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Studies on antitumor Chinese medicines (II) Antitumor constituents of *Stellera chamaejasme* L.

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Abstract

Methanol extracts of Rui-xiang-lang-du, a root of *Stellera chamaejasme* L., were assessed for antitumor activity against murine P388 leukemia *in vivo*, and the extracts were proved to be active in an i.p.-i.p. assay system. By further extraction with petroleum ether, followed by purification of column chromatography using Sephadex LH-20 and HPLC using ODS column, several antitumor constituents were isolated. One of these constituents was identified as Pimelea factor P_2, and it was found to be very active on P388 *in vivo*. Antitumor activity and cytotoxicity against leukemia L1210 *in vivo* and *in vitro* was also investigated.

Keywords Rui-xiang-lang-du, *Stellera chamaejasme* L., Pimelea factor P_2, antitumor activity, P388, L1210.

Abbreviations BDF_1, F_1 of C57BL/6 and DBA/2 mouse ; $CDCl_3$, deuterium chloroform ; DMSO, dimethylsufoxide ; HPLC, high performance liquid chromatography ; IC_{50}, 50 % inhibition concentration ; ILS, increase in life span ; i.p. intraperitoneally ; PBS, Phosphate buffer saline ; RPMI, Roswell Park Memorial Institute ; TLC, thin layer chromatography ; Roh-doku, Lang-du, 狼毒 ; Zuikoh-roh-doku, Rui-xiang-lang-du, 瑞香狼毒.

Introduction

Our studies on search for novel antitumor agents have been performed focusing on Chinese plants, especially traditional Chinese medicinal herbs which are reported to manifest some antitumor effects or are used in clinical practices, but without having yet clarified their active constituents. In the course of our studies, we found that a methanol extract of "Lang-du" (Roh-doku in Japanese) showed significant antitumor activity against murine lymphatic leukemia P388 *in vivo* by our antitumor screening bioassay. However, in traditional Chinese medicine, there are three kinds of species of plants which are called "Lang-du"; roots of *Stellera chamaejasme* L. (Thymelaeaceae), *Euphorbia fischeriana* STEUD. and *Euphorbia ebiateolata* HAYATA (both Euphorbiaceae).[1] Therefore, we attempted to investigate which has the better result on antitumor effects and what the antitumor principles are.

In this study, we investigated the antitumor effect of methanol extracts of *Stellera chamaejasme* L. and *Euphorbia fischeriana* Steud. and isolated several antitumor constituents from *Stellera chamaejasme* L.. We report herein the isolation and purification of one of the active compounds, a daphnane type diterpene, Pimelea factor P_2.

Materials and Methods

General experimental procedure : Open column chromatography using Sephadex LH - 20 (particle size : 25-100 um) was monitored with a Dual path monitor UV - 2 by UV 254 nm and fractionated by fraction collector FRAC - 300 (Pharmacia Fine Chemicals, Sweden) and MeOH was used as an eluting solvent. Thin layer chromatography (TLC) was performed on Merck RP-18 $F_{254}S$ (0.25×20 cm). High performance liquid chromatography (HPLC) was performed

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with JASCO Tri Rotar-VI (column: Intertsil ODS, 4.6×250 mm, Gasukuro Kogyo, Japan, flow rate: 1.0 ml/min, detection: UV 228 nm and Shima-pack Prep-ODS, 20×250 mm, Shimadzu, Japan, flow rate: 7.5 ml/min., detection: UV 228 nm). In both TLC and HPLC, MeOH : H_2O (10 : 1) was used as a developing solvent. UV spectra were obtained with Hitachi UV-2000 spectrophotometer in MeOH solution. FAB-Mass spectra was recorded by JEOL JMS-DX300. Proton nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were recorded with JEOL GSX-270.

Plant materials: In 1988 and 1989, about 60 kg of the dried root of *S. chamaejasme* L. and 2 kg of the dried root of *E. fischeriana* STEUD. were collected in Sichuan or Tibet, China. Identification of the plants collected was made by Beijing Institute for Control of Drugs, Beijing, China.

Extraction procedure: The roots (1 kg) of *S. chamaejasme* L. or *E. fischeriana* STEUD. were chopped and extracted with MeOH (10 ℓ) for 5 hr under refluxing, and the residuals were extracted once again under the same conditions. The extracts were combined and evaporated in vacuum to obtain MeOH extracts.

