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Pheophytin Content and Cytotoxicity of Silkworm Feces Against Jurkat Cells According to Dry Method and Storage Period

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In order to find potential anticancer agents, we extracted pheophytin from silkworm feces according to various dry and storage methods such as sun dry, shade dry, fresh freezing dry and freezing dry after freezing storage (for 1 ~ 3 years). The pheophytin extracts, mainly 10-hydroxypheophytin a, little b, of various storage silkworm feces were analyzed by reversed-phase high-performance liquid chromatography with photodiode array and fluorescence detection. The content of those pheophytin in old silkworm for 3 years (freezing storage and freezing dried in use, or freezing dried and cold storage) was better than others. The cytotoxicity of the pheophytin extracts and ethanol extracts of various storage silkworm feces were measured using Jurkat cells originated from human leukemia, using dye uptake assay (MTT) in order to find effective photodynamic therapeutic agents. The anticancer activity of those pheophytin extracts in various storage methods showed little difference among them. But ethanol extracts of fresh freezing dried silkworm in the current year was good cytotoxic activity than those of any other silkworm feces. With regards to these results, fresh ethanol extracts of silkworm feces were better than old ones. On the other hands, the pheophytin extracts of old silkworm feces contained the highest percentage of pheophytin content and showed good cytotoxicity against cancer cells by changing the pheophytin into pheophobide in the degradative process.

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Introduction

Sunlight-dried feces of silkworm (*Bombyx mori*) obtained from 3 ~ 4 instar larvae have been used in China as the remedy for palsy, blood circulation, removal of fever, healthy eyes, headache, itching, arthritis and etc. It contains phytol (0.25 ~ 0.29%), β-sitosterol (1.5%), cholesterol, ergosterol, tetracosanol, lupeol, bombyrenone, β-sitosterol-β-glucoside, carotene, heteroauxin, Vitamin A, B, E, chlorophyll, amino acid and etc (Cui *et al.*, 2002). In a previous report, the ethanol extracts of the silkworm feces obtained at 3rd day of 5th instar following freezing dry showed more potent anticancer activity than those of mulberry leaves, mulberry branches and samples prepared from other 5th instar larvae (Ahn *et al.*, 2001). Also, the ethanol extracts of the silkworm feces prepared from other larval stages exhibited better fibrinolytic activity than those of mulberry leaves (Ahn *et al.*, 2002). It has been reported that certain chlorophyll derivative (CpD) frcations from TLC of silkworm metabolites were effective for photodynamic therapy (CpD) in test animals, exhibiting killing of ascites tumors (Lee *et al.*, 1990). The essential CpD component was determined to be mainly 10-hydroxypheophytin a, little b, chlorophyllide and pheophorbide including a ~ d forms of chlorophyll by reversed-phase HPLC with photodiode array and fluorescence detection (Braumann and Grimme, 1981). Several authors have also suggested the existence of an Mg-releasing enzyme (Mg-dechelatase), which would catalyze the removal of Mg^{2+} ion from the tetrapyrrolic ring (Almela *et al.*, 2000). The 7th day of 5th instar silkworm larvae feces contained more pheophytin than others when calculated

by total amount of silkworm feces (Ahn *et al.*, 2002). The result indicates a direct relationship between ingestion of mulberry leaves by silkworm larvae and chlorophyll (pheophytin) content. Porphyrins and their analogues of silkworm feces have possibility as photosensitizers in the clinical treatment and these tetrapyrrolic macrocycles were reported to exhibit absorption bands in the red region (600 ~ 800 nm) of the visible spectrum that is endowed with an especially high penetration power into human tissue (Lim *et al.*, 2003).

In this study, we report upon the storage method and period (year) of the feces to select them for photodynamic therapy as a potential anticancer agent on cancer cells.

Materials and Methods

Preparation of test solution

Silkworm feces were collected at 4th day of 5th instar larvae from May 2000 to May 2002. The various silkworm feces according to storage and dry methods were as follows: freezing for 3 years and dry, freezing for 2 years and dry, freezing for 1 year and dry, freezing dried before 3 years and cold storage, freezing dried before 3 years and room temperature storage. The dried sample (50 g) was extracted with 625 ml of distilled water by incubation at 50°C for 2.5 hrs with mechanical stirring. The extract was filtered through four layers of gauze and the filtrate was then centrifuged for 10 min at 7,000 g in a Suprazik centrifuge (Hanil, Incheon, Korea). The pellet, which contained most of the chloroplast material, was suspended in 435 ml of methanol and 188 ml of $CHCl_3$ and incubated at 50 for 1.5 hrs with mechanical stirring. An aliquot of these extracts was investigated their spectra maxima (UV max). These extracts were concentrated by evaporator (Buchi Co., Ltd.) and resuspended in chloroform and methanol (1:9) for HPLC (Spectra system FL3000). The pheophytin (pheophytin a and 10-hydroxypheophytin a) obtained from Department of Kangwon University, was used as a retention time standard. All procedures were performed under shading light.

