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Antitumor Activity of the Aqueous-alcoholic Extracts from Unripe Cotton Ball of *Gossypium indicum*

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The present study investigated the antitumor activity of the aqueous-alcoholic extracts from unripe cotton balls of *Gossypium indicum*. An Exposure of murine B16 melanoma and L1210 lymphoma cells to the extracts resulted in their severe deaths in time- and concentration-dependent manners. Of the extracts, hydrophilic fractions were most efficacious for the antitumor activity and found to contain certain amounts of catechin and its derivatives. The hydrophilic extract fraction C36B2-8 had approximately 10 times more cytotoxic effects on B12 and L1210 cells than on isolated murine thymocytes. High concentrations (>150 μg/ml) of C 36B3-8 mainly induced necrotic cell death. At low concentrations (<100 μg/ml), however, C 36B3-8 induced not only necrosis but also apoptosis of the two tumor cell lines, which was proved by the TUNEL staining and DNA fragmentation techniques. The data indicate that certain ingredients of the cotton ball extract of *G. indicum* have an antitumor activity.

Key words : *Gossypium indicum*, Cotton ball, Anticancer activity, Apoptosis, Necrosis, B16 melanoma, L1210 leukemia, Thymocytes

INTRODUCTION

G. indicum has been used as a folk remedy and shown to have antitussive, antibiotic, antiviral and antitumor effects. Many chemical components have been identified and characterized in the *G. indicum,* including gossypol, flavonoids, phenolinic acids, salicylic acid, betaine, saponin and their derivatives. Of these chemical components, gossypol, a polyphenolic aldehyde, has been intensively studied as an antifertility agent (Comhaire, 1994) and an antitumor agent (Tuszynski and Cossu, 1984; Shidaifat *et al.*, 1996; Gilbert *et al.*, 1995; Hu *et al.*, 1993). Gossypol is mainly distributed in the root and seed. In contrast, little study has been done regarding the antitumor activity of cotton balls.

Unripe cotton ball contains sugar-like components with a sweet taste. Our preliminary study has shown that the extracts of unripe cotton ball have cytotoxic effects on the tumor cells in *in vivo* and *in vitro* models. In the present study we investigated the mechanism of the antitumor effect of the unripe cotton ball extracts.

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MATERIALS AND METHODS

Materials

Diaminobenzidine, catechin, epicatechin and epigallocatechin, proteinase K, isopropanol and ammonium acetate, phenol/chloroform/isoamyl alcohol, sodium dodesyl sulfate, RNAse A, paraformaldehyde were purchased from Sigma Chemical Co (St. Louis, MO, USA). RPMI1640 and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Terminal deoxynucleotidyl transferase and biotinylated dUTP were purchased from Boeringer Mannheim (Ottweiler, Germany). Extra-avidin Peroxidase were purchased from BioMakor (Rehovot, Israel). [^{3}H]thymidine were purchased from Dupont NEN (Boston, MA, USA).

Extraction and fractionation of cotton balls

Unripe cotton balls (50 kg) harvested from the farm of Ko-chang, Jeonbuk Korea, July 1996, were extracted with methanol under reflux. The methanol (MeOH) extracts were vacuum-concentrated and subsequently extracted with n-butanol (BuOH). BuOH-soluble fraction (304 g) was subjected to silica column chromato-

graphy with eththylacetate (EtOAc):methanol(MeOH): H_2O (10:1:0.1) mixture. Seven fractions were collected (C36B1~C36B7). Fraction C36B2 (24 g) was further subjected to silica column chromatography with $CHCl_3$: MeOH:H_2O (10:1.2:0.1, v/v) mixture. Eleven fractions were collected (C36B2-1~C36B2-11).

Cell culture

Murine B16 melanoma and L1210 leukemia cells were maintained in RPMI1640 supplemented with 10 % FBS. Thymocytes were isolated from the thymus of mouse (ICR, 5~6 weeks old) according to the previous report (Kang *et al.*, 1992) and cultured for 2 days before the experiment. In brief, mouse was sacrificed by spinal cord dislocation. Thymus was isolated and left in RPMI 1640 supplemented with 10% FBS containing antibiotics and 20 μM mercaptoethanol. Thymus was then minced with scissors and triturated with pipet. The triturate was left for 5 minutes on the table and then the supernatant was taken for the culture.

