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Bio-assay guided isolation and identification of anti-Alzheimer active compounds from the root of *Angelica sinensis*

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ABSTRACT

Activity-directed fractionation and purification processes were employed to identify the anti-Alzheimer active compounds from the root of *Angelica sinensis*. In this study, the ability of *Angelica* root to inhibit the aggregated amyloid β-peptide (agg $A\beta_{1-40}$) induced damage of differentiated PC-12 cells (dPC-12), a well-known cell model for Alzheimer disease, was investigated. Air-dried roots of *A. sinensis* were extracted with methanol and then separated into ethyl acetate, *n*-butanol and water layers. Among them, only the ethyl acetate layer showed strong activity and therefore, subjected to separation and purification using various chromatographic techniques. Four compounds showing potent activity were identified by comparing spectral data (UV, NMR, and ESI-MS) with literature values to be *Z*-ligustilide, 11-angeloylsenkyunolide F, coniferyl ferulate and ferulic acid. They were found to significantly inhibit $A\beta_{1-40}$ toxicity on dPC-12 cells at lower concentrations (1–10 μg/ml), but at high concentrations (>50 μg/ml) they were toxic to the dPC-12 cells, except 11-angeloylsenkyunolide F. DPPH scavenging activity of the extracts and isolated compounds have also been carried out to find the possible mechanism of the activity.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease that affects cognitive function in the elderly. Amyloid β-peptide (Aβ) has been identified as a possible source of oxidative stress in the AD brain because it can acquire a free radical state that contributes to its toxic effects. Aβ-induced cytotoxicity has been shown to be caused by the intracellular accumulation of reactive oxygen species (ROS), ultimately leading to the peroxidation of membrane lipids and to a cell death (Kumar, Dunlop, & Richardson, 1994). Although the precise mechanisms by which Aβ induces neurotoxicity are still unknown, modulation of Aβ insult has been speculated to be an important preventive and neuroprotective approach to control the onset of AD (Kim, Park, & Kim, 2001). Thus the use of antioxidants has been recognised as an effective method in minimising pathological and toxic effects associated with Aβ-induced oxidative stress. As a result of strong interest in discovering compounds with Aβ-toxicity-modulating properties and antioxidative effects, we screened various phytochemical extracts and selected a methanol extract of *Angelica sinensis*. However, plant derived drug discovery against AD is not well explored. Only *Ginkgo biloba* L. (Oken, Storzbach, & Kaye, 1998), *Huperzia serrata* (Thunb. Ex Murray) Trevis. (Skolnick, 1997) and salvianolic acid B (Durairajan et al., 2008) have been extensively investigated as natural therapeutic agents for the treatment of AD patients.

The roots of *A. sinensis* (Oliv.) Diels, also known in Chinese as Dang Gui or Dong Guai, a well-known herb belonging to the Umbelliferae family, has been used for more than 2000 years in China for various ailments. They have also been widely used as health foods for women's care in Asia (Tieraona, 2005), and were marketed in Europe and America as a dietary supplement (Deng et al., 2006). Previous phytochemical studies on Dang Gui have resulted in the isolation and identification of a variety of constituent classes, including phthalides, polysaccharides, lipids, polyacetylenes, aromatic compounds, terpenes, amino acids, trace elements, and vitamins (Deng et al., 2006). In recent years, roots of Chinese Dang Gui have drawn the attention of researchers and consumers due to their nutritional and health benefits (anti-ulcer, antitumor, radioprotective action and immunostimulating activity) (Yang, Zhao, Li, Wang, & Lv, 2008). Recently, the protective effects of *A. sinensis* extract on amyloid β-peptide-induced neurotoxicity was studied (Huang, Lin, & Chiang, in press); however the active constituents responsible for the activity were not clear. *Z*-Ligustilide, the major constituent of *A. sinensis* was found to protect hydrogen peroxide-induced injury in PC-12 cells (Yu, Du, Wang, & Qian, 2008). On the other hand, ferulic acid, another important constituent of *A. sinsensis*, was well studied in prevention and/or treatment of disorders linked to oxidative stress, including Alzheimer's disease (Yan et al., 2001). Research has mainly focused on extracts

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level or the major compounds present in *A. sinensis*, therefore, the aim of the present study was to investigate the ability of *A. sinensis* root extracts to protect PC-12 cells from $A\beta_{1-40}$-induced cell death and subsequently, to isolate active constituents through bioassay-guided fractionation techniques.

