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Isolation of Agrimoniin, an Antitumor Constituent, from the Roots of *Agrimonia pilosa* LEDEB.

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The antitumor activities of various extracts from the roots of *Agrimonia pilosa* LEDEB. were studied. Each extract was given intraperitoneally to mice once at 4 d before the intraperitoneal inoculation of mouse mammary carcinoma MM2 cells, and the non-sugar fractions with median polarity showed antitumor activity. Agrimoniin was isolated from the antitumor active fractions. Agrimoniin itself showed antitumor activity when given as a pre- or posttreatment; a single dose of 10 to 30 mg/kg of agrimoniin resulted in almost complete rejection of the tumor by the test mice. Agrimoniin also possessed a high degree of cytotoxicity but this activity was significantly reduced by the addition of serum to the culture medium.

Keywords——*Agrimonia pilosa*; agrimoniin; antitumor activity; MM2

Agrimonia pilosa LEDEB. (Rosaseae; Japanese name, Kinmizuhiki) has been traditionally used as an antidiarrheic, a hemostatic, and an antiparasitic in Japan and China.[1] Furthermore, a description of the effectiveness of the plant against some carcinomas appears in "Honzokomoku"[2] and the plant is used for cancer therapy in China today.[3] In a previous paper,[4] we reported that the methanol extract from roots of *Agrimonia pilosa* LEDEB. showed some host-mediated antitumor activities against several transplantable rodent tumors. The present paper describes the antitumor activities of various extracts from *Agrimoniae* root and the isolation of agrimoniin as an antitumor constituent.

The fractionation of the methanol extract (I) of *Agrimoniae* root (Chart 1) was based on antitumor activity assay of each fraction by intraperitoneal injection into mice 4 d before intraperitoneal inoculation of mouse mammary carcinoma MM2 cells (Table I). Extract I was separated by Diaion HP-20 column chromatography into a sugar and amino acid rich fraction (II) and a non-sugar fraction (III). Fraction III showed potent antitumor activity but the activity of fraction II was less than that of extract I. Fraction III was further subjected to celite column chromatography and a second Diaion column chromatography. The antitumor activity was observed in fractions with median polarity, which were obtained in high yield. Fraction IX was fractionated by Toyopearl HW40S column chromatography based on the results of thin layer chromatography (TLC). The life span of mice injected with the fractions eluted with methanol (IX-1—9) was clearly prolonged, but only the final fraction eluted with aqueous acetone (IX-11) caused clear-cut tumor cell rejection by most of the test mice. The last fraction was therefore purified by high performance liquid chromatography (HPLC) using a Shodex Polymerpak D2014 and agrimoniin was obtained. Tables I and II show that the antitumor activity of agrimoniin is potent both as a pre- and posttreatment, and is higher than that of the initial methanol extract I.

Agrimoniin is a tannin which has been recently isolated from the roots of *Agrimonia japonica* (MIQ) KOIDZ. and *Potentilla Kleiniana* WIGHT *et* ARNOTT by Okuda *et al.*[5] It is

