Evaluation of Polyphenolic Acid Esters as Potential Antioxidants

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Six polyphenolic acid esters were synthesized and their antioxidative properties were evaluated in three model systems [2,2′-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-induced lipid peroxidation system, and the dye-bleaching assay of peroxynitrite radical]. Among these compounds, we found that compounds 4 [3,4-dihydroxy-benzoic acid-(2-phenoxyethyl ester)], and 5 [3,4-dihydroxy-cinnamic acid-(2-phenoxyethyl ester)] provided comparable activity to caffeic acid phenethyl ester (CAPE) in the DPPH model. Compound 3 [2,5-dihydroxy-benzoic acid-(2-phenoxyethyl ester)], was found to be more active than CAPE in the AAPH system, it also displayed about 2-fold greater activity than CAPE in the peroxynitrite radical model. These results suggest that these phenolic acid ester derivatives, with their potent anti-oxidant activities, may have useful applications as antioxidants.

Key words polyphenolic acid ester; caffeic acid analogue; antioxidant; free radical scavenging activity

Reactive oxygen species (ROS), including free radicals, superoxide anions and hydroxyl radicals, are the intermediates of regular pathway of aerobic metabolism. ROS generated from normal metabolism or exogenous insults lead to peroxidation of membrane lipids and cellular damage of proteins and DNA. To prevent the harmful effects of ROS, cells are equipped with enzymatic systems including catalases, peroxidases, and superoxide dismutases (SOD) and molecular antioxidants, such as ROS scavengers, glutathione, vitamin C, E, and other antioxidative molecules. The cellular redox homeostasis is well-maintained in normal cases; however, oxidative stress occurs when redox status within the cell is altered. This imbalance may be due to either overproduction of ROS or a deficiency in one or more antioxidant systems. The uncontrolled oxidative stress initiates a series of harmful biochemical events which are associated with diverse pathological processes. These processes can lead to various cellular damages and diseases.[1,2]

Polyphenols, existing ubiquitously in nature, are commonly used as food additives and folk medicine in many countries. Interest in polyphenols has increased because many of them exhibit a broad spectrum of biological activities including anti-inflammatory, antiviral, antiatherogenic, antibacterial, as well as anticancer effects.[3,4] These activities are associated, to a great extent, to their antioxidant properties, though other mechanisms may also be involved. Caffeic acid phenethyl ester (CAPE), a polyphenolic acid ester, has been identified as one of the major active components of honeybee propolis.[5] The ability of CAPE to alter oxidative processes is well documented.[6,7] It has been shown to induce selectively apoptosis on transformed cell lines, to chelate metal ions, to scavenge free radicals, and to inhibit enzymes that are involved in free radical and lipid hydroperoxidation formation such as lipoxygenases and cyclooxygenase-2 (COX-2).[6—8] Therefore, CAPE can be used to prevent the lipid oxidation in foods, reduce cellular damages initiated by free radicals, and prevent diseases related to inflammation.

We have recently focused on the synthetic antioxidants with diverse anti-oxidative functionalities.[9—11] Based on the biological activities of CAPE, we are interested in study the CAPE-related polyphenolic acid esters. It has been shown that the antioxidative activity of polyphenolic compounds is determined by their molecular structure.[12—14] The position of hydroxylation, for instance, will influence the ability of the delocalization of unpaired electrons to stabilize the formed radical after reaction with the initiator radical.[15] Previous studies by many research groups demonstrated that the catechol moiety, with the 3,4-dihydroxyl configuration, is important for the free radical scavenging activity for this type of phenolic compounds.[16—18] In addition, the effect of conjugated ethylenic side chain of the related phenolic compounds on their antiradical activities is not very clear. Several studies indicate that the conjugated ethylenic side chain is essential for optimal antioxidative activity. Contrary to this, other studies suggest that the structure is not required for the activity.[13,16,19] Thus, it is worthy to compare the antioxidative activity of the synthesized polyphenolic acid esters with different dihydroxyl positions on the benzoic acid moiety. We prepared six phenoxyethyl esters of polyphenolic acid derivatives and the efficacy of these compounds as radical scavengers was analyzed by their reactivity toward the 2-2-diphenyl-1-picrylhydrazyl radical (DPPH).[20,21] In addition, their potency as antioxidants was evaluated by the ferric thiocyanate assay using a Tween-emulsified linoleic acid oxidation system induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH).[22—24] The relative antioxidative activity of these compounds was also evaluated by peroxynitrite scavenging assay.[25,26]

