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Cytotoxic Activities of Various Fractions Extracted from Some Pharmaceutical Insect Relatives

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This research was performed to screen the cytotoxic activities of some pharmaceutical insect relatives. Cytotoxic activities of total extract and fractions of hexane, ethyl acetate, methanol, water and boiling water were extracted from four pharmaceutical insect relatives: the Chinese gall, the cicada slough, the hornet nest and the batryticated silkworm. These extracts were investigated against the cancer cell lines of L1210, P388 and SNU-1 *in vitro* tests. Results showed that, ED_{50} against the cancer cell lines of L1210, P388 and SNU-1 were 0.55, 0.50, and 0.83 μg/ml in the ethyl acetate fraction from the Chinese gall; 1.07, 2.19, and 2.24 μg/ml in the ethyl acetate fraction, 1.51, 1.26, and 1.45 μg/ml in the water fraction and 1.48, 2.29, and 1.29 μg/ml in the boiling water fraction from the cicada slough; 3.31, 2.00, and 6.61 μg/ml in the water fraction from the hornet nest and 13.80, 19.95, and 31.62 μg/ml in the hexane fraction and 33.88, 21.88, and 25.12 μg/ml in the ethyl acetate fraction from the batryticated silkworm, respectively. All of the fractions mentioned above showed high cytotoxic activities and could be suggested for further studies *in vivo* tests.

Key words : Cytotoxic activities, Pharmaceutical insects, Chinese gall, Cicada slough, Hornet nest, Batryticated silkworm, Cancer cell lines (L1210, P388 and SNU-1)

INTRODUCTION

Pharmaceutical insects have been used in traditional Chinese medicine (TCM) for several thousands years. However, the pharmacological research, particularly about the anticancer function of pharmaceutical insects has been reported little and late. In the mid-1980s, the cytotoxic activities of pharmaceutical insects against cancer cells were reported for the first time (Kosuge *et al.*, 1985), in which the water and methanol fractions of six species of insects and some species of other animals and plants were studied with Ehrlich ascites carcinoma and HeLa cancer cells. After that, the cytotoxic effects of water fractions extracted from eleven species of insects and some species of other animals and plants were tested with JTC-26 and HE-1 cancer cells (Sato, 1989). Recently, the cytotoxic activities of water, methanol and ethyl acetate fractions of twelve species of insects and some species of other animals and plants were investigated with HeLa-S3, Colon26, SW1116, PC-3, B16, G361, Li-7, HepG2, LL and Detroit55 cancer cells (Takatsuki *et al.*, 1996). Other than these, no report about the cytotoxic activities of pharmaceutical insects has been found yet.

This research was performed to investigate the cytotoxic activities of some pharmaceutical insect relatives (some substances related to insects are called as insect relatives including gall, shellac, honey, feces, exuviae, nests and abnormal insect bodies parasitized by fungi). Since some pharmaceutical insects are used as one of the ingredients in certain TCM anticancer prescriptions, we presumed that they may have an anticancer function. However, the exact anticancer activities of various fractions extracted from these insects should be confirmed by some modern methods of pharmacology and pharmaceutical chemistry. Therefore, each of the four insect relatives used in TCM for treating cancers was extracted as total extract, hexane, ethyl acetate, methanol, water and boiling water fractions and the cytotoxicity of every fraction against the cancer cells of L1210, P388 and SNU-1 was examined *in vitro* tests.

MATERIALS AND METHODS

Insect materials

Chinese gall (the gall formed by the Chinese gall aphid (*Melaphis chinensis* Bell) (*Homoptera*: *Erioso-*

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matidae) on the plant leaves of Chinese sumac (*Rhus chinensis* Mill)), cicada slough (the exuviae of the black cicada (*Cryptotympana pustulata* Fabricius) (*Homoptera*: *Cicadiae*) after emergence from the nymph), hornet nest (the nest built by the large yellow wasp (Polistes mandarinus Saussure) (*Hymenoptera*: *Vaspidae*)) and batryticated silkworm (the larve of the silkworm (*Bombyx mori* L.) (*Lepidoptera*: *Bombycidae*) parasitized by the fungus species of *Beauveria bassiana* (Bal.s) Vuill) were purchased from traditional Chinese drug stores in Jilin, Jilin, China, May 11, 1996.

