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Detection of Anticancer Activity from the Root of *Angelica gigas In Vitro*

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Anticancer activity of a fraction of the ethanol extract from the root of Korean angelica (*Angelica gigas* Nakai) was recognized in human cancer cell lines HeLa S_3, K-562, and Hep G_2. The extract blocked the phorbol ester-inducing megakaryocytic differentiation of K-562 cells, which indicated the modification of protein kinase C (PKC) activity. *In vitro* assay showed the activation of PKC by the extract. An effective fraction of the *Angelica gigas* extract, of which R_f value was 0.64 in a thin layer chromatography, was a different component from those of European angelicas. The ED_{50} value of the fraction was 8, 9, and 16 μg/ml against HeLa S_3, Hep G_2 and K-562 cells, respectively, while the fraction showed higher ED_{50} values against normal cell lines.

To screen anticancer activity from natural products, cytotoxicity tests on human cancer cell lines has been widely used (1, 19). *In vitro* screening of anticancer activity may be divided into the clonogenic assay and the short-term chemosensitivity test using MTT or SRB. The latter is more widely used (15, 23). The cytotoxicity test only for the screening of anticancer drugs is not an effective method because most drugs developed based upon the cytotoxicity have displayed numerous side effects. Recently many researchers endeavoured to find inhibitors of enzymes specifically related to the signal transduction of cancer cells (10, 19) and substances inhibiting the expression of oncogenes (5, 9, 25). On the other hand potential supplements from oriental medicines can be used for cancer chemotherapy in order to eliminate the side effects of conventional anticancer drugs (13).

In this paper, considering the damage to hematopoietic cells by conventional anticancer drugs, among hundreds of oriental medicines, we selected Korean angelica root, rhemannia root and deer antler because these have been used predominantly for hematogenesis, and tested primarily Korean angelica for the anticancer effect. Korean angelica (*Angelica gigas* Nakai), which belongs to Umbelliferae, is a herb used specifically for anemia in traditional Korean medicine. The major components of *Angelica gigas* root are coumarins such as decursin, decursinol, nodakenetin, umbelliferone, nodakenin, and β-sitosterol (20). However pharmaceutical efficacy of those components have been reported limitedly. The ether extract of *Angelica gigas* root induced excitement of the ectomized intestinal tract and uterus of a rabbit, and enhanced blood pressure and respiration of rabbits (2). Both purified decursin and decursinol showed inhibiting effect on the ectomized heart of a frog and on the spontaneous action of mice (3). However the hematogenetic function was not shown from decursin, the main component of *Angelica gigas*, though the total extract of *Angelica gigas* displayed hematopoietic effect to some degree (6).

In this study, *Angelica gigas* root was selected and tested for *in vitro* anticancer activity, and the potential of an F3 fraction was examined as a novel anticancer drug without minimal side effects.

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Key words: Root of Angelica gigas, bleb assay, anticancer activity, protein kinase C activator

MATERIALS AND METHODS

Materials

The fresh roots of Korean angelica (*Angelica gigas*) and rhemannia (*Rehmannia glutinosa var. purpurea*) were dried in a dark place. Deer antlers (*Cornu cervi*) from Siberia, China and New Zealand were bought at a Korean herb shop. K-562 (ATCC CCL 243), Hep G_2 (ATCC HB 8065), HeLa S_3 (ATCC CCL 2.2), Vero (ATCC CCL 81) and MRC-5 (ATCC CCL 171) cell lines were obtained from the American Type Culture Collection (Rocksville, Maryland, U.S.A.). Fetal bovine serum (FBS) and anti-

biotics (penicillin and streptomycin) were purchased from Hyclone (Logan, UT, USA). Phorbol 12, 13-dibutyrate and staurosporine were from Sigma (St. Louis, MO, USA), Protein kinase C assay kit was from Promega (Madison, WI, USA), and TLC plate (Alugram Sil G/UV_{254}) was from Macherey-Nagel Düren (Düren, Germany). Coumarine standards of angelicin, scopoletin, umbelliferone, and xanthotoxin were from Carl Roth (Karlsruhe, Germany).