[^{3}H]Thymidine uptake

Cells cultured on the 24 well plate were incubated 15~24 hours with drugs and [^{3}H]thymidine (0.3 μCi/well) when the cells were 30% confluent on the plates. Media were removed from the cells and replaced with 0.5 ml methanol for 5 minutes. Cells were washed with 0.5 ml phosphate buffered saline after removal of methanol and incubated 10 minutes with 200 μl of 10% trichloroacetic acid. Cells were solubilized with 0.2 N NaOH and 0.5% sodium dodecyl sulfate solution. The solution was neutralized with 1 N HCl. Radioactivity was measured using a -counter (Beckman Instrumental Inc., Fullerton, CA, USA).

Trypan blue exclusion

L1210 cells and thymocytes in suspension and B16 cells on the dishes were added with 0.1% trypan blue in KRH and counted on a haemocytometer. Those cells unable to exclude trypan blue have lost their membrane integrity and are defined as nonviable.

Analysis of DNA fragmentation

DNA fragmentation was determined as previously described (van Lookeren Campagne *et al.*, 1995). In brief, cells were washed with 10 mM Tris-HCl buffer (pH 7.4) and harvested in 10-fold volume of the same buffer containing 30 mM EDTA (TE buffer). Immediately after this, sodium dodesyl sulfate (0.5%) and proteinase K (200 μg/ml) were added and then incubated at 37°C overnight. For the extraction of DNA, equal measures of phenol/chloroform/isoamyl alcohol (25:14:1; v/v/v, Sigma) were added to the samples and the mixture was gently stirred, followed by centrifugation at 2,000 g for 5 min. The DNA in the phenol supernatant was precipitated with 2-fold isopropanol containing 1 M ammonium acetate, followed by centrifugation at 2,000 g for 5 min. The pellet was washed in 70% ethanol and treated with RNase A (500 μg/ml) at 37°C for 1 h. The entire contents were dissolved in TE buffer and the nucleic acid and protein contents were measured in a spectrophotometer at wavelengths of 260 and 275 nm, respectively. Only samples yielding a nucleic acid to protein ratio of >1.8:1 were used for DNA fragmentation analysis. 15 μg of DNA or 1 mg of a 123 bp DNA ladder (Gibco BRL, Gaithersburg, MD) was mixed with 4 ml of a 5 x concentrated sucrose layer mix [500 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH8.0), 0.5% SDS, 32% sucrose, 0.25 % Orange G (BDH, Poole, UK)] and used for electrophoresis on agarose (1.5%, Boehringer Mannheim, Germany) containing ethidium bromide (0.5 mg/ml). DNA was visualized by a UV (302 nm) transluminator and the gels were photographed with a polaroid camera.

In situ labeling of free 3'-hydroxyl ends on DNA

Visualization of fragmented DNA in cells was done as previously described by Gavrieli *et al.* (1992). In brief, cells were fixed 5 minutes with 2% buffered paraformaldehyde and, to inactivate the endogenous peroxidase, covered with 2% H_2O_2 for 5 min at room temperature. Cells were incubated 60 min at 37°C with terminal deoxynucleotidyl transferase (0.05 U/μl) and biotinylated dUTP (0.01 nmol/μl). The reaction product was visualized with Extra-avidin Peroxidase (30 min, 37°C) and diaminobenzidine. Apoptotic cells were recognized by their dark nuclear staining.

RESULTS

Fig. 1 illustrates the procedures of aqueous-alcoholic extraction of *G. indicum*. [^{3}H]Thymidine incorporation data showed that, of the extracts examined, hydrophilic fractions such as C36B2-7, 8, and 9 appeared to be most potent: LD50 values for the inhibitory activity of aqueous-alcoholic extracts of C36B2-8 on B16 melanoma and L1210 leukemia cells were less than 40 g/ml (Fig. 2). Thus, further studies were carried out using the fraction C36B2-8. Morphological observation under light microscopy showed concentration- and time-dependent necrotic and apoptotic deaths of the cells. Fig. 3 shows that B16 and L1210 cells underwent necrotic death at high concentrations (each >100 g/ml) of C36B2-8.

