

Effects of (+)-Eudesmin from the Stem Bark of *Magnolia kobus* DC. var. *borealis* Sarg. on Neurite Outgrowth in PC12 Cells

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(+)-Eudesmin [4,8-bis(3,4-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane] was isolated from the stem bark of *Magnolia kobus* DC. var. *borealis* Sarg. and found to have neuritogenic activity. 50 μ M (+)-eudesmin induced neurite outgrowth and enhanced nerve growth factor (NGF)-mediated neurite outgrowth from PC12 cells. At this concentration, (+)-eudesmin also enhanced NGF-induced neurite-bearing activity and this activity was partially blocked by various protein kinase inhibitors. These included PD98059, a mitogen-activated protein kinase (MAPK) kinase inhibitor. GF109203X, a protein kinase C (PKC) inhibitor and H89, a protein kinase A (PKA) inhibitor. These results suggest that (+)-eudesmin can induce neurite outgrowth from PC12 cells by stimulating up-stream MAPK, PKC and PKA pathways.

Key words: (+)-Eudesmin, *Magnolia kobus*, Neurite outgrowth, PC12 cells

INTRODUCTION

Neurotrophic factors such as nerve growth factors (NGF) stimulate neurite outgrowth in neuronal cells and play an important role in survival and maintenance in the central nervous system (Patrick *et al.*, 1996). It was therefore, proposed that NGF and NGF-like agents could be used therapeutically in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Connor and Dragunow, 1998).

The chemical components of *Magnolia kobus* DC. var. *borealis* Sarg. (*M. kobus*, Magnoliaceae) have antioxidative and antibacterial activities (Kim, 1999). The major components of *M. kobus* are lignans (eudesmin, epieudesmin, yangambin, kobusine, sesamin and phillygenin), sesquiterpenes (kobusimin A and 9-oxonerolidol) and essential oils (d-limonene, p-cymene, l-camphor and d-nerolidol) (Kim, 1999; Fujita *et al.*, 1975; Iida *et al.*, 1982). Eudesmin and kobusine have been shown to have antioxidant

activity (Kim, 1999).

Rat adrenal pheochromocytoma PC12 cell lines differentiated and showed de novo extension of neurites in the presence of NGF. Therefore, these cells have been used as a model system for sympathetic neuron-like cells (Gysbers and Rathbone, 1992).

As a part of our ongoing search for neurotrophic agents from natural resources, we found that a methanol (MeOH) extract from the stem bark of *M. kobus* induced neurite outgrowth in PC12 cells. In this study, therefore, the MeOH extract was subjected to bioactivity-guided fractionations to isolate the bioactive components. The final purified bioactive substance, which induced neurite outgrowth of PC12 cells, was identified as a lignan derivative, 4,8-bis(3,4-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane [(+)-eudesmin] (Fig. 1), and its neuritogenic mechanism was elucidated in part.

MATERIALS AND METHODS

Materials

The stem barks of *M. kobus* were collected from Jinju, Kyungnam, Korea and a voucher specimen was deposited at the herbarium of the Division of Wood Chemistry and

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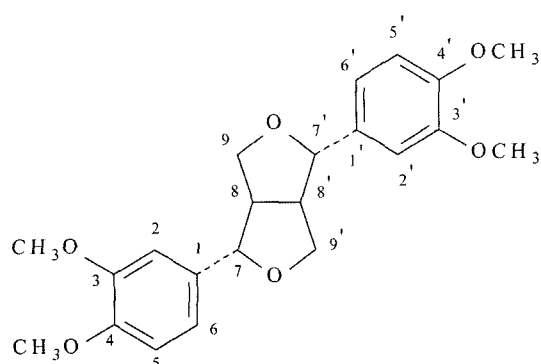


Fig. 1. Chemical structure of (+)-eudesmin

Microbiology, Korea Forest Research Institute (Seoul, Korea). RPMI 1640, donor horse serum (HS) and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, U.S.A.). 2.5S NGF, poly-L-lysine, antibiotics, PD98059, GF109203X and H89 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Separation and identification of (+)-eudesmin

