

A Study on the Effects of Herbal Acupuncture of *Holotrichia* (蠚) for Anti-tumor and Increase in Immunity on various Tumor-Induced Models

Joon Mu Lee* · Ji Yong Ha**

* Dept. of Point Location, College of Oriental Medicine, Sangji University

** Dept. of Pathology, College of Oriental Medicine, Sangji University

I . Introduction

Holotrichia(*Citri Reticulatae Virride Pericarpium*) is a sun dried maggot (Scarabaeidae) which is often found in a rotten bird. *Holotrichia* has characteristics of salty, slightly warm, and enters the liver channel. Since it was introduced in *Shinnong Materia Medica*, it has been used for the purpose of breaking blood stasis, promoting lactation, treating gout, tetanus, suppressing pain by removing stasis, stopping cough, brightening eyes,

and etc. Nonetheless, the main purpose of *Holotrichia* has been for removing the blood stasis. It's often believed to be effective against the liver cancer in the folk remedies.

In Oriental medicine, the occurrence of tumor is believed to be influenced by external factors such as wind, cold, heat, fire, and damp as well as interior factors like anger, tenderness, excess thought, sadness, afraidness, stifling, and surprise. Other factors that contribute to the onset of cancer are improper diet, excess work, excess sexual activity, and so forth.

The strength and weakness of 'righteous qi' plays important role in onset of a disease and various interior and exterior factors entering the body provide conditions for outbreak of an illness. Therefore, physical condition and status of visceral organs are the basic factors that determine whether our body is healthy enough to fight off, defend, and recover from an illness.

Sudden death of a cell is generally categorized into necrosis and apoptosis. Necrosis is accompanied by pathological connotations such as destruction of the cell membrane, swelling, and lysis. Apoptosis is characterized by increase of calcium density in the cytoplasm, condensation of chromatin, activation of serine protease, abscission of nucleus, and accompanies apoptotic body which is removed by the neighboring cells through phagocytosis, thus it doesn't incur inflammation of the tissues. Popular anti-cancer drugs and chemotherapies given these days are known to lead apoptosis at an insufficient density to impede ordinary metabolism of the body. These modern treatment techniques must face the problems of selecting specific cancer cells and prohibit cancer cells to be tolerant to the treatment.

Based on all these factors, this study is aimed at closely examining *Holotrichia* by analyzing its efficacies and components, and provide an opportunity for a wider clinical usage. In this experiment, typical pharmacological studies were carried out, anti-cancer effects were measured utilizing existing carcinogenic models, established methods to reduce the side effects, and focused on developing a medium to increase immunity.

II. Experiment Method

1. Materials

(1) Animals

Animals used in the experiment were 4 weeks old ICR type and C57BL/6 type sterile mice weighing approximately 25 grams, supplied by Korea Research Institute of Bioscience & Biotechnology - Laboratory Animal Division. The animals were given solid pellet feed (Samfeed Inc., Korea) free of antibiotics and plenty of water, and were adapted to the laboratory thermohygrostat (Myungjin Machinery, MJ-721cs, Korea) for one week before conducting the experiment.

Table I. Composition of Pellet

Component	Composition(%)
Crude protein	22.1
Crude fat	3.5
Crude fiber	5.0
Crude ash	8.0
Ca	0.6
P	0.4
Others	60.4
Total	100.0

(2) Experiment Herbs

Holotrichias used in the experiment were categorized into either Korean or Chinese origin. Only the complete Holotrichias were used and due to transportation limitation, all of Chinese origins were in a dried form whereas Korean origins were fresh.

2. Method

(1) Manufacturing of herbal extract

(a) Water extraction alcohol precipitation method⁵⁾

600g of Korean Holotrichia and 300g of Chinese Holotrichia were each inserted to a round flask with 2000ml of distilled water and decocted for 3 hours. Then

the decoction was decompressed using a rotary evaporator (Yamato, Japan) and distilled water was added to reach the total volume to 200ml and cooled to a room temperature. Then ethanol was added to make 75% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered.

Remaining fluid was again decompressed with the rotary evaporator and added 100ml of distilled water. Then ethanol was added to make 85% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered. Remaining fluid was once again decompressed with the rotary evaporator and added 100ml of distilled water. Then ethanol was added to make 95% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered. Remaining fluid was decompressed with the rotary evaporator and added 100ml of saline solution and the pH balance was adjusted to 7.2-7.4 with 0.1N of NaOH. Vacuum filtrator (Millipore, USA) with membrane filter (0.45 μ m, 25mm diameter) was pressure sterilized before filtering and the solution was filtered again with syringe filter (0.25 μ m, Whatman, U.S.A) and pressure

sterilized again before used as an herbal acupuncture extract.

(b) Alcohol extraction water precipitation method⁵⁾

600g of Korean Holotrichia and 300g of Chinese Holotrichia were each inserted to a 2000ml beaker and soaked with 1000ml of 100% ethyl alcohol for a day and decocted in the heating mental for 3 hours. Then the distilled water was added to make 75% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered.

Remaining fluid was again decompressed with the rotary evaporator and ethanol was added to make 85% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered. Remaining fluid was once again decompressed with the rotary evaporator and added ethanol to make 95% ethanol solution and stored in the refrigerator at 4°C for a day and the formed precipitate was filtered. Remaining fluid was decompressed with the rotary evaporator until all the moisture was removed and dissolved with 100ml of ethanol. The solution was filtered with syringe-filter (0.25 μ m, Whatman, U.S.A, fat soluble) and

pressure sterilized again before used as an herbal acupuncture extract.

(c) Distillation method⁶⁾

600g of Korean Holotrichia and 300g of Chinese Holotrichia were each inserted to a round flask with 2000ml of distilled water and decocted for 3 hours. Distilled fluid was extracted for 2 hours using the cooling condenser and the vacuum filtrator (Millipore, USA) with membrane filter (0.45 μ m, 25mm diameter) which was pressure sterilized before filtering and the remaining solution of 300ml was sterilized under a high pressure before used as an herbal acupuncture extract.

(2) Disposition of herbal extract and separation of groups

Normal group didn't receive any proceeding, control group was given 0.15ml of 0.9% saline solution on the points of Ren 12 and ST36 that correspond to the human body. Hd group was given Korean Holotrichia extract made by the distillation method, Jd group was given Chinese Holotrichia extract made by the distillation method, Hw was given Korean Holotrichia extract made with the water extraction alcohol precipitation method, Jw was given

Chinese Holotrichia extract made with the water extraction alcohol precipitation method, Ha was given Korean Holotrichia extract made with the alcohol extraction water precipitation method, Ja Ha was given Chinese Holotrichia extract made with the alcohol extraction water precipitation method. For C57BL/6 mice, additional group of Hwt was assembled to inject Korean Holotrichia extract made with the water extraction alcohol precipitation method directly on the tumor area.

For groups with cancer induced by DMBA, Holotrichia was classified as either Korean or Chinese origin and administered Holotrichia extract made with the water extraction alcohol precipitation method and the saline solution on BL18. Another group was given 0.2ml orally and conducted a reproducibility experiment.

All the herbal extract was administered with 1ml insulin syringe (Becton Dickinson, 29G 1/2, USA).

(3) Composition of Culture ground

(a) Standard culture ground

RPMI 1640 (Gibco, USA), 2g of sodium bicarbonate (Shinyo-pure Chemicals Co., LTD., Japan), 4ml of

fungizone (Gibco, USA), 1ml of penicillin G (100,000 units/ml), and 1ml of streptomycine (100mg/ml, Sigma, USA) were added into the distilled water to make 1000ml. pH balance was adjusted to 7.2 and was filtered with 0.22 μ m disposable sterile bottle top filter (Corning, USA) before the usage.

(b) Mixed culture ground

FBS (fetal bovine serum, Gibco. USA) was under inactivation for 30 minutes at the temperature of 56°C and adjusted to be 10% density of the standard culture ground before used to culture the cancer cells.

