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# Apoptotic Activity of Insect Pathogenic Fungus *Paecilomyces japonica* Toward Human Acute Leukemia Jurkat T Cells is Associated with Mitochondria-Dependent Caspase-3 Activation Regulated by Bcl-2

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**Abstract** The antitumor activity of the insect pathogenic fungus *Paecilomyces japonica* has been attributed to apoptotic cell death. However, the mechanism underlying the induced apoptosis has not yet been elucidated. In this study, we for the first time show that mitochondria-dependent caspase-3 activation was associated with the apoptotic activity of *P. japonica* in human acute leukemia Jurkat T cells. When Jurkat T cells were treated with the ethyl acetate extract of *P. japonica* at concentrations ranging from 2–6 μg/ml, apoptotic cell death, accompanied by several biochemical events such as caspase-9 activation, caspase-3 activation, degradation of poly (ADP-ribose) polymerase (PARP), and apoptotic DNA fragmentation, was induced in a dose-dependent manner. In addition, the release of cytochrome c from mitochondria was detected. Under these conditions, the expression of Fas and Fas-ligand (FasL) remained unchanged. Ethyl acetate extract-induced mitochondrial cytochrome c release, caspase-3 activation, PARP cleavage, and apoptotic DNA fragmentation were suppressed by the ectopic expression of Bcl-2, which is known to block mitochondrial cytochrome c release. Accordingly, these results demonstrate that *P. japonica*-induced apoptotic cell death is mediated by a cytochrome c-dependent caspase-3 activation pathway that can be interrupted by Bcl-2.

**Key words:** *Paecilomyces japonica*, antitumor activity, apoptosis, cytochrome c-dependent caspase-3 activation, Bcl-2, Jurkat T cell line

The genus *Cordyceps*, which includes about 300 species, is the largest single genus of insect parasites in the division of Ascomycota and belongs to the family Clavicipitacea that comprises order Clavicipitales [4, 17, 27]. Insects infected by *Cordyceps* mainly include Hemiptera, Diptera, Lepidoptera, Hymenoptera, and Coleoptera [17]. The infection of these insects occurs most commonly in the larval or pupal stages. Since the insect body dies as the infected fungus grows and produces toxins in it, it has been proposed that *Cordyceps* has potential as biological control agents against insect pests [2, 32].

*Cordyceps* possesses a sexual stage as well as a nonsexual stage throughout its life cycle. It has been generally accepted that *Cordyceps* can produce ascospores for the sexual reproductive structure and conidia for the nonsexual reproductive structure [7, 11]. While *Cordyceps* has been classified based largely on its morphological characteristics, the nonsexual stages of *Cordyceps* have also been assigned to a number of unique genuses, such as *Acremonium*, *Akanthomyces*, *Cephalosporium*, *Hirsutella*, *Hymenostilbe*, *Isaria*, *Nomuraea*, *Paecilomyces*, *Paraisaria*, *Pseudogilbellular*, *Sporothrix*, *Stilbella*, *Verticillium*, and *Beauveria* [11, 28].

Several *Cordyceps* species are used as traditional medicine in China, Japan, and Korea [3]. Recently, the mass production of these strains through artificial cultivation has been successfully accomplished. This allows several *Cordyceps* species to be supplied for public demand, and employed as a target for identifying a new anticancer and immunomodulating drug. In Korea, *Paecilomyces japonica* is one of the *Cordyceps* species that has been artificially cultivated on a large scale using silkworm pupas and is currently being consumed as a functional food believed to be effective in cancer prevention along with related therapy. However, little is known about the mechanism underlying the antitumor activity of *Paecilomyces japonica*. In a previous study, we demonstrated that an aqueous extract of *P. japonica* artificially cultivated on the silkworm pupa exhibited cytotoxicity against tumor cells such as Jurkat, U937, HL-60, HepG2, and BW5147.G.1.4 [21]. When the aqueous extract was fractionated further by sequential organic solvent extractions using n-hexane and ethyl acetate, the ethyl

