# HPLC Analysis of Juzen-taiho-to and Its Variant Formulations and Their Antimetastatic Efficacies

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**Our previous study demonstrated that the oral administration of Juzen-taiho-to resulted in a significant inhibition of the liver metastasis of colon 26-L5 cells as compared with the untreated control, without side effects. We attempted to investigate the relationship between the HPLC pattern (referred to as the fingerprint) of the formulation and its component crude drugs and the inhibition of tumor metastasis in order to obtain the optimal efficacy and constant quality of the formulation. Two Juzen-taiho-to formulations (batches #1 and # 2), which were individually prepared using the same 10 crude drugs and the same preparation procedure, showed similar anti-metastatic effects and absorbance patterns by HPLC analysis. Some variant formulations of Juzen-taiho-to, in which one crude drug was substituted with other crude drugs from different sources or places of origin, exhibited reduced efficacy as compared with the original formulation, as well as differences in the fingerprint pattern compared with the original formulation. Juzen (Naimo-Ogi→Kibana-Ogi), a variant formulation with the substitution of Astragali radix of a different origin and place of harvest, showed significant inhibition of the liver metastasis of tumor cells and a HPLC fingerprint pattern similar to that of the original formulation. Thus, HPLC fingerprint analysis of Kampo medicines may provide a useful basis for obtaining their optimal efficacy as well as constant quality of the formulation, although it has some problems and limitations, such as detectability by and sensitivity to UV absorbance.**

**Key words** Juzen-taiho-to; variant formulation; liver metastasis; colon 26-L5; HPLC pattern analysis

Juzen-taiho-to, a Kampo Japanese herbal medicine, is a nourishing agent, so-called "ho-zai" (in Japanese), for improving disturbances and imbalances in the homeostatic condition of the body as diagnosed by Kampo medicine. It is administered to patients in various weakened conditions, such as post-surgery patients and patients with chronic illnesses. The general symptoms it can alleviate are extreme fatigue, pale complexion, loss of appetite, dry or scaly skin, night sweating, and dryness of the mouth. Recently, it has often been administered to cancer patients. Several studies have shown that Juzen-taiho-to has various biological activities: namely, the enhancement of phagocytosis,[1)] cytokine induction,[2,3)] antibody production,[4)] induction of mitogenic activity of spleen cells,[5)] an anti-tumor effect when combined with surgical excision,[6)] augmentation of anti-tumor activities with or without other drugs,[7,8)] and protection from the deleterious effects of anti-cancer drugs[9)] as well as radiation-induced immunosuppression and bone marrow toxicity.[10,11)]

We have previously reported that Juzen-taiho-to effectively prevented weakly malignant QR-32 fibrosarcoma cells from growing progressively upon co-implantation with a gelatin sponge, and that it may act to induce antioxidative substances, in addition to augmenting host-mediated immune responses.[12)] Also, the oral administration of Juzen-taiho-to inhibited the liver metastasis produced by an intraportal injection of the liver-metastatic variant of murine colon 26 carcinoma (colon 26-L5) cells in a dose-dependent manner,[13,14)] and enhanced the survival rate through the activation of macrophages and T-cells rather than NK cells in the immune system.[13)]

On the other hand, since Kampo formulations are complex mixtures of natural crude drugs, it is necessary to control the quality of the formulations and their component crude drugs and also their processing procedures to obtain reproducibility of the formulation and efficacy. However, to our knowledge, these problems have not been studied in detail, although much information about Kampo formulations and their constituents is available in Japanese and Chinese archaic writings. In the present study, we attempted to analyze the HPLC pattern of Juzen-taiho-to and its variant formulations, in which one crude drug in Juzen-taiho-to was substituted with related crude drugs. We thereby tried to confirm the preparation of a constant formulation. We also investigated the inhibitory effect of the various formulations on the liver metastasis of colon 26-L5 carcinoma cells in order to analyze the relationship between the constituents of formulations and their efficacy.

