A Cytotoxic Activity of *Panax Ginseng* Extract Against Some Cancer Cells *In Vivo* and *In Vitro*.

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Abstract: This study was devised to observe the cytotoxic activities of petroleum-ether extract of *Panax ginseng* root(crude Gx) and its partially purified fraction from silicic acid column chromatography(7:3 GX) against sarcoma-180(S-180) and Walker carcinosarcoma 256(Walker 256) *in vivo*, and murine leukemic lymphocytes(L_{1210}) and human rectal cancer cell(HRT-18) and human colon cancer cells(HT-29 and HCT-48) *in vitro*.

Each cell-line was cultured in medium containing serial concentrations of the crude Gx or 7:3 Gx *in vitro*. A highly lipid soluble compound in the extract of *Panax ginseng* root was cytocidal to murine leukemic cells and human colon and rectal cancer cells *in vitro*. In the meantime, ginseng saponin derivatives did not cytotoxic effects at its corresponding concentration. The growth rates of the cancer cells in medium containing ginseng extracts were inhibited gradually to a significant degree roughly in proportion to the increase of the extract concentration. The cytotoxic activity of 7:3 Gx was about 3 times more potent than that of crude Gx, one unit of cytotoxic activity against L_{1210} cells being equivalent to 2.54 μg and 0.88 μg for the crude Gx and 7:3 Gx, respectively. The Rf value of the active compound on silica-gel thin layer chromatography with petroleum-ether/ethyl ether/acetic acid mixture(90:10:1, v/v/v) as a developing solvent was 0.23.

The survival times of mice inoculated with S-180 cells were extended about 1.5 to 2 times by the 7:3 Gx treatment compared with their control group. The significantly decreased hemoglobin values of rats after inoculation with Walker 256 were recovered to normal range by oral administration of the crude Gx.

The synthetic levels of protein, DNA and RNA in human colon and rectal cancer cells were significantly diminished by treatment with the crude Gx, which can explain a part of the origin of its anticancer activity.

Introduction

Panax ginseng has been extensively used in the traditional oriental medicine as a restorative, tonic and prophylactic agent[1]. The pharmacological effect of ginseng and its extract reported during past ten years are omnivorously numerous and still controversial[2-5]. Recently, several reports regarding to anticancer effects of *Panax ginseng* has accumulated[6-9]. These studies emphasized the fact that the anticancer activities might be due to a glycoside group called ginsenoside or panax saponin which has a water soluble characteristic[10-11].

However, the authors and collaborates demonstrated that a highly lipid soluble component in extract of *Panax ginseng* roots contains a considerable cytotoxic activities against murine leukemic cells *in vitro*[12].

In such a context, the present study was conducted *in vitro* to evaluate the cytotoxic activitives of the lipid soluble ginseng extracts against L_{1210} cells in comparison with the saponin derivatives purified from *Panax ginseng*. Accordingly, the petroleum-ether extract showed remarkable cytotoxic activities *in vitro*, which was subject to *in vivo* experiment to test their clinical effectiveness as anticancer

agents by measuring the change of the survival time and the recovery rate of the hematopoietic derangement in cancer bearing mice or rats after treatment with the ginseng extracts. Additionally, biochemical events responsible for the expression of its anticancer activity was pursued by analyzing the influence of petroleum-ether extract of ginseng on the synthesis of protein, DNA and RNA of human colon cancer cells *in vitro* biosynthetic labelling.

Materials and Method

Materials

Dried ginseng roots(first grade, six years old) were purchased from a local herb shop in Seoul. Panax-diol, panax-triol, diol saponin and triol-saponin were a gift from the Korean Ginseng and Tobacco Research Institute.

