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Synthesis of Caffeic Acid Phenethyl Ester Derivatives, and Their Cytoprotective and Neuritogenic Activities in PC12 Cells

Haiming Shi,*[,∥,†] Dongsheng Xie,[∥,‡] Ruoling Yang,[§] and Yaqian Cheng[#]

[†]Institute of Food and Nutraceutical Science, SJTU-Rich Research Institute of Nutrition and Skin Science, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

[‡]School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China

[§]Department of Pharmacy, School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China

[#]College of Chemistry & Material Engineering, Wenzhou University, Zhejiang, Wenzhou 325035, China

ABSTRACT: Twenty-one caffeic acid phenethyl ester (CAPE) derivatives were synthesized, and characterized by IR, HR-MS, ^{1}H and ^{13}C NMR analyses. All compounds were evaluated for their cytoprotective effects against H_2O_2-induced cytotoxicity and neuritogenic activities in the neurite outgrowth in PC12 cells. Compounds **1** and **20** exhibited stronger cytoprotective activities than their parent compound CAPE at 4 nM. Compounds **1**, **4**, **12** and **13** showed potential neuritogenic activities at 0.5 nM, while compounds **19** and **20** induced neurite outgrowth at 10 nM. The results from this study suggested that CAPE and its derivatives may be potential functional food ingredients for the prevention of neurodegenerative diseases.

KEYWORDS: *Cytoprotective activity, neuritogenic activity, CAPE, synthesis, H_2O_2, PC12 cells*

INTRODUCTION

Age-associated human neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, have become a major global health problem. It is well-known that oxidative stress plays a critical role in the pathogenesis of these diseases.[1] Recently, natural antioxidants have been proven to benefit neurodegenerative diseases for their potential in preventing oxidative damage in neural cells.[2,3] In addition, some dietary components could mimic or enhance the neuritogenic activity of nerve growth factor (NGF),[4,5] which is essential for maintaining neural cell functions. Foods rich in potential cytoprotective and neuritogenic components may reduce the risk of neurodegenerative diseases.

Caffeic acid phenethyl ester (CAPE), an active component first isolated from propolis, possessed antitumor,[6,7] anti-inflammatory,[8,9] antioxidant,[10,11] and immunomodulatory[12] activities. *In vitro* and *in vivo* studies showed that CAPE can be easily metabolized to caffeic acid or phase II metabolites including glucuronidation, sulfation and methylation of CAPE.[13−15] There is limited data relating the concentration of CAPE in plasma and tissue after oral administration. Although the metabolism of CAPE *in vivo* is unclear, several studies have proven that CAPE possessed neuroprotective effects on HT22 mouse hippocampal cells against oxidative stress induced by acrolein,[16] dopaminergic neurons in rat organotypic midbrain slice cultures from IFN-γ/LPS-induced injury,[17] and dopaminergic neuronal loss induced by 6-hydroxydopamine in rats.[18] Considering the above beneficial effects of CAPE on the neurons/nervous system and its possibility of crossing the blood−brain barrier,[19] CAPE might be a promising candidate for reducing the risk of neurodegenerative diseases. Furthermore, in a continuation of our research on bioactive compounds from propolis,[20,21] CAPE showed an interesting neurite outgrowth-promoting activity in NGF-mediated PC12 cells at 10 nM concentration.

To date, there is little study on cytoprotective and neuritogenic activities for CAPE derivatives. This study synthesized several groups of CAPE derivatives and investigated their cytoprotective and neuritogenic activities in PC12 cells. The results from this study may enhance our understanding of the structure−activity relationships for CAPE derivatives, and approaches to improve the physicochemical and biological properties for CAPE derivatives.

MATERIALS AND METHODS

Materials. All chemical reagents were purchased from Aladdin Reagent Company (Shanghai, China). Silica gel (200−300 mesh, Qingdao Marine Chemical Co. Ltd.) was used in open column chromatography. All solvents used for isolation and UPLC-Q-TOFMS analysis were of analytical grade and chromatographic grade, respectively. Differentiated and undifferentiated PC12 (rat adrenal pheochromocytoma) cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). DMEM and 1 × PBS were purchased from Hyclone (Greeley, CO, USA). Horse serum and fetal bovine serum were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). DMSO was obtained from Applichem Co. (Darmstadt, Germany). Hydrogen peroxide was purchased from Sinopharm (Beijing, China). Ultrapure water was prepared by a Millipore ultra-Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ (Millipore, Billerica, MA, USA) and was used for all experiments.

Instruments. Melting point was determined on an XT4A digital micromelting point apparatus (Tianjin, China). UV data were recorded on a Shimadzu UV-1800 spectrophotometer (Kyoto,

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Japan). IR spectra were determined with a Nicolet 6700 FT-IR spectrophotometer (Madison, Wisconsin, USA). NMR spectra were obtained on Bruker Advance DRX-300, DRX-400 spectrometers (Rheinstetten, Germany) and the chemical shifts are given in δ (ppm) values with reference to TMS. The coupling constants (J values) are reported in Hz. HRMS were carried out on Waters Xevo G_2 Q-TOF mass spectrometer (Milford, Massachusetts, USA).

General Synthetic of Procedure CAPE Derivatives. *Method A for Compounds 1−4.* To a mixture of caffeic acid (1 mmol) in 10 mL anhydrous DME was added thionyl chloride (6 mmol) under nitrogen atmosphere. This mixture was heated to reflux for 12 h and then distilled under vacuum to remove the excess thionyl chloride completely. The intermediate was redissolved with 10 mL anhydrous DME, and to this solution was added alcohol (1 mmol). This mixture was heated to reflux for 4 h before evaporation. The residue was purified by column chromatography and/or recrystallization to give target compounds.

Modified Method A for Compounds 5−13. To a mixture of caffeic acid or its analogues (1 mmol) in 5−10 mL alcohol was added thionyl chloride (3−6 mmol) under nitrogen atmosphere. This mixture was heated slowly to 65 °C and stirred for 4 h. After distillation of most of alcohol under high vacuum the residue was purified by column chromatography and/or recrystallization to give target compounds.

Method B for Compounds 14 and 15. To a mixture of caffeic acid or its analogue (1 mmol), potassium carbonate (1.1 mmol) and 5 mL DMF was added benzyl bromide (1 mmol). This mixture was stirred at room temperature for 12 h. Fifteen mL water was added and the suspension was extracted with diethyl ether (10 mL × 3). The combined organic layer was washed with aqueous $NaHCO_3$ and brine, dried, evaporated and purified by recrystallization to give target compounds.

