Phenolic Diterpenes Derived from *Hyptis incana* Induce Apoptosis and G_2/M Arrest of Neuroblastoma Cells

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Abstract. *Background: Neuroblastoma is one of the most commonly encountered solid tumors in the pediatric age group, and the prognosis of patients with advanced neuroblastoma is very poor. In this study, the antitumor effects of five phenolic diterpenes derived from Hyptis incana (Lamiaceae), a Brazilian medicinal plant, were examined on neuroblastoma cells. Materials and Methods: Cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptotic nuclear shrinkage was monitored by Hoechst 33342 staining. The cell-cycle status was evaluated by flow cytometry and protein alterations were monitored by western blotting. Differentiated cells were photographed and counted in a randomized fashion. Results: All of the examined compounds exhibited significant cytotoxicity towards the neuroblastoma cells. In particular, 7-ethoxyrosmanol had a high degree of efficacy. Nuclear condensation and degradation of procaspase-3 and -9 were observed after treatment of the cells with these compounds. Moreover, phenolic diterpenes induced cell-cycle arrest in the G_2/M phase. Rosmanol and epirosmanol tended to induce differentiation. Conclusion: Phenolic diterpenes isolated from H. incana have multiple antitumor effects on neuroblastoma cells.*

Neuroblastoma is the most common extracranial tumor in children. It has been reported that at the time of diagnosis, 40% of all patients with neuroblastoma have stage 4 disease, when the tumor has already disseminated to distant organs (1). The 5-year event-free survival rate of patients diagnosed with neuroblastoma with distant metastasis is 35%, even after multimodal treatment with strong chemotherapy, surgical resection, radiotherapy and bone marrow transplantation (1). On the other hand, in some cases of neuroblastoma, the outcome is favorable, with the tumors often regressing spontaneously, even if dissemination has already occurred (special stage: 4S). However, the mechanisms underlying such spontaneous regression are still poorly understood, although some reports have suggested the involvement of apoptosis and differentiation (2, 3).

In the chemotherapy of patients with advanced neuroblastoma, drugs known to induce apoptosis and cell-cycle arrest, such as cisplatin and vincristine, respectively, are mainly used. Patients receiving these strong chemotherapeutic drugs often develop severe side-effects, which limits the continuation of the treatment. In addition, cell differentiation therapy is often employed for patients with neuroblastoma, with 13-*cis*-retinoic acid used as the inducer of differentiation, and significant efficacy has been reported (4). However, 13-*cis*-retinoic acid treatment has been reported to be frequently associated with adverse effects such as advanced bone age, hypertriglyceridemia and hypercalcemia (5, 6).

A representative phenolic diterpene, carnosol, has been reported to induce apoptosis of leukemia cells (7) and to induce G_2/M-phase cell-cycle arrest in colon and prostate cancer cells (8, 9). We obtained five carnosol analogs from the extracts of *Hyptis incana* (Brazilian name: Salva-de-marajo; Lamiaceae), a traditional medicinal plant used as a diaphoretic and emmenagogue in Brazil. The antitumor effects of these phenolic diterpenes have yet to be fully elucidated. Moreover, there is yet no evidence of the differentiation-inducing effect of carnosol analogs in neuroblastoma cells.

In this study, we evaluated the apoptosis-inducing activity and impact of these five phenolic diterpenes, derived from *H. incana,* on the cell cycle of neuroblastoma cells. Moreover, the differentiation-inducing activities of these compounds were also assessed.

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Key Words: Neuroblastoma cells, phenolic diterpenes, apoptosis, cell-cycle arrest, differentiation, *Hyptic incana*.

Materials and Methods

Test compounds. Five known phenolic diterpenes [7-ethoxyrosmanol (**1**), rosmanol (**2**), isorosmanol (**3**), epirosmanol (**4**), and 7-methoxyrosmanol (**5**)] were isolated and purified from 80% ethanolic extracts of *H. incana* (Figure 1), and their characteristics were compared with their previously described chemical profiles (10, 11).

Cell culture. Human neuroblastoma cell lines IMR-32, LA-N-1 and SK-N-SH were purchased from Riken Bio Resource Center (Ibaraki, Japan). The human neuroblastoma cell line NB-39 was kindly provided by Dr. Toshimitsu Suzuki, Fukushima Medical University. These neuroblastoma cell lines were cultured in RPMI-1640 medium (Life Technologies Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Normal human dermal fibroblasts (NHDFs) and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). These normal cells were cultured in FGM-2 and EGM-2 media (Lonza), respectively. Cells were incubated at 37˚C in a humidified incubator containing an atmosphere of 95% air with 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IMR-32, LA-N-1, SK-N-SH and NB-39 cells were spread onto 96-well plates (1×10^4 cells/100 μl/well) in phenol red-free RPMI-1640 medium with 10% FBS. NHDFs and HUVECs were spread onto 96-well plates (2×10^4 cells/100 μl/well) containing the corresponding culture medium. Cells were incubated for 24 h, and then the cells were exposed to the test compounds (final concentration: 3×10^{-8}–1×10^{-4} M) for 48 h. MTT solution [0.5% in phosphate-buffered saline (PBS)] was added at a volume of 10 μL into the well. After additional incubation for 3 h, 100 μl of stop solution (0.04 M hydrochloride dissolved in isopropanol) was added. The derived blue formazan was then completely dispersed by pipetting, and the absorbance was measured at 570 nm (peak) and 655 nm (trough). Cell viability was calculated by comparing the absorbance with that of the vehicle (DMSO)-treated control.

