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Bioorganic & Medicinal Chemistry 13 (2005) 1791–1797
Bioorganic & Medicinal Chemistry

Evaluation of caffeic acid amide analogues as anti-platelet aggregation and anti-oxidative agents

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Received 18 September 2004; revised 26 November 2004; accepted 26 November 2004
Available online 22 December 2004

Abstract—A series of amides of caffeic acid were synthesized and evaluated for their anti-platelet and anti-oxidative activities. *N*-(2-Bromo-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (**12**) and *N*-(3-Bromo-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (**13**) exhibited potent inhibitory activity (IC_{50} = 5.8 and 6.7 μM, respectively) against arachidonic acid-induced (AA) platelet aggregation, comparable with invalid caffeic acid. Most of the synthesized caffeic acid anilides exhibited the promising anti-platelet aggregation in AA-induced assay and anti-oxidative activities. This study also exhibited that caffeic anilides displayed more potent anti-oxidative activity in the radical scavenging activity assay than trolox and vitamin E.

1. Introduction

The abnormal expression of platelets promotes the occurrence of many cardiovascular diseases as well as some inflammatory responses. Our previous studies suggested that many intracellular factors, especially some lipid mediators, that is arachidonic acid (AA), thromboxane A_2 (TXA_2) and platelet activating factor (PAF), could induce the activation of platelets.[1] These lipid mediators may contribute to pathogenesis of various cardiovascular disorders that can be amplified by platelet activation. Moreover, it was reported that the thromboxane A_2, a platelet aggregation agent, derived from arachidonic acid metabolic pathway would be affected by inhibitors of oxygenase and/or by some antioxidants.[1] These evidences motivate us to progress two main topics including the investigation of anti-platelet and anti-oxidative agents from natural sources and related synthetic derivatives.

Recently, we found that the EtOH extract of *Aster geratoides* Turcz. exhibited anti-platelet aggregation activity. Further investigating the partitioned EtOAc layer by bioassay-directed fractionations led to the isolation of 3,4-dihydroxycinnamic acid (caffeic acid, **1**), nonacosane, α-spinasterol, apigenin, quercetin-3-*O*-rutinoside and several aliphatic acids. Except flavonoids, caffeic acid and its derivatives are also widely distributed in the plant kingdom. In deed, their esters of organic acid and glycosides exhibit a broad spectrum of biological activities including anti-oxidative,[2–4] anti-inflammatory,[5,6] antiviral[7–9] and anticancer effect.[10–13] Although many caffeic acid derived ester analogues display the potential activities, but the ester groups in caffeic acid are metabolically very labile and limited for their use.[14,15] Several modified caffeic acid amides are recently demonstrated for anti-lipooxidation and exhibiting more stable characteristics.[16] Furthermore, some caffeic acid amide analogues, such as *N*-caffeoyl-β-phenethylamine was reported to have inhibitory effects on PG synthetase and to be potential for the inhibition of arachidonate 5-lipoxygenase.[17] These findings prompted us to synthesize a series of corresponding caffeic acid amides **2-17** for the purpose to extend the pharmacological activities, such as anti-platelet and anti-oxidative responses.

2. Results and discussion

Using dicyclohexyl carbodiimide (DCC) as a condensing reagent, we have carried out the reaction of the caffeic acid with a variety of aniline under reflux THF to give the corresponding amide products in good yield (Table 1).

Keywords: Caffeic anilides; Arachidonic acid; Anti-platelet aggregation; Anti-oxidation.
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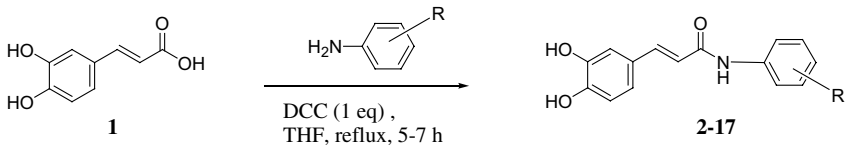
doi:10.1016/j.bmc.2004.11.055

Table 1. Reactions of caffeic acid (**1**) with a variety of anilines



Entry	R	Reaction time (h)	Product (% Yield)
1	H	7	**2** (95)
2	2-OH	7	**3** (99)
3	3-OH	7	**4** (94)
4	4-OH	6	**5** (86)
5	2-F	7	**6** (97)
6	3-F	7	**7** (84)
7	4-F	7	**8** (69)
8	2-Cl	7	**9** (57)
9	3-Cl	7	**10** (91)
10	4-Cl	6	**11** (97)
11	2-Br	7	**12** (83)
12	3-Br	7	**13** (99)
13	4-Br	5	**14** (74)
14	3-CN	5	**15** (35)
15	2-CO_2Et	7	**16** (84)
16	3,4-Dimethoxy	6.5	**17** (73)