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a etate extract appeared to contain the most cytotoxic a tivity, and the $IC_{50}$ value for various tumor cells ranged fi )m 1.5 to 10 μg/ml. It was also demonstrated that the c totoxic activity of *P. japonica* toward human acute leukemia J rkat T cells was attributable to induced apoptosis. Since tl e $IC_{50}$ value of the ethyl acetate extract of *P. japonica* is c mparable with the recently reported cytotoxicity of several n ıtural compounds against tumor cells [5, 12, 16, 21, 22, 2 ɔ], it is likely that the ethyl acetate fraction of *P. japonica* is a potent chemotherapeutic agent. In the present study, to e ucidate the mechanisms underlying the apoptosis of tumor c lls induced by *P. japonica*, we investigated whether the ethyl a etate extract-induced apoptosis accompanied mitochondria- c pendent activation of the caspase cascade and whether it c ɔuld be protected by the ectopic expression of anti- a ɔoptotic protein, Bcl-2. The results showed that the ethyl a etate extract-induced apoptotic cell death was mediated by r itochondrial cytochrome c release with resultant activation c caspase-9 and -3, and cleavage of poly (ADP-ribose) p ɔlymerase (PARP), which was suppressed by Bcl-2.

## I IATERIALS AND METHODS

### I licroorganism and Culture Condition

T he *P. japonica* was obtained from the Korea Rural I evelopment Administration. The strain was periodically s ıbcultured on a Potato Dextrose Agar (PDA) Medium ( Difco Laboratories, Detroit, U.S.A.) and stored at 4°C. To p repare the seed culture, the strain grown on a PDA ı ıedium was cultivated with shaking in a Potato Dextrose l roth (Difco Laboratories, Detroit, U.S.A.) for 7 days at 25°C. l or large-scale cultivation, 100 ml of the seed culture was i ıoculated into a two liter polypropylene bag containing 500 g ( f sterilized silkworm pupas, and incubated at 25°C for 15 days ı nder 70% relative humidity (RH). The culture was incubated s equentially at 20°C under 90-95% RH with exposure to l ght for a period of 20 days to induce the fruiting body.

### Iuman Jurkat T Cell Culture

The human acute leukemia Jurkat T cell line E6.1 was upplied by Dr. Albert A. Nordin (Gerontology Research enter, NIA/NIH, Baltimore, MD, U.S.A.). The Jurkat T cell lones JT/Neo and JT/Bcl-2 were supplied by Dr. Dennis aub (Gerontology Research Center, NIA/NIH, Baltimore, MD, U.S.A.). The Jurkat T cells were maintained in RPMI 640 (Life Technologies, Gaithersburg, MD, U.S.A.) containing 0% FBS (UBI, Lake Placid, NY, U.S.A.), 20 mM HEPES pH 7.0), $5\times10^{-5}$ M 2-ME, and 100 μg/ml gentamycin.

### reparation of Antitumor Component from *P. japonica* y Ethyl Acetate Extraction

o purify the active component exhibiting antitumor activity rom *P. japonica*, 30 g of the lyophilized *P. japonica* cultured on the silkworm pupas was suspended in 300 ml of distilled water and incubated in a boiling water bath for 3 h. The aqueous solution was centrifuged at 12,000 rpm to remove the mycelium. This water soluble fraction was extracted with the same volume of n-hexane three times, and then the remaining aqueous phase was extracted with ethyl acetate three times. From 200 ml of the aqueous solution, approximately 16.2 mg of the ethyl acetate fraction was recovered as previously described [21].

### Cytotoxicity Assay

The cytotoxic effect of the ethyl acetate extract from *P. japonica* on Jurkat T cells was analyzed by an MTT assay, reflecting the cell viability, as described elsewhere [12, 21, 22]. For the MTT assay, Jurkat T cells ($5\times10^4$) were added to a serial dilution of the ethyl acetate extract in 96-well plates. After incubation for 20 h, 50 μl of the MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well and then 150 μl of DMSO was added to dissolve the colored formazan crystal produced from the MTT. The OD values of the solutions were measured at 540 nm using a plate reader.

### DNA Fragmentation Analysis

The apoptotic DNA fragmentation induced in the Jurkat T cells following treatment with the ethyl acetate extract was determined as described elsewhere [9]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) for 20 min on ice. After centrifugation for 15 min at 14,000 rpm, the supernatant was collected and treated for 2 h at 50°C with proteinase K and subsequently with RNase for 4 h at 37°C. After extraction with an equal volume of buffer-saturated phenol, the DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 0.5 M NaCl and visualized following electrophoresis on a 1.2% agarose gel.