The antiproliferative activity of C36B2-8, which was assessed by measuring [^{3}H]thymidine incorporation, was found to be more selective to tumor cells such as B16 and L1210 cells than thymocyte in primary culture:

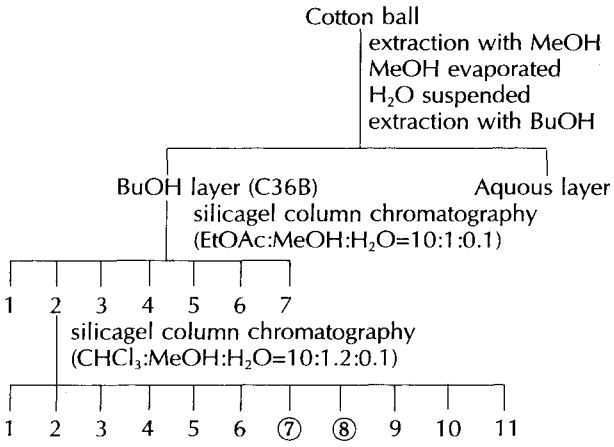

Fig. 1. Extraction of *Gossypium indicum*. #⑦: C36B2-7, ⑧: C36B2-8

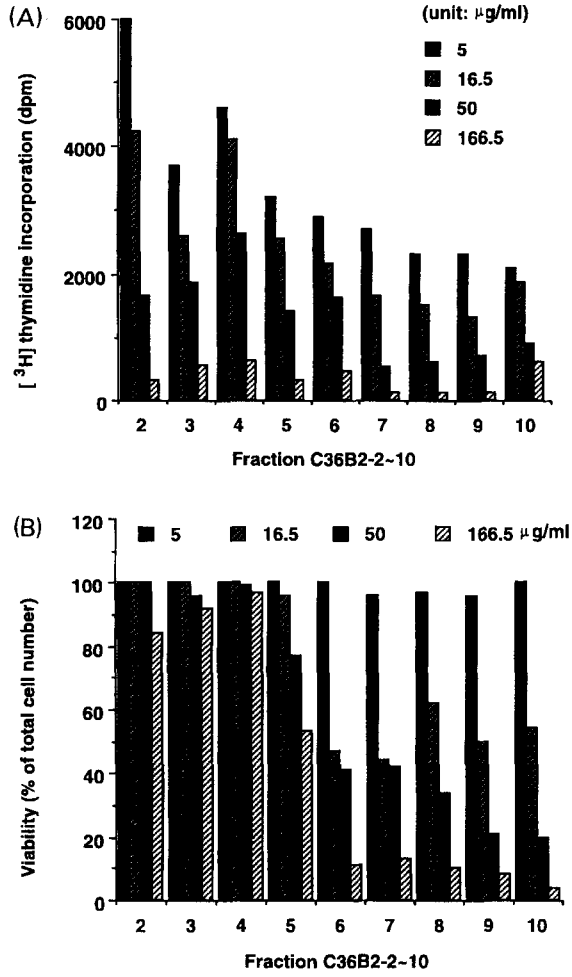

Fig. 2. Cytotoxicity of the extracts of *Gossypium indicum* on B16 and L1210 cells. A) B16 cells were incubated for 15~24 hours with [^{3}H]thymidine (0.3 μCi/well) in the presence and absence of various concentrations of each extract. B) Viability of L1210 cells were assessed by counting the cells exclusing trypan blue after incubation with various concentrations of each extract (N=4).

LD50 values for the inhibition by C36B2-8 of [^{3}H]thymidine incorporation into B16 cells, L1210 cells, and thymocyte were 8.4±2.5, 22±7.2, and >100 g/ml (Fig. 4). For comparison, LD_{50} values for the growth inhibition of L1210 cells by the known natural antitumor reagents catechin and epicatechin were 91±13.2 and 107±18.9 g/ml, respectively (Fig. 4, each N=3).

