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ANTITUMOR PRINCIPLES OF *STELLERA CHAMAEJASME* L.

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Methanol extract of *Stellera chamaejasme* L. was assessed for antitumor activity by an antitumor active bioassay against murine leukemia P388 *in vivo*. The bioassay-directed separation of the extract furnished seven diterpene compounds (stellerarin, stelleramacrin, gnidimacrin, pimelea factor P_2 , subtoxin, huratoxin, simplexin) and two biflavanone compounds (neochemaejasmin A and B). Among them, gnidimacrin, stellerarin and stelleramacrin (a novel compound) were found to have high antitumor and cytotoxic activities against P388, L1210 and K562 *in vivo* and *in vitro*. The results suggested that the diterpene compounds were the potent antitumor principles of *Stellera chamaejasme* L.

Key words: Antitumor agents, Stellerarin, Stelleramacrin, Gnidimacrin, Pimelea factor P_2 , Subtoxin, Huratoxin, Simplexin, Neochemaejasmin A, Neochemaejasmin B. Plant, *Stellera chamaejasme* L.

In the studies on novel antitumor agents from traditional Chinese Medicinal herbs, we have reported that a methanol extract of *Stellera chamaejasme* L. (瑞香狼毒) showed significant antitumor activity against murine leukemia P388 *in vivo*.[1] From the methanol extract, six antitumor active compounds including a novel compound named stelleramacrin have been isolated to be diterpene (compounds).[2] Gnidimacrin, the most active compound was found to manifest high but also solid tumors *in vivo* and strong cell growth inhibitory activity against human leukemia or stomach cancer cell lines *in vitro*.[3] The unique mechanism of the antitumor activity of gnidimacrin was studied.[4]

In the continuing studies, we found that an other compound showed high antitumor activity and named it stellerarin. It was isolated from the plant for the first time and identified to be phorbol-12-benzoate-13-decanoate, a tigliane type diterpene.

We report herein the isolation and the antitumor activities of two biflavanones and seven diterpenes including a novel compounds isolated from *Stellera chamaejasme* L. and discuss the antitumor principles of the plant.

MATERIALS AND METHODS

Materials

Chemicals

Dried rhizomes of *Stellera chamaejasme* L. were collected in the east Tibet, China. Identification of the plant was made by the Beijing Institute for Control of Drugs, Beijing, China. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] purchased from Sigma Chemical Co. Ltd., USA was dissolved in Ca^{++} and Mg^{++} — free PBS [PBS(-)] at a concentration of 2% (2mg/ml).

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Animals

Five week-old female BDF_1 mice supplied by Japan SLC Co. Ltd, were used.

Tumors

Murine leukemia P388 and L1210 were inoculated intraperitoneally (ip) into the mice *in vivo*. The human cancer cell line, leukemia K562 and the murine cancer cell line, leukemia L1210 were used *in vitro*.

Isolation and Identification

Dried rhizomes of *Stellera chamaejasme L.* were ground into particles and then extracted with methanol under refluxing for 5 hr. After the extract was evaporated the solvent, it was extracted with petroleum ether in a Soxhlet extractor for 5 hr. The concentrated petroleum ether extract was subjected to an open column chromatography (50 × 100 mm, silica gel 60, Merck, USA) by elution systems of hexane: ethylacetate (4: 1, 2:1, 1: 1, 1: 4). The active fraction (Fraction IV) eluted with hexane: ethylacetate (1:4) was subjected to a reversed phase high-performance liquid chromato-graphy (HPLC; column: Inertsil ODS, 22 × 250 mm, GL Science Inc. Japan) with MeOH-H_2O (10: 1) as eluent, then the active compoundswere isolated. The isolation procedure was shown in Figure 1.

The chemical structures of the compounds were identified by ultraviolet (UV) absorption, mass spectrometry, ^{1}H and ^{13}C-nuclear magnetic resonance (NMR) spectrometry.

Typical retention time (*Rt*) values of the compounds identified to be neochemaejasmin A, neochemaejasmin B, stelleramacrin, gnidimacrin, stellerarin simplexin, subtoxin A, huratoxin, pimelea factor P_2 were 3. 87, 5. 40, 6. 03, 9. 12, 10. 04, 14. 15, 16. 46, 20. 11 and 21.14 min, respectively, when analysed by a reversed phase HPLC (Inertsil ODS, 4. 6 × 250 mm, GL Science Inc. Japan; MeOH: H_2O=10: 1; flow rate: 1.0 ml/min).

