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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

plates with anisaldehyde-sulfuric acid reagent or NP-PEG reagent [18]. Two fractions, Nn-M-B-VII and Nn-M-B-IX, eluted with 75% methanol in water, significantly decreased $A\beta_{1-40}$-induced differentiated PC-12 (dPC-12) cell death. Rechromatography of the Nn-M-B-VII on reverse phase open column chromatography (eluted with methanol in water from 50% to 100%), followed by preparative C18 HPLC [Atlantis T3 Prep C18 column, 150 × 19 mm, mobile phase consisted of water (A) and methanol (B) using a linear gradient elution from 5% B in 0 minutes to 100% B in 60 minutes, flow rate 8 mL/min] yielded 12 mg rutin. Rechromatography of the Nn-M-B-IX on reverse phase open column chromatography (eluted with methanol in water from 50% to 100%), and the earlier fractions (eluted with 50% methanol) were subjected to Sephadex LH-20 (methanol) and followed by preparative C18 HPLC [Atlantis T3 Prep C18 column, 150 × 19 mm, mobile phase consisted of water (A) and methanol (B) using a linear gradient elution from 5% B in 0 minutes to 100% B in 90 minutes, flow rate 8 mL/min] yielded 6 mg orientin and 4 mg isoorientin. The latter fractions (eluted with 75% methanol) were subjected to preparative C18 HPLC [Atlantis T3 Prep C18 column, 150 × 19 mm, mobile phase consisted of water (A) and methanol (B) using a linear gradient elution from 5% B in 0 minutes to 100% B in 90 minutes, flow rate 8 mL/min] yielded 1.5 mg hyperoside and 5 mg isoquercetrin. All compounds, known in the literature, were identified by comparing spectral data (UV, NMR, and ESI-MS) with reported values.

2.3. 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 HS, 5% heat-inactivated 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 hours. Subsequently, cells were incubated in serum-free DMEM with 50 ng/mL NGF (NGF–DMEM) for 4 days [19], 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, Billerica, MA, USA). The solution was held at 4°C for 60 hours and then incubated at 37°C for 8 hours with gentle mixing every 2 hours to accelerate aggregation. After aggregation, the solution was separated into aliquots (10 μL) in sterile Eppendorf tubes and stored at −20°C. Prior to use, $A\beta_{1-40}$ was shifted to a 37°C water bath for 8 hours.

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, Fremont, CA, USA) or 4 μM agg $A\beta_{1-40}$ from another supplier (BioSource International) and different concentrations of samples. The mixture was incubated for another 24 hours, and the viability of dPC-12 cells was determined using the MTT assay according to the method of Choi et al [20] with slight modifications. At the end 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 hour at 37°C; then, the medium was removed, and 300 μL dimethyl sulfoxide (DMSO) was applied to the well to dissolve the formazan derived from the mitochondrial cleavage of the tetrazolium ring by live cells. The absorbance of solubilized MTT formazan products was measured at 570 nm using a microplate reader from Synergy HT (Bio-TEK, Winooski, VT, USA).

2.4. DPPH radical scavenging activity

The antioxidant activity of the extracts and compounds, based on the scavenging activity of the stable DPPH free radical, was determined according to the method of Leung et al [21] with minor modifications. Test samples (50 μL) were added to 200 μL of 0.1 mM DPPH–methanol solution. Absorbance at 520 nm was determined after 30 minutes, and the radical scavenging activity was obtained from the following equation: Radical scavenging activity (%) = {[Ab − (A − As)]/Ab} × 100 (where Ab is the absorbance without sample, A is the absorbance with extract and DPPH, and As is the absorbance with extract only).

2.5. HPLC analysis

HPLC analysis was performed on Hitachi HPLC system (Hitachi, Tokyo, Japan) Waters Atlantis T3 C18 column (4.6 mm × 150 mm, 3 μm; Waters, Milford, MA, USA) with a compatible guard column, and the mobile phase consisted of water containing 0.5% acetic acid (A) and methanol (B) using a gradient program of 10–30% B in 1–15 minutes, 30–40% B in 15–35 minutes, 40–50% B in 35–45 minutes, 50–100% B in 45–55 minutes and 100% B in 60 minutes. The solvent flow rate was 0.8 mL/min, and the column temperature was set at 30°C. UV 254 nm was used as the measuring wavelength.

2.6. Statistical analysis

All data were expressed as mean ± standard deviation. Significant differences were determined using Student t test, where differences were considered significant if $p < 0.05$. All samples were measured in triplicate.