## Materials and Methods

Preparation of Juzen-taiho-to and Its Variant Formulations Juzen-taiho-to (TJ-48), obtained from Tsumura & Co., Ltd., Tokyo, is composed of ten crude drugs (Table 1), of which the quality is controlled by Japanese Pharmacopoeia XIII. Juzen-taiho-to was prepared as follows: a mixture of Astragali Radix (3.0 g), Cinnamomi Cortex (3.0 g), Rehmanniae Radix (3.0 g), Paeoniae Radix (3.0 g), Cnidii Rhizoma (3.0 g), Atractylodis Lanceae Rhizoma (3.0 g), Angelicae Radix (3.0 g), Ginseng Radix (3.0 g), Hoelen (3.0 g), and Glycyrrhizae Radix (1.5 g) was added to 285 ml of water and extracted at 100 °C for 1 h. The extracted solution was filtrated and spray-dried to obtain a dry extract powder (2.3 g). Variant formulations of Juzen-taiho-to, in which one crude drug in Juzen-taiho-to was substituted with related crude drugs of different botanical origin or harvesting place (Table 2), were also prepared by the same procedures described above. The extracted powders were dissolved in distilled water and administered orally to mice at the appropriate dose for 7 d before tumor inoculation.

**Analysis of Juzen-taiho-to and Its Component Crude Drugs by HPLC** We carried out HPLC pattern analysis of the formulation in order to assess the constancy of the formulation. Juzen-taiho-to of the dosage described in Table 1 was extracted with $H_2O$–EtOH (9 : 1, 200 ml), filtered, and analyzed by HPLC (HP-1090, Hewlett–Packard) under the following conditions (Fig.

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1): Column, TSK gel 80 Ts octadecyl silica (ODS) (4.6×250 mm); mobile phase, 10 mM phosphoric acid: $CH_3CN$ (linear gradient, 95 : 5→20 : 80, for 1 h); flow rate, 0.8 ml/min; oven temperature, 40 °C; injection volume, 5 $\mu$l. Substituted crude drugs of Juzen-taiho-to were pulverized, and the pulverized material (500 mg) was refluxed with 250 ml of water for 1 h. The solution was filtered and analyzed by HPLC in a similar way. The HPLC profiles are from a single monitor (220 nm) and contour plot (190—400 nm) using a photo-diode array system as a detector.

