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Antitumor Activity of Pedunculagin, one of the Ellagitannin

Jee Hun Chang[1], Jang Hyun Cho[1], Ha Hyung Kim[1], Kwang Pyo Lee[1], Min Won Lee[2], Seong Sun Han[3] and Do Ik Lee[1]

[1]Division of Immunology, [2]Division of Pharmacognosy, College of Pharmacy, Chung Ang University, Seoul 156-756, Korea and [3]Division of Microbiology, College of Pharmacy, Chung Buk National University, Cheong Ju, Chung Buk 360-763, Korea

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As a part of trials to develop the antitumor agent from tannins isolated from plants, the antitumor activity of pedunculagin, an ellagitannin, isolated from *Alnus hirsuta* var. *microphylla* was examined *in vitro* and *in vivo*. *In vitro*, the cytotoxicity was determined by 0.4% trypan blue dye exclusion method. Pedunculagin showed the dose-dependent cytotoxicity against human chronic myelogenous leukemia (K-562), human promyelocytic leukemia (HL-60), mouse lymphoid neoplasm (P388), mouse lymphocytic leukemia (L1210) and mouse sarcoma 180 (S 180) cell lines. ED_{50} values (ED_{50}) of each cell line were 5.30, 0.92, 2.78, 9.35 and 1.38 μg/ml respectively. The most sensitive cell line was HL-60. *In vivo*, pedunculagin was administered to ICR mouse with the doses of 50 and 100 μg/kg intraperitoneally once at 20 days before S180 inoculation. Pedunculagin showed the antitumor activity and its T/C ratio (%) was 120.82% in the group of both concentrations.

Key Words : Pedunculagin, Antitumor activity, Ellagitannin, T/C ratio

INTRODUCTION

The surgical operation, radiological remedy, immunotherapy, medical therapy and so on have been applied in treating cancers. Due to its metastasis, it is difficult to suppress cancer cell growth with local treatment only. Therefore, systemic treatment-medical therapy-has been carried out fundamentally at the same time (Gilman *et al.*, 1988; Dipiro *et al.*, 1989).

Recently, the studies of antitumor agents have been directed to make up for defects, such as drug delivery systems (Bodor *et al.*, 1987; Poste *et al.*, 1982; Mazer *et al.*, 1990) and prodrug-form (Anders *et al.*, 1987; Gogate *et al.*, 1987).

On the other hand, the efforts of developing antitumor agents from plants have been tried (Han *et al.*, 1991; Ahn *et al.*, 1992; Zahang *et al.*, 1980; Ikekawa *et al.*, 1968; Ikekawa *et al.*, 1969). So, effective antitumor agents were developed (Arai *et al.*, 1971; Torikai *et al.*, 1978) and their mechanisms were generally known (Yukio *et al.*, 1981; Maeda *et al.*, 1971; Bomford *et al.*, 1977; Komatsu *et al.*, 1969).

This study was scheduled and progressed with a view to developing a new antitumor agent from tannins isolated from *Alnus hirsuta* var. *microphylla*..

Tannins are polyhydric phenols found in plants, and polymolecular substances linked with proteins and alkaloids (M.W. = 500~3,000). Tannins are classified chemically into hydrolyzable tannin and condensed tannin. Hydrolyzable tannin is classified furthermore into gallotannin in which only galloyl radical is combined with saccharide and ellagitannin which is metabolized to ellagic acid by hydrolysis (Lyu *et al.*, 1989).

Tannins are widely distributed in plants, and are significant to human because of their usage as tanning agents, dyes, and drugs in addition to their wide distribution as astringent components in many foodstuffs (Shirahata *et al.*, 1985), and reported to possess a variety of biological activities such as lowering effect of the blood urea nitrogen (Shibutai *et al.*, 1983), inhibiting effect of the activity of ACE (Inokuchi *et al.*, 1985; Uchida *et al.*, 1987), antiviral effect (Lee *et al.*, 1992; Nonaka *et al.*, 1990), DNA-breaking effect (Shirahata *et al.*, 1985; Shirahata *et al.*, 1989), psychotropic effect (Ueki *et al.*, 1986) and antibacterial effect (Serit *et al.*, 1991).