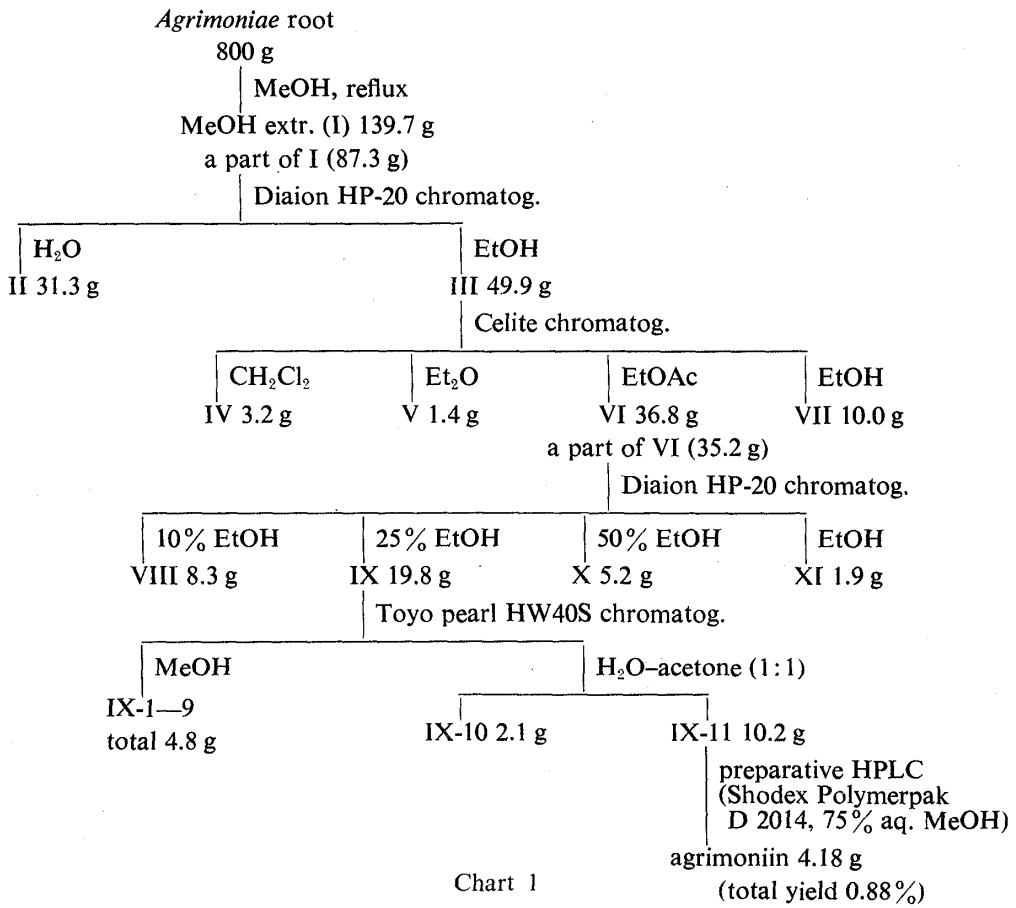

Chart 1

TABLE I. Antitumor Effect of Pretreatment with Extracts from Roots of *Agrimonia pilosa* on MM2 in Mice

Exp. No.	Extract No.	Dose (mg/kg)	Survival days[a] (mean±S.D.)	% ILS[b]	60-d survivors[c] No take	Regressing
1	I	30	29	35	1	4
		10	24.3±5.5	13	0	3
		3	18.8±0.7	−12	0	0
	II	30	25.6±3.9	19	0	1
		10	23.0±3.5	7	0	1
		3	21.8±1.4	2	0	0
	III	30	—	—	6	0
		10	22.0±0.5	2	0	1
		3	20.3±0.6	−5	0	0
	IV	30	19.1±1.6	−11	0	0
		10	17.2±0.6	−20	0	0
		3	30.7±8.4	43	0	1
	V	30	—	—	6	0
		10	27.5±5.3	28	0	0
		3	33.3±7.4	55	0	3
	VI	30	—	—	6	0
		10	25.0±1.8	16	0	1
		3	21.0±1.5	−2	0	0
	VII	30	19.0±0.5	−12	0	1
		10	22.0±1.7	2	0	0
		3	30.7±5.4	43	0	0
	Control		21.5±1.1	—	0	0
2	VI	30	—	—	6	0

TABLE I. (continued)

Exp. No.	Extract No.	Dose (mg/kg)	Survival days[a] (mean±S.D.)	% ILS[b]	60-d survivors[c] No take	Regressing
2	VIII	30	18.0±0.5	−17	3	1
		10	30.7±7.3	41	0	0
		3	31.6±7.9	46	0	1
	IX	30	—	—	5	1
		10	19.5±1.5	−10	2	2
		3	26.8±5.6	24	0	1
	X	30	—	—	6	0
		10	26.5±6.5	22	0	2
		3	28.4±7.2	31	0	1
	XI	30	35.3±7.4	63	0	0
		10	26.8±7.3	24	1	0
		3	33.3±7.7	54	0	0
	Control		21.7±1.3	—	0	0
3	IX	30	—	—	6	0
	IX-1	30	24.7±1.9	5	0	3
		10	30.5±6.5	30	0	1
		3	30.6±2.8	31	0	1
	IX-3	30	38.3±13.1	64	0	4
		10	23.2±1.7	−1	1	0
		3	29.4±3.2	26	0	3
	IX-5	30	21.4±1.3	−9	1	0
		10	36.0±7.2	54	0	2
		3	31.8±5.8	36	0	1
	IX-6	30	24.5±1.7	5	0	0
		10	34.3±10.0	46	0	3
		3	23.8±1.3	2	0	0
	IX-7	30	32.8±8.3	40	0	2
		10	38.0±15.0	62	0	4
		3	33.5±12.5	43	0	4
	IX-8	30	26.5±2.1	13	0	2
		10	44.7±8.5	91	0	0
		3	23.0±1.5	−2	0	0
	IX-9	30	21.6±0.6	−8	0	1
		10	22.5±1.8	−4	0	0
		3	26.0±1.8	11	0	0
	IX-10	30	38.3±10.2	63	1	2
		10	24.2±2.1	3	0	1
		3	22.8±1.7	−3	0	2
	IX-11	30	18.0	−23	5	0
		10	—	—	2	4
		3	21.0±1.7	−10	0	3
	Control		23.4±1.2	—	0	0
4	IX-11	30	—	—	6	0
	Agrimoniin	30	—	—	6	0
		10	—	—	5	1
		3	19.7±1.5	6	0	3
		1	19.0±0.4	3	0	2
	Control		18.5±0.3	—	0	0

