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# Cytotoxic Activity of *Bombyx mori* and *Morus alba* Derived Materials against Human Tumor Cell Lines

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**Abstract** : The cytotoxic activity of MeOH extracts of the freeze-dried silkworm (*Bombyx mori*)-derived materials (4th instar larvae, female and male pupae, virgin female and male adults), dried *Beauveria bassiana*-infected silkworm larvae, dried feces from the 4th instar larvae of *B. mori*, and dried mulberry (*Morus alba*)-derived materials (leaves, fruits, root barks) *in vitro* was evaluated by sulforhodamine B assay, using the five human solid A 549 lung, SK-OV-2 ovarian, SK-MEL-2 melanoma, XF-498 CNS and HCT-15 colon tumor cell lines. The responses varied with both cell line and material used. The 70% hot MeOH extract of *B. mori* feces (BFH) revealed potent cytotoxic activity against model tumor cell lines whereas moderate activity was observed from the MeOH extract of *B. mori* feces, *M. alba* root barks, and *M. alba* fruits. The other test materials were ineffective. Because of its potent cytotoxic activity, the activity of each solvent fraction from the BFH was determined. Chloroform and ethyl acetate fractions showed the most potent cytotoxic activity. In conclusion, our results may be an indication of at least one of the pharmacological actions of *B. mori* feces, *M. alba* root barks, and *M. alba* fruits.
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## Introduction

Chemical carcinogenesis can be divided into two-defined stages, initiation and promotion. The initiators and the promoters of carcinogenesis are both found in human environments.[1,2] Recent investigations on enviromental carcinogenesis have indicated that naturally ocurring tumor promoters play a great role in the development of human cancer than initiators.[3,4] Therefore, tumor-promoting inhibitors in the two-stage carcinogenesis may be highly effective on cancer control. Since the establishment of the short-term *in vitro* colorimetric assay with reproducible results by National Cancer Institute (NCI), this assay has been used to detect tumor promoters in the environment.[5-7]

Current cancer chemotherapy is primarily dependent upon repeated administrations of synthetic anti-cancer agents. Their continued or repeated use has led to the development of resistance to the agents in the tumor cell lines[8-10] and adverse effects on human health such as alopecia, leucopenia, sterility and secondary malignancies in clinical trials.[11] Decreasing efficacy and increasing concern over possible adverse effects of chemotherapeutic agents have brought about the need for the development of new types of selective alternatives with lower toxic and more inhibitory effects.

Natural products such as plants, animals and microorganisms constitute a rich source of bioactive chemicals.[12-15] Since many of them are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer anti-cancer agents. Additionally, some of natural product-derived materials are found to be effective against cancer cells resistant to current chemotherapeutic agents.[8] Therefore, much efforts have been focused on natural products for potentially useful products as commercial anti-cancer agents or as lead compounds. However, relatively little work has been carried out on the anti-tumor activities of animal-derived materials and their related products such as feces compared to those of plant origin,[16-18] microorganism origin[19] and food origin.[20,21]

In the laboratory study described herein, we assessed the cytotoxic activity of the silkworm (*Bombyx mori*)- and the mulberry (*Morus alba*)-derived materials to develop potentially new safer types of anti-tumor agents.

Key words : cytotoxic activity, tumor cell, silkworm feces, *Bombyx mori, Morus alba, Beauveria bassiana*
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## Materials and Methods

### Chemicals

Sulforhodamine B (SRB), benzylpenicillin potassium, and streptomycin sulfate were purchased from Sigma (St. Louis, USA) Chemical. Fetal bovine serum and RPMI 1640 (Gaithersburg, USA) were supplied by Gibco. All other chemicals were of reagent grade.

### Tumor cell lines and culture conditions

Five human tumor cell lines used in this study were SK-MEL-2 human melanoma, A549 non-small cell lung, SK-OV-3 ovarian, HCT-15 colon, and XF-498 CNS tumor cell lines. They have been maintained in the laboratory as stocks in RPMI 1640 supplemented with 10% fetal bovine serum. Cell cultures were passaged once or twice weekly using trypsin-EDTA to detach the cells from their culture flasks.

### Test materials and sample preparation

Test materials used in this study are listed in Table 1. The economic importance of the silkworm (*Bombyx mori*)- and the muberry (*Morus alba*)-derived materials is described in detail elsewhere.[14] *B. mori* larvae were reared on *M. alba* leaves. The dried Beauveria bassiana-infected *B. mori* larvae and *M. alba* root barks were purchased from a market in Seoul, Korea. *M. alba* leaves and fruits were collected at the College of Agriculture and Life Sciences, Seoul National University, Suwon, Korea. They were finely powdered using a blender. Each sample was extracted twice with MeOH (50 g/400 m*l*) at room temperature for 2 days and filtered (Toyo filter paper No. 2, Toyo Roshi, Japan). The combined filtrate was concentrated *in vacuo* at 35°C using a rotary vacuum evaporator. The yield of MeOH extracts is shown in Table 1.

