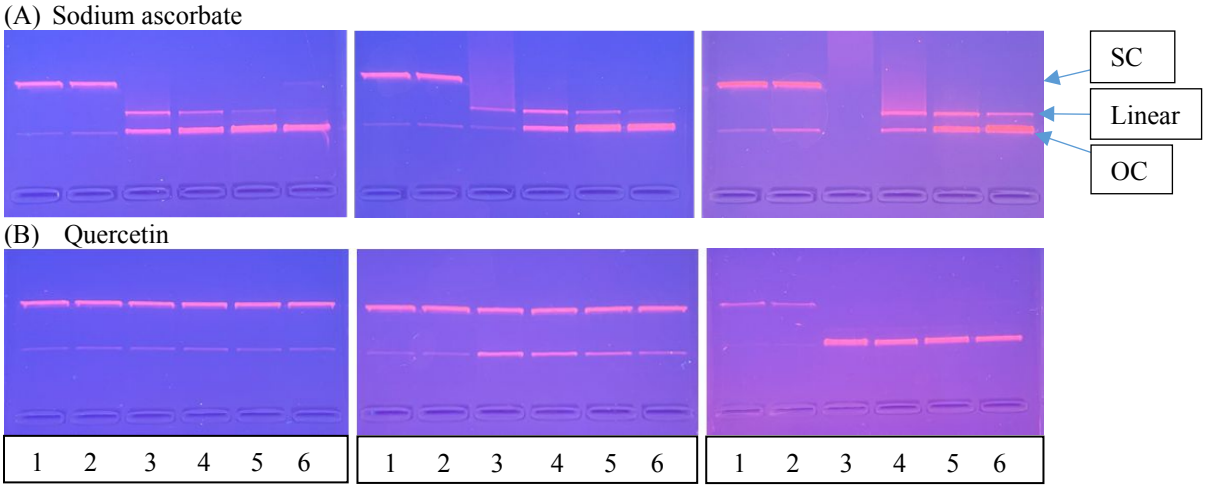


Figure 3. The impact of pH on the pro-oxidant properties of antioxidant standards. The left panels display samples at pH 4, the middle panels at pH 7, and the right panels at pH 9. In the quercetin tests, 1% DMSO was used for the controls and the sample solutions. In each panel, lane 1 contains native DNA, and lane 2 contains DNA + Cu(II) ions. Lanes 3-6 show DNA + Cu(II) ions + antioxidants at various concentrations: 41.7 μM, 20.8 μM, 10.4 μM, and 5.2 μM, respectively. (A) Sodium ascorbate; (B) Quercetin.
*Note: Supercoiled (SC) DNA refers to native, intact DNA. In contrast, the nicked open circular (OC) form and the cleaved linear form represent damaged DNA molecules.

Table 4. Summary of the effects of pH on the PO activities of hydrophilic and hydrophobic antioxidants.

Solubility	**Antioxidant**	**pH 4**	**pH 7**	**pH 9**
Hydrophilic AO	Sodium ascorbate	Strong PO in 41.7–20.8 μM Strong PO in 10.4–5.2 μM	Severe PO at 41.7 μM Strong PO in 20.8–5.2 μM	Severe PO in 41.7–10.4 μM Strong PO at 5.2 μM
	Compound **3**	No PO	No PO	No PO
Hydrophobic AO	Quercetin	No PO	Mild PO in 41.7–5.2 μM	Strong PO in 41.7–5.2 μM
	Compound **1**	No PO	No PO	No PO
	Compound **2**	No PO	No PO	No PO
	Compound **4**	No PO	No PO	No PO

Based on the results obtained with sodium ascorbate and quercetin in our study, the effects of pH on their AO and PO activities exhibited similar trends. Specifically, as the pH increases, both AO and PO activities become stronger. At the same pH levels, higher concentrations lead to more pronounced AO and PO activities, with this trend becoming particularly evident at elevated pH levels. When comparing the AO activities (Figure 1A) with the PO effects (Figure 3A) across a concentration range from 5.2 μM to 41.7 μM in buffers at pH 4, pH 7, and pH 9, it is clear that the 41.7 μM concentration exhibits the most significant protective AO activities while also causing the strongest damaging PO effects. Notably, the concentration effect on AO and PO activities is much more pronounced at pH 9 than at pH 7 and pH 4. This indicates that both sodium ascorbate and quercetin exhibit concentration (dose)-dependent and pH-dependent AO and PO activities.

In comparison, neither our hydrophilic tetrameric antioxidant (compound **3**) nor the hydrophobic tetramers (compounds **1**, **2**, and **4**) showed any significant PO activity under any of the three tested pH conditions (see Table 4 and S6).

In the copper-ion-mediated pro-oxidant (PO) assay, antioxidants engage in multiple competing processes. These include chelating Cu(II), reducing either chelated or free Cu(II), and potentially scavenging OH radicals generated from pro-oxidant activities. The effect of Cu(II) chelation can result in either antioxidant or pro-oxidant actions, depending on the fate of the cupric ions. If the chelated Cu(II) ions remain unreduced, AO activity prevails, as chelation prevents these ions from engaging in pro-oxidant events. Conversely, if Cu(II) is reduced, it leads to increased production of OH radicals (PO activity).[73] These processes are closely linked to pH levels, as chelation of Cu(II) ions is more effective through deprotonated phenoxide than its neutral counterpart.[73,74,75] This suggests that an increase in pH enhances the chelation of Cu(II) ions by increasing the availability of phenoxides. In addition, as pH rises, the reduction of Cu(II) ions is also promoted, driven by the ease of electron donation from phenoxides. Taken together, elevated pH fosters efficient Cu(II) chelation and rapid Cu(II) reduction. Our findings, obtained with sodium ascorbate and quercetin, reinforce the notion that higher pH levels enhance both Cu(II) chelation and Cu(II) reduction, resulting in significantly stronger pro-oxidant effects.