Murine leukemic lymphocyte(L_{1210}), murine ascitic sarcoma 180 and Walker carcinosarcoma 258 were a gift from the Research Institute for Microbilal Diseases, Osaka University. Human rectal cancer cell(HRT-18) and human colon cancer cells(HT-29 and HCT-48) were donated by Y.S. Kim in College of Medicine, California University, U.S.A. Each cell-line and experimental animal have been maintained in our laboratory and used for the experiment. Experimental animals; Swiss mice(strain A) and albino rat(Sprague-Dawley strain) were a contribution from Dr. Park in Seoul National University, Seoul, Korea. The Fisher's medium, Dulbecco's modified Eagle Medium, horse serum, fetal bovine serum and trypsin were purchased from the Grand Island Biological Co. Silicic acid(BioSil A, 100-200 mesh) from the Bio-Rad Laboratories; Silica-gel thin layer chromatography sheet from E. Merck, Darmstadt; millipore filter disc(GS 0.22 nm) and its accessories were from the Millipore corp; Coulter counter(model ZBI) and its accessories were goods of Coulter Electronic LTD. All other chemicals used were of the highest purity available commercially. Amersham provided (^{3}H) leucine, (^{3}H) thymidine, (^{3}H) uridine, and Insta-gel is a product of Packard.

Preparation and partial purification of the ginseng extracts.

The *Panax ginseng* roots were finely pulverized using a motar and 3 grams of the powder were subjected to extraction with petroleum ether for 12 to 15 hours in a Soxhlet apparatus. The crude extract prepared from the above(Crude Gx) was thoroughly dried by vacuum evaporation using a rotary evaporator under a stream of nitrogen gas and stored at 4℃.

Extractions with other organic solvents were performed in the same fashion as the case of petroleum-ether. Only water extract was made by boiling the powder of ginseng in distilled water for 8 hours, after which the mixture was centrifuged and the supernatant was sterilized by autoclaving for 20 minutes at 120℃. The petroleum-ether extract of ginseng was used to test cytotoxic activity in all experiments unless otherwise stated. The crude Gx was then subjected to silicic acid column chromatography for further purification. After 15g of silicic acid were activated in an oven by heating at 120℃ for two hours, which was followed by equilibration in 50 m*l* of chloroform, the sludgy was poured into a glass column(2×17 cm). The column was washed with 50 m*l* of chloroform and 50 m*l* of petroleum-ether in order. The crude Gx(100-200 mg, dry weight) dissolved in a minimal amount of petroleum-ether was loaded onto the column, and stepwise elution was performed with 50 m*l* of petroleum-ether, 90:10(v/v) petroleum-ether/ethyl ether mixture, 80:20(v/v) petroleum-ether/ethyl ether mixture and with 70:30(v/v) petroleum-ether/ethyl ether mixture. Of the fractions separated, 70:30(v/v) fraction(7:3 Gx) was selected for further purification for its highest specific activity, and was dried in vacuum evaporator.

Each fraction obtained from silicic acid column chromatography was chromatographed by thin layer plate of silica-gel using petroleum-ether/ethyl ether/acetic acid mix-

ture(90:10; 1, v/v/v) as a developing solvent. The chromatograms were recognized as fluorescent spots at 375 nm immediately after spraying 0.01% aqueous solution of rhodamine 6G as described by Marinette(1964). The spots might also be recognized visually by their pink color development after the rhodamine solution dried.

Cell-culture

L_{1210} cells were cultured in the Fisher's medium and HT-29, HCT-48 and HRT-18 cells were cultured in the DMEM by Fischer and Sartorelli method[13]. Sarcoma 180 cells were maintained in Swiss mice by transplanting them every ten days after intraperitoneal injection of the cell to mice. Walker 256 cells were maintained in rat by transplanting them every ten days after hypodermic injection of the cell under the chest of Sprague-Dawley.

In vitro cytotoxic activity determination.

To test the cytotoxic activities of extracts, the solutions were filtered through a millipore disc under a sterile condition, then the filtrate was diluted with sterile distilled water. When a small aliquot of this solution was added to the culture medium to the needed concentrations, the final dilution used in the cell culture was such that concentration of ethanol did not exceed 0.2%. Greater concentrations of ethanol are krown to inhibit cell growth itself. One unit of cytotoxic activity was arbitrarily defined as the amount of drug in one m*l* culture medium which causes a two fold increase in the apparent doubling time of cell-lines tested. In practice, the assay was carried out as follows; The cells of each cell-line were grown in media containing serial concentrations of the ginseng extracts, and the cell populations of each culture were counted after 24 hours of incubation employing Coulter Counter. Then, the dose corresponding to the midpoint between the logarithm of the cell number at 0 hours and after 24 hours of incubation was estimated from a plot of the logarithm of cell number *vs.* concentration of drug. Growth curves of each cell-line treated with sequential concentrations of petroleum-ether extract or saponin derivatives from ginseng were determined by counting the cell populations at regular intervals in the course of incubation.

