Spirafolide from Bay Leaf (*Laurus nobilis*) Prevents Dopamine-induced Apoptosis by Decreasing Reactive Oxygen Species Production in Human Neuroblastoma SH-SY5Y Cells

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Reactive oxygen species (ROS) are important mediators in many neurodegenerative diseases including Alzheimer’s disease and Parkinson’s disease. This study tested the neuroprotective effects of spirafolide, a compound purified from the leaves of *Laurus nobilis* L. (Lauraceae), against dopamine (DA)-induced apoptosis in human neuroblastoma SH-SY5Y cells. Following a 24-h exposure of cells to DA (final conc., 0.6 mM), we observed a marked increase in apoptosis, increased generation of ROS and decreased cell viability. Pretreatment of the cells for 24 h with spirafolide (0.4, 2, and 10 µM) before exposure to DA notably increased cell survival (*p* < 0.01) and lowered intracellular ROS levels (*p* < 0.01). These results indicate that spirafolide has neuroprotective effects against DA toxicity. These effects may contribute to the treatment of neurodegenerative diseases.

**Key words:** *Laurus nobilis*, Spirafolide, Reactive oxygen species, Apoptosis, SH-SY5Y cells

INTRODUCTION

The neurotoxic effects of exogenous dopamine have been studied using primary cultures and several cell lines (Asanuma et al., 2003). Increased oxidative deamination of dopamine enhances the production of hydrogen peroxide (H₂O₂), which can be converted into cytotoxic hydroxyl free radicals (Cohen, 1990). In addition, studies using neurotoxic compounds such as 6-hydroxydopamine have demonstrated that neuronal cell death is regulated by reactive oxygen species (ROS) generation, mitochondrial inhibition, and oxidative stress-related signals (Chalovich et al., 2006; Hanrott et al., 2006). Dopaminergic neurons are now believed to be involved in oxidative stress by generating intracellular ROS, which results in the activation of apoptosis. The overproduction of ROS induces severe damage to cellular function, is involved in apoptotic signals, and may contribute to the pathophysiology of diseases such as Parkinson’s disease and Alzheimer’s disease (Di Monte et al., 1986). Currently, downregulation of intracellular ROS production of apoptotic cascades are new strategies for preventing or treating neurodegeneration, and a great deal of attention has been focused on natural products that modulate apoptosis and ROS production.

*Laurus nobilis* (bay leaf, Lauraceae) is a folk medicine in Europe (Hisashi et al., 2000) where it is widely used as an antiseptic, insecticide, and stomachic, and to treat rheumatism. Spirafolide of *L. nobilis* inhibits ethanol elevation in rat blood (Yoshikawa et al., 2000) and nitric oxide (NO) production (De Marino et al., 2005) and has antimycobacterial properties (Luna-Herrera et al., 2007). However, it has not been determined whether *L. nobilis* has any neuroprotective effects against dopamine (DA)-induced apoptosis.
To fill this gap in our knowledge, we purified a sesquiterpene lactone spirafolide (\(\text{Furo}[2,3-g][3]\text{benzoxepin}-2(3H)\text{-one},3a,4,5a,10a,10b\text{-hexahydro-5a,10-di-methyl-3-methylene-,(3aS,5aS,10aS,10bS)}) from the leaves of \(L. \ nobilis\) and studied its cytotoxicity and determined whether it can inhibit DA-induced ROS generation and apoptosis in neuronal SH-SY5Y cells.

**MATERIALS AND METHODS**

**Plant material**

The leaves of \(L. \ nobilis\) were purchased from Orient Forest Agricultural & Food Products Foreign Trade, Turkey. The plant was identified by Dr. Yeong-han Kwon (Korea National Arboretum). Voucher specimens (NPRI-Q003) were deposited in the Natural Products Research Institute herbarium, Seoul National University, Korea.

**Extraction and isolation**

The dried leaves of \(L. \ nobilis\) (40.0 kg) were extracted with methanol (3 × 20 L, 24 h each) at room temperature and concentrated under vacuum at 40°C to yield a brown residue (2.4 kg). The residue was partitioned between \(n\)-hexane and 10% aqueous methanol (2 L each). An aliquot (37.5 g) of the \(n\)-hexane fraction (318.7 g) was separated by vacuum flash chromatography (\(\Phi\)10 × 50 cm; silica gel 70-230 mesh, 1 kg) using 10% gradient mixtures of \(n\)-hexane-ethyl acetate to create ten 1.5-L fractions. An aliquot (418.8 mg) of the fraction eluted with 20% EtOAc/\(n\)-hexane fraction (6.08 g) was separated by semi-preparative high-performance liquid chromatography (HPLC; YMC-silica column, 25 × 1 cm) with 15% EtOAc/\(n\)-hexane to yield spirafolide (Fig. 1). This compound was further purified using reversed-phase HPLC (YMC-ODS-A column, 25 × 1 cm) with 10% aqueous methanol to yield 9.1 mg of spirafolide with purities exceeding 98.2%, as determined by analytical HPLC. Spirafolide was characterized by combined analyses of high-resolution mass spectroscopy and one- and two-dimensional nuclear magnetic resonance (NMR). Identify of the compound was confirmed by comparison of its spectral data with literature values (Hashemi-Nejad et al., 1990; Barla et al., 2007).

**Reagents**

Dopamine, dichlorofluorescein diacetate (DCFH-DA), trypsinethylenediaminetetraacetic acid (EDTA), Apomorphine (#A4393), Annexin V-fluorescein isothiocyanate (FITC), and propidium iodide (PI) were purchased from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were acquired from Gibco-BRL. Penicillin and streptomycin were obtained from Meiji Seika.

**Cell culture**

Human neuroblastoma SH-SY5Y cells were cultured by growing them in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO\(_2\) atmosphere.

**Cell viability assays**

Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assays. SH-SY5Y cells (5 × 10\(^4\) cells/mL) were plated to 96-well plates (100 \(\mu\)L/well). After 12 h, they were treated with various concentrations (0.08, 0.4, 2, 10, 25, and 50 \(\mu\)M) of spirafolide for another 48 h and then incubated with MTT (250 \(\mu\)g/mL) at 37°C in phenol-red free medium. After 4 h, the MTT solution was removed and replaced with 250 \(\mu\)L of dimethyl sulfoxide (DMSO). Cell viability was determined by quantifying the production of the colored MTT metabolite formazan. This was achieved by measuring absorbance at 595 nm. Values obtained from untreated control cells were considered to represent 100% viability.

**Effects of spirafolide on DA-induced apoptotic cell death**

Apoptotic cells were distinguished from non-apoptotic ones by their DNA content, as determined by their lower PI staining intensity (Zhang and Zhao, 2003). For experiments, the cells were seeded at a density of \(3 \times 10^5\) cells/well in 6-well plates and cultured for 24 h. After the cells were pretreated with spirafolide (0.4-10 \(\mu\)M containing 0.5% dimethyl sulfoxide (DMSO) for 24 h, the medium was replaced with fresh medium containing DA at selected concentrations and incubated for another 24 h. The cellular DNA content was detected by flow cytometry (FACS) via the determination of PI. Briefly, cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and fixed with 70% cold ethanol overnight. Cells were then centrifuged and the ethanol was removed by washing thor-