The lack of PO activity in our tetramers is likely due, in part, to the presence of hindered phenolic units substituted with electron-donating methoxy groups at the *ortho* positions of the phenolic OH groups. The steric crowding around these OH groups may hinder effective interaction with transition metal ions. Furthermore, the steric hindrance is not affected by pH, which explains why our antioxidants derived from hindered phenols do not exhibit pH-dependent copper ion-mediated PO effects.

Our uniquely designed tetrameric antioxidants may also have the potential to trap Cu^{2+} ions due to the presence of electronegative nitrogen and oxygen atoms in the scaffold. This trapping could help limit the PO effects. Our study suggests that the PO effects might be eliminated if antioxidants incorporate hindered phenolic unit(s) that impede metal ion binding and/or possess metal chelating properties elsewhere in the molecule (other than the phenolic OH group).

The availability of potent antioxidants that do not exhibit pH-dependent and/or concentration-dependent pro-oxidant side effects will significantly enhance their bioavailability. This advancement will offer substantial benefits across various biological and medicinal applications, leading to transformative impacts in these fields.

4. Conclusion

Antioxidant and pro-oxidant properties can vary under different conditions. In this study, we examined how antioxidants with multiple hindered phenol rings are influenced by pH levels and concentrations. Our results indicated that the hydrophilic and hydrophobic tetrameric antioxidants we prepared, along with sodium ascorbate and quercetin standards, demonstrate concentration-dependent antioxidant activities. Moreover, the antioxidant activities increased with higher pH levels, showing the effectiveness order: pH 9 > pH 7 > pH 4. This indicates that antioxidant activity is influenced by both concentration and pH.

In the copper ion-mediated PO assays, sodium ascorbate and quercetin produced strong PO effects at high concentrations and milder effects at low concentrations. Their PO effects were also dependent on pH, with the order being pH 9 > pH 7 > pH 4. At the same pH level, stronger PO effects were observed as the concentration increased, indicating that both concentration and pH influence PO activity. In contrast, our tetrameric antioxidants, synthesized with phenolic building blocks substituted

with electron-donating methoxy group(s) at the *ortho* position(s) to the phenolic OH, did not show any significant PO effects, regardless of concentration and pH conditions, although they showed strong AO activities.

Our results indicate that sodium ascorbate and quercetin, well-known natural antioxidants, increase both AO and PO activities as pH levels and concentrations rise. In contrast, our synthesized tetrameric antioxidants, which contain hindered phenolic units, enhance their AO activities with increasing pH and concentrations but do not exhibit any PO effects regardless of concentration and pH levels. Furthermore, the hydrophilic and hydrophobic tetramers do not demonstrate a crossover in activity from AO to PO or vice versa under all test concentrations and pH conditions.

Abbreviations

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride
AO: Antioxidant
BB: Building block
$CDCl_3$: Chloroform
COOH: Carboxyl (or carboxylic)
$CuCl_2$: Copper(II) chloride
Cu(I): Cuprous ion
Cu(II): Cupric ion
DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene
DIPEA: N,N-diisopropylethylamine
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DPPH: 2,2-diphenyl-1-picrylhydrazyl
EDG: Electron donating group
EDTA: Ethylenediaminetetraacetic acid
Fe(II): Ferrous ion
Fe(III): Ferric ion
h: Hour
HCl: Hydrochloric acid
H_2O_2: Hydrogen peroxide
HPLC: High-performance liquid chromatography
HR-MS: High-resolution mass spectrometry
IC50: Half-maximal inhibitory concentration
IUPAC: International union of pure and applied chemistry
LC/MS(ESI): Liquid chromatography/mass spectrometry (electrospray ionization)
min: Minute
$NaBH(OAC)_3$: Sodium triacetoxyborohydride
NMR: Nuclear magnetic resonance
O_2: Oxygen
OC: Open circular
$OH^{\bullet}$: Hydroxyl radical
OH: Hydroxyl
pBR: Plasmid Bolivar Rodriguez
PBS: Phosphate-buffered saline
PO: Pro-oxidant

Q-TOF: Quadruple time-of-flight
SC: Supercoiled
TBDMS: Tert-butyldimethylsilyl
TAE: Tris-Acetate-Ethylenediaminetetraacetic acid
TEA: Triethyl amine
THF: Tetrahydrofuran
TLC: Thin layer chromatography
TOF: Time-of-flight
UPLC: Ultra-performance liquid chromatography
UV: Ultraviolet

Data Availability Statement: All data collected from this study are presented either in the text or in the Supplementary Information.

Author Contributions: Conceptualization, C.Y.L.; methodology, C.Y.L.; investigation, C.A.B., B.A., J.H.K., Y.G., N.L., and R.L.U.; data curation, C.A.B., B.A., J.H.K., Y.G., and R.L.U.; formal analysis, C.A.B., B.A., and R.L.U.; validation, C.Y.L.; supervision, C.Y.L. and R.L.U.; funding acquisition, C.Y.L. and R.L.U.; writing—original draft preparation, C.Y.L.; writing—review and editing, C.Y.L. and R.L.U.

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Data Availability Statement: All the data obtained from this study are shown in either text or Supplementary Information.