The most active 70% hot MeOH extract (100 g) of *B. mori* feces (BFH) was sequentially partitioned into hexane (8.1 g), chloroform (23.1 g), ethyl acetate (7.3 g), butanol (20 g) and water-soluble (42.5 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by a rotary vacuum evaporator at 35°C, and water portion was freeze-dried.

**Table 1. The silkworm (*Bombyx mori*)- and the mulberry (*Morus alba*)-derived materials tested**

| Material | Abbreviation | Yield (%) |
|---|---|---|
| Feces of *B. mori* larvae, dried[a] | BFH[b] | 12.5 |
| (Bombycis Faeces) dried[a] | BF | 8.1 |
| *B. mori* 4th larvae, freeze-dried | BV | 7.3 |
| (Bombycis Vermiculus) | | |
| *Beauveria bassiana*-infected BV, dried[a] | BB | 25.1 |
| (Bombyx Batryticatus) | | |
| *B. mori* pupae (♂), freeze-dried | BPM | 9.2 |
| (♀), freeze-dried | BPF | 8.9 |
| *B. mori* adults (♂), freeze-dried | BAM | 7.6 |
| (♀), freeze-dried | BAF | 7.3 |
| *M. alba* root barks, dried[a] | MRC | 8.2 |
| (Mori Radicis Cortex) | | |
| *M. alba* fruits, dried[a] | MFR | 3.6 |
| (Mori Fructus) | | |
| *M. alba* leaves, dried[a] | MFO | 20.1 |
| (Mori Folium) | | |

[a]Dried in an oven at 50°C for 3 days.
[b]Extracted with 70% hot MeOH for 2 hr at 65°C.

### Bioassy

SRB assay is applied for the measurement of the cytotoxicity of the test materials against model tumor cell lines.[5-7] The rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations ($1\sim2\times10^4$ cells/well) into 96 well microtiter plates. After incubation for 24 hr, the materials dissolved in culture medium were applied to the culture wells in triplicate followed by incubating for 48 hr at 37°C under 5% $CO_2$ atmosophere. The cultures fixed with cold TCA were stained by 0.4% SRB dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered tris base by gyrotory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). All tests were replicated three times. Fifty percent inhibitory dosage ($ED_{50}$) was defined as the dosage which reduced absorbance by 50% of untreated wells as of the control in the SRB assay.

## Results and Discussion

The cytotoxic effects of the test materials against the 5 human solid tomor cell lines are given in Table 2. The responses varied with both material and cell line used (Table 2). In a test with *B. mori* feces, the BFH extract revealed

**Table 2. Cytotoxic activity of methanol extracts from *Bombyx mori*- and Morus *alba*-derived materials**

| Material[a] | $ED_{50}$ (μg/m*l*) | | | | |
|---|---|---|---|---|---|
| | A549 | SK-OV-3 | SK-MEL-2 | XF-498 | HCT-15 |
| BFH | 90 | 142 | 80 | 94 | 45 |
| BF | 190 | 170 | 150 | 160 | 130 |
| BV | >300 | >300 | >300 | >300 | >300 |
| BB | >300 | >300 | >300 | 138 | >300 |
| BPM | >300 | >300 | >300 | >300 | >300 |
| BPF | >300 | >300 | >300 | >300 | >300 |
| BAM | >300 | >300 | >300 | >300 | >300 |
| BAF | >300 | >300 | >300 | >300 | >300 |
| MRC | 170 | 170 | 180 | 170 | 180 |
| MFR | 150 | 127 | 97 | 250 | 212 |
| MFO | >300 | >300 | >300 | >300 | >300 |

[a]For explanation, see Table 1.

potent cytotoxic activity against tumor cell lines used ($ED_{50}$, 45~142 μg/m*l*) whereas moderate activity was observed from the BF extract ($ED_{50}$, 130~190 μg/m*l*).

For tests with *B. mori*-derived materials, the BV, BPM, BPF, BAM and BAF extracts showed very weak cytotoxic activity against tumor cell lines used ($ED_{50}$, >300 μg/m*l*). $ED_{50}$ values of the BB extract against the cell lines with an exception of XF-498 ($ED_{50}$, 138 μg/m*l*) were >300 μg/m*l* (Table 2).

In tests with *M. alba*-derived materials against tumor cell lines used, moderate cytotoxic activity was observed in the MRC and MFR extracts with $ED_{50}$ values of 100~180 and 97~250 μg/m*l*, respectively. However, the MFO extract exhibited very weak activity ($ED_{50}$, >300 μg/m*l*) with an exception of HCT-15 ($ED_{50}$, 190 μg/m*l*) (Table 2).

