SAFETY EVALUATION OF *PANAX GINSENG* EXTRACTS: A LACK OF MUTAGENICITY IN THE SALMONELLA TYPHIMURIUM AND CHINESE HAMSTER V79 CELLS

I.P. Lee, H.C. Yun, N. Stammer, and R. Langenbach
National Institute of Environmental Health Sciences
Research Triangle Park, NC 27709, U.S.A.

INTRODUCTION

Panax ginseng has been used extensively not solely as therapeutic agents but also as a variety of foods in Asia for centuries (Papove and Goldwag, 1973; Petkov, 1975). In recent times, the use of *Panax ginseng* has become world wide. Although innumerable research papers have described its possible therapeutic effects and pharmacologic agents, precise mechanisms of action of ginseng remain unelucidated. Furthermore, potential mutagenic or carcinogenic effects of ginseng have not been evaluated previously. Therefore, this study was undertaken to determine whether *Panax ginseng* (C.A. Meyer) extracts possess genotoxic effects at both prokaryotic and eukaryotic cell levels.

MATERIALS AND METHODS

Chemicals. Red *Panax ginseng* (C.A. Meyer) powder was obtained from Korea Ginseng and Tobacco Research Institute, Seoul, Korea. Nicotinamide adenine dinucleotide (NADP) and glucose-6-phosphate (G-6-P), ouabain, 6-thioguanine (6-TG), L-histidine, L-amino acids standards, ethylene glycol-bis(B-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), collagenase type 1, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company and Fisher Scientific Company, respectively.

Extraction procedure of panax ginseng (C.A. Meyer). Red *panax ginseng* powder (180g dry weight) was either extracted with 1.81 of distilled water or 1.81 of ethylacetate at 80° and 48° for 24 hrs, respectively Fig. 1. Subsequently, aqueous and solvent.

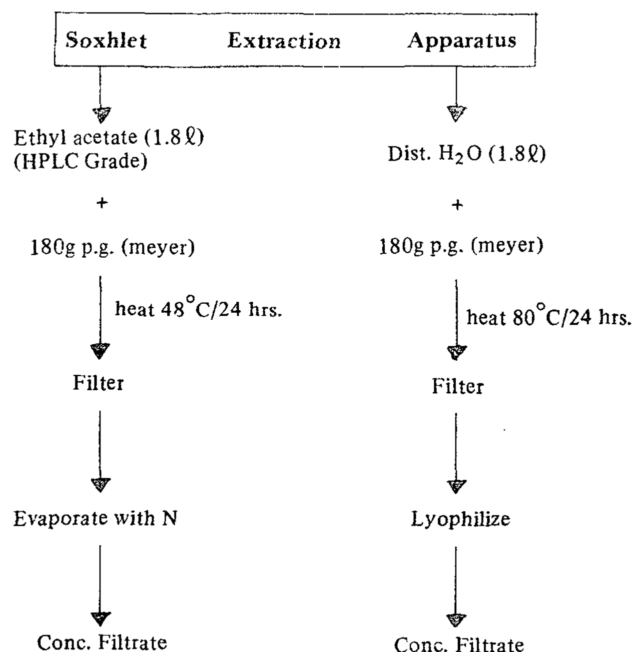

Fig. 1. Extraction procedure of *panax ginseng* C.A. Meyer

Tester strains. *Salmonella typhimurium* strains TA 98 and TA 100 were provided by B. N. Ames (Berkley, California) and stored at $-80°$ as recommended (Ames et al., 1975). Strains were checked for identity and responsiveness at the time of each experiment by the method of Zeiger et al. (1981).