Further studies were carried out to study if C36B2-8 could exert the antitumor activity via apoptosis. Following the treatment of C36B2-8, cells seemed to underwent either early necrosis or delayed apoptosis depending on the concentration of C36B2-8. Fig. 5 shows the *in situ* DNA end-labeling of B16 and L1210 cells incubated for 20 hours with C36B2-8 (50 g/ml). Most of B16 cells and L1210 showed positive in the DNA fragmentation. However, L1210 also showed a diffused pattern of the DNA fragmentation, which may imply a necrotic cell death.

We further studied fragmentation of internucleosomal DNA. Fragmentation into a DNA ladder appeared approximately at 12 h after treatment with C36B2-8, with concomitant appearance of shrunken floating cells (data not shown). Incubation with C36B2-8 for 20 hours resulted in a DNA fragmentation in B16 and L1210 cells (Fig. 6).

DISCUSSION

In the present study we have found that the extracts of unripe cotton ball of the *G. indicum* has antitumor activity in two murine tumor cell lines, i.e., B12 melanoma cells cultured onto a plate and L1210 leukemic cells in suspension. In the *G. indicum,* gossypol mainly distributed in the root and seed has been recognized as a major and pharmacologically active ingredient. Gossypol, a yellow pigment from the cotton plant, has been shown to produce antisteroidogenic activity *in vivo* and has been extensively investigated as a male contraceptive agent (Comhaire, 1994). The antitumor effects of gossypol was first observed in melanoma and colon carcinoma cells (Tuszynski and Cossu, 1984). Since then, gossypol has been shown to be capable of inhibiting the proliferation of a wide range of cancer cells *in vivo* and *in vitro* (Shidaifat *et al.*, 1996, Gilbert *et al.*, 1995, Hu *et al.*, 1993). To our knowledge, this report is the first to show the antitumor activity of unripe cotton ball extract.

Many of chemotherapeutic agents are known to induce apoptotic cell death (Hale *et al.*, 1996; Dixon *et al.*, 1997). Pharmacological manipulation of apoptosis may manipulate the equilibrium between the rates of cell division and cell death, influencing the anomalous accumulation of neoplastic cells and has far-reaching implications for new directions in cancer therapy (Dixon *et al.*, 1997). The aqueous-alcoholic ex-