Fractionation procedure: The dried MeOH extract of *S. chamaejasme* L. (MM, 153.6 g) was further extracted with petroleum ether in Soxlet extractor for 10 hr to give the petroleum ether fraction (MMP, 15.8 g). One gram of MMP was subjected to open column chromatography (column 4 ×60 cm) on Sephadex LH-20 using MeOH as an eluting solvent. Part III (MMP-III) which is the next fraction closest to the second peak monitored by UV 245 nm absorption was isolated to concentrate the antitumor activity *in vivo* into this fraction (Fig. 1). When a fraction was collected (999 drops each), the fraction between Fr. No. 52 and 63 was yielded as MMP-III (Fig. 2).

Further purification was carried out by a combination of preparative TLC and HPLC. MMP-III was developed on reverse phase TLC plates using MeOH : H_2O (10 : 1) as a solvent and detected by fluorescence or iodine vapor. Then more than twelve bands were detected and among them, active substances giving bands Rf values

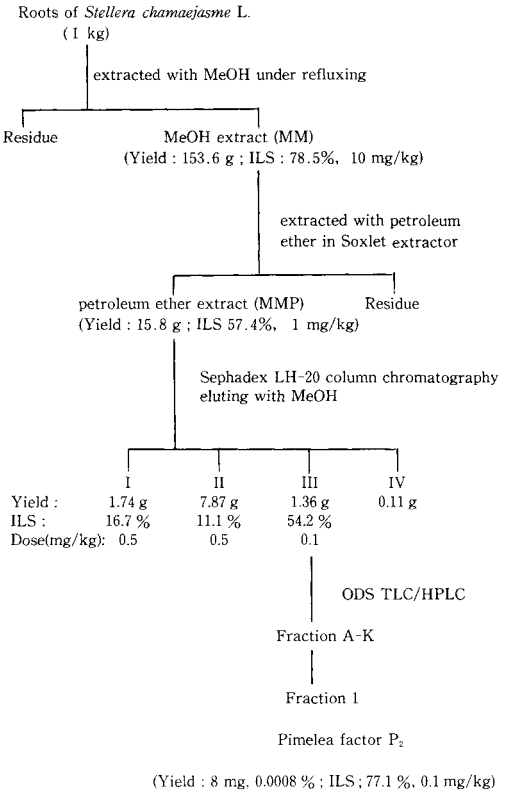

Fig. 1 Isolation procedure and antitumor activities on P388 of constituents of *Stellera chamaejasme* L.

around 0.25 and 0.35 were collected, and the powders of TLC plates were extracted with MeOH.

Finally the extracts were purified by HPLC developing with MeOH : H_2O (10 : 1) as a mobile phase. More than 11 peaks were obtained and isolated by this preparative HPLC, and the activity of each fraction was determined by *in vivo* bioassays. Fractions E, I and k were highly active by both *in vivo* and *in vitro* bioassays. Fraction I showing a band around Rf 0.25 on the TLC plate gave a peak having a retension time about 20 minutes in HPLC. It was further purified by repeated HPLC and isolated as a single compound. Chemical analysis of the compound thus obtained was made by UV, mass spectra, proton and ^{13}C-NMR spectra, and it was found to be identical with a daphnane type diterpene, Pimelea factor P_2. Its yield was approximately 0.0008 % from the original plant' s root.

Fraction E gave a band around Rf 0.35 on

TLC, its retention time in HPLC was about 9 minutes, and retention time of Fraction K was about 26 minutes. But both were isolated in very small amounts, in which Fraction E gave a yield of about 0.0001 % and Fraction K gave a yield of about 0.00005 % from the plant.

Characterization of Pimelea factor P_2 : $C_{37}H_{50}O_9$: FAB - Mass spectra m / z 638 ; UV λ_{max} (MeOH) 229 (ε 14400), 272 (ε 1260) ; ^{1}H - NMR (270MHz, $CDCl_3$) δ 0,83 (3H,d,J = 7Hz), 1.06 (3H,d, J = 7Hz), 1.44 (3H,d,J = 7Hz), 1.74 (3H,s), 2.89 (1H, d,J = 3Hz),3.11 (1H,d,J = 11Hz), 3.35 (1H,s) 3.77 (1H,d,J = 14Hz), 3.89 (1H,d,J = 12Hz), 4.11(1H,s), 4.28 (1H,d,J = 3Hz), 4.85 (1H,s), 4.97 (1H,s), 5.07 (1H,d,J = 5Hz), 7.48 (2H,m), 7.61 (1H,m), 8.05 (2H, m) ; ^{13}C - NMR (67.5MHz, $CDCl_3$) δ 12.53, 14.40, 18.83, 20.12, 21.27, 24.62, 26.62, 27.75, 27.89, 29.16, 33.48, 35.23, 36.26, 36.40, 36.66, 37.87 47.25, 50.55, 60.85, 64.09, 66.06, 73.52, 80.00, 81.11, 82.47, 82.87, 83.32, 110.75, 119.66, 128.66 (2XC), 129.67 (2XC), 129.81, 133.49, 146.67, 167.67.