Antitumor Tests

In vivo test against murine tumors

The antitumor activity of the compounds was evaluated by the antitumor bioassay against murine leukemia P388 and L1210 *in vivo*. P388 (10^6 cells) or L1210 (10^5 cells) were inoculated ip into 6 mice for each group, respectively. From 24 hr after the tumor implantation, the mice were administered by an ip injection of each compound dissolved in 0.1 ml of 1% DMSO-saline once a day for 9 days, respectively. The control group was ip injected 0.1ml of 1% DMSO-saline once a day for 9 days. The experiments were repeated 3 times. Survival days of the mice were observed and the evaluation was determinated by increase in life span (ILS). Mean survival days of the treatment group was then compared with that of the control group:

$$ILS = \left(\frac{\text{Mean survival days of treatment group}}{\text{Mean survival days of control group}} - 1\right) \times 100\%$$

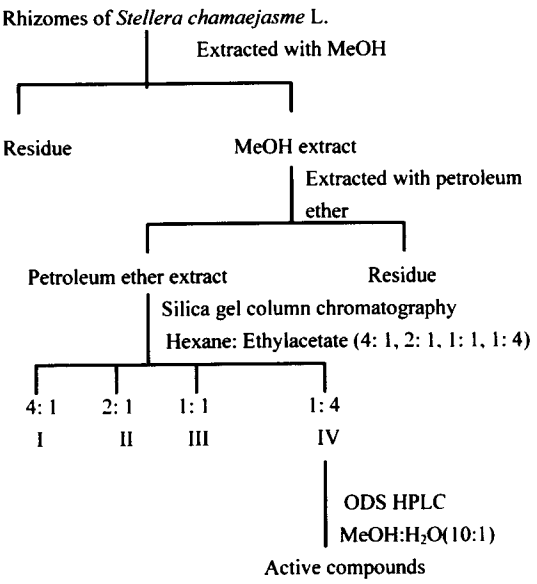


Fig 1. Isolation procedure of the antitumor active compounds from *Stellera chamaejasme* L.

in vitro cytotoxic activity test (MTT assay)

The cytotoxic activity of the compounds against cancer cell lines *in vitro* was determinated by an MTT-based chemosensitivity assay. The cells were cultured

by RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal bovine serum. The cells (0.5 - 1 × 10^4 cells/ml) were plated in a volume of 180 μl in a 96-well microplate and incubated at 37 ℃ in an atmosphere of 5% CO_2 -incubator for 2 hr pre-incubation. Twenty μl of each compound dissolved in RPMI-1640 in different concentrations or RPMI-1640 only as the control were added to each well, respectively and each concentration contains 4 wells. Then the cells were cultured in the CO_2 -incubator for 4 days. Thereafter, MTT (50 μl) was added to each well and the plates were further re-incubated for 4 hr. After the incubation, the plates were centrifuged (2000 rpm, 15 minutes) prior to removal of the medium and MTT, and then the MTT-formazan crystals were dissolved in DMSO (200 μl/well). Thereafter, glycine buffer (25 μl/well, 0.1 M, pH 10.5) was added to each well and the absorbency was measured at 570 nm in a muti-well plate reader (BIO-RAD Laboratory Co., Ltd., Japan). Evaluation of the activities was expressed in terms of the drug concentration required to inhibit 50% of the cell-growth (IC_{50}). It is estimated as the absorbency value equal to 50% of that cells in the control wells. The experiments were repeated at least 3 times.

RESULTS

Isolation and Characterization of the Compounds

The isolation and purification of the active compounds were carried out under monitoration of the antitumor activity against murine leukemia P388 *in vivo*. The isolation procedure was shown in Figure 1. The antitumor activities of each extracts or fractions were shown in Table 1. The methanol extract of the rhizomes of *Stellera chamaejasme* L. showed 79% of ILS at a dosage of 10 mg/kg. Following further extraction by petroleum ether, ILS of the petroleum ether extract was 57% at a dosage of 1 mg/kg. After the fractionation by a silica gel column chromatography, ILSs of Fraction I, II and III were shown 6%, 39% and 36% at the dosage of 1 mg/kg, respectively and ILS of Fraction IV was 65% at a dosage of 0.1 mg/kg.

Finally, nine antitumor active compounds were isolated from Fraction IV by the purification of the preparative HPLC. The chemical structures of these compounds were decided by analyses of UV spectra, mass spectra, ^{1}H-and ^{13}C-NMR spectra and comparison with the spectra data published.

