ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2017 Vol. 27. No. 8. 951~957 DOI : https://doi.org/10.5352/JLS.2017.27.8.951

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Machilus Thunbergii Water Extract Induces Cytotoxic Effect against Human Acute Jurkat T Lymphoma

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Received April 24, 2017 / Revised June 2, 2017 / Accepted June 8, 2017

To understand the cytotoxic activity of *Machilus thunbergii*, which has been used as a traditional oriental medicine, the mechanism underlying the cytotoxic effect of its extract on human acute Jurkat T cells was investigated. The methanol extract of roots (3 kg) of *M. thunbergii* was evaporated, dissolved in, and then extracted by water. The water-extracted active substance was designated MTWE. When Jurkat T cells were treated with MTWE at concentrations of 0, 25, 50, and 100 μg/ml, the apoptotic phenomenon of cells accompanying several subsequent biochemical reactions, such as mitochondrial cytochrome c release, activation of caspase-3, and ICAD degradation, was detected in the Jurkat T cells. Moverover. the expression of Bcl-xL, which is a suppressor for mitochondrial cytochrome c release pathway, was reduced in the Jurkat T cells. As DUSP6, a growth suppressor of cancer cells, ranged from 0, 25, 50, 100 μg/ml of MTWE, the expression level was elevated in the Jurkat T cells. The apoptotic morphological change of the nuclei was observed by DAPI staining. Although the potential involvement of the other factors and DUSP6 is currently being investigated in more detail, these findings support the notion that MTWE is able to achieve the apoptosis of Jurkat T cells, and it seems that MTWE is useful as a method of evaluating a chemotherapeutic agent or tonic materials for human acute leukemia.

Key words : Apoptosis, cytotoxicity, Jurkat T cells, Machilus thunbergii

Introduction

Cancer is a diverse collection of life-threatening diseases that is caused by abnormal and invasive cell proliferation. For a normal cell to evolve into a disease-causing cancer is a lengthy process. Most emerging cancers are likely to be eliminated by the immune system before they are detectable or cause any symptoms. Only a minority of cancers defeat the immune system and progress to cause the diseases that are so feared. Cancers of immune system cells are known as leukemias when they involve circulating cells. T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological cancer that is mainly diagnosed in children and arises from the malignant transformation of T cell progenitors [17]. In treating leukemias, physicians resort to surgery, radiation, bone marrow transplantation and cytotoxic drugs, sometimes referred to slash, burn, and poison. Although these treatments give remission of cure to some patients, more often they are limited by the incomplete elimination of cancer cells and the deleterious side-effects such as reduced intellectual capacity, osteonecrosis and growth deficiencies, infertility, and an increased risk of secondary tumor development later in life. Therefore, integration of novel targeted therapies into contemporary T-ALL treatment protocols will be required to increase the quality of life for pediatric T-ALL survivors [2]. Natural products have played a significant role in drug discovery and specifically in the development of new tonic agents; more than 79.8% of the anticancer drugs introduced from 1981 to 2008 were natural products, semi-synthetic analogs, or synthetic compounds based on natural-product pharmacophores [3].

Machilus thunbergii belongs to the Lauraceae family and is one of the most commonly distributed in Asia. The bark of *M. thunbergii* SIEB. *et* ZUCC. (Lauraceae) has been used as a folk medicine for the treatment of leg edema, abdominal