(4) Cultivation of cancer cells

To induce abdominal cancer in the ICR type mice, sarcoma-180 cells were provided by Korean Cell Line Bank. To induce a tumor in the C57BL/6 type mice, B-16 cells of succeeding generation were provided by Yonsei University, Wonju campus, Department of microbiology.

(5) Induction of cancer

For ICR mice, the sarcoma-180 cells were administered on the abdominal area to induce an abdominal cancer and for

C57BL/6 mice, B16 cells were injected on the upper limbs to induce a tumor. In addition, 0.7mg/ml (body weight of 25g as standard) of DMBA (7,12 dimethylbenz(α)anythracene, Sigma, USA) mixed with the corn oil were orally administered twice a week for 4 weeks to induce a cancer⁷⁾.

(6) Observation of survival time

The sarcoma-180 cells removed from the abdomen of succeeding generation ICR type mice were washed twice centrifugally in PBS (phosphate buffered saline, pH 7.2) before injecting 4×10^6 cells/0.2ml/each on the abdomen of all the groups of ICR type mice. It was followed by 21 days of injecting Holotrichia extract and observed the onset of abdominal cancer for 30 days. Mice having no signs of the abdominal cancer were deleted from the survival time calculation. Median survival time equation devised by Geran and others⁸⁾ was used to calculate increase of life span.

$$\text{Median survival time} = \frac{x+y}{2}$$

$$\% \text{ increase in survival time} = \frac{T-C}{C} \times 100$$

X : First time (day) when the surviving number of mice became 1/2

Y : First time (day) minus 1 from when the surviving number of mice became 1/2

If the total number of mice is an odd number, median survival time is X/2

T : median survival time of the experiment group (day)

C : median survival time of the control group (day)

(7) Measurement of inhibition rate on tumor

Succeeding generation B-16 cells were adjusted to 1×10^4 cells/ml and injected 0.2 ml on the right upper limb of every C57BL/6 type mice groups. After the tumor reached a certain size, saline solution and Holotrichia extract were administered. After 21 days, the mice were sacrificed by fracturing the cervical vertebrae and the tumor was removed and cleaned before weighted using an electronic scale (A&D company, ER-182A, Japan). By weighing the tumor, inhibition rate on tumor was determined. All groups received saline

and Holotrichia extract injection on the points corresponding to Ren 12 and ST 36 and only Hwt group received direct injection of Korean Holotrichia extract made by the water extraction alcohol precipitation method on the tumor growing area.

(8) Measurement of *In vitro* cytotoxicity

To measure the *In vitro* cytotoxicity of various types of Holotrichia extract, modified MTT method^{9,10} developed by Mosmann was conducted.

The sarcoma-180 cells in the exponential multiplier was adjusted to 1×10^4 cells/ml, and inserted $180 \mu\text{l}$ of cell floating liquid and $20 \mu\text{l}$ of Holotrichia herbal extract into 96 well microtitration plate (Falcon, USA). The Holotrichia herbal extract was diluted into half density and filtered using $0.22 \mu\text{m}$ syringe filter before applying onto the 96 well microtitration plate. For 3-4 days, the cells were cultured in 37°C , 5% CO_2 incubator and examined with the phase microscope for the growth of cells untreated with Holotrichia herbal extract. When the cells in the control well grows to a certain size, culture medium was removed and placed $20 \mu\text{l}$ of MTT solution (5mg/ml in PBS, Sigma, USA)

then continued cultivation in 37°C , 5% CO_2 incubator for 3 hours. Then $100 \mu\text{l}$ of 0.04M HCl (in propan-2-ol) was added to react with MTT solution in order to yield blue colored formazan crystallization. This crystallization was completely dissolved and measured optical density within 30 minutes at 540 nm using ELISA reader (Emax precision microplate reader, Molecular devices, USA). Wavelength of 650nm was used as reference wavelength. Each experiment used 4 wells and averaged the value. The experiment was repeated twice and the optical density of the experiment groups and control group were compared. Survival rate was determined using the following equation.

$$\text{Viability} = \frac{[\text{average optical density of expt. group} - \text{standard optical density}]}{[\text{average optical density of control group} - \text{standard optical density}] \times 100(\%)}$$

IC_{50} (50% inhibition concentration)^{11,12} value was figured based on the survival rate. That is, considering IC_{50} as 50% reduction in the optical density according to the MTT examination, X axis being the density of herbal extract and Y axis

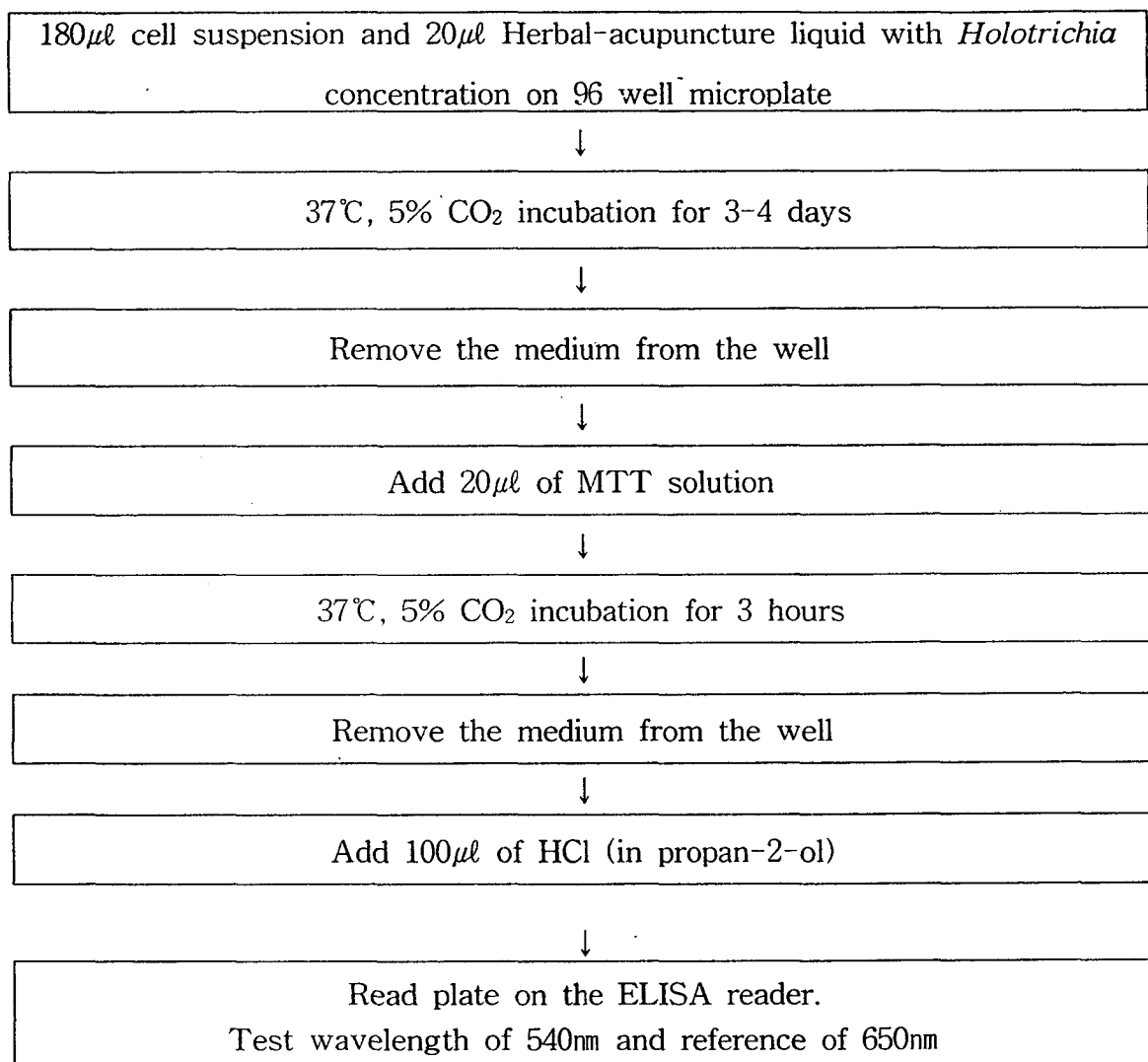
being the survival rate calculated from the optical density, IC_{50} value was yielded by multiple session equation.