Hoechst 33342 staining. IMR-32 cells were spread onto 6-well plates (1×10^5 cells/2 ml/well) in RPMI-1640, containing 10% FBS and incubated for 24 h at 37˚C in a humidified incubator containing an atmosphere of 95% air with 5% CO_2. Thereafter, the cells were exposed to the test compounds (1×10^{-5}–1×10^{-4} M) for 48 h and Hoechst 33342 solution (final concentration: 0.001%) was applied for 15 min for staining the nuclei. Phase-contrast and fluorescent images were then captured under a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Cell-cycle analysis (flow cytometry). IMR-32 cells were spread onto 6-well plates (1×10^6 cells/well) in RPMI-1640, containing 10% FBS and incubated for 24 h at 37˚C in a humidified incubator containing 95% air with 5% CO_2. Thereafter, the cells were exposed to each of the test compounds (1×10^{-5}–1×10^{-4} M) for 48 h. Cells were harvested by pipetting and fixed in 70% ice-cold ethanol. RNase solution (0.025%) was added to the cells which were then incubated for 30 min at 37˚C. Thereafter, 0.05% propidium iodide solution at 1/10th the volume of RNase solution was added and cells were further incubated for 30 min at 4˚C. The amount of DNA in the cells was measured by flow cytometry (FC500; Beckman Coulter, Brea, CA, USA) using the FL3 range.

Western blotting. IMR-32 cells were spread onto a 60-mm dish (2×10^6 cells/dish) in RPMI-1640, containing 10% FBS and incubated for 24 h at 37˚C in a humidified incubator containing 95% air with 5% CO_2. Thereafter, the cells were exposed to each of the test compounds at 1×10^{-4} M for 0-48 h (0 h: untreated). The cells were then collected, washed with Tris-buffered saline (TBS) and lysed in an extraction buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP-40, 10% glycerol, protease inhibitor cocktail I (1:200; Sigma-Aldrich, Saint Louis, MO, USA), phosphatase inhibitor cocktail II (1:100; Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The cells were disrupted twice by sonication for 30 s, and the supernatants were obtained by centrifugation at 15,000 rpm (20,630 ×*g*) for 10 min at 0˚C. The supernatants were mixed with 3×sample buffer (0.24 M Tris-HCl (pH 6.8), 9% sodium dodecyl sulfate (SDS), 30% glycerol, 15% 2-mercaptoethanol and traces of bromophenol blue) at 2:1, and boiled for 3 min at 100˚C. The supernatants, as loading samples, were obtained by centrifugation at 15,000 rpm (20,630 ×g) for 1 min at 0˚C. Equal amounts (20 μg) of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (GE Healthcare, Tokyo, Japan). After blocking by 5% skim milk, the membranes were probed with the primary antibodies [anti-caspase-3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-caspase-9 (1:1000; Cell Signaling Technology), anti-cyclin B (1:1000; BD Biosciences, San Jose, CA, USA), anti-cell division control protein 2 (CDC2) (1:2500; BD Biosciences) and anti-β-tubulin (1:10000; Sigma-Aldrich)], overnight at 4˚C. After washes, the membranes were incubated with horse radish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were detected with an enhanced chemiluminescence (ECL) system (GE Healthcare).

Differentitation. NB-39 cells were spread onto human fibronectin-coated 12-well plates (1×10^4 cells/well) in RPMI-1640 containing 1% FBS and incubated for 24 h at 37˚C in a humidified incubator, containing 95% air with 5% CO_2. Thereafter, the cells were exposed to each of the test compounds (1×10^{-8}–1×10^{-6} M) for 96 h. The cells were then observed and photographed under a microscope (IX-71; Olympus). The images were randomized and the total number of cells, and the numbers of cells possessing neurites or other differentiation features, cells showing neurite outgrowth (over the soma length), cells with multiple neurites, cells with branched neuritis, and Schwann-like cells were counted in a blinded fashion.

Statistical analysis. Data are presented as the means±standard errors of the means (SEM) (n=3). Significance testing was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's test for comparing three or more data, or Student's *t*-test for comparing two sets.

Results

Cytotoxicity. The cytotoxicities of the five phenolic diterpenes derived from *H. incana* towards neuroblastoma and normal cells were assessed by the MTT assay (Figure 2). All five of the compounds induced significant cell death of the neuroblastoma cell lines at 100 μM after 48 h of application. 7-Ethoxyrosmanol exibited the most potent cytotoxicity towards NB-39, LA-N-1, and SK-N-SH cells

