Original Research Article

Effects of oxyresveratrol and its derivatives on cultured P19-derived neurons

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Abstract

Purpose: To modify the structure of oxyresveratrol and evaluate the obtained derivatives for effects on neuronal cells.
Methods: Electron-withdrawing groups were selectively introduced to the aromatic ring of the core stilbene structure. Oxyresveratrol and derivatives were then evaluated for their ability to enhance the survival of P19 derived neuronal cells by XTT method, in comparison with the widely known antioxidants, Trolox and ascorbic acid. Phase-contrast microscopic images of the neurons under various conditions were also taken and analyzed.
Results: Oxyresveratrol, at a very low concentration (1 ng/mL), enhanced the survival of neurons in both normal and serum-deprivation conditions. Higher activity was observed for the 5-formylated and t 5-carboxylated products. The potencies of these polyoxygenated stilbenes were far greater than those of Trolox and ascorbic acid. These observations were supported by results from the examination of the phase-contrast micrographs of the neuronal cells.
Conclusion: Oxyresveratrol and some derivatives prepared in this study demonstrate significant cell protective activity and may be of therapeutic value, but further investigations in animals are required to verify their neuroprotective potentials.

Keywords: Oxyresveratrol, Artocarpus lacucha, Artocarpus lakoocha, Polyoxygenated stilbenes, Cell protection, Neuron

INTRODUCTION

As the world's ageing population continues to grow at an unprecedented rate, the number of people affected by neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) is rising rapidly [1-3]. Although the etiology of these ageing-related diseases is still unclear, oxidative stress has been implicated in their pathogenesis [4,5].

Oxyresveratrol (2,4,3`,5`-tetrahydroxystilbene, 1) is a phytoalexin found in large quantities in several members of the family Moraceae, for example, *Artocarpus lacucha* Buch.-Ham. (formerly known as *A. lakoocha* Roxb.) and *Morus alba* L. [6,7]. Our previous studies have shown that 1 possesses a wide range of biological activities, for example, anti-herpetic activity, inhibitory effect on tyrosinase and protective activity against DNA damage [8-10]. In recent years, considerable amounts of research

have been published on the possible neuroprotective effects of 1. Studies have shown that 1 could protect cultured neurons from β-amyloid-induced toxicity and defend neuroblastoma cells against 6-hydroxydopamine, suggesting potential applications for AD and PD, respectively [11,12]. The compound was also studied for protective effects against cerebral ischemia *in vivo* [13], and traumatic injury *in vitro* [14]. Its antioxidant activity has been investigated in several models [7,15,16].

P19 embryonal carcinoma cells have been shown to differentiate into neurons when cultured in the presence of all *trans* retinoic acid and cytosine arabinoside (Ara-C), and they have been recently employed as a tool in neurological research [17].

In this communication, we wish to report the effects of oxyresveratrol (1) and some derivatives (2 – 4) on the cell viability of P19-derived neuronal cells.

EXPERIMENTAL

General

Melting points (uncorrected) were measured on a Fisher-Johns hot stage melting point apparatus. IR spectra were recorded using UATR (Universal Attenuated Total Reflectance) on a Perkin Elmer system 2000 FT-IR or Jasco A-30 Spectrophotometer. High resolution mass spectra were taken on a MicroTOF instrument (APCI or ESI) in positive or negative mode. ^{1}H and ^{13}C-NMR were recorded on a Bruker Avance III 300 MHz spectrometer or Bruker 400 spectrometer. All chemical shift values were reported as δ (ppm) with the solvent signal as reference, and coupling constant values J were measured in Hz.

Plant material and isolation of oxyresveratrol (1)

The collection and botanical identification of the heartwood of *Artocarpus lacucha* Buch.-Ham. (*A. lakoocha* Roxb.), and the isolation and chemical identification of **1** were carried out as previously described [18].

Synthesis of compounds 2 - 4

Compounds **2** – **4** were prepared through the intermediates **1a** – **1f**, following the previously reported synthetic routes [16].

Synthesis of 3′,5′-dihydroxy-2,4-diisopropoxystilbene (1a) and 2,3′,4,5′-tetraisopropoxystilbene (1b)

K_2CO_3 (5.67 g, 41 mmol) and 2-bromopropane (6.2 mL, 66.3 mmol) were added to a solution of DMF (20 mL) containing compound **1** (2 g, 8.20 mmol). Then 2-bromopropane (3.1 mL, 33.1 mmol) was added every 24 h. The reaction mixture was stirred at 55 °C for 3 days and monitored by TLC. After completion, water (20 mL) was added and the reaction was extracted with EtOAc (3 x 20 mL). The organic phase was washed with water (7 x 20 mL) and brine, dried over Na_2SO_4, filtered and concentrated. Column chromatographic purification with gradient EtOAc/hexane gave **1a** (0.52 g, 19 %) and **1b** (1.21 g, 35 %).

Synthesis of 3′,5′-diacetoxy-2,4-diisopropoxystilbene (1c)

To the solution of CH_2Cl_2 (8 mL) containing compound **1a** (212 mg, 0.65 mmol), Et_3N (0.2 mL, 1.44 mmol) and acetic anhydride (0.13 mL, 1.37 mmol) were added and stirred for 2 h at room temperature. Then the reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic phase was washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* to give a crude product. Purification with column chromatography eluting with 20 % EtOAc in hexanes gave **1c** (163 mg, 61 %).