3. Results and discussion

3.1. Extraction and isolation of bioactive compounds

Solvent extraction of 70% methanol extract followed by open column chromatography of the n-butanol extract of *N. nucifera* yielded two active fractions. The 70% methanol extract was suspended in water and first extracted with hexane, ethyl acetate, and then with n-butanol to obtain hexane, ethyl acetate, n-butanol, and water extracts. Tables 1 and 2 summarize the activity of different extracts and fractions, respectively. The ability of Nn-M-B to protect dPC-12 cells from $A\beta_{1-40}$-induced cell death was higher than that of Nn-M, Nn-M-H, Nn-M-EA, and Nn-M-W extracts. Hence, Nn-M-B

extract was subjected to fractionation on an open Sephadex LH-20 column, and eluted with water and methanol mixtures. Two active fractions, Nn-M-B-VII and Nn-M-B-IX, were obtained from Sephadex LH-20 column chromatography. On further purification of these active fractions using various column chromatographic techniques, the following five flavonoids were obtained: rutin (**1**), orientin (**2**), isoorientin (**3**), isoquercetrin (**4**) and hyperoside (**5**). Figure 1 schematically represents extraction and fractionation scheme of *N. nucifera* seed embryo.

3.2. *Structure determination of isolated compounds*

The chemical structure confirmation of the components from the *N. nucifera* was accomplished by comparing UV, ESI-MS, ^{1}H, and ^{13}C NMR data obtained to published data.

Table 1 – Effects of different extracts of *Nelumbo nucifera* on inhibition of agg $A\beta_{1-40}$ induced cytotoxicity and dPC-12 cells viability.

Sample	Concentration of sample (μg/mL)	Inhibition of $A\beta_{1-40}$ cytotoxicity	Toxicity
		Cell viability (%)[a]	
Control		100 ± 1.0	100.0 ± 1.2
$A\beta_{1-40}$[b]	2μM	55.6 ± 2.5	50.2 ± 2.2
Sal B[c]	50	73.3 ± 1.6***	102.4 ± 2.4
	100	84.9 ± 2.3***	103.9 ± 1.3*
EGb 761[c]	50	71.5 ± 1.9***	103.3 ± 3.5
	100	82.4 ± 2.6***	106.2 ± 3.2*
Nn-M	25	62.2 ± 2.6*	99.7 ± 5.2
	50	64.1 ± 3.8**	107.2 ± 5.4
	100	55.5 ± 2.7	78.3 ± 8.8***
Nn-M-H	25	50.3 ± 1.3*	91.0 ± 3.2**
	50	53.0 ± 1.5	91.4 ± 7.2*
	100	57.8 ± 3.3	96.6 ± 2.7
Nn-M-EA	25	59.3 ± 3.3	96.1 ± 1.9*
	50	63.8 ± 5.3*	84.1 ± 3.5**
	100	64.2 ± 3.4**	75.3 ± 8.3***
Nn-M-B	25	61.8 ± 2.6*	99.5 ± 0.1
	50	65.8 ± 3.4**	99.7 ± 3.0
	100	69.3 ± 1.3***	89.1 ± 1.6**
Nn-M-W	25	57.8 ± 1.5	99.3 ± 1.0
	50	60.1 ± 1.4*	96.9 ± 9.4
	100	64.2 ± 0.9***	98.9 ± 4.4

Aβ = beta-amyloid; dPC-12 cells = differentiated PC-12 cells; EGb 761 = a standard extract of *Ginkgo biloba* L; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SAL B = salvianolic acid B from *Salvia miltiorrhiza* Bunge.

[a] Cell viability was measured by MTT reduction assay. Cell viability (%) represents three replicates per treatment and each treatment was repeated three times. In "inhibition of Aβ cytotoxicity," each data was compared with Aβ group, and statistical analysis was performed by Student *t* test. In "toxicity" of sample alone towards dPC-12 cells, each data was compared with control group, and statistical analysis was performed by Student *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

[b] $A\beta_{1-40}$ purchased from AnaSpec (San Jose, CA, USA).

[c] Sal B and EGb 761 are the positive controls.

Table 2 – Effects of Sephadex LH-20 fractions Nn-M-B of *Nelumbo nucifera* on inhibition of agg $A\beta_{1-40}$ induced cytotoxicity and dPC-12 cells viability.