Chromatography

The HPLC equipment consisted of a Thermo Spectra Products (San Jose, USA) liquid chromatographic system equipped with P4000 operated from a Spectrasytem controller. Spectrasystem fluorescence FL3000 and a Spectrasystem UV 3000 photodiode UV-Vis detector were used on line. The fluorescence detector was operated at 430 nm (excitation) and 670 nm (emission). Spectral data from PAD system were recorded between 430 and 670 nm. Analytic separations were performed on a 5 μm Luna C^{18} column (25 cm × 0.4 cm, I. D.) (Phenomex, USA). Semipreparative separations were carried out on Luna C^{18} column. Silkworm feces pigments were eluted using a linear gradient in 30 min from 100% solvent A, (80% methanol in 1M ammonium acetate) to 100% solvent B (80% methanol in acetone). Solvent B was maintained until the pigments were completely eluted. The flow-rate was 0.8 ml/min with the analytic column. Isolated pigments were identified according to the retention time of standard references (Braumann and Grimme, 1981).

Measurement of cytotoxicity

The cytotoxicities of the crude insect test solutions were tested against Jurkat cell line (human leukemia cell) using MTT [3-(4,5-(dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide) kit solution (Boehringer Mannheim) treated light irradiation (10 min), as described previously (Carmichael *et al.*, 1987). And measurements were performed in triplicate. The IC_{50} (50% inhibitory concentration; mg/ml) was defined as the fraction of extract that caused a 50% inhibition of cell viability compared with the control.

Light illumination

The light source was a 200 W halogen lamp (MVI Micro Video Instruments Inc., MA, USA) attenuated by a 515 nm cut off filter according to previously described methods (Lim *et al.*, 2003).

Analysis of mineral content and amino acid composition

Mineral content was analyzed by atomic absorption spectroscopy and phosphorus content was determined by colorimetric method, which utilizes ammonium molybdate, hydroquinone, and sodium sulfate (Kim *et al.*, 2001). Amino acid Compositional analysis was carried out by derivatization of the first N-terminal amino acids with phenylisothiocyanate (PITC) followed by RP-HPLC (Williams *et al.*, 1998).

Results and Discussion

Pheophytin content of silkworm feces according to dry method and storage period

Chlorophyll derivatives include a broad spectrum of green-grey-brown pigments of very different polarities. The pheophytin extracts (mainly 10-hydroxypheophytin a, little b) obtained from silkworm feces prepared by different dry methods and storage periods were analyzed with photodiode array and fluorescence detection using a linear gradient from ammonium acetate to acetone in 80% methanol.

As the results, the old stored dried feces throughout the

action of chlorophyllase and Mg dechelatazation by native fermentation, such as the freezing dried feces stored 4°C for 3 years, contained relatively more pheophytin content per mg than silkworm feces obtained by other storage method and period (year). The older silkworm feces had more content than fresh ones in terms of total silkworm feces pheophytin amount (g/50 g crude silkworm feces) (3 years > 2 years > 1 year). The result indicated that the pheophytin extracts of old silkworm feces contained the highest percentage of pheophytin and better cytotoxicity against cancer cells (Table 1).

Mineral content

Mineral, moisture, fat, protein and ash content of silk-

Table 1. HPLC analysis of pheophytin a and 10-hydroxypheophytin a of the extracts of silkworm feces according to various storage

Storage Period and Dry method	Pheophytin extract (g/50g crude silkworm feces)	Pheophytin extract 1 mg		Pheophytin a and 10-pheophytin a (mg/50 g crude silkworm feces)
		Pheophytin a (μg)	10-hydroxypheophytin a (μg)	
Pheophytin standard (1mg/ml)		140.58	127.42	
1. Freezing for 3 years and dry	2.8	9.94	8.24	50.62
2. Freezing for 2 years and dry	1.65	0.47	10.10	17.44
3. Freezing for 1 year and dry	2.8	1.61	1.77	9.49
4. Freezing dried before 3 years and cold storage	3.4	14.68	7.15	74.22
5. Freezing dried before 3 years and R. T. storage	2.7	10.77	3.83	39.42

Table 2. Analysis of inorganic components of silkworm feces (except heavy metal)