Size distribution curves of L_{1210} treated with ginseng extracts were compared with the control containing no addition of extracts from ginseng after 0, 12, 24 hours of incubation by Coulter counter at Manometer or "stop at full scale" count mode settings.

Biosynthetic labeling of DNA, RNA and protein.

Biosynthetic labeling of DNA, RNA and protein in subconfluent monolayers with 2.5 μCi(^{3}H) thymidine, (^{3}H) uridine and (^{3}H) Leucine each per 35 mm tissue culture plate was performed for 2, 6, 10 and 24 hours after 24-48 hours in serum-free medium. Appropriate cultures were incubated with serum, or with serum as well as the addition of Crude Gx at concentrations of 50μg/ m *l* for HRT-18, HCT-48 and HT-29. Fetal bovine serum was added to a final concentration of 5%(v/v). Cell cultures were brought quickly to 0℃ after the termination of incubation, and washed twice with the ice cold phosphate buffered saline(0.2g KCl, 0.2g KH_2PO_4, 8g NaCl and 2.16g $Na_2HPO_4 \cdot 7H_2O$/ *l*, pH 7.4, calcium and magnesium free). Cells were scraped off dishes or removed from captubes in 1 m*l* of phosphate buffered saline and transferred to test tubes. After sonication for 15 sec. three times, protein determination by Lowry method was followed. Same amount of protein was transferred to conical centrifuge tube and made 10% in trichloroacetic acid and washed twice with 10% trichloroacetic acid solution. Precipitates were suspended in 1 m*l* deionized water, After the addition of 5 m*l* of Insta-gel, redioactivity was counted in a Packard TRI-CARB 4530 model scintillation counter.

The effects of petroleum ether extracts of ginseng on the survival time of sarcoma-180 bearing mice.

Twenty swiss mice weighing from 20 to 25g were divided into two groups(each group comprises of 10 mice). A control group represents S-180 cells bearing mice without 7:3 Gx treat-

ment, and 7:3 treated groups were treated with 7:3 Gx after the inoculation with S-180 cells. Sarcoma 180 cells were introduced into the control group and the crude Gx treated group of mice about 1×10^6 cells per head by intraperitoneal injection. The 7:3 Gx treated group was injected 7:3 Gx(5.0 mg/head/day) intraperitoneally every day for the duration of 10 days from the third day after inoculation with S-180 cells. Then the survival times of two groups were compared.

The effect of the crude Gx on hemoglobin values of Walker 256 bearing rats.

Seventy rats weighing about 180g on the average were divided into seven groups(each group contained 10 rats); a normal group which received neither inoculation of Walker 256 cells nor other treatment and six experimental groups. The experimental groups divided again into three control group inoculated with Walker 256 cells and three groups treated with the crude Gx after inoculation of Walker 256 cells. Rats in the Gx group were given 10 mg of the crude Gx per day by oral administration for 3, 6 and 9 days, respectively. On the third, sixth and ninth day after the ingestion of the crude Gx, each Gx group and its control groups were sacrificed, and hemoglobin values of rats of each group were determined by the AOAC procedure[14].

Results and discussion

Comparison of cytotoxic activities of each extracts with six solvents.

The data obtained with each extract are summarized in Table 1. The cytotoxic activity yield was highest with acetone, but petroleum-ether provided an extract with the highest specific activity. For this reason, the petroleum-ether extract was used selectively for the later study.

Partial purification of cytotoxic compound from ginseng.

