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A Study on the Antitumor Activity of *Panax ginseng*

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Abstract□*Panax ginseng* has been extensively used in the traditional oriental medicine as a restorative, tonic and prophylactic agent. Recently, several reports regarding to anticancer effects of *Panax ginseng* has accumulated. 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. 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 (L1210, P388) and human cancer cells (HRT-18, HT-29, HCT-48). This sutdy was devised to observe the cytotoxic activities of petroleum-ether extract of *Panax ginseng* roots (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 (L1210) and human rectal cancer cells (HRT-18) and human colon cancer cells (HT-29 and HCT-48) *in vitro*. Each cell-line was cultured in medium containing serial concentration 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 have 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 L1210 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. While, the Panaxydol and Panaxynol as active compounds were purified from petroleum-ether extract of *Panax ginseng* root by Drs. Ahn and Kim, and author found out that the one unit of cytotoxic activity of the Panaxydol and Panaxynol against L1210 cells being equivalent to 0.26 μg and 0.39 μg, respectively. 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 prophylatic agent.[1-3] The pharmacological effects of ginseng and its extract reported during past eighteen years are various and controversial.[4-7] Very extensive pharmacological activities were reported in some purified ginsenosides with respect to central nervous system, gonadotropism, antistress, RNA synthesis, DNA, lipid and protein synthesis.[8-11]

Both ginsenoside Rb1 (protopanaxdiol group ginseng saponin) and Rg1 (protopanaxtriol group ginseng saponin) are among the most important pharmacologically active components.[12,13] but they have

only part of the pharmacological properties of ginseng which have been noted.

Recently, several reports regarding to the anticancer effects of *Panax ginseng* accumulated,[14,15] but these studies were incomplete as only crude extracts were used and systematic data on the anticancer active component were not included. From these studies, it was emphasized that the anticancer activities might be due to a glycoside group called ginsenoside or panax saponin on the *Panax ginseng* which was soluble in water.[16,17] However, we demonstrated that a highly lipid soluble component in the extract of *Panax ginseng* root had a considerable cytotoxic activities against some cancer cells.[18-20]

Therefore, this study was carried out in vitro to determine the cytotoxic activities of the lipid soluble ginseng extracts (crude GX and 7 : 3 GX) against several cancer cell lines (leukemic and human colon) in comparison with those of the saponin derivatives purified from *Panax ginseng*. Then, the crude GX and 7 : 3 GX which had shown very significant activities *in vitro* were applied *in vivo* test to study their effectiveness as anticancer agents or the supportive anticancer agents to 5-FU by measuring the survival time and recovery rate of the hematopoietic derangement on cancer bearing mice or rats after treatment with the ginseng extracts.

Materials

Dried Korean ginseng root (first grade, six year 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. 5-Fluorouracil was purchased from drug store in Seoul. Cancer cells: murine leukemic lympocyte (L1210, P388), murine ascitic sarcoma 180 and Walker carcinosarcoma 256 were a gift from the Research Institute for microbial Diseases, Osaka University. Experimental animals; DBA/2, C57BL/6 were also a gift from the Osaka University. Swiss mice (strain A) and Albino rat(Sprague-Dawley strain) were a gift from Dr. Park (Seoul National University), and human rectal cancer cell HRT-18 and human sigmoid colon cancer cell HCT-48 and HT-29 were a contribution from Prof. Kim, Y.S. Medical School, University of California, San Francisco, U.S.A. Each cell-line and experimental animal were maintained in our labolatory and used for the experiment. The Fischer's medium, Dulbecco's Modified Eagle Medium (DMEM) and horse serum were purchased from the Grand Island Biological Co.; silicic acid (Bio-Sil A, 100~200 mesh) from the Bio-Rad Laboratories; millipore filter discs (GS. 0.22 μm) and accessories were from the Millipore Corp. Coulter counter (model ZBI) and its accessories were goods of Coulter Electronic LTD.