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pain and abdominal in Korea [6]. It has been reported that compounds such lignans, alkaloids, flavonoids, butanolides, essential oils, and machilin derived from *M. thunbergii* have marked anti-oxidative activity with hepatoprotective, anti-bacterial acivities [5, 19, 21], anti-inflammatory activities in RAW 264.7 cells [18] and neuroprotective activity against glutamate-induced neurotoxicity [13], *in vitro* osteoblast differentiation [11]. Magnolol and honokiol, a phenolic component of water extracts of the stem bark of *Magnolia officinalis* has multiple pharmacological effects [8, 10]. Moreover, an ingredients of this medicinal herb has been reported to have anti-oncogenic properties against A549 cells, HeLa, B16F10 metastatic melanoma cells, F9 mice teratocarcinoma cells and human promyelocytic leukemia HL-60 cells [6, 16]. Targeting specific molecules is a promising cancer treatment because certain types of cancer cells are dependent on specific oncogenes. Although there have been studies on the anti-cancer activity of *M. thunbergii*, however, its activity in T acute lymphoblastic leukemia (T-ALL) is still unclear. Therefore, in the present study, we investigated a role for the anti-cancer activity of *M. thunbergii* extract using biochemical assay in the human acute leukemia, Jurkat T cells of T-ALL cell lines.

Materials and Methods

Sample preparation from *M. thunbergii*

M. thunbergii was purchased from Hyun-dae Pharmacological Company (Busan, Korea). A sample has been deposited in the author's laboratory. The dried roots (3 kg) from *M. thunbergii* were extracted with 100% methanol. The methanol extract was evaporated (295.2 *g*), resuspended in water, and then freeze-dryed by freezer. The cytotoxic compound was designated as MTWE.

Cell culture

The human T cell lymphoma line, Jurkat T E6.1 cells were obtained from Albert A Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA). The Jurkat T E6.1 cells were grown in RPMI1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin. Male Balb/c mice were purchased from Samtako, Inc. (Osan, Korea). Mice used in all experiments were 12 weeks old. These mice were housed in a specific pathogen-free facility with appropriate temperature and humidity and allowed free access to food and water. The mice for this study (DEU-R2013-002) were approved by the Institutional Animal Care and Use Committee at Dong-eui University. Mice were killed by cervical dislocation, and spleens were aseptically removed and stripped of fat. Single-cell suspensions were obtained by grinding the spleens with a syringe plunger against a fine steel mesh. Erythrocytes were lysed with ammonium chloride haemolysis buffer (0.8% NH_4Cl with 0.1 mM EDTA) and then washed twice in complete RPMI-1640 Medium. Splenocytes (5×10^5 cells/well) were plated in triplicate in 96-well culture plates and cultured in RPMI-1640 Medium supplemented with 5% foetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity assay with Jurkat E6.1 T cell

Cytotoxicity of MTWE against Jurkat T cell was determined by Cell Titer 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, USA). Briefly, the cells (5×10^5) were added to a serial dilution of the MTWE in 96-well plates. After 24 hr, 5% MTS was added in a 96-well plate and incubated for 2 hr before reading at a wavelength of 490 nm by a microplate reader (Bio-Rad, USA). Jurkat T cells (5×10^5 cells/well) were treated with phytohemagglutinin (PHA, 12.5 μg/ml, Sigma Aldrich, St. Louis, MO, USA) to stimulate T cells cytotoxicity in the absence or presence of MTWE and incubated in 96-well culture plates (Corning, NY, USA) for 24 hr.

Immunoblotting

Cells pretreated with or without MTWE were lysed with lysis buffer [137 mM NaCl, 15 mM EDTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM 3-N-morpholino-propanesulfonic acid (MOPS, Sigma, USA), and 2.5 μg/ml proteinase inhibitor E-64, pH 7.2]. Protein concentration was measured by BCA (Peirce, USA). After 12% SDS-PAGE, the samples were transferred onto immobilon-P membranes. The membranes were soaked in a blocking solution (5% skim milk and 0.2% Tween 20-PBS) for 1 hr, and then incubated with anti-cytochrome c (Pharmingen), anti Bcl-xL, anti-caspase-3, anti-DUSP6, anti-ICAD and anti-β-actin (Santa Cruz Biotechnology, Inc., USA). After being washed with Tween 20-PBS, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 hr. Specific bands were visualized by an ECL method (ECL+Amersham Biosciences, Arlington Heights, IL, USA). The relative amount of cytochrome c, caspase-3,

Bcl-xL, DUSP6 and ICAD compared with that in the control was calculated by measuring the band density of cytochrome c, caspase-3, Bcl-xL, DUSP6 and ICAD and normalized to β-actin density.

