

RESEARCH ARTICLE

Neuritogenic and antioxidant activities of nordentatin from *Clausena harmandiana*

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Abstract

Nordentatin, a member of coumarins from natural sources, was identified from root bark of *Clausena harmandiana* and previously tested by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS) indicating its potent antioxidant and inhibition of lipid peroxidation. This lead to further isolate of its derivatives, dentatin and xanthoxyletin, and evaluate their activities on neuritogenic and neurotoxic using cultured P19 neurons and antioxidant effects. Nordentatin showed no neurotoxicity on the cultured cells ($IC_{50} > 10 \mu M$) while dentatin and xanthoxyletin expressed neurotoxicity at 1 nM (viability $62.28 \pm 12.11\%$ and $43.02 \pm 10.31\%$, respectively). Phase-contrast micrographs showed that nordentatin promoted neurites outgrowth of the neurons. The neurons treated with nordentatin displayed significantly more branching numbers of the neurites than the control ($p < 0.05$). It can be concluded that nordentatin is a remarkable neuritogen from natural product.

Introduction

Various pathological conditions including cancer, arthritis, arteriosclerosis, heart disease, inflammation, brain dysfunction and acceleration of the ageing process appear to have etiological relation to the active oxygen-induced and free radical-mediated oxidation of biomolecules. Antioxidant agents are substances that can prevent or delay oxidative damage of lipids, proteins and nucleic acids by reactive oxygen species, for example hydroxyl, peroxy, hypochlorous radicals, etc. The most abundant antioxidants in

plants are polyphenol compounds. These polyphenols, most of which are flavonoids, are present mainly in ester and glycoside form. Thus, interest in natural antioxidant has increased considerably, and various plants are proposed to be antioxidant activity according to isolated bioactive compounds. *Clausena harmandiana* (Rutaceae), available mainly in the Northeastern part of Thailand, was used in folk medicine for treatments of stomach ache and fever. Its root and root bark contain coumarins and carba-

zole alkaloids (Wangboonskul *et al*, 1984; Chaichantipyuth *et al*, 1988). Coumarins and carbazoles isolated from *C. harmandiana* and other species of this genus have been reported to exhibit diverse biological activities, such as antiplasmodial activity (Sunthikawinsakul *et al*, 2003), antimicrobial and antifungal activities (Yenjai *et al*, 2000). The screening data for antioxidant capacity of the isolated coumarins and carbazole alkaloids from *C. harmandiana* were done by our group. The result revealed that one of coumarin compounds exhibited significant antioxidant activity by DPPH assay. In addition, there are very few reports on phyto-chemistry of *C. Harmandiana* (Tian-Shung *et al*, 1986; Sunthikawinsakul *et al*, 2003; Ito *et al*, 2005). Furthermore, a recent report revealed that pyrano-coumarin derivative showed anti-HBV and cytotoxic activity (Chung-Ren *et al*, 2009). Due to above evidences together with our previous research, it was decided to isolate bioactive compounds from the root bark of *C. harmandiana* and screen for their effects on cultured P19 neurons.

The P19 embryonal carcinoma cell is a pluripotent stem cell line which is differentiated into neurons by retinoic acid (Jones-Villeneuve *et al*, 1982). The P19-derived neurons are irreversibly post-mitotic and exhibit many characteristics of mature CNS neurons containing particular neurotransmitters such as γ -amino-butyric acid (MacPherson and McBurney, 1995) and acetylcholine (Jones-Villeneuve *et al*, 1983). The differentiated P19 neuroglial cultures have been used for neurotoxicity evaluation of cysteinyl-catechols (Montine *et al*, 1997), neuro-protective activity of geldanamycins (Tadtong *et al*, 2007), and blue-color butterfly pea petal, rambutan pericarp, and red-color lotus anther (Tadtong *et al*, 2012). Our interest is focusing on evaluating the neuritogenicity on P19-derived neurons of natural products isolated from *C. harmandiana* as nordentatin, dentatin, and xanthoxyletin.

Materials and Methods

Plant material: The roots of *C. harmandiana* were collected in April 2010 from Kalasin Province. The plant was identified voucher specimen (KKU21145) and deposited at the herbarium of Khon Kaen University.

Chemicals: P19 cell line ATCC CRL-1857 was from American Type Culture Collection, U.S.A, and alpha minimal essential medium (α -MEM), fetal bovine serum (FBS), newborn calf serum (NCS), and antibiotics-antimycotic solution from Gibco, U.S.A. All *trans*-retinoic acid, cytosine-1- β -D-arabinoside, porcine trypsin, poly-L-lysine (MW > 3×10^5), XTT (2,3-bis(2-methoxy-4-nitro-5-sulphonyl)-2H-tetrazolium-5-carboxani-lide sodium) and phenazine metho-sulfate (PMS) were obtained from Sigma, U.S.A. Dimethylsulfoxide (DMSO) was purchased from Merck, Germany. 6-well and 96-well plates were purchased from Corning, U.S.A.