Sample	Concentration of sample (μg/mL)	Inhibition of $A\beta_{1-40}$ cytotoxicity	Toxicity
		Cell viability (%)[a]	
Control		100 ± 1.1	100.0 ± 1.0
$A\beta_{1-40}$[b]	4μM	51.6 ± 1.2	52.1 ± 1.1
Sal B[c]	25	62.2 ± 2.1***	101.5 ± 1.7
	50	69.2 ± 2.2***	103.7 ± 2.3
EGb 761[c]	25	59.8 ± 0.7***	102.1 ± 1.8
	50	67.2 ± 1.2***	103.4 ± 2.1
Nn-M-B	10	55.3 ± 2.2	99.7 ± 1.6
	25	60.7 ± 2.1**	99.5 ± 2.7
	50	62.7 ± 1.1***	91.6 ± 3.2**
Nn-M-B-I	10	55.3 ± 2.4	84.7 ± 3.8***
	25	50.3 ± 3.9	78.3 ± 5.3***
	50	30.4 ± 5.1***	58.9 ± 4.9***
Nn-M-B-II	10	55.7 ± 1.4*	98.9 ± 1.5
	25	58.8 ± 1.8*	96.7 ± 3.2
	50	61.5 ± 2.3**	90.2 ± 4.2*
Nn-M-B-III	10	57.6 ± 2.5*	96.7 ± 2.3
	25	58.8 ± 2.3*	90.4 ± 2.9*
	50	54.6 ± 3.3	82.3 ± 3.6***
Nn-M-B-IV	10	54.3 ± 2.2	97.7 ± 2.4
	25	55.7 ± 2.8	88.5 ± 3.5*
	50	49.2 ± 3.5	76.7 ± 6.5***
Nn-M-B-V	10	56.1 ± 2.6	100.7 ± 2.1
	25	57.2 ± 2.8	100.2 ± 2.1
	50	58.9 ± 2.5*	93.4 ± 2.5*
Nn-M-B-VI	10	56.3 ± 2.4*	93.5 ± 2.3*
	25	57.9 ± 3.1*	90.5 ± 2.1**
	50	50.2 ± 3.1	78.1 ± 3.4***
Nn-M-B-VII	10	57.4 ± 1.8*	99.6 ± 2.6
	25	61.3 ± 2.1**	98.9 ± 2.3
	50	71.2 ± 2.4***	95.2 ± 3.4
Nn-M-B-VIII	10	59.2 ± 1.8**	97.8 ± 2.2
	25	58.9 ± 2.3*	84.5 ± 4.7**
	50	48.9 ± 3.4	63.6 ± 5.5***
Nn-M-B-IX	10	58.1 ± 1.9*	99.5 ± 2.4
	25	63.2 ± 2.5**	98.4 ± 1.8
	50	71.1 ± 3.6***	97.6 ± 2.5
Nn-M-B-X	10	56.7 ± 2.3*	90.4 ± 3.1*
	25	56.3 ± 3.1	81.6 ± 3.4***
	50	45.3 ± 4.7	67.3 ± 4.7***
Nn-M-B-XI	10	57.8 ± 1.7*	97.8 ± 2.7
	25	62.1 ± 2.9**	95.6 ± 3.8
	50	60.7 ± 3.8**	88.5 ± 3.6*

Aβ = beta-amyloid; dPC-12 cells = differentiated PC-12 cells; EGb 761 = a standard extract of *Ginkgo biloba* L; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SAL B = salvianolic acid B from *Salvia miltiorrhiza* Bunge.

[a] Cell viability was measured by MTT reduction assay. Cell viability (%) represents three replicates per treatment and each treatment was repeated three times. In "inhibition of Aβ cytotoxicity," each data was compared with Aβ group, and statistical analysis was performed by Student *t* test. In "toxicity" of sample alone towards dPC-12 cells, each data was compared with control group, and statistical analysis was performed by Student *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

[b] $A\beta_{1-40}$ purchased from BioSource International (Camarillo, CA, USA).

[c] Sal B and EGb 761 are the positive controls.