In the recent study, tannins were reported to have the antitumor activity (Yoshida *et al.*, 1991; Gali *et al.*, 1992; Yoshiki *et al.*, 1992; Miyamoto *et al.*, 1993a;

Correspondence to: Do Ik Lee, Division of Immunology, College of Pharmacy, Chung Ang University, 221 Huk Suk Dong, Dong Jak Gu, Seoul 156-756, Korea

Miyamoto *et al.*, 1993b; Miyamoto *et al.*, 1993c). Among them, Yoshiki *et al.* (1992) reported that 129 tannins and relative compounds showed the antitumor activity, but all of the tannins were found to be inactive (ED_{50}>10 μg/ml) against A549, HCT-8, KB and TE671 cell lines.

Moreover, Miyamoto *et al.* (1993a) reported that ellagitannins showed IL-1 induction and that 21 ellagitannins had the antitumor activity in mouse bearing S180 (Miyamoto *et al.*, 1993b). In addition, ellagitannin was reported to have DNA breakage activity (Shirahata *et al.*, 1985) and pedunculagin was reported to have anti-HIV activity (Lee *et al.*, 1992).

In this paper, the direct cytotoxicity of pedunculagin, an ellagitannin, isolated from *Alnus hirsuta* var. *microphylla*- against K-562, HL-60, P388, L1210 and S180 and the lengthening effect of the life span in mice bearing S180 were examined and discussed.

MATERIALS AND METHODS

Materials

Pedunculagin was isolated from *Alnus hirsuta* var. *microphylla*. Its structure is shown in Fig. 1. Fetal bovine serum (FBS) was obtained from Hyclone Co., Ltd.. RPMI1640 media, trypan blue, dimethyl sulfoxide (DMSO) were purchased from Sigma Co., Ltd..

In vitro assay

Cell culture: K-562, HL-60, P388, L1210 and S180 cell lines were subcultured from Chong-Keun Dang Co., Ltd. and used. Cell lines were routinely maintained in the CO_2 incubator (95% air and 5% CO_2) at 37°C RPMI1640 medium supplemented with 10% heat-inactivated (56°C, 30 min.) FBS was used in this study. Cell lines were kept under LN_2 until use and used at early passage as possible.

Assay method: Four milliliters of cell suspension in logarithmic phase was inoculated into each well of 12 well plate, and treated with the concentrations of 1, 5, 10 and 20 μg/ml of pedunculagin. After treatment, cells were incubated for further 48 hours (hr). Living cells were counted at each 24 and 48 hr on hemacytometer by 0.4% trypan blue dye exclusion method.

Data calculation: The value of ED_{50} which was the concentration of a test compound to inhibit the growth of tumor cells by 50% to control was determined with protocol of National Cancer Institute (NCI), USA (Thayer., 1971).

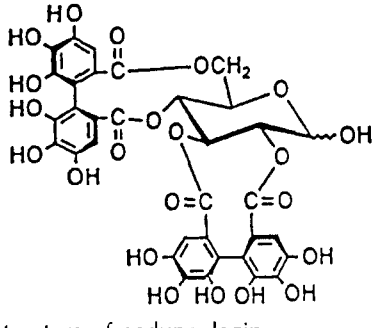


Fig. 1. The structure of pedunculagin

In vivo assay

Animal: ICR mice used for this study were purchased from Dae-Ryuk Co.. 3-week-old ICR mice were purchased. After raising for 1 week, ICR mice were used for the antitumor test. Male (20-25 g) ICR mice were devided into groups of 8 mice for each *in vivo* assay.

Tumors: S180 tumor cell line was kindly provided by Sun-Kyung Industrial Co. (SKI) where S180 had been maintained in the ascites form.