All the caffeic acid and its synthesized amides (**2**–**17**) derivatives were tested for their bioactivities against platelet aggregation induced by arachidonic acid (AA), U46619 (an analogue of TXA_2), platelet activating factor (PAF) and MDA formation induced by AA.[1] The results was summarized in Table 2. Platelet aggregation and AA-induced MDA formation all could be inhibited by free caffeic acid at high dose. These data correlated to the observation in reported article by Koshihara et al.[18] Quite pronounced results were also observed that all the synthesized caffeic acid amides derivatives exhibited obvious inhibitory activity on AA-induced platelet aggregation and MDA formation, and some analogs (**11**, **14**, **16**, **17**) also inhibited U46619-induced and/or PAF-induced platelet aggregation. Inspection of the anti-platelet aggregation induced by AA test, caffeic anilides with halogens **6**–**14** or ester groups **16** were more active than those with hydroxy **3**–**5** or methoxy group **17**. The order of caffeic acid with aniline substituted group for the anti-platelet aggregation activity is Br > Cl, F, CO_2Et > OH > OMe > CN. These results imply that caffeic anilides with halogens or esters, which are belonging to hydrophobic groups rather than the responding hydrophilic groups as hydroxy or methoxy, would increase anti-platelet aggregation activity. Notably, caffeic acid anilides **12** and **13** exhibited potent inhibitory activity (IC_{50} = 5.8 and 6.7 μM, respectively) against AA-induced platelet aggregation, and **14** and **16** have promising inhibition in all four mediator-induced assays (AA, U46619, PAF and MDA).

Table 2. The IC_{50} (μM) value[23] of caffeic acid amides on the aggregation of washed rabbit platelets induced by arachidonic acid (AA), U46619, PAF and anti-MDA formation

Sample	Anti-AA-induced	Anti-U46619-induced	Anti-PAF-induced	Anti-MDA formation
Aspirin				15.5 ± 2.3
Caffeic acid **1**	>100	>100	>100	>50
2	56.1 ± 1.4	>100	>100	6.7 ± 0.1
3	59.4 ± 7.4	104.3 ± 9.9	>100	5.8 ± 0.4
4	64.4 ± 1.3	>100	>100	23.0 ± 4.4
5	25.1 ± 0.3	>100	>100	19.6 ± 1.2
6	21.4 ± 1.8	>100	>100	8.7 ± 2.8
7	26.0 ± 4.8	>100	>100	2.7 ± 0.2
8	22.0 ± 4.4	>100	>100	2.3 ± 0.0
9	18.6 ± 5.1	>100	>100	3.1 ± 3.0
10	41.6 ± 7.4	>100	>100	22.1 ± 1.1
11	25.5 ± 2.9	95.9 ± 6.7	>100	3.5 ± 2.3
12	5.8 ± 0.6	>100	>100	9.3 ± 3.3
13	6.7 ± 0.5	>100	>100	8.2 ± 2.4
14	16.4 ± 5.7	66.0 ± 1.2	69.6 ± 9.7	11.6 ± 4.6
15	64.1 ± 1.6	>100	>100	17.2 ± 1.7
16	17.9 ± 5.3	37.8 ± 12.3	58.0 ± 2.8	10.6 ± 3.6
17	60.2 ± 5.9	56.1 ± 1.4	>100	47.7 ± 9.8

Table 3. Scavenging activity of caffeic acid amides for DPPH and ABST radical assay[19–22]

Compound	ABTS radical scavenging activity[a]	DPPH radical scavenging activity in ethanolic-water buffer system[b]	DPPH radical scavenging activity in anhydrous ethanolic system
Trolox	18.9 ± 1.9	41.7 ± 1.4	34.3 ± 4.2
Vit. E	91.0 ± 1.0	110.6 ± 11.9	101.7 ± 5.0
Caffeic acid **1**	11.0 ± 5.5	15.5 ± 4.9	27.4 ± 1.0
2	12.2 ± 7.3	20.7 ± 3.2	22.6 ± 1.0
3	8.4 ± 0.5	20.7 ± 2.3	27.3 ± 1.3
4	44.0 ± 28.0	38.1 ± 10.5	26.9 ± 2.5
5	5.2 ± 3.3	17.3 ± 0.6	20.5 ± 1.7
6	13.7 ± 4.1	16.3 ± 0.7	18.9 ± 1.1
7	12.2 ± 2.8	15.5 ± 1.4	18.8 ± 1.7
8	12.3 ± 2.5	16.0 ± 1.8	19.2 ± 0.9
9	12.1 ± 1.9	14.4 ± 2.3	17.2 ± 0.8
10	11.2 ± 3.8	13.8 ± 2.7	17.5 ± 1.5
11	15.1 ± 1.7	16.2 ± 0.6	20.8 ± 2.0
12	10.7 ± 1.7	12.7 ± 0.5	17.6 ± 2.2
13	11.8 ± 1.7	11.1 ± 2.2	16.4 ± 1.4
14	12.5 ± 2.4	11.9 ± 0.5	18.9 ± 2.2
15	11.2 ± 3.4	19.4 ± 0.8	19.4 ± 1.7
16	14.3 ± 0.8	13.8 ± 0.4	17.3 ± 0.4
17	13.5 ± 2.2	18.1 ± 2.3	22.1 ± 1.9

Data are shown as EC_{50} (μM ± SD).