In our study, significant differences between cytotoxic activities of the test materials. The BFH and BF extracts were effective against cell lines used whereas the extracts of the BV and MFO as food of *B. mori* larvae exhibited very weak activity. These results suggest that certain metabolites induced by the intestitinal microbial enzymes of *B. mori* larvae or the detoxifying enzymes of *B. mori* larvae such as mixed-function oxidase, hydrolase and glutathione S-transferase systems might be involved in the phenomenon. Significance of intestinal microbial enzymes and detoxifying enzymes in xenobiotics metabolism have been well reviewed by Hentages[22] and Mitsuoka,[23] and Hodgson[24] and Wood,[25] respectively.

Because of its potent cytotoxic activity against tumor cell lines used, the activity of each solvent fraction from the BFH extract was evaluated (Table 3). Chloroform and ethyl acetate fractions showed potent cytotoxic activity. Moderate cytotoxic activity was produced from hexane and butanol fractions whereas water fraction exhibited very weak activity. Although the active principles of *B. mori* feces remain unknown at present, the feces is known to be composed of organic materials (83~90%), histidine, leucine, lysine, phytole (0.25~0.29%), β-sitosterol (1.5%), cholesterol, ergosterol, tetracosanol, lupeol, vitamins A and B, chlorophyll, and nucleic acids.[14]

*B. mori*- and *M. alba*-derived materials have long been considered in East Asia to have natural medicinal properties.[14] For example, *B. mori* feces is known to be effective against urticaria, metrorrhagia, headache, polydipsia, ophthalmitis, and gastrospasm. More recently, chlorophyll derivatives (CpDs A, B, C and D) which have the specificity as a photosensitizer for tumor cells of human and mouse were isolated from *B. mori* feces.[26] Barclay and Perdue[16] suggested that the most promising botanicals as sources of novel plant-based anti-tumor agents for use at present (1976) and in the future are species of the families Cephalotaxaceae, Podocarpaceae, Taxaceae, Aunonaceae, Menispermaceae, Thymelaeaceae, Celestraceae, Celestraceae, Euphorbiaceae, Rutaceae, Simanubaceae, Apocynaceae, and Liliaece. In our studty, *B. mori* feces and *M. alba* (Family Moraceae) root barks showed potent cytotoxic activity against the five tumor cell lines, suggesting an indication of at least one of their pharmacological actions. The strong cytotoxic activity of these materials confirms their superiority and usefulness on an anti-tumor agent. Additionally, natural product-derived materials are found to be effective against cancer cells resistant to current chemotherapeutic agents.[8]

Based upon our results and these earlier findings, *B. mori* feces- and *M. alba*-derived materials may be useful for developing new types of anti-tumor agents.

**Table 3. Cytotoxic activity of solvent fractions of hot MeOH extract from feces of the silkworm larvae[a]**

| Fraction | $ED_{50}$ (μg/m*l*) | | | | |
|---|---|---|---|---|---|
| | A549 | SK-OV-3 | SK-MEL-2 | XF-498 | HCT-15 |
| Hexane | 109 | 96 | 90 | 97 | 79 |
| Chloroform | 57 | 41 | 43 | 44 | 52 |
| Ethyl acetate | 74 | 62 | 47 | 53 | 48 |
| Butanol | 163 | 152 | 80 | 67 | 130 |
| Water | >200 | >200 | >200 | >200 | >200 |

[a]Extracted with 70% hot MeOH for 2 hr at 65°C.

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## 누에 및 뽕나무 유래 물질의 人間 癌細胞株에 대한 細胞毒性

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**초 록** : 凍結乾燥한 누에 유래(4齡幼蟲, 암 · 수 번데기, 암 · 수 成蟲) 및 건조 뽕나무 유래 재료(잎, 오디, 桑白皮)의 메탄올 추출물, 白殭蠶 및 누에 4齡幼蟲 蠶糞의 메탄올 추출물의 5종 人間 癌細胞株(A549 lung, SK-OV-2 ovarian, SK-MEL-2 melanoma, XF-498 CNS, HCT-15 colon tumor cell lines)에 대한 細胞毒性을 sulforhodamine B법을 이용하여 *in vitro* 검정하였다. 공시시료중 蠶糞의 70% 메탄올 熱湯抽出物은 이들 癌細胞株에 대하여 강한 細胞毒性을 나타내었으나, 蠶糞의 메탄올 추출물 및 오디와 桑白皮의 메탄올 추출물은 중간 정도의 활성을 보였다. 기타 물질들은 이들 癌細胞株에 거의 독성을 보이지 않았다. 70% 메탄올 熱湯抽出物이 강한 細胞毒性을 나타내어, 용매 분획한 결과 클로로포름과 에틸아세테이트 획분이 癌細胞株에 대하여 가장 강한 細胞毒性을 보였다. 결론적으로, 蠶糞, 桑白皮 및 오디의 抗癌活性은 이들의 약리작용의 일부를 설명할 수 있을 것으로 생각된다.

찾는말 : 세포독성, 암세포주, 잠분, 누에, 뽕나무, 백강잠
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