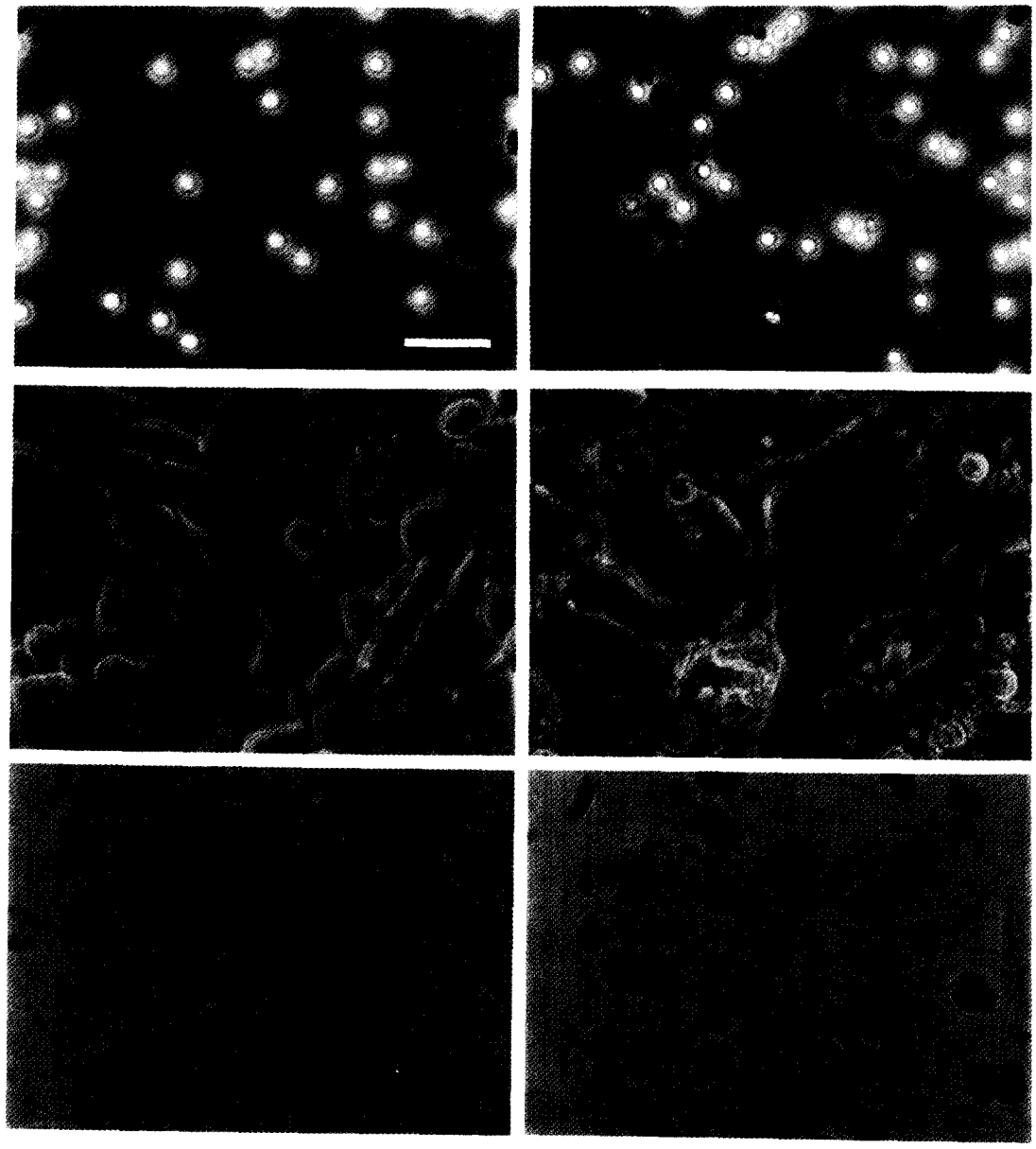

Fig. 3. Cytotoxicity of C36B2-8 on B16, L1210, and thymocytes. Cells (upper, thymocyte as a control; middle, B16; lower, L1210) were incubated for 15~24 hours in the presence (right) and absence (left) of C36B2-8 (150 μg/ml for thymocytes; 50 μg/ml for B16 and L1210 cells). L1210 cells and thymocytes were incubated for 5 minutes with 0.1% trypan blue and photographed. Bar=30 μm. Magnification=×400.

tracts of unripe cotton ball were found to exert an antitumor activity via induction of apoptotic cell death as demonstrated by positive TUNEL staining and DNA fragmentation. The in situ labelling of DNA fragmentation (TUNEL staining) is very much useful to show the DNA fragmentation in single cells. However, positive TUNEL staining sometimes occurs in necrotic cell death as a diffused staining. The aqueous-alcoholic extracts of unripe cotton ball might also induce necrotic cell death to some extent: necrotic cell death was evidenced by the rapid (less than 3 hours exposure to the drug) leakage of LDH and rupture of the cell membrane. The most widely accepted biochemical marker of apoptosis is DNA breakdown into DNA fragments of approximaely 180- to 200-bp integers. These fragments are believed to occur as a result of DNA cleavage at the linker regions between nucleosomes and are visualized as DNA ladders by conventional agarose gel electrophoresis. Thus, the tumor cells used in the present study are thought to undergo either early necrosis or delayed apotosis following an exposure to the unripe cotton ball extract.

We identified catechin and (-)-epicatechin in the unripe cotton ball extract (data not shown). Although