[a] The final concentration of ABTS ethanolic solution was 2.0×10^{-4} M.

[b] The final concentration of DPPH ethanolic-water buffer solution was 1.0×10^{-4} M.

As above mentioned, the thromboxane A_2, a platelet aggregation agent would be affected by some antioxidants.[1] The evaluation of caffeic anilides derivatives for anti-oxidation was also described herein. Free radical scavenging is generally the accepted mechanism for antioxidants inhibiting lipid oxidation. Two stable radicals including 2,2-diphenyl-1-picrylhydrazyl (DPPH)[19,20] and 2′2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)[21,22] were employed. DPPH has a strong absorption at 517 nm and is a stable nitrogen-centered free radical which can accept an electron or hydrogen radical converting into a stable, diamagnetic molecule. ABTS can react with H_2O_2 or hydroxyl radical to generate blue/green ABTS radical cation ($ABTS^{\cdot+}$) with absorption at 734 nm. Addition of antioxidant to preformed $ABTS^{\cdot+}$ reduces it to ABTS and the decrease in absorption is also correlated with the scavenging action. Because $ABTS^{\cdot+}$ and DPPH are two different radicals with different reactivity in oxi-reduction, they are served as two probes for our search for the free radical scavenging action of caffeic acid and its amides.

As shown in Table 3, these caffeic acid derivatives displayed potent anti-oxidative activity in the ABTS and DPPH free radical scavenging assay. All of synthetic caffeic acid amides derivatives as well as caffeic acid itself exhibited the lower EC_{50}'s values than those of the standard antioxidants, trolox and vitamin E. However, no obvious difference for the free radical scavenging activity was found between caffeic acid and caffeic acid amides. These findings were not consistent with the results reported from literature[16] that caffeic acid amides were more effective than caffeic acid in the microsomal lipid peroxidation. To investigate the affinity of hydrophilic or lipophilic for caffeic acid and its amides on antioxidant activity, we designed the more lipophilic solvent system in DPPH radical-scavenging assay. Thus, ethanol–water with 100 μL acetate buffer system was replaced by the anhydrous ethanol for an attempt to increase lipophilic affinity in the DPPH assay. Except for **4**, no obvious difference of radical scavenge activity for the synthetic caffeic acid amides (**2**–**17**) were found between ethanol–water with buffer and anhydrous ethanol solvent system (Table 3). The anti-oxidative activity of caffeic acid (**1**) was obviously decreased in the modified solvent system, compared with the ethanol–water buffer. Moreover, the results assayed by the anhydrous ethanol solvent system displayed caffeic acid anilides possessing the higher scavenging activity than those of caffeic acid (Table 3). On the basis of the anhydrous ethanol solvent, the anti-oxidative activity for the synthetic caffeic acid anilides would meaningfully increase, in the order of substituted halogen > H > OH.

By both ABTS and DPPH with ethanol–water buffer system assays, the order of radical scavenging ability was exhibited as caffeic acid anilides **5** > **3** > **4**, corresponding to substituted 4′ > 2′ > 3′-aminophenol, respectively. These evidences would reveal that caffeic anilides with the resonance electron-donating group at C2′ and/or C4′ position increase electron density to C1′, then to the N position, and therefore, enhance the electron donating of *N*-anilide to stabilize the corresponding caffeoyl radical conformers responsible for the higher activity. Not only found in the anti-oxidation but also in the anti-platelet aggregation, caffeic anilide **4** has the lowest activity (as shown in Table 2) compared with **5** and **3**.

3. Conclusion

A series of caffeic anilides were synthesized and evaluated for their anti-platelet aggregation and anti-oxidative activity. Basing on aforementioned evidences, the synthesized amide analogues permit them to possess

potent anti-platelet aggregation activity than those of original caffeic acid. More promising results were found on the basis of anti-oxidative assay revealing that caffeic acid and its anilides seems to have more potent activity than positive control agents, trolox and vitamin E. Preliminary structure–activity relationships (SAR) studies were also noted. The results of anti-oxidation and anti-platelet aggregation for **3**, **4** and **5** concluded that caffeic anilides with the electron-donating group at C2′ and/or C4′ position would enhance the anti-platelet aggregation and anti-oxidative activities. Most of the synthesized caffeic acid anilides exhibited the promising anti-platelet aggregation in AA-induced assay and anti-oxidative activities, deducing that the AA might be inhibited by the other antioxidants.