Among the four fractions from silicic acid column chromatography, the cytotoxic active compound against cancer cells existed predominently in 7:3 fraction(Table 2). The silica gel thin layer chromatograms of crude Gx and each fraction from the column chromatography are shown in Fig.1. Based on these data, the Rf value of cytotoxic active compound on silica gel TLC with petroleum-ether/ethyl ether/acetic acid mixture(90:10:1, v/v/v) as a developing solvent was calculated to 0.23(Fig. 1).

Table 1. *Comparison of each extract with six solvents.*

Solvent	Weight yield (mg/g)	Activity yield (unit/g)	Specific activity (unit/mg)
Water	500	825	1.6
Ethanol	74	1370	18.5
Chloroform	8.4	1830	220
Ethyl ether	7.7	1890	246
Acetone	9.9	4100*	420
Petroleum -ether	4.4	2350	530*

Relative cytotoxic activity of crude Gx, 7:3 Gx and saponin derivatives from ginseng.

One unit of cytotoxic activity against L_{1210} cells was equivalent to 2.54μg and 0.88μg for

Table 2. *The cytotoxic activity of each fraction separated by silicic acid column chromatography.*

Fraction	Specific activity (unit/mg)	Total activity (units)	Recovery (%)
crude GX	530	50880	0
10 : 0 GX	0	0	0
9 : 1 GX	0	0	0
8 : 2 GX	477	5088	10
7 : 3 GX	2094	24420	48

crude GX; petroleum ether extract from *Panax ginseng*.
10:0 GX; only petroleum ether fraction.
9:1 GX; petroleum-ether/ethyl-ether=9:1 fraction.
8:2 GX; petroleum-ether/ethyl-ether=8:1 fraction.
7:3 GX; petroleum-ether/ethyl-ether=7:3 fraction.

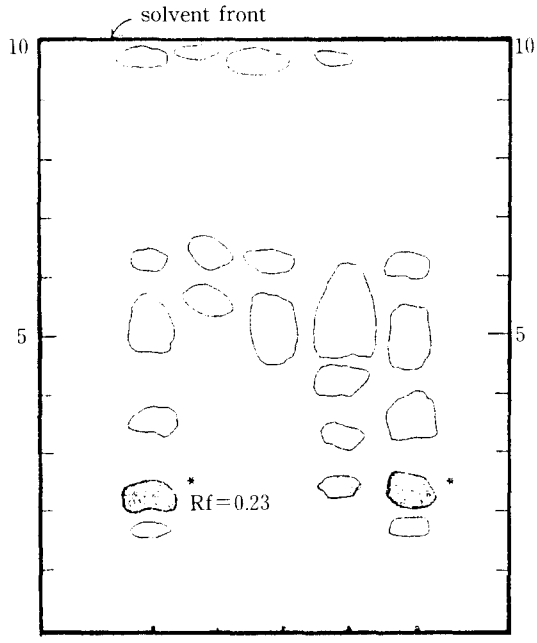

Fig.1. *Silica-gel thin layer chromatograms of crude GX and each fraction by silicic acid column chromatography.*
A: petroleum-ether/ethyl-ether=10:0 fraction
B: petroleum-ether/ethyl-ether=9:1 fraction
C: petroleum-ether/ethyl-ether=8:2 fraction
D: petroleum-ether/ethyl-ether=7:3 fraction
*: active compound

the crude Gx and 7:3 Gx respectively, so that the activity of the 7:3 Gx is about three times more potent than that of the crude Gx(Fig. 2-A). While, the cytotoxic activities of panax-diol, panax-triol, diol saponin and triol saponin against L_{1210} cells were not detected in those concentrations and even in ten times higher concentrations(Fig.2-B).

Characterization of cytotoxic effect of 7:3 Gx.

Fig.3 shows that the inhibition effects of the crude Gx and 7:3 Gx on the growth rate of L_{1210} cells *in vitro* are dependent on the incubation time and the concentrations of the extract. The growth inhibitions induced by the crude Gx or 7:3 Gx against L_{1210} were not found remarkable during the first 11 hours of incubation period, but definitely significant inhibition was found after 12 hours of incubation roughly in proportion to the dose of each extract. Meaningfully, twelve hours corresponds to the doubling time of L_{1210}.