Synthesis of 3′,5′-diacetoxy-5-formyl-2,4-diisopropoxystilbene (1d)

$POCl_3$ (0.61 mL, 6.64 mmol) was stirred with dry DMF (5 mL) at room temperature for 2 h under argon. The solution of **1c** (273 mg, 0.66 mmol) in dry DMF (5 mL) was added at 0 °C. Then the reaction mixture was allowed to warm to room temperature and stirred overnight. After completion of the reaction, cool water (10 mL) was added and the reaction mixture was extracted with EtOAc (3 x 10 mL). The organic layer was washed with water (7 x 10 mL) and brine, dried with Na_2SO_4 and concentrated in vacuo to give a crude product, which was purified by preparative TLC (20 % EtOAc/hexanes) to furnish **1d** (185 mg, 64 %).

Synthesis of 3′,5′-diacetoxy-5-carboxy-2,4-diisopropoxystilbene (1e)

A solution of water (0.5 mL) containing $NaClO_2$ (135 mg, 1.49 mmol) and $NaH_2PO_4 \cdot 2H_2O$ (237 mg, 1.52 mmol) was added to a solution of acetone (0.5 mL) containing compound **1d** (82

mg, 0.19 mmol) and 2-methyl-2-butene (0.06 mL, 0.71 mmol) and stirred for 1h at room temperature. After completion, the reaction mixture was diluted with water (5 mL) and extracted with EtOAc (3 x 5 mL). The organic layer was washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* to give a crude product, which was purified by column chromatography on silica (40 % EtOAc/hexanes) to furnish **1e** (68 mg, 78 %).

Synthesis of 2′-formyl-2,3′,4,5′-tetraisopropoxystilbene (1f)

$POCl_3$ (0.31 mL, 3.38 mmol) was stirred with dry DMF (8 mL) at room temperature for 2 h under argon. A solution of **1b** (400 mg, 0.97 mmol) in dry DMF (8 mL) was added at 0 °C and the reaction mixture was further stirred overnight. After completion of the reaction, cool water (15 mL) was added and extracted with EtOAc (3 x 15 mL). The organic layer was washed with water (7 x 15 mL) and brine, dried over Na_2SO_4, filtered and followed by removal of the solvent under reduced pressure to give a crude product, which was purified by column chromatography on silica (15 % EtOAc/hexanes) to furnish **1f** (344 mg, 79 %).

Synthesis of 5-formyl-2,3′,4,5′-tetrahydroxy-stilbene (2)

A solution of BCl_3 (1.69 mL, 1.69 mmol) was added to a solution of **1d** (121 mg, 0.27 mmol) in CH_2Cl_2 (4 mL) at −78 °C under argon. Then it was allowed to warm to room temperature and stirred overnight. Water (5 mL) was then added and the reaction mixture was extracted with EtOAc (3 x 5mL). The EtOAc was washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* to give a crude product which was purified by preparative TLC (40 % EtOAc/hexanes) to give **2** (42 mg, 57 %).

Synthesis of 5-carboxy-2,3′,4,5′-tetrahydroxy-stilbene (3)

A solution of BCl_3 (0.85 mL, 0.85 mmol) was added to a solution of **1e** (65 mg, 0.14 mmol) in CH_2Cl_2 (2 mL) at −78 °C under argon. Then the reaction was allowed to warm to room temperature and stirred overnight. Water (5 mL) was added and the mixture was extracted with EtOAc (3 x 5 mL).

The organic layer was washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* to give a crude product which was purified by Sephadex LH20 (methanol) to give **3** (31 mg, 76 %).

Synthesis of 2′-formyl-2,3′,4,5′-tetrahydroxy-stilbene (4)

Compound **1f** (292 mg, 0.66 mmol) was dissolved in 10 mL of CH_2Cl_2. To this solution, BCl_3 (5.32 mL, 5.32 mmol) was added at -78 °C under argon. The reaction was allowed to warm up to room temperature and stirred overnight. Then, water (10 mL) was added, and the reaction was extracted with EtOAc (3 x 10 mL). The EtOAc phase was washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* to give a crude product which was purified by using preparative TLC (40 % EtOAc/hexanes) to give **4** (100 mg, 56 %).

Evaluation of superoxide free radical anion scavenging activity

The compounds were determined according to established protocols. The assay was based on the capacity of the test sample to inhibit the reduction of nitroblue tetrazolium (NBT) in the riboflavin-light-NBT system. A 96-well microplate was used. The reaction mixture (200 µL) in each well contained 20 µL of 50 mM potassium phosphate buffer,100 µL of 266 µM riboflavin, 20 µL of 1 mM EDTA, 20µL of 750 µM NBT and 40 µL of sample solution. The production of blue formazan was monitored by measuring the increase in absorbance at 570 nm after a 10-min illumination with a fluorescent lamp. The entire reaction proceeded in a closed box lined with aluminum foil. A similar reaction mixture was kept in the dark and served as the blank.

Assays for effects on P19-derived neurons

Compounds were evaluated for their effects on P19-derived neurons following previously reported protocols [17].

Cell culture

Murine embryonal carcinoma cells, P19 ATCC CRL-1857, obtained from the American Type Culture Collection, USA, were grown in alpha minimal essential medium (α-MEM) with 7.5 % new born calf serum (NCS), 2.5 % fetal bovine serum (FBS), and 1 % antibiotics-antimycotic solution (P19GM) in a 5 % CO_2 humidified atmosphere, at 37 °C. Cells in monolayer cultures were maintained in exponential growth by subculturing every 2 days.