Treatment: Pedunculagin was prepared in sterile distilled water. Fifty and 100 μg/kg of pedunculagin were administered intraperitoneally once at 20 days before S180 inoculation. After 20 days of pedunculagin administration, 0.1 ml of washed S180 cells (10^6 cells/ml) was implanted in the peritoneal cavity of mice, and their life spans were examined and recorded.

Data calculation: ICR mice were examined for 30 days after S180 inoculation. Their life spans were recorded. At 30 days after S180 inoculation, survivors were divided into No-takes (Failure of individual implants) group and S180 bearing group. Mice divided into No-takes and mice that died within the 10th day were excluded from calculation by the protocol of NCI, USA (Cancer Chemotherapy National Service Center, 1972).

T/C ratio (%) was calculated with mean survival time (MST) computed according to the equation below (Hyun, 1993).

$$\text{T/C ratio (\%)} = \frac{\text{MST of Treated Group}}{\text{MST of Control Group}} \times 100$$

RESULTS AND DISCUSSION

In vitro

Pedunculagin was examined for the cytotoxicity on the growth of five cultured tumor cell lines.

Initial cell number of K-562 was 3.94×10^4 cells/ml, and its ED_{50} to pedunculagin was 5.30 μg/ml (Table I). Pedunculagin showed the dose-dependent cytotoxicity against K-562 (Fig. 2).

Initial cell number of HL-60 was 7.00×10^4 cells/ml, and its ED_{50} to pedunculagin was 0.92 μg/ml (Table I). Pedunculagin showed the significant cytotoxicities in all concentration at 48 hr (Growth ratio<50%), but

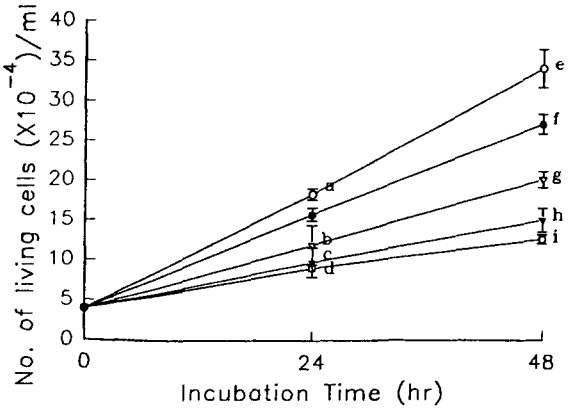


Fig. 2. The effect of pedunculagin on the growth of K-562 cells. ○: control ●: 1 μg/ml ▽: 5 μg/ml ▼: 10 μg/ml □: 20 μg/ml, 3.95×10^4 cells/ml were cultured in 5% CO_2 incubator at 37°C for 48 hr, a vs b and e vs f: $p<0.05$, a vs c, d and e vs g, h, i: $p<0.01$ (Student's t-test), The values were given as Mean±S.E. of n=4.

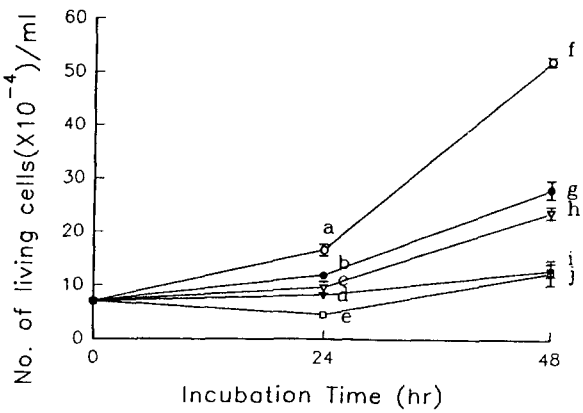


Fig. 3. The effect of pedunculagin on the growth of HL-60 cells. ○: control ●: 1 μg/ml ▽: 5 μg/ml ▼: 10μg/ml □: 20 μg/ml, 7.00×10^4 cells/ml were cultured in 5% CO_2 incubator at 37°C for 48 hr, a vs b, c, d, e and f vs g, h, i, j: $p<0.01$ (Student's t-test). The values were given as Mean±S.E. of n=4.

