We have recently reported that inhibition of tumor necrosis factor-α (TNF-α) release is a useful method for screening of cancer preventive agents,1) because TNF-α and other inflammatory cytokines stimulate tumor promotion and progression of initiated cells as well as premalignant cells.2,3) Since TNF-α and interleukin-1 (IL-1), as endogenous tumor promoters, activate nuclear factor κB (NF-κB),4,5) inhibitors of NF-κB activation are also assumed to be cancer preventive agents.6) However, in a practical sense, it is quantitatively easier to measure inhibition of TNF-α release from cells treated with a tumor promoter such as okadaic acid than inhibition of NF-κB activation using a gel mobility shift assay.7)

Based on the study of green tea as cancer preventive agent in the United States8) and as cancer preventive beverage in Japan,9) we decided to take a closer look at Japanese herbal medicine for other likely cancer preventives. One classical Japanese herb, *Geranium thunbergii* Sieb. et Zucc. (Genno-shoko in Japanese), is used as an antidiarrheic10); it contains geraniin, which has been intensively studied as a representative tannin.11) And the bark of *Acer nikoense* (Megusurino-ki in Japanese), which contains diarylheptanoids, is sold as a folk medicine for eye and liver diseases in Japan.12) The Forestry Experiment Station of Saitama Prefecture succeeded in culturing young plants of *A. nikoense* collaborated with us on the scientific utilization of the plant. We received four water extracts—leaves, bark, small branches, and timber—from the Station and subjected them to inhibition of TNF-α release assay using BALB/3T3 cells treated with okadaic acid 13): The leaves showed the strongest activity among the four extracts. Each extract was subjected to high-performance liquid chromatography (HPLC) with CAPCELL PAK C18, and geraniin and corilagin were identified in the active leaf extract (Fig. 1).

In 1980, Okuda et al. reported that geraniin is the main

---

**New TNF-α Releasing Inhibitors, Geraniin and Corilagin, in Leaves of Acer nikoense, Megusurino-ki**

Sachiko Okabe, a Masami Suganuma, a Yoko Imayoshi, b Shoko Taniguchi, b Takashi Yoshida, b and Hirota Fujiki,* a

Saitama Cancer Center, a Ina, Kitadachi-gun, Saitama 362–0806, Japan and Faculty of Pharmaceutical Sciences, Okayama University, b Tsushima, Okayama 700–8530, Japan. Received May 28, 2001; accepted July 12, 2001

The success of green tea as a cancer preventive is based on evidence that green tea contains tannins and antioxidants, does not show toxicity in humans and has long traditional use in Asia. In the light of this, herbal medicines are now also attracting attention as potential sources of cancer preventive agents. Using the inhibition of TNF-α release assay, we studied Acer nikoense (Megusurino-ki in Japanese), one of the herbal medicines. The inhibitory activity of TNF-α release was found in the leaf extract rather than the bark extract, and the main active constituents were identified as geraniin and corilagin, which are present in another Japanese traditional herb, *Geranium thunbergii* (Genno-shoko). The IC₅₀ values of TNF-α release inhibition were 43 μM for geraniin and 76 μM for corilagin, whereas that for (−)-epigallocatechin gallate (EGCG) was 26 μM. Treatment with geraniin prior to application of okadaic acid, a tumor promoter on mouse skin initiated with 7,12-dimethylbenz(a)anthracene, reduced the percentage of tumor-bearing mice from 80.0 to 40.0% and the average numbers of tumors per mouse from 3.8 to 1.1 in week 20. Thus, geraniin has slightly weaker inhibitory activity than EGCG. Since geraniin and corilagin have been well investigated as representative tannins, we discuss here the new possibility of classical herbal medicine in the development of preventive agents for cancer and other life-style related diseases.

Key words cancer chemoprevention; tannin; green tea, Genno-shoko; tumor promotion; Acer nikoense

---

![Fig. 1. Structures of Geraniin, Corilagin and (−)-Epigallocatechin Gallate (EGCG)](image-url)
tannin in several species of Geranium (Geraniaceae) and Euphorbia (Euphorbiaceae), and that hydrolysis of geraniin produces corilagin.\textsuperscript{14} In various biochemical and biological functions of tannins,\textsuperscript{15} geraniin and corilagin function in a manner similar to (−)-epigallocatechin gallate (EGCG), the main constituent of green tea polyphenols, and this led us to study geraniin as a cancer preventive agent. We found that, like EGCG, repeated topical applications of geraniin inhibited tumor promotion of okadaic acid in a two-stage carcinogenesis experiment on mouse skin. Based on these results, geraniin and the leaf extract of A. nikoense are proposed as new cancer preventive agents. This study will demonstrate that investigation of Japanese herbal medicine can lead to breakthroughs in cancer chemoprevention.

**MATERIALS AND METHODS**

**Extraction** Leaves (6 g), bark (6 g), small branches (6 g), and timber (12 g) were each separately boiled with 300 ml distilled water for 10 min. After cooling to room temperature, the extracts were each filtered with gauze and then stored in a refrigerator (4 °C). All extracts were obtained from the Forestry Experiment Station of Saitama Prefecture.