Method C for Compounds 16 and 17. A mixture of caffeic acid (1 mmol), phenethylamine/4-hydroxyphenethylamine (1 mmol), triethylamine (1.5 mmol) and 4 mL DMF were stirred at 0 °C for 10 min, to which was added a solution of BOP (1 mmol) in 4 mL DCM. After stirred for 30 min at 0 °C, the mixture was allowed to warm to room temperature and stirred for 20 h. Fifteen g crushed ice and 25 mL chloroform were added to the mixture, organic layer was separated and washed with 10% hydrochloric acid and brine, dried, evaporated and purified by column chromatography and recrystallization to give target compounds.

Method D for Compounds 18 and 19. A mixture of alcohol (10 mmol), meldrum's acid (12 mmol) and 40 mL 1, 4-dioxane was heated to reflux for 6 h before evaporation. The residue was purified by column chromatography to give the intermediate malonic acid mono ester. A mixture of malonic acid mono ester (5 mmol), 3,4-dihydroxybenzaldehyde/4-nitrobenzaldehyde (5 mmol), 5 mL pyridine and 0.5 mL piperidine was stirred for 15 h at room temperature before evaporation. The residue was redissolved with 50 mL ethyl acetate and washed with 5% hydrochloric acid, brine, dried, evaporated and purified column chromatography and recrystallization to give target compounds.

Caffeic Acid Cinnamyl Ester (1). The title compound was prepared according to method A as white powder (45 mg, 15.2% yield). mp 159−160 °C; IR (KBr) ν_{max} 3477, 3318, 1682, 1602, 1535, 1280, 1184 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.55 (1H, d, J = 16.0 Hz, H-β), 7.21−7.35 (5H, m, H-2′, 3′, 4′, 5′, 6′), 7.02 (1H, d, J = 1.2 Hz, H-2), 6.95 (1H, dd, J = 1.2, 8.0 Hz, H-6), 6.81 (1H, d, J = 8.0 Hz, H-5), 6.70 (1H, d, J = 16.0 Hz, H-γ'), 6.35 (1H, dt, J = 6.4, 16.0 Hz, H-β'), 6.31 (1H, d, J = 16.0 Hz, H-α), 4.79 (2H, d, J = 6.4 Hz, H-α'); HRESIMS $[M - H]^-$ m/z 295.0966 (Calcd for $C_{18}H_{15}O_4$, 295.0970).

Caffeic Acid Phenylpropyl Ester (2). The title compound was prepared according to method A as light yellow powder (47 mg, 15.8% yield). mp 121−123 °C; IR (KBr) ν_{max} 3491, 3338, 1683, 1603, 1535, 1278, 1183 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 9.56 (1H, s, −OH), 9.13 (1H, s, −OH), 7.45 (1H, d, J = 15.9 Hz, H-β), 7.15−7.30 (5H, m, H-2′, 3′, 4′, 5′, 6′), 7.03 (1H, d, J = 2.1 Hz, H-2), 6.98 (1H, dd, J = 2.1, 8.1 Hz, H-6), 6.75 (1H, d, J = 8.1 Hz, H-5), 6.24 (1H, d, J = 15.9 Hz, H-α), 4.09 (2H, t, J = 6.6 Hz, H-α'), 2.68 (2H, t, J = 6.6 Hz, H-γ'), 1.94 (2H, quintet, H-β'); HRESIMS $[M - H]^-$ m/z 297.1124 (Calcd for $C_{18}H_{17}O_4$, 297.1127).

Caffeic Acid 4-Hydroxyphenethyl Ester (3). The title compound was prepared according to method A as light yellow powder (60 mg, 20.0% yield). mp 153−155 °C; IR (KBr) ν_{max} 3495, 3266, 1698, 1605, 1513, 1283, 1204, 1170 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.89 (1H, d, J = 16.0 Hz, H-β), 7.46 (1H, d, J = 1.6 Hz, H-2), 7.40 (2H, d, J = 8.0 Hz, H-2′, 6′), 7.25 (2H, d, J = 8.0 Hz, H-3′, 5′), 7.16 (1H, dd, J = 1.6, 8.4 Hz, H-6), 7.00 (1H, d, J = 8.4 Hz, H-5), 6.55 (1H, d, J = 16.0 Hz, H-α), 3.87 (2H, t, J = 7.2 Hz, H-α'), 3.22 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 299.0916 (Calcd for $C_{17}H_{15}O_5$, 299.0919).

Caffeic Acid Nonyl Ester (4). The title compound was prepared according to method A as white powder (43 mg, 14.0% yield). mp 107−109 °C; IR (KBr) ν_{max} 3488, 3333, 1686, 1604, 1536, 1278, 1184 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.53 (1H, d, J = 16.0 Hz, H-β), 7.06 (1H, d, J = 2.0 Hz, H-2), 6.95 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.82 (1H, d, J = 8.0 Hz, H-5), 6.21 (1H, d, J = 16.0 Hz, H-α), 4.13 (2H, t, J = 6.8 Hz, H-1′), 1.63 (2H, m, H-2′), 1.20−1.32 (12H, m, H-3′-H-8′), 2.55 (3H, t, J = 6.8 Hz, H-9′); HRESIMS $[M - H]^-$ m/z 305.1739 (Calcd for $C_{18}H_{25}O_4$, 305.1753).

Ferulic Acid Phenethyl Ester (5). The title compound was prepared according to modified method A as light yellow gum (195 mg, 65.4% yield). IR (KBr) ν_{max} 3418, 1682, 1603, 1516, 1270, 1172 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.60 (1H, d, J = 16.0 Hz, H-β), 7.22−7.34 (5H, m, H-2′, 3′, 4′, 5′, 6′), 7.06 (1H, dd, J = 2.0, 8.4 Hz, H-6), 7.01 (1H, d, J = 2.0 Hz, H-2), 6.91 (1H, d, J = 8.4 Hz, H-5), 6.27 (1H, d, J = 16.0 Hz, H-α), 4.42 (2H, t, J = 7.2 Hz, H-α'), 3.91 (3H, s, 3−OCH_3), 3.02 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 297.1125 (Calcd for $C_{18}H_{17}O_4$, 297.1127).