2. Materials and methods

2.1. Materials and chemicals

A. sinensis were purchased from Chung Ching Tang Pharmaceutical Co., Ltd. (Taipei, Taiwan). ^{1}H NMR spectra were obtained on a Bruker DMX-300, 500 MHz instrument (Bruker Instruments, Billerica, MA, USA), and the ESI-MS spectra were acquired with a Linear Ion trap LXQ (ThermoFinnigan, San Jose, CA, USA). HPLC analysis was performed on an Hitachi HPLC system (Hitachi, Tokyo, Japan). Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), normal phase Silica gel (Merck Si 60, Darmstadt, Germany), reverse-phase Silica gel (Cosmosil 75 C18-OPN, Nacalai Tesque, Inc, Kyoto, Japan) were used as the adsorbents for open column chromatography. Silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), foetal bovine serum (FBS), nonessential amino acids (NEAA) and a penicillin/streptomycin mixture were purchased from Gibco-Invitrogen (Grand Island, NY, USA) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and nerve growth factor (NGF) were purchased from Sigma (St. Louis, MO) and the fragment of Aβ peptide ($A\beta_{1-40}$) was purchased from AnaSpec (San Jose, CA, USA) and also from Biosource (Camarillo, CA, USA). EGb 761 was purchased from Schwabe Pharmaceuticals (Karlsruhe, Germany). Sal B was a gift sample from Professor M.-S. Shiao of the Department of Medical Research and Education of Veterans General Hospital, Taipei, Taiwan. A rat pheochromocytoma cell line (PC-12, CRL-1721) was purchased from American Tissue Culture Catalogue (Manassas, MD, USA). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) was purchased from Aldrich Co. (Milwaukee, WI, USA). All solvents used for chromatography were of HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Extraction and isolation of bioactive compounds

A. sinensis root (2 kg) were laid in a 45 °C dryer for 24 h, cut into small pieces and extracted with methanol (6 L × 3 times) overnight and then filtered through Whatman No. 1 filter paper (Whatman, Maidstone, England). The methanol filtrates were collected and concentrated under reduced pressure by a rotary evaporator at 40 °C to dryness producing 428 g of menthol extract (AS-M). Part of the methanol extract was reserved for activity assays whilst the rest of the extract was suspended in water and partitioned with ethyl acetate and *n*-BuOH (up to decoloration of the organic solvents), followed by concentration to yield 60.3 g of the ethyl acetate extract (AS-M-EA), 26.3 g of the *n*-butanol extract (AS-M-B) and 332.1 g of the water extract (AS-M-W) respectively. The neuroprotective activities of all the extracts were estimated by the "Inhibition of agg $A\beta_{1-40}$-induced differentiated PC-12 cell death" model (Choi et al., 2006). Among these only the AS-M-EA extract showed strong activity when compared with other fractions.