Antitumor bioassay : Six female BDF_1 mice weighing 18 ± 3 g supplied by Japan SLC Co. Ltd. (Shizuoka, Japan) were used in each group. Murine lymphatic leukemia P388 or L1210 maintained in this Research Institute by a weekly passage was used. Antitumor bioassay method was as followed : P388, 1.0×10^6 cells/mouse or L1210, 1.0×10^5 cells / mouse was implanted intraperitoneally. The test-sample was dissolved in physiological saline containing 5 % DMSO in the case of the crude extracts or 1 % DMSO in the case of pure compounds. The control group was injected intraperitoneally 5 % or 1 % DMSO-saline. Treatment started from 24 hr after the tumor implantation, and the test-sample was administered once daily for five days for P388 or nine days for L1210. Increase in Life Span (ILS) of the treatment group is determined comparing mean survival days (MSD) of the treatment group with that of the control group[2] by the following formula :

$$\text{ILS}(\%)=\frac{T-C}{C}\times100$$

T : MSD of treatment group
C : MSD of control group

Cytotoxic activity test in vitro : A cultured murine leukemia L1210 cell line maintained in this Research Institute was cultured in a RPMI-1640 medium supplemented by 10 % fetal bovine serum. The cell suspension (180 μl) at a concentration of 4.0×10^4 cells/ml without or with the test-sample dissolved in 20 μl of the medium were plated in a 96-well microtiter plate and incubated for 72 hr at 37°C in a humidified atmosphere of 5 % CO_2- incubator. The first row of 8 wells contained the medium only in order to blank the plate reader. After the incubation, 50 μl of 2 mg/ml MTT (Sigma Chemicals Co. Ltd., U.S.A.) in PBS was added to each well and further reincubated for 4 hr. After centrifugation, the supernatant was aspirated and formazan crystals formed were dissolved in 200 μl of DMSO and 25 μl of glycine buffer (0.1M, pH 10.5) by complete mixing using a micromixer. The plate was read on a microplate reader (Immuno Reader NJ-2000, Inter Med. Tokyo, Japan) at 540 nm. Results are expressed in terms of the drug concentration required to inhibit 50 % of the cell-glowth (IC_{50}). It is estimated as the absorbance value equal to 50 % of that cells in the control wells.[3,4]

Results and Discussion

There are three kinds of plants being used in clinical practices of traditional Chinese medicines as "Lang-du" : *S. chamaejasme* L. (Thymelaeaceae), *E. fischeriana* STEUD. and *E. ebiateolata* HAYATA (both Euphorbiaceae).[1] "Lang-du" was mentioned to have certain effects for the clinical treatment of some cancerous diseases in ancient or traditional Chinese medical book.[1,5] The root of S. *chamaejasme* L. is known as "Rui-xiang-lang-du" in Chinese (Zuikoh-roh-doku in Japanese), and several studies on the antitumor effects of "Rui-xiang-lang-du" have been reported by Chinese scientists.[6,7] However, what the antitumor active principles are has been unknown. In order to clarify the antitumor constituents of the plant, isolation and purification studies were carried out under monitoration of the antitumor activity against murine lymphatic leukemia P388 *in vivo*.

MeOH extracts from, the roots of S. *chamae-*

jasme L. and *E. fischeriana* STEUD. were tested for antitumor activity. The extract of *S. chamaejasme* L. showed a higher activity (ILS : 78.5 % and 56.8 %)against P388 in i.p.-i.p. assay system than that of *E. fischeriana* STEUD. (ILS : 41.2% and 27.4 %) at the same dose (10 mg/kg and 5 mg/kg), as shown in Table I. This result indicated that *S. chamaejasme* L. was more active against the tumor than other species of "Lang-du". Therefore, the isolation and purification of active components contained in the MeOH extract of *S. chamejasme* L. were undertaken by index of the antitumor activity as shown in Fig. 1. The extract was found to become more active (ILS : 57.4 %, at the dose of 1 mg/kg) by further extraction of the MeOH extract with petroleum ether.

