

NEURITOGENIC ACTIVITY OF THAI PLANT EXTRACTS

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ABSTRACT: Neuritogenic activity of three Thai medicinal plant extracts were evaluated on cultured P19-derived neurons. All of the extracts, butterfly pea (*Clitoria ternatea* L.) petal, lotus (*Nelumbo nucifera* Gaertn.) anther, and rambutan (*Nephelium lappaceum* L.) pericarp, at a very low concentration of 1 ng/mL enhanced the survival of cultured neurons (% viability more than 100%) by MTT reduction assay. The extracts were further evaluated for their neuritogenicity at 1 ng/mL. The phase-contrast micrographs revealed that all of the extracts possessed neurogenic activity by promoting the neurite outgrowth of the cultured neurons.

Keywords: neuritogenic activity, neurite outgrowth, *Clitoria ternatea* L., *Nelumbo nucifera* Gaertn., *Nephelium lappaceum* L., P19 derived-neurons

INTRODUCTION

Polyphenolic compounds, including flavonoids, anthocyanins, and tannins are remarkable biologically active compounds [1-3]. Their biological activities such as antioxidation, antimicrobial and have been recognized [1-4]. Moreover, polyphenolic compounds are able to prevented cardiovascular diseases and exhibited anticarcinogenic, anti-inflammation, and neuroprotective activities [5]. The P19 embryonal carcinoma cell is a pluripotent stem cell line which is differentiated into neurons by retinoic acid [6]. The P19-derived neurons are irreversibly postmitotic and exhibit many characteristics of mature CNS neurons containing particular neurotransmitters such as γ -aminobutyric acid [7] and acetylcholine [8]. The differentiated P19 neuroglial cultures have been used for neurotoxicity evaluation of cysteinylcatechols [9] and neuroprotective activity of geldanamycins [10]. In this study, we hypothesized that the phenolic compounds from plant extract would enhance neurons' survival and/or promote neurite outgrowth which can be used to prevent neurodegenerative disorders. According to those reasons we mentioned above, our interest is focusing on evaluating the neuritogenicity on P19-derived neurons of phenolic compounds containing plants, blue-color butterfly pea petal [11], rambutan pericarp [4], and red-color lotus anther [12].

MATERIALS AND METHODS

Plant materials

The fruits of *N. lappaceum* L., Family Sapindaceae were collected from Rayong Province, Thailand

during June-July, 2007. The petals of *Clitoria ternatea* L., Family Papilionaceae and the anthers from red *Nelumbo nucifera* Gaertn., Family Nymphaeaceae were collected from Nakhon nayok Province, Thailand during June-July, 2010.

Extraction

The pulverized dried peels of *N. lappaceum* L. were successively extracted by maceration with methanol. The filtrates were pooled and evaporated under reduced pressure at the temperature not exceeding 40°C to give the methanol extract.

The dried blue petals of *Clitoria ternatea* L. were successively extracted with ethanol. The filtrates were pooled and evaporated under reduced pressure at the temperature not exceeding 40°C to give the ethanol extract.

The dried anthers of red color *Nelumbo nucifera* Gaertn. were successively extracted with ethanol. The filtrates were pooled and evaporated under reduced pressure at the temperature not exceeding 40°C to give the ethanol extract.

The Biological effects assay on cultured P19-derived neuron

Cell culture

P19 cells were grown in alpha minimal essential medium (α -MEM) supplemented with 7.5% newborn calf serum (NCS), 2.5% fetal bovine serum (FBS), and 1% antibiotics-antimycotic solution in a 5% CO₂ humidified atmosphere, at 37 °C. Cells in monolayer cultures were maintained in exponential growth by subculturing every 2 days [6].

Differentiation of P19 cells into P19-derived neurons

Exponentially grown cultures were trypsinized and dissociated into single cells. P19 cells (2×10^6 cells/mL) were then suspended in 10 mL α -MEM

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supplemented with 5% FBS, 1% antibiotics-antimycotic solution and 0.5 μ M all *trans*-retinoic acid (RA) and seeded onto a 100-mm bacteriological culture dish. The cells formed large aggregates in suspension. After 4 days of RA treatment, aggregates were dissociated by 5-mL glass measuring pipette, re-plated on poly-L-lysine-pre-coated multi-well plates (multi-well plates were coated with 50 μ g/mL poly-L-lysine dissolved in PBS for overnight and sterilized under UV light for 30 min) at 7×10^4 cells/mL (150 μ L/well in 96-well plate and 1.5 mL/well in 6-well plate), in α -MEM supplemented with 10% FBS, and 1% antibiotics-antimycotic solution and incubated for 24 h. Cytosine-1- β -D-arabinoside or Ara-C (10 μ M) was added at day one after plating and the medium was changed every 2-3 days. The differentiated neuronal cells, P19 derived-neurons, cholinergic neurons, were used after day 14 of the differentiation process [6-7].