Methods

1. Preparation and partial purification of the ginseng extracts

The *Panax ginseng* roots were finely pulverized using a mortar and 3 grams of the powder were subjected to extraction with petroleum ether for 12 to 15 hours by 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 in a refrigerator. The crude GX was then subjected to a silicic acid column chromatography for partial purification. After 15g of silicic acid were activated in an oven by heating at 120℃ for two hours, 50 m*l* of chloroform was added to it, and slurry was poured into a glass column(2×17 cm). The prepared column was washed with 50 m*l* of chloroform, and then with 50 m*l* of petroleum ether. The crude GX(100~200 mg, dry weight) dissolved in small amount of petroleum ether was loaded to the column, and stepwise elution was processed 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 twice with 70 : 30(v/v) petroleum ether-ethyl ether mixture. From each seperated fraction, 70 : 30(v/v) fraction(7 : 3 GX) was selected for futher study, and the eluate was dried by vacuum evaporation. For the experiments, the crude GX and 7 : 3 GX were dissolved in small amount of absolute ethanol and diluted with water to the desired concentration.

2. Thin layer chromatography

Crude GX and each fraction obtained from silicic acid column chromatography were chromatographed by thin plate of silica-gel using petrolium-ether/ethyl-ether/acetic acid mixture (90 : 10 : 1, v/v/v) as a developing solvent. Chromatograms were recognized as fluorescent spots at 375 nm immediately after spraying 0.01% aqueous solution of rhodamine 6G as described by Marinetti.[21] The spots could also be recognized by their pink color even after the rhodamine solution dried. The area of silica-gel corresponding to necessary band was scraped off to test the cytotoxic activity against cancer cells, and the lipid was eluted with ethyl-ether three times. Only a trace amount of rhodamine 6G was found in the eluent.

3. Cell culture

L1210 cells were cultured in the Fischer's medium by Fischer & Sartorelli method.[22] 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 chest of the Albino rat.

Human colon cancer cell lines HRT-18, HCT-48, and HT-29 were cultured by the procedure of Hwang (Hwang *et al.*, 1986). Cancer cells were grown in DMEM containing 5% fetal bovine serum, penicillin (100 unit/m*l*) and streptomycin (100 μg/m*l*) in T-75 flask or 35 mn petridish at 37℃ under 5% CO_2 tension. Mouse embryo 10~12 days old was minced, and then treated with trypsin. Mouse embryo cells were cultured in DMEM containing 5% fetal bovine serum at 37℃ under 5% CO_2 tension.

4. Quantification of cytotoxic potency

One unit of cytotoxic activity was arbitrarily defined as the amount of drug in one m*l* of culture medium which causes a two-fold increase in the apparent doubling time of each cell-line. In practice, the assay was carried out as follows; P388 or L1210 cells were grown in media containing serial concentrations of the ginseng extracts and the cell populations of each culture medium were counted after 24 hours of incubation by using the Coulter counter. Then, the dose corresponding to the midpoint between the logarithm of control cell number at 0 hours and 24 hours of incubation was estimated to one unit from a plot of the logarithm of cell number vs. concentration of drug.

Growth curves of P388 and L1210 cells treated with various concentrations of ginseng extract were determined by counting the cell populations at regular intervals in the course of incubation. Size ditribution curves of P388 and L1210 cells treated with various concentrations of ginseng extract were determined in comparison with those of control cells after 48 hours of incubation by using the analyzer at "Manometer" count mode setting.