Differentiation of P19 cells into neurons

The exponentially grown cultures indicated the cells density by trypan blue exclusion assay. Then, neuronal differentiation was induced by

seeding 2×10^6 cells/mL cell in a 100 mm bacteriological grade culture dish containing 10 mL α-MEM supplemented with 5 % FBS, 1 % antibiotic-antimycotic solution, and 0.5 µM all *trans*-retinoic acid (RA). Under these conditions, cells did not adhere to dishes but instead formed large aggregates in suspension (neurospheres). After 4 days of RA treatment, aggregates were dissociated with glass pipette, washed, and then resuspended on poly-L-lysine-precoated 96-well plates (plates previously coated with 50 µg/mL poly-L-lysine in PBS for overnight and sterilized under UV light for 30 min) at a cell density of 7×10^4 cells/mL (150 µL/well) in α-MEM supplemented with 10 % FBS and 1 % antibiotics-antimycotic solution, and incubated for 24 h. Ara-C (10 µM) was added at day one after plating to inhibit the proliferation of nonneuronal cells. The medium was changed every 2 - 3 days. The differentiated P19-derived neurons were used after day 14 of the differentiation process.

Assay for cell viability under normal condition

The assay was performed in triplicate. After 14 days of differentiation process, the medium (α-MEM supplemented with 10 % FBS, 10 µM Ara-C and 1 % antibiotics-antimycotic solution) was removed, and replaced with a DMSO solution of test compound in P19SM (α-MEM supplemented with 10 % FBS). Thereafter, a 1 % antibiotic-antimycotic solution containing 10 µM Ara-C was added to give a final concentration of the test sample at 1, 10 or 100 ng/mL. The final DMSO content in each well was kept at 0.5 %, which had no effect on the cells. The blank control wells were filled with the corresponding medium (P19SM, 10 µM Ara-C, and 1 % antibiotics-antimycotic solution). The cells were incubated for 18 h at 37 °C. Then, the medium was removed, and 50 µL of XTT solution (1 mg/mL XTT in α-MEM and 25 µM phenazinemethosulfate) was added. After incubation at 37 °C for 4 h, 100 µL of PBS was added. The OD value was determined on a microplate reader at 450 nm. The data were expressed as the mean ± SEM (n = 3), with the medium as a control representing 100 % cell viability. The concentration that enhanced the survival of cultured neurons more than control will be further investigated for neuroprotective activity.

Assay for cell viability under serum-deprivation condition

The assays were carried out in a 96-well plate and performed in triplicate. For serum deprivation condition, the cells were fed with medium without FBS (α-MEM supplemented with 10 µM Ara-C, and 1 % antibiotic-antimycotic solution), and a DMSO solution of test compound was added. DMSO (0.5 %) was used as the control. The cells were incubated for 18 h at 37 °C. Cell viability was assayed by the XTT reduction method.

Statistical analysis

A mean viability of the cells was statistically analyzed by Student's *t*-test to compare the statistical significance between either the control or oxidative stress conditions and experimental groups. Differences were considered significant only at $p < 0.05$.

RESULTS

Chemistry

Compounds **2**-**4** were prepared from **1** following earlier reported protocols with slight modification [16], as shown in Figure 1. The synthetic route began with the reaction of oxyresveratrol (**1**) with 2-bromopropane to give di- and tetra-O-isopropyl products (**1a** and **1b**). Compound **1a** was then O-acetylated on ring B to give **1c**. This intermediate (**1c**) was formylated at C-5 by Vilsmeier-Haack reaction to give **1d** which, after deprotection with BCl_3, furnished compound **2**. Oxidation of **1d** by $NaClO_2$ gave the corresponding **1e** which was subsequently converted into **3**, after BCl_3-mediated cleavage of all the O-protecting groups. Finally, compound **4** was obtained from the Lewis-catalyzed hydrolysis reaction of **1f**, which was earlier prepared from **1b** through the Vilsmeier-Haack formylation at C-2`.