The active AS-M-EA extract (32.6 g) was subjected to open column chromatography on normal phase silica gel and eluted with a solvent mixture of hexane/ethyl acetate (10:0–0:10, v/v) and finally eluted with methanol affording 10 major fractions. TLC analysis was performed on silica gel using hexane/ethyl acetate in various proportions as the mobile phase. Compounds were visualised under UV light (254 and 365 nm) or by spraying the plates with anisaldehyde-sulfuric acid reagent (Wagner, Bladt, & Zgainski, 1984). Fractions eluted with hexane/ethyl acetate (8:2) (AS-M-EA-Fr.3) and hexane/ethyl acetate (1:1) (AS-M-EA-Fr.6) significantly decreased $A\beta_{1-40}$ induced dPC-12 cells death. Re-chromatography of the AS-M-EA-Fr.3 on Sephadex LH-20 (methanol), followed by silica gel column chromatography (hexane:ethyl acetate), reverse-phase open column chromatography (90% methanol in water) and by semi-preparative reverse-phase HPLC (Spherisorb S5 ODS-2, 250 × 8 mm, methanol:water = 85:15, v/v, flow rate 2 ml/min), yielded 45 mg of *Z*-ligustilide (**1**) and 7 mg of 11-angeloylsenkyunolide F (**2**) as active compounds. Re-chromatography of the AS-M-EA-Fr.6 on Sephadex LH-20 (methanol), followed by silica gel column chromatography (hexane:ethyl acetate), and semi-preparative C18 HPLC (Spherisorb S5 ODS-2, 250 × 8 mm, methanol:water = 70:30, v/v, flow rate 2 ml/min) yielded 22 mg of coniferyl ferulate (**3**) and 48 mg of ferulic acid (**4**) as active compounds. All the compounds, known in literature, were identified by comparing spectral data (UV, NMR, and ESI-MS) with reported values.

2.3. HPLC analysis

HPLC analysis was performed on a Hitachi HPLC system (Hitachi, Tokyo, Japan) Alltima C18 column (4.6 mm × 250 mm, 5 μm, Deerfield, IL, USA) with a compatible guard column and the mobile phase consisted of water containing 0.5% (v/v) acetic acid (A) and ACN (B) using a gradient programme of 20% B in 0–6 min, 20–50% B in 6–14 min, 50% B in 14–20 min, 50–75% B in 20–25 min, 75–100% B in 25–35 min and 100% B in 35–45 min. The solvent flow rate was 1 ml/min and the column temperature was set at 30 °C. UV 290 nm was used as the measuring wavelength.

2.4. Assay for agg $A\beta_{1-40}$-induced cytotoxicity and dPC-12 cells viability

PC-12 cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum (HS), 5% heat-inactivated foetal bovine serum (FBS), 1% NEAA and a mixture of 1% penicillin/streptomycin. The cells were cultured on 100-mm cell culture dishes (Falcon, NY, USA) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The medium was refreshed approximately three times a week and cells were subcultured when the cultures were 80–90% confluent (split ratio 1:4). PC-12 cells were seeded in collagen-coated 24 well culture plates (1×10^4 cells/well) with complete DMEM for 24 h. Subsequently, cells were incubated in serum-free DMEM with 50 ng/ml NGF (NGF-DMEM) for 4 days (Li & Buccafusco, 2003), with replenishment of the NGF every second day.

$A\beta_{1-40}$ was prepared as a 0.5 mM (1 mg/460 μl) stock solution in milli-Q water and filtered through a 0.22 μm filter (Millipore, USA). The solution was held at 4 °C for 60 h and then incubated at 37 °C for 8 h with gentle mixing every 2 h to accelerate aggregation. After aggregation, the solution was separated into aliquots (10 μl) in sterile Eppendorf tubes and stored at −20 °C. Before use, $A\beta_{1-40}$ was shifted to a 37 °C water bath for 8 h.

PC-12 cells were differentiated by 50 ng/ml NGF for 4 days and the culture medium was replaced by fresh serum-free DMEM (without NGF) with or without 10 μM agg $A\beta_{1-40}$ from one supplier (AnaSpec, CA, USA) or 4 μM agg $A\beta_{1-40}$ from another supplier (Biosource, CA, USA) and different concentrations of samples. They were then incubated for another 24 h and the viability of dPC-12 cells was determined by the MTT assay according to the method of Choi et al. (2006) with slight modifications. At the termination of the incubation period, the culture medium was replaced by fresh serum-free DMEM which contained 0.5 mg/ml MTT. The cells were incubated for 1 h at 37 °C, the medium was removed and 300 μl