Arch. Pharm. Res. 8(1), 31~38(1985)

Effects of Antitumor Polysaccharides from *Forsythia Corea* on the Immune Function (I)

Chang-Kiu Moon, Kwang-Sik Park, Soo-Hwan Lee, Bae-Jin Ha and Byeong Gon-Lee
College of Pharmacy, Seoul National University, Seoul 151, Korea
(Received March 29, 1985)

Abstract □ Polysaccharide fractions were prepared from *Forsythia Corea* by several different extraction schedules. The fractions obtained were designated as ForCo A, ForCo B and ForCo C respectively. ForCo C was further purified through Sephadex G 200 column chromatography and obtained two subfractions (Coreana A, Coreana B). ForCo C showed marked antitumor activity against sarcoma 180 and its activity was dose-dependent. Coreana A, purified from ForCo C, showed somewhat higher antitumor activity. ForCo C increased the number of circulating leucocytes and peritoneal exudate cells, but didn't show any significant effects on the phagocytic activity and total serum protein level. Chemical analysis showed that ForCo C was composed of glucose, galactose, xylose and arabinose. A close relationship between antitumor activities and polysaccharide contents was observed. These results indicate strongly that antitumor active principle of *Forsythia Corea* is polysaccharide.

Keywords □ Polysaccharide, *Forsythia Corea*, Antitumor activity, Immune function, Chemical analysis

Cancer is not a single disease, but a large number of disease that are expressed as the abnormal and continued growth of cells of a given tissue. Although cancer hazard remains one of our most pressing health problems, the events that lead to the development of cancer, and the factors that control it, are poorly understood.

Furthermore, conventional treatment programs for tumor patients such as surgical method, radiation therapy and chemotherapy, are faced with some limitations in effects.

Therefore, development of a substance or a method which enhances or potentiates such a resistance inherent in the human body must be one of the most important means of opening a new way for the arrest of cancer.[1]

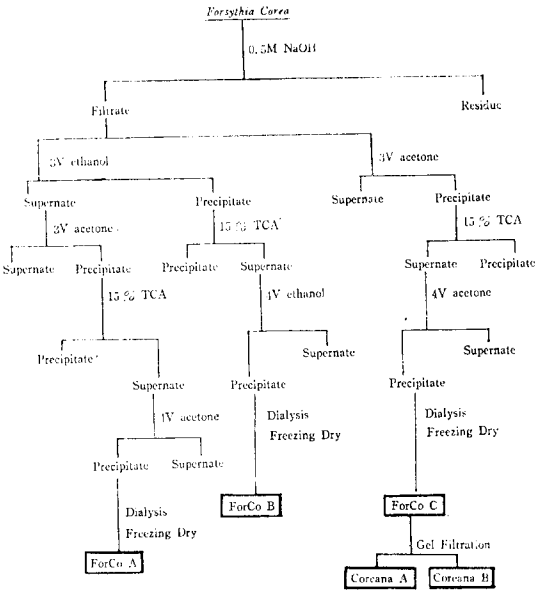
In these aspects, the polysaccharides with antitumor activity have been reported in a variety of natural sources. It is well known that Lentinan,[2,3] polysaccharide from *Lentinus edodes*, completely regresses sarcoma 180 solid form transplanted s.c. in ICR mice and the mechanism of antitumor activity has something to do with Immune Function.

Lentinan enhances antibody dependent cell-mediated cytotxicity through helper T-lymphocytes.[4] It has the characteristics of macrophage activator.[5] Also, it had been reported to enhance the cytotxic and cytostatic activities of peritoneal exudate cells against various tumor cells,[6] to stimulate complement system.[7] Recently, many other Immune Functions of Lentinan have been reported and studies on Immunomechanism are going on.

From ancient, a number of Medicinal herbs have been used for treatment of malignant tumor patients in orient and "Dong Eui Bo Gam", the classic handbook of Korean traditional medicine, written by Huh Jun, described the systemic treatment of various tumors and some conditions resembling tumors e.g. inflammatory masses and indurations with some oriental herb medicines.