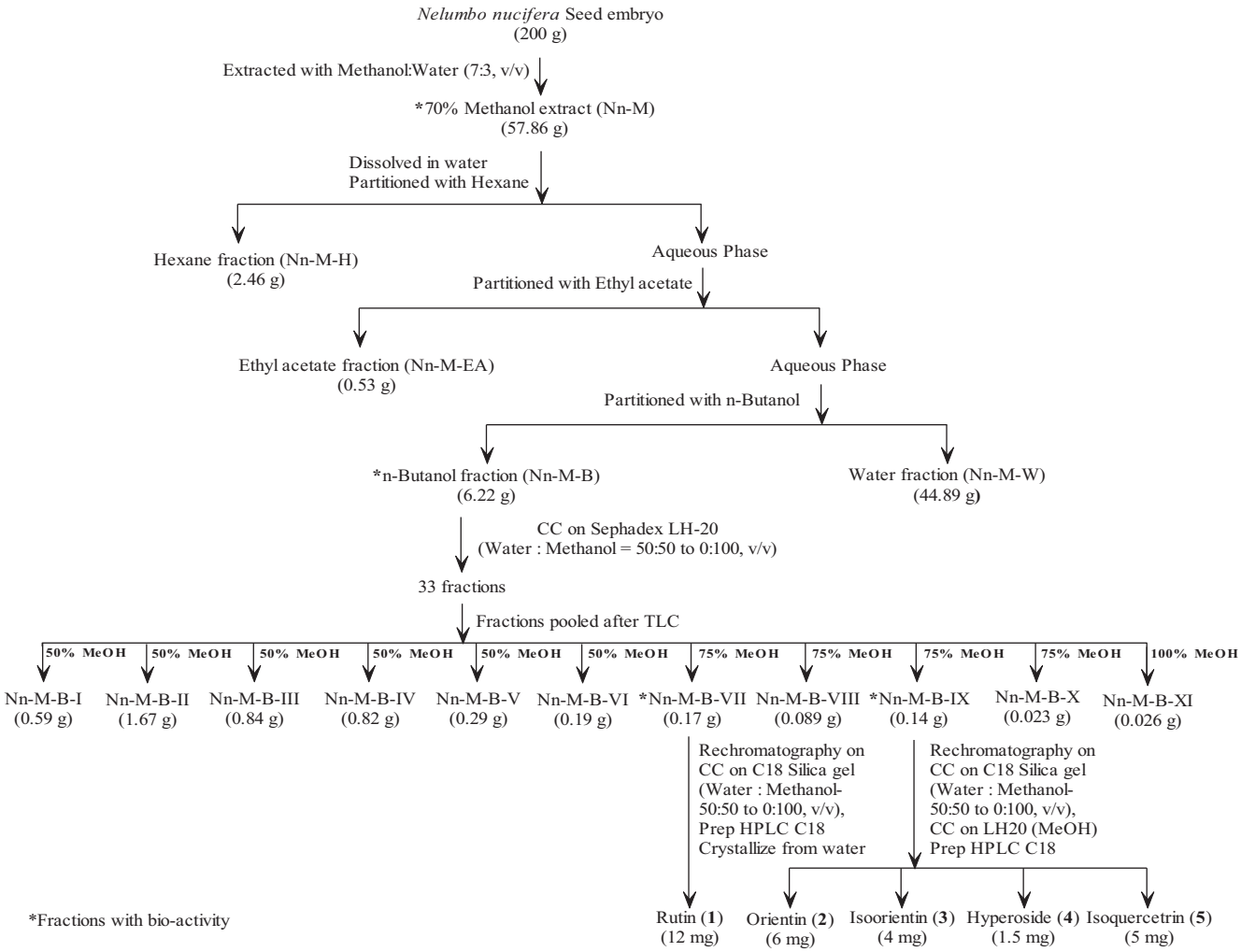

Figure 1 – Extraction and fractionation scheme of *Nelumbo nucifera* seed embryo. CC = Column Chromatography ;HPLC = high-performance liquid chromatography; TLC = thin-layer chromatography.

Rutin (**1**) was isolated and crystallized from water as yellow crystals, UV λ_{max} (CH_3OH) nm: 207, 259 sh, 261, 356 sh. ESI-MS, *m/z* 609 [M–H]; NMR spectra matched that of commercially available standard compound.

Orientin (**2**), yellow powder, UV λ_{max} (CH_3OH) nm: 349, 247. ESI-MS: 447 [M–H]; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 6.63 (1H, s, H-3), 6.26 (1H, s, H-6), 7.41 (1H, d, J = 2.1 Hz, H-2′), 6.85 (1H, d, J = 8.4 Hz, H-5′), 7.52 (1H, dd, J = 2.1, 8.0 Hz, H-6′), 4.66 (1H, d, J = 10.0 Hz, H-1″), 3.84 (1H, t, J = 9.0, H-2″), 3.24 (2H, m, H-3″, 5″), 3.36 (1H, m, H-4″), 3.78 (1H, dd, J = 11.4, 1.2 Hz, H-6a″), 3.52 (1H, m, H-6b″). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 164.05 (C-2), 102.36 (C-3), 181.98 (C-4), 162.50 (C- 5), 98.06 (C-6), 160.34 (C-7), 104.51 (C-8), 155.95 (C-9), 103.99 (C-10), 121.98 (C-1′), 114.04 (C-2′), 145.77 (C-3′), 149.77 (C-4′), 115.61 (C-5′), 119.32 (C-6′), 73.35 (C-1″), 70.73 (C-2″), 78.73 (C-3″), 70.66 (C-4″), 81.96 (C-5″), 61.60 (C-6″), and agree well with the reported data [22].

Isoorientin (**3**), yellow powder, UV λ_{max} (CH_3OH) nm: 350, 213. ESI-MS: 447 [M–H]; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 6.66 (1H, s, H-3), 6.47 (1H, s, H-6), 7.40 (1H, d, J = 2.1 Hz, H-2′), 6.88 (1H, d, J = 8.2 Hz, H-5′), 7.41 (1H, dd, J = 2.2, 8.2 Hz, H-6′), 4.57 (1H, d, J = 9.8 Hz, H-1″), 4.02 (1H, pt, J = 9.1, H-2″), 3.19 (1H, pt, J = 8.7, H-3″), 3.13 (1H, pt, J = 8.8, H-4″), 3.26 (1H, m, H-5″), 3.67 (1H, dd, J = 10.8, 2.0, H-6a″), 3.52 (1H, m, H-6b″). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 163.63 (C-2), 102.79 (C-3), 181.88 (C-4), 160.67 (C- 5), 108.85 (C-6), 163.22 (C-7), 93.48 (C-8), 156.18 (C-9), 103.40 (C-10), 121.42(C-1′), 113.30 (C-2′), 145.73 (C-3′), 149.68 (C-4′), 116.04 (C-5′), 118.96 (C-6′), 73.03 (C-1″), 70.60 (C-2″), 78.93 (C-3″), 70.19 (C-4″), 81.55 (C-5″), 61.48 (C-6″), and agree well with the reported data [22].