DAPI staining

Cells were washed with PBS and were treated with 95% ethanol for 1hr at 4℃ as a fixative. After washing the sample, RNase (12.5 μg) in 1.12% sodium citrate buffer (pH 8.45) was added at 37°C for 30 min. MTWE-treated jurkat T cells were stained with DAPI (4 μg/ml) in 100% methanol for 15 min at 37°C and observed under the microscope with ultraviolet (UV) excitation at 300-500 nm (Microphot-FX, Nikon, Tokyo, Japan). Cells with nuclei that contained clear condensed chromatin or cells with fragmented nuclei were scored as apoptosis.

Statistical analysis

For statistical analysis, the ex vivo and in vitro assays were performed independently at least three times. The P values were determined through one-way ANOVA with less than 0.05 considered to be statistically significant. The P-values are represented as an asterisk (*) or (**).

Results and Discussion

MTWE has the cytotoxicity against Jurkat T cells

We tested cell apoptosis in Jurkat T cell stimulated with MTWE. Cells (5×10^5) treated at concentration ranging 0, 25, 50, 100 μg/ml MTWE were incubated for 24 hr and carried out with MTS assay. The cells (5×10^5) were incubated with MTWE solution containing 0, 25, 50, 100 μg/ml concentration in 96-well plate for 24 hr. The MTWE (25.0 μg/ml) treated-cells showed the cell viability about 95% and the MTWE (100.0 μg/ml) treated-cells showed the cell viability less than 50% (Fig. 1A). To see whether MTWE suppresses T cell proliferation, Jurkat T cells were treated with PHA in the presence or absence of MTWE. As shown in Fig. 1A, MTWE inhibited T cell proliferation. Splenocytes (5×10^5) were incubated with MTWE containing 0, 25, 50, 100, 500 μg/ml concentration for normal cell cytotoxicity for 24 hr. The MTWE (25, 50, 100 μg/ml) treated-splenocytes showed the cell viability similar to control and the MTWE (500 μg/ml) treated-cells showed the cell viability less than 87%(Fig. 1B). This result suggested that no cytotoxicity showed at concentration ranged from 0, 25, 50, 100 μg/ml MTWE. This means that MTWE induces the apoptosis of Jurkat T cells. Although it has been reported that *M. thunbergii* has been used for tonic materials or fork remedies in several countries including Korea [6], but little is known that biochemicals in *M. thunbergii* shows potential cytotoxicity against leukemic cells, and especially signaling pathways during apoptosis is still unknown. In this research, we have first demonstrated that a partial extracted phytochemical ingredient des-