Isoferulic Acid Phenethyl Ester (6). The title compound was prepared according to modified method A as white powder (202 mg, 67.7% yield). mp 79−81 °C; IR (KBr) ν_{max} 3384, 1686, 1600, 1262, 1184, 1165 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.58 (1H, d, J = 16.0 Hz, H-β), 7.22−7.34 (5H, m, H-2′, 3′, 4′, 5′, 6′), 7.13 (1H, d, J = 2.0 Hz, H-2), 7.01 (1H, dd, J = 2.0, 8.4 Hz, H-6), 6.83 (1H, d, J = 8.4 Hz, H-5), 6.27 (1H, d, J = 16.0 Hz, H-α), 4.41 (2H, t, J = 7.2 Hz, H-α'), 3.91 (3H, s, 3−OCH_3), 3.01 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 297.1120 (Calcd for $C_{18}H_{17}O_4$, 297.1127).

4-Methoxy Cinnamic Acid Phenethyl Ester (7). The title compound was prepared according to modified method A as light yellow gum (194 mg, 68.7% yield). IR (KBr) ν_{max} 2930, 1708, 1631, 1515, 1263, 1216, 1150 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.63 (1H, d, J = 16.0 Hz, H-β), 7.47 (2H, d, J = 8.8 Hz, H-2, 6), 7.24−7.32 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.90 (2H, d, J = 8.8 Hz, H-3, 5), 6.30 (1H, d, J = 16.0 Hz, H-α), 4.41 (2H, t, J = 7.2 Hz, H-α'), 3.83 (3H, s, 4−OCH_3), 3.02 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 281.1176 (Calcd for $C_{18}H_{17}O_3$, 281.1178)

3,4-Dimethoxycinnamic Acid Phenethyl Ester (8). The title compound was prepared according to modified method A as white powder (165 mg, 52.8% yield). mp 89−91 °C; IR (KBr) ν_{max} 2916, 2849, 1702, 1635, 1515, 1258, 1236, 1139 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.62 (1H, d, J = 16.0 Hz, H-β), 7.28 (5H, m, H-2′, 3′, 4′, 5′, 6′), 7.09 (1H, dd, J = 2.0, 8.4 Hz, H-6), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.86 (1H, d, J = 8.4 Hz, H-5), 6.30 (1H, d, J = 16.0 Hz, H-α), 4.43 (2H, t, J = 7.2 Hz, H-α'), 3.90 (6H, s, 3, 4−OCH_3), 3.02 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 311.1300 (Calcd for $C_{19}H_{19}O_4$, 311.1283).

p-Coumaric Acid Phenethyl Ester (9). The title compound was prepared according to modified method A as white powder (157 mg, 58.5% yield). mp 90−92 °C; IR (KBr) ν_{max} 3279, 1681, 1631, 1582, 1515, 1278, 1206, 1170 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.62 (1H, d, J = 16.0 Hz, H-β), 7.39 (2H, d, J = 8.4 Hz, H-2, 6), 7.22−7.33 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.85 (2H, d, J = 8.8 Hz, H-3, 5), 6.27 (1H, d, J = 16.0 Hz, H-α), 4.42 (2H, t, J = 7.2 Hz, H-α'), 3.02 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 267.1016 (Calcd for $C_{17}H_{15}O_3$, 267.1021).

Cinnamic Acid Phenethyl Ester (10). The title compound was prepared according to modified method A as white powder (145 mg, 57.5% yield). mp 55−56 °C; IR (KBr) ν_{max} 1710, 1637, 1496, 1313,

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1205, 1173 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.67 (1H, d, J = 16.0 Hz, H-β), 7.35−7.51 (5H, m, H-2, 3, 4, 5, 6), 7.21−7.33 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.42 (1H, d, J = 16.0 Hz, H-α), 4.42 (2H, t, J = 7.2 Hz, H-α'), 3.01 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 251.1067 (Calcd for $C_{17}H_{15}O_2$, 251.1072).

*Sinapic Acid Phenethyl Ester (**11**).* The title compound was prepared according to modified method A as light yellow oil (216 mg, 65.8% yield). IR (KBr) ν_{max} 3419, 1704, 1633, 1514, 1283, 1218, 1153 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.58 (1H, d, J = 16.0 Hz, H-β), 7.22−7.34 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.76 (2H, s, H-2, 6), 6.29 (1H, d, J = 16.0 Hz, H-α), 4.43 (2H, t, J = 7.2 Hz, H-α'), 3.91 (6H, s, 3, 5−OCH_3), 3.02 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 327.1228 (Calcd for $C_{19}H_{19}O_5$, 327.1232).

*Caffeic Acid 3-Bromophenethyl Ester (**12**).* The title compound was prepared according to modified method A as white powder (42 mg, 11.6% yield). mp 120−122 °C; IR (KBr) ν_{max} 3472, 3316, 1686, 1604, 1528, 1278, 1180 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 9.59 (1H, s), 9.12 (1H, s), 7.52 (1H, m, H-2′), 7.44 (1H, d, J = 16.0 Hz, H-β), 7.42 (1H, m, H-6′), 7.28 (2H, m, H-4′, 5′), 7.03 (1H, d, J = 2.0 Hz, H-2), 6.98 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.75 (1H, d, J = 8.0 Hz, H-5), 6.22 (1H, d, J = 16.0 Hz, H-α), 4.32 (2H, t, J = 6.8 Hz, H-α'), 2.95 (2H, t, J = 6.8 Hz, H-β'); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.4, 148.5, 145.6, 145.3, 141.1, 131.7, 130.5, 129.3, 128.1, 125.4, 121.6, 121.4, 115.7, 114.8, 113.7, 63.9, 34.0; HRESIMS $[M - H]^-$ m/z 361.0079 (Calcd for $C_{17}H_{14}O_4Br$, 361.0075).

*Caffeic Acid 2-Bromophenethyl Ester (**13**).* The title compound was prepared according to modified method A as white powder (68 mg, 18.7% yield). mp 137−139 °C; IR (KBr) ν_{max} 3465, 3301, 1694, 1607, 1529, 1280, 1188 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 9.59 (1H, s), 9.15 (1H, s), 7.61 (1H, dd, J = 7.6, 0.8 Hz, H-3′), 7.46 (1H, d, J = 16.0 Hz, H-β), 7.41 (1H, dd, J = 7.6, 1.6 Hz, H-6′), 7.34 (1H, dt, J = 7.6, 0.8 Hz, H-5′), 7.11 (1H, dt, J = 7.6, 1.6 Hz, H-4′), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.98 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.76 (1H, d, J = 8.0 Hz, H-5), 6.22 (1H, d, J = 16.0 Hz, H-α), 4.34 (2H, t, J = 6.8 Hz, H-α'), 3.09 (2H, t, J = 6.8 Hz, H-β'); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.4, 148.5, 145.6, 145.3, 137.2, 132.6, 131.4, 128.8, 127.9, 125.4, 124.0, 121.4, 115.7, 114.8, 113.7, 62.7, 34.7; HRESIMS $[M - H]^-$ m/z 361.0073 (Calcd for $C_{17}H_{14}O_4Br$, 361.0075).