Stellerarin and gnidimacrin were isolated from the plant for the first time. Stellerarin, a tiglinane-type diterpene, was characterized to be a phorbol-12-benzoate-13-decanoate. Gnidimacrin was characterized to be a daphnane-type diterpene. Stelleramacrin, a novel compound, was characterized to be 3-debenzoyl-gnidimacrin. Four compounds as diterpenes were identified to be pimelea factor P_2, subtoxin A, huratoxin and simplexin. Two compounds as biflavanones were identified to be neochemaejasmin A and B.[6-8] The chemical structures of these compounds were shown in Figure 2.

Table 1. Antitumor activity of the extracts of Stellera chamaejasme L. against murine leukemia P388 in vivo

Extracts	Dose (mg/kg)	Survival days (Mean ± SD)	ILS (%)
Control	-	8.5 ± 0.5	-
MeOH extract	10	15.2 ± 1.8*	79
Petroleum ether extract	1	13.3 ± 1.6*	57
Fraction I	1	9.0 ± 0.7	6
Fraction II	1	11.8 ± 1.5*	39
Fraction III	1	11.6 ± 1.5*	36
Fraction IV	0.1	14.0 ± 1.7*	65

* : $P < 0.001$ vs control

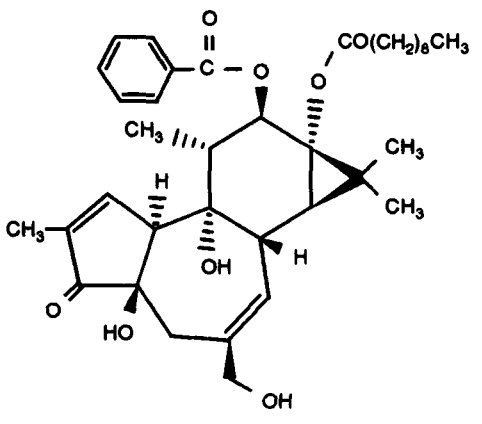
Antitumor Activity of the Compounds

Antitumor activities of the compounds isolated from *Stellera chamaejasme* L. were examined *in vivo* and *in vitro* bioassay system using murine and human leukemia cell lines.

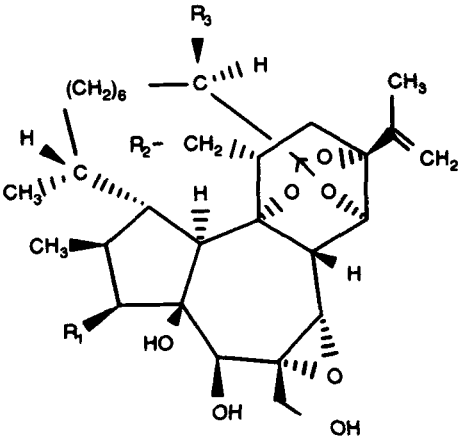
Antitumor Activity against Murine Leukemia in vivo

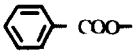
The antitumor activities of eight compounds against murine leukemia P388 and L1210 *in vivo* were tested. The results were shown in Table 2 and Table 3. In the P388 system, ILS of gnidimacrin was 79% at a dosage of 0.02 mg/kg; ILS of pimelea factor P_2 was 74% at a dosage of 0.1 mg/kg; ILSs of stelleramacrin, subtoxin A, huratoxin and simplexin were 70%, 51%, 32% and 14% at a dosage of 0.5 mg/kg, respectively. ILSs of neochamaejasmin A and B were 8% and 23% at a dosage of 2.0 mg/kg, respectively.

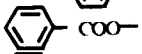
These compounds were also tested by L1210system. ILS of gnidimacrin was 80% at a dosage of 0.03 mg/kg. ILS of pimelea factor P_2 was 51% at a dosage of 0.1mg/kg, ILSs of stelleramacrin, subtoxin A, huratoxin and simplexin were 65%, 8%, 8% and 19% at the dosage of 0.5 mg/kg, respectively.