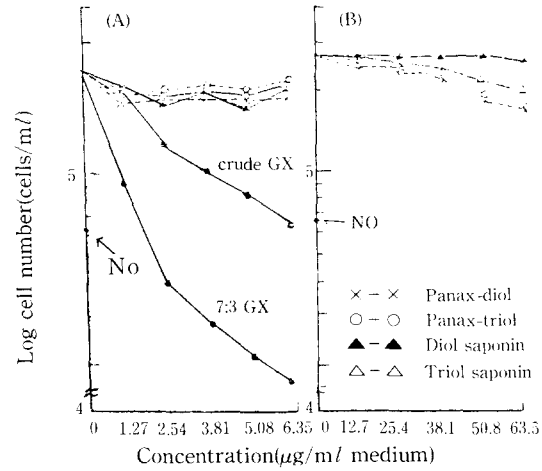

Fig.2. *Dose response curves of petroleum-ether extracts or saponin derivatives of Panax ginseng on the growth of L_{1210} cells after 24 hours of incubation.*
crude GX; unpurified petroleum-ether extract of ginseng root.
7:3 GX; partially purified fraction from petroleum-ether extract of ginseng root by silicic acid column chromatography.
saponin derivatives; Panax-diol, Panax-triol, Diol saponin, Triol saponin.
N_0; initial cell number.

HRT-18 cells, the control group without

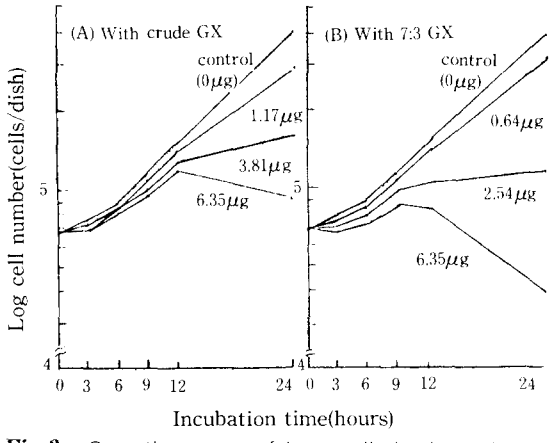

Fig.3. *Growth curves of L_{1210} cells in the culture medium containing various amount of the crude GX(A) and 7:3 GX(B).*
crude GX and 7:3 GX; see Fig.2.

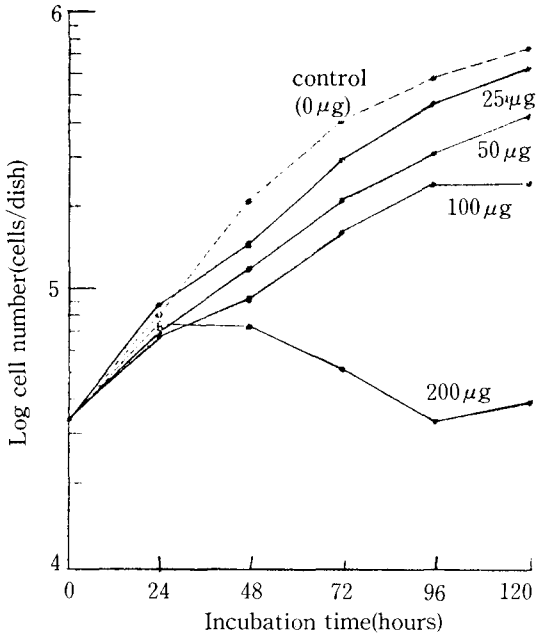

Fig.4. *Growth curves of HRT-18 cells in the culture medium containing various amount of ginseng extract.*

crude Gx and the crude Gx groups containing 25 μg, 50μg, 100μg and 200μg/m*l* each, were cultured for 5 days, and growth curves thus obtained are as illustrated in Fig.4. Accordingly, there was not found discriminable differences in growth pattern until 24 hours of incubation between the control group and crude Gx groups, which was, however, followed by a gradual decrease in the growth rate after 48 hours of incubation period with the elapse of time in proportion to the increase of crude Gx addition. Especially in crude Gx group containg 200μg/m*l*, the proliferative activity was markedly reduced so that the cell number remained at the initial level throughout the experimental period. The same experiment was performed for HCT-48 and HT-29, and the same trend was obtained as for HRT-18.