Six C3H/He mice were intraperitoneally administered with each extract or agrimoniin once at 4 d before the tumor cell (5×10^5) intraperitoneal inoculation. *a*) Excluding 60-d survivors. *b*) $[(T-C)/C] \times 100$; T, mean survival days of treated mice; C, mean survival days of non-treated control mice. *c*) No take: number of mice which completely rejected the tumor cell inoculation. Regressing: number of mice in which the peritoneal cavity tumor grew until about 30 d but was decreased or regressed on day 60.

TABLE II. Antitumor Effect of Posttreatment with Extracts from Roots of *Agrimonia pilosa* on MM2 in Mice

Extract	Dose (mg/kg)	Survival days[a] (mean±S.D.)	% ILS[b]	60-d survivors[c] No take	Regressing
I	30	22.8±0.8	7	2	0
	10	31.7±5.1	49	0	0
	3	25.5±2.3	20	0	0
IX-11	30	—	—	1	5
	10	37.5±15.5	76	0	4
	3	23.0±2.1	8	0	2
Agrimoniin	30	53	149	4	1
	10	22	3	3	2
	3	21.2±0.5	0	0	1
Control		21.3±0.7	—	0	0

Six C3H/He mice were intraperitoneally administered with each extract or agrimoniin three times on days 1, 4, and 7 after the intraperitoneal tumor cell (5×10^5) inoculation. *a*—*c*) Described in the footnotes of Table I.

TABLE III. Cytotoxic Activity of Extract I and Agrimoniin against MM2 Cells *in Vitro*

Extract	IC_{50}[a] (μg/ml) Serum (−)	Serum (+)
I	7.3	88.0
Agrimoniin	2.6	62.5

Cells (2×10^5) were treated with graded concentrations of MeOH extract I or agrimoniin in the absence or presence of fetal calf serum for 2 h at 37 °C and cultured for 2 d. *a*) 50% cell growth inhibiting concentration.

known that Agrimonia pilosa is rich in tannins[6] and it has been suggested that most of the effects of the plant on diseases are due to these constituents. The present study indicates that the antitumor effect of *Agrimonia pilosa* LEDEB. is mainly due to agrimoniin, which is contained in large amounts in this plant. This tannin was highly cytotoxic towards MM2 cells *in vitro* but the activity was significantly diminished to about 4% of the initial value by addition of calf serum to the culture (Table III). Consequently, it is difficult to explain the antitumor activity of agrimoniin simply in terms of direct cytotoxic activity, because the tannin may bind rapidly, *e.g.*, to proteins, in the host animals. Our previous paper[4] showed that the methanol extract from roots of *Agrimonia pilosa* LEDEB. has antitumor activity, and after intraperitoneal administration of the extract, the white blood cells and peritoneal exudate cells (which possess the capacity to take up [^{3}H]thymidine and have cytostatic activity, respectively) were increased. In the present study, agrimoniin showed a strong antitumor activity even when given to mice as a single dose 4 d before the tumor cell inoculation.

From these results, it is suggested that agrimoniin is the main antitumor active constituent of *Agrimonia pilosa* LEDEB., and mechanism of its action may involve both host-mediated actions and direct cytotoxicity to tumor cells.

Experimental

Optical rotation was measured on a JASCO DIP-360 digital polarimeter. Infrared (IR) spectra were recorded on a Hitachi 270-30 infrared spectrometer. Ultraviolet (UV) spectra were recorded on a Hitachi 642 digital

spectrophotometer. Fast atom bombardment mass spectra (FAB-MS) were recorded on a VG Analytical ZAB-HF spectrometer. Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM-FX200 spectrometer (200 MHz, acetone-d_6 for ^{1}H-NMR and 50 MHz, MeOH-d_4 for ^{13}C-NMR). Diaion HP-20 (Mitsubishi Chemical Industries), Celite No. 545 (Kokusan Chemical Works), and Toyopearl HW-40S (Toyo Soda Mfg.) were used for column chromatography. TLC was performed on Kieselgel 60 F_{254} (Merck) with BuOH–AcOH–H_2O (4 : 1 : 2) and spots were detected by measuring the UV absorption at 254 nm and by spraying 10% H_2SO_4 and heating. Analytical and preparative HPLC were carried out on a JASCO Trirotar liquid chromatograph with a JASCO UVIDEC-100-II UV detector and a Shimadzu RID-2A RI detector.