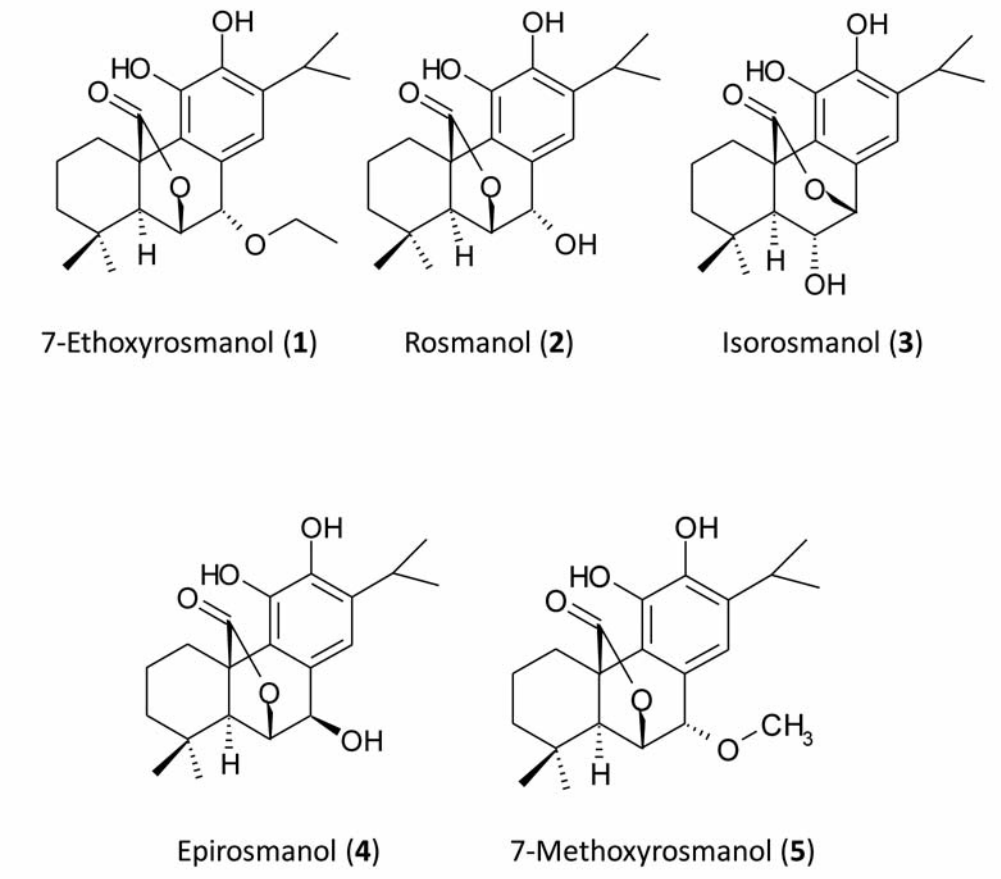


Figure 1. *Chemical structures of compounds* ***1-5*** *isolated from Hyptis incana*.

among the test compounds, with 50% inhibitory concentration (IC_{50}) values of 2.87×10^{-5}, 1.48×10^{-5}, and 5.5×10^{-6} M, respectively. In contrast to 7-ethoxyrosmanol, which induced cell death on the HUVECs, as well as on neuroblastoma cells, isorosmanol tended to be selectively cytotoxic towards neuroblastoma cell lines as compared to normal cells at 3×10^{-5} M.

Apoptosis. To clarify whether the cell death induced by these compounds was apoptosis, we observed the morphological features of apoptosis. Examination after Hoechst 33342 staining showed that all of the phenolic diterpenes induced cell and nuclear shrinkage after treatment of cells for 48 h at 10^{-4} M (Figure 3). Molecular apoptotic features were also observed on western blot analysis. Both procaspase-3 and -9 were degraded after 24 or 48 h application of all the test compounds (Figure 4).

Cell cycle arrest. Cell cycle analysis was performed by flow cytometry (Figure 5). Significant G_2/M-phase cell-cycle arrest was induced by 7-ethoxyrosmanol, rosmanol, isorosmanol, and epirosmanol after exposure at 10^{-5}–10^{-4} M for 48 h. A tendency towards an increase of the G_2/M cell population was observed in the cells treated with 7-methoxyrosmanol. In addition, the G_0/G_1 cell population was significantly reduced after the application of 7-ethoxyrosmanol. CDC2 and cyclin B are known to play important roles in cell-cycle progression in the G_2/M phase. All of the phenolic diterpenes down-regulated the protein levels of both CDC2 and cyclin B (Figure 4).

Differentiation. In the cell differentiation study, we used NB-39 cells, an undifferentiated type of neuroblastoma cell line (12). Sub-cytotoxic concentrations of phenolic diterpenes were applied to the cells for 96 h and the morphological changes were observed. No significant alterations were observed in any of the parameters (differentiated, elongated, multiple, branched, and glial) after application of each of the test compounds and of 13-*cis*-retinoic acid (10^{-6} M), as a positive control. However, a tendency towards an increase in the number of elongated neurites was observed after application of rosmanol at 10^{-7} M, and a tendency towards