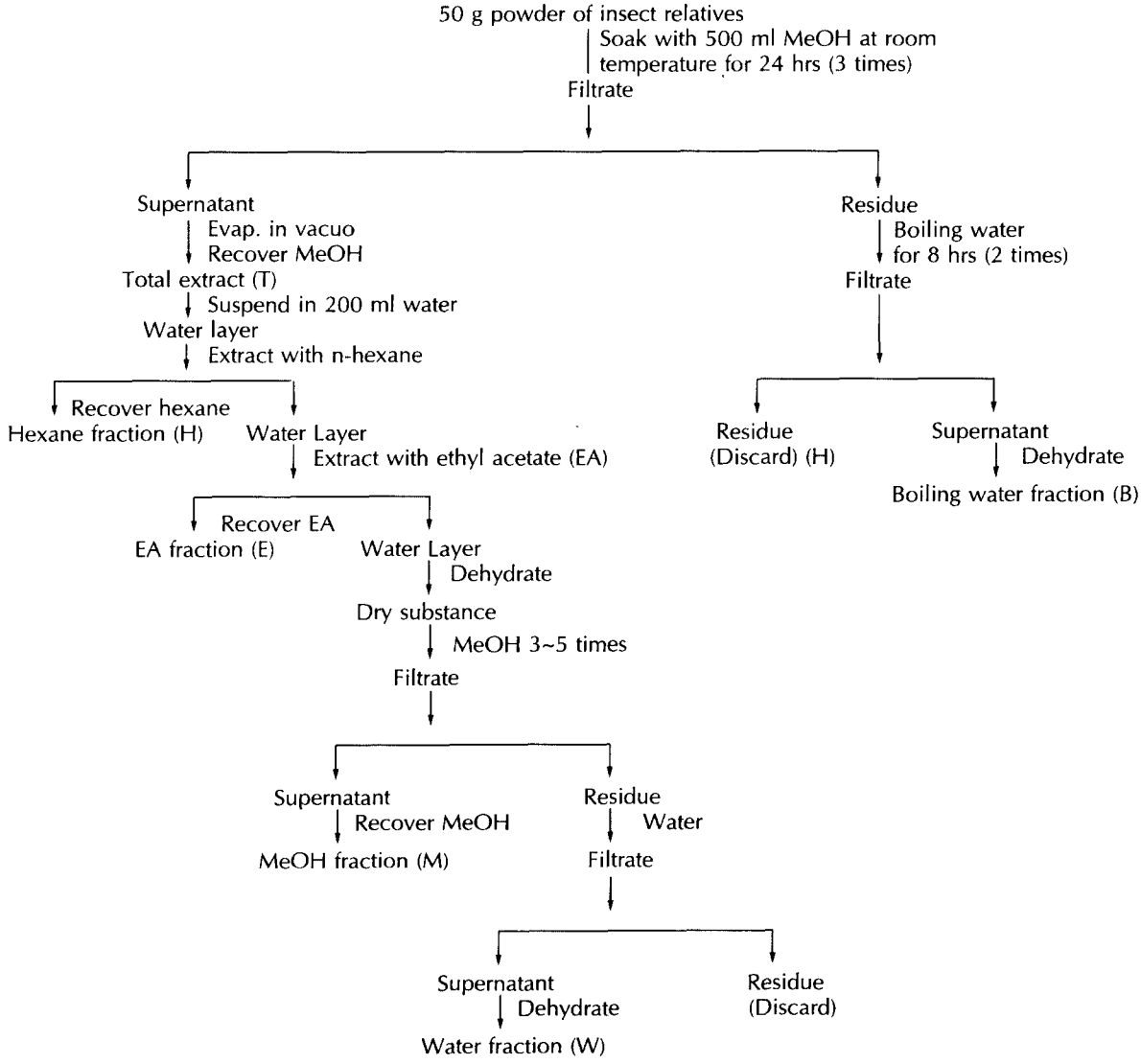
Reagents

The ethyl acetate, $NaHCO_3$ and HCl were the products of Kanto Chemical Co. (Tokyo, Japan), the hexane was that of Wako Pure Chemical Industries (Osaka, Japan) and the methanol was from Jin Chemical & Pharm Co. (Duk San, Korea). The RPMI 1640 medium, fetal calf serum (FCS), phosphate buffered saline (PBS), penicillin G sodium and streptomycin sulfate were purchased from Gibco Laboratories (New York, USA). However, the ethylenediaminetetraacetate (EDTA), dimethyl sulphoxide (DMSO) and trypan blue were purchased from Sigma Chemical Co. (St. Louis, USA).

Sample preparation

After air drying at room temperature, each insect relative material was ground into powder. Fifty grams of powder of every insect relative was treated with the process shown in Scheme 1.