5. Biosynthetic labeling of DNA, RNA and protein

Biosynthetic labeling of DNA, RNA and protein was performed as follows. Cells preincubated in serum free medium for 24 hours were placed in serum free medium, serum added medium, serum and Ginseng added medium or nutritionally varied mediums containings (3H) Thymidine or (3H) Uridine or (3H) Leucine for 2~24 hours. Ginseng petroleum ether extract was added to human colon cancer cells at 50 μg/m*l* of medium and to L1210 at 3 μg/m*l* of medium. Nutritionally varied mediums were 5 kinds as follows. Medium 1; 1% D-Glucose, 126 mg/*l* Arg, 2.4 mg/*l* Cys, 290 mg/*l* Gln, 42 mg/*l* His, 52.5 mg/*l* Ile, 52.4 mg/*l* *Lue, 72.5 mg/l* Lys, 15 mg/*l* Met, 33 mg/*l* Phe, 47.6 mg/*l* Thr, 10.2 mg/*l* Trp, 36 mg/*l* Tyr, 46.8 mg/*l* Val, 8.9 mg/*l* Ala, 15 mg/*l* Asn, 13.3 mg/*l* Asp, 14.7 mg/*l* Glu, 7.5 mg/*l* Gly, 11.5 mg/*l* Plo, 10.5 mg/*l* Ser, Serum. Medium 2; Glutamine eliminated Medium 1. Medium 3; Non-essential amino acid eliminated Medium 1. Medium 4; Glucose eliminated Medium 1. Medium 5; Medium 1 where glucose was replaced by galactose. After the termination of the biosynthetic labelling, cells were briefly shifted to 0℃ and washed with ice cold phosphate buffered saline (PBS), and scraped off the dishes in 1 m*l* of ice cold PBS. Sonication was applied for 15 sec 13 times and protein determination by Lowry method[23] was followed. Aliquots containing equal amount of protein was made 10% in trichloroacetic acid (TCA). Precipitates were suspended in 1 m*l* of deionized water, and 5 m*l* of Instagel was added to count radio-acti-

vity.

6. 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 were divided again into three control groups 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 and9 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 preceure.[24]

Results and Discussion

The cytotoxic activities 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.

One unit of cytotoxic activity against L1210 cells was equivalent to 2.54 μg and 0.88 μg for 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. 1−A). While, the cytotoxic activities of panax-diol, panax-triol, diol saponin and triol saponin aganist L1210 cells were not detected in those concentration (Fig. 1−B).

Fig. 2 shows that the inhibition effects of the crude GX and 7 : 3 GX on the growth rate of L1210 cells *in vitro* are dependent on the incubation time and the concentrations of the extract. The growth

Table 1. Comparision of cytotoxic activity of each extract by various 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*

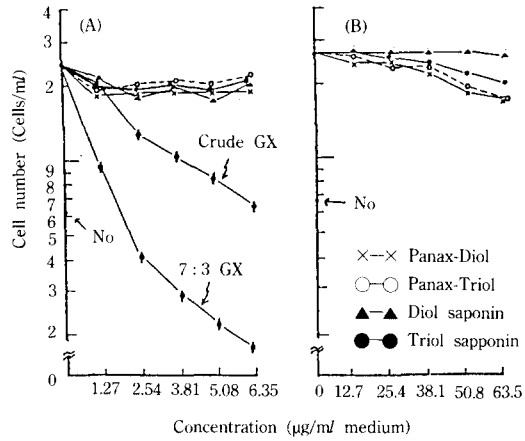

Fig. 1. Dose response curves of petroleum ether extracts or saponin derivatives of *Panax ginseng* on the growth of L1210 cells after 24 hours of incubation. Crude GX; unpurified petroleum ether extract of ginseng root. 7 : 3 GX; partially purified fraction from petroleum ether extract of ginseng root by silicic acid column chromatography. Saponin derivatives; Panax-Diol, Panax-Triol, Diol saponin, Triol saponin. No; initial cell number.

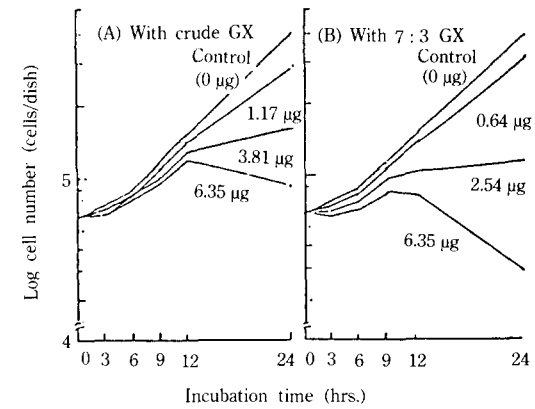

Fig. 2. Growth curves of L1210 cells in the culture medium containing various amount of the crude GX(A) and 7 : 3 GX(B).