1a: *3′,5′-Dihydroxy-2,4-diisopropoxystilbene*: brown oil; IR (UATR, cm^{-1}): 3379, 2976, 1598; ^{1}H-NMR (400 MHz, CDCl$_3$), *δ* (ppm): 7.43 (d, *J* = 8.4 Hz, 1H), 7.31 (d, *J* = 16.4 Hz, 1H), 6.84 (d, *J* = 16.4 Hz, 1H), 6.56 (d, *J* = 2.1 Hz, 2H), 6.48 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.45 (d, *J* = 2.2 Hz, 1H), 6.25 (br s, 1H), 4.52 (m, 2H), 1.35 (d, *J* = 6 Hz, 6H), 1.33 (d, *J* = 6 Hz, 6H); ^{13}C-NMR (100 MHz, CDCl$_3$), *δ* (ppm): 158.6, 157.0, 156.5, 141.0, 127.4, 126.1, 124.3, 120.3, 107.5, 105.8, 103.2, 101.7, 71.2, 70.1, 22.1, 22.0; TOF-HRMS *m/z* [M − H]⁻, calcd for $C_{20}H_{23}O_4$: 327.1602; found: 327.1594. These physical and spectroscopic properties are in agreement with earlier reported values [16].
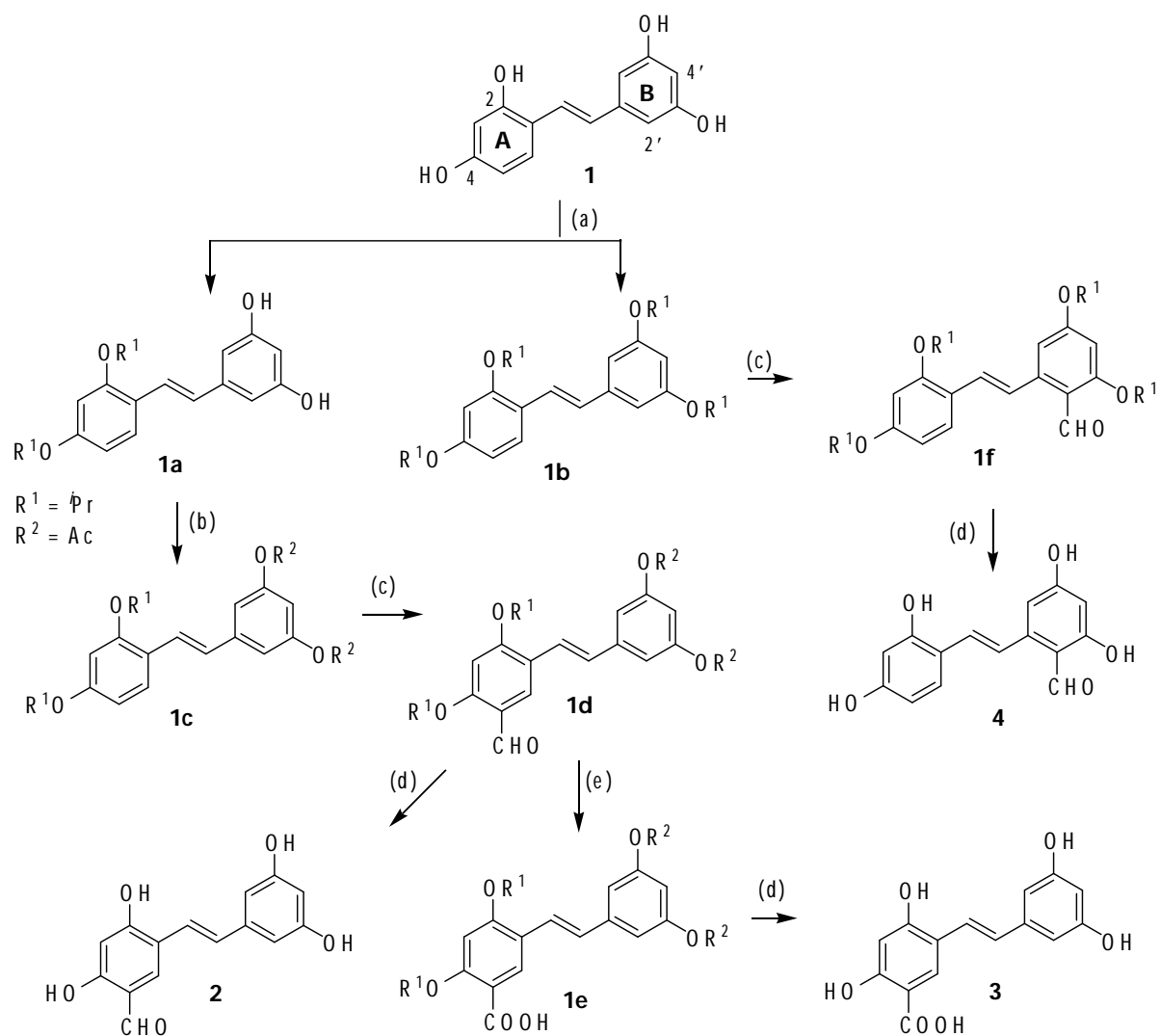
Figure 1: Reagents and conditions: (a) 2-bromopropane, K_2CO_3, DMF, 55 °C; (b) Ac_2O, Et_3N, CH_2Cl_2, rt; (c) $POCl_3$, DMF, 0 °C, Ar; (d) ; BCl_3, CH_2Cl_2, −78 °C→ rt, Ar; (e) $NaClO_2$, $NaH_2PO_4 \bullet 2H_2O$, 2-methyl-2-butene, acetone, rt

1b: *2,3′,4,5′-Tetraisopropoxystilbene*: white solid, mp 58–60 °C; IR (UATR, cm^{-1}): 2976, 1585; ^{1}H-NMR (300 MHz, CDCl$_3$), *δ* (ppm): 7.46 (d, *J* = 8.4 Hz, 1H), 7.33 (d, *J* = 16.4 Hz, 1H), 6.91 (d, *J* = 16.4 Hz, 1H), 6.62 (d, *J* = 2.2 Hz, 2H), 6.49 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.45 (d, *J* = 3.9 Hz, 1H), 6.34 (t, *J* = 2.1 Hz, 1H), 4.55 (m, 4H), 1.37 (d, *J* = 6 Hz, 6H), 1.35 (d, *J* = 6 Hz, 12H), 1.34 (d, *J* = 6 Hz, 6H); ^{13}C-NMR (75 MHz, CDCl$_3$), *δ* (ppm): 159.1, 158.6, 156.6, 140.5, 127.4, 126.7, 124.2, 120.4, 107.3, 106.4, 103.0, 102.5, 71.0, 69.9, 69.8, 22.2, 22.1; TOF-HRMS *m/z* [M + H]$^+$, calcd for C$_{26}$H$_{37}$O$_4$: 413.2686; found: 413.2679. These physical and spectroscopic properties are in agreement with earlier reported values [16].

