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# Primary Screening for Growth Inhibitors of L1210 Cells from Oriental Herbs·

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# 한약재로 부터 L1210 세포 생장 억제물질의 검색

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## Abstract

In order to obtain anticancer substances from natural products, extracts of dry herbs, which have long been used to treat cancer or cancer-like diseases in oriental countries, were screened. Extracts were made with hot water and/or organic solvents. With the extracts we treated murine leukemic L1210 cells growing in Fischer's medium. After 48 hours of incubation, cells were counted and concentrations of dry extracts to achieve 50 percent inhibition of the control growth, $ED_{50}$ values, were determined. Among the 38 species of medicinal plants tested, water extracts of six species showed $ED_{50}$ values of substantially low. Further extraction with organic solvents could reduce their $ED_{50}$ values within the range of the NCI quality control limit. The promising species as potential sources of anti-cancer substances included *Cinnamomum cassia, Citrus trifoliata, Coptis japonica, Panax ginseng, Phellodendron amurense,* and *Scutellaria baikalensis.*

## Introduction

Natural products, which are able to produce a wide variety of chemical entities of novel structure, appeared to be a promising sources for new types of compounds to test for antitumor activity. The antitumor activity of plant materials has been known for many centuries. Plant preparations were prescribed for what is through to have been cancer as early at 1500 B.C. and have continued to be employed.[1]

Plant products were introduced during 1957 into the HCI (National Cancer Institute) screening program.[2] Due to the increased interest in the screening of plant materials in the late 1950's, the WARF institute, Inc. contracted with the NCI in August 1961 to prepare crude plant extracts. Thousands of plant extracts were submitted for screening ever since. The NCI's record of "active plants" (extracts which showed a significant inhibitory effct in experimental tumor systems) was compared with plants reported in folklore to have medicinal or poisonous properties.[3] The occurrence of active plants was found to be higher in plants reported in folk

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literature than in plants collected at random, suggesting a correlation between plants used in folklore and those with anticancer activity.

In the present study, extracts were made from 38 different dry herbs purchased from drug stores of oriental medicine. The selection of herbs was based on the descriptions related to treatments of cancer-like diseases appeared in old books of oriental medicines.[4,5] Among the herbs tested, organic solvent extracts of six species inhibited the growth of murine leukemic L1210 cells substantially.

## Materials and Methods

### Herbs

Thirty eight kinds of dry herbs (medicinal plants) which have been listed in Table 1 were purchased from local drug stores of oriental medicine. The herbs having Korean local names were reidentified in scientific names based on illustrations appeared in Makino's classical flora book.[6]

### Water extraction

One part of dry herbs and 10 parts of distilled water were put into a screw-capped bottle, and water soluble components in the plant material were extracted for 10 hours at 100°C. After the extraction, solid materials were removed by filtration followed by centrifugation at 12000 rpm (17400 x g) for 10 minutes. The supernatant was then sterilized by milipore filtration before added to the culture solution.

The concentration of soluble material in the extract was determined by dispensing 10 ml of extract into a weighing dish, drying for three hours at 100°C, keeping in a desicator overnight, and weighing.

### Fractionation

Dry herbal material of 20 to 30 grams was subjected to extract first with petroleum ether via soxhelet extractor for eight hours. The soluble portion was taken by filtration, evaporated in vacuum, dissolved in 95% ethanol, and diluted with distilled water for test (sample A). The marc was dried in air and extracted with 95% reagent ethanol via soxhelet extractor for 14 hours, and then discarded. The soluble portion was concentrated in vacuum to about 100 ml volume, taken a quarter and concentrated to paste in vacuum (sample B). The remainder was concentrated to paste in vacuum and partitioned between chloroform-water (1:1). The chloroform part was evaporated in vacuum and the solid dissolved in 95% ethanol for test (sample C). The water part was also concentrated in vacuum (sample D). The basic principle of the fractionation procedure was from Statz and Coon[2], and the whole process was summarized in Figure 1.

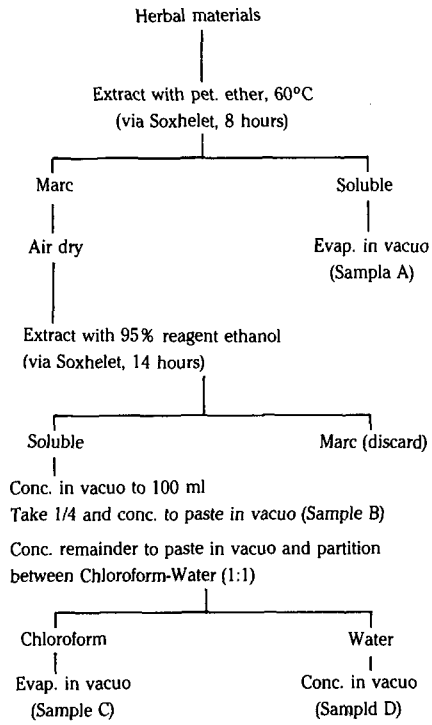


**Fig. 1. Modified fractionation procedure for plant.**

### Cancer Cell

Murine leukemic lymphoblast, L1210, was obtained from Perman's laboratory, University of Wisconsin. The cells are round and grow by binary division. They have doubling time of 12 to 18 hours under favorable conditions. The culture has been maintained by growing in screw-capped tubes (20 x 150 mm) at 37°C and transferring twice a week.