Preparation of Extracts

The root of *Angelica gigas, Rehmannia glutinosa,* and *Cornu cervi* were broken into pieces, and 20g of each broken sample was extracted with 200ml of 70% ethanol (EtOH) by shaking at 50°C for 24 hours, and the extract was filtered through Whatman No. 1 filter paper. In the case of aqueous extraction, each broken sample was extrcted with 200ml of boiling D.W for 4 hours. All samples were dried and an equal amount of the dried samples were dissolved in phosphate buffered saline (PBS) solution containing 5% DMSO to make 50mg/ml in the final concentration and stored at 4°C in a dark place.

Maintenance of Cell Lines

K-562 cells were cultured in RPMI-1640 (Gibco-BRL, Grand Island, NY, U.S.A.) medium supplemented with 10% fetal bovine serum and 1% antibiotics solution (penicillin and streptomycin, Hyclone), Hep G_2, HeLa S_3, MRC-5, and Vero cells were cultured in Dulbecco's Modified Essential Medium (Gibco-BRL) with the same supplements as above. Cells were cultured on 25cm^2 T-flasks in a CO_2 incubator supplied with 5% CO_2 and 95% humid air at 37°C and subcultured every 3 days for at least 2 weeks before the treatment of samples. When Hep G_2, HeLa S_3, MRC-5, and Vero cells were subcultured, 0.25% trypsin was used to detach the cells from the T-flasks.

Bleb-inhibition Assay with K-562 Cells

K-562 cells were diluted to 1×10^5cells/ml and 100 µl of cell suspension was dispensed in each well of a 96-well plate. Each 10 µl of sample extracts (50mg/ml) was added into the wells and cells were incubated for 1 hour at 37°C. Phorbol 12, 13-dibutyrate (PDBu) was then added to make the final concentration of 0.1 µg/ml. After 30min, bleb-bearing K-562 cells were counted under a microscope and compared with the positive and the negative controls treated with 1nM staurosporine and without respectively.

Protein Kinase C Assay

Protein kinase C (PKC) activity was determined using a non-isotopic Promega assay kit, which contained protein kinase C isolated from rat brain and the fluorescent-tagging peptide (P-L-S-R-T-L-S-V-A-A-K) as a substrate. Total reaction volume was 40 µl and each tube contained 20mM HEPES, pH 7.4, 1.5mM $CaCl_2$, 1mM DTT, 10mM $MgCl_2$, 1mM ATP, phosphatidylserine (0.2 mg/ml), 20ng peptide substrate, 10ng enzyme, and various extracts of indicated amounts. Enzyme reaction was performed at 30°C for 20min and stopped by adding 1.5% phosphoric acid. The reaction solutions were loaded onto the wells positioned at the center of 0.8% agarose gel and electrophoresed in order to separate phosphorylated and non-phosphorylated peptide substrates based upon the net charge difference. The resultant bands shown under a UV illuminator were excised, and resolved for fluorometric or spectrometric detection at 570nm.

Thin Layer Chromatography

Ethylether/toluene (1:1) was used as a solvent for the separation of hydrophobic compounds on the TLC plate of Alugram Sil G/UV_{254}. After samples were separated and dried, bands were shown under UV light and R_f values were estimated. To obtain fractions, the resultant spots were scratched from the plate, extracted with ethanol, evaporated at reduced pressure and used for further studies. R_f values of coumarin standards, such as angelicin, scopoletin, umbelliferone, and xanthotoxin were compared with the fractions from *Angelica gigas* extracts.

Determination of Cytotoxicity

To determine the cytotoxicity of extracts on human cancer cell lines, initially 5×10^4 cells/ml of cells were incubated for 1 day, then treated with F3 fraction, and further cultured for 3 days. Cytotoxicity was then determined using ED_{50}, the concentration of samples displaying 50% survival compared to the untreated control. Viable cells were counted indirectly by SRB method using a 96 well-plate (23).