Table I. The cytotoxicity of pedunculagin on growth of cell lines

Cell Lines	Growth ratio[a](%) at different concentrations (μg/ml)					ED_{50}[b] (μg/ml)
	Control	1	5	10	20	
HL-60	100	46.67	37.22	13.33	12.22	0.92
K-562	100	77.11	53.57	36.65	28.69	5.30
L-1210	100	78.42	55.70	50.40	40.74	9.35
P388	100	68.13	40.79	33.21	-4.23	2.78
S-180	100	62.50	9.45	1.83	-7.01	1.38

a: $Y(\%) = \frac{T\text{-}Co}{C\text{-}Co} \times 100$

Y: growth ratio for each dose of testing substances

T: mean cell count for each dose of testing substances after 48 hr incubation

C: mean cell count for control after 48 hr incubation

Co: mean cell count at the start of incubation

b: ED_{50} was the effective dose which inhibited growth to 50% of control group

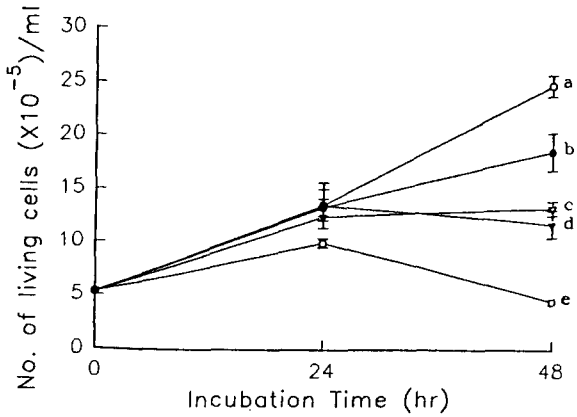


Fig. 4. The effect of pedunculagin on the growth of P388 cells. ○: control ●: 1 μg/ml ▽: 5 μg/ml ▼: 10 μg/ml □: 20 μg/ml, 5.29×10^5 cells/ml were cultured in 5% CO_2 incubator at 37°C for 48 hr, a vs b: $p<0.05$, a vs c, d, e: $p<0.01$ (Student's t-test), The values were given as Mean±S.E. of n=4

there was little difference in growth ratio between the group of 10 and 20 μg/ml (Fig. 3).

Initial cell number of P388 was 5.29×10^5 cells/ml, and its ED_{50} to pedunculagin was 2.98 μg/ml (Table I). Pedunculagin didn't show the dose-dependent cytotoxicity against P388 (Fig. 4).

Initial cell number of L1210 was 2.55×10^5 cells/ml, and its ED_{50} to pedunculagin was 9.35 μg/ml (Table I). Pedunculagin showed the dose-dependent cytotoxicity against L1210 (Fig. 5).

Initial cell number of S180 was 5.80×10^4 cells/ml, and its ED_{50} to pedunculagin was 1.38 μg/ml (Table I). Pedunculagin showed the dose-dependent cytotoxicity against S180. Especially growth ratio of the group of 20 μg/ml was -7.01% (Fig. 6).

Pedunculagin showed the cytotoxicity against K-562, HL-60, P388, L1210 and S180 (ED_{50}<10 μg/ml). HL-60 was the most sensitive (ED_{50}=0.92 μg/ml), and L1210 was the most insensitive (ED_{50}=9.35 μg/ml) to pedunculagin.

Bae *et al.* (1994) reported that bisabolangelone isolated from Angelicae Koreanae Radix showed the cytotoxicity against L1210 and HL-60, and ED_{50} were 1.20 and 2.30 μg/ml respectively. Pedunculagin showed the stronger cytotoxicity against HL-60 than bisabolangelone, and the weaker cytotoxicity against L1210 than bisabolangelone.

Baik *et al.* (1993) also reported that ar-turmerone isolated from curcuma species showed the cytotoxicity against L1210, HL-60 and K-562. And, ED_{50} were all above 10 μg/ml.

