Suppression of mouse skin tumor promotion and induction of apoptosis in HL-60 cells by Alpinia oxyphylla Miquel (Zingiberaceae)

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There have been considerable efforts to search for naturally occurring substances for the intervention of carcinogenesis. Many components from dietary or medicinal plants have been identified that possess substantial chemopreventive properties. An example is curcumin (Curcuma longa Linn., Zingiberaceae), which has been shown to inhibit tumor promotion in experimental carcinogenesis. *Alpinia oxyphylla* Miquel, another plant of the ginger family used in oriental herbal medicine, contains diarylheptanoids whose structures are analogous to that of curcumin. In the present study, we have tested *A. oxyphylla* for its ability to suppress tumor promotion. Thus, topical application of the methanolic extract of dried fruits of *A. oxyphylla* significantly ameliorated 12-O-tetradecanoylphorbol-13-acetate (TPA)–induced skin tumor promotion as well as ear edema in female ICR mice. In another study, treatment of HL-60 cells with the methanolic extract of *A. oxyphylla* significantly reduced the viability of the cells and also inhibited DNA synthesis. Microscopic examination of the treated cells showed characteristic morphology of apoptosis. Furthermore, cells treated with the extract of *A. oxyphylla* exhibited internucleosomal DNA fragmentation in time- and concentration-dependent manners. TPA-stimulated generation of superoxide anion in differentiated HL-60 cells was also blunted by *A. oxyphylla*. Taken together, these findings suggest that *A. oxyphylla* possesses potential chemopreventive and antitumorigenic activities.

Introduction

There has been growing evidence that consumption of certain foods, including dark green leafy vegetables and yellow fruits, can decrease the risk for various forms of human cancer. Besides antioxidative vitamins, some dietary phytochemicals were shown to possess inhibitory effects against chemically induced carcinogenesis and mutagenesis. One such agent is curcumin, a yellow color pigment from the rhizome of *Curcuma longa* Linn., *Zingiberaceae,* which has been shown to inhibit tumor promotion in experimental carcinogenesis. *Alpinia oxyphylla* Miquel, another plant of the ginger family used in oriental herbal medicine, contains diarylheptanoids whose structures are analogous to that of curcumin. In the present study, we have tested *A. oxyphylla* for its ability to suppress tumor promotion. Thus, topical application of the methanolic extract of dried fruits of *A. oxyphylla* significantly ameliorated 12-O-tetradecanoylphorbol-13-acetate (TPA)–induced skin tumor promotion as well as ear edema in female ICR mice. In another study, treatment of HL-60 cells with the methanolic extract of *A. oxyphylla* significantly reduced the viability of the cells and also inhibited DNA synthesis. Microscopic examination of the treated cells showed characteristic morphology of apoptosis. Furthermore, cells treated with the extract of *A. oxyphylla* exhibited internucleosomal DNA fragmentation in time- and concentration-dependent manners. TPA-stimulated generation of superoxide anion in differentiated HL-60 cells was also blunted by *A. oxyphylla*. Taken together, these findings suggest that *A. oxyphylla* possesses potential chemopreventive and antitumorigenic activities.

Materials and methods

**Chemicals**

TPA was purchased from Alex Biochemicals (San Diego, CA). RPMI 1640, fetal bovine serum, gentamicin, and trypsin blue were products of Gibco BRL (Grand Island, NY). [3H]Thymidine was obtained from Amersham (Arlington Heights, IL). 7,12-Dimethylbenz[a]anthracene (DMBA), cytochrome c, sodium dodecylsulfate (SDS), proteinase K were purchased from Sigma (St Louis, MO). Other chemicals and solvents used were of highest analytical grade.

**Preparation of the Alpinia oxyphylla extract**

Powdered fruits of *A. oxyphylla* were obtained from a local market in Seoul. Two hundred grams of the powder was extracted with methanol (2 l) for 15 h at room temperature. The solution was filtered and washed with *n*-hexane (1 l). The methanolic extract was evaporated to dryness under vacuum and the resulting residue was partitioned between chloroform (1 l) and an equal volume of water. The organic layer was collected and concentrated in vacuo. HPLC analysis showed that the methanolic extract contains ~1.3% (w/w) Yukuchinones A and B.

**Induction of mouse ear edema**

Young female ICR mice of ~7 weeks of age were treated on the right ear with acetone or a test material in acetone, 30 min prior to application of TPA (2 nmol). Control animals received acetone in lieu of TPA. The mice were killed 5 h later, and punched discs (7 mm in diameter) from treated and

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control ears were weighed. The increase in the weight of ear edema was a measure of inflammation.

**Induction of mouse skin carcinogenesis**

Groups of 25–30 female ICR mice were treated on their shaven backs with a single topical application of DMBA (0.2 µmol) in 0.2 ml acetone or the solvent alone. At 1 week after initiation, 15 nmol of TPA was topically applied twice weekly until termination of the experiment. Starting 1 week following the promoter treatment, tumors of at least 1 mm in diameter were counted every week, and the results were expressed as the percentage of tumor-bearing mice (incidence) and the average number of tumors per mouse (multiplicity).