*Caffeic Acid Benzyl Ester (**14**).* The title compound was prepared according to method B as white powder (148 mg, 54.8% yield). mp 151−153 °C; IR (KBr) ν_{max} 3466, 3327, 1689, 1601, 1535, 1277, 1177 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.52 (1H, d, J = 16.0 Hz, H-β), 7.26−7.34 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.97 (1H, d, J = 1.2 Hz, H-2), 6.86 (1H, dd, J = 1.2, 8.0 Hz, H-6), 6.74 (1H, d, J = 8.0 Hz, H-5), 6.20 (1H, d, J = 16.0 Hz, H-α), 5.15 (2H, s, H-α'); HRESIMS $[M - H]^-$ m/z 269.0810 (Calcd for $C_{16}H_{13}O_4$, 269.0814).

*p-Coumaric Acid Benzyl Ester (**15**).* The title compound was prepared according to method B as white powder (213 mg, 83.8% yield). mp 90−92 °C; IR (KBr) ν_{max} 3401, 1691, 1599, 1517, 1279, 1203, 1170 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.60 (1H, d, J = 16.0 Hz, H-β), 7.34 (2H, d, J = 8.4 Hz, H-2, 6), 7.26−7.33 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.76 (2H, d, J = 8.4 Hz, H-3, 5), 6.27 (1H, d, J = 16.0 Hz, H-α), 5.18 (2H, s, H-α'); HRESIMS $[M - H]^-$ m/z 253.0860 (Calcd for $C_{16}H_{13}O_3$, 253.0865).

*Caffeic Acid Phenylacetamide (**16**).* The title compound was prepared according to method C as light yellow powder (81 mg, 28.6% yield). mp 153−155 °C; IR (KBr) ν_{max} 3488, 3346, 1649, 1602, 1551, 1326, 1287, 1189 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.34 (1H, d, J = 16.0 Hz, H-β), 7.13−7.24 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.93 (1H, d, J = 1.6 Hz, H-2), 6.82 (1H, dd, J = 1.6, 8.4 Hz, H-6), 6.71 (1H, d, J = 8.4 Hz, H-5), 6.16 (1H, d, J = 16.0 Hz, H-α), 3.48 (2H, t, J = 7.2 Hz, H-α'), 2.78 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 282.1125 (Calcd for $C_{17}H_{16}NO_3$, 282.1130).

*Caffeic Acid 4-Hydroxyphenylacetamide (**17**).* The title compound was prepared according to method C as light yellow powder (35 mg, 11.7% yield). mp 211−212 °C; IR (KBr) ν_{max} 3369, 1646, 1604, 1516, 1241 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.37 (1H, d, J = 15.6 Hz, H-β), 7.05 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.99 (1H, d, J = 2.0 Hz, H-2), 6.89 (1H, dd, J = 2.0, 8.4 Hz, H-6), 6.75 (1H, d, J = 8.4 Hz, H-5), 6.70 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.33 (1H, d, J = 15.6 Hz, H-α), 3.45 (2H, t, J = 7.2 Hz, H-α'), 2.74 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 298.1081 (Calcd for $C_{17}H_{16}NO_4$, 298.1079).

*Caffeic Acid 3,4-Dihydroxyphenethyl Ester (**18**).* The title compound was prepared according to method D as a white powder (648 mg, 41.0% yield for Knoevenagel condensation). mp 118−120 °C; IR (KBr) ν_{max} 3339, 1680, 1600, 1525, 1278, 1186 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.52 (1H, d, J = 16.0 Hz, H-β), 7.03 (1H, d, J = 2.0 Hz, H-2), 6.94 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.78 (1H, d, J = 8.0 Hz, H-5), 6.70 (1H, d, J = 2.0 Hz, H-2′), 6.70 (1H, d, J = 8.0 Hz, H-5′), 6.58 (1H, dd, J = 2.0, 8.0 Hz, H-6′), 4.29 (2H, t, J = 7.2 Hz, H-α'), 2.84 (2H, t, J = 7.2 Hz, H-β'); HRESIMS $[M - H]^-$ m/z 315.0862 (Calcd for $C_{17}H_{15}O_6$, 315.0869).

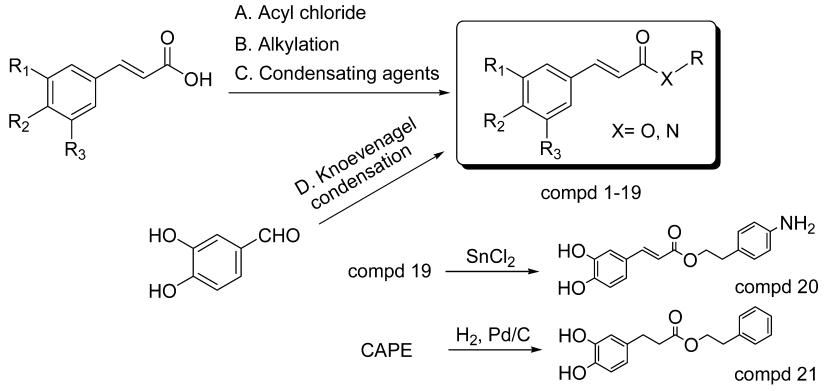
*Caffeic Acid 4-Nitrophenethyl Ester (**19**).* The title compound was prepared according to method D as a light yellow powder (305 mg, 18.5% yield for Knoevenagel condensation). mp 172−174 °C; IR (KBr) ν_{max} 3418, 1691, 1596, 1507, 1340, 1277, 1167 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 9.55 (1H, s), 9.11 (1H, s), 8.17 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.58 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.44 (1H, d, J = 16.0 Hz, H-β), 7.03 (1H, d, J = 2.0 Hz, H-2), 6.98 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.76 (1H, d, J = 8.0 Hz, H-5), 6.21 (1H, d, J = 16.0 Hz, H-α), 4.39 (2H, t, J = 6.4 Hz, H-α'), 3.11 (2H, t, J = 6.4 Hz, H-β'); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.4, 148.5, 146.7, 146.3, 145.6, 145.4, 130.2, 125.4, 123.4, 121.4, 115.7, 114.9, 113.6, 63.5, 34.2; HRESIMS $[M - H]^-$ m/z 328.0823 (Calcd for $C_{17}H_{14}NO_6$, 328.0821).