Moreover, the order of anti-platelet aggregation for caffeic acid with aniline substituted group was showed as Br > Cl, F, CO_2Et > OH > OMe > CN. These evidences also imply the importance of soft and hydrophobic characteristics for the aniline substituted group, and further conclude that the solubility effect might be more important than withdrawing-donating electron effect of the substitute groups in the anti-platelet aggregation assay. For the detailed SAR studies, more caffeic acid derivatives and bioassays on the anti-platelet aggregation and anti-oxidation remain to be investigated.

4. Experimental

Infrared spectra were recorded with an FT-IR spectrometer Analect RFX-65. ^1H and ^{13}C NMR spectra were measured for samples in $CDCl_3$ with an FT-NMR spectrometer Bruker AC-300 at 300 and 75 MHz, respectively. Mass spectra were recorded with a spectrometer JEOL JMS-D-100. High resolution mass spectra were measured with a mass spectrometer JEOL TMS-HX 110. The silica gel used for flash column chromatography was made by Merck (60 H), and precoated Silica gel (Merck 60F-254) plates were used for TLC. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. All reagents were of reagent grade.

4.1. General procedure for the synthesis of caffeic anilides

A mixture of the caffeic acid (180 mg, 1 mmol), the dicyclohexyl carbodiimide (DCC, 206 mg, 1 mmol) and aniline (1 mmol) was stirred in THF at reflux temperature for 7 h. The solvent was removed under vacuum. The residue was then purified by flash chromatography using hexane and hexane/ethyl acetate (2:1 → 1:1) as the eluent.

4.1.1. 3-(3,4-Dihydroxy-phenyl)-*N*-(2-fluoro-phenyl)-acrylamide (6). Yellow solid (263.8 mg, 97%); mp 199–200 °C; IR (film) 3262, 1663, 1602, 1525, 1491, 1443, 1350, 1277, 1195, 1159, 1114, 973, 936, 851, 811, 777, 679, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.58 (1H, d, J = 15.7 Hz), 6.77–6.83 (1H, m), 6.85 (1H, d, J = 8.1 Hz), 6.98 (1H, dd, J = 8.1, 1.9 Hz), 7.10 (1H, d, J = 1.9 Hz), 7.26–7.39 (2H, m), 7.56 (1H, d, J = 15.7 Hz), 7.78–7.83 (1H, m), 9.45 (1H, br s); ^{13}C NMR δ 107.0 (1C, d, J = 27 Hz), 110.3 (1C, d, J = 22 Hz), 115.0, 115.6 (1C, d, J = 2 Hz), 116.3, 119.1, 122.0, 128.0, 130.9 (1C, d, J = 9 Hz), 142.3 (1C, d, J = 11 Hz), 142.6, 146.3, 148.3, 163.7 (1C, d, J = 239 Hz), 165.2; EIMS (relative intensity) m/z 273 (M^+, 23), 164 (10), 163 (100), 145 (9), 135 (22), 134 (37), 117 (15), 111 (17), 89 (34), 83 (10), 77 (13), 63 (10); exact mass calcd for $C_{15}H_{12}FNO_3$ m/z 273.0801, EI-HRMS m/z 273.0793.

4.1.2. 3-(3,4-Dihydroxy-phenyl)-*N*-(3-fluoro-phenyl)-acrylamide (7). Light yellow solid (229.8 mg, 84%); mp 191–192 °C; IR (film) 3261, 1662, 1597, 1525, 1488, 1455, 1350, 1304, 1282, 1259, 1185, 1113, 975, 849, 811, 753, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.85 (1H, d, J = 15.5 Hz), 6.85 (1H, d, J = 8.2 Hz), 7.00 (1H, dd, J = 8.2, 2.0 Hz), 7.07–7.19 (4H, m), 7.57 (1H, d, J = 15.5 Hz), 8.38–8.43 (1H, m), 8.96 (1H, br s); ^{13}C NMR δ 115.0, 115.7 (1C, d, J = 20 Hz), 116.3, 119.2, 122.0, 123.3, 124.9 (1C, d, J = 8 Hz), 125.1 (1C, d, J = 3 Hz), 128.2, 128.2 (1C, d, J = 11 Hz), 142.7, 146.3, 148.2, 153.8 (1C, d, J = 242 Hz), 165.2; EIMS (relative intensity) m/z 273 (M^+, 43), 164 (10), 163 (100), 162 (38), 135 (19), 134 (37), 117 (13), 111 (61), 89 (23), 83 (11); exact mass calcd for $C_{15}H_{12}FNO_3$ m/z 273.0801, EI-HRMS m/z 273.0806.