When the petroleum ether extract (MMP) was purified by open column chromatography of Sephadex LH-20 eluting by MeOH, four main fractions were obtained monitoring by UV 254 nm, and the antitumor activity was concentrated into the third fraction (MMP - III) (Fig. 2). It showed more marked antitumor activity than the other three fractions summarized in Fig. 1. The active fraction MMP-III was further purified by a combination of reverse phase TLC and preparative HPLC using ODS column eluting with MeOH : H_2O (10 : 1) to afford several active fractions as shown in Fig. 3. The fraction I giving retention time at about 20 minutes was isolated as a pure compound which gave an Rf value of about 0.25 by development of TLC eluting at the same condition with ODS plate. The antitumor active compound thus isolated was identified with a daphnane type diterpene, Pimelea factor P_2 (Fig. 4), by analyses of UV spectra, mass spectra, ^1H- and ^{13}C - NMR spectra in detailed comparison with spectra data published.[8,12]

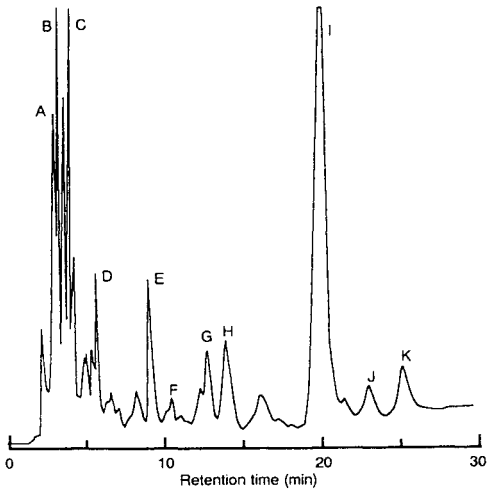

Fig. 3 HPLC separation of MMP-III. Operation condition : column, Inertsil ODS (4.6 × 250 mm) ; eluent, MeOH / H_2O (10 : 1) ; flow rate, 1.0 ml/min. ; detection, UV 228 nm.

Pimelea factor P_2 had been isolated previously as a new irritant diterpene ester from the *Genera pimelea,*[8] but then it did not describe antitumor activity in an earlier investigation.[10] However, in other studies, Pimelea factor P_2 also had been isolated from many species of the Thymelaeaceae family, such as *Pimelea prostrata*[11]

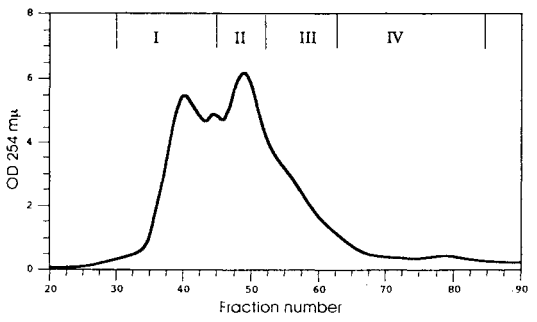

Fig. 2 Column chromatography of MMP by Sephadex LH-20. Operation condition : column, 4 ×60 cm ; eluent, MeOH ; detection, UV 254 nm.

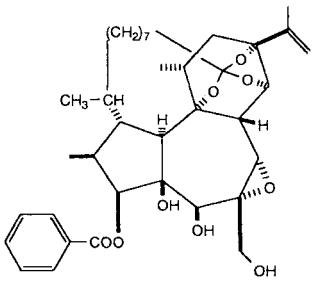

Fig. 4 Chemical structure of Pimelea factor P_2.

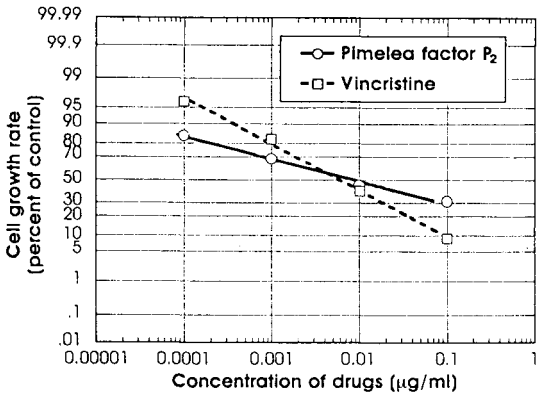

Fig. 5 Effect of Pimelea factor P_2 on the growth of L1210 cell line comparing with vincristine. 4 $\times 10^4$ cells/ml were incubated for 72 hr with the test-sample and proliferation was measured by MTT assay.