Preparation of S-9, S-9 mix and rat hepatocyte isolation. S-9 was prepared from Aroclor 1254-induced Sprague-Dawley male rat liver according to Ames method (Ames et al., 1975), with the exception that the homogenization was performed using Polytron (Brinkmann Instruments). Aiquots (1.5ml) were dispensed in 5ml crew cap vials, sealed with Parafilm, and frozen in dry ice before storage at $-80°$. Each ml of S-9 mix contained the following: 0.5ml buffered salts (0.3ml $Na_2HPO_4.7H_2O$), 330mM; 0.1ml KCl, 330mM; and 0.1ml $MgCl_2.6H_2O$, 80mM; NADP (4umoles) and G-6-P (5umoles) dissolved in 0.37ml of distilled H_2O and filtersterilized (0.22u Millipore filter); and 0.13ml rat liver S-9 Immediately before testing, the buffered salts, NADP, and G-6-P solution were combined with the S-9. Primary rat hepatocytes from 6-week-old Sprague-Dawley male rats were perfused at 37° through the protal vein for 3min at 10ml/min with 0.5mM EGTA/20mM N-2-hydroxyethyl-piperazine-N'-2-ethansulfonic acid (HEPES) in Ca^{+2} and Mg^{+2} free HBSS. Once the perfusion was started, the subhepatic inferior vena cava was severed. A solution of collagenase type I (100units/ml) in William's Medium E was then perfused for 10min at 5-8ml/min. The liver was combed with a metal hair comb into the collagenase solution and the cells were centrifuged at 50 x g for 5min at 4°. The cells were suspended and grown in William's Medium E plus 10% heat-inactivated fetal calf serum supplemented with 2mM L-glutamine, penicillin (100 units/ml) and gentamycin (50units/ml). The viability of the hepatocytes by trypan blue exclusion averaged greater than 90%. The maximal number of viable hepatocytes was attached by 3hr. after seeding. After this time, the medium was changed and 8ml of fresh medium containing a test compound was added. In culture experiments the hepatocytes were seeded into 25cm^2 flasks that had been seeded with 2 x 10^5 V79 cells 18hrs earlier. The plating efficiency of the hepatocytes was 20-25% was determined 24hrs after seeding.

S. typhimurium mutagenesis assay. The methods used are those described by Ames et al. (1975). To 2ml of molten top agar (45°) up to 0.1ml of the test chemical dissolved in H_2O or DMSO, 0.1ml of an overnight culture of Salmonella (kept on ice), and 0.5ml of either S-9 mix or Na_2PO_4 buffer (100mM, pH7.4) were added, in that order. The tubes were mixed, and poured onto agar plates (Vogel and Bonner, 1956). ACE plates were prepared in triplicate. All operations were performed in biological hood (Class 2, type B) equipped with amber fluorescent light cover. The plates were incubated 40hr at 37°, after which they were scored for his^+ revertants. All plates were examined under magnification for evidence of toxicity, as determined by a decrease in density of the background lawn.

V79 mutagenesis assay. The cell-mediated V79 assay described by Langenbach et al. (1981) was used with some modification. T-25 flasks were seeded with 2 x 10^5 V79 cells 18 hrs prior to addition of either with or without 5 x 10^6 hepatocytes. After 3 hr period, to allow hepatocyte attachment, the medium and floating cells were gently removed, and culture extracts were concentrated by lyophilization and evaporation with Nz, respectively (Fig. 1).

Amino acid analysis of Panax ginseng extracts. An aliquote of aqueous extracts of *Panax ginseng* was precipitated with 10% trichloroacetic acid and subjected to molecular filtration with an Amicon filter (MW exclusion of 1,000). The supernatant was acid hydrolysed with the PICO-TAG system using 6N HC1. The HPLC hardware consited of two Waters M6000A pumps controlled by an M660 or M7720 gradient controller, an M440 fixed wavelength UV detector, and M710B WISP autosampler and an M730 data module integrator/plotter. The column was an application-specified reverse-phase PICO-TAG column, 3.9 x 150mm, and was placed in a column heater (waters) held at 38 ± 1°C. The

eluants were blanketed with helium via a Waters Eluent Stabilization system. The usual mobile phase system consisted of two eluants: A=0.14M sodium acetate, pH 6.4 containing 0.05% triethylamine (v/v) and B=60% acetonitrile, 40% water. The gradient was run from 10 to 51% B at the flow rate of 1ml/min using a convex curve. An additional step at 100% B was used to wash the column prior to returning to initial conditions for reequilibration. The lyophilized reside was dissolved in 250μl phosphate buffer (pH 6.5) and analyzed.