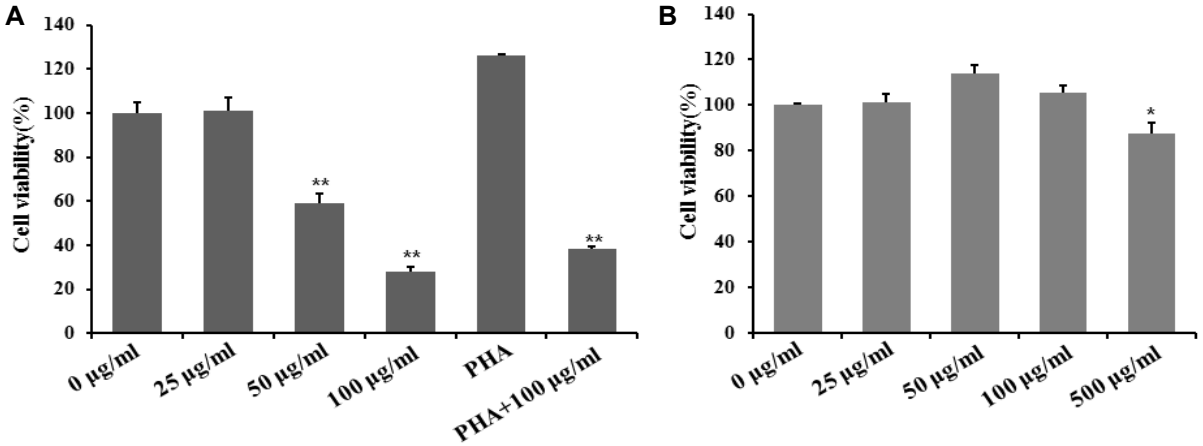

Fig. 1. Effect of MTWE on cell viability in Jurkat T cells and splenocytes. MTWE shows cytotoxicity dose-dependently in Jurkat T cells. Continuously growing Jurkat T cells (5×10^5 cells/well) (A) and splenocytes (5×10^5 cells/well) (B) were incubated with indicated concentrations of MTWE in a 96-well plate for 24 hr and further incubated with MTS for 4 hr. Jurkat T cells (5×10^5 cells/well) were treated with PHA (12.5 ug/ml) in the absence or presence of MTWE and incubated in 96-well culture plates for 24 hr. The cells were sequentially processed to assess the colored formazan crystal produced from MTS as an index of cell viability. Data were given as means of values from three independent assays. Level of significance was identified statistically (*, $p<0.05$; **, $p<0.01$) using ANOVA test.

ignated as MTWE from *M. thunbergii* induces human acute Jurkat T cell apoptosis.

Apoptosis pathway triggered by MTWE is involved in mitochondrial cytochrome c release and caspase-3 activation

We investigated the apoptosis mechanism by utilizing dose-dependent treated Jurkat T cells stimulated by MTWE to find molecular mediators. Significant differences from antibody reaction related to mitochondrial cytochrome c release pathway [14] were detected in control cell compared to MTWE-treated cells. Release level of mitochondrial cytochrome c to cytosol was increased in cells incubated with 0, 25, 50, 100 μg/ml of MTWE (Fig. 2A, Fig. 2B). Subsequently, released cytochrome c is related to switching caspase 3, one of downstream partners of cytochrome c related to apoptotic pathway. The amount of caspase 3 was increased in the range of 0, 25, 50, 100 μg/ml of MTWE in Jurkat T cells (Fig. 2C, Fig. 2D). In the present results, in Jurkat T cells exposed to MTWE, mitochondrial cytochrome c release and activation of caspase-3 were detected. Several reports suggest that caspase-3 can be activated through active caspase-8 in turn can cleave Bid, leading to cytochrome c release from mitochondria [12] and setting up a self-amplification loop to amplify caspase-9. Anyway, these findings support the notion that apoptotic signaling of MTWE in Jurkat T cell is regulated by mitochondrial cytochrome c release pathway.

MTWE decreases protein levels of B-cell lymphoma-extra large (Bcl-xL)

Anti-apoptotic Bcl-2 protein family including Bcl-2 itself and Bcl-xL acts as key regulators in the intrinsic or mitochondrial apoptosis pathway [22]. To determine the mechanism involved, we examined the effect of the MTWE on Bcl-2 family anti-apoptotic signaling molecule. MTWE reduced levels of the pro-survival protein Bcl-xL under dose-dependent concentration (Fig. 3). Major function of Bcl-xL is known to suppress mitochondrial cytochrome c release [7, 20]. Therefore, these experiments suggest that MTWE is involved in facilitation the activation of mitochondria-dependent death-signaling pathway in Jurkat T cells, and subsequent cascade events.