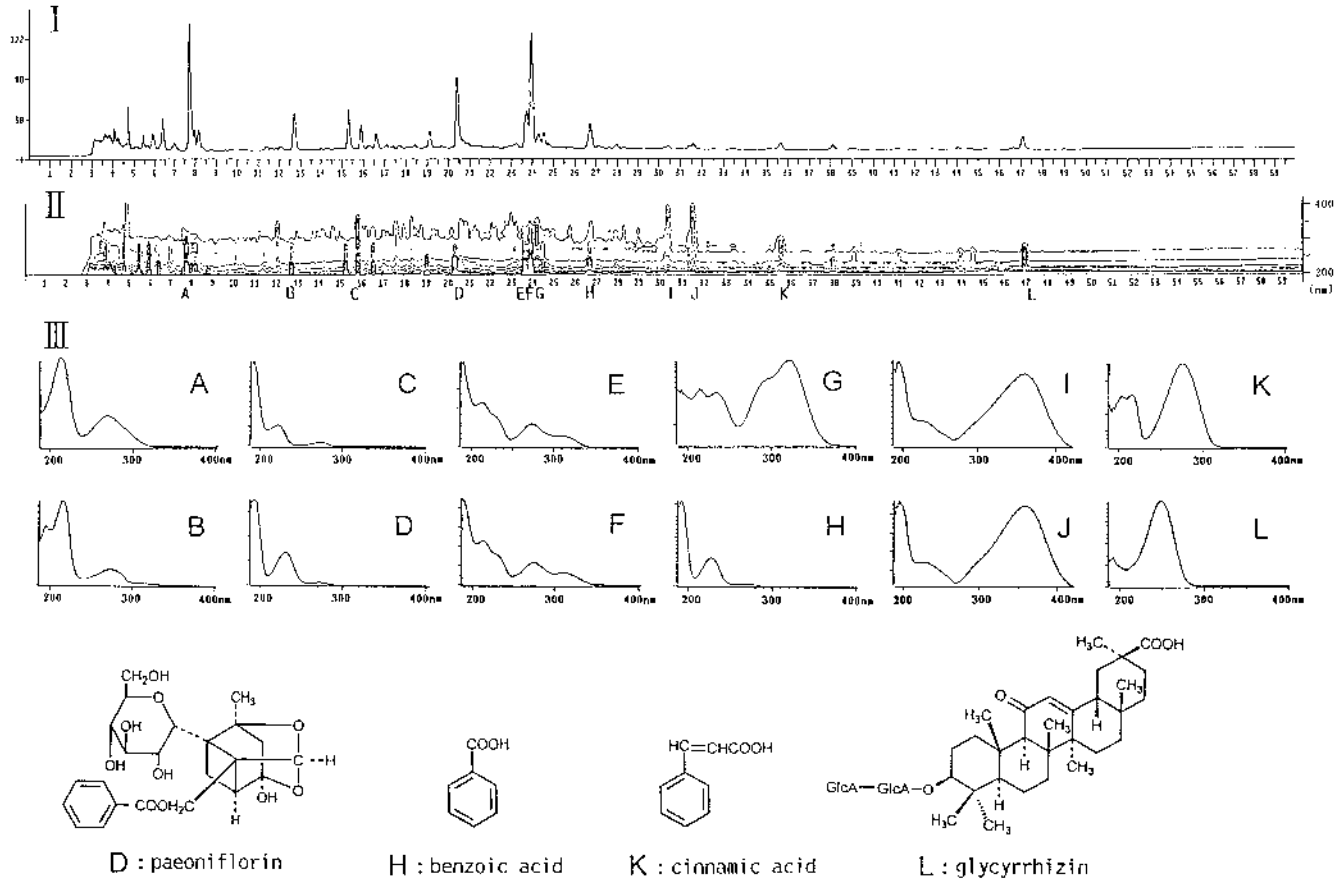


Fig. 1. HPLC Profile of Juzen-taiho-to and UV Spectra of Its Constituent Crude Drugs

I: HPLC pattern analyzed by absorbance at 220 nm. II: Contour plot of HPLC pattern by UV absorbance (190—400 nm). III: UV spectra of main peaks, origins of peak [A: Angelicae Radix, Ginseng Radix; B: Angelicae Radix; C: Atractylodis Lanceae Rhizoma, Angelicae Radix; D: Paeoniae Radix(paeoniflorin); E: Astragali Radix, Glycyrrhizae Radix; F: Glycyrrhizae Radix, Paeoniae Radix; G: Cnidii Radix, Angelicae Radix, H: Paeoniae Radix (benzoic acid); I: Glycyrrhizae Radix, Atractylodis Lanceae Rhizoma; J: Glycyrrhizae Radix; K: Cinnamomi Cortex (cinnamic acid); L: Glycyrrhizae Radix (glycyrrhizin)].

Table 1. The Botanical Origins and Harvesting Seasons of Crude Drugs of Juzen-taiho-to

| Crude drugs | Japanese name | Botanical origin[a] (Family name) | Harvesting | Place | Ratio (g) |
|---|---|---|---|---|---|
| Astragali Radix | Ogi | *Astragalus mongholicus* Bunge[b] or *A. membranaceus* Bunge (Leguminosae) | Autumn | China | 3.0 |
| Cinnamomi Cortex | Keihi | *Cinnamomum cassia* Blume[b] or other species of the same genus (Lauraceae) | Mainly autumn | China | 3.0 |
| Rehmanniae Radix | Jio | *Rehmannia glutinosa* Liboschitz[b] or *R. glutinosa* var. Liboschitz var. *purpurea* Makino (Scrophulariaceae) | Autumn | China | 3.0 |
| Paeoniae Radix | Shakuyaku | *Paeonia lactiflora* Palla[b] or allied plants (Paeoniaceae) | Autumn | China | 3.0 |
| Cnidii Rhizoma | Senkyu | *Cnidium officinale* Makino[b] (Umbelliferae) | Summer | Japan | 3.0 |
| Angelicae Radix | Toki | *Angelica acutiloba* Kitagawa[b] or allied plants (Umbelliferae) | Autumn | Japan | 3.0 |
| Atractylodis Lanceae Rhizoma or Atractylodis Rhizoma | Sojutsu Byakujutsu | *Atractylodes lancea* De Candolle[b] or *A. chinensis* Koidzumi (Compositae) *Atractylodes japonica* Koidzumi *et* Kitamura or *A. ovata* De Candolle | Autumn | China | 3.0 |
| Ginseng Radix | Ninjin | *Panax ginseng* C.A. Meyer[b] (Araliaceae) | Autumn | China | 3.0 |
| Hoelen | Bukuryo | The sclerotium of *Poria cocos* Wolf[b] (Polyporaceae) | Autumn to winter | China | 3.0 |
| Glycyrrhizae Radix | Kanzo | *Glycyrrhiza uralensis* Fischier[b], *G. grabra* Linne, or other species of the same genus (Leguminosae) | Autumn | China | 1.5 |