### Staining of Jurkat T Cells with 4,6-Diamidino-2-Phenylindol (DAPI)

After exposure to the ethyl acetate extract of *P. japonica*, the Jurkat T cells were stained with DAPI (Sigma, St. Louis, MO, U.S.A.) and analyzed using a fluorescence microscope (Microphot-FX, Nikon, Tokyo, Japan) to assess nuclear morphological change. Approximately $1\times10^6$ cells were suspended in 100 μl of PBS containing 2% FBS, and then 200 μl of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS containing 2% FBS, and resuspended with 12.5 μg of RNase in 250 μl of 1.12% sodium citrate buffer (pH 8.45). The incubation was continued at 37°C for 30 min before staining the cellular DNA with 250 μl of DAPI (4 μg/ml) for 20 min at room temperature.

### Preparation of Cell Lysates and Western Blot Analysis

The cellular lysates were prepared by suspending $5\times10^6$ Jurkat T cells in 200 μl of a lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 2.5 μg/ml proteinase inhibitor E-64, and pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. An equivalent amount of protein lysate (20-30 μg) was denatured with an SDS sample buffer, and subjected to electrophoresis on a 4-12% SDS gradient polyacrylamide gel with MOPS buffer. For the detection of caspase-3 activation and mitochondrial cytochrome c release, the protein lysates were electrophoresed on a 10% SDS polyacrylamide gel with MES buffer. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, U.S.A.), and then probed with individual antibodies. The monoclonal anti-caspase-3, anti-caspase-9, anti-Fas, or anti-FasL antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.), and the anti-cytochrome c antibody was purchased from Pharmingen (San Diego, CA, U.S.A.). The anti-PARP, anti-Bcl-2, and anti-Bcl-xL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The detection of each protein was carried out using an ECL Western blotting kit (Amersham, Arlington Heights, IL, U.S.A.) detection solution as described elsewhere [6, 20, 22, 30].

### Detection of Mitochondrial Cytochrome c Release in Cytosolic Protein Extracts

To assess the mitochondrial cytochrome c release in the Jurkat T cells following the ethyl acetate extract treatment, cytosolic protein extracts were prepared, as previously described [9]. Briefly, approximately $5\times10^6$ cells treated with the ethyl acetate from *P. japonica* were washed twice with cold PBS and then suspended in 0.5 ml of a lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2.5 μg/ml E-64, 20 mM HEPES, pH 7.2). The cells were allowed to swell on ice for 30 min and were then homogenized with a Dounce homogenizer with 50 strokes. The homogenates were centrifuged at 3,500 rpm for 10 min at 4°C, and the supernatants were centrifuged at 13,700 rpm for 15 min at 4°C. The supernatants were harvested as cytosolic extracts free of mitochondria, and analyzed for mitochondrial cytochrome c release.

## RESULTS AND DISCUSSION

### Apoptotic Effect of Ethyl Acetate Extract of *P. japonica* on Jurkat T Cells

To elucidate the mechanisms underlying the apoptotic cell death induced by the ethyl acetate extract of *P. japonica*, the apoptotic activity of the ethyl acetate extract toward the Jurkat T cell line E6.1 was investigated. When the cells were treated with the ethyl acetate extract at various concentrations ranging from 1 to 6 μg/ml for 24 h, the cell viability determined by an MTT assay declined in a dose-dependent manner. Although the cell viability in the presence of 1 μg/ml of the ethyl acetate extract was sustained at the level of 68%, the viability in the presence of 2, 4, and 6 μg/ml appeared to be 60, 56, and 52%, respectively (Fig. 1A). Under these conditions, apoptotic