The air-dried stem barks of *M. kobus* (5 kg) were extracted with EtOH, and then the dried EtOH extract (510 g) was partitioned in turn with CH_2Cl_2 , Et_2O , EtOAc and BuOH. The Et_2O extract (85 g) was chromatographed over Sephadex LH-20 (Pharmacia, 60×4.5 cm; MeOH-EtOH=3:7, v/v) and silica gel column (40-100 mesh, 27.5 × 7.0 cm, Fischer Scientific; benzene-EtOAc=8:1, v/v) to obtain eight fractions (MKBE-2-1 - MKBE-2-8). The MKBE-2-5 fraction (3.3 g) was further chromatographed over Sephadex LH-20 (20-100 μm , 50×4.5 cm, MeOH) to obtain four fractions (MKBE-2-5-1 - MKBE-2-5-4). (+)-Eudesmin (120 mg) was isolated from the MKBE-2-5-2 fraction. Optical rotation was obtained using a JASCO p-1020 polarimeter (JASCO, Ltd., Tokyo, Japan). ^1H - and ^{13}C -NMR, ^1H - ^1H correlation spectroscopy (COSY), NOE spectroscopy (NOESY) and heteronuclear multiple quantum coherence (HMQC) NMR spectra were recorded on a Varian UI 500 spectrophotometer (Varian, Inc., Palo Alto, U.S.A.). EI-MS was measured on a JEOL JMS-600W mass spectrophotometer (JEOL, Ltd., Yamagata, Japan).

Cell culture and assay for neurite outgrowth

PC12 cells were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated HS and 5% heat-inactivated FBS (Greene and Tischler, 1976). The morphology of PC12 cells was observed according to the method described previously (Sano and Kitajima 1998). PC12 cells were dissociated by incubation with 1 mM EGTA in phosphate-buffered saline for 1 h and were seeded in 24-well culture plates (ca. 2×10^4 cells/well) coated with

poly-L-lysine. After 24 h, the medium was changed to an appropriate test medium for neurite-bearing containing 2% HS and 1% FBS. Neurite outgrowth and neurite-bearing from PC12 cells were monitored under a phase-contrast microscope. Processes with lengths equivalent to one or more diameters of a cell body were scored as neuritis. A minimum of 100 cells were examined for each data point. 2 ng/mL 2.5S NGF was used for neurite-bearing and 30 ng/mL 2.5S NGF for neurite outgrowth. The data was analyzed by one-way ANOVA followed by Tukey's test for significance.

RESULTS AND DISCUSSION

A bioactive component, which induced neurite outgrowth in PC12 cells, was isolated from *M. kobus* and identified as a 4,8-bis(3,4-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane [(+)-eudesmin] (Fig. 1); mp. 107-108°C. $[\alpha]_D^{22} +50.94$ ($c = 0.59$, acetone). EI-MS m/z : 386 (M^+), 249, 219, 189, 177, 165. ^1H -NMR (500 MHz, acetone- d_6): δ 3.10-3.14 (2H, *m*, H-8, H-8'), 3.82 (6H, *s*, 4- and 4'-OCH₃), 3.84 (6H, *s*, 3- and 3'-OCH₃), 3.85 (2H, *dd*, $J = 4.0, 11.0$ Hz, H-9b, H-9b'), 4.25 (2H, *dd*, $J = 5.5, 8.0$ Hz, H-9a, H-9a'), 4.73 (2H, *d*, $J = 4.5$ Hz, H-7, H-7'), 6.92 (2H, *d*, $J = 8.5$ Hz, H-5, H-5'), 6.94 (2H, *dd*, $J = 2.0, 6.0$ Hz, H-6, H-6'), 7.02 (2H, *d*, $J = 1.0$ Hz, H-2, H-2'). ^{13}C -NMR (125 MHz, acetone- d_6): δ 54.63 (C-8, 8'), 55.56 (C-OCH₃×4), 71.67 (C-9, 9'), 85.85 (C-7, 7'), 110.46 (C-2, 2'), 112.11 (C-5, 5'), 118.46 (C-6, 6'), 134.81 (C-1, 1'), 149.21 (C-4, 4'), 149.85 (C-3, 3'). ^1H - ^1H COSY correlations: H-7/H-7'→H-8/H-8', H-9a/H-9a'→H-9b/H-9b'/H-8/H-8'. HMBC correlations: H-7→C-2/C-6/C-8/C-9, H-9→C-7/C-8. ^1H -NMR and ^{13}C -NMR spectra, and physical constants of (+)-eudesmin were consistent with previous reports (Iida *et al.*, 1982).

When cultured in the absence of NGF for 24-48 h, PC12 cells had no neurites. The addition of NGF (30-40 ng/ml) induced neurite outgrowth from over 95% of PC12 cells (data not shown) as was seen by other groups (Middlemiss *et al.*, 1995; Hayakawa *et al.*, 1999). 20-100 μM (+)-eudesmin alone induced neurite-bearing activity in a concentration-dependent manner in PC12 cells incubated for 24-36 h in a normal medium including 10% HS and 5% FBS (Fig. 2).