Refer to the flow chart for the sequential order. (Figure 1).

(9) Preparation of Spleen cells

Sacrifice the mice by fracturing the cervical spine and apply an alcohol on the abdominal area. The spleen is removed in a germ free condition and remove all the excess tissues around the

Figure 1. Flow chart for measurement of *In vitro* cytotoxicity



spleen and washed twice with the standard culture ground at 4°C. Then the spleen is squashed with cell dissociation sieve-tissue grinder kit (Sigma, USA) and washed thrice with the standard culture ground after removing tissue fragments. Using sterilized distilled water to induce hypotonic shock¹³⁾ for complete hemolysis and washed twice with 10× HBSS (Gibco, USA), washed again with the standard culture ground and spread the spleen cells on the mixed culture ground.

(10) Proliferative reaction of lymphocyte

Spleen cells were cultured for 48 hours until the concentration reached 2×10^6 cell/well and divided into 96 well plates and each well received 100 μ l of sample. Then the cells were cultured for 24 hours in the CO₂ incubator and injected 10 μ l of Premix WST-1 solution (Takara, Japan) and cultured for another 4 hours in the CO₂ incubator. The proliferative reaction of lymphocyte was measured with ELISA Reader at 450nm by measuring the optical density.

(11) Production and Measurement of Interleukin-2

Spleen cells were spread on the mixed

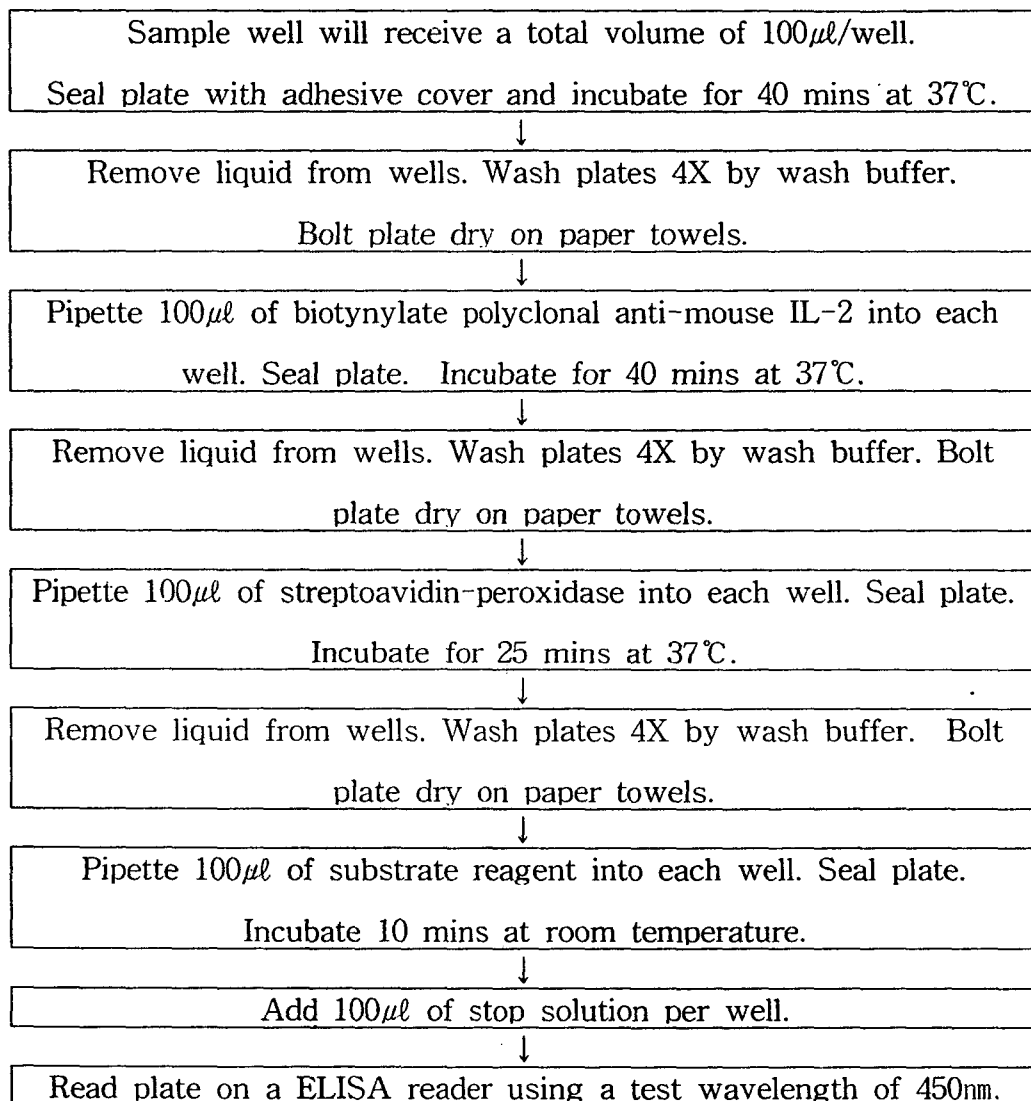
culture ground with 10% of FBS included and added concanavalin-A (Sigma, USA) at the density of 100 μ g/ml. Then the productivity of interleukin-2 was measured following 24 hours of culturing at 37°C, in 5% CO₂ incubator and collected the top layer for the measurement.

Measurement of mice interleukin-2 was done by using intertest-2X Kit (Genzyme, USA). Intertest-2X kit is ELISA Kit to measure the mice IL-2 by using a solid immune enzyme. It measures the optical density at the wavelength of 450nm and compute the volume of IL-2 in the body from the standard curve. Each well received 100 μ l of solution, sealed and incubated for 40 minutes at 37°C. Then the reactive liquid was removed and washed with a buffer four times and the moisture was removed with the paper towel. 100 μ l of biotinylated polyclonal anti-mouse IL-2 was placed onto each well and incubated again for 40 minutes at 37°C. Above cleaning procedures were repeated and this time, 100 μ l of streptoavidin-peroxidase was placed onto each well and incubated for 25 minutes at 37°C. The procedures were once again repeated and substituted with 100 μ l of substrate mix

and incubated for 10 minutes. Lastly, 100 μl of stop solution was added to each well and the optical density was measured with ELISA reader at 450nm.

Refer to the flow chart for the sequential order. (Figure 2).

Figure 2. Flow chart for measurement of IL-2 production



III. Experiment Results

1. Measurement of Survival Time

To investigate the anti-cancer effects of Holotrichia herbal extract, the mice were induced with an abdominal cancer by the sarcoma-180 cells. Normal group didn't receive any treatment, control group was injected with 0.9% saline solution, and experiment groups were administered with the herbal extract on the points corresponding with Ren 12 and ST 36. The normal group showed median survival time⁸⁾ of 22.5 days and

the control group's median survival time increased to 24 days.