Isolation of Agrimoniin from *Agrimonia pilosa* LEDEB.——The dried and powdered roots (800 g) of *Agrimonia pilosa* LEDEB. collected around this university in early autumn were extracted three times with 4.5 l of MeOH for 4 h under reflux. The MeOH solution was cooled, filtered, and dried *in vacuo* to give a brown residue (139.7 g). The fractionation of the MeOH extract was carried out based on the antitumor activity of each extract as an indicator, by the method described below. The MeOH extract (87.3 g) was chromatographed over Diaion HP-20 (7 × 35 cm) and eluted with H_2O (9 l) and EtOH (3 l). The eluate with EtOH was dried *in vacuo* to give a brown colored residue (49.9 g). The residue was then subjected to celite column chromatography (7 × 20 cm) and eluted with CH_2Cl_2 (3 l), Et_2O (3 l), EtOAc (3 l), and EtOH (2 l) successively. Eluates with each solvent were dried *in vacuo*. The EtOAc extract (36.8 g), which was antitumor active, was obtained in high yield. This extract (35.2 g) was further subjected to column chromatography on Diaion HP-20 (5 × 35 cm) with aqueous EtOH (initially 10% EtOH, then the concentration of EtOH was increased). The eluates with 25% EtOH were combined and dried *in vacuo* to give a residue (19.8 g), which was chromatographed on Toyopearl HW-40S (2.5 × 30 cm) with MeOH (650 ml) and 50% aqueous acetone (320 ml) to afford eleven fractions based on TLC monitoring. Fraction IX-11 (10.2 g), which was most effective among these fractions, was purified by preparative HPLC to give agrimoniin (4.18 g, yield 0.88%). The preparative HPLC conditions: column, Shodex Polymerpak D 2014 (2 cm i.d. × 50 cm); mobile phase, 75% aqueous MeOH; flow rate, 3.5 ml/min; temp., room temperature; detection, IR and UV 335 nm; t_R, 40 min.

Agrimoniin——A pale brown amorphous powder, $[\alpha]_D^{24}$ +140° (c=1.94, EtOH). *Anal.* Calcd for $C_{82}H_{54}O_{52} \cdot 3H_2O$: C, 51.16; H, 3.14. Found: C, 51.29; H, 3.17. FAB-MS m/z: 1893 [$(M+Na)^+$]; this product was identified as agrimoniin by comparison of the ^{1}H-NMR, ^{13}C-NMR, UV, and IR spectral data and analytical HPLC behavior with those of an authentic sample. The analytical HPLC conditions: column, Waters μ-Bondapak C_{18} (3.8 mm i.d. × 30 cm); mobile phase, 0.1 M H_3PO_4–0.1 M KH_2PO_4–EtOAc–EtOH (40 : 40 : 5 : 15); flow rate, 1 ml/min; detection, UV 280 nm; temp., room temperature; t_R, 16.3 min.

Antitumor Experiment in Mice——Mouse mammary carcinoma MM2 was maintained by intraperitoneal passage at weekly intervals in C3H/He mice (Shizuoka Laboratory Animal Center). Mice were intraperitoneally inoculated with tumor cells (5×10^5) on day 0 and the intraperitoneal treatment was performed on day −4 for pretreatment and on days 1, 4, and 7 for posttreatment. Sixty days after the tumor cell inoculation, survivors were killed and autopsied.

Assay of Direct Cytotoxic Activity against MM2 Cells——A 2 ml aliquot of tumor cells (2×10^5 cells/ml) prepared in RPMI 1640 medium (Nissui Seiyaku) with or without 10% fetal calf serum (Gibco) was supplemented with graded concentration of extract or agrimoniin and incubated at 37 °C for 2 h. Then, the cells were washed with Hanks' solution and further incubated in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 μM mercaptoethanol at 37 °C in a humidified CO_2 incubator (Ikemoto Rika) for 48 h. The viable cells were assayed by a dye exclusion method with 0.2% Trypan Blue.

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