RESULTS AND DISCUSSION

Primary Screening for Anticancer Drugs

The anticancer activity of oriental medicines which are known to have hematopoietic effect was tested, based upon the morphological change of K-562 cells. When K-562 cells were treated with phorbol ester which is generally known as a potent tumor-promoting agent, the membrane surface of K-562 cells was transformed to megakaryocytic morphology (8). This morphological change, sometimes referred to as the bleb formation, seems to reflect an initial cancer-forming step by way of abnormal protein kinase C activation because the intracellular acceptor for phorbol ester is protein kinase C. Therefore bleb inhibition assay could be used for the screening of PKC inhibitors or anticancer drugs (16). Among herbs frequently used in oriental medicine, Korean angelica root (*Angelica gigas*), rhemannia root (*Rhemannia gluticosa*), paeony root (*Paeonia lactiflora*), and cnidium rhizome (*Cnidium officinale*) have been

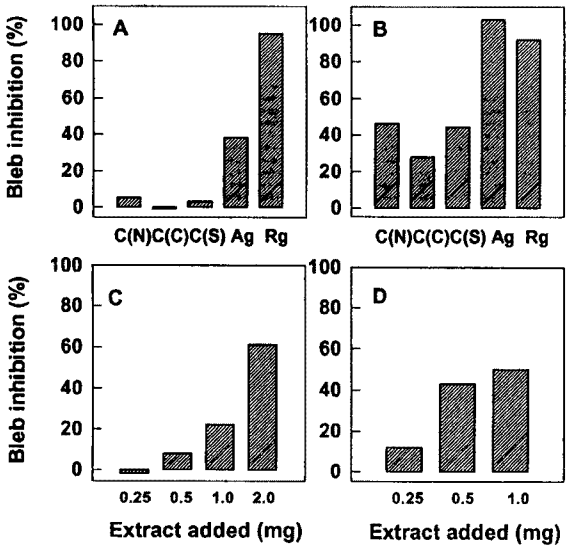

Fig. 1. Bleb inhibition of the aqueous and EtOH extracts from *Cornu cervi, Angelica gigas* and *Rhemannia gluticosa* on K-562 cells.

A: Aqueous extract. B: EtOH extract. C: EtOH extract of *Angelica gigas* D: EtOH extract of *Rhemannia gluticosa*

C(N), *Cornu cervi* from New Zealand; C(C), *Cornu cervi* from China; C(S), *Cornu cervi* from Siberia; Ag, *Angelica gigas;* Rg, *Rhemannia gluticosa*

used to treat anemia and deer antlers (*Cornu cervi*) have frequently been used as a supplement. Therefore if a novel anticancer drug could be developed from these herbs, it may be possible to overcome the side effects of conventional anticancer drugs. Here we tested the bleb-inhibiting effect of water- and ethanol-extracts of *Angelica gigas, Rhemannia gluticosa,* and three kinds of *Cornu cervi.* The results showed that ethanol extracts of *Angelica gigas* and *Rhemannia gluticosa* inhibited completely the bleb-forming activity of phorbol ester on K-562 cells (Fig. 1A) and the aqueous extract of *Rhemannia gluticosa* inhibited the bleb formation as well (Fig. 1B). In some leukemic cells such as K-562 and HL-60, phorbol ester induces blebs on the plasma membrane and activates PKC in early stage (12, 24). Therefore a substance which can block bleb-forming activity is likely to modulate PKC activity. In fact, the higher the concentration of ethanol extract of *Angelica gigas* and *Rhemannia gluticosa* used, the higher the bleb-inhibiting activity was seen to be (Fig. 1C, D). The antagonistic activity of the extract against phorbol ester was confirmed in the addition of the ethanol extract following the phorbol ester treatment as well as in the simultaneous addition of the ethanol extract and phorbol ester (data not shown). These results indicated that substances competing with phorbol ester may present in the ethanol extract of *Angelica gigas* and *Rhemannia gluticosa.*