Media. Petri dishes (Falcon #1028, muta-assay dishes contained 25ml of Vogel-Bonner medium E (Vogel and Bonner, 1956) in 1.5% Bacto-Difco purified agar supplemented with 0.25% glucose and 12.5 umole d-biotin per liter. Top agar was prepared by adding 10ml of a 0.5mM L-histidine-0.5mM d-biotin solution, 2.5ml of a 20% glucose solution, and 2ml of a 50X concentration of Vogel-Bonner salts to 100ml of 0.6% agar. Overnight cultures were grown in nutrient broth (Oxoid #2) to a density of 1 x 10^9 cells/ml. William Medium E, Hanks balanced salt solution (HBSS), L-glutamine, penicillin, gentamycin, trypsin/EDTA, trypan blue, and fetal bovine serum were obtained from Grand Biological Company and Sterile System, Inc., Logan, Utah, respectively.

Medium containing the ginseng extracts were added. There was usually 20-25% attachment of hepatocytes. After a 24hr after addition of ginseng extracts, the cells were dissociated with 0.05% trypsin/0.02% EDTA in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS, pH7.4). V79 cells were differentially enumerated from hepatocytes by size difference with a hemacytometer. Since the adherence of hepatocytes to the flask surface is greater than that of V79 cells, th gently enzyme treatment allowed the rapid and selective removal of the V79 cells while leaving the hepatocytes on the flask surfce. The 10^5 V79 cells were seeded per 60mm Petri dish to determine mutation frequency. Cloning efficiency is determined by counting the number of colonies in five Giemsa-stained dishes per point 6-7 days after seeding 100 cells in 4ml of medium (total volume, 5ml). The frequencies of ouabain-resistant mutants (Baker et al., 1974) determined from 15 dishes for each dose of the chemical tested with an experiment, by counting colonies 14-16 days after seeding 10^5 cells in 4ml of medium per Petri dish (total volume, 5ml). Ouaban in 1ml of medium was added to a final concentration of 1mM at 2 days after seeding (Huberman and Sachs, 1976). The mutation frequency for resistance to ouabain was calculated per 10^6 cell survivors, based on the cloning efficiency and number of cells seeded for mutant selection.

Mutation frequency for resistance to 6-thioguanine was carried out essentially same as that of ouabain assay with exception of the number of V79 cells per dish (2 x 10^4 cells are seeded per 60mm dish) and the expression period following the test chemical exposure was 6 days prior to the addition of 6-thioguanine (final concentration of 30uM/dish). Mutation frequency scoring and the cloning efficiency were determined as was done for the ouabain assay. MNNG was used as a positive control.

RESULTS AND DISCUSSION

The results showed that the solvent extracts at all doses were not mutagenic but were cytotoxic at the highest concentration (1g equivalent) (Table 1). In contrast, the revertant frequencies in TA 98 strain induced by the aqueous extracts either with and without S9 were 38 ± 0.3 (control), 64 ± 3.3 (0.004g), 74 ± 3.6 (0.02g), 82 ± 5.2 (0.1g), and 25 ± 3.7 (control), 52 ± 3.6 (0.04g), 65 ± 3.5 (0.02g), and 75 ± 6.8 (0.1g), respectively (Table 2). Thus, the revertant frequencies at all concentrations of the aqueous extracts were statistically significant ($P<0.005$-0.015). Likewise, the revertant frequencies in TA 100 strain either with and without S9 were 121 ± 7.8 (control), 217 ± 4.8 (0.004g), 273 ± 38 0.02g), 332 ± 4.4 (0.1g), and 128 ± 7.4 (control), 268 ± 5.8 (0.004g), 327 ± 14 (0.02g), and 405 ± 42 (0.1g), respectively (Table 2). Thus, the revertant frequencies in all the aqueous extracts were statistically significant as compared to the con-