In this point of view, we started to screen the antitumor activities of polysaccharides[8,9,10] from some plants which were described to have potent antitumor activities. Based on the previous results, we found that the polysaccharides from *Forsythia Corea* had most potent antitumor activity.[10] Deep study on this polysaccharide was partially carried out. Active principles of *Forsythia Corea* were isolated and purified with gel filtration using Sephadex G-200, and its chemical composition was analyzed.

In order to elucidate the mechanism of its antitumor activity, effects on Immune Function, such as circulating leukocyte count, peritoneal exudate cell count, phagocytic activity, immunoorgan weight and the total serum protein level, were primarily examined.



Scheme 1. Flow sheet for fractionation of Polysaccharides from *Forsythia Corea.*

EXPERIMENTAL METHODS

Materials

Forsythia Corea was commercially obtained from Kyung-Dong Herbdrug Market in Seoul, Korea. C57BL and DBA mice were obtained by the courtesy of Japan CLEA. Male BDF_1 and ICR mice were obtained from the Experimental Animal Breeding Center of Seoul National University. Sarcoma 180 and Leukemia P388 were maintained by serial i.p. passage in ICR and BDF_1 mice respectively.

Preparation of Polysaccharide

Polysaccharide fractions were obtained from *Forsythia Corea* with the modified method of Caldes et.al.[11] In this case, two different polar solvents were used for the fractionation with different order as shown in scheme I. Three fraction obtained were designated as ForCo A, ForCo B and ForCo C.

Purification of Crude Polysaccharide, ForCo C

Sephadex G 200 medium gel was prepared and packed onto a column with 4 cm (ID) × 90 cm (L).[12]

300 mg of ForCo C was dissolved in 20 ml of distilled water and non soluble material was removed by filtration. The filtrate was applied onto the top of the column bed. The elution rate was 10 ml/hr, and hydrostatic pressure was maintained 10 cm H_2O by using Mariotte flask. Eluent was collected in a glass tube per 30 minutes, and anthrone reaction was accom-

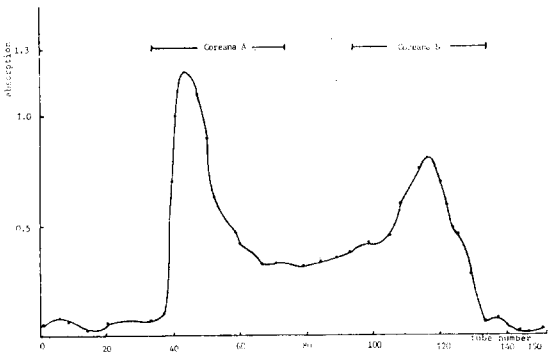


Fig. 1: Column chromatographic separation of Coreana A and Coreana B on Sephadex G-200.

plished for odd numbered frations. Absorption intensity at 625nm was checked and the gel filtration pattern is shown in Fig. 1. The anthrone test positive fractions were concentrated, dialyzed against distilled water for three days, and lyophilyzed. The first anthrone test positive fraction was designated as Coreana A and the second fraction as Coreana B.

Antitumor test

1) Solid tumor inhibition test

Antitumor test was carried out by the same way as that reported on the antitumor activity of LENTINAN.[2] Male ICR mice about 20 g were implanted s.c. with 1×10^6 cells of sarcoma 180 into the left groin at 24 hours before the start of sample administration. Samples were administerred once a day for ten days by i.p. injection with each dose. On the 21 th or 28 th day after tumor implantation, the mice were sacrificed and the solid tumors were excised and inhibition ratios were calculated from their weights.

2) Survival test

BDF1 mice (DBA×C57BL) were inoculated i.p. with leukemia P 388 cell suspension, 1×10^6 cell/0.1 ml. Test sample (ForCo C) was injected i.p. for consecutive ten days, starting on the first day after tumor implantation. Saline was administerred to control group. The life span of the mice was observed. Mean survival days were calculated according to the guide line of National Cancer Institute of U.S.A.[13]

Number of Circulating Leucocyte[14]

The effect of ForCo C on the number of circulating leucocytes was examined by the follow ing method. Each group consisted of 12 ICR mice and was given i.p. injection of 0.1 ml ForCo C (10 mg/kg/day) for consecutive ten days. Blood was collected from the retroorbital plexus, on lst, 2 nd, 4 th, and 7 th day after the last sample injection. Collected blood was diluted with citrate saline, stained by Turk's reagent and the number of nucleated cells was countered in haemacytometer chamber. Triple counting per sample was carried out and the mean value of results was calculated. The number was compared with that obtained from control mice.