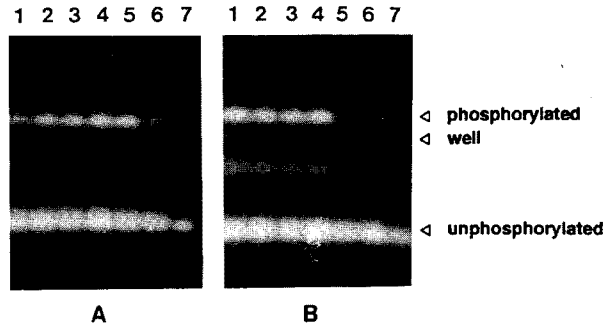

Fig. 2. Protein kinase C assay by agarose gel electrophoresis. After the enzyme reaction, the unphosphorylated peptide and phosphorylated peptide as a sustrate and a product, respectively, were separated by electrophoresis.

A: Reactants of PKC with phosphatidylserine. Lane 1-5, each *Angelica gigas* EtOH extract of 0.1, 0.2, 0.3, 0.4, and 0.5mg was added into the 40 μl reaction mixture respectively; Lane 6, control; Lane 7, sub strate peptide unreacted.

B: Reactants of PKC without phosphatidylserine; Lane 1-4, each *Angelica gigas* EtOH extract of 0.2, 0.3, 0.4, and 0.5mg respectively; Lane 5, PDBu 0.1 μg/ml; Lane 6, staurosporine 1nM; Lane 7, control.

Effect of the Ethanol Extract of *Angelica gigas* on PKC Activity

To estimate the effect of ethanol extracts of *Angelica gigas* and *Rhemannia gluticosa* on PKC activity *in vitro,* a non-isotopic PKC assay kit (Promega, Madison, WI, U.S.A.) was used, which contained protein kinase C isolated from rat brain and fluorescent-tagging peptide (P-L-S-R-T-L-S-V-A-A-K) as a substrate. Surprisingly *Angelica gigas* ethanol extract activated PKC activity potently *in vitro* at the same concentration as in complete bleb-inhibition on K-562 (Fig. 2), but *Rhemannia gluticosa* extract inhibited PKC activity (data not shown). Futhermore PKC activity was activated whether phosphatidylserine was added (Fig. 2A) or not (Fig. 2B). Both PDBu, a PKC activator, and staurosporine, a PKC inhibitor, were tested simultaneously as controls (Fig. 2B).

Up to date, many modulators of PKC activity have been found from natural products (4). Among the modulators, PKC inhibitors have recently been focused on in the development of novel anticancer drugs because of their abilities to reduce the multi-drug resistance (MDR) (21, 22). On the other hand, PKC activators like phorbol esters have long been recognized as carcinogen but interestingly bryostatin, a PKC activator isolated from a *bryozoan,* showed potent anticancer activity (17). In *Angelica gigas* ethanol extract, there may exist an anticancer compound similar to bryostatin which displayed potent anticancer activity by way of activating PKC by different mechanism from phorbol esters (4, 7, 11, 14).

TLC Fraction with PKC Modulation Activity

In Korean angelica (*Angelica gigas* Nakai) ethanol extract, the main components are coumarins, a kind of

Table 1. Bleb-inhibition of the fraction of *Angelica gigas* on K-562 cells.

Fractions/standards		R_f	Bleb-inhibition*
Fractions from *Angelica gigas*	F1	0.79	-
	F2	0.74	-
	F3	0.64	+++
	F4	0.55	-
	F5	0.47	-
	F6	0.40	-
	F7	0.33	-
	F8	0.28	-
	F9	0.19	-
Total EtOH Extract			+++
Staurosporine			+++
Standards from *Angelica officinale*	Xanthotoxin	0.53	-
	Scopoletin	0.26	-
	Umbelliferone	0.41	-
	Angelicin	0.66	-