Table 1. Ethylacetate extracts of *panax ginseng*

Dose	His$^+$ Revertants$^+$			
	TA 98		TA 100	
	-S9	+S9 (A1254)	-S9	+S9 (A1254)
Control (DMSO)	24.7 ± 0.9	35 ± 4.0	140 ± 11	133 ± 3.8
0.01g	31.7 ± 6.0	42 ± 4.2	140 ± 7.9	135 ± 7.8
0.1g	29 ± 3.1	38.2 ± 2.5	141 ± 14	109 ± 0.7
1.0 g	20.3 ± 3.1	29 ± 3.8	100± 3.2*	61.4 ± 3.5*

+ Mean ± S.E.M (N = 3)
* P <0.001 – 0.02

Table 2. Aqueous extracts of *panax ginseng*

Dose	His$^+$ Revertants$^+$			
	TA 98		TA 100	
	-S	+S (A1254)	-S	+S(A1254)
Control (H_2O)	25 ± 3.7	38 ± 0.3	128 ± 7.4	121 ± 7.8
0.004g	52 ± 3.6*	64 ± 3.3*	268 ± 5.8*	217 ± 4.8*
0.02g	65 ± 3.5*	74 ± 3.6*	327 ± 14*	273 ± 21*
0.1g	75 ± 6.8*	82 ± 5.2*	405 ± 42*	332 ± 4.4*

† Mean ± S.E. (N = 3)
* P <0.0002 – 0.02

trols ($p < 0.0002$-0.02). However, the differences in the revertant frequencies between the plates with and without S9 mix in both tester strains were not statistically significant. The significant increase in the number of the revertant colonies of all tester strains exposed to all concentrations of aqueous extracts appeared to be dose dependent over the spontaneous rate.

The results of the amino acid analysis of the acid hydrolysate of 1,000 molecular weight exlusion supernatant is shown in Figure 2. Total of sixteen amino acids are identified. Arginine and glycine content were the highest (39 and 13.8% of the total). L-histidine was only 1.74% of the total and represented 437.5ug in the acid hydrolysate of 1,000M. Wt. exclusion supernatant (0.1g equivalent) (Table 3). Free L-histidine levels were not performed. Instead, effects of free L-histidine on revertant frequencies of TA 98 and TA100 tester strains as a function of free L-histidine concentration were studied and demonstrated that his+ revertant frequencies increase with increasing L-histidine concentrations in the media in the both tester strains (Figs. 3 and 4). From these results, free L-histidine in the aqueous extract equivalent to 0.1g was estimated to be approximately 80-90ug (approximately 1/5 of the total L-histidine in the acid hydrolysate). Therefore, the dose dependent increase of his$^+$ revertants appears to be attributed to the presence of free L-histidine in the aqueous extract of *Panax ginseng*.

V79 cytotoxicity of aquesou extract of P. ginseng. Preliminary study to determine the effects of aqueous extract of *P. ginseng* at 0.8, 4, 7.5, 15 and 20 x 10^{-2} g equivalent on V79 cell