Ten mg of each fraction was suspended with 0.2 ml DMSO and diluted with RPMI 1640 (pH=7.2~7.4, adjusted with 1 N HCl or 10% $NaHCO_3$) to the con-



Scheme 1. Extraction of different fractions from each insect relative.

centrations of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} g/ml, respectively. The final concentration of DMSO in each test solution was 0.1%. All solutions were sterilized by passing through 0.2 μm membrane filters (Cai *et al.*, 1995).

Cell culture

The cancer cell lines used in these cytotoxicity tests were mouse lymphocytic leukemia L1210, mouse lymphoid neoplasm P388 (Chang *et al.*, 1995; Baek *et al.*, 1995) and human gastric cancer SNU-1 (Chung *et al.*, 1994; Jeong *et al.*, 1994). The culture medium was RPMI 1640 (pH=7.2~7.4) containing 10% FCS and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml) and was changed twice a week (Bae *et al.*, 1994). The cells were grown in 25 cm^2 tissue culture flasks (Corning, New York, USA) and maintained in a humidified CO_2 incubator (95% air and 5% CO_2) at 37°C. Generally, the cell lines were kept under liquid nitrogen until use and at as early a passage as possible (Kim *et al.*, 1994).

Cytotoxicity assay *in vitro*

The 100 μl of each sample was put in a well of 96-well flat bottom tissue culture plate (Corning, New York, USA). And then, 100 μl cancer cell suspension in a logarithmic phase (Living cell ratio>95%) of L 1210, P388 and SNU-1 with the initial concentrations of 4.98×10^4, 5.85×10^4 and 4.61×10^4 cells/ml, respectively, was inoculated into each well. After 72 hrs of incubation for L1210 and P388 (Kondo *et al.*, 1991) and 96 hrs for SNU-1 (Chung *et al.*, 1994), living cells were counted on a hemacytometer by 0.4% trypan blue dye exclusion method (Chang *et al.*, 1995). Since both L1210 and P388 are suspensible cells, the cell number was obtained directly from the cell suspension. Conversely, SNU-1 was grown as a monolayer on the bottom of the wells. First, we discarded the medium solution from the well, and then put 100 μl EDTA-PBS in each well. After approximately ten min incubation, the SNU-1 cell suspension was obtained by repeatedly drawing and stirring with a pipette. Eight replicated wells containing RPMI 1640 (pH=7.2~7.4) medium with 0.1% DMSO were served as the control.

Data calculation

The growth ratio of cancer cells under different concentrations of each insect fraction was calculated by the following formula (Chang *et al.*, 1995):

$$Y(\%)=(T-Co)/(C-Co)\times 100$$

Where

Y=Growth ratio of cancer cell in each sample concentration

T=Mean cell count of treatment after 72 or 96 hrs incubation

C=Mean cell count of control after 72 or 96 hrs incubation

Co=Mean cell count at the start of incubation

The value of ED_{50} which was the concentration of a test sample to inhibit the growth ratio of cancer cells by 50% (Thayer *et al.*, 1971) was determined with the regression equation composed by a logarithmic value of four graded concentrations (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} g/ml) and the growth ratio of cancer cells in every fraction. All data represented the average values of the three wells.

RESULTS AND DISCUSSION

Chinese gall

As shown in Fig. 1, ED_{50} of the total extract against cancer cell lines of L1210, P388 and SNU-1 were 0. 52, 0.46, and 2.04 μg/ml, respectively. The strongest inhibition was obtained from the fraction of ethyl acetate. Their ED_{50} were only 0.55, 0.50, and 0.83 μg/ml, successively, 3.63, 2.40, and 14.79 μg/ml in the methanol fraction and 2.18, 1.66, and 16.60 μg/ml in the water fraction. The weakest inhibition occurred in the hexane fraction. The ED_{50} against these three cancer cells were 50.12, 30.20, and 66.07 μg/ml and was followed by the boiling water fraction, which were 7.24, 3.31, and 25.12 μg/ml, respectively. The results of further extraction suggested that nearly every fraction of Chinese gall displayed inhibitory activities to these cancer cells. However, the ethyl acetate fraction should be subjected to further research.