1c: *3′,5′-Diacetoxy-2,4-diisopropoxystilbene*: white solid, mp 65–67 °C; IR (UATR, cm^{-1}): 2978, 1768, 1599; ^{1}H-NMR (300 MHz, CDCl$_3$), *δ* (ppm): 7.41 (d, *J* = 8.4 Hz, 1H), 7.34 (d, *J* = 16.5 Hz, 1H), 7.08 (d, *J* = 2.1 Hz, 2H), 6.94 (d, *J* =

16.2 Hz, 1H), 6.78 (t, *J* = 2.1 Hz, 1H), 6.46 (dd, *J* = 8.1, 2.4 Hz, 1H), 6.45 (br s, 1H), 4.51 (m, 2H), 2.25 (s, 6H), 1.36 (d, *J* = 6 Hz, 6H), 1.31 (d, *J* = 6 Hz, 6H); ^{13}C-NMR (75 MHz, CDCl$_3$), *δ* (ppm): 168.7, 158.8, 156.6, 151.0, 140.7, 127.6, 125.8, 124.6, 119.4, 116.3, 113.3, 106.9, 102.5, 70.6, 69.7, 21.9, 21.8, 20.8; TOF-HRMS *m/z* [M + Na]$^+$, calcd for C$_{24}$H$_{28}$NaO$_6$: 435.1778; found: 435.1796. These physical and spectroscopic properties are in agreement with earlier reported values [16].

1d: *3′,5′-Diacetoxy-5-formyl-2,4-diisopropoxystilbene*: viscous yellow oil; IR (UATR, cm^{-1}): 2979, 1768, 1671, 1593; ^{1}H-NMR (300 MHz, CDCl$_3$), *δ* (ppm): 10.32 (s, 1H), 8.03 (s, 1H), 7.27 (d, *J* = 16.5 Hz, 1H), 7.08 (d, *J* = 1.8 Hz, 2H), 7.03 (d, *J* = 16.5 Hz, 1H), 6.81 (t, *J* = 2.1 Hz, 1H), 6.44 (s, 1H), 4.66 (m, 2H), 2.29 (s, 6H), 1.43 (d, *J* = 6 Hz, 6H), 1.40 (d, *J* = 6 Hz, 6H); ^{13}C-NMR (75 MHz, CDCl$_3$), *δ* (ppm): 188.1, 168.7, 161.8, 161.4,

151.0, 140.1, 126.6, 126.3, 124.2, 119.6, 118.8, 116.4, 113.8, 98.1, 71.2, 70.9, 21.67, 21.66, 20.8; TOF-HRMS *m/z* [M + Na]$^+$, calcd for $C_{25}H_{28}NaO_7$: 463.1727; found: 463.1743. These physical and spectroscopic properties are in agreement with earlier reported values [16].

1e: *3',5'-Diacetoxy-5-carboxy-2,4-diisopropoxy-stilbene*: white solid, mp 106–108 °C; IR (UATR, cm^{-1}): 3267, 2980, 1769, 1732, 1601; ^{1}H-NMR (300 MHz, CDCl$_3$), *δ* (ppm): 8.35 (s, 1H), 7.26 (d, *J* = 16.5 Hz, 1H), 7.09 (d, *J* = 2.1 Hz, 2H), 7.08 (d, *J* = 16.5 Hz, 1H), 6.82 (t, *J* = 2.1 Hz, 1H), 6.50 (s, 1H), 4.83 (sept, *J* = 6 Hz, 1H), 4.65 (sept, *J* = 6 Hz, 1H), 2.31 (s, 6H), 1.51 (d, *J* = 6 Hz, 6H), 1.45 (d, *J* = 6 Hz, 6H); ^{13}C-NMR (75 MHz, CDCl$_3$), *δ* (ppm): 168.9, 165.3, 160.2, 157.2, 151.2, 140.1, 132.1, 127.5, 123.9, 121.4, 116.8, 114.2, 110.9, 99.0, 74.2, 71.4, 21.92, 21.88, 21.0; TOF-HRMS *m/z* [M + Na]$^+$, calcd for $C_{25}H_{28}NaO_8$: 479.1676; found: 479.1695. These physical and spectroscopic properties are in agreement with earlier reported values [16].

1f: *2'-Formyl-2,3',4,5'-tetraisopropoxystilbene*: viscous yellow oil; IR (UATR, cm^{-1}): 2976, 1671, 1586; ^{1}H-NMR (300 MHz, CDCl$_3$), *δ* (ppm): 10.53 (s, 1H), 8.08 (d, *J* = 16.4 Hz, 1H), 7.60 (d, *J* = 8.6 Hz, 1H), 7.30 (d, *J* = 16.4 Hz, 1H), 6.75 (d, *J* = 2.1 Hz, 1H), 6.50 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.44 (d, *J* = 2.4 Hz, 1H), 6.35 (d, *J* = 2.1 Hz, 1H), 4.69 (sept, *J* = 6 Hz, 1H), 4.48–4.63 (m, 3H), 1.33–1.41 (m, 24H); ^{13}C-NMR (75 MHz, CDCl$_3$), *δ* (ppm): 191.1, 163.6, 162.7, 158.9, 156.7, 143.9, 128.1, 127.3, 125.4, 120.4, 116.8, 107.4, 105.0, 102.9, 100.1, 71.4, 70.9, 70.1, 69.9, 22.2, 22.04, 21.98; TOF-HRMS *m/z* [M + H]$^+$, calcd for $C_{27}H_{37}O_5$: 441.2635; found: 441.2639. These physical and spectroscopic properties are in agreement with earlier reported values [16].