To pursue the metabolic events led to cytotoxic activity displayed as above, DNA, RNA and protein synthesis profile was obser-

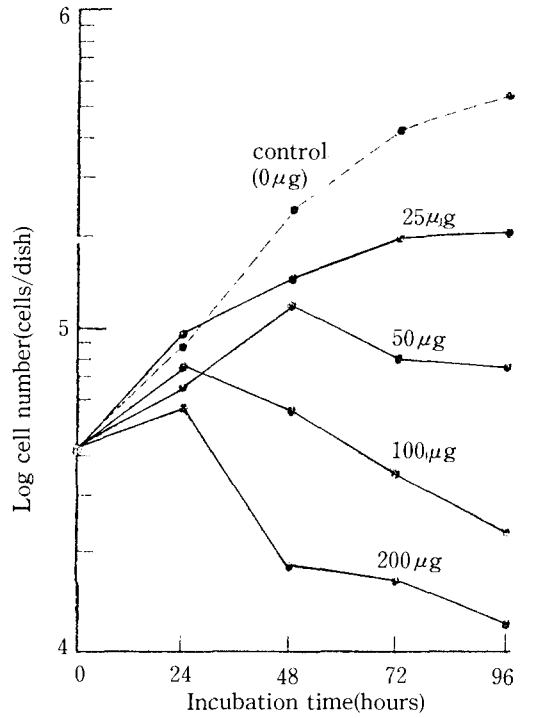

Fig.5. *Growth curves of HCT-48 cells in the culture medium containing various amount of ginseng extract.*

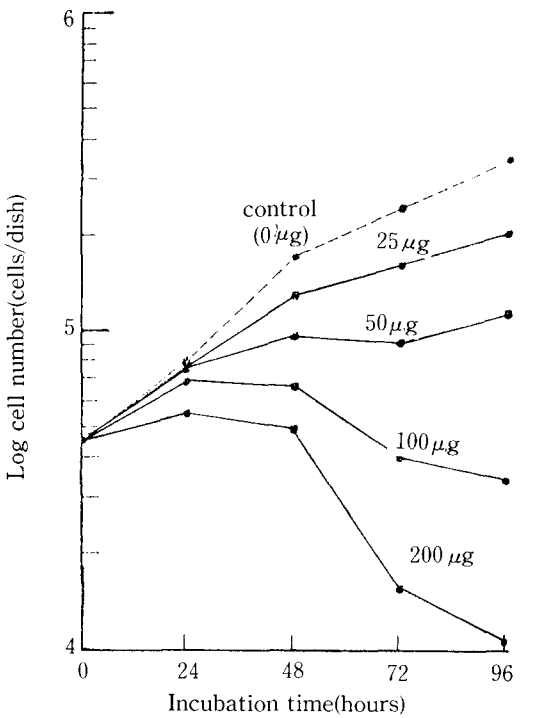

Fig.6. *Growth curves of HT-29 cells in the culture medium containing various amount of ginseng extract.*

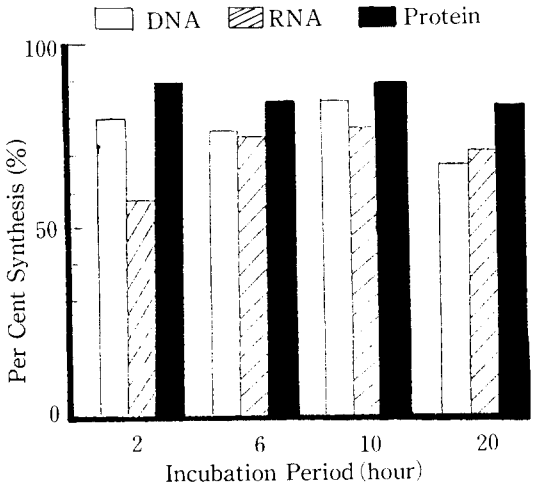

Fig.7. *DNA, RNA and protein synthesis profile of HRT-18 in the presence of 50μg/ml of petroleum-ether extract of ginseng Biosynthetic labeling with tritium labeled thymidine, uridine and leucine was performed for indicated period. The degree of macromolecular synthesis is represented as per cent synthesis to ginseng free culture.*