In addition, 20-100 μM (+)-eudesmin induced neurite outgrowth from PC12 cells incubated in normal medium for 96 h. 50 μM (+)-eudesmin significantly induced neurite elongation (Fig. 3B). This concentration of (+)-eudesmin in association with NGF (2 ng/mL) also enhanced neurite outgrowth compared with (+)-eudesmin treatment alone (Fig. 3D). PC12 cells exposed to (+)-eudesmin (50 μM) and/or (+)-eudesmin associated with NGF (2 ng/mL) also formed long neurites, which extended to the neighboring cells over distances of 50 μm . However, when PC12 cells

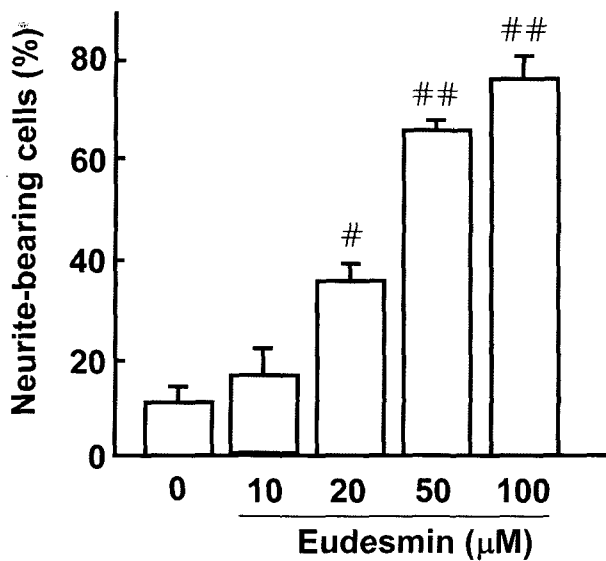


Fig. 2. Effects of (+)-eudesmin on neurite-bearing activity in PC12 cells. The number of neurite-bearing cells was expressed as a percentage of maximum number in response to NGF (30 ng/mL, 100%) in the absence of (+)-eudesmin. The results represent mean \pm SEM of four experiments. Statistically significant difference is indicated in the figure: # $p < 0.01$, ## $p < 0.001$, compared to the untreated cells (ANOVA followed by Tukey's test).

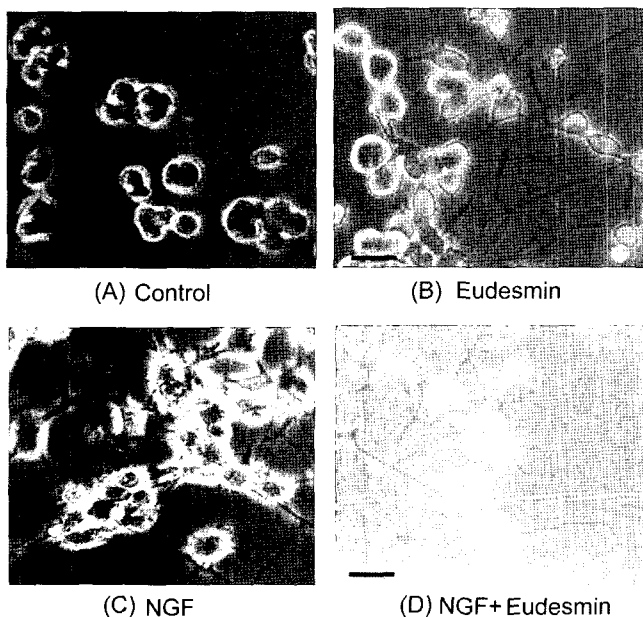


Fig. 3. Effects of (+)-eudesmin and nerve growth factor (NGF) on the morphology of PC12 cells. PC12 cells were treated for 96 h without (A, C) or with (B, D) (+)-eudesmin (50 μ M) in the absence (A, B) or presence (C, D) of NGF (2 ng/mL), respectively. Scale bar 50 μ m.

treated with (+)-eudesmin were cultured in serum (HS and FBS)-free media, the cells started the apoptotic process earlier compared with the untreated PC12 cells. These results suggested that (+)-eudesmin was neuritogenic in

