

Inhibitory Effect of *Capsella bursa-pastoris* Extract on Growth of Ehrlich Solid Tumor in Mice¹

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SUMMARY

The treatment of ICR mice with i.p. injections (0.14 g/kg/day) of the extract of *Capsella bursa-pastoris* herb (*Cruciferae*) caused 50 to 80% inhibition of the solid growth of Ehrlich tumor cells that had been inoculated into the s.c. tissue of the animals. The tumor lumps in the treated mice showed multifocal necroses and the infiltration of host fibrous tissue cells. Experiments were also performed to isolate and identify the active component for the antitumor action, and an acidic substance was isolated in crystalline form from the herb extract. This acidic substance was identified as fumaric acid and was effective in inhibiting the growth of Ehrlich solid tumor at a dose of 10 mg/kg/day. The 50% lethal dose (i.p.) of this acid was 266 mg/kg.

INTRODUCTION

CBP² (*Cruciferae*) has been eaten and used medicinally in China and Japan for many centuries, and our previous studies indicated that the extract of this herb has various kinds of pharmacological activities, such as diuretic, anti-inflammatory, antiulcer, and oxytocic (2-5). It was also found that the administration of the herb extract inhibited the induction of hepatomas in the rats fed 3'-methyl-4-dimethylaminoazobenzene (1). In the present study, we found that the herb extract inhibits the growth of Ehrlich solid tumor inoculated into the s.c. tissue of mice. Then, experiments were carried out to isolate and identify the active component of the herb extract that is responsible for the antitumor action.

MATERIALS AND METHODS

The herb CBP which had been harvested in spring and dried in the shade was treated with boiling 95% ethanol under a reflux condenser for 24 hr. The contents of the flask were filtered, and the filtrate was applied to a charcoal column. Elution was made with 95% ethanol, and the effluent was concentrated in a rotary evaporator. The residue was used as CBP extract for the study on Ehrlich tumor growth in mice.

Animals used were male ICR mice obtained from Nippon Rat Co., Saitama, Japan. The basal diet for feeding animals was a semisynthetic diet, CE-2 (6), obtained from CLEA Inc., Tokyo, Japan. Mice, 5 to 7 weeks of age, were inoculated with 2×10^6 Ehrlich cells into the s.c. tissue of the left inguinal region of the animals. From the 4th day after the inoculation, mice were given i.p. injections of a suspension of CBP extract in 0.5% carboxymethylcellulose:0.9% NaCl solution at the dose of 0.14 g/kg/day for 25 days. Then mice were killed and the lumps of the solid tumor were taken out and weighed. The weights of tumor lumps were compared to those from the control animals, which had been given only the vehicle, and also to those from the positive control animals, which had been given the suspension of 6-mercaptopurine, a potent antitumor agent, 12 mg/kg/day in the vehicle. 6-Mercaptopurine was obtained from Kojin Co., Ltd., Tokyo, Japan. The tumor samples for microscopic examination were fixed in 10% formaldehyde solution, embedded in paraffin, and stained with hematoxylin and eosin.

Isolation of the active component from the herb extract was carried out using antitumor activity as a guide. The dried herb was treated with boiling 70% ethanol under a reflux condenser for 24 hr. The contents of the flask were filtered, and the filtrate was concentrated in a rotary evaporator. The tarry brown residue was dried under sulfuric acid in an evacuated desiccator. Absolute ethanol was added to the residue, and the mixture was warmed and kneaded thoroughly with a spatula. This extraction with absolute ethanol was repeated 3 times. The ethanol solution thus obtained was filtered, and the filtrate was concentrated. The concentrate was dissolved in water (1:50, v/v), and the aqueous solution was filtered. The aqueous solution was strongly acidic (pH 3 to 4). To this aqueous solution 1/9 volume of 10 N sulfuric acid was added, then 2 volumes of ethyl ether were added in a separatory funnel, and the mixture was shaken mechanically. This extraction with ethyl ether was repeated 3 times. Acidic substances free of amino acids (as shown on paper chromatography using bromocresol green and ninhydrin reagents as indicators) were extracted. The ether solution was concentrated and the residue was dissolved in water. The aqueous solution was decolorized with charcoal that had been activated at 150° for 60 min and then filtered, concentrated, and put in the refrigerator. A crystalline precipitate formed on the following day. Further purification of the crystalline precipitate was made by repetition of the treatment with charcoal and recrystallization in water. Identification of the crystalline substance was made by comparing it to authentic carbox-

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² The abbreviations used are: CBP, *Capsella bursa-pastoris*; IR, infrared; NMR, nuclear magnetic resonance.