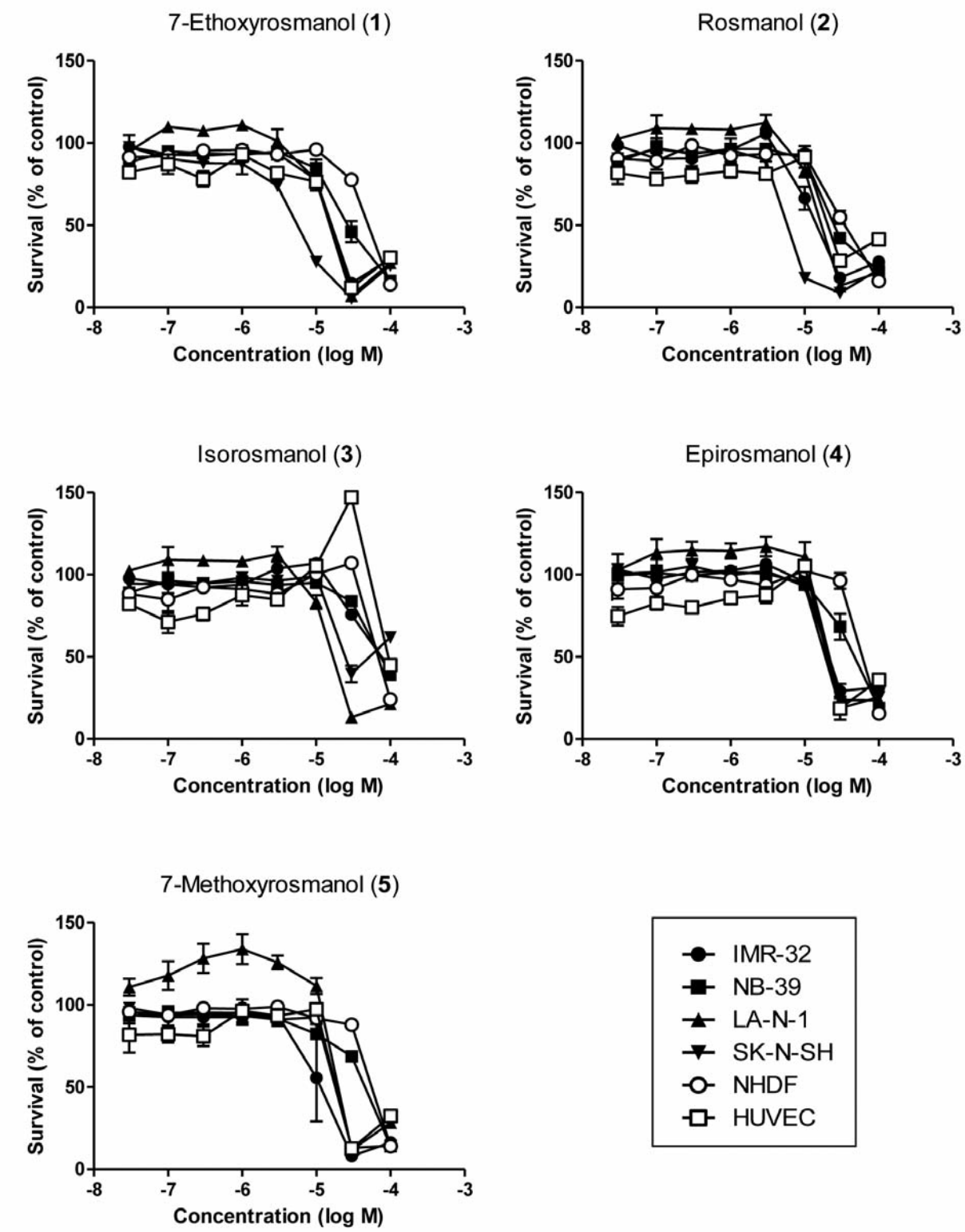


Figure 2. *Cytotoxicity of the phenolic diterpenes towards neuroblastoma cells and normal cells. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IMR-32, NB-39, LA-N-1 and SK-N-SH neuroblastoma cells and normal human dermal fibroblasts (NHDFs), and human umbilical vein endothelial cells (HUVECs), were treated with the indicated concentrations of the phenolic diterpenes or dimethylsulfoxide (vehicle control) for 48 h. Each plot shows the survival rate relative to that of the vehicle control (mean±SEM, n=3).*