*Caffeic Acid 4-Aminophenethyl Ester (**20**).* A mixture of caffeic acid 4-nitrophenethyl ester (60 mg, 0.091 mmol), stannic chloride dihydrate (123 mg, 0.55 mmol) and 5 mL of ethanol was heated to reflux for 10 h before being cooled to room temperature. Ten milliliters of saturated Na_2CO_3 was added, and the suspension was extracted with ethyl acetate (10 mL × 3). The combined organic layer was dried, evaporated and purified by column chromatography to give the title compound as a light yellow powder (34 mg, 62.3% yield). mp 173−175 °C; IR (KBr) ν_{max} 3432, 1692, 1598, 1515, 1280, 1175 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 9.57 (1H, s), 9.12 (1H, s), 7.45 (1H, d, J = 16.0 Hz, H-β), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.99 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.91 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.76 (1H, d, J = 8.0 Hz, H-5), 6.50 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.23 (1H, d, J = 16.0 Hz, H-α), 4.20 (2H, t, J = 7.2 Hz, H-α'), 2.75 (2H, t, J = 7.2 Hz, H-β'); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.0, 148.9, 147.5, 146.0, 145.5, 129.7, 126.0, 125.1, 121.8, 116.2, 115.3, 114.5, 65.4, 34.3; HRESIMS $[M - H]^-$ m/z 298.1075 (Calcd for $C_{17}H_{16}NO_4$, 298.1079).

*3,4-Dihydroxyphenyl Propionic Acid Phenethyl Ester (**21**).* To a solution of caffeic acid phenethyl ester (50 mg, 0.176 mmol) in 10 mL of ethyl acetate was added 10% Pd/C (5 mg) under nitrogen atmosphere. The reaction flask was vacuumed and backfilled with hydrogen and stirred at room temperature for 12 h. The mixture was filtered and evaporated to give the titled compound as yellow gum (50 mg, 99.4% yield). IR (KBr) ν_{max} 3400, 1709, 1605, 1519, 1352, 1282, 1192 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.18−7.30 (5H, m, H-2′, 3′, 4′, 5′, 6′), 6.74 (1H, d, J = 8.0 Hz, H-5), 6.63 (1H, d, J = 2.0 Hz, H-2), 6.57 (1H, dd, J = 2.0, 8.0 Hz, H-6), 4.28 (2H, t, J = 7.2 Hz, H-α'), 2.91 (2H, t, J = 7.2 Hz, H-β'), 2.79 (2H, t, J = 7.8 Hz, H-α), 2.55 (2H, t, J = 7.8 Hz, H-β); HRESIMS $[M - H]^-$ m/z 285.1120 (Calcd for $C_{17}H_{17}O_4$, 285.1127).

*Cytotoxicities of CAPE and Its Derivatives **1**-**21** in PC12 Cells.* Differentiated PC12 cells were maintained in DMEM medium supplemented with 1% L-glutamine and 10% fetal bovine serum at 37 °C under a humidified 5% CO_2 atmosphere. Each test compound with appropriate concentration was dissolved in DMSO and diluted with serum-free media. Before treatment, cells were seeded on a 96 well plate at the density of 5 × 10^3 cells/well and cultured for 24 h. Then the medium was changed and cells were treated with CAPE and its derivatives at 4 and 40 nM for 24 h, respectively. Then, 20 μL of MTT solution (final concentration, 0.5 mg/mL) were added to each well, and cells were incubated for 4 h. The resultant formazan product was dissolved by the addition of 150 μL of DMSO. The absorbance was measured at the wavelength of 570 nm using a Thermo Scientific

Scheme 1. Synthesis of CAPE Derivatives[a]



Compounds	R_1	R_2	R_3	R	X	Method
1	OH	OH	H	$C_6H_5CH{=}CHCH_2$	O	A
2	OH	OH	H	$C_6H_5CH_2CH_2CH_2$	O	A
3	OH	OH	H	4-OH-$C_6H_5CH_2CH_2$	O	A
4	OH	OH	H	n-nonyl	O	A
5	OCH_3	OH	H	$C_6H_5CH_2CH_2$	O	A
6	OH	OCH_3	H	$C_6H_5CH_2CH_2$	O	A
7	H	OCH_3	H	$C_6H_5CH_2CH_2$	O	A
8	OCH_3	OCH_3	H	$C_6H_5CH_2CH_2$	O	A
9	H	OH	H	$C_6H_5CH_2CH_2$	O	A
10	H	H	H	$C_6H_5CH_2CH_2$	O	A
11	OCH_3	OH	OCH_3	$C_6H_5CH_2CH_2$	O	A
12	OH	OH	H	3-Br-$C_6H_5CH_2CH_2$	O	A
13	OH	OH	H	2-Br-$C_6H_5CH_2CH_2$	O	A
14	OH	OH	H	$C_6H_5CH_2$	O	B
15	H	OH	H	$C_6H_5CH_2$	O	B
16	OH	OH	H	$C_6H_5CH_2CH_2$	N	C
17	OH	OH	H	4-OH-$C_6H_5CH_2CH_2$	N	C
18	OH	OH	H	3,4-di-OH-$C_6H_5CH_2CH_2$	O	D
19	OH	OH	H	4-NO_2-$C_6H_5CH_2CH_2$	O	D

[a]Reagents and conditions: Method A: (1) caffeic acid, $SOCl_2$, DME, reflux, 12 h, (2) acyl chloride of caffeic acid, alcohol, DME, reflux, 4 h; Modified method A: caffeic acid or its analogues, excess alcohol, $SOCl_2$, 65 °C, 4 h. Method B: caffeic acid or its analogues, benzyl bromide, K_2CO_3, DMF, RT, 12 h. Method C: caffeic acid, phenethylamines/4-hydroxyphenethylamine, triethylamine, BOP, DMF/DCM, 0 °C to RT, 20 h. Method D: (1) alcohol, meldrum's acid, 1,4-dioxane, reflux, 6 h; (2) malonic acid mono ester, 3,4-dihydroxybenzaldehyde/4-nitrobenzaldehyde, pyridine, piperidine, RT, 15 h. RT stands for ambient temperature.