*a*) The botanical origins are based on Japanese Pharmacopoeia XIII. *b*) These crude drugs were used in the Juzen-taiho-to (TJ-48).

Table 2. Variant Formulations of Juzen-taiho-to and the Substituted Crude Drugs

| Juzen-taiho-to and its variant formulations[a)] | Crude drugs | | Botanical origin | Harvesting | Place |
|---|---|---|---|---|---|
| | | Substituted with: | | | |
| Juzen-taiho-to | Atractylodis Lanceae Rhizoma (Sojutsu) | | *Atractylodes lancea* De Candolle (Hosoba-Okera) | Autumn | China |
| Juzen (Sojutsu→Byakujutsu/Okera) | | Atractylodis Rhizoma (Byakujutsu) | *Atractylodes japonica* Koizumi *et* Kitamura (Okera) | Autumn | Korea |
| Juzen (Sojutsu→Byakujutsu/Obana-Okera) | | Atractylodis Rhizoma (Byakujutsu) | *Atractylodes obata* De Candolle (Obana-Okera) | Autumn | China |
| Juzen-taiho-to | Angelicae Radix (Toki) | | *Angelica acutiloba* Kitagawa (Yamato-Toki) | Autumn | Japan (Nara) |
| Juzen (Yamato-Toki→Hokkai-Toki) | | Angelicae Radix (Toki) | *Angelica acutiloba* Kitagawa var. *sugiyamae* Hikino (Hokkai-Toki) | Autumn | Japan (Hokkaido) |
| Juzen-taiho-to | Astragali Radix (Ogi) | | *Astragalus mongholicus* Bunge (Naimo-Ogi) | Autumn | China |
| Juzen (Naimo-Ogi→Kibana-Ogi) | | Astragali Radix (Ogi) | *Astragalus membranaceus* Bunge (Kibana-Ogi) | Autumn | Japan |
| Juzen (Naimo-Ogi→Shingi) | | Hedysari Radix[b)] (Shingi) | *Hedysarum polybotrys* Hand.-Mazz | Autumn | China |

*a*) A crude drug (Japanese name) in Juzen-taiho-to was substituted with the related crude drug as indicated. *b*) This is not listed in Japanese Pharmacopoeia XIII, but is sometimes used in Japan (based on some other regulation). ( ) Japanese name.

**Animals** Specific pathogen-free female BALB/c mice, 6 weeks old, were purchased from Japan SLC, Hamamatsu, Japan. The mice were maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar airflow conditions. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Tumor Cells** The liver metastatic cell line of the colon 26 carcinoma (colon 26-L5) was obtained by the *in vivo* selection method of Fidler.[15,16] Colon 26-L5 cells were maintained as monolayer cultures in RPMI-1640 supplemented with 7.5% fetal bovine serum (FBS) and 2 mM L-glutamine.

**Assay for Experimental Liver Metastasis of Tumor Cells** Log-phase cell cultures of colon 26-L5 cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI-1640, and resuspended at appropriate concentrations in PBS. BALB/c mice under ether anesthesia underwent laparotomy by an upper median incision, the duodenal loop was exposed, and an injection of colon 26-L5 ($1—2\times10^4$/200 $\mu$l) cells was made into the portal vein through a 29-gauge needle attached to a 1-ml syringe. A sterile absorbable cotton sponge was placed over the injection site as the needle was withdrawn to prevent bleeding and peritoneal dissemination of the tumor cells. The mice were killed 19 d after tumor inoculation, and the number of metastatic colonies in each liver was macroscopically counted. The liver weight was recorded to evaluate tumor metastasis, as previously described.[17,18]