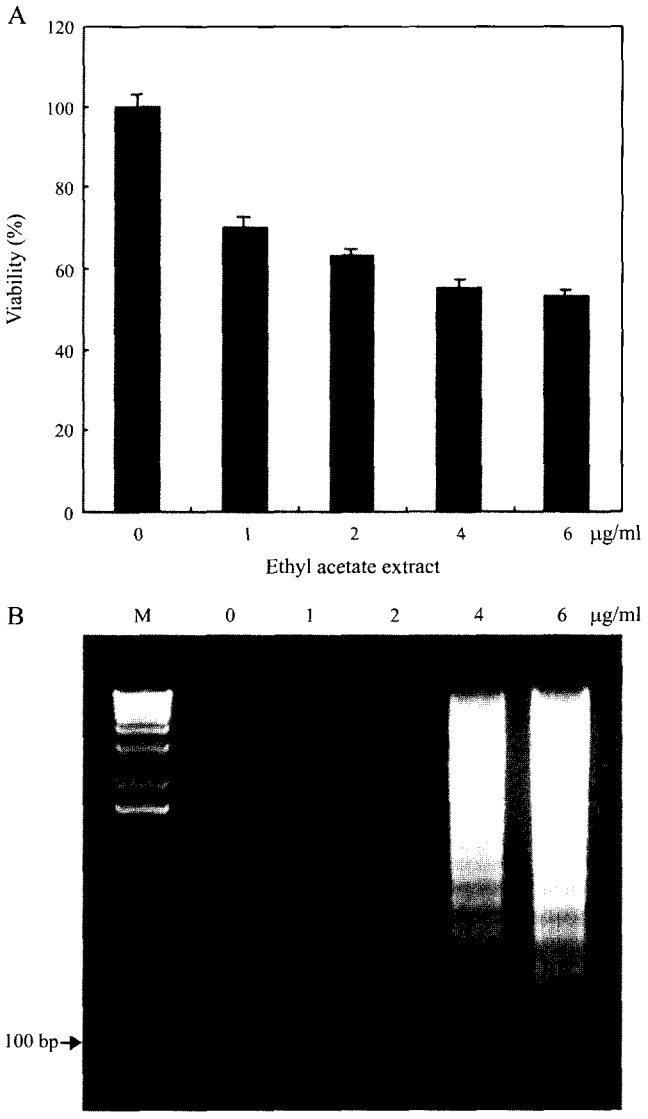


**Fig. 1.** Apoptotic activity of ethyl acetate extract from *P. japonica* toward cell viability (A) and apoptotic DNA fragmentation (B) in Jurkat T cells.

Continuously growing Jurkat T cells ($5\times10^4$) were incubated with the indicated concentrations of the ethyl acetate extract in a 96-well plate for 24 h, and the final 4 h was incubated with MTT. The cells were sequentially processed to assess the colored formazan crystals produced from the MTT as an index of cell viability. Equivalent cultures were prepared and the cells were collected for analysis of the apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis.

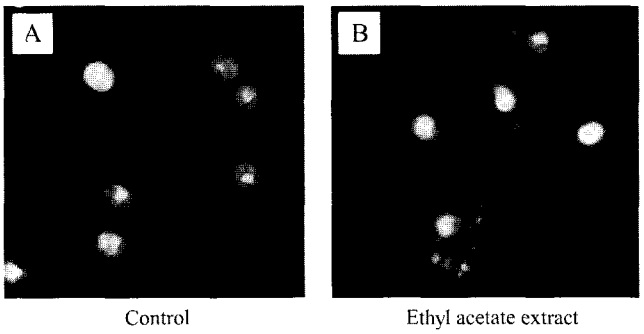


**Fig. 2.** Fluorescence photomicrographs of nuclear apoptotic change in Jurkat T cells following treatment with ethyl acetate extract.
After the Jurkat T cells were incubated in the absence (A) or presence (B) of 4 μg/ml of the ethyl acetate extract for 24 h, the cells were fixed with cold ethanol and then stained with DAPI to assess nuclear morphological changes resulting from the induced apoptosis.

DNA fragmentation was also induced in a dose-dependent manner (Fig. 1B). DNA fragmentation was barely detectable in the presence of 1-2 μg/ml, yet easily detectable at a concentration of 4-6 μg/ml. To assess the apoptotic change in the nuclear morphology of the Jurkat T cells following treatment with the ethyl acetate extract, the cells treated with 4 μg/ml of the ethyl acetate extract for 24 h were stained with DAPI. As shown in Fig. 2, typical apoptotic bodies were detectable among the Jurkat T cells treated with the ethyl acetate extract. These results demonstrate that the active fraction in the ethyl acetate extract from *P. japonica* is able to induce apoptotic cell death, and suggest that the cytotoxic effect of the ethyl acetate extract is attributable to induced apoptosis.