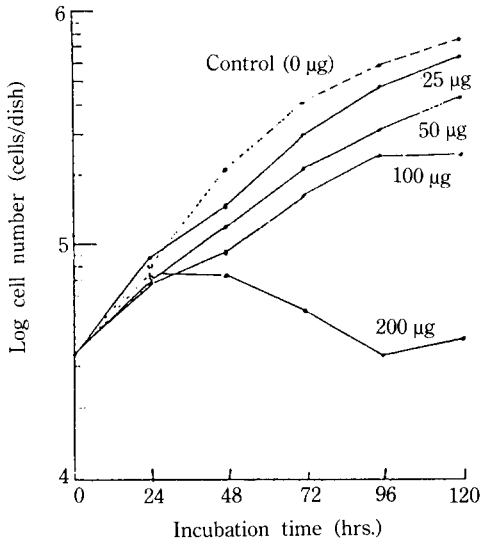

Fig. 3. Growth curves of HRT-18 cells in the culture medium containing various amount of ginseng extract.

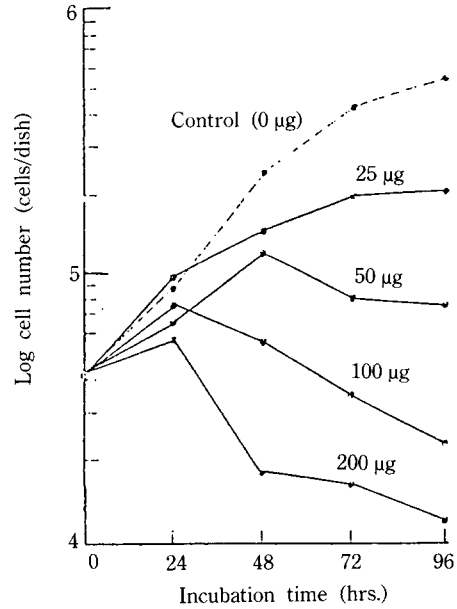

Fig. 4. Growth curves of HCT-48 cells in the culture, medium containing various amount of ginseng extract.

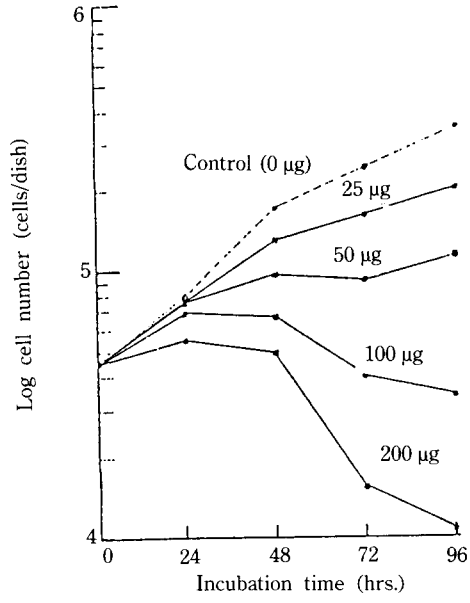

Fig. 5. Growth curves of HT-29 cells in the culture medium containing various amount of ginseng extract.

inhibitions induced by the crude GX or 7 : 3 GX against L1210 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 L1210.

HRT-18 cells, the control group without crude GX and the crude GX groups containing 25 μg, 50 μg, 100 μg, and 200 μg/ml each, were cultured for 5 days, and growth curves thus obtained are as illustrated in Fig. 3. 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 containing 200 μg/ml, 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 (Fig. 4 and Fig. 5).

The effect of panaxynol and panaxydol administered on the some cancer cells cultured *in vitro* have been examined as shown in Fig. 6 and Fig. 7.