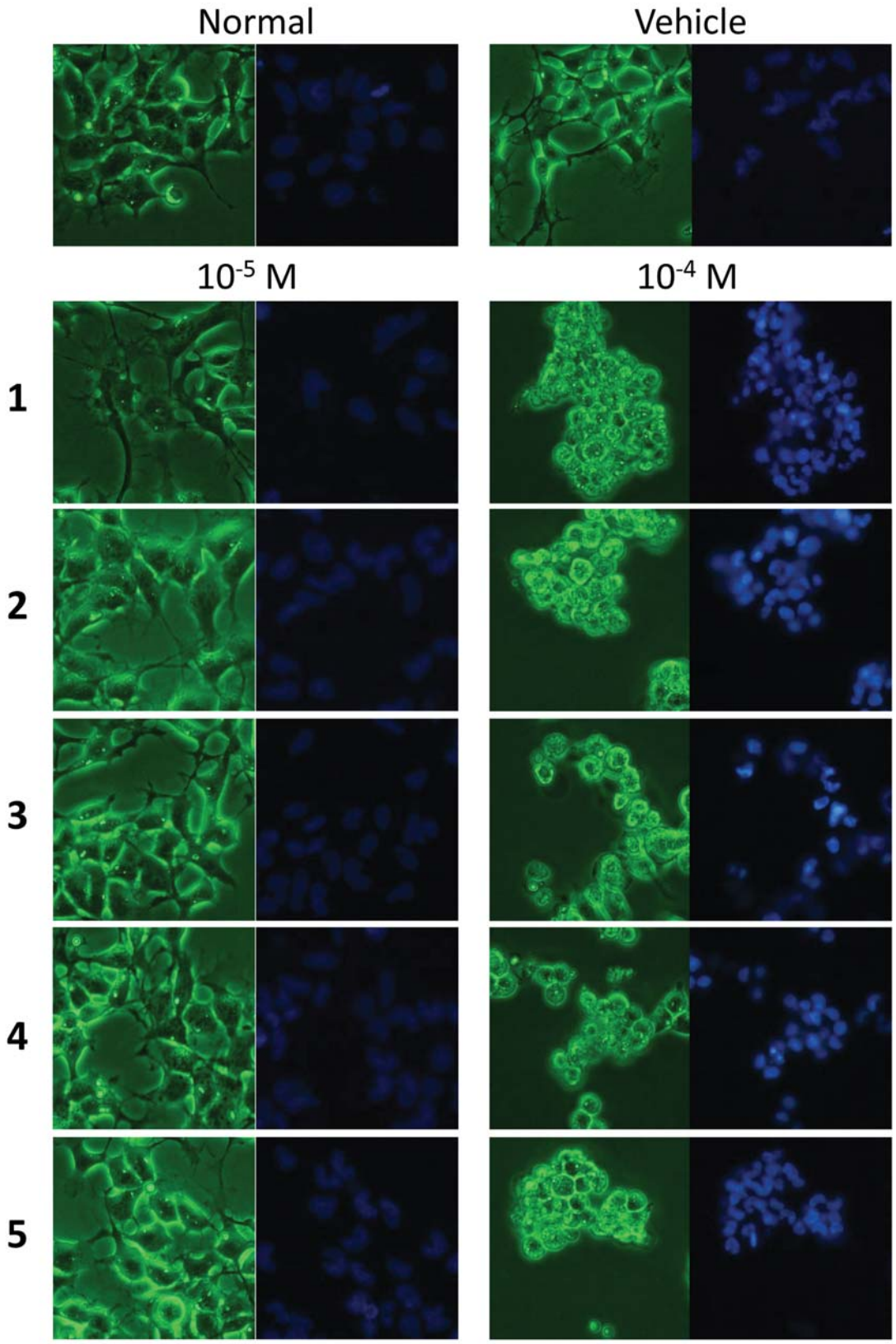


Figure 3. *Morphological features of apoptosis observed using Hoechst 33342 staining. IMR-32 cells were exposed to phenolic diterpenes (**1-5**) for 48 h, and phase-contrast images (left) and fluorescence images (right) were obtained.*