DUSP6 is involved in apoptosis pathway triggered by MTWE

Ectopic expression of *DUSP6*/MKP-3, a pivotal negative feedback regulator of the RAS-ERK pathway, in either pancreatic or lung cancer cells resulted in the suppression of cell growth and apoptosis related to restrain oncogenic ERK signalling [4, 15]. Thus, we performed western blotting for DUSP6. As DUSP6 ranged from 0, 25, 50, 100 μg/ml of MTWE, the expression level was elevated in Jurkat T cells (Fig. 4). DUSP6 expression levels were found to be weaker in most lung cancer cell lines, and DUSP6 restoration suppressed cellular growth [15]. However, the potential involvement between the other factors and DUSP6 is currently be-

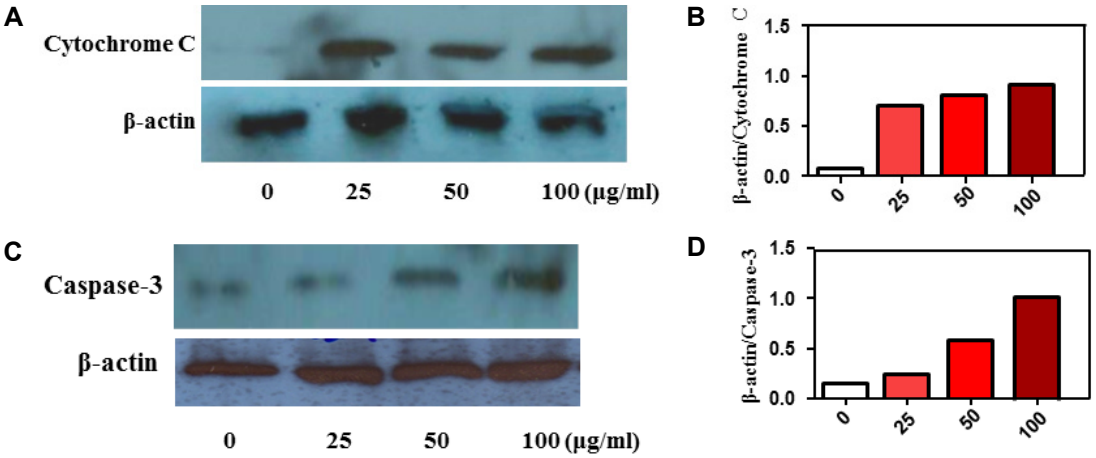

Fig. 2. Effect of MTWE on cytochrome C and caspase-3 expression in Jurkat T cells. MTWE induced-cell death is through mitochondrial cytochrome c release. (A, C) The cells (5×10^5) were incubated with indicated concentrations of MTWE for 24 hr and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 12% SDS polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in materials and methods using the ECL Western blot detection system. Cytochrome c (A), caspase-3 activation (C), and β-actin. (B, D) The relative intensity compared with the control level was normalized by the amount of β-actin (histograms).

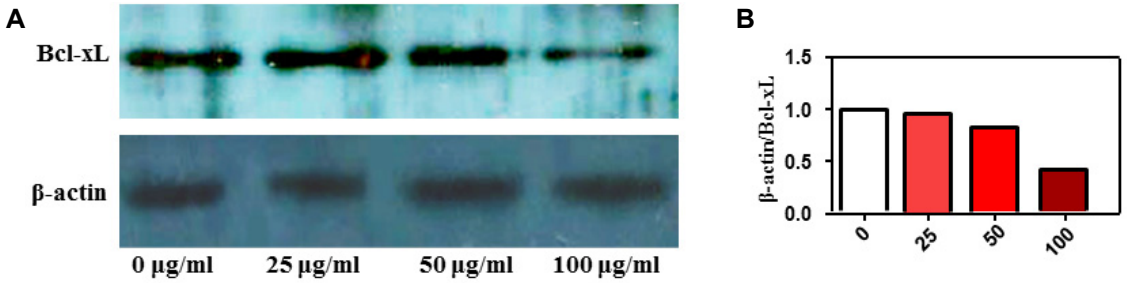

Fig. 3. Effect of MTWE on Bcl-xL expression in Jurkat T cells. MTWE facilitates the attenuation of Bcl-xL expression in Jurkat T cells. (A) Jurkat T cells (5×10^5 cells) were incubated with indicated concentrations of MTWE in microplate for 20 hr. The cells were further incubated with DMSO for 4 hr to solve the colored formazan crystal produced from MTS as an index of cell viability. (B) The relative intensity compared with the control level was normalized by the amount of β-actin (histograms).