MATERIALS AND METHODS

Chemicals 3,4-Dihydroxy benzoic acid, 3,5-dihydroxy benzoic acid, 3,4-dihydroxy cinnamic acid, triphenylphosphine (TPP) were purchased from E. Merck (Darmstadt, F.R.G.). 2,4-Dihydroxy benzoic acid, 2,5-dihydroxy benzoic acid, 3,4-dimethoxy cinnamic acid, 2-phenoxy ethanol, diisopropyl azodicarboxylate (DIAD), DPPH, AAPH, linoleic acid, polyoxyethylenesorbitan monolaureate (Tween 20), and ferrous chloride tetrahydrate were purchased from Aldrich-Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium dihydrogen phosphate monohydrate, anhydrous sodium hydrogen phosphate, and ammonium thiocyanate were purchased from

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Fischer Scientific, Inc. (Hampton, NH, U.S.A.). All other reagents and solvents were of analytical, spectrometric grade.

Apparatus Synthesized compounds were purified by chromatography on a silica gel 60 (230—400 mesh; Merck) column and identified by thin-layer chromatography (TLC), MS, NMR, and elementary analysis. Melting points (mp) were taken on a BUCHI 530 apparatus. TLC was performed on precoated silica gel F_{254} plates (Merck) using a 254-nm UV lamp to visualize the compounds. IR spectra were recorded on a Shimadzu Forurier Transform Infrared Spectrophotometer FTIR-8700 using Nujol as mulling agent or performed neat; only the most significant absorption bands are reported (ν_{max} cm^{-1}). ^{1}H- and ^{13}C-NMR data were acquired at room temperature on a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Where necessary, deuterium exchange experiments were used to obtained proton shift assignments. Mass spectra were recorded on a JEOL JMS-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78 °C in the presence of P_2O_5 for at least 12 h unless otherwise specified. Elemental analyses were obtained using a Perkin–Elmer 2400 Elemental Analyzer. A Perkin–Elmer Lambda 40 UV/VIS spectrophotometer was used in the DPPH scavenging, ferric thiocyanate lipid peroxidation, and pyrogallol red bleaching assays.

General Synthetic Procedure for Polyphenolic Acid Ester Derivatives The esters were synthesized from polyphenolic acid and 2-phenoxy ethanol by Mitsunobu esterification using DIAD and TPP as coupling reagents[28] as shown in Fig. 1. Briefly, to a solution of 3,4-dihydroxy cinnamic acid (6 mmol) and 2-phenoxy ethanol (6 mmol) in dry tetrahydrofuran (15 ml) were added TPP (6 mmol) and DIAD (6 mmol) at 0 °C. After stirring at room temperature for 48 h, the reaction was worked up by removal of the solvent, and the residue was partitioned between ethyl acetate and saturated $NaHCO_3$. The organic phase was washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by flash chromatography on a silica gel column using a mixture of *n*-hexane/ethyl acetate (3 : 1 or 2 : 1) as eluent. Yields are between 41—90%.

3,5-Dihydroxy-benzoic acid-(2-phenoxy-ethyl ester) (**1**): mp 111—112 °C; yield 42%. ^{1}H-NMR (DMSO-d_6) δ: 9.62, 9.58 (1H each s, OH), 7.31—6.41 (8H, m, ArH), 4.52 (2H, t, J=8.4 Hz, $COOCH_2$), 4.27 (2H, t, J=8.4 Hz, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 165.8, 158.6, 158.3, 131.3, 129.6, 121.0, 114.7, 107.4, 107.3, 65.9, 63.3. IR ν_{max} (cm^{-1}): 3336, 3064, 1697, 1087. MS (EI^+) *m/z*: 275 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15; Found: C, 65.33; H, 5.30.