**Statistical Analysis** Significance of differences between the groups was determined by the Mann–Whitney U-test.

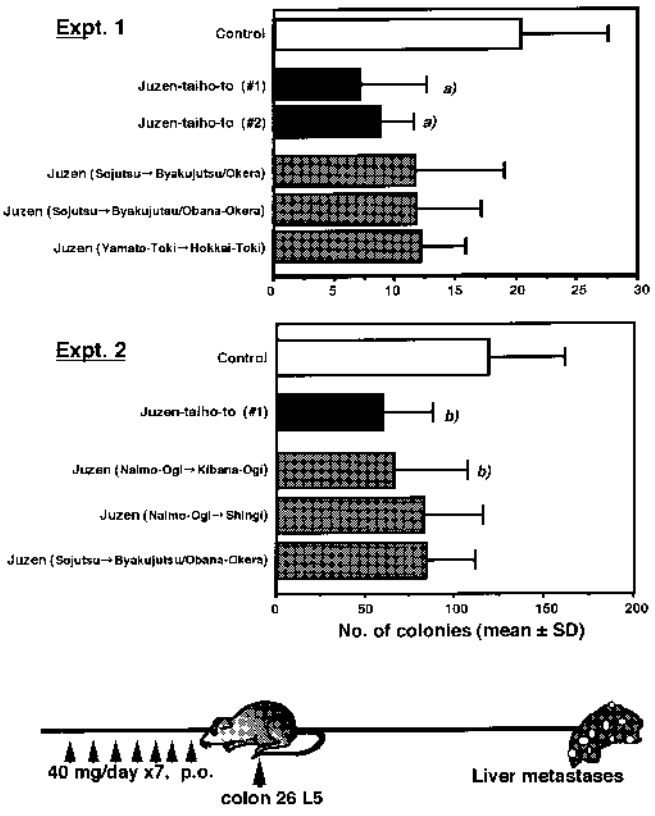


Fig. 2. Effect of Oral Administration of Juzen-taiho-to and Its Variant Formulations on Experimental Liver Metastasis Produced by the Intraportal Injection of Colon 26-L5 Carcinoma Cells

Five BALB/c mice per group were inoculated intraportally with colon 26-L5 cell ($1\times10^4$ in expt.1 or $2\times10^4$ in expt. 2). Juzen-taiho-to and its variant formulations (40 mg/mouse) were orally administered for 7 d before tumor inoculation. Nineteen days after tumor inoculation, mice were sacrificed and the number of liver colonies was manually counted. *a*), $p<0.01$; *b*), $p<0.05$ as compared to untreated controls.

## Results and Discussion

Herbal prescriptions, including Kampo medicines, have become recognized by the scientific medical system and have become increasingly popular. Since Kampo formulations are generally prepared from the combination of many crude drugs, they may have combined effects which differ from the sum of effects of the individual constituent crude drugs. They must have an acceptable efficacy and quality when used as therapeutic medicines. Formulations prepared from crude drugs with different qualities would have different biological activities and efficacies. Therefore, it is necessary to control and confirm the quality of the formulations and their constituent crude drugs, because their quality may vary with the origins of crude drugs and the place of harvest, *etc*. In Japan, the quality of individual crude drugs is controlled by Japanese Pharmacopoeia XIII, which regulates the botanical origin, the crude drug test of foreign matter, loss by drying, total ash, acid-insoluble ash, extract content, essential oil content, and microscopic examination.

For the purpose of obtaining proper formulations with constant quality and efficacy, we first attempted HPLC pattern analysis of Juzen-taiho-to by using their chemically de-