Isoquercetrin (**4**), pale yellow powder, UV λ_{max} (CH_3OH) nm: 206, 349. ESI-MS, *m/z* 463 [M–H]; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 6.20 (1H, d, J = 2.0 Hz, H-6), 6.39 (1H, d, J = 2.1 Hz, H-8), 7.71 (1H, d, J = 2.2 Hz, H-2′), 6.86 (1H, d, J = 8.5 Hz, H-5′), 7.58 (1H, dd, J = 2.2, 8.5 Hz, H-6′), 5.26 (1H, d, J = 7.6 Hz, H-1″), 4.47 (1H, t, J = 9.1,7.7 Hz, H-2″), 3.41 (1H, t, J = 9.0, 8.7 Hz, H-3″), 3.33 (1H, t, J = 9.5, 8.9 Hz, H-4″), 3.21 (1H, ddd, J = 9.6, 5.3, 2.3 Hz, H-5″), 3.70 (1H, dd, J = 11.9, 2.3 Hz, H- H-6a″), 3.56 (1H, dd, J = 11.9, 5.3 Hz, H- H-6b″). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 159.0 (C-2), 135.6 (C-3), 179.5 (C-4), 163.1(C-5), 99.9 (C-6), 166.2 (C-7), 94.7 (C-8), 158.5 (C-9), 105.7 (C-10), 123.1 (C-1′), 117.5 (C-2′), 145.9 (C-3′), 149.9 (C-4′), 116.0 (C-5′), 123.2 (C-6′), 104.3 (C-1″), 75.7 (C-2″), 78.1 (C-3″), 71.2 (C-4″), 78.4 (C-5″), 62.6 (C-6″), and agree well with the reported data [23].

Hyperoside (5) was isolated and crystallized from methanol as pale yellow crystal, UV λ_{max} (CH_3OH) nm: 256, 305, 361. ESI-MS, *m/z* 463 [M−H]. Comparing with the reported data, the ^1H NMR and ^{13}C NMR data were in agreement with those reported in the literature [24].

The structures of the compounds isolated from *N. nucifera* are shown in Figure 2.

3.3. *Prevention of agg $A\beta_{1-40}$-induced cytotoxicity and dPC-12 cells viability*

In order to ascertain the neuroprotective effects and the beneficial influences on neurodegenerative disease of *N. nucifera*, we used an *in vitro* "Inhibition of agg $A\beta_{1-40}$-induced dPC-12 cell death" model to estimate the neuroprotective effect. The PC-12 cell model has been used for studies of anti-Alzheimer's or antiaging drug development [19]. The PC-12 is a useful model to study cellular signal transduction, neuronal differentiation, and neurodegenerative disease [25]. We used an MTT assay to evaluate the cytotoxicity of agg $A\beta_{1-40}$ toward dPC-12 cells. The MTT assay is an indicator of the mitochondrial activity of living cells, because mitochondria play an important role in neuronal cell survival and death. In our study, we found that exposure of dPC-12 cells to agg $A\beta_{1-40}$ for 24 hours induced a concentration-dependent loss of cell viability, and 10 μM agg $A\beta_{1-40}$ from one supplier (AnaSpec) or 4 μM agg $A\beta_{1-40}$ from another supplier (BioSource International) caused approximately 50% dPC-12 cell death. Sal B (salvianolic acid B from *Salvia miltiorrhiza* Bunge) and EGb 761 (a standard extract of *G. biloba* L.) have been used as positive control, because several studies indicated that both Sal B and EGb 761 can inhibit Aβ aggregation and fibril formation [17], which can reduce neurotoxicity from agg Aβ.

Nn-M-B significantly inhibited agg $A\beta_{1-40}$-induced cytotoxicity at the concentration of 100 μg/mL. As shown in Table 1, Nn-M-B had the most neuroprotective activity among the extracts. The cell viability, expressed as percent of untreated control in the absence versus the presence of Nn-M-B (100 μg/mL) in dPC-12 cells with 2 μM agg $A\beta_{1-40}$, was 55.6 ± 2.5% versus 69.3 ± 13%. By contrast, as shown in Table 2, the active fractions Nn-M-B-VII and Nn-M-B-IX were found to significantly inhibit $A\beta_{1-40}$ toxicity on dPC-12 cells at 50 μg/mL, and cell viability, expressed as percent of untreated control in the absence versus presence of the fractions in dPC-12 cells with 4 μM agg $A\beta_{1-40}$, was 51.6 ± 1.2% versus 71.2 ± 2.4% and 71.1 ± 3.6%, respectively. It was also observed that the active fractions were nontoxic at lower concentrations, but at high concentrations (>50 μg/mL) they were toxic to the dPC-12 cells (data not shown).