Chemicals

P19 cell line was obtained from American Type Culture Collection, USA. Alpha minimal essential medium (α -MEM), fetal bovine serum (FBS), and antibiotics-antimycotic solution were purchased from Gibco, USA. Newborn calf serum was purchased from JRSscientific, USA. All *trans*-retinoic acid, cytosine-1- β -D-arabinoside, 1:250 porcine trypsin, and poly-L-lysine (MW > 300,000) were obtained from Sigma, USA. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Invitrogen, USA. Dimethylsulfoxide (DMSO) analytical grade was purchased from Merck, Germany. 6-Well and 96-well plates were purchased from Corning, USA.

Viability assay

The assay was carried out on P19-derived neurons cultured in a 96-well plate. The assay was performed in triplicate. After 14 days of differentiation process, the α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution was removed and DMSO solutions of the extracts, diluted with the α -MEM supplemented with 10% FBS and 1% antibiotics-antimycotic solution in the presence of 10 μ M Ara-C were added to give the concentrations of 0.001, 0.01, 0.1, 1, and 10 μ g/mL. The concentration of DMSO was added to the cultures at 0.5%. The α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution was added into control wells. The cells were incubated for 18 h at 37 °C. Then 150 μ L of the medium was removed, and 100 μ L of MTT solution (1 mg/mL MTT in α -MEM) was

added. After incubated at 37 °C for 4 hours, the MTT solution was removed, and 200 μ L of DMSO was added. The OD value was determined on a microplate reader at 570 nm. The data were expressed as the mean $\pm$ SD ($n = 3$), with the medium as a control representing 100% cell viability. The concentration that enhanced survival of cultured neurons more than control will be further investigated for neuritogenicity.

Neuritogenicity assay

The assay was carried out with P19-derived neurons cultured in a 6-well plate. After 14 days of differentiation process, the α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution was removed and DMSO solution of the extracts, diluted with the α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution, were added to give the final concentration of the extract at concentration that enhanced survival of cultured neurons more than control. The concentration of DMSO was added to the cultures at 0.5%. The α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution was added into control wells. The cells were incubated for 18 h at 37 °C. The morphology under a phase-contrast microscope was observed. The appearance of P19-derived neurons was compared to the control and measured for the length and number of neurites. Average length and number of neurites of 30 neurons from the assay were measured. The data were expressed as the mean $\pm$ SD.

Statistical analysis

Average length and branching numbers of the neurites were statistical analyzed by Student's *t*-test to compare the statistical significance between the control and experimental groups. Differences were considered significant only when the *p*-value was less than 0.05.

RESULTS AND DISCUSSION

Viability assay of the cultured P19-derived neurons by MTT reduction method revealed that rambutan pericarp extract, butterfly pea petal extract, and lotus anther extract at 1-100 ng/mL enhanced viability of the cultured neurons (%viability more than 100%) as shown in Figure 1. Interestingly, the butterfly pea petal extract showed no neurotoxicity on cultured P19-derived neurons (IC₅₀ more than 10 μ g/mL) while rambutan pericarp extract and lotus anther extract showed stronger neurotoxicity. These extracts at concentration of 1 ng/mL were further evaluated for the neuritogenicity on cultured