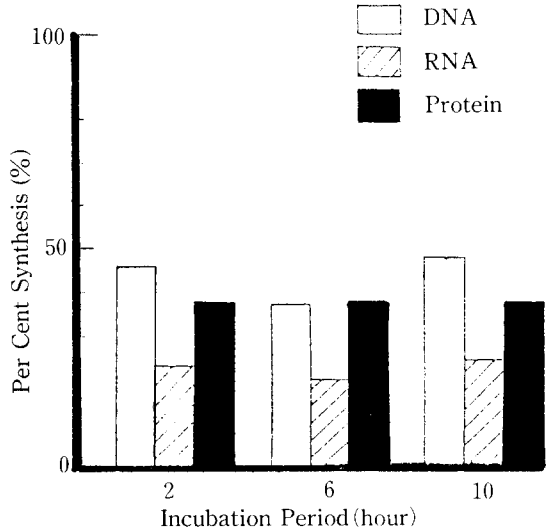

Fig.8. *DNA, RNA and protein synthesis profile of HCT-48 in the presence of 50μg/ml of petroleum-ether extract of ginseng. Biosynthetic labeling with tritium labeled thymidine, uridine and leucine was performed for indicated period. The degree of macromolecular synthesis is represented as per cent synthesis to ginseng free culture.*

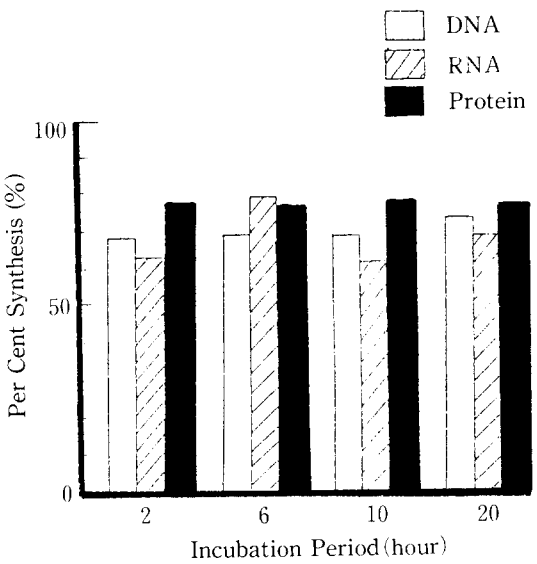

Fig.9. *DNA, RNA and protein synthesis profile of HT-29 in the presence of 50μg/ml of petroleum-ether extract of ginseng. Biosynthetic labeling with tritium labeled thymidine, uridine and leucine was performed for indicated period. The degree of macromolecular synthesis is represented as per cent synthesis to ginseng free culture.*

ved under given concentrations of crude Gx. In HRT-18, DNA, RNA and protein synthesis was reduced 20%, 42% and 11% respectively. and in HCT-48, 53%, 78% and 60% respectively, and in HT-29, 32%, 38% and 23% respectively at a concentration of 50μg of crude Gx/m*l* after 2 hours of incubation compared with the control group.

Influence of 7:3 Gx on the cell size distribution.

Fig.10 shows that the changes of the cell size by the action of the ginseng extracts seems to be related to the decrease of cell number during the incubation. The overall changes of size distribution of L_{1210} treated with 6.35μg of 7:3 Gx per m*l* of culture medium were determined after 0, 12 and 24 hours of incubation. The peaks of each size distribution curve were analyzed in order to compare the sized of the cells affected by 7:3 Gx were greatly changed to less than 40 cubic microns(c.m.) within 24 hours of incuba-

