Cytoprotective Activity of Chemical Constituents Isolated from *Streptomyces* sp.

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**Abstract:** Four flavonoids including kaempferol (1), isoscutellarin (2), umbelliferone (3) and cichorin (4) have been isolated from *Streptomyces* sp. Tc052, an endophyte in the root tissue of *Alpinia galanga* Swartz. The evaluation for protective effect of compounds 1-4 against glutamate-induced cytotoxicity in hippocampal HT22 cell line was conducted. Compounds 1 and 2 showed significant effective protection ratios of 62.4±2.8 and 55.3±3.4%, respectively, at a concentration of 100 µM, whereas compounds 3-4 were inactive. Compounds 1 and 2 showed potent scavenging effects on DPPH radical exhibiting IC₅₀ value of 60.74 and 75.65 µM, respectively. These results suggest that compounds 1 and 2 may possess the neuroprotective activity against oxidative cellular injuries.

**Keywords:** *Alpinia galanga*, cytoprotective activity, endophytic *Streptomyces*, flavonoids, HT22 cells

INTRODUCTION

The organism *Streptomyces* sp. Tc052 was isolated from the root tissue of *Alpinia galanga* Swartz (Zingiberaeaceae). It produced flavonoids; kaempferol, isoscutellarin, umbelliferone and cichorin (Fig. 1), which had antimicrobial activities (Taechowisan et al., 2008). Flavanoids have been reported antioxidative stress in human hepatocarcinoma HepG2 cells (Wolfe and Liu, 2007) and also human lymphocytes (Martini et al., 2004).

Oxidative stress, defined as the accumulation of Reactive Oxygen Species (ROS) caused by enhanced ROS production or by suppression of ROS destruction, play an important role in a variety of neurodegenerative disorders of central nervous system, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and ischemia (Coyle and Puttfarken, 1993; Satoh et al., 2006; Satoh and Lipton, 2007). It is also well known that the accumulation of ROS in neurons results in lipid peroxidation, protein oxidation, DNA damage and finally cell death. Thus, antioxidant is one of the potential targets of drug development for neuroprotection (Satoh et al., 2006; Satoh and Lipton, 2007). As a model of oxidative stress-induced cell death, we studied the glutamate-induced cytotoxicity in HT22 cells, a neuronal cell line that subeloned from the mouse HT4 hippocampal cell line and has been used as a useful model for studying the mechanism of oxidative glutamate toxicity (Davis and Maher, 1994). Glutamate, the major neurotransmitter in the mammalian brain, is known to induce oxidative stress by inhibiting cysteine uptake, which subsequently leads to depletion of glutathione levels (Davis and Maher, 1994), increased production of ROS and elevated Ca²⁺ levels (Sagara et al., 2002). However, excitotoxicity is not involved in the cell death since glutamate receptor antagonists

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did not have any effects (Davis and Mahar, 1994). Thus, the aim of this study was to find substances for the protection of the cytotoxic effect of glutamate in hippocampal HT22 cells from the flavonoids isolated from Streptomyces sp. Tc 052 and would be valuable for potential therapeutic use.

MATERIALS AND METHODS

Extraction and Purification of Active Compounds

Spores of Streptomyces sp. Tc052 were used to inoculate 250 plates of ISP-2 and incubated for 14 days at 28°C. The culture medium was then cut into small pieces that were extracted with ethyl acetate (3-500 mL). This organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (980 mg). The solid was separated by column chromatography using silica gel 60 (Merck, 0.040-0.063 mm) and CHCl₃:MeOH (20:1, 20:2, 20:3, 20:4 and 20:5) as the eluent to give active fractions, A-4 and A-5. Fraction A-4 (385 mg) and A-5 (420 mg) were purified by reversed-phase Sep-Pak® eluting with 10% MeOH in H₂O to give compound 1 and 2 of fraction A-4 and compound 3 and 4 of fraction A-5.

Cell Culture

The mouse hippocampal HT22 cells, which is a subclone of the HT4 hippocampal cell line were received from the Cell Line Bank of the Chinese Academy of Sciences and were maintained at 1 x 10⁷ cell mL⁻¹ culture in DMEM supplemented with 10% heat inactivated FBS, penicillin G (100 IU mL⁻¹), streptomycin (100 μg mL⁻¹) and L-glutamine (2 mm) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The experiment was performed with three groups: the control, glutamate-treated and sample pre-treated groups. Cells of the control group received neither glutamate nor the sample treatment. Those of the glutamate-treated group were incubated with glutamate at a concentration of 5 mm for 12 h and those of the sample pre-treated group received sample treatment for 1 h prior to exposure to glutamate. The samples were dissolved initially in DMSO concentration in each experimental and control well was kept constant at 0.1% and this final concentration showed no relevant effects of DMSO on cellular growth and survival in the assay.