### Involvement of Mitochondrial Cytochrome c Release with Resultant Activation of Caspase-3 in the Ethyl Acetate Extract-Induced Apoptosis

Previous studies have demonstrated that the activation of caspase-3 through the proteolytic degradation of a 32-kDa pro-enzyme into a 19-kDa activated form is often required for apoptosis induced by many different stimuli [15]. To determine whether the ethyl acetate extract-induced apoptosis in the Jurkat T cells was accompanied by caspase-3 activation, changes in the pro-caspase-3 level and active caspase-3 level were investigated by Western blot analysis after treatment of the Jurkat T cells with the ethyl acetate extract. As shown in Fig. 3A, the expression of pro-caspase-3 was easily detectable in the continuously growing Jurkat T cells. Although there was no detectable change in the level of pro-caspase-3 (32 kDa) with 1-2 μg/ml of the ethyl acetate extract, the level declined in a concentration-dependent manner in the presence of the ethyl acetate extract ranging from 4-6 μg/ml. In addition, at concentrations of 4-6 μg/ml of the ethyl acetate extract, active caspase-3 (19 kDa) appeared to increase concomitantly with a reduction in the pro-caspase-3 level.

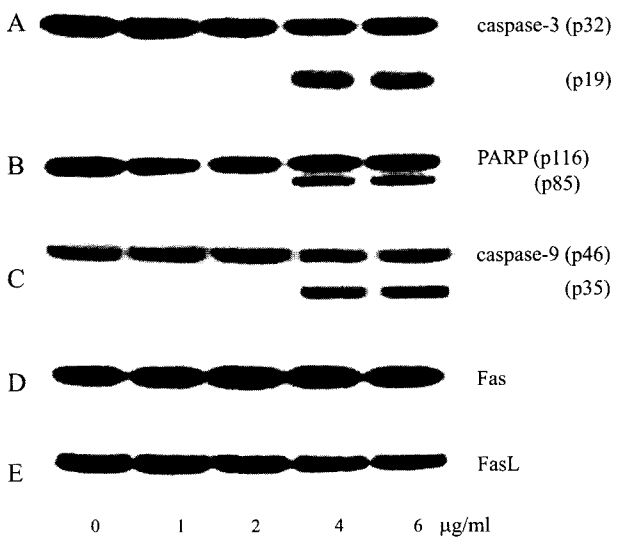


**Fig. 3.** Western blot analysis of activation of caspase-3 (A), cleavage of poly (ADP-ribose) polymerase (PARP) (B), activation of caspase-9 (C), and expression level of Fas (D) and FasL (E) in Jurkat T cells after treatment with ethyl acetate extract.
The cells (~$5\times10^6$ cells) were incubated at a concentration of $4\times10^5$/ml with the indicated concentrations of the ethyl acetate extract for 24 h and prepared for the cell lysates. Equivalent amounts of the cell lysates were electrophoresed on 4-12% SDS gradient polyacrylamide gels and electrotransferred to an Immobilon-P membrane. The membrane was probed with anti-caspase-3, anti-PARP, anti-caspase-9, anti-Fas, or anti-FasL, and then with a horse-radish peroxidase conjugated secondary antibody. The detection of each protein was performed using an ECL Western blotting detection system.

As a downstream target of active caspase-3 during the induction of apoptosis, poly (ADP-ribose) polymerase (PARP) has been reported to be cleaved into two fragments [13]. When the degradation of PARP was investigated by Western blot analysis, the cleavage of PARP was also detected relative to the activation of caspase-3 in the presence of 4-6 μg/ml of the ethyl acetate extract (Fig. 3B). Since activation of caspase-9 is known to be an upstream event of caspase-3 activation [14], a conversion of pro-caspase-9 (46 kDa) to active caspase-9 (35 kDa) was also investigated by Western blot analysis. The activation of caspase-9 was detected in the presence of the ethyl acetate extract ranging from 4-6 μg/ml (Fig. 3C). Under the same conditions, there was no alteration in the Fas and Fas ligand (FasL) levels (Figs. 3D and E), which can be upregulated in tumor cells following treatment with anticancer drugs and thus induce apoptotic cell death [8, 18]. These results demonstrated that the apoptotic DNA fragmentation in the Jurkat T cells induced by the ethyl acetate extract of *P. japonica* accompanied activation of caspase-9 and -3, and subsequent degradation of PARP.