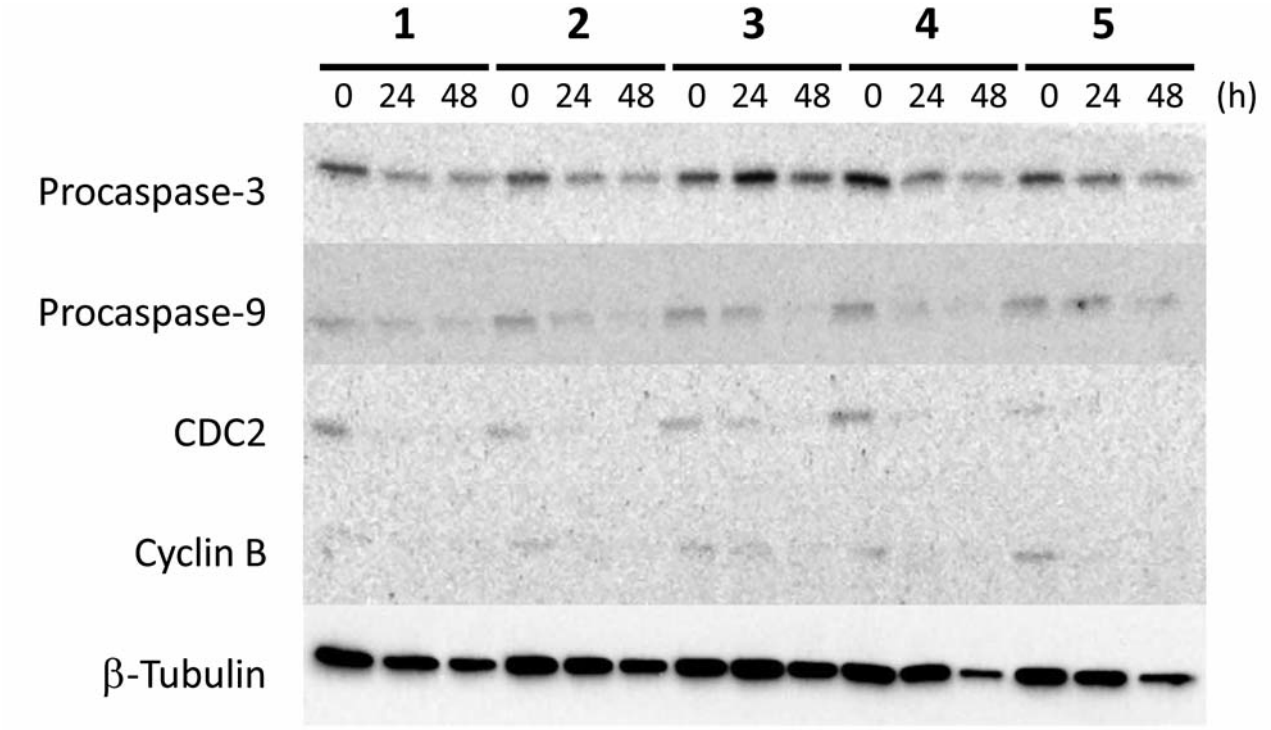


Figure 4. *Western blot analysis of apoptosis- and cell cycle-related proteins. IMR-32 cells were exposed to 10^{-4} M of each of the test compounds (**1-5**) for 24 or 48 h and procaspase-3, procaspase-9, CDC2, cyclin B, and β-tubulin were detected by western blot analysis.*

an increase in the number of Schwann-like cells was observed after treatment with epirosmanol at each of the test concentrations (10^{-8} M–10^{-6} M) (Figure 6).

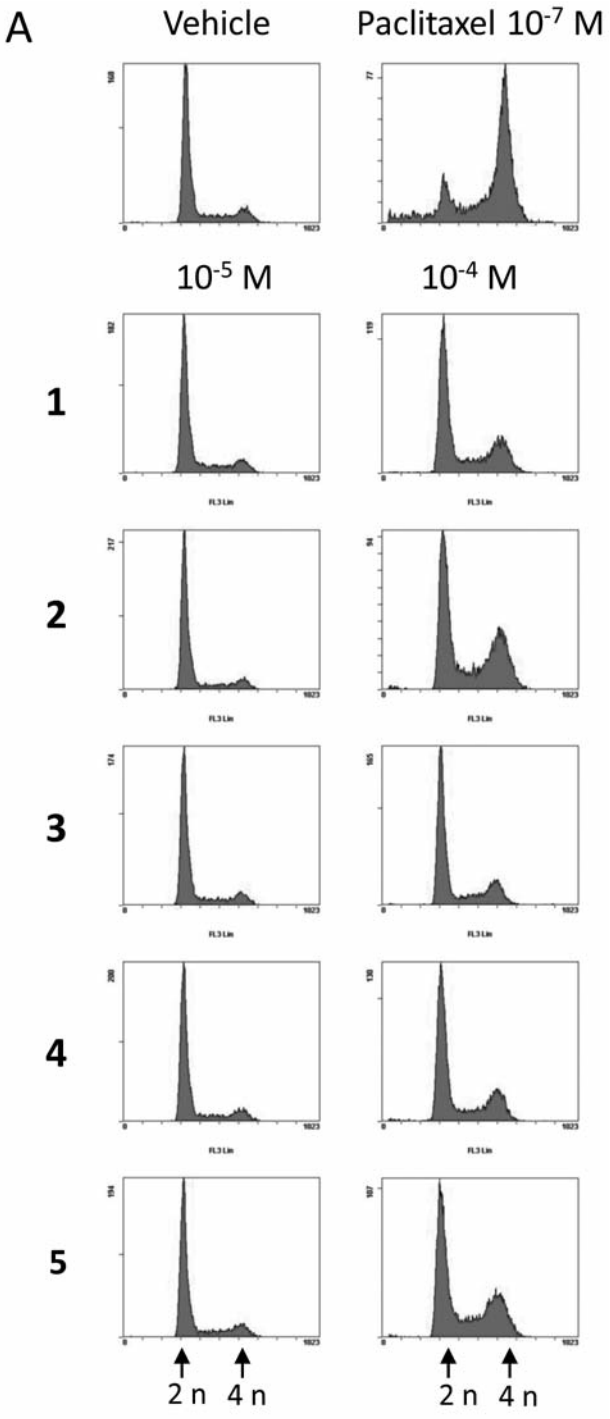
Discussion

In this study, all five of the phenolic diterpenes, derived from *H. incana,* exhibited multiple anticancer activities towards neuroblastoma cells, including induction of apoptosis, G_2/M-phase cell-cycle arrest, and a tendency to induce differentiation. The antitumor activities of these phenolic diterpenes on neuroblastoma cells represent novel, hitherto unreported, knowledge.

All five phenolic diterpenes induced significant cytotoxicity towards the neuroblastoma cells, in a dose-dependent manner. In a previous study, isorosmanol has been demonstrated by sulforhodamine B (SRB) assay to inhibit cell growth of ovarian, lung and breast cancer cell lines (13). Rosmanol and 7-methoxyrosmanol have been shown, by the MTT assay, to exert cytotoxicity towards cervical, lung, breast and colon cancer cell lines (14, 15). The reported effective concentration ranges are quite similar to our data. On the other hand, no antitumor effects of 7-ethoxyrosmanol and epirosmanol have been previously reported. Intriguingly, isorosmanol was selectively cytotoxic towards neuroblastoma cells as compared to normal cells at 3×10^{-5} M. It may represent a lead compound for developing efficient and safe chemotherapeutic agents for the treatment of neuroblastoma.