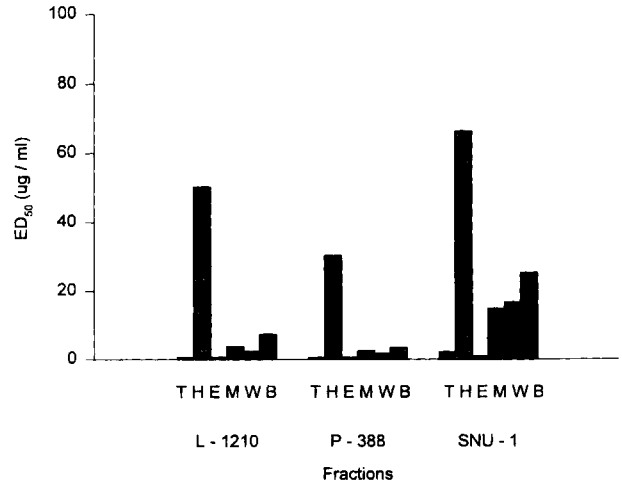


Fig. 1. ED_{50} values of six fractions extracted from the Chinese gall against the L1210, P388 and SNU-1 cancer cell lines. T: Total extract, H: Hexane fraction, E: Ethyl acetate fraction, M: Methanol fraction, W: Water fraction, B: Boiling water fraction.

The results we obtained were similar with those obtained by Kosuge *et al.*, (1985). The inhibitions of both the water and methanol fractions extracted from the gall against the HeLa cancer cell were quite strong. Their inhibitory ratios with the concentration of 100 were more than 75%. However, they did not test for the fraction of ethyl acetate. Our results were also similar with those of Takatsuki *et al.*, (1996), where every fraction of gall displayed inhibitory activities. In his experiments, the IC_{50} of the water, methanol and ethyl acetate against HeLa-S3 were 30, 50, and 63, respectively. Our results were slightly different in that the strongest inhibition in his experiment occurred in the water fraction, where the ethyl acetate produced the strongest inhibition in our tests. The reason may be that the sensitivities among different cancer cell lines to the same samples were different.

Cicada slough

The experiments reported here (Fig. 2) demonstrated that all of the fractions extracted from the cicada slough displayed cytotoxic activities. The ED_{50} against L1210, P388 and SNU-1 were 1.07, 2.19, and 2.24 μg/ml in the ethyl acetate fraction, 1.51, 1.26, and 1.45 μg/ml in the water fraction and 1.48, 2.29, and 1.29 μg/ml in the boiling water fraction, respectively. However, the weakest one among the six fractions was the total extract and its ED_{50} against these three cancer cell lines were 25.12, 20.42, and 45.71 μg/ml, respectively. It was followed by the hexane fraction and the methanol fraction, where the ED_{50} were 19.95, 10.47, and 33.88 μg/ml in the former and 7.24, 7.27, and 11.48 μg/ml in the latter. As a result, the fractions of ethyl acetate, water and boiling water extracted from the cicada slough are suggested for further studies in vivo tests.

The results from the water fraction in our test showed quite strong cytotoxic activities, which were the same as those tested by the cancer cell lines of B16 and G361 (Takatsuki *et al.*, 1996), but different from the HeLa (Kosuge *et al.*, 1985). Moreover, just as the result obtained by Takatsuki *et al.*, (1996), the ethyl acetate fraction in our experiments also showed strong cytotoxic activity. Furthermore, the fraction of boiling water expressed quite a strong inhibition to the cancer cell lines of L1210, P388 and SNU-1 in our tests.

Hornet nest

ED_{50} of every fraction extracted from the hornet nest against the cancer cell lines of L1210, P388 and SNU-1 were illustrated in Fig. 3. The ED^{50} of the water fraction against these three cancer cell lines were 3.31, 2.00, and 6.61 μg/ml, respectively, and then, 18.62, 25.12, and 43.65 μg/ml in the boiling water fraction, 36.31, 23.99, and 15.85 μg/ml in the ethyl acetate fraction and 70.79, 29.51, and 32.20 μg/ml in the hexane fraction. Certainly, the water fraction is the best choice.

The water fraction showed very strong cytotoxic activity in our tests. These results were completely different from those of Kosuge *et al.*, (1985) and Takatsuki *et al.*, (1996). We thought that perhaps some substances that may affect the cytotoxic activities of the water fraction were separated into some other fractions, since the insects in our experiments were extracted as six fractions instead of two or three fractions.

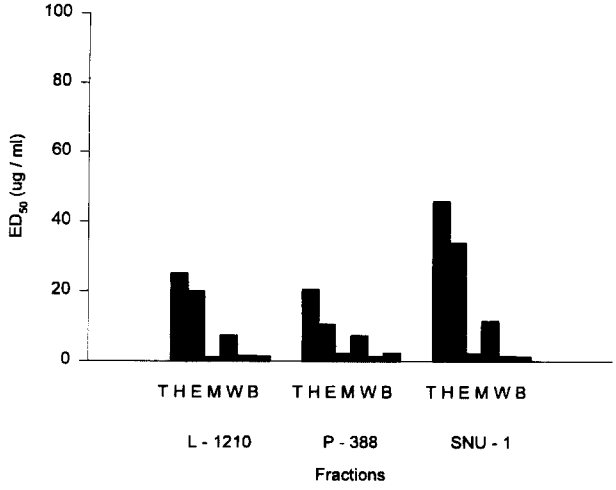


Fig. 2. ED_{50} values of six fractions extracted from the cicada slough against the L1210, P388 and SNU-1 cancer cell lines. T: Total extract, H: Hexane fraction, E: Ethyl acetate fraction, M: Methanol fraction, W: Water fraction, B: Boiling water fraction.