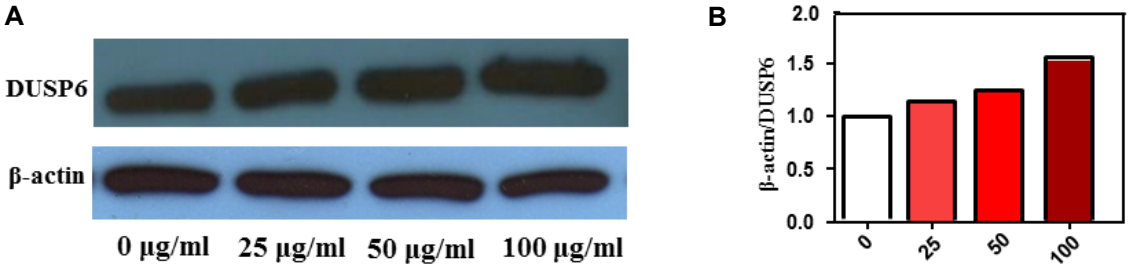

Fig. 4. Effect of MTWE on DUSP 6 expression in Jurkat T cells. MTWE facilitates the enhancement of DUSP6 expression in Jurkat T cells. (A) The cells (5×10^5) were incubated with indicated concentrations of MTWE for 24 hr and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 12% SDS polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in materials and methods using the ECL Western blot detection system. (B) The relative intensity compared with the control level was normalized by the amount of β-actin (histograms).

ing investigated in detail. Anyway, these findings support the notion that MTWE is able to carry out apoptosis of Jurkat T cells and it seems that MTWE is used as the evaluation for chemotherapeutic agent. Collectively, these results demonstrate that MTWE, a partially purified constituent from *M. thunbergii*, induces apoptosis of Jurkat T cells via mitochondria dependent pathway by release of mitochondria cytochrome c, and activation of caspase-3. These things will be helpful and useful for evaluation of its chemotherapeutic potency and tonic material.

MTWE induces apoptosis of jurkat T cells

Caspases, a family of cysteine proteases, contribute to a diverse range of functions in cell including apoptosis. Caspase 3 in particular is a key factor in canonical apoptotic signaling promoting both the cytosolic and nuclear alterations required for cellular disassembly [9]. Caspase 3 activates caspase-activated DNase (CAD) by proteolytic inactivation of the inhibitor of CAD (ICAD) to promote DNA fragmentation [9]. So, we performed Western blotting for ICAD, which is one of the downstream targets of active-caspase 3. Protein level of ICAD ranged from 0, 25, 50, 100 ug/ml of MTWE was reduced (Fig. 5A, Fig. 5B). To address the apoptotic characteristics including chromatin crescent formation, DNA fragmentation and apoptotic body formation [1], we stained jurkat T cells with DAPI. In DAPI staining, nuclear morphology of cells treated with 50 ug/ml of MTWE showed fragmented morphology and apoptotic body compared to untreated-control cells (Fig. 5C, arrow). The cells undergoing apoptosis evidently may increase when the concentration of MTWE is elevated and reaction time is prolonged. In order to elucidate the mechanisms of inhibitory effects of MTWE on jurkat T lymphoma growth, we determined the incidence of MTWE-mediated apoptosis in jurkat T lymphomas by cell cytotoxic assay, biochemical assay and DAPI staining on the basis of molecular and morphological level. The results demonstrated that MTWE-treated jurkat T cells were characteristic by typical apoptotic al-