**Cell culture**

HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 5 µg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO₂–95% air.

**Assessment of cell viability and DNA synthesis**

Exponentially growing cells were suspended at a density of 5×10⁵ cells/ml in RPMI 1640 medium containing 5% fetal bovine serum and treated with various amounts of methanolic extract of *Alpinia oxyphylla* in 0.1% dimethyl sulfoxide (DMSO). Controls were treated with DMSO alone. After 5 h incubation at 37°C, cells were washed with and resuspended in phosphate buffered saline (PBS) containing 0.2% trypan blue. The blue and white cells were counted under a microscope using a hemocytometer. To assess the cell proliferation, [methyl-³H]thymidine (sp. act. 54.0 Ci/mmol) was added at a final concentration of 1 µCi/ml and incubated for 5 h. The cells were then harvested, washed with PBS and incubated with 1.3% DMSO and incubated for 6 days at 37°C in a humidified atmosphere of 5% CO₂. Cells were harvested by centrifugation, washed with and resuspended in PBS at a density of 1×10⁵ cells/ml. Cells were preincubated with or without the methanolic extract of *Alpinia oxyphylla* for 15 min, and TPA (8 µmol) was added to the reaction mixture. After 30 min incubation at 37°C, superoxide generation was determined by measuring absorbance of reduced cytochrome c at 550 nm.

**Results**

The anti-tumorigenic potential of *Alpinia oxyphylla* was evaluated in a two-stage mouse skin carcinogenesis model with DMBA as an initiator and TPA as a promoter. When given 30 min prior to TPA application, each topical dose of 2 mg methanolic extract of *A. oxyphylla* reduced both the incidence and the multiplicity of skin papillomas by >60% at the 22nd week (Figure 1). The higher dose (10 mg) almost completely suppressed the papilloma formation. TPA-induced inflammation on mouse ear was also inhibited by 56% with the extract (Table I).

In another study, HL-60 cells were incubated with the methanolic extract of *A. oxyphylla* and the viability of treated cells was compared with that of solvent-treated controls. As shown in Figure 2, treatment of cells with 10, 20, 33 and 67 µg/ml of the extract for 5 h resulted in 27, 54, 74 and 82% inhibition of viability, respectively. We also assessed the effect on 1-(4'-hydroxy-3'-methoxyphenyl)-7-phenyl-3-heptanone (Yakuchinone A) and 1-(4'-hydroxy-3'-methoxyphenyl)-7-

![Fig. 1. Inhibitory effects of *Alpinia oxyphylla* on mouse skin tumor promotion. Female ICR mice were subjected to topical application of either 2 (○) or 10 mg (■) of the methanolic extract of *A. oxyphylla* dissolved in acetone or vehicle alone (○) 30 min prior to each TPA treatment twice a week. ■, the group initiated with DMBA followed by twice weekly application of the methanolic extract (10 mg) without TPA promotion.](image)

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<th>Treatment</th>
<th>Weight per punch (mg)</th>
<th>% inhibition</th>
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<td>Acetone</td>
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<td>10.8 ± 0.35*</td>
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Female ICR mice were treated topically on the right ear with acetone, TPA (2 nmol), or TPA (2 nmol) and a test material in acetone. Five hours later, the animals were killed by cervical dislocation. Ear punches (7 mm in diameter) were weighed. Data are expressed as the means ± SE obtained from eight animals per group.

*P < 0.001 (versus TPA alone).

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Phenylethyl-1-en-3-one (Yakuchinone B), major diarylheptanoids contained in *A. oxyphylla*, on the viability of HL-60 cells. After treatment with 100 µM Yakuchinone A and Yakuchinone B, the cell viability was decreased by 69 and 79%, respectively (J.-Y.Kang et al., unpublished data). Effects of *A. oxyphylla* on the rate of DNA synthesis were determined by measuring incorporation of [³H]thymidine in cultured HL-60 cells. In parallel with the suppression of cell viability shown in Figure 2, DNA synthesis was decreased dose-dependently in cells treated with *A. oxyphylla*. After 5 h incubation with 3, 10, 20, 33 and
Tumor suppression and induction of apoptosis by *Alpinia oxyphylla*

**Fig. 2.** Concentration-dependent inhibition of viability of HL-60 cells by the methanolic extract of *Alpinia oxyphylla*. Incubation conditions and other experimental details are described under Materials and methods.

**Fig. 3.** Inhibitory effects of *Alpinia oxyphylla* on DNA synthesis in HL-60 cells. Data represent the means ± SE obtained from four separate determinations.

At a concentration of 62 µg/ml of the extract, incorporation of [3H]thymidine was reduced by 34, 55, 72, 84 and 94%, respectively (Figure 3).