Number of Peritoneal Exudate Cells[15]

Each group consisted of 12 ICR mice and was injected i.p. with 0.1 ml ForCo C (10mg/kg/day) for three consecutive days. 4 mice in each group were sacrificed by cervical dislocation on 1 st, 2 nd, and 4 th day after the last sample injection. The peritoneal cavity of sacrificed mouse was washed with 5 ml saline and the ascitic fluid was collected. The number of total PEC was counted in a haemacytometer chamber by using Turk's solution.

Carbon Clearance Test[16,17]

ICR mice of test groups were treated i.p. with saline. Two days later on the last sample injection, phagocytic activity was measured. For the preparation of test particle, colloidal carbon (Pellican Ink, Black number 17) was diluted 1/6 with 1 % gelatin and kept in a water bath at 40 °C for all the time of the experiment. Injection was executed via the lateral tail vein by using a 1 ml syringe with 26 gauge needle at the dose of 0.01 ml of colloidal carbon solution per gram of mouse. This corresponded to approximately 16 mg carbon per 100 g body weight of mouse. In order to dilute the vein, xylene was used. At the interval of 5 min., 15 min., 30 min., and 40 min., 20 μl of blood sample was obtained from the retroorbital plexus. The collected blood samples were expelled into each vial containing 2 ml sodium carbonate, and then the contents were well mixed for the lysis of red blood cells. The absorbance of the

colloidal carbon contained in blood was measured directly in the spectrophotometer using water blank at 600 nm. From these results, phagocytic index and corrected phagocytic index were calculated.

Lymphoid Organ Weight[18]

Male ICR mice of test groups were treated i.p. with ForCo C (10 mg/kg/day) for consecutive ten days and those of control group with saline. On the 8th day after the last sample injection, mice were weighed and sacrificed by cervical dislocation. Liver, spleen, and thymus were removed and weighed and compared with the results obtained from control mice.

The Level of Total Serum Protein[19]

Male ICR mice of test groups were treated i.p. with ForCo C (10 mg/kg/day) for consecutive ten days and those of control group with saline. On the third day after the last sample injection, blood was collected from tail vein and centrifuged with microcentrifuge at 12,000 g for 2 minutes. Total serum protein levels were determined with Biuret reaction and BSA solution was used as standard.

Chemical Analysis

1) Total polysaccharide content[20]

The contents of the total polysaccharide were determined by anthrone test and glucose solution was used as standard.

2) Identification of monosaccharide with high performance thin layer chromatography.[21]

5 mg of ForCo C was completely hydrolyzed in the reflux reaction apparatus with 10 ml of 4 N trifluoroacetic acid. After hydrolysis, trifluoroacetic acid was evaporated completely in a rotary vacuum pump. And then, 1 ml of distilled water was added. Sample and reference monosaccharides were chromatographed on a HPTLC plate using the solvent system. Developed plate was sprayed with anilline phosphate spraying reagent and heated at 100°C for 2-5 minutes. The spots were visualized in red-brown for pentoses and brown for hexoses.

Table I: Running condition of gas chromatography.

Column	3% OV-1	130°C~180°C
Temperature	column	rate; 3°C/min
	injector	250°C
Flow rate	air	0.5kg/cm^2
	hydrogen	0.5kg/cm^2
	nitrogen	50ml/min.
Sensitivity	attenuate	3
	range	2
Model	Shimadzu RPR-G1	

3) Determination of monosaccharide content with gaschromatography

gaschromatographic analysis of monosaccharide was performd according to Mitruka.[22] The procedure was schemed as follows; 5 mg of For Co C was hydrolyzed in the reflux reaction apparatus with 10 ml 4 N of trifluoroacetic acid in a boiling water bath. After trifluoroacetic acid was evaporated completely by rotary vacuum pump, 1 ml of pyridine was added. Then, methylsilanization was performed by addition of TMCS, and HMDS with vigorous mixing. After 15 minutes, the sample was ready for analysis. GC conditions are shown in Table I.