2: *5-Formyl-2,3',4,5'-tetrahydroxystilbene*: yellow solid, decomposed >205 °C; IR (UATR, cm^{-1}): 3208, 1626; ^{1}H-NMR (300 MHz, methanol-*d*$_4$), *δ* (ppm): 9.69 (s, 1H), 7.76 (s, 1H), 7.27 (d, *J* = 16.5 Hz, 1H), 6.95 (d, *J* = 16.5 Hz, 1H), 6.47 (d, *J* = 2.4 Hz, 2H), 6.25 (s, 1H), 6.16 (t, *J* = 2.1 Hz, 1H); ^{13}C-NMR (75 MHz, methanol-*d*$_4$), *δ* (ppm): 194.9, 167.8, 164.8, 159.6, 141.6, 133.2, 128.4, 123.8, 121.0, 115.5, 105.9, 103.7, 102.7; TOF-HRMS *m/z* [M − H]$^-$, calcd for $C_{15}H_{11}O_5$: 271.0612; found: 271.0603. These physical and spectroscopic properties are in agreement with earlier reported values [16].

3: *5-Carboxy-2,3',4,5'-tetrahydroxystilbene*: yellow solid, decomposed >230 °C; IR (UATR, cm^{-1}): 3337, 1616; ^{1}H-NMR (300 MHz, methanol-*d*$_4$), *δ* (ppm): 8.01 (s, 1H), 7.24 (d, *J* = 16.5 Hz, 1H), 6.92 (d, *J* = 16.5 Hz, 1H), 6.49 (d, *J* = 2.1

Hz, 2H), 6.36 (s, 1H), 6.19 (t, *J* = 2.1 Hz, 1H); ^{13}C-NMR (75 MHz, methanol-*d*$_4$), *δ* (ppm): 173.5, 164.2, 162.8, 159.6, 141.6, 130.0, 128.5, 123.8, 118.9, 106.3, 105.8, 103.3, 102.7; TOF-HRMS *m/z* [M − H]$^-$, calcd for $C_{15}H_{11}O_6$: 287.0561; found: 287.0549. These physical and spectroscopic properties are in agreement with earlier reported values [16].

4: *2'-Formyl-2,3',4,5'-tetrahydroxystilbene*: yellow solid, decomposed >218 °C; IR (UATR, cm^{-1}): 3337, 1602; ^{1}H-NMR (300 MHz, methanol-*d*$_4$), *δ* (ppm): 10.17 (s, 1H), 7.55 (d, *J* = 15.9 Hz, 1H), 7.38 (d, *J* = 9 Hz, 1H), 7.23 (d, *J* = 15.9 Hz, 1H), 6.56 (d, *J* = 2 Hz, 1H), 6.33 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.32 (br s, 1H), 6.14 (d, *J* = 2 Hz, 1H); ^{13}C-NMR (75 MHz, methanol-*d*$_4$), *δ* (ppm): 194.3, 167.3, 167.2, 160.1, 158.0, 148.0, 131.8, 129.6, 120.4, 117.2, 112.8, 108.5, 107.4, 103.6, 102.0; TOF-HRMS *m/z* [M − H]$^-$, calcd for $C_{15}H_{11}O_5$: 271.0612; found: 271.0607. These physical and spectroscopic properties are in agreement with earlier reported values [16].

Biological evaluation

The effect of compounds **1** – **4** on superoxide radical scavenging activity and cell viability of P19-derived neuronal cells in normal condition are shown in Table 1 while the effect of stilbenes **1** – **3** on the survival of the neurons cultured under serum-deprivation condition are displayed in Table 2 and Figure 2. Phase-contrast micrographs of neurons in the corresponding experiments are illustrated in Figure 3.

It is clear from Figure 2 that pretreatment of the neurons with 1 ng/mL oxyresveratrol (**1**) could significantly reduce cell death, bringing the cell survival back to the level of 67.6%. It should be noted that compound **2** displayed statistically higher activity than **1** ($p < 0.05$). On the contrary, the widely used antioxidants Trolox and ascorbic acid showed much lower activity than **1**, **2** or **3**, as reflected from their much greater amounts (15 and 10 μg/mL, respectively) required for the activity and the less percentages of cell survival (65.4 and 69.3 %, respectively). To further confirm the biological effects of these compounds (**1** – **3**), phase-contrast images of the neurons under various conditions were taken, and the results are illustrated in Figure 3. Figure 3 (a and b) show the neuronal cells growing in normal and serum-starvation condition, respectively. It can be seen from Figure 3 (b) that nutrient starvation caused cell death and reduced neurite growth. These adverse effects were prevented by adding antioxidant ascorbic acid (10 μg/mL) or Trolox (15 μg/mL) to the medium,

Table 1: Superoxide scavenging activity of compounds **1 – 4** and their effects on P19-derived cell viability at different concentrations