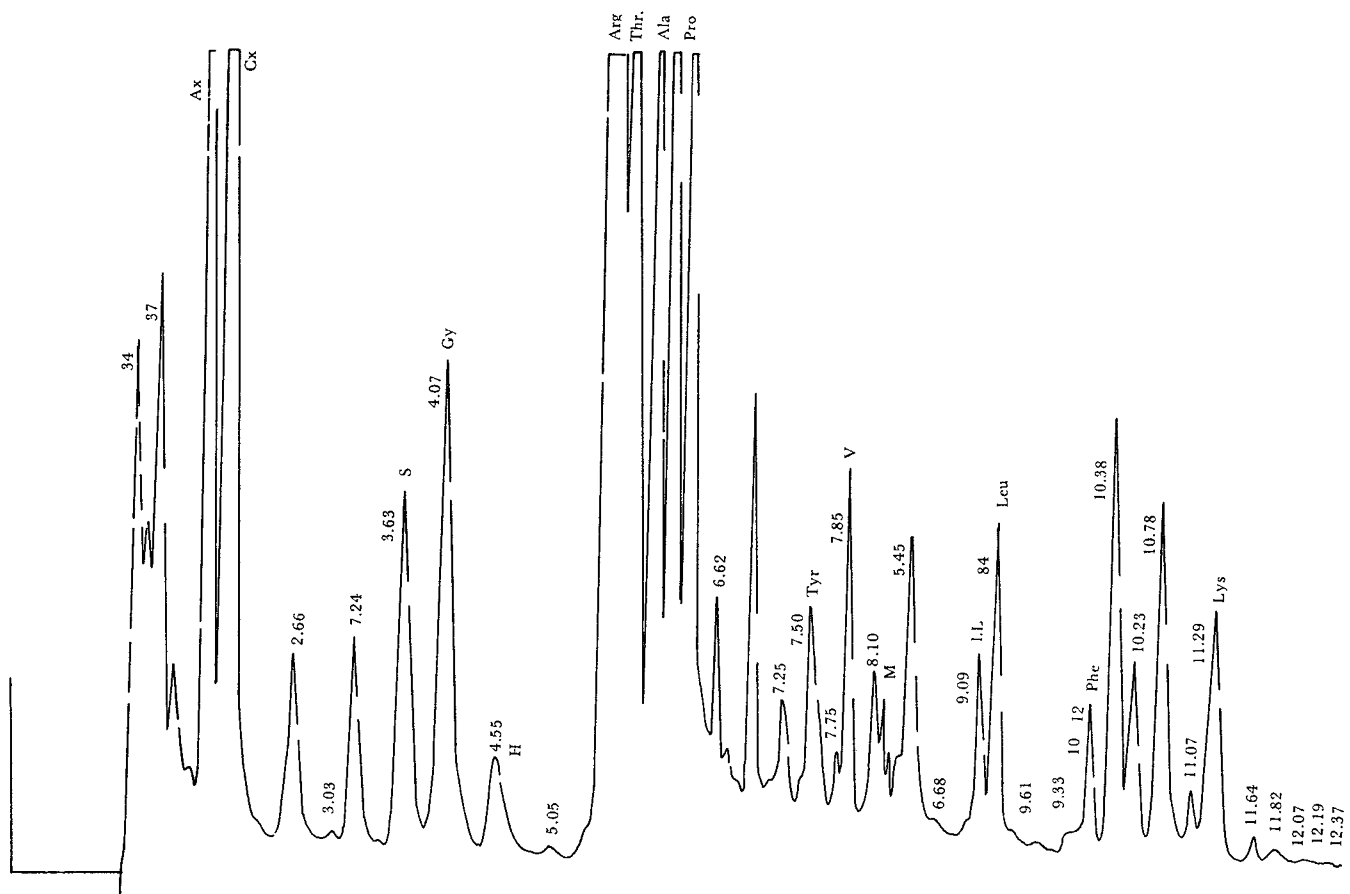

Fig. 2. HPLC Separation of amino acids

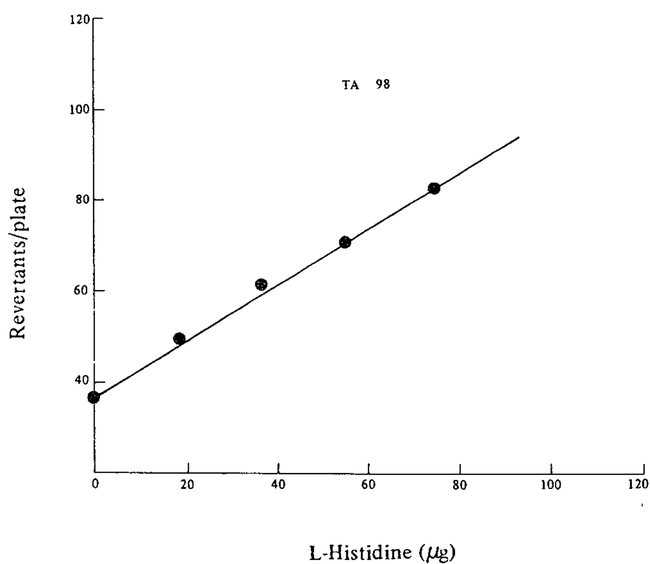

Fig. 3. Effect of L-Histidine on his^+ revertant frequencies in TA 98 tester strain