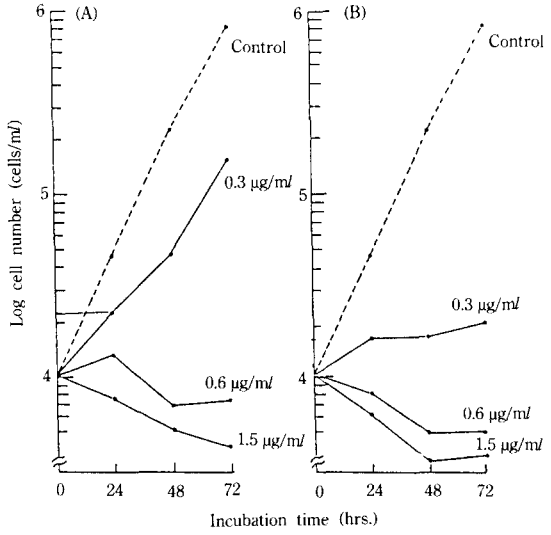

Fig. 6. (A) The effect of panaxynol on the growth of L1210 cells.
(B) The effect of panaxynol on the growth rate of L1210 cells.

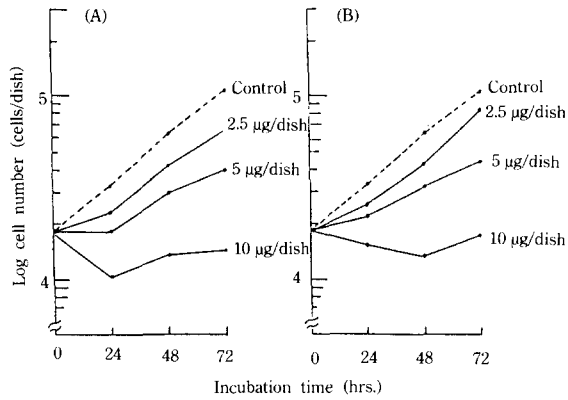

Fig. 7. (A) The effect of panaxynol on the growth of HT-29 cells.
(B) The effect of panaxynol on the growth rate of HT-29 cells.

Mouse leukemic cells (L1210 and P388) and human colon cancer cells (HT-29) were used for the experiment.

The doubling times of the L1210, P388 and HT-29 cells in control culture medium were 12, 12 and 24 hours, respectively.

The growth rate for each kind of cancer cells was inhibited in proportion to the concentration of

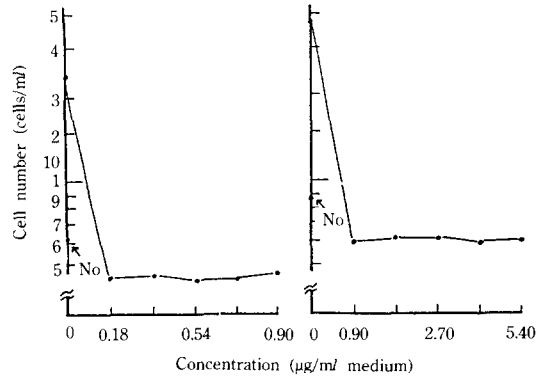

Fig. 8. Dose response curves of 5-FU on the growth of L1210 cells after 24 hours of incubation. No; initial cell number.

panaxynol or panaxydol.

The cytotoxic activities of panaxynol and panaxydol on the mouse leukemic cells were more sensitive than on human colon cancer cells. And the sensitivity of cytotoxic activity was some what different in different cancer cell lines.

For the comparison of the above results with already well known anticancer agent, same experiments were carried out with a synthetic pyrimidine analogue, 5-FU, which had been used frequently in the cancer chemotherapy because of its known action as an inhibitor of thymidylate synthetase and RNA synthesis. The does response curves of 5-FU against L1210 cells and growth curves of the cell after treated with 5-FU showed different aspects from those of the ginseng extracts. 5-FU showed constant cytotoxic activities above its mininum dose necessary for the activity independently of its concentration (Fig. 8). Furthermore, multification of L 1210 cells in a culture medium was strongly inhibited by 5-FU even in the begining of incubation.

Table 2 shows that the decrease of Hb values of rats after inoculation with Walker 256 can be prevented by treating the rats with the crude GX. In all exprimental groups, significantly decreased Hb values of rats after inoculation with the cell were corrected to normal values by oral administration of the crude GX for the duration of 3, 6 or 9 days. This means that the ginseng extract is expected to prevent the anemic condition caused by multification of the cancer cells in a rat.