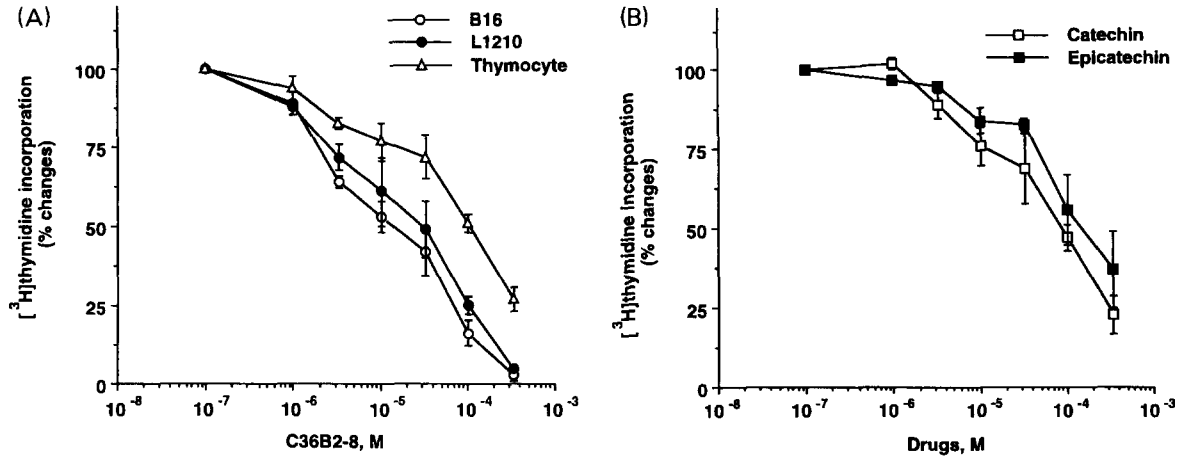

Fig. 4. [^{3}H]Thymidine incorporation. Cells were incubated for 15~24 hours with [^{3}H]thymidine (0.3 μCi/well) in the presence of various concentrations of C36B2-8 (A) or catechin and epicatechin (B). Data were expressed as mean±S.D. from the representative of 3 separate experiments.