2,4-Dihydroxy-benzoic acid-(2-phenoxy-ethyl ester) (**2**): mp 118—121 °C; yield 45%. ^{1}H-NMR: (DMSO-d_6) δ: 10.6 (1H, s, OH), 10.4 (1H, s, OH), 7.62—6.28 (8H, m, ArH), 4.57 (2H, t, J=3.8 Hz, $COOCH_2$), 4.30 (2H, t, J=3.8 Hz, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 169.0, 164.4, 162.8, 158.3, 131.8, 129.6, 121.0, 114.8, 108.5, 104.0, 102.6, 65.8, 63.3. IR ν_{max} (cm^{-1}): 3373, 2964, 1668, 1109. MS (EI^+) *m/z*: 275 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15; Found: C, 65.34; H, 5.16.

2, 5-Dihydroxy-benzoic acid-(2-phenoxy-ethyl ester) (**3**): A pale yellow oil; yield 41%. ^{1}H-NMR: (DMSO-d_6) δ: 9.87 (1H, s, OH), 9.20 (1H, s, OH), 7.29—6.79 (8H, m, ArH), 4.60 (2H, t, J=3.4 Hz, $COOCH_2$), 4.32 (2H, t, J=3.4 Hz, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 168.7, 158.3, 153.4, 149.7, 129.6, 124.1, 121.0, 118.3, 114.3, 112.5, 65.7, 63.7. IR ν_{max} (cm^{-1}): 3415, 3105, 1670, 1050. MS *m/z*: 275 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15; Found: C, 64.50; H, 5.10.

3,4-Dihydroxy-benzoic acid-(2-phenoxy-ethyl ester) (**4**): mp 118—120 °C; yield 45%. IR ν_{max} (cm^{-1}): 3296, 2927, 1683, 1128. ^{1}H-NMR: (DMSO-d_6) δ: 7.39—6.81 (m, 8H, ArH), 4.53 (t, J=3.9 Hz, 2H, $COOCH_2$), 4.30 (t, J=3.9 Hz, 2H, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 165.6, 158.2, 156.5, 150.5, 145.0, 129.5, 121.9, 120.8, 116.2, 115.3, 114.5, 65.7, 62.7. MS *m/z*: 275 (EI^+) (M^+). *Anal.* Calcd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15; Found: C, 65.55; H, 5.43.

3,4-Dihydroxy-cinnamic acid-(2-phenoxy-ethyl ester) (**5**): mp 127—128 °C; yield 44%. ^{1}H-NMR: (DMSO-d_6) δ: 9.60 (1H, s, OH), 9.13 (1H, s, OH), 7.63—6.91 (8H, m, ArH), 6.74 (1H, d, J=15.6 Hz, COCH), 6.29 (1H, d, J=15.6 Hz, CH), 4.42 (2H, t, J=8.1 Hz, $COOCH_2$), 4.22 (2H, t, J=8.1 Hz, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 166.3, 158.1, 148.3, 145.4, 145.3, 132.1, 131.8, 131.3, 129.3, 128.6, 125.3, 121.2, 120.7, 65.7, 62.3. IR ν_{max} (cm^{-1}): 3210, 2990, 1720, 1120. MS (EI^+) *m/z*: 301 (M^+). *Anal.* Calcd for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37; Found: C, 67.90; H, 5.46.

3,4-Dimethoxy-cinnamic acid-(2-phenoxy-ethyl ester) (**6**): mp 109—111 °C; yield 90%. ^{1}H-NMR: (DMSO-d_6) δ: 7.59 (1H, d, J=15.6 Hz, COCH), 7.36—6.94 (8H, m, ArH), 6.61 (1H, d, J=15.6 Hz, CH), 4.44 (2H, t, J=3.3 Hz, $COOCH_2$), 4.23 (2H, t, J=3.3 Hz, OCH_2), 3.78 (6H, s, CH3). ^{13}C-NMR (DMSO-d_6) δ: 166.5, 158.3, 151.2, 149.2, 145.1, 129.6, 126.9, 123.1, 120.9, 115.4, 114.6, 111.7, 110.7, 65.9, 62.6, 55.8. IR ν_{max} (cm^{-1}): 2954, 1716, 1153. MS (EI^+) *m/z*: 328 (M^+). *Anal.* Calcd for $C_{19}H_{20}O_5$: C, 69.50; H, 6.14; Found: C, 69.44; H, 6.03.