4.1.3. 3-(3,4-Dihydroxy-phenyl)-*N*-(4-fluoro-phenyl)-acrylamide (8). Yellow solid (188.1 mg, 69%); mp 173–175 °C; IR (film) 3268, 1660, 1598, 1508, 1444, 1408, 1355, 1284, 1214, 1184, 1158, 1114, 1004, 975, 836, 813, 777, 515 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.60 (1H, d, J = 15.5 Hz), 6.85 (1H, d, J = 8.2 Hz), 6.96 (1H, dd, J = 8.2, 2.0 Hz), 7.03–7.11 (3H, m), 7.57 (1H, d, J = 15.5 Hz), 7.74–7.80 (2H, m), 8.33 (2H, br s), 9.40 (1H, br s); ^{13}C NMR δ 114.9, 115.9 (1C, d, J = 23 Hz), 116.3, 119.4, 121.8 (1C, d, J = 7 Hz), 121.8, 128.1, 136.9 (1C, d, J = 3 Hz), 142.1, 146.2, 148.1, 159.5 (1C, d, J = 238 Hz), 164.9; EIMS (relative intensity) m/z 273 (M^+, 32), 164 (11), 163 (100), 162 (18), 135 (22), 134 (31), 117 (15), 111 (40), 110 (10), 89 (34), 83 (11), 78 (10), 77 (13); exact mass calcd for $C_{15}H_{12}FNO_3$ m/z 273.0801, EI-HRMS m/z 273.0799.

4.1.4. *N*-(2-Chloro-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (9). Yellow solid (165.9 mg, 57%); mp 186–188 °C; IR (film) 3253, 1663, 1592, 1524, 1439, 1352, 1285, 1181, 1113, 975, 811, 753, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.86 (1H, d, J = 8.2 Hz), 6.89 (1H, d, J = 15.3 Hz), 7.02 (1H, dd, J = 8.2, 2.0 Hz), 7.10 (1H, td, J = 7.7, 1.3 Hz), 7.16 (1H, d, J = 2.0 Hz), 7.31 (1H, td, J = 7.7, 1.3 Hz), 7.43 (1H, dd, J = 8.2, 1.3 Hz), 7.59 (1H, d, J = 15.4 Hz), 8.26 (2H, br s), 8.36 (1H, dd, J = 8.2, 2.0 Hz), 8.68 (1H, br s); ^{13}C NMR δ 115.1, 116.3, 119.1, 122.1, 124.0, 124.6, 125.6, 128.1, 128.2, 130.0, 136.5, 143.0, 146.2, 148.3, 165.2; EIMS (relative intensity) m/z 291 (8), 289 (M^+, 26), 164 (10), 163 (100), 162 (41), 135 (19), 134 (37), 127 (28), 117 (13), 89 (27), 78 (6), 77 (8); exact mass calcd for $C_{15}H_{12}ClNO_3$ m/z 289.0506, EI-HRMS m/z 289.0500.

4.1.5. *N*-(3-Chloro-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (10). Light yellow solid (263 mg, 91%); mp 178–180 °C; IR (film) 3288, 1690, 1663, 1593, 1523,

1481, 1444, 1424, 1350, 1283, 1183, 1114, 1077, 1004, 975, 906, 880, 850, 811, 778, 680, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.60 (1H, d, J = 15.5 Hz), 6.85 (1H, d, J = 7.9 Hz), 6.95–7.13 (3H, m), 7.28 (1H, t, J = 7.9 Hz), 7.58 (1H, d, J = 15.5 Hz), 7.60 (1H, d, J = 15.5 Hz), 8.00–8.02 (1H, m), 9.47 (1H, br s); ^{13}C NMR 115.0, 116.3, 118.3, 119.0, 119.9, 122.0, 123.8, 127.9, 130.9, 134.6, 141.7, 142.8, 146.2, 148.2, 165.4; EIMS (relative intensity) m/z 291 (8), 289 (M^+, 23), 164 (10), 163 (100), 162 (35), 135 (17), 134 (29), 127 (18), 117 (11), 89 (22), 77 (7); exact mass calcd for $C_{15}H_{12}ClNO_3$ m/z 289.0506, EI-HRMS m/z 289.0497.