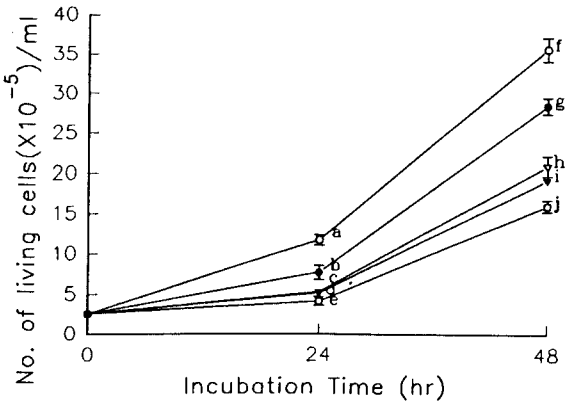


Fig. 5. The effect of pedunculagin on the growth of L-1210 cells. ○: control ●: 1 μg/ml ▽: 5 μg/ml ▼: 10 μg/ml □: 20 μg/ml, 2.55×10^5 cells/ml were cultured in 5% CO_2 incubator at 37°C for 48 hr, a vs b: $p<0.05$, a vs c, d, e and f vs g, h, i, j: $p<0.01$ (Student's t-test), The values were given as Mean±S.E. of n=4

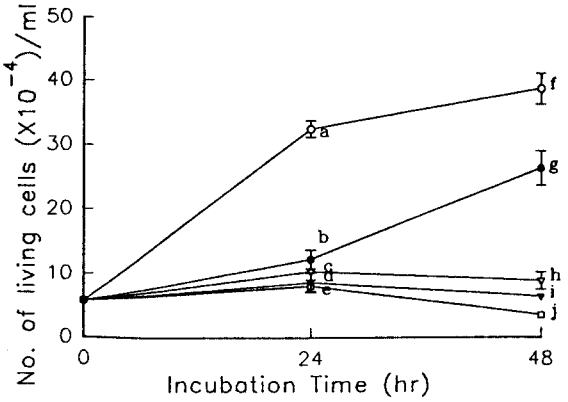


Fig. 6. The effect of pedunculagin on the growth of S-180 cells. ○: control ●: 1 μg/ml ▽: 5 μg/ml ▼: 10 μg/ml □: 20 μg/ml, 5.80×10^4 cells/ml were cultured in 5% CO_2 incubator at 37 for 48 hr, a vs b, c, d, e and f vs h, i, j: $p<0.01$, f vs g: $p<0.05$ (Student's t-test), The values were given as Mean±S.E. of n=4.

Lee *et al.* (1986) reported that antitumor substances were isolated from *Lithospermum erythrorhizon, Curcuma domestica, Tricosanthes kirilowii, Panax ginseng and Liriodendron tulipifera,* and that those substances showed the cytotoxicity against L1210. And, ED_{50} were all above 10 μg/ml.

In view of the results so far achieved, pedunculagin was regarded to have the significant cytotoxicity against K-562, HL-60, P388, L1210 and S180.

In vivo

Pedunculagin was examined for the ability of tumor immunity to host animal *in vivo* on the basis of the data shown *in vitro.* Not thinking of the metabolism *in vivo* of pedunculagin at the dose used *in vitro,* pedunculagin was administered with the doses of 50 and 100 μg intraperitoneally once at 20 days before S180 inoculation. It was quite little concentration than the order of mg generally used.

Table II. Mean suvival time and T/C ratio of each group.