Determination of the Radical Scavenging Activity DPPH assay was performed as described.[20,21] Briefly, the stable radical DPPH in ethanol (500 μM, 2 ml) was added to 2 ml of the test compounds at different concentrations in ethanol. The final concentration of the test compounds in the reaction mixtures were 12.5, 25, 37.5, and 50 μM. Each mixture was then shaken vigorously and held for 30 min at room temperature and in the dark. The decrease in absorbance of DPPH at 517 nm was measured. Ethanol was used as a blank solution and DPPH solution in ethanol served as the control. The percentage of remaining DPPH was then calculated, and the radical-scavenging effects of the tested compounds were compared in terms of EC_{50} (the concentration needed to reduce 50% of the initial amount of DPPH and expressed as

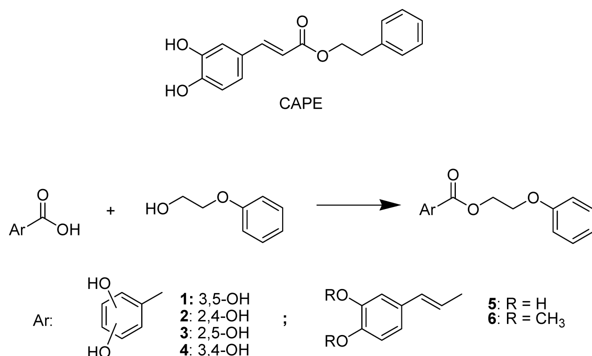

Fig. 1. CAPE Structure and General Synthetic Scheme for Polyphenolic Acid Esters

the molar ratio of each compound to the radical). All tests were performed in triplicate.

Determination of Antioxidative Activity The antioxidative activity was evaluated by the ferric thiocyanate assay using AAPH-induced lipid peroxidation of a Tween-emulsified linoleic acid system.[22—24] Briefly, 0.2 ml of distilled water, 0.5 ml of 0.2 M phosphate buffer (pH 7.0), and 0.5 ml of 0.25% Tween-20 (in buffer solution) were mixed with 0.5 ml of 2.5% (w/v) linoleic acid in ethanol. The mixture was then stirred for 1 min. The peroxidation was initiated by the addition of 50 μl of AAPH solution (0.1 M). The stock solution of antioxidant or test compounds in DMSO (final concentrations for the test compounds and DMSO are 10^{-4} M and 0.1%, respectively) was then added, and the reaction was carried out at 37 °C for 375 min in the dark. The degree of inhibition of oxidation was measured by the ferric thiocyanate method for each interval of 75, 150, 225, 300, and 375 min. To 0.1 ml of peroxidation reaction mixture at each interval, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 2×10^{-2} M freshly prepared $FeCl_2$ (in 3.5% aqueous HCl) were added. Precisely 3 min after addition, the absorbance of the red complex $[Fe(SCN)]^{2+}$ was measured at 500 nm. The control for the assay was prepared in the same manner by mixing all of the chemicals and reagents except the test compound. All tests were performed in triplicate.

Determination of the Scavenging Effect on Peroxynitrite Peroxynitrite synthesis was carried out as described.[27] Briefly, acidified hydrogen peroxide (1 M in 0.7 M HCl, 20 ml) and sodium nitrite (0.2 M, 20 ml) solution were drawn into two separate syringes. The contents of both syringes were simultaneously injected into an ice-cooled beaker containing 1.5 M potassium hydroxide (40 ml). Manganese dioxide was added to the solution to remove excess hydrogen peroxide. The solution was filtered and the concentration of the resulting stock was determined spectrophotometrically at 302 nm (ε=1670 M^{-1} cm^{-1}). The typical yield of freshly prepared peroxynitrite was 30 mM. The peroxynitrite was diluted in 0.1 M NaOH. The pyrogallol red (PR) dye bleaching assay of peroxynitrite was carried out according to the reported methods.[25,26] Experiments were conducted at 25 °C in 50 mM phosphate-buffered saline containing 0.1 mM diethylenetriaminepentaacetic acid, 90 mM NaCl, and 5 mM KCl, pH 7.4. Blanks using DMSO alone in the absence of test compounds and peroxynitrite allowed to degrade for 5 min in phosphate-buffered saline, pH 7.4, were also examined. There was no interference by DMSO and degraded peroxynitrite on the PR. Peroxynitrite induced the bleaching of PR dye, which was measured at 542 nm (ε=24000 M^{-1} cm^{-1}). Consumption of PR (50 μM) in the presence and absence of test compounds (1.25—125 μM) was measured over a range of peroxynitrite concentrations (0—62.5 μM). The ratios of rate constants k_A/k_{PR}, which represent the relative antioxidant activities, were determined by plotting D_0/D_A against $[\text{antioxidant}]_0/[PR]_0$. k_A and k_{PR} are the rate constants for reaction of peroxynitrite with the antioxidants and PR, respectively. D_0 and D_A are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the antioxidant compounds, respectively.