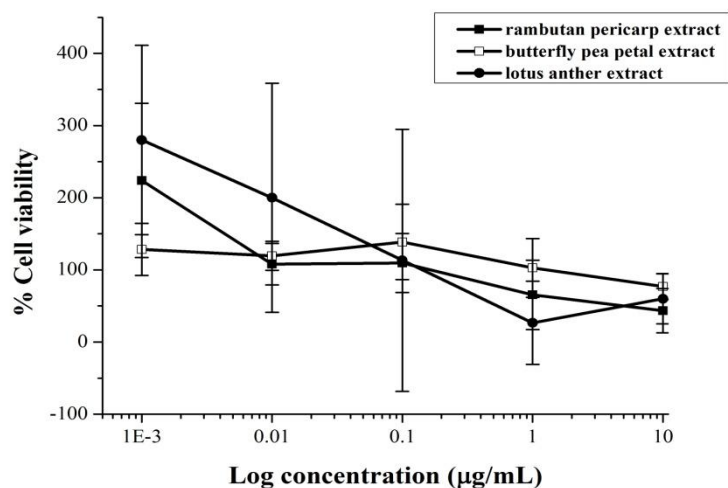


Figure 1 Viability of the P19-derived neurons after incubated with rambutan pericarp extract, butterfly pea petal extract, and lotus anther extract at various concentrations for 18 h. The data are expressed as mean $\pm$ SD ($n = 3$, each n run in triplicate).

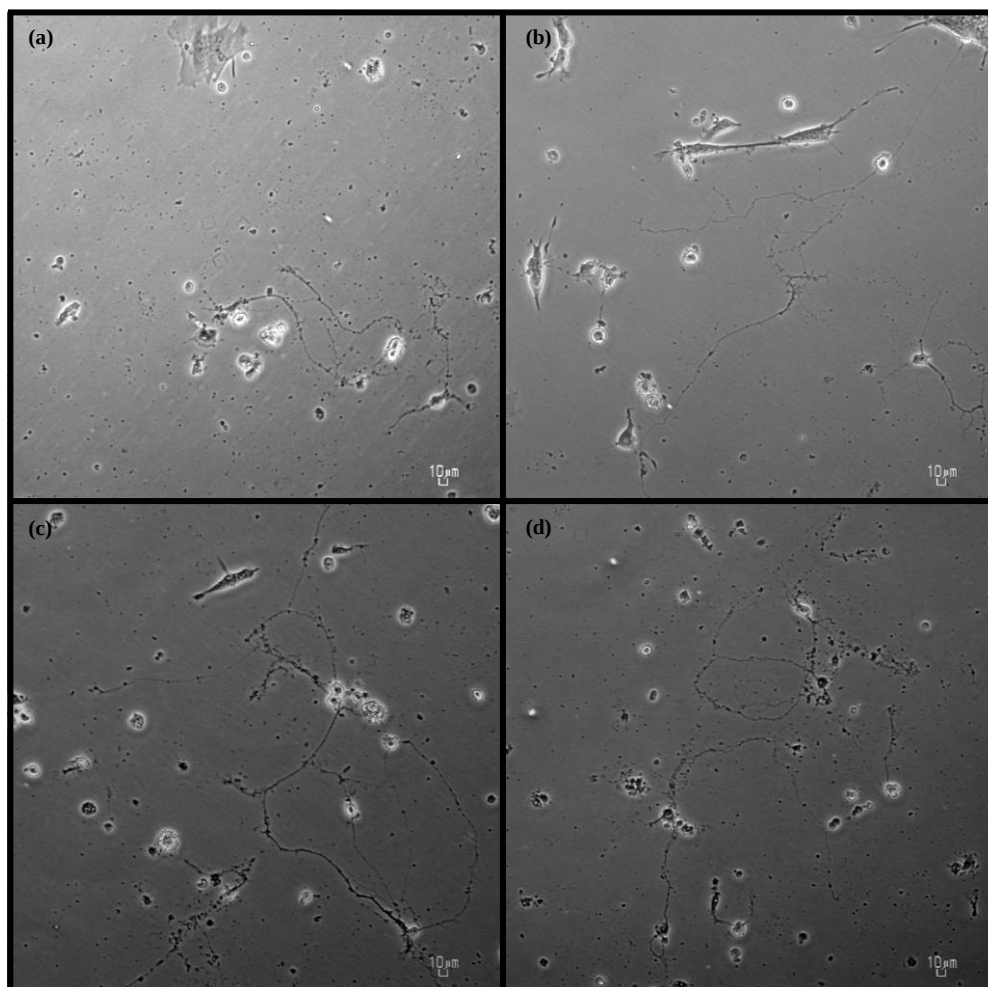


Figure 2 Neuritogenicity of rambutan pericarp extract, butterfly pea petal extract, and lotus anther extract at concentration of 1 ng/mL on P19-derived neurons culture. Phase-contrast micrographs of cultured P19-derived neurons incubated for 18 h and treated with: (a) no compounds, (b) rambutan pericarp extract, (c) butterfly pea petal extract, and (d) lotus anther extract. Bar = 10 μ m at 100 $\times$.