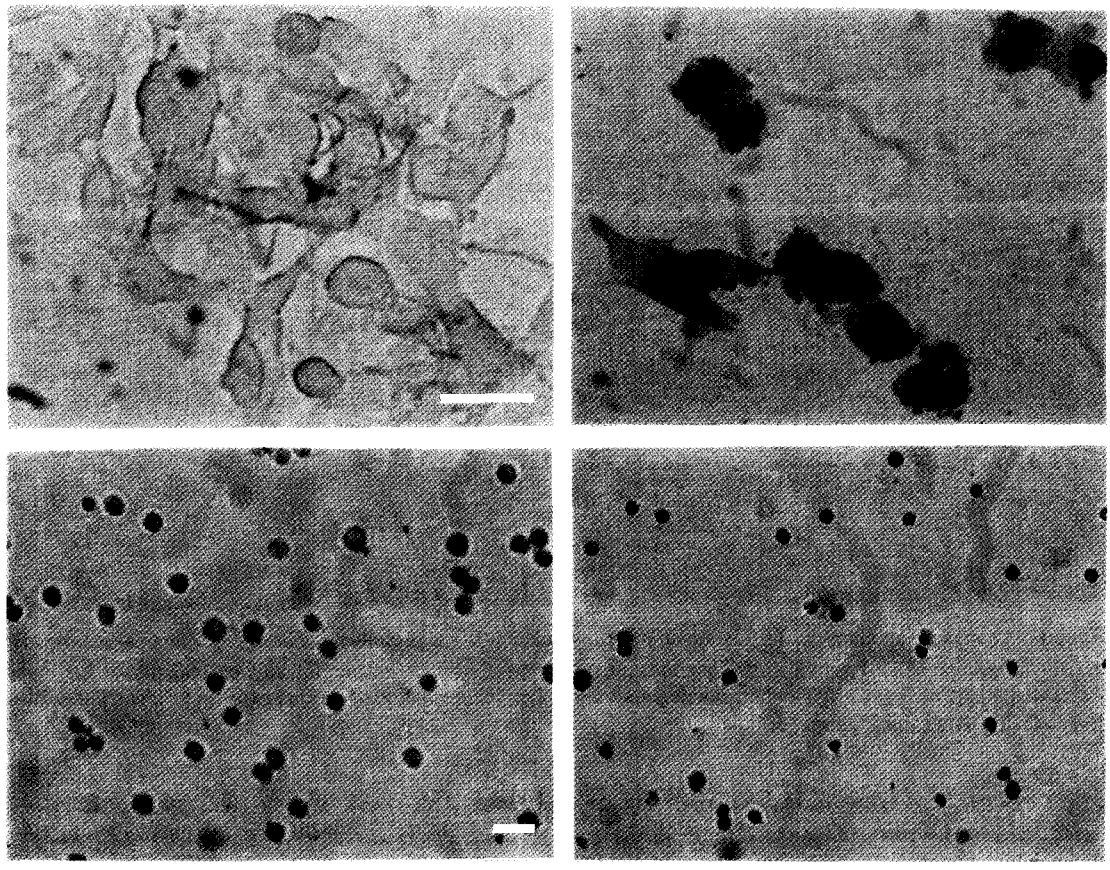

Fig. 5. TUNEL staining. B16 (upper) and L1210 cells (lower) were incubated for 20 hours in the presence (right) and absence (left) of C36B2-8. DNA end-labeling was carried out using biotin-16-dUTP and terminal deoxynucleotidyl transferase. Bar=30 μm. Magnification: x 400 for B16 cells, x 200 for L1210 cells.

we did not completely identify the effective chemical compounds in this study, we consider that the antitumor activity of the extract is in part attributable to the catechin and its derivatives existing in the extract fraction C36B2-8. Catechin and its derivatives mainly found in green tea, dried unfermented leaves of Camellia sinensis (family Theacea), have been reported to have antitumor activity (Kumari *et al.*, 1996; Yin *et al.*, 1994; Gensler *et al.*, 1996. Valcic *et al.*, 1996 Stoner *et al.*, 1995. Liao *et al.*, 1995 Yamane *et al.*,

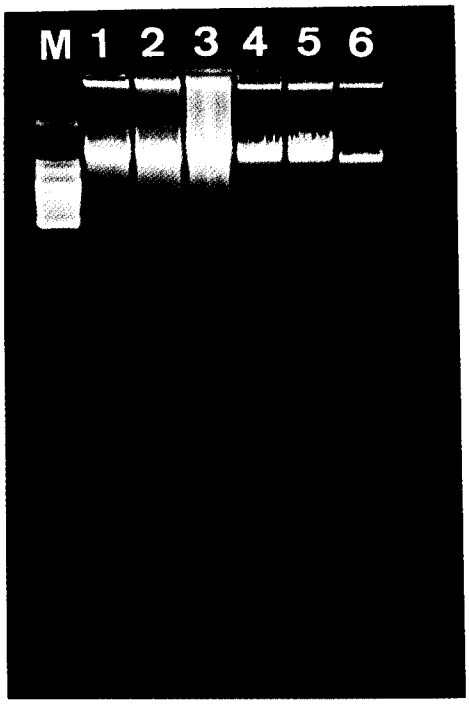

Fig. 6. DNA fragmentation in B16 cells and L1210 cells treated with C36B2-8. B16 and L1210 cells were incubated for 20 hours with C36B2-8. DNA was extracted from the cells (B16 cells (lane 1-3) and L1210 cells (lane 4-6)) and electrophoresed on the agarose-gel: Lane M, 123 bp marker; Lane 1, control; Lane 2, 50 μg/ml; Lane 3, 100 μg/ml; Lane 4, control; Lane 5, 50 μg/ml; Lane 6, 100 μg/ml.

1995). Our *in vitro* study, however, showed that catechin or (-)-epicatechin alone had much less (approximately 10-fold less) antitumor activity than the unripe cotton ball extract. Therefore, it is still possible that catechin and/or its derivatives synergistically enhance the antitumor activity of other active ingredients in the extract. It is also plausible that a unknown active ingradient(s) may exist in the extracts.

Antiproliferating chemotherapeutic drugs must have a selective cytotoxicity on tumor cells. In general, a rough correlation exists between very rapidly growing cells and their sensitivity to the antitumor drugs. For example, slow growing cells, such as mammary adenocarcinoma, are least sensitive, requiring higher concentrations of antitumor drugs. For comparison, we have studied the effects of C36B2-8 on the proliferation of normal thymocyte, which proliferates rapidly like tumor cells (Kang *et al.,* 1992). At present, we do not know what causes the selective effect of C36B2-8. Obviously, more studies are needed to identify the active component(s) of the extract and understand the mechanism of the selective antitumor activity of the extract.

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