**Inhibition of TNF-α Release from BALB/3T3 Cells** BALB/3T3 cells (2×10⁵/0.5 ml) were preincubated with lyophilized material (10—500 μg) of various extracts for 1 h, and then treated with 0.2 μM okadaic acid for another 24 h. Concentration of TNF-α in medium was determined by ELISA (Genzyme, MA, U.S.A.), as described previously.\textsuperscript{13}

**Determination of Geraniin and Corilagin by HPLC** Reversed-phase HPLC was performed on a CAPCELL PAK C18 UG120 column (4.6×250 mm) using UV detector (280 nm). The mobile phase used was a mixture of 0.1 mM H₃PO₄ : 0.1 mM KH₂PO₄ : CH₃CN (9 : 9 : 2) at a flow rate of 1.0 ml/min at 40 °C. Contents of geraniin and corilagin in extracts were determined by comparison of each peak area of the HPLC pattern with that of standard specimens. Compounds used for the experiments were obtained from Gera-nium thunbergii\textsuperscript{13} and recrystallized from CH₃CN–H₂O and H₂O, respectively.

**Inhibition of Tumor Promotion on CD-1 Mouse Skin** Inhibition of tumor promotion in a two-stage carcinogenesis experiment on mouse skin was conducted as described previously.\textsuperscript{16} Initiation was achieved by a single topical application of 100 μg of 7,12-dimethylbenz(α)anthracene (DMBA), and tumor promotion was conducted by repeated applications of okadaic acid (1.0 μg/0.1 ml), twice a week. Geraniin (5 mg per application) was applied topically 15 min before each application of okadaic acid. Inhibition of tumor promotion was estimated by decrease in percentage of tumor-bearing mice and in average numbers of tumor per mouse. CD-1 female mice, 7 weeks old, were obtained from Charles River Japan Inc. (Kanagawa), and each experimental group consisted of 10 mice.

**RESULTS AND DISCUSSION**

**Inhibition of TNF-α Release from BALB/3T3 Cells** The tumor promoter okadaic acid induces TNF-α release from BALB/3T3 cells mediated through NF-κB activation.\textsuperscript{13} We previously reported that pretreatment with various cancer preventive agents, such as EGCG, sulindac or tamoxifen, inhibited TNF-α release with IC₅₀ values of 5.8 to 28 μM.\textsuperscript{11} In this experiment, lyophilized extracts of leaves, bark, small branches, and timber of A. nikoense were subjected to inhibition of TNF-α release assay (Fig. 2): The leaf extract inhibited TNF-α release most strongly, with IC₅₀ value of 260 μg/ml; the other three extracts did not inhibit it significantly (Table 1). The activity of the leaf extract suggested that its constituents were different from the other three extracts. In fact, the constituents in the leaves and bark of A. nikoense are known to be clearly distinct. Various investigators reported that the leaves contain β-amyrin, β-amyrin acetate, β-sitosteryl glucoside, quercetin, quercitrin, ellagic acid, geraniin and elaeocarpusin; the bark, scopoletin, (+)-rhododendrol, cleomiscosin, aquillochin, acerogenin, aceroseide, β-sitosterol and β-sitosterol glucoside.\textsuperscript{12,17,18} The most distinguishable feature of the constituents between the leaves and bark of A. nikoense was that leaves are rich in geraniin and corilagin,\textsuperscript{19} which are tannins identical to those isolated from G. thunbergii (Genno-shoko).\textsuperscript{10} With this in mind, we next subjected the lyophilized extracts to HPLC.

**Determination of Geraniin and Corilagin by HPLC** The leaf extract contained geraniin and corilagin as the main constituents (Table 1). Since the leaves were extracted in boiling water for 10 min, we assume that geraniin is easily hydrolyzed to corilagin, since the corilagin content was much higher than that of geraniin in the leaf extract, which also contained small amounts of elaeocarpusin and ellagic acid (data not shown). The other three extracts (bark, small branches, and timber) contained far smaller amounts of geraniin and corilagin.

**Inhibition of TNF-α Release by Geraniin and Corilag-
BALB/3T3 cells dose-dependently, with IC50 values of 26 μM for EGCG, geraniin, and corilagin showed 74, 42 and 34% inhibition, respectively, at a concentration of 5 μM. The following should also be noted: Geraniin is quickly hydrolysed during hot water extraction of the herb, while corilagin is relatively stable,14) seasonal variations affect the content of geraniin and corilagin; and the leaves of A. nikoense are more efficacious than the bark now commercially sold. In summary, our screening for cancer preventive agents found that the main constituents of A. nikoense leaf extract are active compounds—geraniin and corilagin—that inhibited TNF-α release. This study reevaluates the significance of geraniin, long known to be a representative tannin, as a new cancer preventive agent. More important, these results with traditional herbal medicine could open the door to a new frontier in the search for preventive agents of cancer and other lifestyle-related diseases.20)

Acknowledgments This work was supported by the following Grants-in-Aid: for Cancer Research from the Ministry of Health and Welfare, Scientific Research on Priority Areas for Cancer Research from the Ministry of Education, Science, Sports and Culture, Japan: for a Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health and Welfare, of Japan: for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare, Japan: for Selectively Applied and Developed Research from Saitama Prefecture, Japan: and by the Smoking Research Fund. We thank Yoshiaki Kitaoka at the Department of Agriculture and Forestry, Saitama Prefecture and Minoru Uchida and Masashi Nakamura at the Forestry Experiment Station of Saitama Prefecture for their collaboration. We also express our thanks to Dr. Takuo Okuda for his fruitful discussion.

REFERENCES