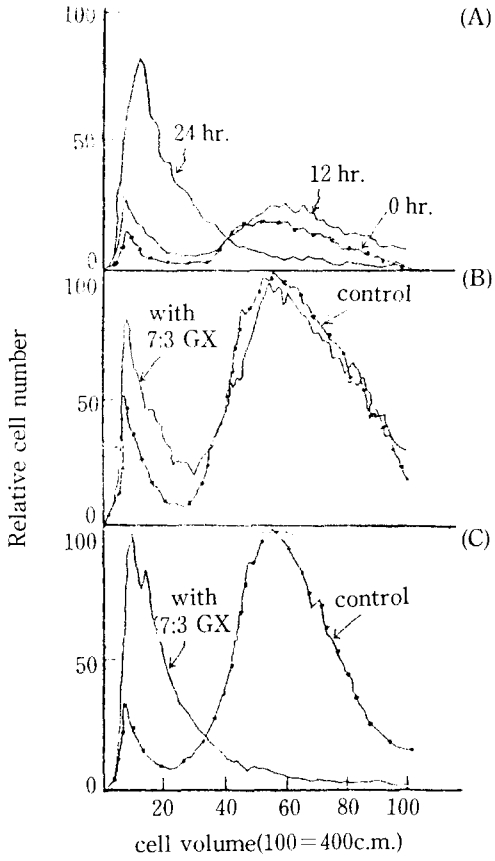

Fig.10. *Size distribution curves of L_{1210} cells incubated with or without 6.35 μg of 7:3 GX per ml of culture medium.*
(A) Overall changes of size distribution curves of L_{1210} cells incubated with 7:3 GX for 12 hours.
(B) Peaks of size distribution curves of L_{1210} cells incubated with or without 7:3 GX for 12 hours.
(C) Peaks of size distribution curves of L_{1210} cells incubated with or without 7:3 GX for 24 hours.

tion, while those of the control cells were maintained at 140 c.m. during incubation. detailed mechanism about this phenomena is interestingly under study in view of the cell morphology.

Effect of 7:3 Gx on survival time and hemoglobin level.

Survival times of mice inoculated with S-180 cells were significantly increased in the partially purified 7:3 Gx treated group. About 80% of S-180 bearing mice in the 7:3 Gx treated group survied more than 18 days after inoculation of the cancer cells, while none of the control group survived after 18 days(Table 3).

Table 4. *Changes of hemoglobin values of rats inoculated with Walker 256 cells by treatment with crude petroleum-ether extract of ginseng root**

Group	With Walker 256	With Walker 256 & GX
Control**	9.53±1.80	9.53±1.80
1	6.79±2.33	11.57±0.34
2	5.15±1.15	12.45±1.00
3	6.29±1.66	8.95±0.80

* Each 10 mg of crude petroleum-ether extract of ginseng root per day was treated to rat by oral administration for the duration of 3, 6 and 9 days after inoculation with Walker 256 cells, respectively.
**Control; Normal rats which received neither inoculation with Walker 256 cells nor crude GX treatment.
Group 1; 3-day group after administration of Walker 256 or Walker 256 & crude GX.
Group 2; 6-day group after administration.
Group 3; 9-day group after administration.

Table 3. *Changes of survival times of Swiss mice inoculated with Sarcoma-180 cells by treatment with 7:3 GX.*

Term(day) / Group	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	30	over
Control					20		40		100									
7 : 3 GX**							10				30		40	50		60	80*	

*; % of dead animal.
**; Treated group with 7:3 GX(5.0 mg/head/day) after inoculated with Sarcoma-180 cells.

Therefore, on the average, the survival times of mice bearing the cancer were extended about 1.5 to 2 fold by treating them with the 7 : 3 Gx compared with their control group. From the above results, it is concluded that the 7:3 Gx have the potential anti-cancer activities *in vivo* against some cancer cells.

Table 4 illustrates that the decrease of hemoglobin value fo rat after inoculation of Walker 256 can be recovered by treating the rats with the crude Gx. In all experimental groups, significantly decreased hemoglobin values of rats after inoculation of the cancer cell were corrected to normal values by oral administration of the crude Gx for the duration of 3, 6 and 9 days. This means that the ginseng extract has the ability to ameliorate the hematologic derangement frequently encountered on the proliferation of the cancer cells in a rat.

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