4.1.6. *N*-(4-Chloro-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (11). Light yellow solid (282 mg, 97%); mp 198–200 °C; IR (film) 3249, 1655, 1648, 1596, 1560, 1522, 1491, 1399, 1283, 1242, 1185, 1174, 1116, 1091, 975, 811 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.57 (1H, d, J = 15.4 Hz), 6.85 (1H, d, J = 8.2 Hz), 6.98 (1H, dd, J = 8.2, 1.9 Hz), 7.31 (1H, dt, J = 9.0, 2.5 Hz), 7.55 (1H, d, J = 15.4 Hz), 7.77 (1H, dd, J = 9.0, 2.5 Hz), 9.35 (1H, br s); ^{13}C NMR δ 115.0, 116.3, 119.2, 121.5, 121.9, 128.1, 128.3, 129.4, 139.4, 142.4, 146.2, 148.2, 165.0; EIMS (relative intensity) m/z 291 (8), 289 (M^+, 22), 164 (10), 163 (100), 162 (20), 135 (19), 134 (24), 129 (11), 127 (35), 117 (12), 89 (26), 78 (6), 77 (9); exact mass calcd for $C_{15}H_{12}ClNO_3$ m/z 289.0506, EI-HRMS m/z 289.0504.

4.1.7. *N*-(2-Bromo-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (12). Yellow solid (275.9 mg, 83%); mp 166–168 °C; IR (film) 3259, 2931, 2854, 1660, 1593, 1521, 1435, 1365, 1284, 1181, 1114, 975, 849, 811, 752, 733, 661, 563, 522 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.86 (1H, d, J = 15.2 Hz), 6.86 (1H, d, J = 8.2 Hz), 7.00–7.07 (2H, m), 7.16 (1H, d, J = 2.0 Hz), 7.32–7.38 (1H, m), 7.59 (1H, d, J = 15.4 Hz), 7.59–7.61 (1H, m), 8.24–8.27 (1H, m), 8.58 (1H, br s); ^{13}C NMR δ 114.7, 115.0, 115.8, 118.5, 121.7, 124.3, 125.9, 127.5, 128.3, 132.8, 137.1, 142.7, 145.7, 147.8, 164.8; EIMS (relative intensity) m/z 335 (19), 333 (M^+, 20), 254 (10), 173 (27), 171 (26), 164 (14), 163 (100), 162 (67), 145 (11), 135 (25), 134 (44), 127 (10), 117 (16), 89 (28), 77 (10); exact mass calcd for $C_{15}H_{12}BrNO_3$ m/z 333.0001, EI-HRMS m/z 333.0006.

4.1.8. *N*-(3-Bromo-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (13). Light yellow solid (330 mg, 99%); mp 160–162 °C; IR (film) 3259, 2931, 2853, 1662, 1588, 1525, 1477, 1444, 1420, 1374, 1282, 1181, 1113, 1070, 1044, 974, 849, 810, 775, 679, 590, 563, 521 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.57 (1H, d, J = 15.5 Hz), 6.85 (1H, d, J = 8.2 Hz), 6.98 (1H, dd, J = 8.2, 1.8 Hz), 7.10 (1H, d, J = 1.8 Hz), 7.21–7.26 (2H, m), 7.56 (1H, d, J = 15.5 Hz), 7.60–7.63 (1H, m), 8.15 (1H, d, J = 1.9 Hz), 9.43 (1H, br s); ^{13}C NMR δ 115.0, 116.3, 118.7, 119.0, 122.0, 122.7 (×2), 126.7, 128.0, 131.2, 142.0, 142.7, 146.2, 148.3, 165.2; EIMS (relative intensity) m/z 335 (20), 333 (M^+, 21), 173 (13), 171 (13), 164 (13), 163 (100), 162 (60), 145 (10), 135 (21), 134 (33), 117 (13), 89 (23); exact mass calcd for $C_{15}H_{12}BrNO_3$ m/z 333.0001, EI-HRMS m/z 332.9998.

4.1.9. *N*-(4-Bromo-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (14). Light yellow solid (247.8 mg, 74%); mp 230–232 °C; IR (film) 3266, 1661, 1596, 1528, 1487, 1444, 1395, 1352, 1298, 1283, 1242, 1181, 1114, 1072, 1007, 974, 812, 504 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.59 (1H, d, J = 15.3 Hz), 6.84 (1H, d, J = 8.0 Hz), 6.96 (1H, d, J = 8.0 Hz), 7.11 (1H, s), 7.43 (2H, dd, J = 8.6, 1.3 Hz), 7.57 (1H, d, J = 15.3 Hz), 7.71 (2H, dd, J = 8.6, 1.3 Hz), 8.28 (1H, br s), 9.41 (1H, br s); ^{13}C NMR δ 115.0, 116.0, 116.3, 119.1, 121.9, 122.0, 127.9, 132.3, 139.6, 142.6, 146.1, 148.1, 165.3; EIMS (relative intensity) m/z 333 (M^+, 20), 173 (27), 171 (26), 164 (14), 163 (100), 162 (67), 145 (11), 135 (25), 134 (44), 117 (16), 89 (28); exact mass calcd for $C_{15}H_{12}BrNO_3$ m/z 333.0001, EI-HRMS m/z 333.0006.