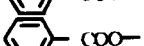


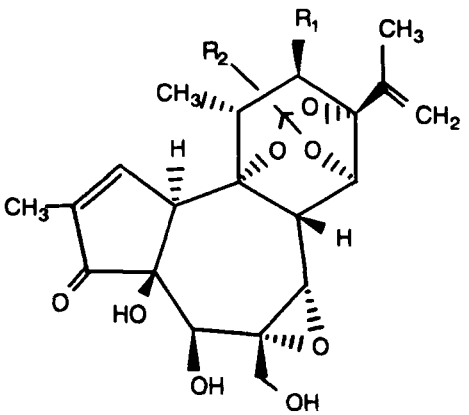
Stellerarin



Stelleramacrin: R_1=OH, R_2=  , R_3=OH

Gnidimacrin: R_1=R_2=  , R_3=OH

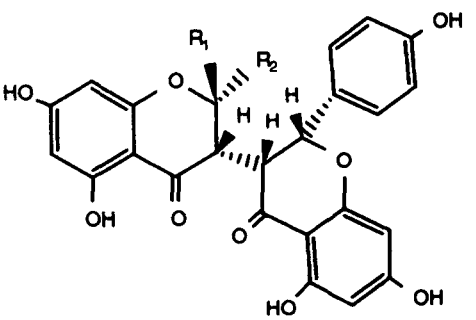
Pimela factor P_2: R_1=  , R_2=R_3=H

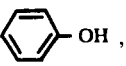


Subtoxin A: R_1=OAc, R_2=$CH_3(CH_2)_8CH$=CH-CH=CH-

Huratoxin: R_1=H, R_2=$CH_3(CH_2)_8CH$=CH-CH=CH-

Simplexin: R_1=H, R_2=$CH_3(CH_2)_8$-



Neochamaejasmin A: R_1=  , R_2=H

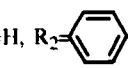
Neochamaejasmin B: R_1=H, R_2= 

Fig 2. Chemical structures of the active compounds from *Stellera chamaejasme* L.

Table 2. Antitumor activity of the compounds against murine leukemia P388 in vivo

Compounds	Dose (mg/kg)	Survival days (Mean ± SD)	ILS (%)
Control	-	8.1 ± 0.8	-
Stelleramacrin	0.5	13.8 ± 1.5**	70
Gnidimacrin	0.02	14.5 ± 1.1**	79
Pimelea factor P_2	0.1	14.1 ± 2.5**	74
Subtoxin A	0.5	12.2 ± 2.2**	51
Huratoxin	0.5	10.7 ± 0.5**	32
Simplexin	0.5	9.2 ± 0.5	14
Neochamae jasmin A	2.0	8.7 ± 0.4	8
Neochamae jasmin B	2.0	10.0 ± 0.9*	23

*: $P < 0.01$, **: $P < 0.001$ vs control

Table 3. Antitumor activity of the compounds against murine leukemia L1210 in vivo

Compounds	Dose (mg/kg)	Survival days (Mean ± SD)	ILS (%)
Control	-	7.4 ± 0.5	-
Stelleramacrin	0.5	12.2 ± 3.2*	65
Gnidimacrin	0.03	13.3 ± 3.7*	80
Pimelea factor P_2	0.1	11.2 ± 2.2*	51
Subtoxin A	0.5	8.0 ± 0.0	8
Huratoxin	0.5	8.0 ± 1.1	8
Simplexin	0.5	8.8 ± 2.1	19

*: $P < 0.001$ vs control

Cytotoxic Activity against Murine and Human Leukemia Cell Lines in vitro

The cytotoxic activity of the compounds against murine leukemia cell line L1210 or human leukemia cell line K562 *in vitro* were tested by the MTT assay. The results were shown in Table 4. Stellerarin and gnidimacrin showed very strong effects to inhibit the cell growth of L1210 or K562. IC_{50} s of stellerarin and gnidimacrin were between 0.0005 — 0.0008 μg/ml in the both cell lines. Stelleramacrin and Pimelea factor P_2 were also showed higher cytotoxic activity. IC_{50} s of stelleramacrin and pimelea factor P_2 were also showed higher cytotoxic activity. IC_{50}s of stelleramacrin and pimelea factor P_2 were between 0.05 — 0.009 μg/ml in the both cell lines. IC_{50} s of subtoxin A, huratoxin and simplexin were between 0.1 — 1.0 μg/ml in the both cell lines. IC_{50} s of two biflavanones, neochamaejasmin A and B were over 10 μg/ml in the both cell lines.

Table 4. Cytotoxic activity of the compounds against murine leukemia L1210 and human leukemia K562 cell lines in vitro

Compounds	IC_{50} (μg/ml)	
	L1210	K562
Stellerarin	0.0006	0.0008
Stelleramacrin	0.05	0.01
Gnidimacrin	0.0008	0.0005
Pimelea factor P_2	0.01	0.009
Subtoxin A	0.2	0.1
Huratoxin	0.7	0.5
Simplexin	1.0	0.8
Neochamaejasmin A	> 10	>10
Neochamaejasmin B	> 10	> 10

DISCUSSION

Stellera chamaejasme L. was mentioned to have certain effects for the clinical treatment of some cancerous or dermatic diseases in the ancient or traditional Chinese medical books.[9] The crude extract of the plant had been clinically used for treatment of some cancers.[10] We reported the methanol extract of *Stellera chamaejasme* L. assessed for antitumor activity by an antitumor active bioassay against murine leukemia P388 *in vivo*.[1] However, what the antitumor principles are has been unknown.