Multiskan MK3 microplate reader (Waltham, MA, USA). Results were expressed as percentage of cell viability (%), assuming control cells as 100%. The cytotoxicities of CAPE and its derivatives in undifferentiated PC12 cells at 0.5, 5, 10, and 20 nM, respectively, were performed following a similar procedure, except that the culture media was changed to DMEM medium supplemented with 1% L-glutamine, 10% horse serum and 5% fetal bovine serum.

Cytoprotective Effects of CAPE and Its Derivatives 1–21 in PC12 Cells. Before treatment, differentiated PC12 cells were seeded on a 96 well plate at the density of 1×10^4 cells/well and cultured for 24 h. The medium was changed, and cells were pretreated for 6 h with 4 nM or 40 nM CAPE and its derivatives, respectively; then the cells were exposed to 200 μM H_2O_2 for another 24 h. Cell viability was determined using the MTT method as described above.

Neuritogenic Effects of CAPE and Its Derivatives 1–21 in PC12 Cells. Undifferentiated PC12 cells (5×10^4 cells/well) were seeded on poly-L-lysine-coated 12-well plates. NGF (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterilized water and then diluted with serum-free media to the final concentration of 50 ng/mL. After 72 h of incubation with NGF, CAPE and its derivatives (0.5 nM or 10 nM), respectively, the neurite outgrowth of cells was observed by inverted microscope (OLYPUS IX71, Tokyo, Japan). Cells with one or more neurites whose lengths were greater than or equal to the length of one cell body were scored as positive. The number of neurite-bearing cells was counted in five randomly selected microscopic fields and expressed as a percentage of the total cells in the field. The neurite length was determined as the mean value for all identified neurite-bearing cells in a field (ImageJ software). At least 100 cells were selected for calculating the neurite length. Each experiment was conducted in triplicate.

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations. Significant differences ($p < 0.05$ or $p < 0.01$) between the means of control and different treatment groups were analyzed by Dunnett's test using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Synthesis of Caffeic Acid Phenethyl Ester Derivatives. Previous studies revealed that bioactivities of CAPE derivatives are mainly affected by the number of hydroxyl groups,[22,23] the methylation degree of the hydroxyl group,[24] the length of the ester moiety,[19] and the conjugate double bonds[25] in CAPE.

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These factors were the basis for designing the targeted compounds for the present study. In addition, selected electron-donating and -withdrawing groups were substituted on the benzene ring of the alcoholic moiety to alter the electronic density or provide additional binding sites.

As shown in Scheme 1, twenty-one CAPE derivatives (compounds **1**−**21**) were prepared, among which compounds **12**, **13** and **20** were first synthesized. Diverse methods were used for synthesis of these derivatives. We obtained caffeic acid esters (method A, compounds **1**−**4**) in 1,2-dichloroethane, which results in fewer side products compared to the frequently used 1,4-dioxane. Moreover, replacement of solvent with excess liquid alcohol lead to better yields of 50−70% in many cases (compounds **5**−**13**). Compounds **14** and **15** were conveniently obtained by alkylation (method B) with benzyl bromide in good yields and purities. Amide derivatives (compounds **16** and **17**) were synthesized by using a condensing agent such as BOP (method C). Some caffeate derivatives (compounds **18** and **19**) hardly unavailable by other methods were prepared by Knoevenagel condensation (method D). Reduction of compound **19** in the presence of stannous chloride gave compound **20** in high yield, and hydrogenation of CAPE afforded compound **21**.

Cytotoxicity of Caffeic Acid Phenethyl Ester Derivatives in PC12 Cells. CAPE and its derivatives were initially assayed for cytotoxic effects in differentiated PC12 cells (4 and 40 nM) and undifferentiated PC12 cells (0.5, 5, 10, and 20 nM), respectively. Differentiated and undifferentiated PC12 cells were incubated with these compounds for 24 h, respectively, and the extent of cell viability was determined using MTT assay. Cell viability less than 90% of control was considered toxic. The results demonstrated that CAPE and its derivatives were nontoxic in differentiated PC12 cells at 4 and 40 nM, respectively. While CAPE and its derivatives showed no toxicity at any tested concentrations except that compounds **4**, **12** and **13** have cytotoxicities at concentrations from 5 nM to 20 nM.

Caffeic Acid Phenethyl Ester Derivatives Protect PC12 Cells against H_2O_2-Induced Oxidative Stress. Reactive oxygen species (ROS) have been involved in the pathogenesis of many neurodegenerative diseases.[26] Hydroxyl radical is the most dangerous ROS and can be generated from H_2O_2 in the presence of transition metals including Fe and Cu. Hydroxyl radical can react rapidly with all types of intracellular biomolecules including lipids, DNA, and protein, eventually leading to cell death. Differentiated PC12 cells were reported to be more sensitive to oxidant stimulus than undifferentiated cells,[27] and be better for cytoprotective study. Thus, the cytoprotective activities of CAPE derivatives against H_2O_2-induced oxidative stress were evaluated on differentiated PC12 cells in the present study.

At the concentrations of 4 nM and 40 nM, all CAPE derivatives did not affect cell survival. Among the tested samples, 4 nM compounds **1** and **20** significantly increased the cell viability to 87.5% and 89.3%, respectively (Figure 1). Their cytoprotective activities were much better than those of the parent compound CAPE under the same concentrations. Compounds **3**, **4**, **12**, **13**, **15**, **16** and **19** showed similar protective capabilities to CAPE within the range of 62.9−81.1% at 4 nM, while compounds **1**−**4**, **6**, 7, **9**, **10**, **12**, **13**, **16**, **19**, and **20** possessed equivalent protective effects as CAPE within the range 61.9−93.9% at 40 nM (Figure 1). Other CAPE