Table 2. Changes of hemoglobin values of rats inoculated with Walker 256 cells by treatment with crude petroleum ether extract of ginseng root[a]

Group		Hb (g %)
Normal		9.53± 1.80
Control	1	6.79± 2.33
	2	5.15± 1.15
	3	6.29± 1.66
GX	1	11.57± 0.34
	2	12.45± 1.00
	3	8.95± 0.80

[a] Each 10 mg of crude petroblem ether extract of ginseng root per day was treated to rat by oral adiministration for the duration of 3, 6 and 9 days after inoculation with Walker 256 cells, respectively.

Normal; Normal rats which received neither inoculation with Walker 256 cells nor crude GX treatment.

Control 1, 2, 3; 3-day, 6-day and 9-day group after inoculation with Walker 256 cells.

GX 1, 2, 3; Treated group with the crude GX for 3, 6, and 9 days after inoculation with Walker 256 cells.

Survival times of mice inoculated with S-180 cells were significantly increased in the 5-FU treated group or the 5-FU & crude GX treated group but very slightly increased in the crude GX treated group (Table 3(a)). As the effect of extended survival time of the mice by treating them with the crude GX was not significant, another test was carried out with the partially purified 7 : GX. About 80% of S-180 bearing mice in the 7 : 3 GX treated group survived more than 18 days after inoculation with the cancer cells, while none of the control group survived after 18 days (Table 3(b)). On the average, the survival times of mice with the cancer were extended about 1.5 to 2 times by treating them with the 7 : 3 GX compard with their control group. This effect is considered to be valid even though it doesn't reach far to the effectiveness of 5-FU.

The effects of ginseng petroleum ether extract on DNA, RNA and protein synthesis were shown in the Fig. 9~12.

When culture was carried out in 50 μg of ginseng petroleum ether extract/m*l* for 20 hours, synthesis of DNA, RNA and protein each was diminished by 31, 29 and 15% in HRT-18 (Fig. 9), 52, 76 and 62% in HCT-48 (Fig. 10), and 27, 30 and 23% in HT-29 (Fig. 11). In case of L1210, 20 hours culture in 3 μg of Ginseng petroleum ether extract/m*l* resulted in the decrease of DNA, RNA and protein synthesis by 39, 48 and 56% (Fig. 12).

From the above results, it is considered that the crude GX and 7 : 3 GX have the potential antican-

Table 3. Changes of survival time of Swiss mice inoculated with sarcoma 180 cells by treatment with ginseng extracts or 5-Fluorouracil

(A) The changes by treatment with 5-FU or crude GX

Group	Term (days)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	30	Over
Control		10[a]	30						70					100					
5-FU		1								10				20					
5-FU & GX								10		20									
GX			10			20		60			90			100					

(B) The changes by treatment with partially purified 7 : 3 GX

Group	Term (days)	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		400	50		60	80

[a] % of dead animal.

7 : GX; Treated group with partially purified fraction from petroleum ether extract of ginseng root by silicic acid column (5.0 mg/head/day) after inoculation with Walker 256 cells.

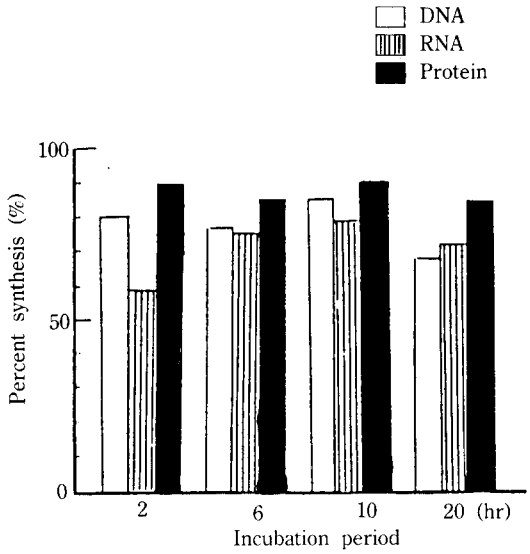

Fig. 9. DNA, RNA and protein synthesis profile of HRT-18 in the presence of 50 μg/ml of petroleum-ether extract of Ginseng.
Biosynthetic labeling with tritium labeled thymidine, uridine and leucine was performed for indicated period. The degree of maeromolecular synthesis is represented as percent synthesis to Ginseng free culture.