MTT Assay

MTT cytotoxicity assay was performed according to the method previously described (Mosmann, 1983). MTT solution was added at a concentration 50 μg mL⁻¹ into each well. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μL DMSO. Optical density at 570 nm was determined with a microplate reader. The optical density of formazan formed in control cells was taken as 100% of viability. Trolox was used as a positive control. EC₅₀ values for protective effects (defined as percentage viability versus the respective control) were calculated by linear regression using mean values and are expressed as Mean±SD of three independent experiments. The results were compared using one way ANOVA and Tukey’s multiple comparison test. Statistically significant differences between groups were defined as having p-values of <0.05. Calculations were performed using SPSS for Window 11.01 (SPSS Inc., Chicago, USA).

DPPH Radical Scavenging Assay

The scavenging action of DPPH radical was measured by (An et al., 2005). The reaction mixture contained 1 mL of 0.1 mM DPPH-ethanol solution, 1 mL of ethanol, 0.95 mL of 0.05 M Tris-HCl buffer (pH 7.4) and 50 mL of either test samples or deionized water (control). Reduction of
the DPPH free radical was measured by reading the absorbance at 517 nm exactly 30 sec after adding the samples. L-Ascorbic acid was used as a positive control. The absorbance of the sample alone was subtracted as the blank from that of the reaction mixture. DPPH radical scavenging activity of the sample was expressed as the IC_{50} value, which required concentration to inhibit DPPH radical formation by 50% determined from the log dose-inhibition curve.

RESULTS AND DISCUSSION

In the present study, we identified the compounds isolated from Streptomyces sp. Te052 for the protection of the cytotoxic effects of glutamate in hippocampal HT22 cells. Phytochemical fractionation of CHCl_{3}:MeOH-soluble fraction led to the isolation of four compounds (1-4).

Structure Elucidation

Purification of A-4 fraction using reversed-phase with 10% MeOH in H_{2}O afforded 65 mg of compound 1 and 48 mg of compound 2 and purification of A-5 fraction using reversed-phase with 10% MeOH in H_{2}O afforded 52 mg of compound 3 and 37 mg of compound 4.

**Compound 1**

Yellow crystals, m.p. 304-305°C. UV λ_{max} (MeOH): 370, 266 nm, (MeOH+NaOMe): 408, 277 nm, (MeOH+AlCl_{3}): 400, 278 nm, (MeOH+AlCl_{3}+HCl): 400, 278 nm, (MeOH+NaOAc): 397, 277 nm. EIMS m/z (rel. abund. %): 286 (3) [M', C_{11}H_{10}O_{4}], 256 (7), 128 (100), 118 (33), 113 (67), 97 (95). ^1H NMR (δ, CD_{3}OD): 7.09 (2H, d, J = 11.4 Hz, H-2', H-6'), 6.74 (2H, d, J = 11.4 Hz, H-3', H-4'), 5.95 (1H, d, J = 3.0 Hz, H-6), 5.14 (1H, d, J = 3.0 Hz, H-8). ^13C NMR (δ, CD_{3}OD): 144.3 (C-2), 136.8 (C-3), 170.9 (C-4), 164.4 (C-5), 99.5 (C-6), 168.0 (C-7), 92.3 (C-8), 149.5 (C-9), 107.8 (C-10), 125.3 (C-1'), 130.9 (C-2'), 116.1 (C-3'), 158.6 (C-4'), 116.1 (C-5'), 130.9 (C-6').

**Compound 2**

Dark yellow crystals, m.p. 300-301°C. UV λ_{max} (MeOH): 282, 332 nm. Degradation occurs with all shift reagents. EIMS m/z (rel. abund%): 286 (100) [M', C_{11}H_{10}O_{4}], 258 (47), 257 (9), 168 (80), 140 (52), 118 (38), 112 (54). ^1H NMR (δ, CD_{3}OD): 7.06 (2H, d, J = 8.5 Hz, H-2', H-6'), 6.77 (2H, d, J = 8.5 Hz, H-3', H-5'). 6.08 (1H, s, H-3), 5.59 (1H, s, H-6).

**Compound 3**

Colorless needles, m.p. 228-229°C. It showed intense blue fluorescence under UV lamp and gave a negative Molisch's test. EIMS m/z (rel. abund. %): 162 (8) [M', C_{10}H_{8}O_{4}], 149 (25), 138 (13), 121 (7), 110 (100), 94 (90), 81 (41), 66 (75), 55 (89). ^1H NMR (δ, CDCl_{3}+CD_{3}OD): 7.59 (1H, d, J = 15.8 Hz, H-4), 7.07 (1H, br.s, H-8), 6.97 (1H, br.d, J = 8.1 Hz, H-6), 6.80 (1H, d, J = 8.1 Hz, H-5), 6.30 (1H, d, J = 15.8 Hz, H-3). ^13C NMR (δ, CDCl_{3}+CD_{3}OD): 167.3 (C-2), 114.3 (C-3), 144.8 (C-4), 147.7 (C-4a), 114.7 (C-5), 121.2 (C-6), 144.2 (C-7), 113.4 (C-8), 145.3 (C-8a).