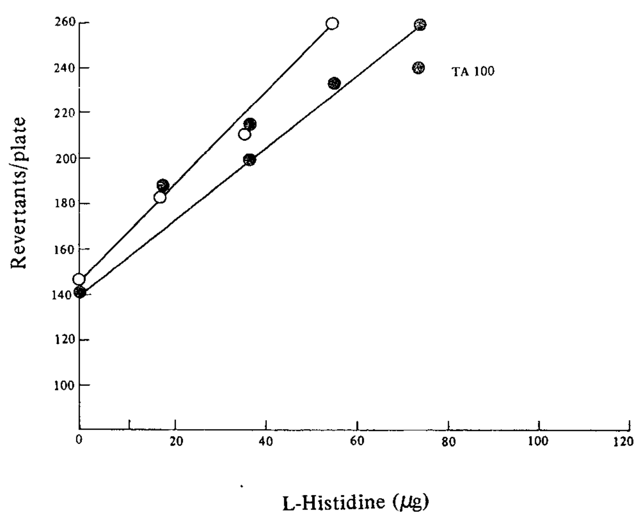

Fig. 4. Effect of L-histidine on his^+ revertant frequencies in TA 100 tester strain

Table 3. Amino Acid Analysis

Sample Position	20	Auto Mode	Wisp Report
Injection Volume	0010		
Number of Injections	1	Injections Remaining	0
Run Time	00: 19	Equilibration Delay	00:01
Non-Default Sys Msg's	6500-0030 PSI		
Wisp Codes Generated:			

Aug. 30, 1984	23:43:29	Chart 2.00 CM/MIN	
		Pun #29	Calc #9
Column		Solvent	Opr Id:

External Standard Quantitation

Peak#	Amount	RT	%
1	469.20300	1.84	7.24 Asp
2	895.34500	2.04	13.82 Glu
3	200.74400	13.691	3.09 Ser
4	299.57700	4.07	4.62 Gly
5	113.14000	4.55	1.74 His
6	2533.30000	5.59	39.10 Arg
7	564.07500	5.79	8.70 Thr
8	366.18700	6.01	5.65 Ala
9	348.14900	6.16	5.37 Pro
10	144.16700	7.50	2.22 Tyr
11	113.46900	7.85	1.75 Val
12	33.94480	8.22	9.52 Met
13	87.42730	9.09	1.34 Ilu
14	137.18500	9.24	2.11 Leu
15	72.27320	10.12	1.11 Phe
16	99.48969	11.29	1.53 Lys
Total	6477.68000		100.00

cytotoxicity indicated that with exception of 2.5 x 10^{-2} g quivalent/106 cells, V79 cell number decreased sharply as a function of time (24 hrs). V79 cells at 0.8, 4, 7.5, 15, and 20 x 10^{-2} g equivalent were 135, 42, 38, 35, and 28% of control, respectively (Fig. 5). In contrast, at the end of 72 hrs at the same concentrations, cytotoxicity was greater (98, 76, 20, 4, and 2% of controls, respectively).

V79 cell mutagenesis with or without hepatocytes. Effects of all doses of aqueous extracts of *P. ginseng* with or without rat hepatocytes failed to demonstrate either ouabain or 6-thioguanine resistant mutants. Therefore, the aqueous extracts of *Panax ginseng* not appear to contain mutagenic compounds or metabolites. The compounds in ethylactate and aqueous extracts responsible for cytotoxicity have not been identified. However, it is speculated that the presence of glycoside and saponin may affect cell membrane and subsequently cuase cell death (Table 4).

In conclusion, these preliminary studies with host-mediated Ame's and V79 Chinese hamster cell tests have failed to demonstrate any mutagenic activity in the extracts of *Panax ginseng* (C.A. Meyer).