Table 1 Neuritogenicity of rambutan pericarp extract, butterfly pea petal extract, and lotus anther extract at concentration of 1 ng/mL on cultured P-19 derived neurons and incubated for 18 h determined under phase-contrast microscope at 100×. The data are expressed as mean $\pm$ SD (from 30 neurons); * $p < 0.05$ versus control.

Compound	Average length (μm) $\pm$ SD	Average number of neurites $\pm$ SD
Untreated	115.90 $\pm$ 61.18	2.50 $\pm$ 1.50
Rambutan pericarp extract	145.68 $\pm$ 74.89	4.63 $\pm$ 1.79*
Butterfly pea petal extract	161.80 $\pm$ 74.04*	3.97 $\pm$ 2.19*
Lotus anther extract	124.20 $\pm$ 45.38	4.07 $\pm$ 1.46*

P19-derived neurons. The phase-contrast micrographs showed that all of the extracts promoted the outgrowth of the neurites of the neurons as shown in Figure 2. All of the neurons treated with the extracts displayed significantly more branching numbers of the neurites than the control ($p < 0.05$) as shown in Table 1. Interestingly, only the neurons treated with butterfly pea petal extract also exhibited significantly longer neurites than the control ($p < 0.05$).

According to the metabolites found in these extracts were phenolic components, anthocyanins and flavonoids from blue-color butterfly pea petal [11], tannins from rambutan pericarp [4], and flavonoids from lotus anther [12], thus, it can be deduced that the neuritogenicity of these extracts was a result of those phenolic compounds contained in the extracts. It can be concluded that anthocyanins, flavonoids, and tannins were remarkable neuritogens from natural products.

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REFERENCES

- Hatano T, Kusuda M, Inada K, Ogawa TO, Shiota S, Tsuchiya T, et al. Effects of tannins and related polyphenols on methicillin-resistant *Staphylococcus aureus*. *Phytochemistry*. 2005 Sep; 66(17): 2047-55.
- Ramassamy C. Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. *Eur J Pharmacol*. 2006; 545: 51-64.
- Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K. Antibacterial action of several tannins against *Staphylococcus aureus*. *J Antimicrob Chemother*. 2001 Oct; 48(4): 487-91.
- Tadtong S, Athikomkulchai S, Worachanon P, Chalongsol P, Chaichanachaichan P, and Sareedenchai V. Antibacterial activities of rambutan peel extract. *J Health Res*. 2011; 25(1): 35-7.
- Mandel S, Youdim MBH. Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. *Free Rad Bio Med*. 2004; 37(3): 304-17.
- Jones-Villeneuve EMV, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol*. 1982; 94: 253-62.
- MacPherson PA, McBurney MW. P19 embryonal carcinoma cells: a source of cultured neurons amenable to genetic manipulation. *Methods*. 1995; 7: 238-52.
- Jones-Villeneuve EMV, Rudnicki MA, Harris JF, McBurney MW. Retinoic acid-induced neuronal differentiation of embryonal carcinoma cells. *Mol Cell Biol*. 1983; 3 (12): 2271-9.
- Montine TJ, Picklo MJ, Amarnath V, Whetsell O, Graham DG. Neurotoxicity of endogenous cysteinylcatechols. *Exp Neuro*. 1997; 148: 26-33.
- Tadtong S, Meksuriyen D, Tanasupawat S, Isobe M, Suwanborirux K. Geldanamycin derivatives and neuroprotective effect on cultured P19-derived neurons. *Bioorg Med Chem Lett*. 2007; 17: 2939-43.
- Kazuma K, Noda N, Suzuki M. Flavonoid composition related to petal color in different lines of *Clitoria ternatea*. *Phytochemistry*. 2003; 64: 1133-9.
- Jung HA, Kim JE, Chung HY, Choi JS. Antioxidant principles of *Nelumbo nucifera* stamens. *Arch Pharm Res*. 2003 Apr; 26(4): 279-85.