Table 1. Free Radical Scavenging Activity of Compounds for DPPH Radical

Compound	EC_{50} (μM)[a]	Inhibition (%)[b]
CAPE	38±1.2	75.3±0.03
1	370±16.6	6.4±0.18
2	305±21.8	9.1±0.21
3	74±3.6	38.5±0.09
4	34±1.5	79.1±0.02
5	37±0.9	75.5±0.04
6	no activity	no activity

a) EC_{50}: The concentration of test compounds needed to reduce DPPH absorption by 50% at 517 nm. The values were calculated from the slope equation of the dose–response curves. Values are from three independent determinations and are expressed as mean±S.D. *b*) Inhibition (%): indicates the percent of inhibition at 50 μM of antioxidant. Results shown represent mean±S.D. from three independent experiments.

RESULTS AND DISCUSSION

Free Radical Scavenging Activity The scavenging DPPH free radical is a common method to evaluate the antioxidative activity of antioxidants. As shown in Table 1, these polyphenolic acid esters displayed various degrees of free radical scavenging activity, with decreasing activity in the following order: **4**≈**5**≈CAPE>**3**>**2**>**1**>**6**. Among them, compounds **4** and **5**, were the most potent, having antiradical effects comparable to that of CAPE. The results indicated that the compounds with *ortho*-dihydroxyl configurations (compounds **4**, **5**) showed greater antiradical activity than *para*-configuration (compound **3**). The compounds with *meta*-dihydroxyl groups (compounds **1**, **2**) or dimethoxylcinnamic acid moiety (compound **6**) gave little or no activity at all. In addition, the results also suggest that the conjugated double bond is dispensable for free radical scavenging activity (compounds **4**, **5**). Furthermore, the replacement of phenethyl in CAPE with phenoxyethyl moiety appears to be no enhancing effect on radical-scavenging activity.

Antioxidative Activity To verify the antioxidative activity of these compounds, the three compounds (compounds **3**—**5**) with significant potent DPPH radical-scanvenging activity were further analyzed for their capacity in inhibiting the lipid peroxidation induced by AAPH. The *in vitro* model using AAPH-induced lipid peroxidation of Tween-emulsified linoleic acid is a common method[22—24] used to measure the antioxidative activity of synthetic antioxidants. In this assay, the oxidation is carried out under conditions similar to biological systems. The inhibitory effects on lipid peroxidation or the antioxidative activity of these compounds are listed in Table 2. We defined the antioxidative activity of these compounds as the relative rate of lipid peroxidation initiated by AAPH radical. The stronger is the antioxidative activity, the smaller is the rate of lipid peroxidation. As shown in Table 2, the inhibitory potency of these compounds is comparable to CAPE with the decreasing order of: **3**>**4**≈CAPE>**5**. Compound **5** with conjugated double bond showed lower antioxidative activity than CAPE. The reduced activity of compound **5** may be resulted from the introduction of phenoxylethyl moiety that altered the hydrophobicity of the compound. It is of interest that compound **3** contains neither conjugated double bond nor catechol groups but possesses the highest activity. One of the explanations for the highest activity of compound **3** might be due to its hydrogen-donat-

Table 2. Antioxidant Activity of the Compounds on AAPH-Induced Lipid Peroxidation of a Tween-Emulsified Linoleic Acid System

Compound	Rate of peroxide formation×10^4 (ΔA_{500}/min)	Inhibition (%)[a]
CAPE	7.61±0.45	73.2
3	4.62±0.54	83.7
4	6.41±0.61	77.4
5	11.21±1.03	60.4

a) Inhibition of peroxidation (%)=(1−rate of test compound/rate of solvent control)×100%. Peroxidation was initiated by the addition of AAPH (0.1 M) solution to the Tween-emulsified linoleic acid mixture. Degree of peroxidation was estimated by measuring absorption at 500 nm for the formation of complex $[Fe(SCN)]^{2+}$. All tests were performed in triplicate. Data shown here represent the slope of the time course-absorption curves of each compound analyzed. The control for the assay was carried out identically but in the absence of the test compound and the slope set as 100%. Results shown represent mean±S.D. from three independent experiments.