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ylic acids with regard to paper chromatography, melting point, IR, NMR and mass spectra, and elementary analysis. In paper chromatography, the filter paper used was Toyo No. 50, obtained from Toyo Roshi Co., Ltd., Tokyo, Japan, and the solvents used were butyl alcohol:CH₂O₂:H₂O (10:2:15, v/v/v) and ethanol:NH₄OH:H₂O (80:5:15, v/v/v). Authentic carboxylic acids were obtained from Wako Pure Chemical Industries, Ltd., Tokyo. IR, NMR, and mass spectra were recorded by Nihon Bunko IR-G IR spectrophotometer, Hitachi Perkin-Elmer 60-MHz NMR spectrophotometer, and Hitachi double-focus RMU-6E mass spectrophotometer, respectively.

RESULTS

Table 1 shows the results of 2 separate experiments on the effect of CBP extract on the solid growth of Ehrlich tumor cells inoculated in mice. The i.p. injections of CBP extract at the dose of 0.14 g/kg/day retarded the growth of Ehrlich solid tumor. The weights of tumor lumps in the mice treated with CBP extract were smaller than those in the control mice treated with the vehicle. 6-Mercaptopurine, a potent antitumor agent, was used as a positive control. Histologically, the Ehrlich tumor cells inoculated into control mice formed a conglomerated mass and sometimes infiltrated into the muscle fibers of the animals, showing a number of mitotic figures (Figs. 1 and 2). In the animals treated with CBP extract, the tumor masses were small in size and were surrounded thickly with the fibrous tissues. Small and focal necroses were scattered throughout the tumor lumps and were accompanied by the infiltration of fibrous tissues. Mitotic figures were rarely observed (Figs. 3 and 4).

The crystalline substance, isolated in the attempt to identify the component responsible for the antitumor activity, showed the same R_F values as those of authentic fumaric acid in paper chromatography, 0.88 and 0.30 in the development with the solvents butyl alcohol:CH₂O₂:H₂O (10:2:15) and ethanol:NH₄OH:H₂O (80:5:15), respectively. The melting point of the crystalline substance, 285–286°, was almost identical to that of authentic fumaric acid, 286–287°. The physical and chemical data of the substance were as fol-

lows: IR (KBr)/cm, 1660, 1425, 1320, 1275, 1234, 1010, 900. NMR in dimethyl sulfoxide-*d*₆ δ ppm from tetramethylsilane, 6.65 (2H, singlet); mass spectrum *m/e*, 116 (M⁺). Elementary analysis was as follows:



Calculated: C 41.39, H 3.47

Found: C 41.66, H 3.52

These data completely coincided with those of authentic fumaric acid, and the crystalline substance was identified as fumaric acid. Table 2 shows the effect of fumaric acid, from either the herb extract or the commercial source, on the growth of Ehrlich solid tumor in mice. The experimental procedures and schedule were similar to those of the experiment with CBP extract except that fumaric acid was dissolved in 0.9% NaCl solution, *i.e.*, without addition of carboxymethylcellulose to the vehicle, and was given to mice at the dose of 40 mg/kg/day. It was shown that fumaric acid from either the herb extract or the commercial source had a similar inhibitory effect on the growth of Ehrlich solid tumor. The dose-response study indicated that the growth of Ehrlich solid tumor was retarded by the administration of fumaric acid, even at the dose of 10 mg/kg/day (Table 3). A toxicity study was also made. A single large dose was lethal to mice and the 50% lethal dose (*i.p.*) was 266 (303 to 233) mg/kg, calculated by the method of Litchfield-Wilcoxon. Animals died in the sedative condition.

DISCUSSION

The growth of Ehrlich tumor inoculated into mice was clearly suppressed by *i.p.* injections of CBP extract. The mode of action of CBP extract seems to be different from

Table 2

Effect of fumaric acid from either CBP herb extract or the commercial source on growth of Ehrlich solid tumor
Each value represents the mean ± S.D. of 15 mice.