3.4. *DPPH radical scavenging activity*

DPPH is usually used as a substrate to evaluate the free radical scavenging activity of antioxidants [26]. The method

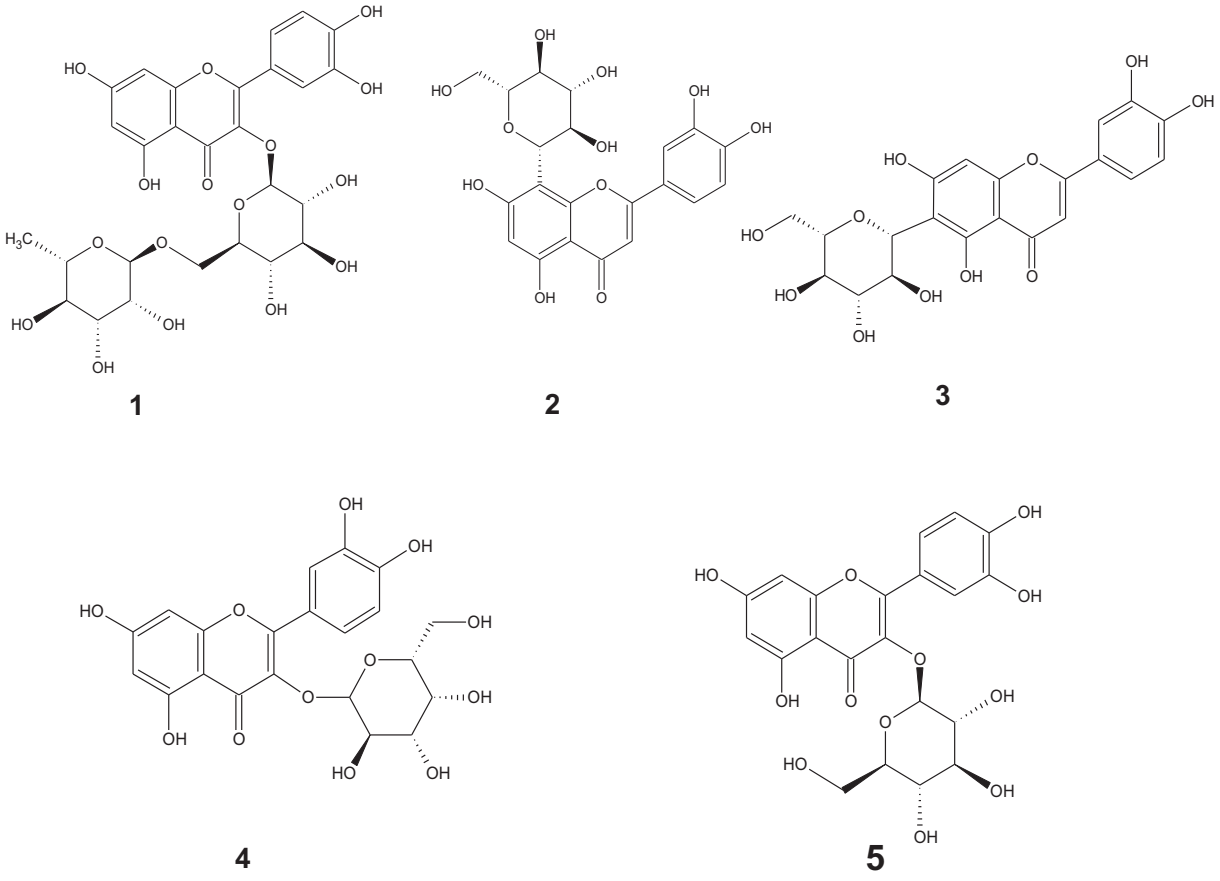

Figure 2 – Structure of bioactive compounds isolated from *Nelumbo nucifera* seed embryo: rutin (1), orientin (2), isoorientin (3), isoquercetrin (4), and hyperoside (5).

is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant owing to the formation of the nonradical form DPPH-H by the reaction. The Nn-M-B and the active fractions showed a concentration-dependent antiradical activity (data not shown) by reducing the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine derivative. Trolox, a known antioxidant, was used as a positive control. All of the fractions from Nn-M-B-VI to Nn-M-B-XI showed potent DPPH free radical scavenging activity, with EC_{50} values ranging from 3.0 μg/mL to 5.5 μg/mL (Table 3).

The generation of ROS and oxidative damage are believed to be involved in the pathogenesis of neurodegenerative disorders. Evidence from the literature indicates that oxidative stress occurs early in the progression of AD, long before the development of senile plaques. The interaction of abnormal mitochondria, redox transition metals, and oxidative stress response elements contributes to the generation of ROS in diseased neurons [27]. Recently, several studies have reported the potential of antioxidants in the inhibition of Aβ deposition using *in vitro* assays and transgenic mouse model studies of AD [28,29]. Several agents, including antioxidants and free radical scavengers, have been shown to be neuroprotective both *in vitro* and *in vivo* against Aβ-induced toxicity [30]. For example, curcumin, resveratrol, rosmarinic acid, nordihydroguaiaretic acid, ferulic acid, tannic acid, and some polyphenols have been shown to inhibit Aβ fibril formation as well as destabilize preformed Aβ fibrils *in vitro* [28]. Our previous investigation of *Angelica sinensis* yielded four anti-Alzheimer compounds with antioxidant activity of the following order: coniferyl ferulate > ferulic acid > Z-ligustilide > 11-angelyolsenkynolide F. Moreover, the positive control used in the present study, Sal B and EGb 761, have strong free radical scavenging effects and can successfully protect neuronal cells against Aβ-induced cell death [26].