Following results are obtained comparing the experiment and control groups:

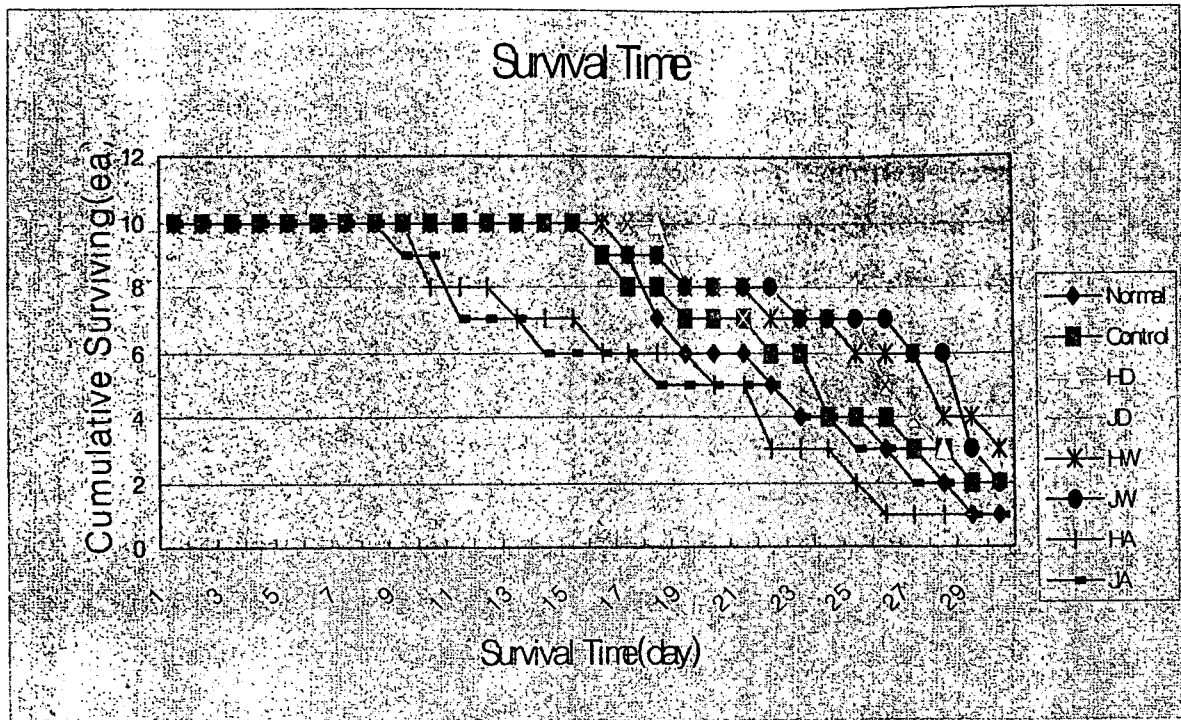
Hd group and Jd group have survival time of 26.5 days which is increase of 10.42%, 28 days for the Hw group at the increase of 16.67%, 29 days for the Jw group at the increase of 20.83%, and Ha and Ja group have survival time of 21 days and 22.5 days respectively, which are shorter than the control group. (Table II, Figure 3)

Table II. Survival Time of the Mice transplanted by Sarcoma-180 Tumor Cell

Group	Route	No. of Animals	Median survival time (day)	ILS ^{a)}
Normal	Non-treated	10	22.5	
Control	Herbal acupuncture	10	24	
Hd	Herbal acupuncture	10	26.5	10.42
Jd	Herbal acupuncture	10	26.5	10.42
Hw	Herbal acupuncture	10	28	16.67
Jw	Herbal acupuncture	10	29	20.83
Ha	Herbal acupuncture	10	21	
Ja	Herbal acupuncture	10	22.5	

a) ILS (Increase of life span): $(\text{treated} - \text{control}) \div \text{control} \times 100$

Figure 3. Survival time of the mice transplanted with sarcoma-180 tumor cells



2. Measurement of inhibition rate on tumor

To measure the inhibition of tumor, the tumor was removed from the C57BL/6 type mice after 21 days of administration of the herbal extract and weighed. Normal group weighed at $2.937 \pm 0.077g$ and the control group at $2.538 \pm 0.005g$, while the groups administered with the herbal extract weighed $2.415 \pm 0.017g$ for the Hd group, $1.995 \pm 0.028g$ for the Jd group, $1.851 \pm 0.027g$ for the Hw group, and $1.827 \pm 0.015g$ for the Jw group, thus showed significant inhibition of the tumor. But the tumor from the

Hwt group weighed $2.520 \pm 0.041g$ which is similar to that of the control group. (Table III, Figure 4)

3. Measurement of *In vitro* cytotoxicity

Considering 1 as the value of IC_{50} of the Holotrichia herbal extract, Holotrichia extract made by the water extraction alcohol precipitation method with Korean was 0.6 and Chinese was 0.375. (Figure 7,8)

Following figures show cytotoxicity on the sarcoma-180 cells according to the place of origin.

- A Study on the Effects of Herbal Acupuncture of Holotrichia (蟾蜍) for Anti-tumor and Increase
in Immunity on various Tumor-Induced Models -

Table III. Inhibition of Holotrichia and Saline on B-16 Tumor in Mice.

Group	Route	No. of Animals	Weight of Tumor (g) ^{a)}	Inhibition rate (%)
Normal	Non-treated	8	2.937 ± 0.077	
Control	Herbal acupuncture	8	2.538 ± 0.005	0
Hd	Herbal acupuncture	8	2.415 ± 0.017*	4.85
Jd	Herabl acupuncture	8	1.995 ± 0.028*	21.39
Hw	Herbal acupuncture	8	1.851 ± 0.027*	37.71
Jw	Herbal acupuncture	8	1.827 ± 0.015*	28.01
Hwt	Herbal acupuncture	8	2.520 ± 0.041	

a) : Mean ± standard deviation

* : P < 0.05

Figure 4. Inhibition of Holotrichia and Saline on B-16 Tumor in Mice.

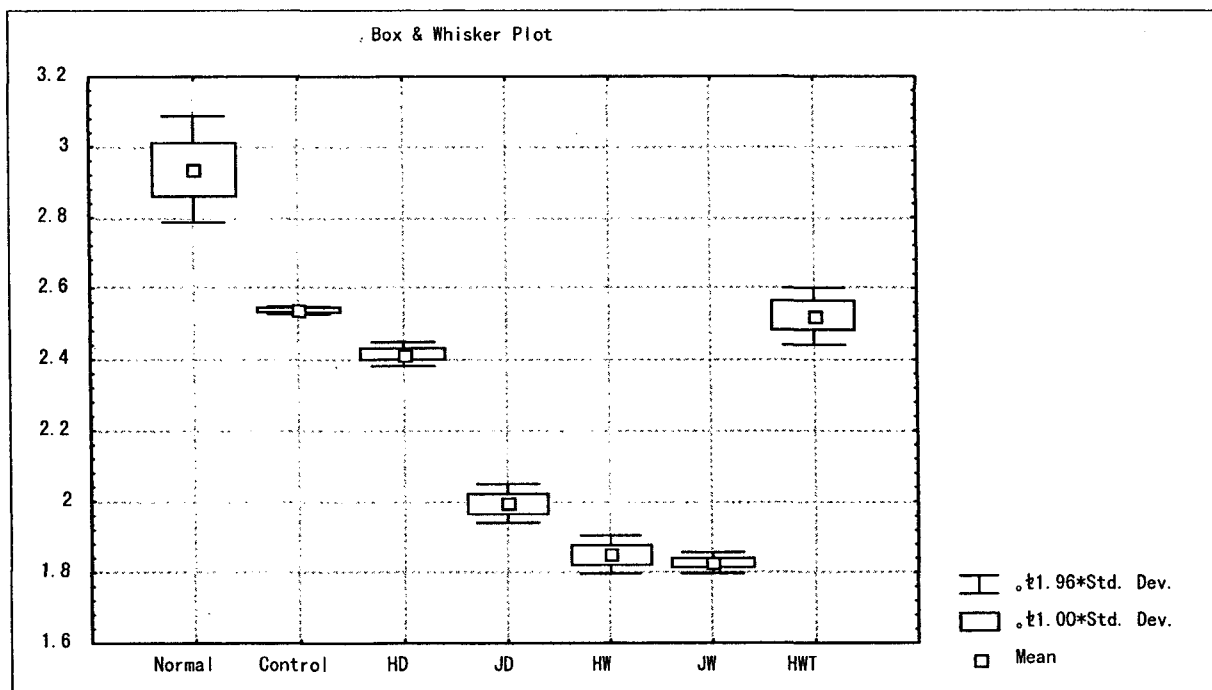


Figure 5. Cytotoxicity of Korean Holotrichia herbal extract made with the water extraction alcohol precipitation method on the S-180 cells