Group of mice	Body wt (g)	Tumor wt (g)
Control	37.8 ± 5.1	10.9 ± 4.8
Fumaric acid from CBP ^a	38.4 ± 3.9	4.4 ± 2.4 ^b
Authentic fumaric acid ^a	41.0 ± 4.6	3.9 ± 2.2 ^b

^a 40 mg/kg/day for 25 days given *i.p.*

^b *p* < 0.01.

Table 3

Dose response of fumaric acid effect on growth of Ehrlich solid tumor

Fumaric acid was from CBP herb extract. Each value represents the mean ± S.D. of 15 mice.

Dose of fumaric acid ^a (mg/kg)	Body wt (g)	Tumor wt (g)
0 (Control)	40.0 ± 3.1	8.6 ± 3.5
2.5	40.8 ± 3.6	9.2 ± 3.0
5	40.0 ± 2.6	6.1 ± 3.6
10	43.5 ± 2.5	5.8 ± 1.6 ^b
20	38.0 ± 3.0	3.4 ± 1.8 ^b
40	38.7 ± 2.3	2.6 ± 1.6 ^b

^a Given *i.p.* for 25 days.

^b *p* < 0.01.

Table 1

Effect of the extract of CBP on growth of Ehrlich solid tumor

Each value represents the mean ± S.D. of 15 mice in Experiment 1 and that of 10 mice in Experiment 2.

Group of mice	Body wt (g)	Tumor wt (g)
Experiment 1		
Control	44.1 ± 6.1	13.5 ± 7.8
CBP-treated ^a	42.5 ± 5.2	3.0 ± 3.1 ^b
6-MP ^c -treated ^d	43.4 ± 5.1	2.2 ± 1.6 ^b
Experiment 2		
Control	44.1 ± 3.6	14.4 ± 3.9
CBP-treated	36.5 ± 3.5	6.4 ± 1.5 ^b
6-MP-treated	41.3 ± 4.1	4.3 ± 3.2 ^b

^a 0.14 g/kg/day for 25 days given *i.p.*

^b *p* < 0.01.

^c 6-MP, 6-mercaptopurine.

^d 12 mg/kg/day for 25 days given *i.p.*

cytotoxicity on tumor cells, for the tumor cells remaining in the CBP extract-treated mice showed neither marked degenerative alterations, such as those seen after treatment with X-ray irradiation, nor any indication that the necrosis of the tumor lump was centering around the small arteries. In a culture system, the presence of CBP extract in the medium had no effect on the viability of Ehrlich tumor cells or on the incorporation of [¹⁴C]leucine, [4-¹⁴C]UTP, and [6-³H]-thymidine into the macromolecules. The characteristic histological features of the tumor lumps remaining in the mice treated with CBP extract were the presence of irregular and multifocal necrosis and the infiltration of fairly fine fibrous tissue. The tumor lumps were sometimes demarcated from the host tissues by the fibrocapsule. In the control mice, the tumor cells exhibited infiltrative growth into the muscle layer with a number of mitotic figures, and the necrosis was seen in the center of the tumor nodule. Following the antitumor activity in the fractionation of the herb extract, fumaric acid was isolated in crystalline form. Fumaric acid did have antitumor activity similar to that of CBP extract. The dose-response study indicated that fumaric acid exhibited antitumor activity at a dose of 10 mg/kg/day, which is comparable to the dose of 6-mercaptopurine, a well-known, potent antitumor agent. Fumaric acid is an intermediate metabolite in the citric acid cycle and 4-carbon dicarboxylic acid cycle in respiration, but its pharmacological activities have not been studied in detail. The antitumor activity seems to be specific to fumaric acid, for other related 4-carbon intermediates, *i.e.*, succinic acid, malic acid, and oxaloacetic acid, showed no significant effect on the growth of Ehrlich solid tumor. Fumaric acid

also has an antiulcer action, one of the pharmacological actions of CBP extract (5). It is expected that fumaric acid plays an important role in the pharmacological activities of the herb extract (4, 5). The present antitumor activity and the inhibitory effect of the herb extract on the induction of hepatoma in rats fed 3'-methyl-4-dimethylaminoazobenzene (1) would be expected to be exhibited through mediation of its pharmacological effects on the animals. Further studies to clarify the detailed features along these lines are in progress.