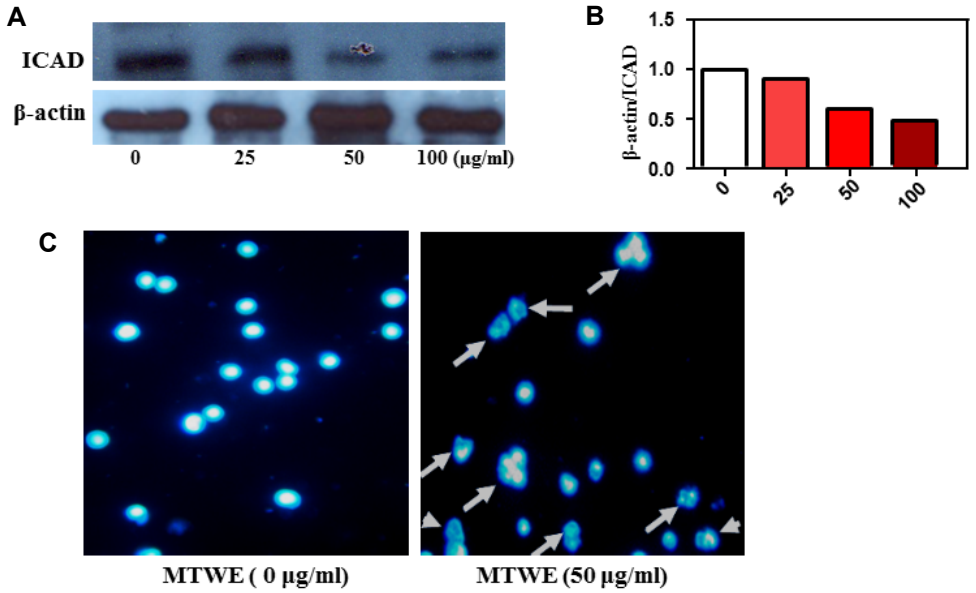

Fig. 5. Effect of MTWE on ICAD expression and apoptotic body formation (arrow) in Jurkat T cells with DAPI staining. (A) MTWE facilitates the enhancement of ICAD expression and the formation of apoptotic body in Jurkat T cells. (B) The relative intensity compared with the control level was normalized by the amount of β-actin (histograms). (C) The cells (1×10^4) were incubated with indicated concentration of MTWE for 24 hr and stained with DAPI.

terations including morphological changes by DAPI staining. Thus, the data above implicated that MTWE inhibits human acute T lymphoma growth by inducing apoptosis.

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초록 : 후박 열수 추출물의 Jurkat T 세포에서 세포사멸 효과

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후박은 전통적으로 동양의학에서 사용되어왔는데 인간 급성 백혈병 세포주인 Jurkat T 세포를 사용하여 후박의 세포독성 관련 기작을 알아보았다. 후박 뿌리(3 kg)를 메탄올로 추출, 증류한 후 내용물을 물에 녹여 동결 건조 후 사용 하였다. 그 활성물질을 MTWE이라 명명하였다. MTWE을 0, 25, 50, 100 μg/ml의 농도로 처리하고 세포사멸 과정을 보았다. 즉, mitochondria cytochrome c 방출, caspase-3의 활성화 및 ICAD 분해를 관찰하였다. 더욱이, mitochondria cytochrome c 방출 억제자인 Bcl-xL이 발현이 감소되는 것을 Jurkat T 세포에서는 확인하였다. 이러한 결과는 MTWE가 mitochondria 신호전달 과정을 통해서 세포사멸을 유도 한다고 할 수 있다. 또한, MTWE를 0, 25, 50, 100 μg/ml 처리에 대한 암세포 성장억제인자인 DUSP6가 증가되는 것을 확인하였고 핵의 apoptotic morphology 변화를 DAPI를 통해 관찰할 수 있었다. 비록 DUSP6와 다른 관련인자들간의 관련성을 찾아야 하지만, 이상의 결과는 MTWE가 T세포에 의한 급성 백혈병을 조절하는데 이용 될 수 있다는 것의 의미한다.