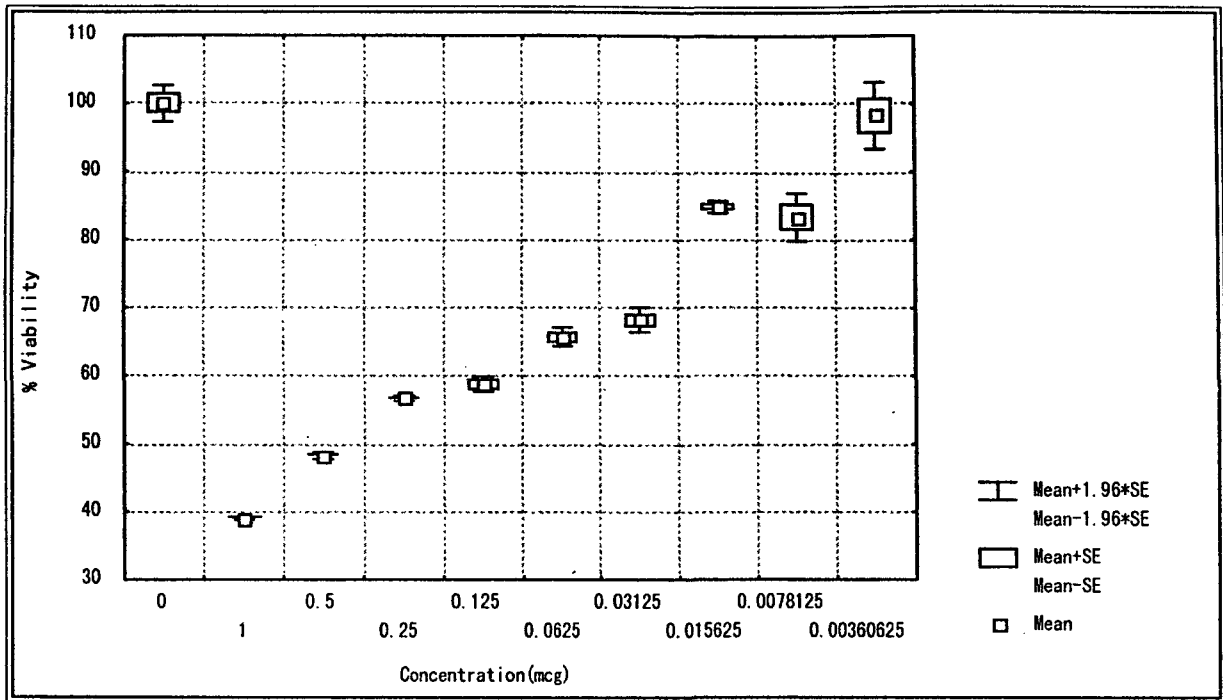


Figure 6. Cytotoxicity of Chinese Holotrichia herbal extract made with the water extraction alcohol precipitation method on the S-180 cells

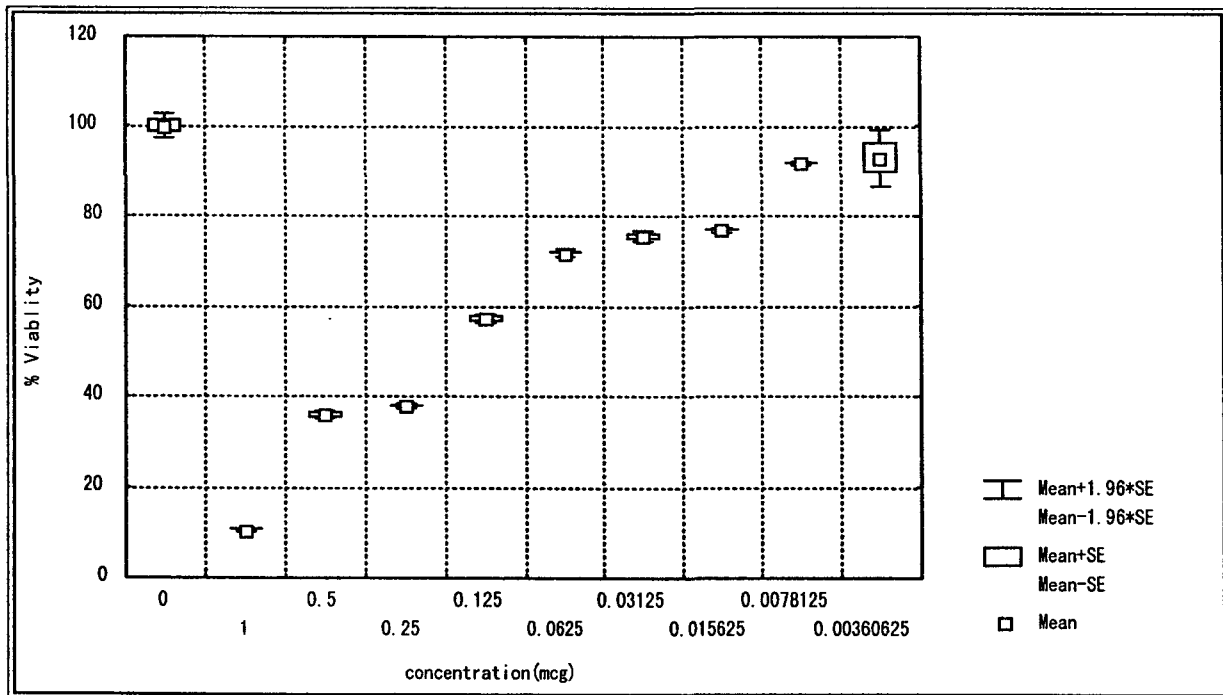


Figure 7. IC₅₀ of Korean Holotrichia herbal extract made with the water extraction alcohol precipitation method on the S-180 cells