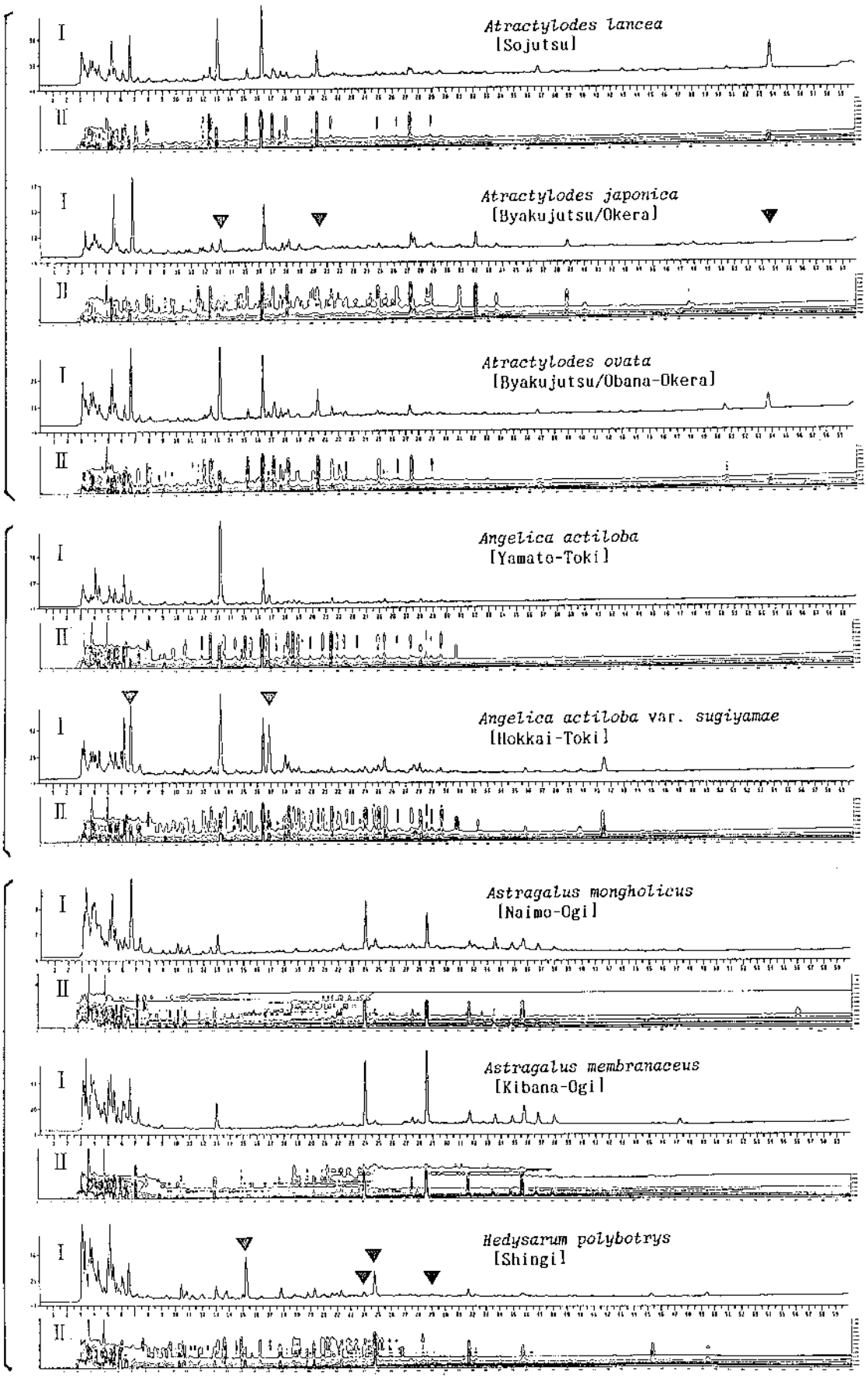


Fig. 3. HPLC Profile and UV Spectra of Substitutable Crude Drugs

I: HPLC pattern analyzed by absorbance at 220 nm. II: Contour plot of HPLC pattern by UV absorbance (190—400 nm). Some significant peaks were indicated by arrowheads on the chromatograms, compared with original standards.

fined components as standard references. Figure 1 shows the HPLC profiles of Juzen-taiho-to by single monitor (220 nm) and contour plot (190—400 nm) using a photo-diode array system as a detector. The contour plot of the UV absorbance intensity of the compounds shows all of the compounds which have detectable UV absorbance in the extracts from the formulation. The origin of each peak of Juzen-taiho-to was identified by comparison with the retention time and with the UV spectra of each extract of crude drug or the chemically defined standard compounds (A—L in Fig. 1). For example, the peaks of paeoniflorin from Paeoniae Radix and glycyrrhizin from Glycyrrhizae Radix were detected at the D and L positions of the contour plot in Fig. 1. Thus, in addition to testing whether the standard compounds have the pharmacological activity of inhibiting liver metastasis or not, this HPLC pattern analysis, the so-called "fingerprint" method, could provide a useful means of identifying the crude drugs and preparing batches of constant formulation. Although many compounds which have no UV absorbance can not be detected by this method, the fingerprint similarity of the formulation may be primarily useful in assessing the homogeneity of the formulation, which should lead to constant efficacy.