Mouse	Control	50 μg/kg	100 μg/kg
MST[a]	24.83±1.40	30.00±0.00[c]	30.00±0.00[d]
T/C(%)b	100	120.82	120.82

*a: MST=mean survival time

*b: $T/C(\%) = \frac{\text{MST of Treated Group}}{\text{MST of Control Group}} \times 100$

*c,d: Significantly different from control, $p<0.01$ (Student's t-test)

*The values were given as Mean±S.E. of n=8

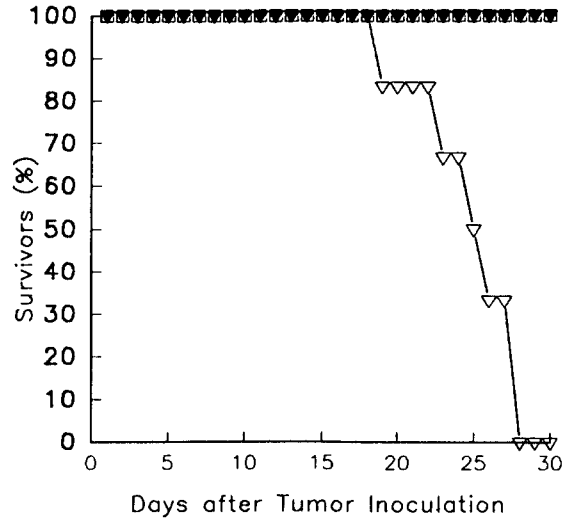
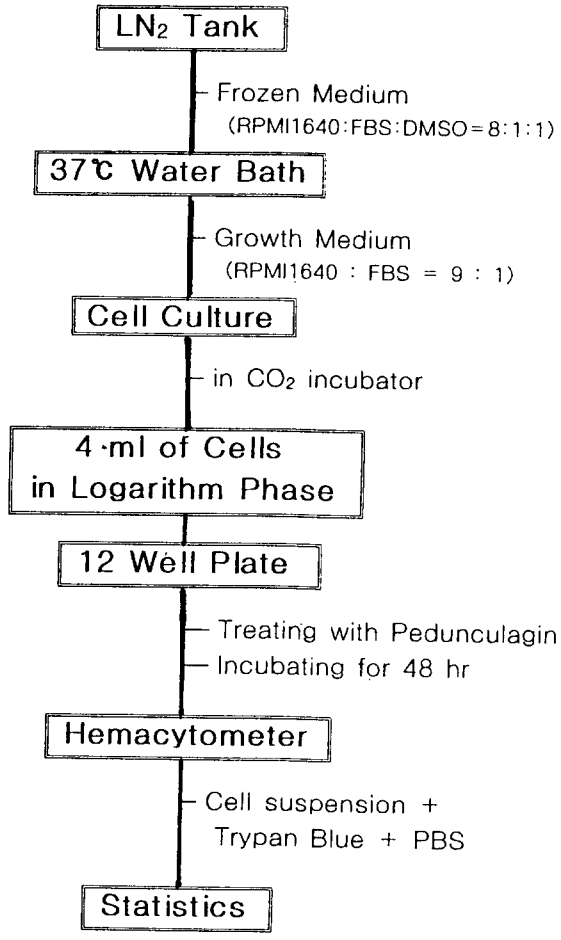


Fig. 7. The effect of pedunculagin treated on the 20th day before inoculation of 10^5 of S-180 cells on lengthening the life span in ICR mouse. ▽: control ▼: 50 μg/kg □: 100 μg/kg, The values were given as survival ratio (%) of n=6 for 30 days.

In the group of 50, 100 μg/kg, one mouse was excluded from calculation, the others were not No-takes. T/C ratio of each group was 120.82 (%) equally, the dose-dependency was not shown (Table II). Pedunculagin have the effect of lengthening the life span in the mouse bearing S180 (Fig. 7).

Taking into account the dose, pedunculagin was thought to have the strong antitumor activity *in vivo.* The dose-dependency was not shown, but it was regared that the range of concentration was so narrow. It was considered that there was no difference in the antitumor activity of pedunculagin between the dose of 50 μg and that of 100 μg. It was considered that the wide scope examinations of pedunculagin must be carried out about the antitumor activity including the route of administration, the time of administration, the sorts of tumor cell line versus host animal and the concentration of drug. As a result, pedunculagin was thought to have the strong antitumor activity by the



Scheme 1. *In vitro* assay.

single dose once 20 days before S180 inoculation which was thought to be quite long time. It was considered that the mechamism of the activity must be examined more detaily in the near future.

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