4.1.10. *N*-(3-Cyano-phenyl)-3-(3,4-dihydroxy-phenyl)-acrylamide (15). Light yellow solid (97 mg, 35%); mp 279–280 °C; IR (film) 3317, 2927, 2856, 2233, 1698, 1668, 1587, 1549, 1484, 1430, 1362, 1284, 1256, 1192, 1169, 1116, 976, 791, 668, 680 cm^{-1}; ^1H NMR (acetone-d_6) δ 6.52 (1H, d, J = 15.5 Hz), 6.78 (1H, d, J = 8.1 Hz), 6.96 (1H, dd, J = 8.1, 1.4 Hz), 7.06 (1H, d, J = 1.8 Hz), 7.40–7.51 (2H, m), 7.55 (1H, d, J = 15.5 Hz), 7.84 (1H, d, J = 7.9 Hz), 8.14 (1H, s); ^{13}C NMR δ 113.7, 115.2, 116.5, 118.0, 119.5, 122.5, 123.8, 125.1, 128.0, 128.2, 131.0, 141.3, 144.3, 146.8, 149.2, 167.5; EIMS (relative intensity) m/z 280 (M^+, 16), 164 (10), 163 (100), 162 (27), 135 (16), 134 (18), 117 (12), 89 (21); exact mass calcd for $C_{16}H_{12}N_2O_3$ m/z 280.0848, EI-HRMS m/z 280.0842.

4.1.11. 2-[3-(3,4-Dihydroxy-phenyl)-acryloylamino]-benzoic acid ethyl ester (16). Yellow solid (273.6 mg, 84%); mp 186–188 °C; IR (film) 3261, 2981, 1680, 1604, 1588, 1525, 1447, 1368, 1258, 1163, 1113, 1090, 1003, 972, 811, 755, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.40 (3H, t, J = 7.2 Hz), 4.41 (2H, q, J = 7.2 Hz), 6.57 (1H, d, J = 15.4 Hz), 6.88 (1H, d, J = 8.1 Hz), 7.07–7.15 (2H, m), 7.22 (1H, d, J = 2.0 Hz), 7.55–7.62 (2H, m), 8.04–8.07 (1H, m), 8.83 (1H, d, J = 8.1 Hz), 11.18 (1H, s); ^{13}C NMR δ 14.3, 62.1, 115.2, 116.1, 116.3, 119.8, 121.0, 122.3, 123.0, 127.8, 131.6, 135.0, 142.8, 143.0, 146.2, 148.4, 165.1, 168.8; EIMS (relative intensity) m/z 327 (M^+, 39), 166 (18), 165 (100), 163 (75), 162 (15), 135 (21), 134 (35), 120 (13), 119 (29), 117 (15), 92 (11), 89 (26); exact mass calcd for $C_{18}H_{17}NO_5$ m/z 327.1107, EI-HRMS m/z 327.1111.

4.1.12. 3-(3,4-Dihydroxy-phenyl)-*N*-(3,4-dimethoxy-phenyl)-acrylamide (17). Light yellow solid (229.6 mg, 73%); mp 200–202 °C; IR (film) 3334, 1693, 1656, 1605, 1513, 1443, 1406, 1259, 1233, 1213, 1154, 1134, 1115, 1023, 976, 851, 810, 770, 668 cm^{-1}; ^1H NMR (acetone-d_6) δ 3.75 (3H, s), 3.76 (3H, s), 6.60 (1H, d, J = 15.5 Hz), 6.84 (1H, d, J = 8.1 Hz), 6.86 (1H, d, J = 8.7 Hz), 6.95 (1H, dd, J = 8.1, 1.8 Hz), 7.10 (1H, d, J = 1.8 Hz), 7.21 (1H, d, J = 8.7 Hz), 7.52 (1H, s), 7.54 (1H, d, J = 15.5 Hz), 9.22 (1H, br s); ^{13}C NMR δ 55.9, 56.4, 105.8, 112.3, 113.2, 114.9, 116.3, 119.6, 121.8, 128.2, 134.1, 141.8, 146.2, 146.6, 148.0, 150.2, 164.9; EIMS (relative intensity) m/z 315 (M^+, 88), 300 (10),

163 (47), 153 (100), 138 (40), 135 (11), 110 (13), 107 (13), 89 (13); exact mass calcd for $C_{17}H_{17}NO_5$ *m/z* 315.1107, EI-HRMS *m/z* 315.1104.