*+++, more than 95%; -, less than 20%

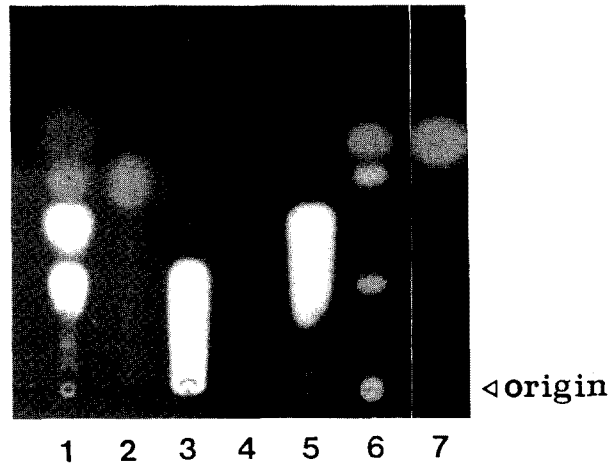

Fig. 3. Thin layer chromatogram of the EtOH extract of *Angelica gigas* and standard coumarins of *Angelica officinale*. Lane 1, Standard coumarin mixture of *Angelica officinale*; Lane 2, Xanthotoxin; Lane 3, Scopoletin; Lane 4, Angelicin; Lane 5, Umbelliferone; Lane 6, *Angelica gigas* EtOH extract; Lane 7, Isolated F3 fraction from Lane 6.

Table 2. ED_{50} of F3 fraction on human cell lines.

Cell lines or strain	ED_{50} (μg/ml)
HeLa S_3 (Human cervical carcinoma)	8
K-562 (Human myelogenous leukemia)	16
Hep G_2 (Human hepatocellular carcinoma)	9
MRC-5 (Human lung fibroblast)	43
Vero (Monkey kidney fibroblast)	42

terpenoids (2). In order to acquire the anticancer fraction, the *Angelica gigas* ethanol extract and coumarin standards from European angelica (*Angelica officinale* Koch) were chromatographed on TLC plate. Out of nine bands only one major band (F3) had predominant bleb-inhibition activity and its R_f value was 0.64 (Table 1). This fraction (F3 fraction) showed only one peak in HPLC (data not shown) and was different from any of the components of *Angelica officinale* (Fig. 3), of which coumarins were well been characterized (26). This result is consistent with the previous report (20), which suggested that *Angelica gigas* was originated separately from European (*Angelica officinale* Koch), Japanese (*Angelica acutiloba* Kitagawa) or Chinese angelica (*Angelica sinensis* Diels) and that its main components were quite different from them.

Cytotoxicity against Human Cancer Cell Lines

Based upon the result that *Angelica gigas* ethanol extract and the F3 fraction on TLC had PKC modulation activity, which is indirectly estimated by the bleb-inhibition test, we checked the cytotoxicity of the F3 fraction against several human cancer cell lines. The cytotoxicity index, ED_{50}, is defined as the concentration at which viable cells become half of the untreated controls after a 3-day-incubation period. ED_{50} values of F3 fraction against HeLa S_3, Hep G_2, and K-562 cells turned out to be 8, 9, and 16 μg/ml respectively. On the other hand, ED_{50} values against MRC-5, normal lung fibroblast, and Vero, monkey kidney fibroblast, were determined to be over 40 μg/ml (Table 2). The low cytotoxicity of the F3 fraction against normal cells indicates that the fraction can be used as a candidate for a novel anticancer drug. The molecular structure of the fraction is under investigation.

The F3 fraction of the root of *Angelica gigas* is very much similar to bryostatin in that they are both PKC activators and demonstrate anticancer activity. Bryostatin, which has been known as a unique compound that showed characteristics of PKC activation and anticancer activity, has been increasingly focused on. The mechanism of action of bryostatin is under investigation compared with other PKC activators (7, 11, 14). The potential of bryostatin as an anticancer drug is under clinical evaluation, and the result of phase I clinical trial of bryostatin seems to be promising (18). Therefore the F3 fraction of *Angelica gigas* which shows similar characteristics to bryostatin has strong potential as a novel anticancer drug.

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