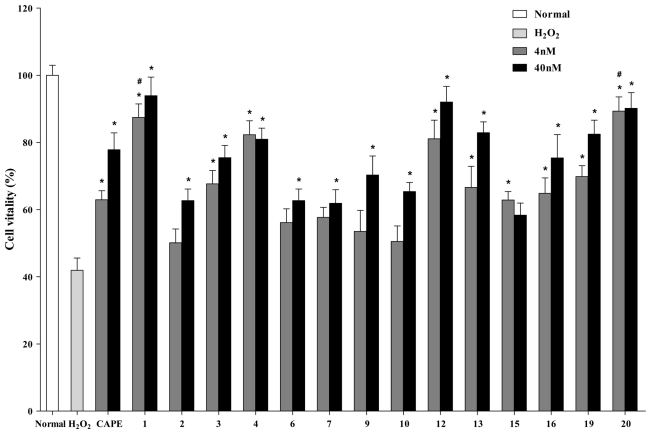

Figure 1. Cytoprotective effects of CAPE and selected CAPE derivatives in PC12 cells under H_2O_2-induced oxidative stress. Differentiated PC12 cells were treated with 4 nM, 40 nM CAPE and its derivatives for 6 h, respectively, and then the cells were exposed to 200 μM H_2O_2 for another 24 h. Cell viability was determined using the MTT method. The viability of normal cells was defined as 100%. Data are shown as mean ± SD (n = 3). (*) $p < 0.05$ compared to H_2O_2-treated group; (#) $p < 0.05$ compared to 4 nM CAPE group.

derivatives demonstrated poorer activities against H_2O_2-indued cytotoxicity (data not shown).

It is noteworthy that introduction of an amino group at C-4′ of the phenethyl ethanol moiety in CAPE could result in a more potent compound. Furthermore, a substitution of phenethyl ethanol with a cinnamyl group could more effectively suppress the toxicity induced by hydrogen peroxide. Our study also showed that the catechol group was not the crucial factor for the protective activities of CAPE derivatives on PC12 cells because compounds **14**, **17**, **18**, and **21** were inactive. Unexpectedly, caffeic acid 4-hydroxyphenethyl ester (**17**) and caffeic acid 3,4-dihydroxyphenethyl ester (**18**) poorly suppressed H_2O_2-induced cytotoxicity in PC12 cells. This phenomenon may be explained by their high hydrophilicities for additional phenolic hydroxyl on the phenethyl ethanol group, resulting in a poor permeability across the cellular membrane.

A series of catechol ring-fluorinated derivatives of CAPE and caffeic acid phenethyl amide (CAPA) were synthesized, respectively, and their cytoprotective effects were evaluated against menadione or H_2O_2-induced cytotoxicity in human umbilical vein endothelial cells (HUVEC).[24,28] One fluorinated derivative of CAPE and three fluorinated analogues of CAPA were found to possess similar cytoprotective activities with their parent compounds. Our results were well in accordance with these previous studies that CAPE derivatives could be considered as a new class of cytoprotective agents.

Caffeic Acid Phenethyl Ester Derivatives Induce Neurite Outgrowth of PC12 Cells. Nerve growth factor (NGF), showing benefits on the growth, differentiation and survival of neuronal cells, was expected to be a promising agent for the treatment of neurodegenerative diseases.[29] But the disadvantages of NGF such as inability to cross the blood−brain barrier and poor stability *in vivo*, drastically limited its application. As a result, discovery of small molecules from natural sources or their derivatives having neuritogenic ability is an alternative approach to prevent neurodegenerative diseases.[30,31]

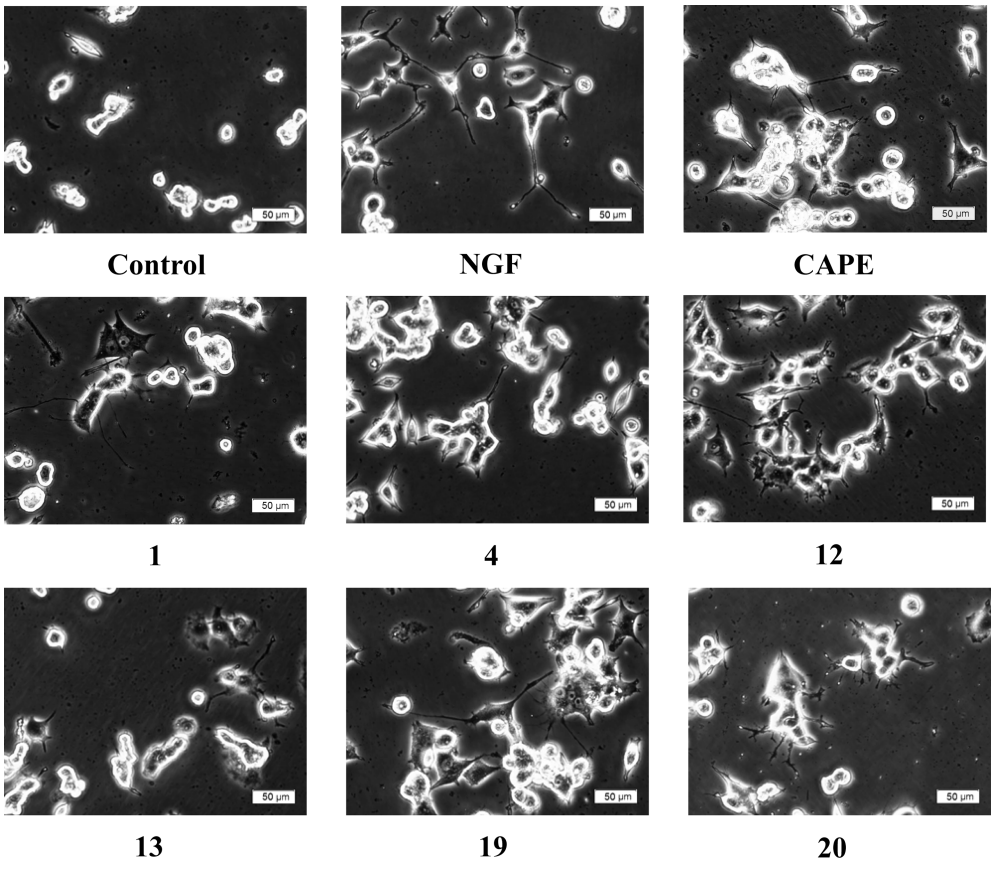

Figure 2. Morphology of PC12 cells treated with CAPE and its derivatives. Undifferentiated PC12 cells were seeded on poly-L-lysine-coated 12 well plates in DMEM medium (1% L-glutamine, 10% horse serum and 5% fetal bovine serum) for 24 h prior to treatment with NGF (50 ng/mL), CAPE (10 nM), and compounds **1** (0.5 nM), **4** (0.5 nM), **12** (0.5 nM), **13** (0.5 nM), **19** (10 nM), and **20** (10 nM) for an additional 72 h. Cell morphology was observed using inverted microscopy.