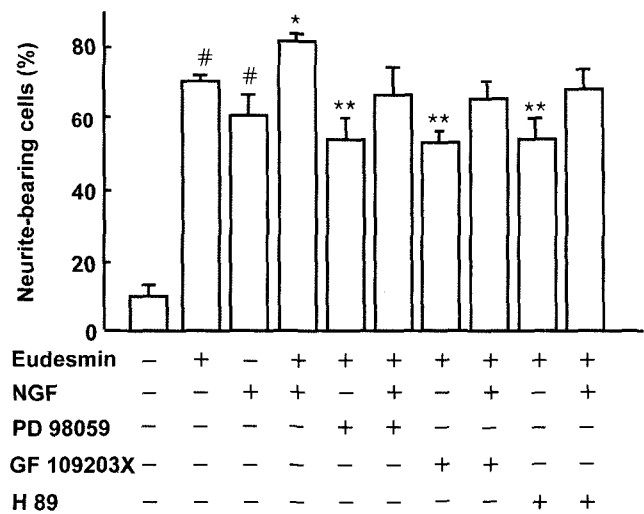


Fig. 4. Effects of PD98059, GF109203X and H89 on (+)-eudesmin-induced neurite-bearing activity in PC12 cells. The number of neurite-bearing cells was expressed as a percentage of maximum number in response to NGF (30 ng/mL, 100%) in the absence of (+)-eudesmin. The concentrations of (+)-eudesmin, NGF, PD98059, GF109203X and H89 were 50 μ M, 2 ng/mL, 30 μ M, 100 nM and 100 nM, respectively. Each point represents mean \pm SEM of four experiments. Statistically significant difference is indicated in the figure: # $p < 0.001$, compared with the untreated cells; * $p < 0.05$, ** $p < 0.01$, compared with (+)-eudesmin-treated cells (ANOVA followed by Tukey's test).

PC12 cells.

Various neurotogenic agents, AIT-082 (100 nM), nerfilin I (0.5 μ g/mL), aroclor 1254 (25 μ g/mL), SR57746A (0.5 μ M) and nordosinone (100 μ M) enhance NGF-mediated neurite outgrowth in PC12 cells (Middlemiss *et al.*, 1995; Hirao *et al.*, 1995; Angus and Contreras, 1995; Pradines *et al.*, 1995; Li *et al.*, 1999). (+)-Eudesmin showed a similar neurotogenic activity.

NGF induces cell differentiation and neurite outgrowth by the formation of large neurites associated with NGF TrkA receptors. This effect of NGF can be mimicked by direct activation with either PKA (Mann *et al.*, 1989; Sanchez *et al.*, 2004) or PKC (Doherty *et al.*, 1988; Leprince *et al.*, 1996; Tsuji *et al.*, 2001). Neurite outgrowth of PC12 cells was also induced by activation of extracellular signal-regulated kinase (ERK) and p38 MAPK (Tsuji *et al.*, 2001). In addition, the activation of TrkA receptor kinase induces phosphorylation of SHC, phospholipase C and phosphatidylinositol 3-kinase (Obermeier *et al.*, 1994; Ashcroft *et al.*, 1999), followed by activation of RAS-ERK cascade (Wood *et al.*, 1992). The activation of RAS-ERK cascade is also sufficient for NGF-induced neuronal differentiation of PC12 cells (Pang *et al.*, 1995; Tsuji *et al.*, 2001). Therefore, induction of neurite outgrowth in PC12 cells has been found to be mediated by the MAPK, PKA and PKC pathways.

50 μ M (+)-eudesmin alone showed a significant neurite-bearing activity in the appropriate test medium for 24-36 h (Fig. 4). However, (+)-eudesmin-induced neurite-bearing activity and its enhancement of NGF-neurite-bearing activity were partially blocked by a representative MAPK/ERK kinase inhibitor PD98059, by a PKC inhibitor GF109203X and by a PKA inhibitor H89 (Fig. 4). This data suggested that (+)-eudesmin-induced neurite outgrowth was partially mediated by amplification of the up-stream steps of MAPK, PKC and PKA in the intracellular signaling pathway. In addition, (+)-eudesmin at concentrations of up to 150 μ M did not exhibit any cell cytotoxicity to PC12 cells according to MTT assays. 20-100 μ M (+)-Eudesmin also had no stimulatory effects on dopamine biosynthesis in PC12 cells (data not shown).

In conclusion, (+)-eudesmin induced neurite outgrowth and enhanced neurite outgrowth in association with NGF in PC12 cells. The *in vivo* applications of (+)-eudesmin and its mechanism need to be studied further.

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