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Original Article

Protective effect of *Nelumbo nucifera* extracts on beta amyloid protein induced apoptosis in PC12 cells, *in vitro* model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. β-Amyloid (Aβ) has been proposed to play a role in the pathogenesis of AD. Deposits of insoluble Aβ are found in the brains of patients with AD and are one of the pathological hallmarks of the disease, but the underlying signaling pathways are poorly understood. In order to develop antidementia agents with potential therapeutic value, we examined the inhibitory effect of the *Nelumbo nucifera* seed embryo extracts on to 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 AD. In the present study, seed embryos of *N. nucifera* were extracted with 70% methanol in water and then separated into hexane, ethyl acetate, *n*-butanol, and water layers. Among them, only the *n*-butanol layer showed strong activity and was therefore subjected to separation on Sephadex LH-20 chromatography. Two fractions showing potent activity were found to significantly inhibit $A\beta_{1-40}$ toxicity on dPC-12 cells in increasing order of concentration (10–50 μg/mL). Further purification and characterization of these active fractions identified them to be flavonoids such as rutin, orientin, isoorientin, isoquercetrin, and hyperoside. 2,2-Diphenyl-1-picrylhydrazyl hydrate scavenging activity of the extracts was also carried out to ascertain the possible mechanism of the activity.

1. Introduction

"Lian zi xin," embryo loti (embryo of the seeds of *Nelumbo nucifera* Gaertner, Nymphaeaceae), has been usually used as a vegetable or, in Chinese traditional herbal medicine, as a sedative, antipyretic, and hemostatic agent [1], indicating that it may possess central nervous effects. In folk medicines, *N. nucifera* seeds are used in the treatment of tissue

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inflammation and cancer, as an antiemetic agent, given to children as a diuretic, and used as a refrigerant [2]. They are also often considered human health immunomodulators [3] and are used as a cooling medicine for skin diseases and leprosy, and are considered to be an antidote to poison [2]. The seeds are reported to possess hepatoprotective and free radical scavenging activity [4], antifertility activity [5], as well as antiproliferative [6] and anti-inflammatory activity [7], and also are reported suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells [6]. For centuries, parts of this plant have been used in Oriental medicine to treat hyperlipidemia and nonalcoholic fatty liver disease [8]. *N. nucifera* is a flavonoid-rich plant containing myricetin, quercetin, kampferol, and isohamnetin [9]. Procyanidins were isolated from the seed pods of *N. nucifera* by Ling et al [10], who have also reported the antioxidant activity of procyanidins. *N. nucifera* seeds also contain alkaloids, saponins, phenolics, and carbohydrates [10,11]. Different parts of *N. nucifera* have been consumed as functional foods and are considered a potential nutraceutical source.

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 [12]. 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 [13]. Thus, the use of antioxidants has been recognized as an effective method in minimizing pathological and toxic effects associated with Aβ-induced oxidative stress. As a result of strong interest to discover compounds with Aβ-toxicity-modulating property and antioxidative effect, we screened various phytochemical extracts and previously reported the anti-Alzheimer compounds from methanol extract of *Angelica sinensis* [14]. However, plant-derived drug discovery against AD is not well explored. Only *Ginkgo biloba* L. [15], *Huperzia serrata* (Thunb. Ex Murray) Trevis [16], and salvianolic acid B [17] have been extensively investigated as natural therapeutic agents for the treatment of AD patients. To the best of our knowledge, there are no published studies on the effects of *N. nucifera* extracts on neurodegenerative diseases. Therefore, we investigated whether *N. nucifera* seed embryo extracts can protect against beta amyloid protein-induced apoptosis in PC12 cells, a well-known cell model for AD.

2. Materials and methods

2.1. Materials and chemicals

N. nucifera seeds were purchased from a local food market near Taipei (Taiwan), and embryos were separated and preserved at −20°C until use. ^{1}H NMR (nuclear magnetic resonance) spectra were obtained on a Bruker DMX-300, 500 MHz instrument (Bruker Instruments, Billerica, MA, USA). The electrospray ionization mass spectrometry (ESI-MS) spectra were acquired with a Linear Ion trap LXQ (Thermo-Finnigan, San Jose, CA, USA). High-performance liquid chromatography (HPLC) analysis was performed on Hitachi HPLC system (Hitachi, Tokyo, Japan). Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), normal phase silica gel (Merck Si 60; Merck, Darmstadt, Germany), and reverse phase silica gels (Cosmosil 75 C18-OPN; Nacalai Tesque, Inc., Kyoto, Japan) were used as adsorbents for open column chromatography. Silica gel 60 F_{254} plates (Merck) were used for thin-layer chromatography (TLC). Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), fetal 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, USA). The fragment of Aβ peptide ($A\beta_{1-40}$) was purchased from AnaSpec (San Jose, CA, USA), also from BioSource International (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 the American Type Culture Collection (ATCC, Manassas, VA, USA). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) was purchased from Aldrich Co. (Milwaukee, WI, USA). All solvents used for chromatography were HPLC grade. All other chemicals were analytical reagent grade.

2.2. Extraction and isolation of bioactive compounds

N. nucifera seed embryo (200 g) were cut into small pieces and extracted with 70% methanol in water (1 L × 3 times) overnight and then filtered by Whatman No. 1 filter paper (Whatman, Maidstone, England, UK). The 70% methanol filtrates were collected and concentrated under reduced pressure by a rotary evaporator at 40°C to dryness, yielding 57.86 g of 70% methanol extract (Nn-M). Part of the Nn-M was reserved for activity assays, and the rest of the extract was suspended in water and partitioned with hexane, ethyl acetate, and *n*-BuOH (up to discoloration of the organic solvents), followed by concentration, yielding 2.46 g of hexane extract (Nn-M-H), 0.53 g of ethyl acetate extract (Nn-M-EA), 6.22 g of *n*-butanol extract (Nn-M-B), and 44.89 g of water extract (Nn-M-W), respectively. The neuroprotective activities of all extracts were estimated by the "Inhibition of agg $A\beta_{1-40}$-induced differentiated PC-12 cell death" model. Among these, only the Nn-M-B extract showed strong activity when compared with other fractions.

The active Nn-M-B extract (6 g) was subjected to open column chromatography on Sephadex LH-20 and eluted with a solvent mixture of water/methanol (50:50–0:100, v/v) and finally eluted with methanol affording 33 fractions and pooled into 11 major factions based on TLC analysis. TLC was performed on silica gel using ethyl acetate/methanol/water/acetic acid (7:1:1:1) as the mobile phase. Compounds were visualized under UV light (254 and 365 nm) or by spraying the

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