The neuroprotective effects of the active fractions Nn-M-B-VII and Nn-M-B-IX rich in flavonoids could be revealed as either antioxidation or inhibition of Aβ-induced cell death.

Table 3 – DPPH radical scavenging activities of Sephadex LH-20 fractions Nn-M-B of *Nelumbo nucifera*.

Test materials	EC_{50}[a] (μg/mL ± SD)
Nn-M-B	13.5 ± 0.68
Nn-M-B-I	12.2 ± 0.44
Nn-M-B-II	39.8 ± 1.21
Nn-M-B-III	9.0 ± 0.31
Nn-M-B-IV	11.5 ± 0.36
Nn-M-B-V	11.7 ± 0.43
Nn-M-B-VI	5.5 ± 0.29
Nn-M-B-VII	5.3 ± 0.32
Nn-M-B-VIII	3.0 ± 0.20
Nn-M-B-IX	3.7 ± 0.24
Nn-M-B-X	3.9 ± 0.32
Nn-M-B-XI	3.8 ± 0.33
Trolox	2.5 ± 0.19

DPPH = 2,2-diphenyl-1-picrylhydrazyl hydrate; SD = standard deviation.

[a] EC_{50} value was determined to be the effective concentration at which DPPH radical was scavenged by 50%. Each value was expressed as the mean ± SD ($n = 3$).

Both active fractions had a strong activity in the prevention of Aβ-induced cell death as well as scavenging free radicals. Recently, naturally occurring flavonoids were marked as potential candidates for the prevention and treatment of AD [31]. EGb 761, a standardized extract of *G. biloba* with a well-defined mixture, contains 24% flavonoids and 6% terpenes. Studies showed that flavonoids present in *G. biloba* extract (10–100 μg/mL) were able to protect hippocampal cells against Aβ peptides or oxidative stress-induced toxicities [32]. In addition, EGb 761 exerts a combination of antioxidative, anti-amyloidogenic, and antiapoptotic effects [33]. Aβ toxicity can also be reduced by the inhibition of Aβ deposition via the antioxidative activity of the soy isoflavone glycitein (100 mg/mL) [34]. Apigenin ameliorates AD-associated learning and memory impairment via inhibiting Aβ deposition and decreasing insoluble Aβ concentrations, inhibiting oxidative stress, and improving the antioxidative enzyme activity of superoxide dismutase and glutathione peroxidase [35]. Green tea flavonoids can also protect against Aβ-induced cytotoxicity in primary rat cortical neurons [36].

The neuroprotective potential of flavonoids in AD is shown not only in Aβ-induced neuronal death models but also in oxidative stress-induced neuronal death. It is reported that oxidative stress was lowered by rutin in SH-SY5Y neuroblastoma cells along with a reduction in malondialdehyde and glutathione disulfide formation [37]. In various cell culture and animal models, flavonoids such as fisetin (25 mg/kg) [38], rutin (0.1–1 μM), and quercetin (0.1–1 μM) [39] inhibited the formation and aggregation of Aβ fibrils and destabilized preformed Aβ fibrils at their effective concentrations. These studies showed that flavonoids have the ability to reduce oxidative stress and Aβ-induced toxicity and to inhibit apoptosis, thus showing therapeutic potential for prevention of or treatment for AD. In the present work, the activity of Nn-M-B-VII could be attributable to the presence of rutin as the major compound, which also correlates with reported results. Another active fraction Nn-M-B-IX mainly contains the C-glycosylflavonoids, orientin and isoorientin. Recently, Lam et al [40] reviewed the medicinal properties of orientin, which include antioxidant, antiaging, antiviral, antibacterial, anti-inflammation, vasodilatation and cardioprotective, radiation protective, neuroprotective, antidepressant-like, antiadipogenesis, and antinociceptive effects. The neuroprotective effect of orientin has also been demonstrated in Aβ-induced mitochondrial dysfunction oxidative-stressed AD mice. Orientin was found to improve cognitive impairment of the AD mice and significantly reduced the levels of the oxidative stress and ROS [41]. The presence of orientin in Nn-M-B-IX could be the reason for the observed activity. However, investigation of isolated compounds separately as well as in combinations to find out the synergistic activity is to be conducted.

3.5. *HPLC analysis of* N. nucifera

The HPLC profiles of active fractions—Nn-M-B, Nn-M-B-VII, and Nn-M-B-IX fractions—are shown in Figure 3. Rutin (**1**) seems to be the most predominant compound in Nn-M-B-VII, and orientin (**2**), isoorientin (**3**), isoquercetrin (**4**), and hyperoside (**5**) appear as major compounds in Nn-M-B-IX.