In order to further investigate the mode of cell death induced by *A. oxyphylla*, we examined the morphological changes of HL-60 cells following treatment with the extract. Characteristic morphological changes, such as membrane blebbings and formation of apoptotic bodies, were observed (Figure 4). Apoptotic bodies were detected at 2 h after treatment and became abundant with increasing concentrations of the extract.

Since one of the most distinct biochemical hallmarks of apoptosis is the cleavage of DNA into multiple internucleosomal fragments of 180–200 bp, nuclear DNA isolated from the treated cells was analyzed by agarose gel electrophoresis. Fragmentation of nuclear DNA was observed at 5 h in a dose-dependent manner with 20–67 mg/ml of the extract (Figure 5A). Internucleosomal DNA fragmentation was observed at 2 h after the treatment with the highest dose used, and increased as a function of time (Figure 5B).

When the methanolic extract of *A. oxyphylla* was present at a concentration of 62 µg/ml in the culture of HL-60 cells differentiated with 1.3% DMSO, TPA-stimulated superoxide generation was attenuated >60% and almost completely abolished with 250 µg/ml of the extract (Figure 6).
Discussion

Antitumor-promotional effects of *A. oxyphylla* were evaluated in ICR mice using a two-stage skin carcinogenesis model. Topical application of the methanolic extract of *A. oxyphylla* significantly suppressed the TPA-induced tumor promotion in mouse skin. The extract also exhibited anti-inflammatory activity as determined by reduction of tumor promoter-induced mouse ear edema. Inflammatory reactions have been considered to occur during the process of tumor promotion and many anti-inflammatory agents are effective in abrogating tumor promotion (22,20,21). Previous studies by Kiuchi et al. (10) have shown that Yakuchinones A and B from *A. oxyphylla* inhibit the formation of prostaglandins and 5-HETE from arachidonic acid. Thus, it seems likely that the inhibitory activity of Yakuchinone A and Yakuchinone B on arachidonic acid metabolism may contribute in part to suppression of mouse skin tumorigenesis by *A. oxyphylla*. According to our preliminary study, both Yakuchinone A and Yakuchinone B significantly ameliorated phorbol ester-induced ear edema and epidermal ornithine decarboxylase. Their possible antitumor-promotional effects are under investigation in this laboratory.

Recent advances in our understanding of biochemical and molecular basis of inflammatory processes revealed that activation of the transcription factor NF-κB by cytokines, reactive oxygen species and a variety of chemical agents, including phorbol ester, mediates inflammatory reactions (22,23). Activation of NF-κB regulates expression of many factors involved in tumor-promotion and is suppressed by certain chemopreventive agents such as curcumin (23–25). Diarylheptanoids contained in *A. oxyphylla* fruits may inhibit activation of NF-κB and the subsequent interaction with responsive DNA sequences.

The antitumor-promotional effect of *A. oxyphylla* may also be attributable to the antioxidative activity of this herb. The extract of *A. oxyphylla* decreased superoxide generation induced by TPA in differentiated HL-60 cells and also suppressed lipid peroxidation in rat brain homogenates (data not shown). Based on these findings, *A. oxyphylla* may inhibit tumor promotion by abrogating oxidative stress, thereby suppressing NF-κB activation mediated by reactive oxygen species.

The results of the present study indicate that the fruits of *A. oxyphylla* possess activities to inhibit cell survival and DNA synthesis in HL-60 cells. Treatment of these cells with the methanolic extract of *A. oxyphylla* also induced apoptosis, as indicated by internucleosomal DNA fragmentation and formation of apoptotic bodies. The methanolic extract of *A. oxyphylla* fruits is known to contain two major diarylheptanoids named as Yakuchinones A and B (18,19), whose structures are analogous to that of curcumin. Our preliminary studies indicate that these diarylheptanoids, as well as curcumin, inhibit growth and proliferation of HL-60 cells and also induce apoptosis in these cells (26). Several investigators have reported that the anti-apoptotic protein Bcl-2 may play a pivotal role in regulation of apoptosis, but the effects are in controversy. Kuo et al. (16) suggested that induction of apoptosis by curcumin is associated with down-regulation of bcl-2 expression at a post-translational level. On the contrary, it was found that 1,25-dihydroxyvitamin D₃ prevented apoptosis but reduced the expression of the bcl-2 gene in HL-60 cells (17). Studies are under way in our laboratory to investigate effects of *A. oxyphylla* and its diarylheptanoids on the expression of bcl-2 and other apoptosis-related genes such as bax and p53 in HL-60 cells.

In summary, this study demonstrates that the methanolic extract of *A. oxyphylla* inhibits promotion of skin tumorigenesis and tumor promoter-induced inflammation in mice. The extract also suppressed the viability and proliferation of HL-60 cells by inducing programmed cell death. Taken together, these results suggest that *A. oxyphylla* possesses chemopreventive and chemotherapeutic potential. Further studies will be required to identify the active anticarcinogenic components of this herb and to elucidate their molecular mechanisms of action.

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References


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