**Compound 4**

Transparent prisms, m.p. 215-216°C. It showed intense blue fluorescence under UV lamp and gave a positive Molisch's test. UV λ_{max} (MeOH): 234, 289, 347 nm, (MeOH+NaOMe): 249, 306, 390 nm, (MeOH+AlCl_{3}): 234, 289, 347 nm, (MeOH+AlCl_{3}+HCl): 234, 289, 347 nm, (MeOH+NaOAc): 252, 282, 352 nm. EIMS m/z (rel. abund %): 340 (2) [M', C_{11}H_{10}O_{4}], 320 (2), 293 (72), 179 (90), 178 (97), 167 (90), 149 (100), 127 (68), 97 (56). ^1H NMR (δ, CDCl_{3}+CD_{3}OD): 7.72 (1H, d, J = 9.5 Hz, H-4), 7.11 (1H, s, H-8), 6.94 (1H, s, H-5), 6.19 (1H, d, J = 9.4 Hz, H-3), 4.88
Fig. 1: Chemical structures of kaempferol (1), isocutelian (2), umbelliferone (3) and cichorin (4)

(1H, d, J = 7.3 Hz, 0 H-1'), 3.31-3.85 (m, 6H, H-2', H-6'). $^{13}$C NMR (δ, CDCl$_3$+CD$_3$OD): 163.0 (C-2), 114.1 (C-3), 144.9 (C-4), 141.3 (C-4a), 113.3 (C-5), 148.9 (C-6), 149.8 (C-7), 104.7 (C-8), 149.99 (C-8a), 102.4 (C-1'), 76.0 (C-2'), 77.8 (C-3'), 74.0 (C-4'), 78.0 (C-5'), 61.7 (C-6').

Compounds 1, 2, 3 and 4 were identified as kaempferol (Markham et al., 1978), isocutelian (Jay and Gornet, 1973), umbelliferone (Yamaguchi, 1970) and cichorin (Abdel-Salam et al., 1986), respectively by comparing their spectral data with those previously published (Fig. 1).

Compounds 1-4 were tested for their cytoprotective activity in the in vitro assay system. Increasing reports have provided evidences implicating oxidative stress as a major pathogenic mechanism in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Emerit et al., 2004). Oxidant over production leads to oxidative molecular damage of the tissue (Zhu et al., 2004). Therefore, protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment neurodegenerative disorders caused by oxidative stress (Behl and Moosmann, 2002; Bastianetto and Quirion, 2002).

Glutamate is the main excitatory neurotransmitter in the CNS, reaching concentrations of 1-10 mM in the synaptic cleft and intraneuronal compartments (Dzubay and Jahr, 1999). Disturbance of glutamate levels is the primary cause of neuronal death in stroke, mechanical trauma and seizure and it is considered to play a role in some chronic neurodegenerative disorders such as Parkinson's or Alzheimer's disease (Lipton, 2004).

HT22 cells, a mouse hippocampal cell line, lacking ionotropic glutamate receptors, the high concentration glutamate inhibits cysteine uptake and depletes intracellular glutathione, which leads to the accumulation of Reactive Oxygen Species (ROS) and ultimately causes cell death (Froissard and Duval, 1994).

To evaluated the in vitro neuroprotective effects of isolated compounds from Streptomyces sp. Te052, protective effects on glutamate-induced cytotoxicity in HT22 cells were tested. Among isolated compounds, compounds 1 (kaempferol) and 2 (isocutelian) exhibited protective effects against glutamate-induced cytotoxicity in HT22 cells, with effective protection ratios of 62.4±2.8% and 55.3±3.4%, respectively, at a concentration of 100 μM (Fig. 2). Trolox, well known for its antioxidative efficiency, was used as a reference substance and showed protect ratio of 92.5±2.5% at a concentration of 100 μM. In addition, to estimate the antioxidative effects of isolated compounds, free radical scavenging activity of compounds 1-4 was evaluated by the interaction with stable free radical DPPH. Among these compounds, compounds 1 and 2 showed potent scavenging effects on DPPH radical exhibiting IC$_{50}$ value of 60.74 and 75.65 μM, respectively. L-Ascorbic acid, well known for its anti-radical efficiency, was used as a positive control and exhibited the IC$_{50}$ value of 72.35 μM.
Fig. 2: The cytoprotective effects of compounds 1 (A) and 2 (B) on glutamate-induced cytotoxicity in HT22 cells. Cytotoxicity was assessed after 12-h incubation period with 100 mM of glutamate in DMEM medium. Each value represents the Mean±SD of three experiments. Significantly different from the control, *p<0.05

In conclusion, four flavonoid compounds (1-4) from Streptomyces sp. Tc052 were isolated. The protective effect of compounds 1 and 2 against glutamate-induced cytotoxicity in mouse hippocampal HT22 cells appears to be related to its free radical scavenging effects and these results suggest that compounds 1 and 2 can be valuable source of potential neuroprotective agents.
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