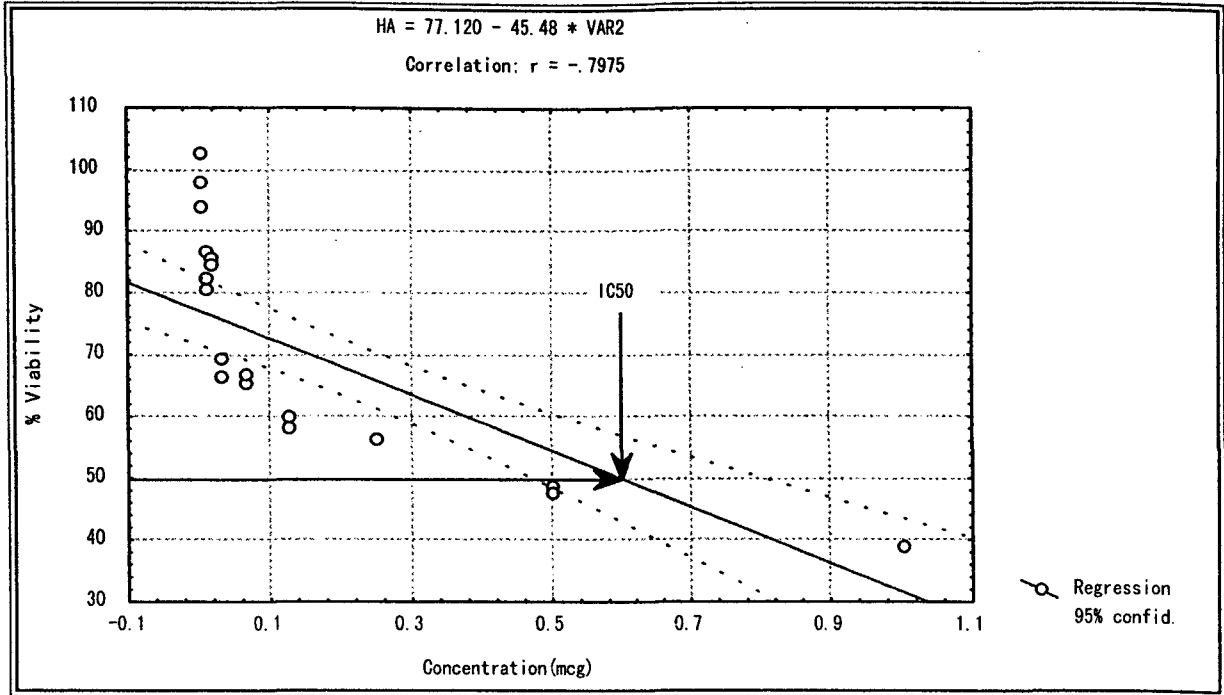
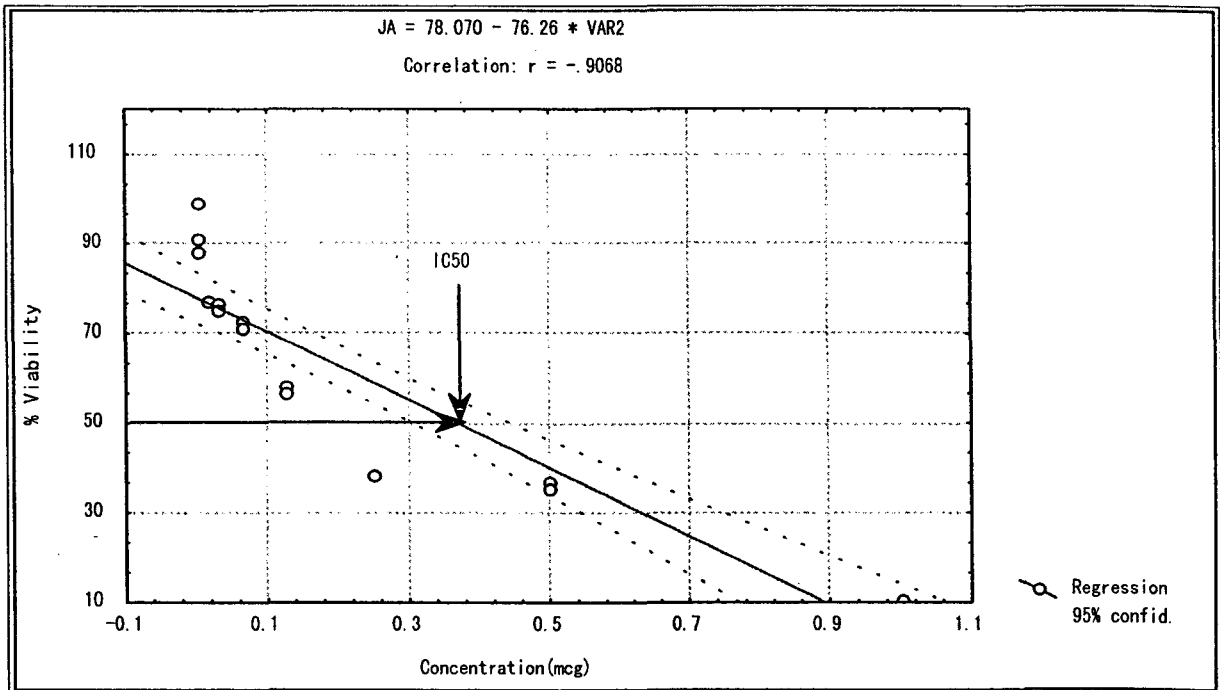


Figure 8. IC₅₀ of Chinese Holotrichia herbal extract made with the precipitation method of water extraction on the S-180 cells



4. Measurement of IL-2 Productivity with oral administration

(1) Increase of IL-2 Productivity in ICR mouse

To measure the IL-2 productivity, 0.2 ml of Holotrichia extract was orally administered for 21 days. As a result,

the control group showed 932.83 ± 36.36 pg/ml and Chinese Holotrichia extract showed 769.68 ± 38.12 pg/ml, thus demonstrated significant decrease. Korean Holotrichia extract had slight increase to 955.66 ± 26.53 pg/ml, but insignificant. (Table IV, Figure 9)

Table IV. Measurement of IL-2 Productivity following oral administration of Holotrichia extract in ICR mice

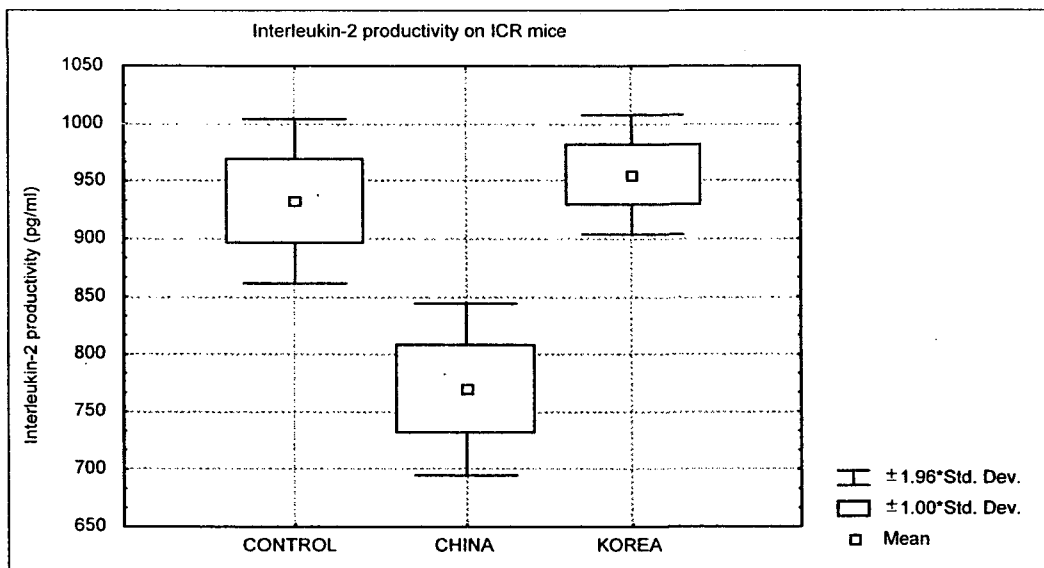
Group	Route	No. of Animals	Interleukin-2(pg/ml) ^{a)}
Control	P.O.	10	932.83 ± 36.36
Chinese Holotrichia	P.O.	10	$769.68 \pm 38.12^*$
Korean Holotrichia	P.O.	10	955.66 ± 26.53

P.O. : Oral administration

a) : Mean \pm standard deviation

* : P<0.05 (vs corresponding control)

Figure 9. Measurement of IL-2 Productivity following oral administration of Holotrichia extract in ICR mice



(2). Increase of IL-2 Productivity in C57BL/6 mouse

To measure the IL-2 productivity, 0.2 ml of Holotrichia extract was orally administered for 21 days. As a result, the control group showed $1268.20 \pm$

72.87pg/ml and Chinese Holotrichia extract showed $1154.00 \pm 62.08\text{pg/ml}$, thus demonstrated significant decrease. Korean Holotrichia extract measured at $1366.40 \pm 48.76\text{pg/ml}$, thus increased significantly. (Table , Figure 10)

Table V. Measurement of IL-2 Productivity following oral administration of Holotrichia extract in C57BL/6 mice