### Culture medium

Fischer's powder medium for leukemic cells of mice

(H-11) was purchased from GIBCO Laboratories, Grand Island, New York, USA. Horse serum was supplied locally by taking fresh blood immediately after the slaughter directly in 500 ml centrifuge tubes. The tubes were stood at room temperature for a couple of hours. After the blood clotted, the tubes were centrifuged for 10 minutes at 5000 rpm. The supernatants were sterilized using Milipore filters and stored at -20°C until use.

To make one liter of the culture medium, a package of Fischer's powder medium (1-liter package) was dissolved in redistilled water. Added 1.125 grams of $NaHCO_3$ and 100 ml of horse serum. Adjusted the pH to 7.2. Filter sterilized and stored in the refrigerator. Just before use added 100 units/ml of penicillin and 0.1 mg/ml of streptomycin.

### Chemicals

A positive control compound, methyl-CCNU (NSC 95441; 1-(2-chloroethyl)-3-(trans-4-methylcyclothexyl)-1-nitrosourea) was obtained from NCI (National Cancer Institute) by the help of K. H. Yang of our department. All other chemicals were of analytical grade.

### Culture preparation

Spinner cultures (cells in logarithmic phase of growth) were prepared in 250-ml screw-capped Ehrlenmeyer flask. The L1210 cells were inoculated to a 50-ml of pre-warmed medium to make an initial concentration of 2 to 5 x $10^5$ cells per ml a day prior to the assay. Through an incubation at 37°C the cell concentration usually reached to 0.8 to 1.0 x $10^6$ cells per ml in 24 hours.

The spinner cultures were diluted with pre-warmed fresh medium to make final concentration of 5.5 x $10^4$ cells per ml immediately before the cells were dispensed into the individual growth tubes. This culture suspension is called "run bottle" and used to distribute 5-ml each in screw-capped culture tubes.

### $ED_{50}$ determination

Exactly 0.5-ml each of the herb extracts containing various amounts of soluble substances was added to the culture tubes having 5-ml each of "run bottle". The same volume of distilled water was added instead of sample extract for control tests. For the positive control tests, different concentrations of methyl-CCNU, a known cytotoxic compound, was used instead of herb extracts. In all cases the total liquid volume per tube was 5.5-ml having final cell concentration around 5 x $10^4$ cells per ml. For each dose duplicate tubes were prepared.

The culture tubes thus prepared were incubated stationally at 37°C for 48 hours. Cell numbers were then counted using a coulter electronic particle counter (Electrozone Celloscope, Particle Data, Inc., Elmhurst, Ill. USA) standardized by hemocytometer. Duplicate counts for each tube were carried, and the mean of four values at one dose level was used for growth ratio determinations.

The value of $ED_{50}$ which is the concentration of the extract which inhibits the growth of cells to the level of 50% of the untreated control was then determined following the procedure described in the NCI manual.

## Results

### Growth of L1210 cells in Fischer's medium

Figure 2 shows a typical growth pattern of L1210 cells in the Fischer's medium without any inhibiting substance added. When the cell concentrations were plotted semilogarithmically against culture time a straight line could be observed at least for 48 hours which was used as

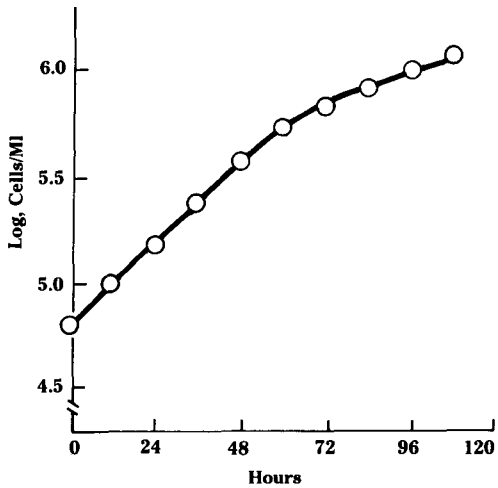


**Fig. 2. Growth of L1210 cells in Fischer's Medium Fortified with Horse Serum and Sodium Bicarbonate. The Cells were Grown Stationary in Screw-capped Tubes at 37°C.**

culture period in this study. The doubling time of L1210 cells during the logarithmic growth period was computed as 15.6 hours.