Features of apoptosis were observed in the neuroblastoma cells after exposure to these phenolic diterpenes. Recently, rosmanol was reported to induce apoptosis of the COLO 205 cell line, a cell line derived from colonic adenocarcinoma, *via* both mitochondrial and death receptor pathways (14). Caspase-8, a key executor of apoptosis *via* the death receptor pathway, is often known to be silenced in malignant neuroblastoma, and the tumor-derived IMR-32 cell line (16). Carnosol, an analog of phenolic diterpenes used in this study, was shown to induce mitochondrial apoptosis *via* B-cell lymphoma 2 (BCL2) down-regulation in B-cell leukemia cells (7). Taken together with the fact that procaspase-9 was degraded after treatment, using each of the five phenolic diterpenes, the apoptosis induced by these compounds is thought to be mainly induced *via* the mitochondrial pathway.

Carnosol induces G_2/M-phase cell-cycle arrest in colon and prostate cancer cell lines (8, 9). Similarly, four out of the five phonolic diterpenes induced significant G_2/M-phase cell-cycle arrest in the neuroblastoma cells. Interestingly, while carnosol was reported to up-regulate cyclin B1 in colon cancer cells (8), all of the phenolic diterpenes used in our



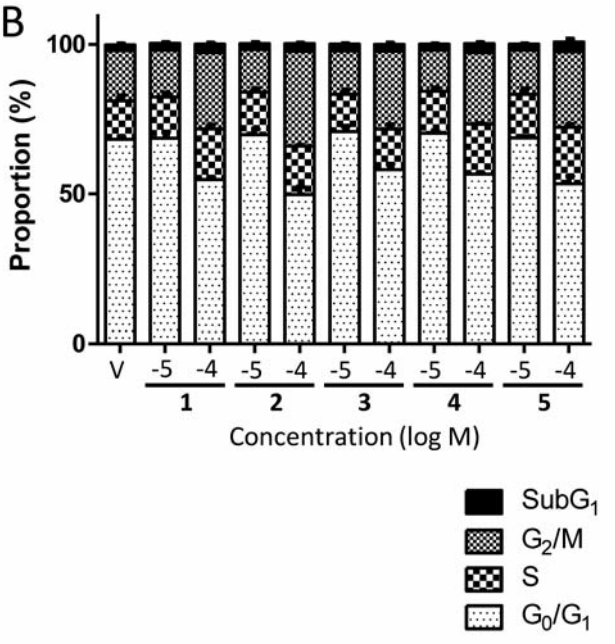
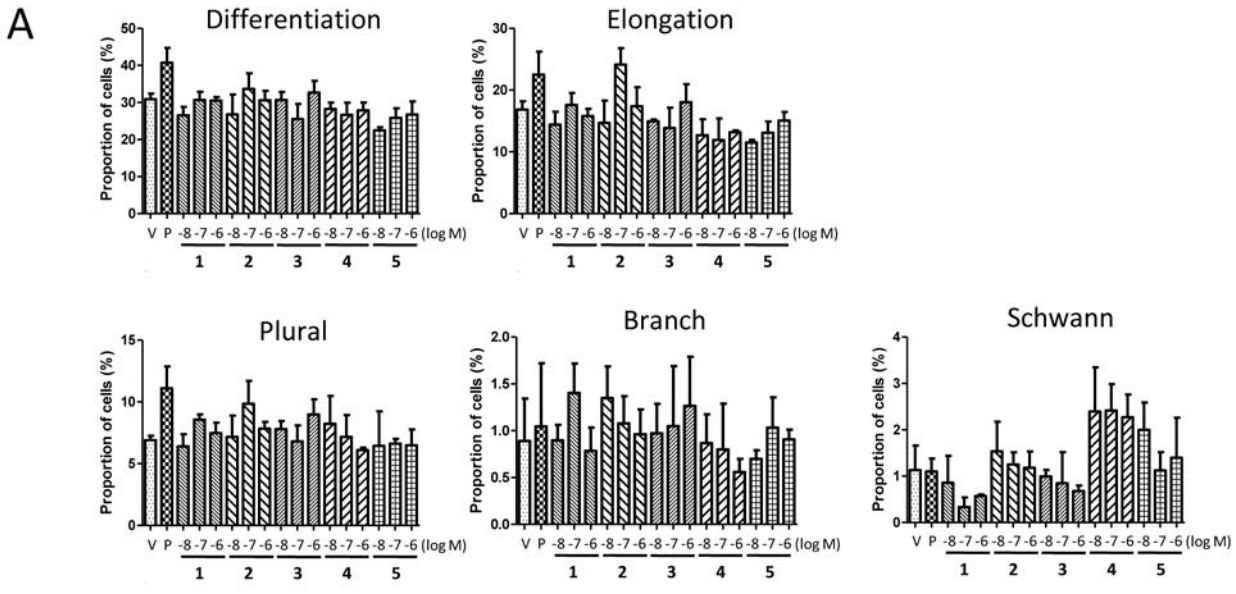


Figure 5. *Analysis of cell-cycle status using flow cytometry. A: Histograms of the flow cytometry are shown in A. IMR-32 cells were exposed to the each of the test compounds (**1-5**) and paclitaxel (positive control) for 48 h, then were compared by flow cytometry. The cell numbers are plotted on the vertical axis, and the cellular DNA contents on the horizontal axis. B: Percentage of cells at the stage of the cell cycle, as analyzed in A.*

study down-regulated the protein levels of cyclin B and CDC2 in the IMR-32 cells. Cyclin B and CDC2 are essential for cell-cycle progression at the G_2 checkpoint (17). The suppression of cyclin B and CDC2 seems to trigger the G_2/M-phase arrest in neuroblastoma cells exposed to these phenolic diterpenes. Moreover, a similar signaling cascade seems to be involved in the induction of cell cycle arrest by all of these compounds.