Statistic Analysis

Student t-test was accomplished for the statistic significance of obtained results.

RESULTS AND DISCUSSION

The antitumor activity of polysaccharides was recognized by the supression of the tumor growth and the prolongation of the life span of the tumor bearing mice.

As shown in Table II, among the fractions of the polysaccharide from *Forsythia Corea*,

Table II: Antitumor activities of Polysaccharides from *Forsythia Corea* against sarcoma 180 implanted s.c. into ICR mice. Solid tumors were resected on 28th day after inoculation.

Sample	N	Dose (mg/kg, day)	Tumor weight (g/mouse)	Inhibition ratio (%)	P value
Control	9	—	6.44±1.74	—	—
Coreana A	8	10, 10	1.04±0.50	83.9	$p<0.01$
Coreana B	9	10, 10	4.04±2.57	37.3	$p<0.05$
ForCo B	8	1, 10	2.77±1.51	57.0	$p<0.01$
ForCo B	9	10, 10	2.92±1.35	54.2	$p<0.01$
ForCo C	9	10, 10	1.87±1.13	71.0	$p<0.01$
Control	8	—	1.30±0.44	—	—
ForCo A	7	1, 10	1.99±1.70	—	N. S
ForCo A	8	10, 10	1.50±0.76	—	N. S
ForCo A	7	30, 10	1.24±0.59	7.7	N. S

Table III: Dose response of antitumor ForCo C against sarcoma 180 implanted s.c.into ICR mice. Tumors were resected on 21 th day after inoculation.

Sample	N	Dose (mg/kg, day)	Tumor weight (g/mouse)	Inhibition ratio (%)	P value
Control	6	—	4.18+1.99	—	—
ForCo C	8	1, 10	3.19+1.72	23.7	N. S
ForCo C	9	30, 10	0.68+0.60	83.7	$p<0.01$
Control	10	—	4.16±2.52	—	—
ForCo C	8	10, 10	0.50±0.38	88.0	$p<0.01$

Table IV: Effects of ForCo C on survival ofBDF1 mice transplanted i.p. with P388 ascites.

Sample	N	Dose (mg/kg, day)	Average survival days	Prolongation ratio (%)
Control	10	—	8.98	—
ForCo C	10	10, 10	9.22	5.0

Coreana A which is the most purified fraction showed the most potent activity, and ForCo A which has very low polysaccharide content didn't show any antitumor activity.

We found that antitumor activity of ForCo C was dose dependent. (Table III)

ForCo C didn't prolong the life span of BDF_1 mice bearing leukemia P388. (Table IV)

The effect of ForCo C on the number of circulating leucocytes in ICR mice is shown in Table V. We could find that the numbers of leucocytes on 1st, 2nd, 4th, and 7th day after the sample administration were significantly increased.

As shown in Table VI, the number of total PEC was significantly increased for one day after the last sample administration. But it was decreased to the level of control group on the second day.

ForCo C didn't show any evident effect on

Table V: Effect of ForCo C on the number of circulating leucocytes. (**cell/mm^3**)

Sample	N	Body weight (g, Mean±S.D)	Dose (mg/kg, day)	1st day	2nd day	4th day	7th day
Control	12	24.3±2.3	—	6,700±1,200	7,500±1,000	7,900±500	6,700±400
ForCo C	12	24.9±1.2	10, 10	11,000±600	8,800±500	12,500±200	10,800±2,200

Table VI: Effect of ForCo C on the number of peritoneal exudate cell (**1×10^6 cells/ml**)

Sample	N	Body weight (g, Mean±S.D)	Dose (mg/kg, day)	1st day	2nd day	4th day
Control	12	24.5±2.6	—	0.86±0.21	1.34±0.26	1.51±0.12
ForCo C	12	22.9±2.6	10, 10	4.89±1.00	1.63±0.42	1.82±0.23