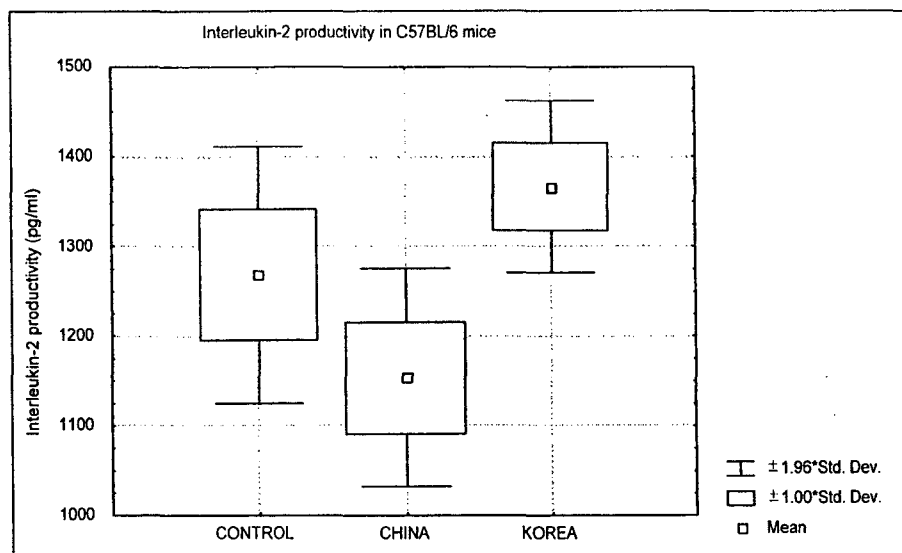
Group	Route	No. of Animals	Interleukin-2(pg/ml) ^{a)}
Control	P.O.	5	1268.20 ± 72.87
Chinese Holotrichia	P.O.	5	$1154.00 \pm 62.08^*$
Korean Holotrichia	P.O.	5	$1366.40 \pm 48.76^*$

P.O. : Oral administration

a) : Mean \pm standard deviation

* : $P < 0.05$ (vs corresponding control)

Figure 10. Measurement of IL-2 Productivity following oral administration of Holotrichia extract in C57BL/6 mice



5. Measurement of Proliferation
reaction of Lymphocyte

On ICR mice treated with DMBA, Holotrichia herbal extract was acupunctured on BL 18 and orally administered for 30 days. As a result, the normal group figured at 804.91 ± 39.72 cpm and the control group measured at $427.85 \pm$

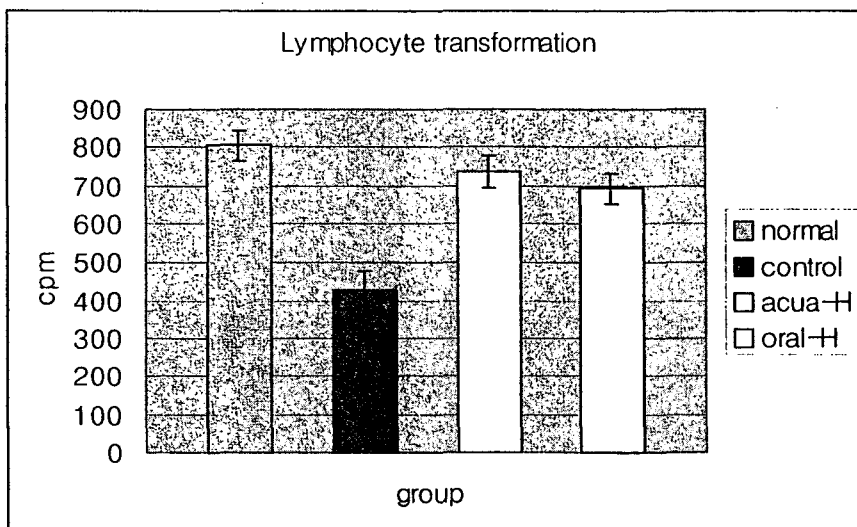
52.44 cpm. For the experiment groups, groups administered with herbal acupuncture had higher figure at 735.27 ± 42.93 cpm versus 692.34 ± 40.75 cpm, thus had more significant result. Significant result was repeated in the reproducibility experiment as well. (Table VI, Figure 11)

Table VI. Effects of Holotrichia on Lymphocyte transformation in mice with DMBA

Treatment	No. of mice	Lymphocyte transformation(cpm)	
		First trial	Second trial
Normal	50	804.91 ± 39.72^c	$871.75 \pm 32.29^{c'}$
Control	50	427.85 ± 52.44^a	$398.33 \pm 47.25^{a'}$
Herbal acupuncture of Holotrichia	50	735.27 ± 42.93^b	$782.04 \pm 35.48^{b'}$
Oral injection of Holotrichia	50	692.34 ± 40.75^b	-

^{abc, a',b',c'} : Means on the same column with different superscripts are significantly different ($p < .05$)

Figure 11. Effects of Holotrichia on Lymphocyte transformation in mice with DMBA



6. Measurement of IL-2 Productivity

On ICR mice treated with DMBA, Holotrichia herbal extract was acupunctured on BL 18 and orally administered for 30 days. As a result, the normal group figured at $579.62 \pm 77.61 \text{ pg/ml}$, the control group measured at $42.59 \pm 9.44 \text{ pg/ml}$, the herbal acupuncture group at $327.27 \pm 55.14 \text{ pg/ml}$, and the oral administration

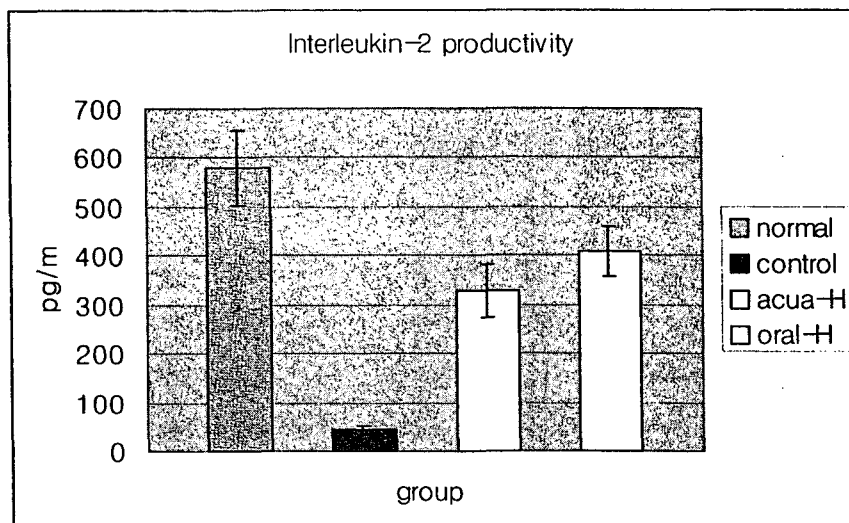
group at $408.35 \pm 50.29 \text{ pg/ml}$. Thus, the herbal acupuncture group and orally administered group showed significant increase in IL-2 productivity when compared to the control group. Significant result was repeated in the reproducibility experiment as well. (Table VII, Figure 12)

Table VII. Effects of Holotrichia on Interleukin-2 productivity in mice with DMBA

Treatment	No. of mice	Interleukin-2 productivity (pg/ml)	
		First trial	Second Trial
Normal	50	579.62 ± 77.61^c	$542.72 \pm 65.37^{c\prime}$
Control	50	42.59 ± 9.44^a	$85.44 \pm 11.27^{a\prime}$
Herbal acupuncture of Holotrichia	50	327.27 ± 55.14^b	$309.75 \pm 60.24^{b\prime}$
Oral injection of Holotrichia	50	408.35 ± 50.29^b	

^{abc, a'b'c'} : Means on the same column with different superscripts are significantly different ($p < .05$)

Figure 12. Effects of Holotrichia on Interleukin-2 productivity in mice with DMBA



IV. Discussion

The purpose of this experiment was focused on exhibiting reproducibility and pellucidity of the Holotrichia herbal acupuncture. This was done by measuring the effectiveness of the herbal acupuncture with injection and oral administration and anti-cancer capability. Examination items range from measuring the survival time, inhibition rate on tumor, *In vitro* cytotoxicity, IL-2 productivity, and proliferative reaction of the lymphocyte.