Extraction and Isolation: The activity-guided fractionation of methylene chloride soluble fraction (50 g) has led to the identification of known cabazole alkaloids and coumarins as heptaphylline [1], 7-methoxy-heptaphylline [2], 2-hydroxy-3-formyl-7-methoxy-carbazole [3], mukonal [4], xanthoxyletin [5], dentatin [6] and nordentatin [7], as shown in Figure 1. All isolated compounds were structurally elucidated by comparison with the authentic samples, which were identical in all respects.

DPPH radical scavenging assay: All these compounds were studied for the ability to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by comparison with a well-known synthetic antioxidant, α -tocopherol (vitamin E). Briefly, a portion of sample solution was mixed with the same volume of 6×10^{-5} M DPPH in methanol and allowed to stand in dark condition at room temperature for 30 min. The absorbance was then measured at 550 nm.

Decreasing of DPPH solution absorbance indicated an increase of DPPH radical scavenging activity. This activity was given as % DPPH radical scavenging that was calculated as follows:

$$\% \text{ scavenging activity} = \frac{1 - A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100$$

where A represents absorbance at 550 nm. The DPPH solution without sample solution was used as the control. All tests were run in triplicate and averaged.

TBAR assay: The lipid peroxidation activity was evaluated using the TBA test. The TBA reaction is based on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malonaldehyde (MDA). Absorbance at 532 nm was determined.

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 (Jones-Villeneuve *et al*, 1982).

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 supplemented with 5% FBS, 1% antibiotics-antimycotic solution and 0.5 μ M of 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-anti-mycotic solution and incubated for 24 h. Cytosine-1- β -D-arabino-side or Ara-C (10 μ M) was added at day 1 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 (Jones-Villeneuve *et al*, 1982, Mac Pherson and McBurney 1995).

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 nordentatin, 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 M. 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 50 μ l of XTT solution (1 mg/ml XTT in α -MEM plus 25 μ M PMS) was added. After incubated at 37°C for 4 h, 100 μ l of PBS was added. The OD value was determined on a microplate reader at 450 nm. The data were expressed as the mean \pm SEM (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 neurotoxicity. Other derivatives were investigated for viability of the neurons at the same concentration that promoted viability of the neurons by nordentatin.

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 SEM.

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.

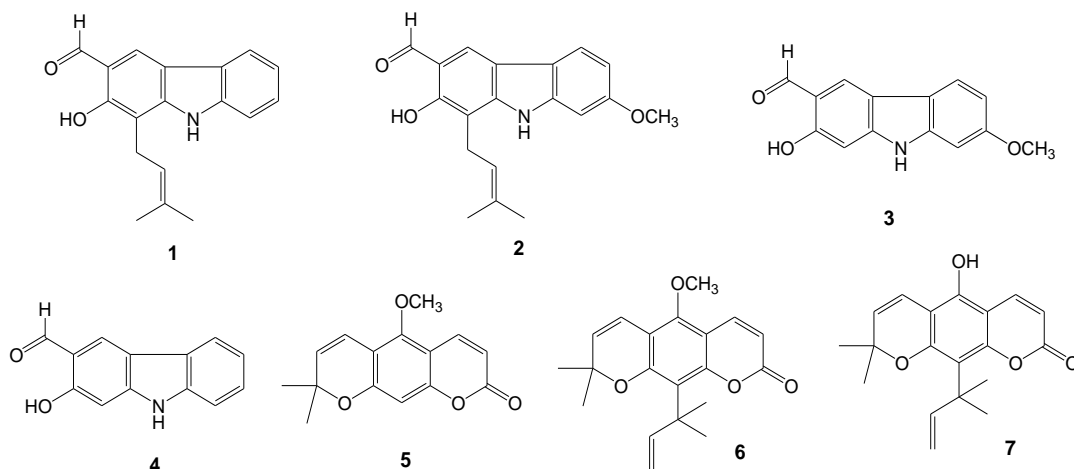


Figure 1 Isolated compounds from *Clausena harmandiana*

Table 1 Antioxidant activities of the isolated compounds from *C. harmandiana*

Compound	DPPH assay IC ₅₀ (μM)	TBAR assay IC ₅₀ (μM)
1	>1000	5.62
2	810.74	1.98
3	>1000	>500
4	>1000	>500
5	>1000	166.01
6	>1000	>500
7	84.32	1.69
Vitamin E	17.10	75.94

Table 2 Neuritogenicity of nordentatin at concentration of 1 nM

Compounds	Average length (μm)±SEM	Average number of neuritis ± SEM
Untreated	81.80 ± 11.55	2.40 ± 0.19
0.5% DMSO	70.02 ± 5.50	2.43 ± 0.23
1 nM nordentatin	71.10 ± 7.51	3.53 ± 0.30* [*]

Neuritogenicity of nordentatin at concentration of 1 nM on cultured P-19 derived neurons and incubated for 18 h determined under phase-contrast microscope at 100x. The data are expressed as mean±SEM (from 30 neurons); **p* < 0.05 versus control; ^{*}*p* < 0.05 versus 0.5%DMSO