Undifferentiated PC12 cells are a useful model for the study of neuronal development *in vitro*, and they can be induced to differentiate into sympathetic-like neurons after exposure to NGF. The effects of CAPE derivatives **1**−**21** on the neurite outgrowth of undifferentiated PC12 cells were evaluated by morphological observations and a quantitative analysis of neurite-bearing cells and neurite length. Among the twenty-one CAPE derivatives, compounds **1**, **4**, **12** and **13** showed potential neuritogenic activities at 0.5 nM, while compounds **19** and **20** induced neurite outgrowth at 10 nM (Figure 2). As shown in Figure 3A, compounds **1**, **4**, **13**, **19** and **20** slightly increased the percent of neurite-bearing cells (<20%) and compound **12** modestly promoted the growth (>20%). The percent of neurite-bearing cells in groups treated with compounds **1**, **4**, **12**, **13**, **19** and **20** was significantly higher than the control. The average of neurite length for PC12 cells treated with compounds **1**, **4**, **12**, **13**, **19** and **20** was in the range of 25.1−37.0 μm, which was significantly higher than the control (18.4 μm) (Figure 3B). Specifically, 0.5 nM compound **12** markedly increased both the percentage of neurite bearing cells (21.5%) and the mean neurite length in those cells (37.0 μm) as compared with those in 10 nM CAPE (15.8% in numbers and 32.2 μm in length). Compounds **19** and **20** possessed similar neuritogenic activities as CAPE at 10 nM. These data suggested that the phenethyl alcohol group and its substitution were critical for maintaining or enhancing the neuritogenic activity of CAPE derivatives. Additional data are required for further structure−activity relationship analysis.

By comparison with the data from the literature,[30,31] the effective concentration of compounds **1**, **4**, **12** and **13** was much lower than that of natural products showing neuritogenic potential, and was comparable with that of tripchlorolide (10^{-10} M),[32] sargaquinoic acid (1.25−100 ng)[33] and catalpol (0.1−1 μg/mL),[34] geniposide (0.1−10 μg/mL),[34] and gardenoside (0.1−10 μg/mL).[34] Compounds **12** and **13** showed cytotoxicity against PC12 cells at 5 nM, which may be originated from the substitution of bromine atom on the aromatic ring. The cytotoxicity might be alleviated by the replacement of other halogen atoms such as fluorine (F) and chlorine (Cl) on the same position. Interestingly, we found that some compounds with significant cytoprotective activities also showed simulative effects on the neurite outgrowth of PC12 cells. These results indicated that the CAPE derivatives were promising candidates for reducing the risk of neurodegenerative diseases. To the best of our knowledge, this is the first report on neuritogenic activities of CAPE derivatives.

In conclusion, twenty-one CAPE derivatives with diverse substitution or carbon skeleton were synthesized. Among them, compounds **1** and **20** significantly protected the PC12 cells against H_2O_2-induced cytotoxicity. Meanwhile, compounds **1**, **4**, **12**, **13**, **19** and **20** remarkably induced the neurite outgrowth. Our study suggested that the presence of the caffeic acid group may be important for keeping the cytoprotective and neuritogenic activities, and the alcohol group and its substitution majorly affected the activities of CAPE derivatives. The results might promote the understanding of the health

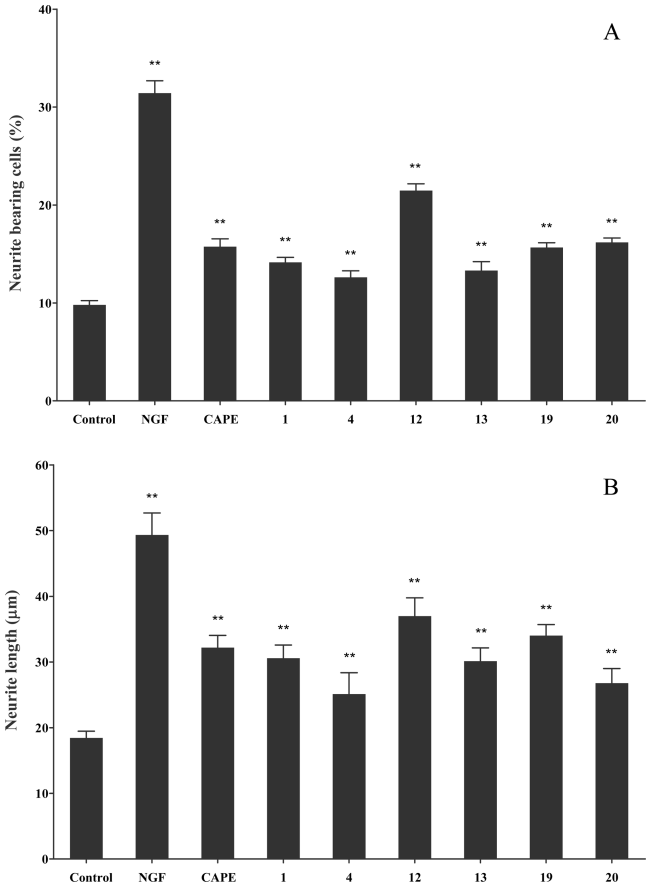

Figure 3. Effects of CAPE and its derivatives on neurite outgrowth in PC12 cells. Undifferentiated PC12 cells were treated with NGF (50 ng/mL), CAPE (10 nM), compounds **1** (0.5 nM), **4** (0.5 nM), **12** (0.5 nM), **13** (0.5 nM), **19** (10 nM) and **20** (10 nM) for 72 h to evaluate their neuritogenic effects, respectively. (A) Neurite bearing cells were counted under inverted microscopy in five random fields. (B) The average of neurite length for all identified neurite bearing cells was analyzed by ImageJ software. At least 100 cells were selected for calculating the neurite length. Data are expressed as mean ± SE (n = 3). (**) $p < 0.01$ represents significant differences compared with that of control.

benefits of CAPE and its derivatives, and may lead to the development of the functional food ingredients for the prevention of neurodegenerative diseases.

AUTHOR INFORMATION

Corresponding Author

*Tel: 86-21-34204041. Fax: 86-21-34204107. E-mail: hmshi@sjtu.edu.cn.

Author Contributions

‖H.S. and D.X. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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