Concentration	Star/Age	Percentage (%)						
Metal name		P	K	Ca	Mg	Na	T-N	Protein
Content	5/3	0.81	2.27	0.66	0.43	0.44	4.73	29.56
	5/6-7	1.68	3.10	0.92	0.39	0.48	3.55	22.19

Table 3. Amino acid content of silkworm feces according to storage methods

Amino acid (%)	1. Freeezing for 3 years and dry	2. Freezing for 2 years and dry	3. Freezing for 1 year and dry	4. Freezing dried before 3 years and cold storage	5. Freezing dried before 3 years and R. T. storage
Cystine	0.210	0.189	0.284	0.200	0.293
Methionine	0.136	0.126	0.164	0.129	0.160
Aspartic acid	1.021	0.939	1.115	0.922	1.132
Threonine	0.451	0.436	0.537	0.432	0.535
Serine	0.502	0.470	0.598	0.466	0.587
Glutamic acid	1.111	0.884	1.151	0.964	1.121
Glycine	0.712	0.659	0.959	0.657	0.963
Alanine	0.664	0.571	0.734	0.583	0.730
Valine	0.483	0.457	0.595	0.461	0.580
Iso-leucine	0.363	0.373	0.410	0.317	0.421
Leucine	0.792	0.772	0.937	0.681	0.936
Tyrosine	0.323	0.237	0.405	0.306	0.348
Phenylalanine	0.587	0.627	0.788	0.466	0.627
Lysine	0.629	0.387	0.695	0.451	0.604
Histidine	0.405	0.224	0.346	0.221	0.342
Arginine	0.495	0.375	0.649	0.373	0.550
Proline	0.468	0.431	0.576	0.486	0.570

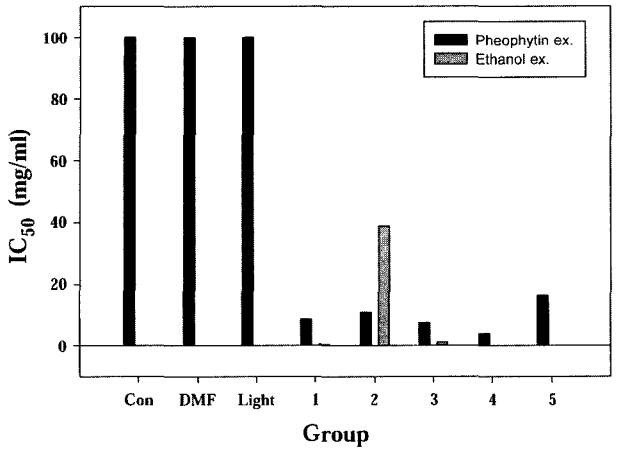

Fig. 1. Cytotoxicity of the pheophytin extracts and ethanol extracts of silkworm feces according to storage method.
1. Freeezing for 3 years and dry, 2. Freezing for 2 years and dry, 3. Freezing for 1 year and dry 4. Freezing dried before 3 years and cold storage, 5. Freezing dried before 3 years and R.T. storage
*DMF, N,N-dimethyl formamide; Light, The light source was a 200 W halogen lamp attenuated by a 515 nm cut off filter.

worm feces were investigated. Silkworm feces contained more proteins and minerals and each minerals and protein contents are as follows: protein (22 ~ 30%), P (0.81 ~ 1.68%), K (2.27 ~ 3.10%), Ca (0.66 ~ 0.92%), Mg (0.39 ~ 0.43%) and Na (0.44 ~ 0.48%) (Table 2). Amino acid content of the feces extracts prepared by different dry method and storage periods showed no obvious trend in the changes of protein and mineral contents in the statistical perspective (Table 3).

Anticancer activity of ethanol and pheophytin extract from silkworm feces by different dry method and storage periods under light irradiation

The anticancer activity against Jurkat cells originated from human leukemia is presented in Fig. 1. The pheophytin extracts prepared by a few storage methods showed a little difference among samples (depending on storage methods). But ethanol extracts of the sample of fresh freezing dried silkworm in the current year showed highest cytotoxic activity among any other silkworm feces. This result indicated that the fresh ethanol extracts of silkworm feces are better than old ones. Therefore, ethanol extracts are enough photodynamic therapeutic (PDT) agent for injection or ointment and further extraction method such as methanol and chloroform extraction is not required for consideration here. But, the ethanol extracts prepared by sun-dry method and stored for a long period have a low cytotoxicity against Jurkat cells.

On the other hands, the pheophytin extracts of old silkworm feces contained the highest percentage of pheophytin and good cytotoxicity against cancer cells changing the pheophytin into pheophobide in the degradative process.

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