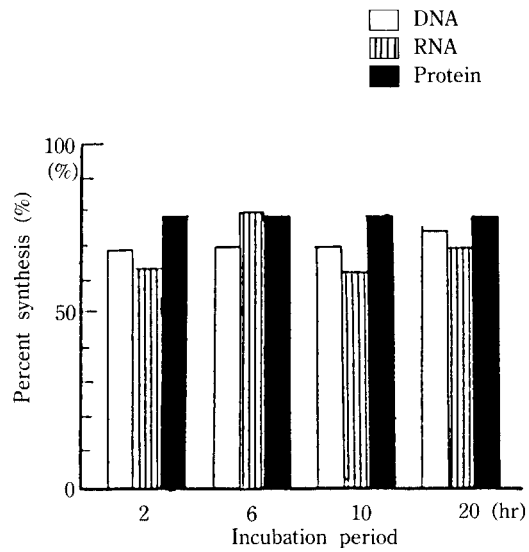

Fig. 11. DNA, RNA and protein synthesis profile of HT-29 in the presence of 50 μg/ml of petroleum-ether extract of Ginseng.

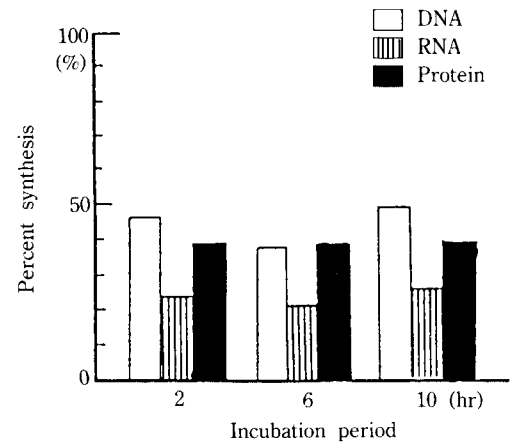

Fig. 10. DNA, RNA and protein synthesis profile of HCT-48 in the presence of 50 μg/ml of petroleum-ether extract of ginseng.
Biosynthetic labeling with tritium labeled thymidine, uridine, and leucine was performe for indicated period. The degree of macromolecular synthesis is represented as percent synthesis to Ginseng free culture.

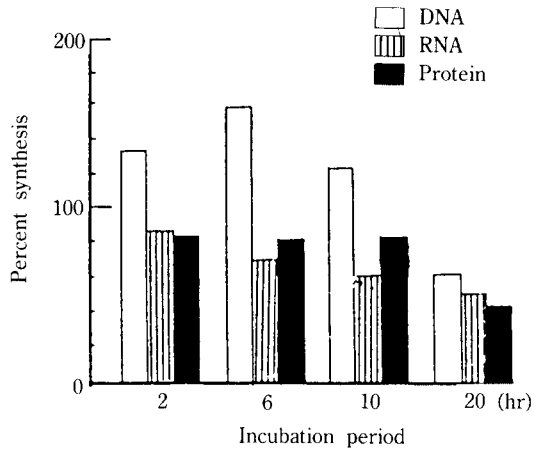

Fig. 12. DNA, RNA and protein synthesis profile of L1210 in the presence of 3 μg/ml of petroleum-ether extract of Ginseng.
Biosynthetic labelling with tritium labeled thymidine, uridine and leucine was performed for indicated period. The degree of macromolecular synthesis is represented as persent synthesis of Ginseng free culture.

cer activities against some cancer cells, but a more finely purified compound of ginseng extract must be prepared in order to establish definite anicancer effect from vivo test.

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