Compound	Superoxide scavenging activity [a]		Cell viability (%) [b]		
	Inhibition at 100 µg/mL	IC$_{50}$ (µM)	1 ng/mL	10 ng/mL	100 ng/mL
1	76.1 ± 3.4	303.1 ± 7.9	115.3 ± 10.5	99.8 ± 4.5	83.6 ± 19.8
2	94.2 ± 3.5	88.3 ± 9.7	107.4 ± 4.7	-	-
3	96.0 ± 3.6	38.6 ± 1.4	109.0 ± 2.1	-	-
4	91.0 ± 6.4	43.4 ± 4.4	83.6 ± 3.1	-	-
Trolox	61.4 ± 3.6	293.5±19.3	-	-	-

[a]Data from a previous report [16]; [b]compared with untreated control

Table 2: Cell protective activity of compounds **1 – 3**

Compound	Mean cell viability (± SEM, %)
1 (1 ng/mL)	67.6 ± 0.9*
2 (1 ng/mL)	75.2 ± 2.0*
3 (1 ng/mL)	72.9 ± 2.0*
Trolox (15 µg/mL)	65.4 ± 5.3*
Ascorbic acid (10 µg/mL)	69.3 ± 3.8*
α-MEM + 10 µM Ara-C + 0.5%DMSO (control of serum-deprivation condition)	40.9 ± 3.6
α-MEM + 10 µM Ara-C (serum deprivation condition)	40.2 ± 6.8
P19SM+ 10 µM Ara-C +0.5%DMSO (control of untreated control)	100.1 ± 1.9
P19SM + 10 µM Ara-C (untreated control)	100.00 ± 0.00

* $P < 0.05$ when compared with serum-deprivation condition and control of serum-deprivation condition; P19SM consists of α-MEM+ 10 %v/v FBS; all culture media contain 1%v/v antibiotic-antimycotic solution
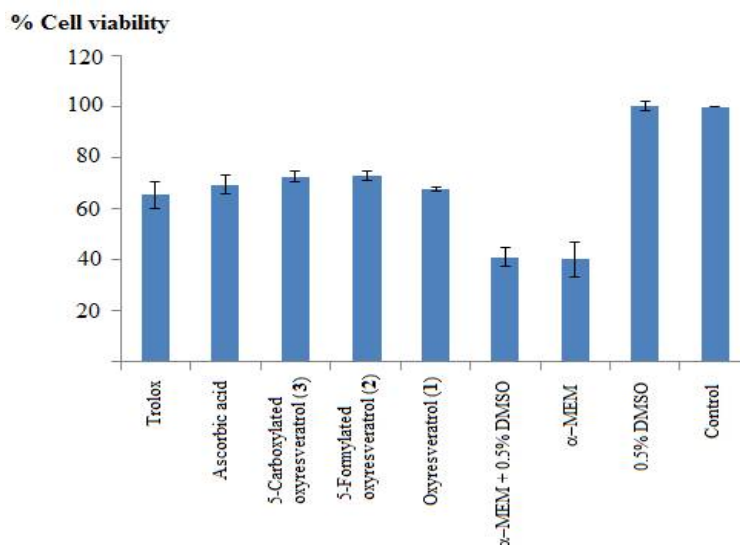
Figure 2: Effect of compounds **1**, **2** and **3** on cell viability of P19-derived neurons under oxidative stress induced by serum deprivation

as depicted in Figure 3 (c and d). Interestingly, at a very much lower concentration (1 ng/mL), stilbenes **1**, **2** and **3**, each, enhanced cell survival, as well as helped to maintain neurite growth, as seen in Figure 3 (e – g).