In order to clarify the antitumor active compounds of the extract, isolation and purification were performed under the guidance of the antitumor bioassay against murine leukemia P388 *in vivo*. As the results, nine antitumor active compounds were isolated by a four-step isolation procedure described as follows: extraction of methanol, further extraction of petroleum ether, purification of the column chromatography and final purification of the high-performance liquid chromatography

The methanol extract of the rhizomes of *Stellera chamaejasme* L. showed high antitumor activity,

which gave 79% of ILS at a dosage of 10 mg/kg. By the further extraction with petroleum ether, ILS of the extracts was 57% at a dosage of 1 mg/kg. Following the fractionation performed by a silica gel column chromatography, ILS of Fraction IV was 65% at a dosage of 0.1 mg/kg. These results indicated that the antitumor activity was enhanced by each step of purification and it was concentrated into Fraction IV.

Thereafter, nine pure compounds [seven diterpenes (stellerarin, stelleramacrin, gnidimacrin, pimelea factor P_2 , subtoxin, huratoxin, simplexin) and two biflavanones (neochemaejasmin A and B)] were purified by a reversed phase (ODS) high-performance liquid chromatography from Fraction IV. The chemical structures of the active compounds were shown in Figure 2.

Stellerarin was identified to be phorbol-12-benzoate-13-decanoate, a tigliane-type diterpene which had been isolated from *Wikstroemia canescens* (Thymelaeaceae),[5] but from *Stellera chamaejasme* L. for the first time. Gnidimacrin, a daphnane-type diterpene compound was also isolated from the plant for the first time. Stelleramacrin, a novel compound was identified to be 3-debenzoyl-gnidimacrin. Pimelea factor P_2, subtoxin A, huratoxin and simplexin identified to be diterpenes had been isolated from many species of Thymelaeaceae including the plant previously.[6,7] Neochemaejasmin A and B identified to be biflavanones had already been reported to be isolated from the plant.[8]

Among the active compounds, gnidimacrin showed the highest antitumor activities against P388 or L1210 *in vivo* by the ip-ip system. The dosage of gnidimacrin to gave similar antitumor effects compared with the crude extract, was only one of the five hundredth of the latter (ILS: 79%, 0.02 vs 10 mg/kg, shown in Table 1 and 2). Gnidimacrin and stellerarin showed very strong effects to inhibit the cell growth of L1210 and K562 *in vitro* at concentrations of 0.0008 — 0.0005 μg/ml. Stelleramacrin and pimelea factor P_2 showed high antitumor activities against P388 and L1210 *in vivo* and cell growth inhibitory effect against human leukemia cell line L1210 and K562 *in vitro*. Subtoxin A and huratoxin showed antitumor activity against P388, but no activity against L1210. Simplexin was almost non-active. Neochemaejasmin B showed a weaker antitumor activity, but neochemaejasmin A was almost no activity against murine leukemia P388.

We isolated seven diterpene compounds and two biflavanone compounds from *Stellera chamaejasme* L. under the guidance of the antitumor bioassay. The diterpenes showed high antitumor activities against the cancer than the biflavanones. The results suggested the diterpenes containing in the plant, such as gnidimacrin and stellerarin are the potent antitumor principles of the plant. The antitumor action of the compounds were due to their direct cell growth inhibitory effects.

We also investigated the antitumor effects of gnidimacrin in the details. A dose-dependent response to inhibit cancer cell lines of gnidimacrin with lower IC_{50} s than that of vincristine and adriamycin was demonstrated.[11] Gnidimacrin induced blebing of the cell surface and arrested the cell cycle transiently to G_2 and finally the G_1 phase. It inhibited phorbol-12,13-dibutyrate (PDBu) binding to K562 cells and directly stimulated protein kinase C (PKC) activity as a PKC activator.[4]

It is well-known that phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have irritant and tumor promotion effects. Stellerarin, a phorbol ester isolated from the plant was found to have high antitumor activity, but not assayed for irritancy.[5] It probably can be expected to be a useful antitumor agent.

In conclusion, we have isolated two biflavonoids and seven diterpenes including a novel compounds from *Stellera chamaejasme* L., and our study suggested that diterpene compounds such as gnidimacrin and stellerarin were the potent antitumor principles of *Stellera chamaejasme* L. It is necessary to do more detail researches on the strong antitumor activities and the unique antitumor mechanism of the active compounds.

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