To evaluate the efficacy of thus prepared Juzen-taiho-to, we next examined the anti-metastatic effect of the oral administration of two Juzen-taiho-to formulations (batches #1 and #2) which were independently prepared using the same 10 crude drugs (Table 1) by the same procedure. The fingerprint analysis of the two batches of Juzen-taiho-to showed similar HPLC profiles to that in Fig. 1. Oral administration of the two Juzen-taiho-to preparations (batches #1 and #2) at the effective dose of 40 mg/day[13] significantly reduced the number of tumor colonies in the liver ($7.2\pm5.4$ and $9.3\pm3.3$ *vs*. $20.4\pm7.1$ of untreated control, respectively), and similar results were observed with both batches of Juzen-taiho-to formulation (Fig. 2, upper panel).

In the usage and preparation of Kampo formulations, some component crude drugs in the formulation are in some cases replaced with related crude drugs. To further examine the effect of the replacement of the original Juzen-taiho-to constituents with different crude drugs on the anti-metastatic action, we prepared variant formulations of Juzen-taiho-to in which one crude drug was substituted with related crude drugs from different sources or places of origin (Table 2). Juzen (Naimo-Ogi→Kibana-Ogi) as well as Juzen-taiho-to (#1) significantly inhibit liver metastasis as compared with the untreated control ($66.7\pm42.0$ and $63.3\pm28.6$ *vs*. $121.8\pm55.1$ of untreated control, respectively, in lower panel of Fig. 2), and also, the HPLC pattern of the root of *Astragalus membranaceus* [Kibana-Ogi] was very similar to that of *Astragalus mongholicus* [Naimo-Ogi] (Fig. 3). In contrast, Juzen (Sojutsu→Byakujutsu/Okera), Juzen (Sojutsu→Byakujutsu/Obana-Okera), Juzen (Yamato-Toki→Hokkai-Toki) and Juzen (Naimo-Ogi→Shingi) had a weaker anti-metastatic effect than the original Juzen-taiho-to (#1). Among these 4 variant formulations with reduced effects, there were some discernible differences in the HPLC fingerprint patterns between the original and substituted crude drugs, except for Atractylodis Rhizoma [Byakujutsu/Obana-Okera] *i.e.* the rhizome of *Atractylodes ovata* [Obana-Okera]. For example, some significant peaks were indicated by arrowheads on the chromatograms in comparison with original standards (Fig. 3). This suggests that the reduced effects of the variant formulations may be associated with the marked differences in the fingerprint pattern of the substituted crude drugs. However, Juzen (Sojutsu→Byakujutsu/Obana-Okera) was less effective in inhibiting the metastasis than the original Juzen-taiho-to ($84.1\pm27.1$ and $63.3\pm28.6$, respectively, in lower panel of Fig. 2), despite the similarity of the HPLC patterns of Sojutsu and Byakujutsu/Obana-Okera (Fig. 3). Therefore, other components in Byakujutsu/Obana-Okera which can not be detected by HPLC analysis, such as polysaccharides and peptides, may be responsible for the reduced efficacy of Juzen (Sojutsu→Byakujutsu/Obana-Okera). Further studies will be needed to examine these points in detail.

In conclusion, the present study demonstrated differential effects of variant formulations of Juzen-taiho-to on tumor metastasis. The reduced anti-metastatic effect of the variant formulations used in this study may be related to the difference in the fingerprint pattern between the original and substituted crude drugs. Thus, HPLC pattern analysis of Kampo medicines may provide a useful basis for obtaining their optimal efficacy as well as constant quality of the formulation, although such analysis has some problems and limitations.

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