To investigate the anti-cancer effects of the Holotrichia herbal extract, mice were induced with abdominal cancer by the sarcoma-180 cells. Normal group didn't receive any treatment, the control group was injected with 0.9% saline solution, and the experiment groups were administered with the herbal extract on the points corresponding with Ren 12 and ST 36. The normal group showed median survival time⁸⁾ of 22.5 days and the control group's median survival time increased to 24 days. Following results were obtained comparing the experiment and control groups.

Hd group and Jd group have survival time of 26.5 days which is increase of

10.42%, 28 days for the Hw group at the increase of 16.67%, 29 days for the Jw group at the increase of 20.83%, and Ha and Ja group have survival time of 21 days and 22.5 days respectively, which are shorter than the control group.

To measure the inhibition of tumor, the tumor was removed from the C57BL/6 type mice after 21 days of administration of the herbal extract and weighed. Normal group weighed at 2.937 ± 0.077 g and the control group at 2.538 ± 0.005 g, while the groups administered with the herbal extract weighed 2.415 ± 0.017 g for the Hd group, 1.995 ± 0.028 g for the Jd group, 1.851 ± 0.027 g for the Hw group, and 1.827 ± 0.015 g for the Jw group, thus showed significant inhibition of the tumor. But the tumor from the Hwt group weighed 2.520 ± 0.041 g which is similar to that of the control group.

Experiment for the *In vitro* cytotoxicity was conducted by MTT assay developed by Mosmann⁹⁾ using the sarcoma-180 cells and HT-29 cells. In general, cytotoxicity of the anti-cancer medication is indicated by the inhibitory concentration (IC_{50}) where the optical density is 50% of the control group. In MTT assay using the sarcoma-180 cells, considering 1 as the value of IC_{50} of the Holotrichia

herbal extract, Holotrichia extract made by the water extraction alcohol precipitation method with Korean Holotrichia was 0.6 and Chinese Holotrichia was 0.375.

The immune response of the body is generally classified into cell mediated immunity where the T helper cell plays vital role, and body fluid immunity that governs production and secretion of the antibody into the blood and body fluid²⁶⁻²⁸⁾. It has been known that the immune response to the tumor is especially done by the cell mediated immune system²⁹⁾. Along with B cell, T helper cell is called as lymphocyte that is distinguished from phagocyte and combined by the red blood cell and CD2 molecule. With NK cells, T helper cells release IL-2 and activate the immune system by stimulating B cell and monocytes²⁷⁾. On ICR mice treated with DMBA, the Holotrichia herbal extract was acupunctured on BL 18 and orally administered for 30 days. As a result, the normal group figured at 804.91 ± 39.72 cpm and the control group measured at 427.85 ± 52.44 cpm.

For the experiment groups, groups administered with herbal acupuncture had higher figure at 735.27 ± 42.93 cpm versus

692.34 ± 40.75 cpm, thus had more significant result. Significant result was repeated in the reproducibility experiment as well.

IL-2, known as the growth factor for the T cell, is produced in the LGLs (large granular lymphocytes) and acts as the growth factor and stimulant for the T cell. It promotes growth and differentiation by stimulating the B-cell and activates macrophage and oligodendrocytes as well as the cells that contain toxins with anti-tumor effects³⁰⁾.

To measure the IL-2 productivity, 0.2 ml of Holotrichia extract was orally administered to the ICR type mice for 21 days. As a result, the control group showed 932.83 ± 36.36 pg/ml and Chinese Holotrichia extract showed 769.68 ± 38.12 pg/ml, thus demonstrated significant decrease. Korean Holotrichia extract had slight increase to 955.66 ± 26.53 pg/ml, but insignificant. For the C57BL/6 mice, the control group showed 1268.20 ± 72.87 pg/ml and Chinese Holotrichia extract showed 1154.00 ± 62.08 pg/ml, thus demonstrated significant decrease. Korean Holotrichia extract measured at 1366.40 ± 48.76 pg/ml, thus increased significantly.

For the ICR mice treated with DMBA, the Holotrichia herbal extract was

acupunctured on BL 18 and orally administered for 30 days. As a result, the normal group figured at $579.62 \pm 77.61 \text{pg/ml}$, the control group measured at $42.59 \pm 9.44 \text{pg/ml}$, herbal acupuncture group at $327.27 \pm 55.14 \text{pg/ml}$, and oral administration group at $408.35 \pm 50.29 \text{pg/ml}$. Thus, the herbal acupuncture group and orally administered group showed significant increase in IL-2 productivity when compared to the control group. Significant result was repeated in the reproducibility experiment as well.

Holotrichia has high potency in activating blood circulation and remove the stasis. It has been popularly used since the Tang dynasty and, nowadays, it is widely used in the folk medicine to treat liver cirrhosis and hepatitis. For the extraction method of the herbal extract, Holotrichia extract made by the water extraction alcohol precipitation method had highest efficacy in the survival time and tumor inhibition experiments. Water extraction alcohol precipitation method displayed consistency in anti-tumor efficacy as the point location varied to Ren 12, St 36, and BL 18. Significant increase in the lymphocyte productivity was displayed with the Holotrichia extract and acupuncture injection, and

the acupuncture injection tend to have better result than the oral administration. In terms of country origin of Holotrichia, Chinese Holotrichia showed better effects in survival time and MTT method while Korean Holotrichia excelled in IL-2 productivity. But the differences in the result was contributed by the drying condition and further research is required to authenticate the result.

V. Conclusion

This experiment was conducted with intention of exhibiting reproducibility and pellucidity of the Holotrichia herbal acupuncture.

This was done by measuring the effectiveness of the herbal acupuncture with injection and oral administration and anti-cancer capability. Examination items range from measuring the survival time, inhibition rate on tumor, *In vitro* cytotoxicity, IL-2 productivity, and proliferative reaction of the lymphocyte. Following results were obtained in the experiment.

1. Regarding each illness condition, reproducibility experiment was done on the points of St 36, Ren 12, and BL 18

to see the changes for the anti-tumor effects.

2. Comparing the efficacy of herbal acupuncture and oral administration, lymphocyte productivity excelled with the herbal acupuncture while the IL-2 productivity bettered with the oral administration.

3. Comparing the survival time based on the extraction methods, Hd and Jd group showed 10.42% increase, Hw group at 16.67%, and Jw group at 20.83% increase compared to the control group.

4. Measuring the tumor inhibition rate, the control group showed 13.59% inhibition rate compared to the normal group, and the Hd group, Jd group, Hw group, and Jw group each showed significant increase of 4.85%, 21.39%, 37.71%, and 28.01%, respectively.

5. Considering 1 as the value of IC_{50} of the Holotrichia herbal extract, Holotrichia extract made by the precipitation method of water extraction with Korean was 0.6 and Chinese was 0.375.

6. In measurement of the IL-2 productivity in the ICR mice, oral administration of Chinese Holotrichia extract showed significant reduction compared to the control group and Korean Holotrichia extract showed insignificant increase. For the C57BL/6 mice, Chinese Holotrichia extract displayed significant decrease while Korean Holotrichia extract showed significant increase.

7. In measurement of the lymphocyte proliferative reaction and IL-2 productivity, 30 days of herbal acupuncture injection on BL 18 showed experiment groups showed significant increase compared to the control group and confirmed in the reproducibility experiment.

Bibliography

1. 이상인: 본초학, 학림사, 서울 1986, p475~476
2. 江蘇新醫學院 ; 中藥大辭典, 上海科學技術出版社, 上海, 1977, p2379~2380
3. 葉志詒 ; 神農本草經贊, 翰成社(本草類上), 서울, 1976, p105
4. 田炳旭, 柳逢夏, 朴東源, 柳基遠 : 癌腫의 病因病理에 관한 文獻的 考察, 대한