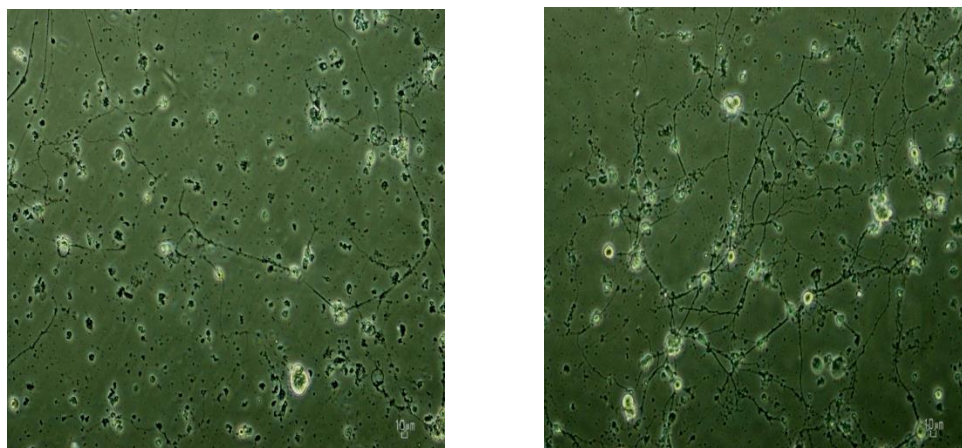


Figure 2 Neuritogenicity of nordentatin at concentration of 1 nM on P19-derived neurons culture. Phase-contrast micrographs of cultured P19-derived neurons incubated for 18 h and treated with: no compound (left) and 1 nM nordentatin (right). Bar = 10 μm at 100x.

Results and Discussion

Preliminary study was done by refluxing the root bark, leaves and stem of *C. harmandiana* with hexane, methylene chloride and methanol for 24 h, respectively. The resulting fractions, nine in total, were tested with DPPH radical scavenging method. The DPPH radical scavenging assay revealed that the methylene chloride soluble fraction

of root bark and methanol soluble fraction of leaves possessed high antioxidant activity with IC₅₀ = 88.3 and 53.8 mg/ml, respectively. Although the leaf extract possesses better antioxidation, however, we chose the root bark for further isolation of the bioactive compounds by underwent activity-guided fractionation due to no chlorophyll. The root bark of *C. harmandiana* was

macerated with methanol and further partitioned with methylene chloride to give 50 g of crude extract. The methylene chloride soluble fraction has led to the isolation of 4 known carbazole alkaloids, heptaphylline **1** (455 mg), 7-methoxyhepta-phylline **2** (502 mg), 2-hydroxy-3-formyl-7-methoxycarbazole **3** (172 mg), and mukonal **4** (45 mg), and coumarins **3**, xanthoxyletin **5** (32 mg), dentatin **6** (1.35 g), and nordentatin **7** (266 mg), as shown in Figure 1.

Antioxidant activity revealed that, in the DPPH assay, compounds **1**, **3**, **4**, **5** and **6** showed less activity with $IC_{50} > 1$ mM, while compounds **2** and **7** showed antioxidant property with $IC_{50} = 810.7 \mu\text{M}$ and $84.3 \mu\text{M}$, respectively (vitamin E; $IC_{50} = 17.9 \mu\text{M}$). In the TBAR assay, all compounds except compounds **3**, **4**, **5** and **6** showed better anti-lipid peroxidation activity than standard vitamin E ($IC_{50} = 75.9 \mu\text{M}$) by which IC_{50} of compounds **1**, **2** and **7** were 5.6, 1.9 and $1.7 \mu\text{M}$, respectively, as shown in Table 1.

The result reveals that the antioxidative components from nature were compounds **1**, **2** and **7** which displayed potent in the primary screening with DPPH and TBAR assays. In addition, compound **7** was the most potent anti-lipid peroxidation in TBAR assay and the most potent antioxidant in DPPH assay. These observations suggested that coumarin system is the more potent biological active chemical structure than that of carbazole alkaloid. Concerning the structure features of the active coumarins, our data indicated that the minimal structure requirements for antioxidant activity is a free 5-OH group on the coumarin core structure. Therefore, we preferred to focus on evaluation of the neuritogenic activity of coumarin core structure, especially nordentatin, **7**, and its

derivatives (compounds **5** and **6**) on the cholinergic cultured P19-derived neuron.

Viability assay of the cultured P19-derived neurons by XTT reduction method revealed that 1 nM nordentatin enhanced viability of the cultured neurons (%viability more than 100%). The viability of cultured P19-derived neurons treated with 1 nM nordentatin was $145.1 \pm 20.6\%$. Interestingly, nordentatin showed no neurotoxicity on cultured P19-derived neurons (IC_{50} more than $10 \mu\text{M}$), while dentatin and xanthoxyletin expressed neurotoxicity at 1 nM (% viability 62.3 ± 12.1 and 43.0 ± 10.3 , respectively). Therefore, only nordentatin was further investigated for its neuritogenic activity at 1 nM concentration on cultured P19-derived neurons. The phase-contrast micrographs showed that nordentatin promoted the neurite outgrowth of the neurons as shown in Figure 2. The neurons treated with the compound displayed significantly more branching numbers of the neurites than the control ($p < 0.05$) as shown in Table 2.

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