Table I Antitumor activities of the methanol extracts of *Stellera chamaejasme* L. and *Euphorbia fischeriana* STEUD. on P388,

Methanol extracts of	Dose (mg/kg)	Mean survival days[a]	ILS[b] (%)
Control		8.50±0.52	−
S. chamaejasme L.	10	15.17±1.84	78.5
	5	13.33±1.51	56.8
E. fischeriana STEUD.	10	12.00±2.56	41.2
	5	10.83±0.98	27.4

Murine lymphatic leukemia P388, 1.0×10^6 cells, were inoculated i.p.. From 24 hr after tumor implantation, mice received i.p. injections of 5 % DMSO-saline (control) or extracts dissolved in 5 % DMSO-saline in the stated doses once daily for five days.
a : Values represent mean±S.D. (n = 6).
b : Increase in Life Span of treatment group comparied with control group.

Table II Antitumor activity of Pimelea factor P_2 on P388.

Compound	Dose (mg/kg)	Mean survival days[a]	ILS[b] (%)
Control		8.00±0.63	−
Pimelea factor P_2	0.1	14.17±2.48	77.1

Murine lymphatic leukemia P388, 1.0×10^6 cells, were inoculated i.p.. From 24 hr after tumor implantation, mice received i.p. injections of 1 % DMSO-saline (control) and Pimelea factor P_2 dissolved in 1 % DMSO-saline in the stated dose once daily for five days.
a : Values represent mean±S.D.(n = 6).
b : Increase in Life Span of treatment group compared with control group.

Table III Cytotoxicity[a] and antitumor activity[b] of Pimelea factor P_2 on L1210.

Compound	*in vitro* IC_{50}[c] (μg/ml)	*in vivo* Dose (mg/kg)	*in vivo* ILS[d] (%)
Pimelea factor P_2	0.007	0.1	48.9
Vincristine	0.005	0.1	68.4

a : L1210, 4.0×10^4 cells/ml with samples dissolved in medium were incubated for 72 hr, and cell growth was meaured by MTT assay.
b : L1210, 1.0×10^5 cells were inoculated i.p.. From 24 hr after tumor implantation, mice received i.p. injection of 1 % DMSO - saline (control) and samples dissolved in 1 % DMSO-saline in the stated doses once daily for nine days, respectively.
c : Drug concentration required to inhibit 50 % of the cell growth of that cells in the control.
d : Increase in Life Span of treatment group compared with control group.

or *Dirca occidentalis* [12] and was demonstrated to have very high antitumor activity against murine leukemia P388.[11,12] It was also isolated from *S. chamaejasme* L. in which only the piscicidal effects was illustrated.[9]

Pimelea factor P_2 isolated in our studies showed 77.1 % of ILS at a dose of 0.1 mg/kg on P388 (Table II)and it also showed a significant antitumor activity against leukemia L1210 *in vivo* and *in vitro* as shown in Table III, although the activity *in vivo* was slightly weaker than vincristine (ILS : 48.9 % vs 68.4 % at the dose of 0.1 mg/kg). The cytotoxicity on L1210 cell line *in vitro* of Pimelea factor P_2 was also remarkably strong. The dose-response of Pimelea factor P_2 to L1210 cell line *in vitro* was approximately similar to vincristine (Fig. 5). Thus, it is suggested that it has almost the same grade of cytotoxicity as vincristine against tumors. So it is demonstrated that Pimelea factor P_2 may play a potent part on antitumor effects of *S. chamaejasme* L.

Further studies on antitumor spectral studies of Pimelea factor P_2 and the chemical structure studies on other active compounds isolated from the plant are in progress.

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和文抄録

瑞香狼毒 (*Stellera chamaejasme* L. の根)のメタノール抽出物はマウス白血病 P388 を用いた腹腔内移植，腹腔内投与による動物実験でかなりの制癌作用を示した。その抽出物を更に石油エーテル抽出し，セファデックス LH-20 のカラムクロマトグラフィーと ODS カラムを用いた高速液体クロマトグラフィーによって精製して数種の制癌成分を分離した。そのうち一つはピメレア因子 P_2 と同定し，それは P388 に対する動物実験で強い活性を示した。また白血病 L1210 に対して動物実験での制癌作用と試験管内での細胞障害作用も研究した。

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