Table VII: Effect of ForCo C on the carbon clearance activity (Phagocytic activity)

Sample	N	Dose (mg/kg, day)	Phagocytic Index	Corrected Phagocytic Index
Control	10	—	0.015±0.003	4.193±0.373
ForCo C	10	10, 10	0.014±0.001	4.508±0.324

Table VIII: Effect of ForCo C on the immunoorgan weight of ICR mice. (g, Mean±S.D)

	Control	ForCo	C P value	Wt. change increase(%)
Body weight (before sample injec.)	14.95±2.70	14.15±2.52	—	—
Body weight (on 17th day)	28.98±1.45	28.15±0.88	—	—
Liver weight	1.53±0.11	1.69±0.16	$p<0.05$	10.0
Spleen weight	0.152±0.019	0.201±0.038	$p<0.01$	32.2
Thymus weight	0.079±0.014	0.095±0.018	$p<0.05$	20.0

Table IX: Effect of ForCo C on the level of total protein.

Sample	N	Dose (mg/kg, day)	Total protein (g/dl)
Control	9	—	6.83±0.25
ForCo C	9	10, 10	6.67±0.12

Table X: Monosaccharide contents of the poly saccharide fraction of ForCo C.

Monosaccharide	glucose	galactose	arabinose	xylose
Content(%)	49.4	18.7	20.9	11.0

Table XI: Polysaccharide contents in each fractions of *Forsythia Corea*.

Fraction	Coreana A	Coreana B	ForCo A	ForCo B	ForCo C
Content(%)	95.0	49.9	20.1	90.7	77.2

the carbon cealrance activity. Corrected phagocytic index was slightly increased, compared with that of control group, but statistically not significant. (Table VII)

Although body weight was not increased, compared with that of control group, the weights of liver, spleen, and thymus were increased

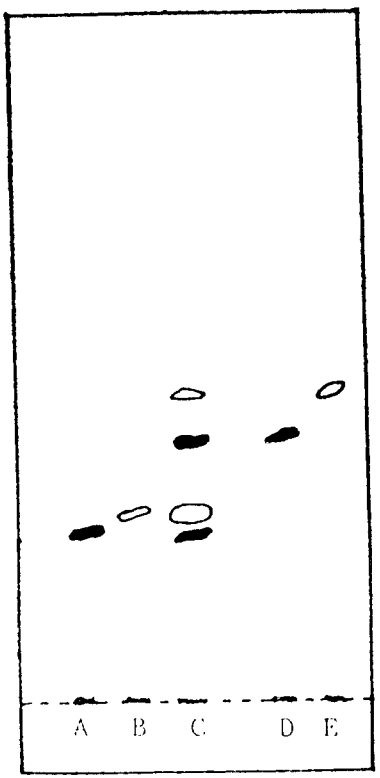


Fig. 2: High performance thin layer chromatogram of monosaccharides from ForCo C.
Developing solvent (EtOAc: Pyridine: Water =120 : 50 : 40)
A: galactose, B: glucose C: ForCo C, D: arabinose E: xylose.

(Table VIII) and there was no significant change of the level of total serun protein (Table IX). Total polysaccharide contents of the polysaccharide fractions from *Forsythia Corea* are shown in Table XI. ForCo C was found to be consisted of glucose, galactose, arabinose and xylose. (Fig. 2) Because glucose solution, which has the most potent intensity in anthrone reaction, was used as standard, the real polysaccharide contents mighs be higher than those exhibited in Table XI.

In the previous study[10], we observed that total polysaccharide fraction from *Forsythia Corea* showed strong antitumor activity against sarcoma 180 solid form, but not against sarcoma 180 ascite form. We found also in this present study, that ForCo C didn't prolong the life span of BDF_1 mice bearing leukemia P388 ascite form. This is the quite similar response pattern with LENTINAN, polysaccharide from *Lentinus edodes* which completely regresses sarcoma 180 solid form.