Although there were no significant differences in the differentiation parameters, the elongation parameter tended to be higher in the group exposed to 10^{-7} M rosmanol than in the positive control group. On the other hand, epirosmanol tended to increase the Schwann-like cell parameter at all tested concentrations (10^{-8}–10^{-6} M). Interestingly, epirosmanol is the stereoisomer of rosmanol. This configurational difference seems to be a determinant of the differentiation pattern of neuroblastoma cells. Although more precise analyses are needed to elucidate the differentiation mechanisms, phenolic diterpene derivatives may affect the pattern of differentiation of neuroblastoma cells.

Previously, we reported that diarylheptanoids, derived from *Alpinia officinarum,* exhibited multiple anticancer effects, including induction of apoptosis, S-phase cell-cycle arrest, and differentiation (18). Phenolic diterpenes, derived from *H. incana,* also possess multimodal anticancer activities. Thus, phenolic diperpenes appear to be useful candidate drugs for chemotherapy of advanced neuroblastoma.



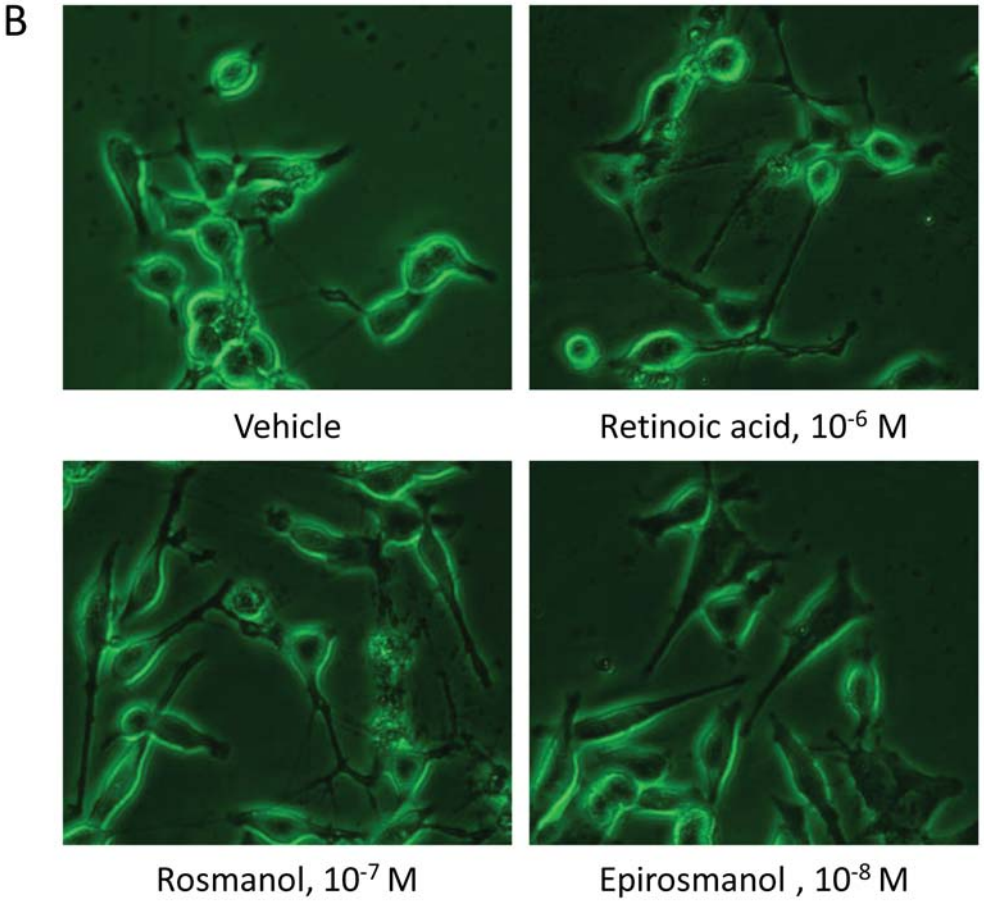


Figure 6. *Morphological analysis of neuronal and glial differentiation of neuroblastoma cells. NB-39 cells were treated with each of the test compounds (**1-5**), 13-cis retinoic acid (positive control), or dimethylsulfoxide (vehicle control) for 96 h. Cells with neurite outgrowth, elongated neurites, multiple neurites, branching neurites, and Schwann cell-like morphology were counted in a blinded manner. A: Vertical axes indicate the numbers of cells expressed as a percentage of the total cell number. V: Vehicle control, P: positive control (13-cis retinoic acid; 10^{-6} M). B: Morphological appearance of cells under phase-contrast microscopy.*

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