Since it is generally accepted that a chemical (most chemotherapeutic agents)-induced apoptotic signaling pathway involves cytochrome c release from mitochondria [14, 23],

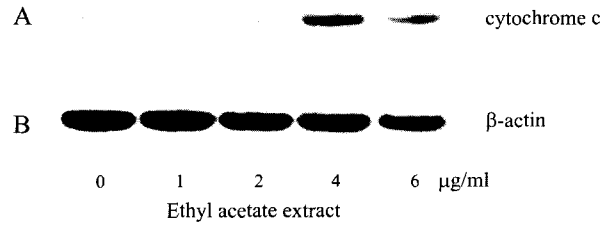


**Fig. 4.** Western blot analysis of mitochondrial cytochrome c release (A) and β-actin (B) in Jurkat T cells after exposure to ethyl acetate extract.
The cells ($5\times10^6$ cells) were incubated at a concentration of $4\times10^5$/ml with the indicated concentrations of the ethyl acetate extract for 24 h and prepared for cytosolic protein extracts. Equivalent amounts of the protein extracts were electrophoresed on 4-12% SDS gradient polyacrylamide gels and electrotransferred to an Immobilon-P membrane. The membrane was probed with anti-cytochrome c or anti-β-actin, and then with a horseradish peroxidase conjugated secondary antibody. The detection of each protein was performed using an ECL Western blotting detection system.

we decided to investigate whether the ethyl acetate extract-induced apoptosis accompanied mitochondrial cytochrome c release. Although there was only barely detectable cytochrome c in the cytosolic fraction of the continuously growing Jurkat T cells, the level of cytosolic cytochrome c released from the mitochondria increased significantly in the presence of the ethyl acetate extract ranging from 4-6 μg/ml (Fig. 4A). However, the level of β-actin in the cytosolic fraction was not altered, regardless of the presence of the ethyl acetate extract (Fig. 4B). Since there was no change in the levels of Fas and FasL during the induced apoptosis of Jurkat T cells by the ethyl acetate extract, these results indicate that the apoptotic signaling pathway leading to caspase-3 activation through the caspase cascade is initiated by the action of cytochrome c released from the mitochondria, but not by the Fas-death signal.

## Effect of Bcl-2 on the Ethyl Acetate Extract-Induced Apoptotic Cell Death

The anti-apoptotic protein Bcl-2 can protect cells from apoptosis induced by diverse signals, such as Fas ligation, ionizing radiation, hypoxia, or chemotherapeutic agents [1, 24, 26, 29]. The anti-apoptotic role of Bcl-2 is initially known to center around its prevention of cytochrome c release from mitochondria [10, 31], resulting in the suppression of a mitochondria-dependent apoptotic pathway [13-15, 19, 23]. Through processing certain forms of apoptosis induced by cytotoxic drugs, it has been proposed that the drug-mediated downregulation of Bcl-2 plays a critical role. However, the change in the expression level of Bcl-2 and its anti-apoptotic role during ethyl acetate extract-induced apoptosis of Jurkat T cells have not yet been elucidated.

To examine whether the ethyl acetate extract modulated the expression level of Bcl-2 and whether the ectopic overexpression of Bcl-2 could negatively regulate the ethyl acetate extract-induced apoptosis, the protein level of Bcl-2 was investigated in Jurkat T cells transfected with the vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2), following exposure to various concentrations of the ethyl acetate extract. As shown in Fig. 5A, the cytotoxicity of the ethyl acetate extract was significantly reduced by overexpression of