Among the polysaccharide fractions, Coreana A, the most purified form, has the most potent antitumor activity. ForCo A, which has very low polysaccharide content, didn't show any activity. These results indicate strongly that antitumor component of *Forsythia Corea* is polysaccharide. Dose dependency of antitumor polysaccharide was confirmed with ForCo C and its optimal dose was 10 mg/kg/day. ForCo B which contains higher polysaccharide contents, showed less potent antitumor activity than ForCo C, it is probably due to different efficacies of ForCo B and ForCo C. Doses of ForCo B(1 or 10 mg/kg/day) which were adopted in this study were found not to be optimal, and effective dose of ForCo B should be optimized in the further studies.

In the studies on the immune function, ForCo C increased the number of circulating leucocytes and peritoneal exudate cells, but didn't show any evident activity on general immune function. Though the effects of ForCo C on general immune function didn't appear to be significant, it doesn't mean that there is no relation between antitumor activity of polysaccharide from *Forsythia Corea* and immune function.

Further studies, such as tumoricidal activity of macrophage on Cr^{51}-labelled tumor[23,24,25], the functions of T lymphocyte[26,27,28], the role of PMN against tumor[29] cell, etc,, are going on. We also tried to improve the polysaccharide purifying technique in order to avoid the conta mination of macromolecules, e.g. incomplate deproteination by TCA.

LITERATURE CITED

1) Maeda, Y.Y., et al., *Int. J. Cancer.* **11**, 153 (1973).
2) Chihara, G., et al., *Cancer Res.* **30**, 2776(1970).
3) Maeda, Y.Y., et al., *Nature.* **229**, 634 (1971).
4) Dennart, G., et al., *J. Natl. Cancer. Inst.* **51**, 1729 (1973).
5) Hamuro, J., et al., *Immunology.* **39**, 551(1980).
6) Maeda, Y.Y., et al., *Gann.* **64**, 351 (1973).
7) Okuda, T. et al., *Nature New Biol.* **238**, 59 (1972).
8) Cha, S.M., *Kor. J. Pharmacog.*, **8**, 1 (1977).
9) Lee, S.H., *Arch. Pharm. Res.* **6**, 123 (1983).
10) Moon, C.K. et al. *Arch. Pharm. Res.*, **8**, 45 (1985).
11) Caldes, G., et al., *J. Gen. Appl. Microbiol.* **27**, 157 (1981).
12) Terrance, G.C., *The Tools of Biochemistry.* John Wiley & Sons, N.Y., 1977. p. 169.
13) Instruction 14, Screening Data Summary. *Interpretation and outline of current screen*, NCI.
14) Brij M. Mitruka., Clinical Biochemical and hem atological reference values...., Massion, N.Y., p.

31, (1981).

15) Barbara, B.M., ed., *Selected methods in cellular Immunology*. W.H. Freeman and Company, (1980).

16) Weir, D.M., ed., *Handbook of Experimental Immunology*. Blackwell. Oxford, p. 31, 25(1973).

17) B. Vernon Robetts, *The Machrophage*. Cambridge Univ. Cambridge. (1972).

18) P.J. Fraker, *Toxi. Appl' Pharm.* **53**, 1 (1980).

19) J.S. Garvey, et al., ed., *Methods in Immunology*. W.A. Benjamin. London, p. 71, (1980).

20) Norris, J.R., *Methods in Microbiology*. **5B**, 242 (1971).

21) Alex Kaplan., Clinical Chemistry; *interpretation and techniques*. Lea & Febiger. Philadelphia, p. 283, (1972).

22) Brij M. Mitruka, *Gaschromatographic Applications in Microbiology and Medicine*. John Wiley & Sons. N.Y. p. 156.

23) Mariko Kurisu, et al., *Cancer Res.* **40**, 3798 (1980).

24) Steven, M.T., et al., *J. Immunology*. **126**, 424 (1981).

25) Carl Nathan, et al., *J. Exp. Med.* **152**, 183 (1980).

26) Howard, D.E., et al., *J. Immunology*. **115**, 356 (1975).

27) Enza Piccolella, et al., *J. Immunology*. **125**, 2082 (1980).

28) Robert, H.W., et al., *J. Immunology*. **124**, 2254 (1980).

29) Kjell, Sveen, *Acta.Path. Microbiol. Scand. Sect. B*, **86**, 237 (1978).