DISCUSSION

The reactivity of the two aromatic rings (**A** and **B**) of **1** toward electrophilic substitution was manipulated by employing different *O*-protecting groups (**1b** and **1c**). In **1b**, ring B was more sensitive to electrophilic attack than ring A, particularly at C-2′ and C-6′ positions, due to the electron donating effects from all of the four *O*-isopropyl ether functionalities. However, in **1c**, the B ring was deactivated by the two *O*-acetyl ester groups, and as a result, ring A was more receptive to electrophiles, specifically at C-5.
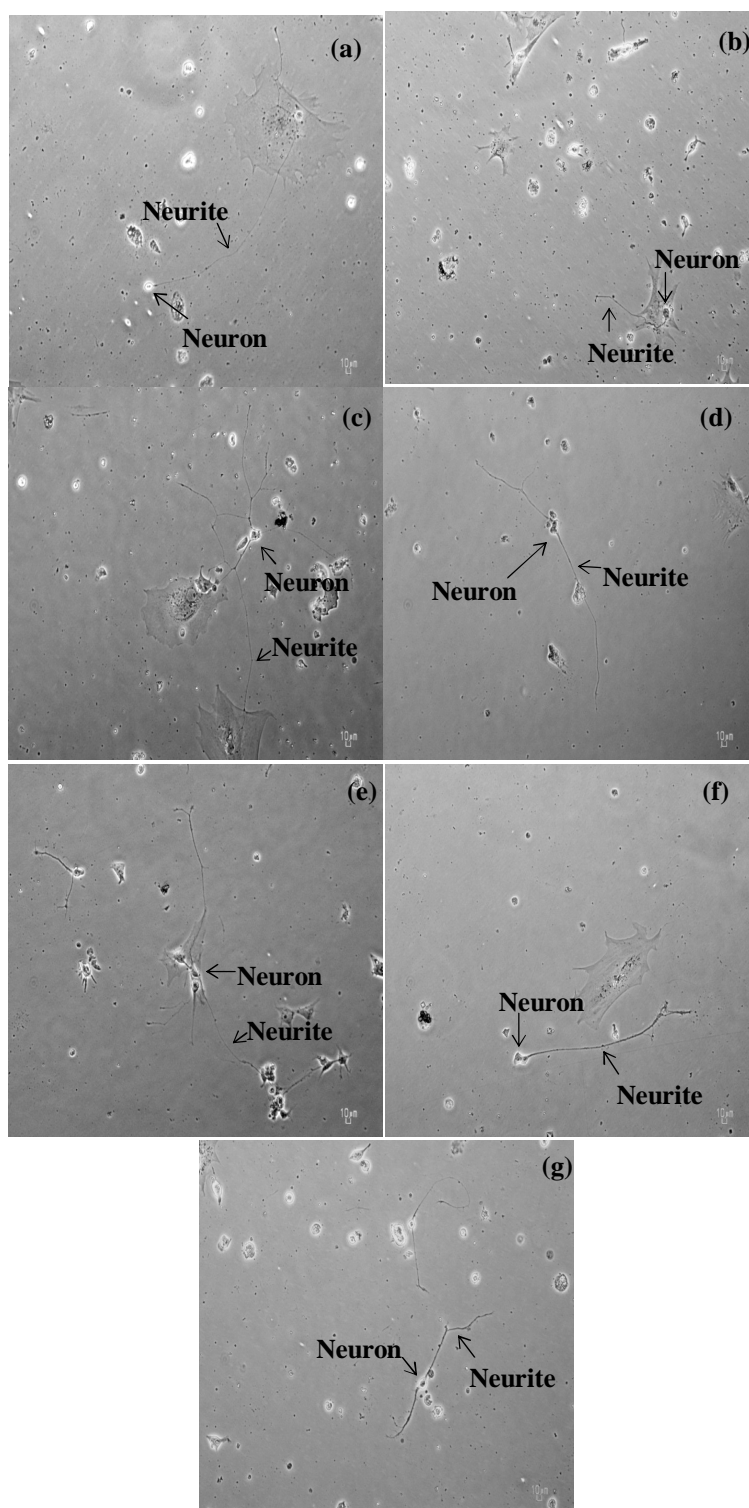
Figure 3: Protective activity of **1**, **2**, and **3** on P19-derived neurons. Phase-contrast images of the neurons incubated for 18 h and treated with: (a) α-MEM with serum (untreated control); (b) α-MEM without serum; (c) 10 µg/mL of ascorbic acid (positive control); (d) 15 µg/mL of Trolox (positive control); (e) 1 ng/mL of **1**; (f) 1 ng/mL of **2**; and (g) 1 ng/mlL of **3**; bar = 10 μm at 100×

This difference in reactivity allowed for selective formylation on the two aromatic rings, and thus enabled the separate preparation of 5-formyloxyresveratrol (**2**) and 2′-formyloxyresveratrol (**4**) through **1d** and **1f**, respectively. 5-Carboxy-oxyresveratrol (**3**) was obtained from **1d** through oxidation with NaClO$_2$ followed by BCl$_3$-catalyzed deprotection. However, our attempts to prepare 2′-carboxy-oxyresveratrol from **1f** in a similar manner were

not successful due to the decomposition of the oxidized product during the deprotection process.

As can be seen from Table 1, oxyresveratrol (**1**) was evaluated for effects on P19-derived neuronal cells at various concentrations, and found to enhance cell viability at 1 ng/ml. When similarly analyzed at this concentration, the 5-formylated and the 5-carboxylated products (**2** and **3**) displayed comparable effects, but the 2′-formylated derivative (**4**) exhibited toxicity and was excluded from the following experiments. In a subsequent study, P19-derived cells were cultured in serum-deprived medium, and the cell viability was measured by the XTT reduction method (Table 2). The oxidative stress induced by nutrient starvation affected cell survival and as a result reduced the cell viability to 40 %, compared with the cells grown in normal condition. Addition of **1**, **2** or **3** to the medium partly restored cell viability.

Nutrient deprivation is known to be a stimulus for oxidative stress, an imbalance of free radical production and the antioxidant defense system. The production of free radicals often begins with the generation of superoxide anions ($O_2^{\bullet\,-}$), which can then act as the precursor for the formation of several other reactive chemical species, such as H_2O_2, $OH^{\bullet}$ and $NO^{\bullet}$. In this study, the ability of these polyoxygenated stilbenes (**1 – 3**) to enhance cell survival appeared to be in parallel with their $O_2^{\bullet\,-}$ scavenging activity. Compounds **2** and **3**, with an additional electron withdrawing group such as CHO or COOH, showed higher superoxide scavenging activity and stronger cell protection activity than the parent compound **1**. However, it should be kept in mind that these compounds **1 – 3** at higher concentrations have the possibility of displaying toxicity, and *in vivo* experiments are thus needed to verify their neuroprotective potential. Recently, we have developed nanostructured carriers for oral delivery of **1** [19], which should be useful for further *in vivo* experiments and clinical trials.

CONCLUSION

Oxyresveratrol and its derivatives, including 5-formylated and 5-carboxylated products, enhance the survival of P19-derived cells in serum starvation condition. Their potencies are much higher than those of the well-known antioxidants, Trolox and ascorbic acid. Although these stilbenes are potentially of therapeutic value, *in vivo* experiments to confirm their neuroprotective potential are still required.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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