4.2. Evaluation of anti-platelet aggregation activity[1]

4.2.1. Preparation of washed platelet suspension. Washed platelets were obtained from rabbits as previously described.[23,24] In brief, rabbit blood was collected from the marginal ear vein into tubes containing one-sixth volume of acid-citrate-dextrose as anticoagulant. The blood was centrifuged at $200 \times g$ for 15 min at room temperature. The platelet-rich plasma was mixed with 1/40 volume of EDTA (final concentration 5 mM) and re-centrifuged at $1000 \times g$ for 12 min. The supernatant was discarded and the platelet pellet was suspended in modified Ca^{2+}-free Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES) with 0.35% bovine serum albumin, heparin (50 unit/mL) and apyrase (1 unit/mL). Following incubation at 37 °C for 20 min, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 mM Ca^{2+}. The platelet numbers were counted by hemacytometer and adjusted to 3.5×10^8 platelets/mL. To eliminate or minimize any possible effects of the solvent, the final concentration of the vehicle dimethyl sulfoxide (DMSO) in the platelet suspension was fixed at 0.5%.

4.2.2. Platelet aggregation. Aggregation was measured by a turbidimetric method.[25] The PACKS-4 aggregrometer (Helena Laboratories, Beaumont, TX, USA) was used. Transmission of washed platelet suspension was assigned 0% aggregation while transmission through Tyrode's buffer was assigned 100% aggregation. Platelets 0.5 mL were pre-incubated with 2.5 μL of the vehicle DMSO (0.5%) to serve as control or caffeic anilides for 2 min and then stimulated with 2.5 μL of stimulants of aggregation such as arachidonic acid, U46619 or PAF.

4.3. Malondialdehyde measurement

Malondialdehyde measurement was modified from the method described by Yagi.[26] Four minutes following challenge with the aggregation stimulants, 0.5 mL of platelet suspension was mixed with equal volumes of cold 20% trichloroacetic acid to stop all reactions. Following centrifugation at $10{,}000 \times g$ for 3 min, the supernatant was reacted with 10 mM 2-thiobarbituric acid in a boiling water bath for 15 min. The malondialdehyder-thiobarbituric acid reactant was extracted by *n*-butanol and the concentration determined by measuring fluorescence (E_x = 532 nm, E_m = 553 nm). The amount of malondialdehyde was calculated according to the standard equation.

4.4. Evaluation of the antioxidant activity by DPPH radical scavenging test in ethanol–buffer system[19,20]

Various concentration of α-tocopheral or test compounds were triply added to well of 96 wells EIA plate, then 100 μL aliquots of 100 mM acetate buffer, 100 μL aliquots of ethanol and 50 μL aliquots of 500 μM DPPH were added sequentially. After 90 min, the absorbance of the remaining DPPH was determined at 517 nm in EIA reader (Power $Wave_x$, Bio-Tex instrument). The scavenging activity was measured as the decrease in absorbance of the DPPH, expressed as a percentage of the absorbance of a control DPPH solution without test compounds.

4.5. Evaluation of the antioxidant activity by DPPH radical scavenging test in ethanol–buffer system[19,20]

Various concentration of α-tocopheral or test compounds were triply added to well of 96 wells EIA plate, then 200 μL aliquots of ethanol and 50 μL aliquots of 500 μM DPPH were added sequentially. After 90 min, the absorbance of the remaining DPPH was determined at 517 nm in EIA reader (Power $Wave_x$, Bio-Tex instrument). The scavenging activity was measured as the decrease in absorbance of the DPPH, expressed as a percentage of the absorbance of a control DPPH solution without test compounds.

4.6. Evaluation of the antioxidant activity by ABTS radical scavenging test[21]

In present study, the antioxidant activity of test compounds was evaluates using an improved $ABTS^{\cdot+}$ scavenging assay described by Re et al.[21] Briefly, the purple $ABTS^{\cdot+}$ solution (7.5 mM) was prepared by mixing ABTS solution with potassium persulfate (2.5 mM) overnight in dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with ethanol or PBS (pH 7.4) to an OD_{734} of 0.70 ± 0.02 for assay. Six minutes after the 5 μL of studied extracts or compounds was added to 1 mL of $ABTS^{\cdot+}$ dilution, the amount of $ABTS^{\cdot+}$ remaining was determined at 734 nm, and the radical scavenging activity was obtained from the following equation:

$$\text{Radical scavenging activity}(\%) = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

The antioxidant activity of extracts or compounds was expressed as EC_{50} which was defined as the concentration (in μM) of extract or compounds required to remove the formed $ABTS^{\cdot+}$ radicals by 50%.

Acknowledgements

Financial support of this work by the National Science Council (NSC 91-2113-M-077-006) and National Research Institute of Chinese Medicine (NRICM-93-DHM-03) of the Republic of China is gratefully acknowledged.

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