**Positive control test**

Following the NCI manual the $ED_{50}$ value of a known anticancer agent, methyl-CCNU, was determined to check reliability of our procedures. The growth ratio for each dose of testing substance, Y, was calculated following the equation,

$$\frac{T - C_0}{C - C_0} \times 100 = Y(\%)$$

where T = mean cell count for each dose of testing substance after 48 hours incubation; C = mean cell count for control after 48 hours incubation; $C_0$ = mean cell count at the start of incubation.

When Y values were plotted against doses of methyl-CCNU semi-logarithmically, a straight line could be obtained as shown in Figure 3. Using the straight line a concentration of methyl-CCNU which could inhibit the growth of L1210 cells by 50% ($ED_{50}$) was estimated as 1.7 ug per ml.

**Growth inhibitors in water extracts**

Following the same procedures used for the positive control test, $ED_{50}$ values for the components of 38 different herbs extracted with water were determined and shown in Table 1. A majority of the samples tested showed $ED_{50}$ values higher than 100 ug per ml suggesting less potentiality as sources of anticancer agents. However, six species, such as *Cinnamomum cassia, Citrus trifoliata, Coptis japonica, Paeonia albiflora, Phellodendron amurense,* and *Scutellaria baikalensis* showed low $ED_{50}$ values and were subjected to further tests.

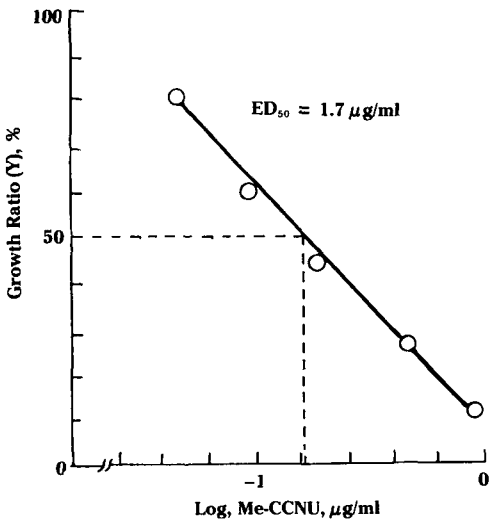


**Fig. 3. $ED_{50}$ Value of Methyl-CCNU on L1210 Cells.**

**Growth inhibitors in solvent extracts**

Herb materials which showed low $ED_{50}$ values were again extracted with organic solvents following the procedures shown in Figure 1. *Panax ginseng* which showed extremely high $ED_{50}$ value in water extract was also included because this herb has been known to contain active material which is soluble in lipid.[7] The results were shown in Table 2. The $ED_{50}$ values for samples A and D of Figure 1 were not shown because they were too high. As can be seen active substances which inhibit the growth of L1210 cells were more effectively extracted by using organic solvents than by water. This was particularly true in the case of *P. ginseng* which showed $ED_{50}$ of less than 2 $\mu$g per ml, whereas it was 1240 $\mu$g per ml when extracted with water. From the results it may be concluded that the herbs listed in Table 2 can be considered as potential sources of anticancer agents.

## Discussion

It is not easy to set up certain level of $ED_{50}$ below which a sample can be considered of clinical significance. When the KB cells originated from human epidemoid carcinoma are used as testing cells to evaluate plant extracts as effective anticancer agents, the $ED_{50}$ should not exceed 20 ug per ml.[8] In the present study, however, murine leukemic L1210 cells were used and no criterion for this type of cells is available. As an alternative way, a positive control test with a known anticancer agent, methyl-CCNU, was performed. Methyl-CCNU is a methylated analog of CCNU (NSC 79037; 1-(2-chloreothyl)-3-cyclohexyl-1-nitrosourea) which is an analog of BCNU (NSC 409962; 1,2-Bis (2-chloroethyl)-1-nitrosourea). Studies on the mechanism of BCNU action had shown alkylating activity in DNA of the cell,[9] and CCNU and methyl-CCNU might have similar activity in DNA. Because of the superior experimental activity in