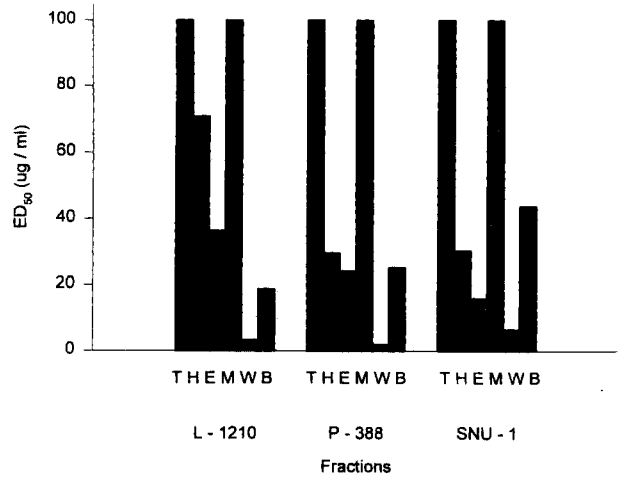


Fig. 3. ED_{50} values of six fractions extracted from the hornet nest against the L1210, P388 and SNU-1 cancer cell lines. T: Total extract, H: Hexane fraction, E: Ethyl acetate fraction, M: Methanol fraction, W: Water fraction, B: Boiling water fraction.

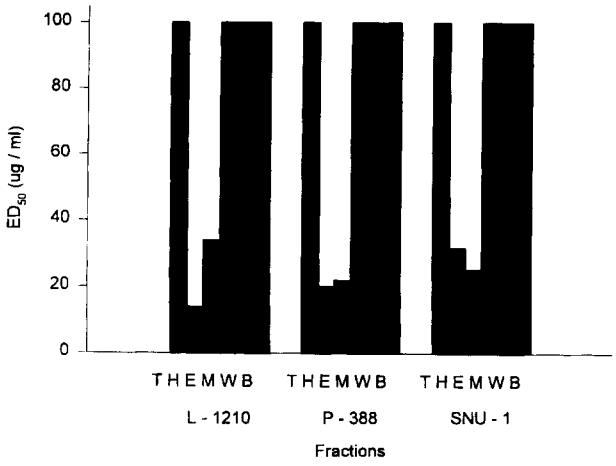


Fig. 4. ED_{50} values of six fractions extracted from the batryticated silkworm against the L1210, P388 and SNU-1 cancer cell lines. T: Total extract, H: Hexane fraction, E: Ethyl acetate fraction, M: Methanol fraction, W: Water fraction, B: Boiling water fraction.

Batryticated silkworm

ED_{50} against L1210, P388 and SNU-1were 13.80, 19.95, and 31.62 μg/ml in the hexane fraction and 33.88, 21.88, and 25.12 μg/ml in the ethyl acetate fraction (Fig. 4). Conversely, all of the ED50 against these three cancer cell lines in the total extract and the fractions of methanol, water and boiling water were beyond 100 μg/ml. For this reason, the fractions of the hexane and ethyl acetate should be considered for further research.

The inhibition rate of the water fraction against JTC-26 and HeLa cancer cells were quite low: only 29% with the concentration of 500 μg/ml (Sato, 1989) and < 25% (100 μg/ml) (Kosuge *et al.*, 1985). The same results occurred in our test. The ED50 of the water fraction was larger than that of 100 μg/ml. In the fraction of ethyl acetate, the results obtained in L1210, P 388 and SNU-1 were very similar to those in HeLa-S3 (IC_{50}=22 μg/ml) (Takatsuk *et al.*, 1996). The fraction of the hexane also showed cytotoxic activity in our tests.

It can, therefore, be concluded that all of the four insect relatives selected with the help of the experiences stemmed from TCM anticancer prescriptions were verified by the present experiments to demonstrate cytotoxic activities. As has been said, the ethyl acetate fraction from Chinese gall, the ethyl acetate, water and boiling water fractions from the cicada slough, the water fraction from the hornet nest and the hexane and ethyl acetate fraction from the batryticated silkworm are suggested for further studies *in vivo* tests.

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