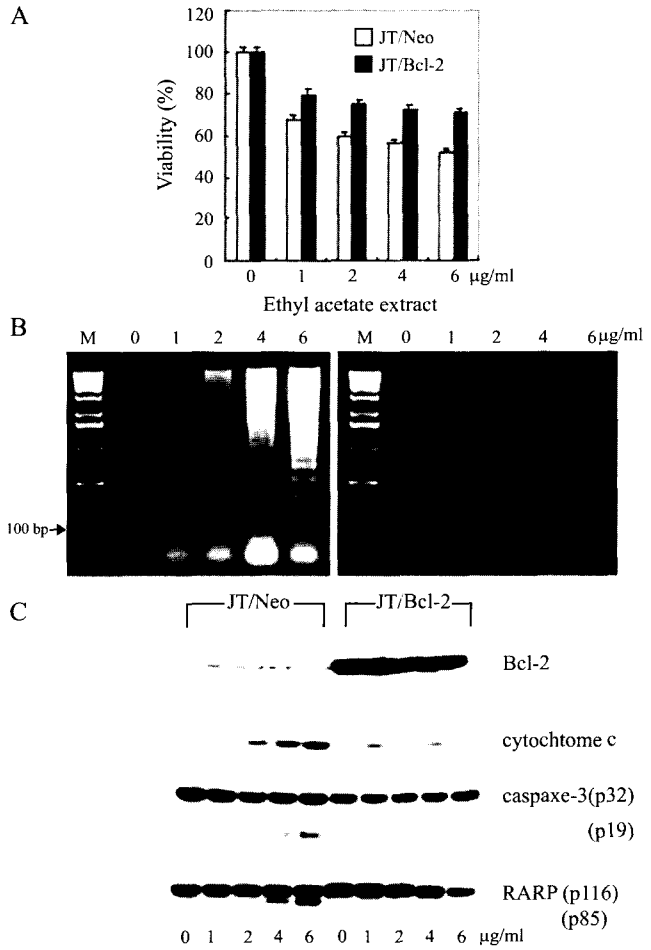


**Fig. 5.** Inhibitory effect of Bcl-2 on cytoxicity (A), apoptotic DNA fragmentation (B), and several apoptotic events, such as mitochondrial cytochrome c release, activation of caspase-3, and cleavage of PARP (C), induced by ethyl acetate extract.
Jurkat T cells overexpressing Bcl-2 and individual control cells were incubated at a density of $5\times10^4$/well with various concentrations of the ethyl acetate extract in 96-well plates. After incubation for 20 h, MTT was added and the mixture was further incubated for an additional 4 h. The cells were then processed to assess the colored formazan crystals produced from the MTT as an index of cell viability. Equivalent cultures were prepared and some of the cells were collected to analyze the apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis, while remaining cells were processed to prepare cytosolic protein extracts or cell lysates. Equivalent amounts of the proteins were electrophoresed on 4-12% SDS gradient polyacrylamide gels and electrotransferred to an Immobilon-P membrane. The membrane was probed with anti-cytochrome c, anti-caspase-3, or anti-PARP, and then with a horse-radish peroxidase conjugated secondary antibody. The detection of each protein was performed using an ECL Western blotting detection system.

B l-2. Similarly, the ethyl acetate extract-induced apoptotic D NA fragmentation was also completely abrogated, indicating th at the protection effect of Bcl-2 on the cytotoxicity of the et nyl acetate extract was mainly due to its inhibition of ap optotic DNA fragmentation (Fig. 5B). However, since o erexpression of Bcl-2 failed to completely block the c totoxicity of the ethyl acetate extract, these results su ggest that the ethyl acetate extract might be able to retard th e cell cycle progression of Jurkat T cells. As shown in F g. 5C, the expression level of Bcl-2, which was easily d tectable in the continuously growing Jurkat T cells (JT/ N eo), did not change after the treatment, and the transfectant o the Bcl-2 gene (JT/Bcl-2) was able to express significantly in creased level of the Bcl-2 protein. Under these conditions, th e ethyl acetate extract-induced apoptotic events, such as th e mitochondrial cytochrome c release, caspase-3 activation, a d subsequent PARP degradation, were completely abrogated. T nese results demonstrate that mitochondrial cytochrome c r lease, activation of caspase-3, and subsequent PARP d gradation, which can be suppressed by Bcl-2, are critical s eps in executing the extract-induced apoptosis in the J rkat T cells. In addition, these results indicate that the a optotic cell death induced by the ethyl acetate extract f om *P. japonica* is negatively regulated by Bcl-2 through i s suppressive role against mitochondrial cytochrome c r lease.

In conclusion, the current study demonstrates that the c totoxicity of the ethyl acetate extract from *P. japonica* t ward Jurkat T cells is due to induced apoptosis, a companying mitochondrial cytochrome c release, caspase-9 a tivation, caspase-3 activation, and cleavage of PARP, a d further indicates that *P. japonica*-induced apoptosis is r ediated by a cytochrome c-dependent caspase-3 activation p athway that can be negatively regulated by Bcl-2.

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