**Table 1. $ED_{50}$ Values of Herbal Components Extracted with Water.**

| Herbs | | Part extracted | $ED_{50}$ (ug/ml) |
|---|---|---|---|
| Scientific name | Local name | | |
| Aconitum koreanum | Baekbooza | Root | >217 |
| Alisma orientale | Taeksa | Tuber | >758 |
| Angelica gigas | Danggwi | Root | >159 |
| Artemisia vulgaris | Aeyup | Leaf | >106 |
| Asparagus cochinchinensis | Chunmoondong | Rhizome | >470 |
| Astragalus membranaceus | Whangi | Root | >117 |
| Atractylis ovata | Baekchool | Rhimzome | >127 |
| Benzoin strychnifolium | Oyack | Root | 210 |
| Cinnamomum cassia | Gepi | Bark | 19 |
| Citrus natsudaidai | Chungpi | Epicarp | 220 |
| Citrus trifoliata | Jisil | Epicarp | 53 |
| Citrus unshiu | Jinpi | Epicarp | >78 |
| Cnidium officinale | Chungoong | Rhizome | >303 |
| Coix Lachryma-Jobi | Iiin | Seed | >235 |
| Coptis japonica | Whangyoun | Root | 25 |
| Dianthus superbus | Goomaek | Root | 480 |
| Euchresta japonica | Sandooguen | Root | 300 |
| Eucommia ulmoides | Doochoong | Fruit | 180 |
| Foeniculum vulgare | Whoehyang | Fruit | >270 |
| Gardenia jasminoides | Chiza | Fruit | 200 |
| Glycyrrhiza uralensis | Gamcho | Root | 960 |
| Hordeum vulgare | Maeka | Cotyledon | >192 |
| Lycium chinense | Googiza | Fruit | >221 |
| Nelumbo uncifera | Yonzayuc | Seed | >845 |
| Opiopogon japonicus | Maekmoondong | Tuber | >512 |
| Oryza sativa | Kyungmi | Seed | >413 |
| Pachyma hoelen | Bokyung | Hypha | 270 |
| Paeonia albiflora | Jakyac | Root | 40 |
| Panax ginseng | Insam | Root | 1240 |
| Patrinia villosa | Paezang | Root | 450 |
| Phellodendron amurense | whangbaek | Bark | 19 |
| Phelloterus littoralis | Bangpoong | Root | 891 |
| Pinellia ternata | Banha | Rhizome | >398 |
| Platycodon glaucum | Gilgyung | Root | >1020 |
| Rehmannia glutinosa | Jiwhang | Root | 439 |
| Scutellaria baikalensis | Whanggum | Root | 76 |
| Sedum japonica | Wasong | Leaf | 400 |
| Zizyphus jujuba | Daejo | Fruit | >669 |

**Table 2. $ED_{50}$ Values of herbal Components Extracted with Water and Organic Solvents.**

| Herbs | $ED_{50}$ in fraction of | | |
|---|---|---|---|
| | Water* | Ethanol** | Chloroform*** |
| *Cinnamomum cassia* | 19 | 6.3 | 4.5 |
| *Citrus trifoliata* | 53 | 36 | 14 |
| *Coptis japonica* | 25 | 1.7 | 2.2 |
| *Panax ginseng* | 1240 | 30 | 1.8 |
| *Phellodendron amurense* | 19 | 2.6 | 3.5 |
| *Scutellaria baikalesis* | 76 | 20 | 4.3 |

* Data from Table 1.

** Sample B, *** Sample C of Fig. 1.

leukemic cell and great lipid solubility than CBNU, methyl-CCNU was selected for clinical trials.[10] Under the NCI protocol, the quality control limit has been tentatively set at a $ED_{50}$ value range of 1.7 to 7.7 ug per ml. When the same compound, methyl-CCNU, was applied to the present testing cells of L1210, the $ED_{50}$ value was 1.7 ug per ml (Fig. 3) which laid in the lower end of the NCI quality control limit range.

Among the 38 species of medicinal plants tested, a half dozen showed $ED_{50}$ values within the NCI quality control limit range when they were extracted effectively with organic solvents except that of *Circus trifoliata* (Table 2). Since most of the citrus fruits are palatable (non-toxic) and trees are locally available, *C. trifoliata* may be also included in the list of herbs for the further studies.

Extraction of active compounds from natural products with hot water is simple and convenient in handling a great variety of sample. However, the process may lose active substances which can solve only in organic solvents as the case of *P. ginseng* (Table 2).

## 요 약


천연물 가운데서 항암물질을 얻을 목적으로 동양에서 오랫동안 암이나 암에 비슷한 질병의 치료에 씌여온 한약재를 물이나 유기용매로서 추출하여 검색하였다. Fischer배지에 자라는 L 1210 세포에 추출물을 첨가한 다음 48시간 후에 세포수를 헤아려 생장을 50% 억제할 수 있는 추출물의 농도를 나타내는 $ED_{50}$값을 측정하였다. 물로서 추출한 38종의 한약재 가운데서 6종이 낮은 $ED_{50}$값을 나타냈다. 이들을 다시 유기용매로서 추출하므로써 $ED_{50}$값이 더욱 떨어져 NCI의 품질관리범위 치역내에 들어갔다. 항암제원으로 유망시되는 한약재로서 *Cinnamomum cassia, Citrus trifoliata, Coptis japonica, Panax ginseng, Phellodendron amurense, Scutellaria baikalensis*를 들 수가 있다.


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