Table 4. Rat hepatocyte-mediated mutagenesis of V79 cells

P. g. Extracts (g. equiv.)		Cloning Efficiency(%) Ouabain	6-TG	Ouabain Mutants/10^6 Survivors	6-TG/10^6 Survirors
Control	V79	96	85	0	0
	V79 + Hepatocytes	84	91	1	0
0.08	V79	108	128	0	0
	V79 + Hepatocytes	73	91	0	0
0.04	V79	54	78	0	0
	V79 + Hepatocytes	59	88	0	0
0.075	V79	44	58	0	0
	V79 + Hepatocytes	42	52	0	0
0.15	V79	26	44	0	0
	V79 + Hepatocytes	22	41	0	0
0.20	V79	12	11	0	0
	V79 + Hepatocytes	11	0	0	0

These values are the mean of two sets of experiments.

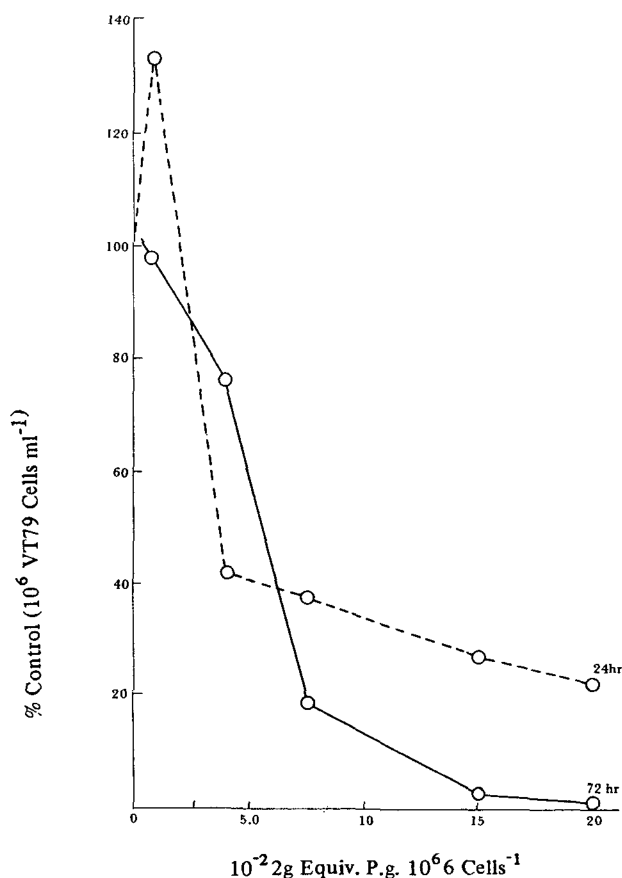

Fig. 5. V79 Cytotoxicity of aqueous extract of *P. ginseng*

Tso: You have been using three types of cells, the bacteria system, the V-79 system, and sperm cell. Is that right? I would like to ask you about one of the conclusions you mentioned. At a certain level, it seems to be at 0.04 gram level, there is a slight cytotoxicity effect. I believe that this level is already known physiologically too high. We never get to that high concentration. But my point of interest is that, have you compared these types of cell to see whether this is a inhibition level which concerns all cell types?

Lee: I think the cell generation may have significant effects on the growth promotion. The V-79 cell happens to have generation time of 12 hours and human mesenchymal cell has generation time of about 8 hours, there are some difference. But I think the promotive effect for cell growth is there. I have not checked all the cell types, so I can't answer you other questions.

Tso: But it seems to me that they are in the same order of magnitude.

Lee: Yes.

Tso: Thank you.

고려인삼의 안전성 평가

I. P. Lee & Z. Matijasevic
미국 N. C., N.I.H.

고려인삼은 아시아 지역에서 수세기동안 강장제 및 치료약으로 광범위하게 사용되어 왔으며, 안전성 및 치료약으로서의 가치는 고대 한의서에 잘 명기되어 있다. 그러나 근래에 들어서 인삼의 복용이 전세계로 확산됨에 따라 안전성 및 인삼의 약리작용에 대한 정확한 기전이 흥미를 끌게 되었다.

따라서 저자는 Salmonella typhimurium tester strain TA 100과 TA 98 및 V-79 Chinese hamster cell line을 이용하여 고려인삼의 안전성을 확인하기 위하여 본 연구를 수행하였다.

지금까지의 결과는 고려인삼이 안전하다는 것을 시사한다.

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