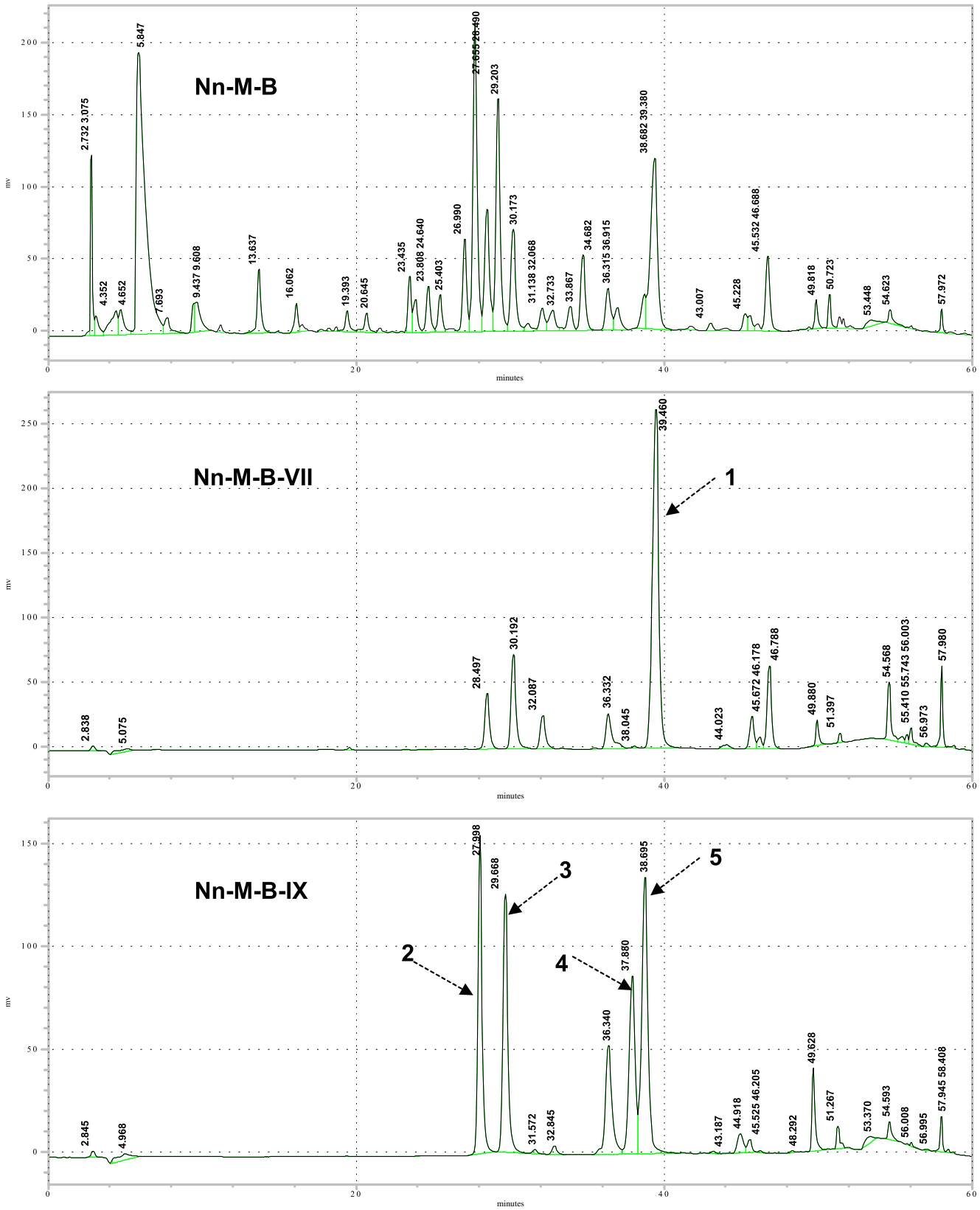

Figure 3 – Typical HPLC profile of the most active fractions of *Nelumbo nucifera* seed embryo. Rutin (1), orientin (2), isoorientin (3), isoquercetrin (4), and hyperoside (5) were detected at 254 nm. HPLC = high-performance liquid chromatography.

4. Conclusion

In the present work, the active fractions obtained from *N. nucifera* seed embryos, Nn-M-B-VII and Nn-M-B-IX, which mainly contain flavonoids, showed strong radical scavenging activity as well as reduced $A\beta_{1-40}$-induced toxicity in dPC-12 cells. The antioxidant properties of these fractions may be the reason for the observed mode of inhibition. Further research is warranted to elucidate the exact mechanism. However, the active fractions showed cytotoxicity at higher concentrations (above 50 μg/mL), so the optimal concentrations for the prevention and treatment of AD have to be considered. The investigations are going on in our laboratory to find out the activity of isolated flavonoid compounds in various *in vitro* assay models.

Conflicts of interest

The authors declare no conflicts of interest.

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