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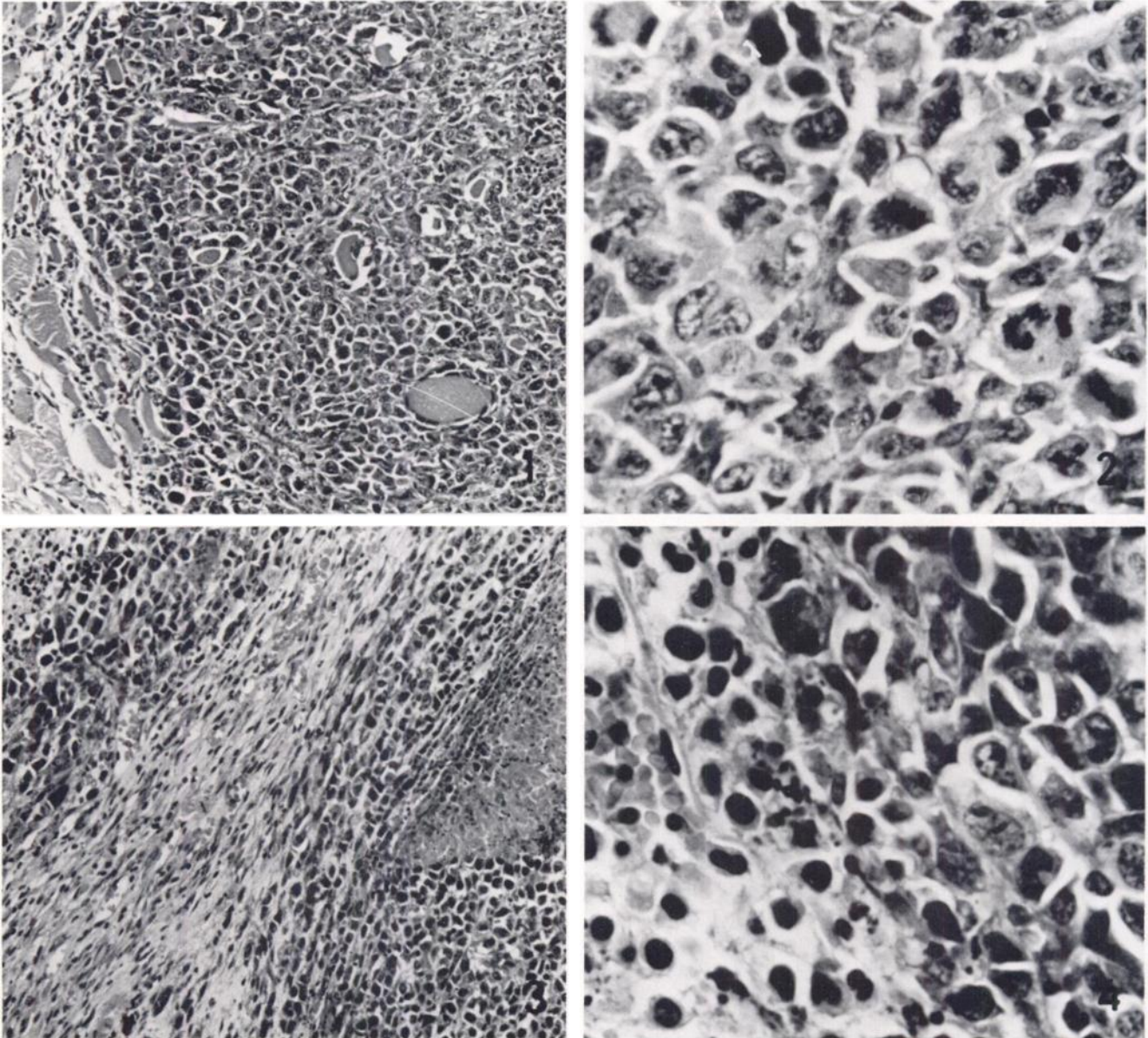


Fig. 1. Ehrlich solid tumor in a control mouse. Tumor cells infiltrate into the muscle fibers of the mouse; some of the destroyed muscle fibers are seen in the tumor cells. H & E, $\times 50$.

Fig. 2. A high magnification of Fig. 1. A number of mitotic figures of tumor cells are seen. H & E, $\times 200$.

Fig. 3. Ehrlich solid tumor in a mouse treated with i.p. injections of CBP extract. A massive, multiple, and small focal necrosis and the fibrous tissue proliferation are seen with tumor cells. They give a mottled appearance to the figure. H & E, $\times 50$.

Fig. 4. A high magnification of Fig. 3. H & E, $\times 200$.