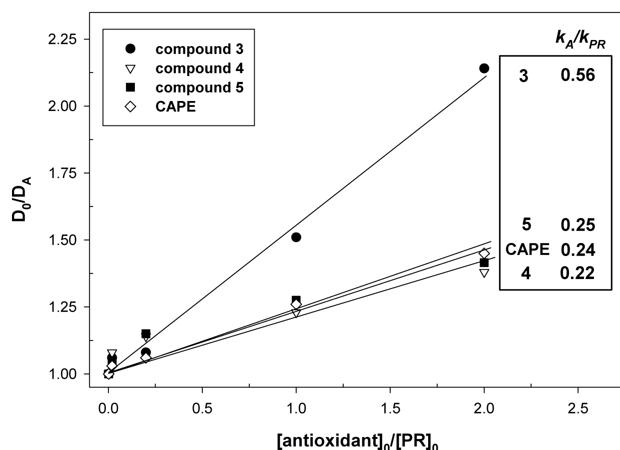

Fig. 2. Plot of D_0/D_A against [Antioxidant]$_0$/[PR]$_0$

Reactions were carried out at room temperature by adding peroxynitrite (12.5 μM) into tubes containing phosphate buffer (50 mM pH 7.0) and 50 mM of [PR]$_0$. Results shown are the mean of two independent experiments.

ing ability. Namely, the neighboring carbonyl group together with *p*-hydroxyl electron donating group to *o*-hydroxyl group in compound **3** could enhance the O–H bond breaking and accelerate the release of hydrogen. However, we can not exclude other potential explanations such as solubility, hydrophobicity, and stability of compound **3**.

Peroxynitrite-Scavenging Activity The interesting evidence for a role of peroxynitrite in biological processes prompted us to investigate the reaction of compounds with peroxynitrite. Studies also demonstrated that polyphenolic and flavonoid compounds exhibited efficiency peroxynitrite-scavenging activity and prevent oxidation of macromolecules elicited by peroxynitrite.[29] In this study, the peroxynitrite-scavenging activities were determined according to the reported method.[25,26] Briefly, peroxynitrite induced the bleaching of PR dye, which was measured at 550 nm. Consumption of PR in the presence and absence of the test compounds was measured over a range of peroxynitrite concentrations (0—62.5 μM). Figure 2 shows the D_0/D_A data for different concentrations of antioxidant plotted against [antioxidant]$_0$/[PR]$_0$. D_0 and D_A are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the tested antioxidant compounds, respectively. k_A and k_{PR} are the rate constants for the reaction of peroxynitrite with the tested antioxidants and PR, respectively. The ratio k_A/k_{PR}, which represents the relative antioxidant activities, was calculated from the slope of the straight line plotting D_0/D_A=against [A]$_0$/[PR]$_0$. The greater is the ratio of k_A/k_{PR}, the more potent is the peroxynitrite-scavenging activity of the tested compounds. Results shown in Fig. 2 indicate that the peroxynitrite-scavenging potency with the order of **3**>**5**≈CAPE≈**4**. Compound **3**, the most potent one, displayed about 2-fold greater activity than CAPE.

Based on the evidence, 2,5- or 3,4-dihydroxyl groups (compounds **3**, **4**, respectively) in benzoic acid moiety confer a marked antioxidative activity. Furthermore, the compounds without the conjugated double bonds (compounds **3**, **4**) gave similar anti-oxidant activity to that of compound **5** and CAPE. These observations also suggest that the conjugated double bond in this type of the polyphenolic structure appears to be independent of antioxidative activity, at least for *in vitro* assays, that is in agreement with some of the previous findings.[13,19]

In conclusion, we synthesized and analyzed the antioxidant capacity of six polyphenolic acid esters. Among them, **3**—**5** are more potent than the other compounds, with their antioxidant efficacy comparable to CAPE. An effective antioxidant should be able to scavenge a wide range of toxic free radical species, these compounds would thus be worthy of further investigations.

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