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Effects of gliotoxin isolated from marine fungus *Aspergillus sp.* on apoptosis in human cervical cancer cell and human chondrosarcoma cell

Graduate School of Chosun University

College of Natural Sciences

Nguyen Van Tinh

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해양진균 *Aspergillus sp.* 로부터 분리된 gliotoxin 의 인간 자궁경부암 및 연골육종 세포에서의 세포사멸 효과

2012 년 8 월 24 일

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LIST OF ABBREVIATION

Apaf-1	Apoptotic protease-activating factor-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
BH1-4	Bcl-2 homology domain 1-4
Caspase	Cysteine-dependent aspartate-directed peoteases
Cyt c	Cytochorome c
DAPI	4,6-diamidi-no-2-phenylindile
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
FBS	Fetal bovine serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PI	Propidium iodide

Rnase A	Ribonuclease A
RT-PCR	Reverse transcription polymerase chain reaction
TAE	Tris-acetate
TBST	Tris-buffered saline-Tween 20

초 록

해양진균 *Aspergillus sp.* 로부터 분리된 gliotoxin의 인간 자궁경부암 및 연골육종 세포에서의 세포사멸 효과

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Gliotoxin은 항암활성을 포함한 다양한 생리활성을 도출하는 해양 진균 *Aspergillus* sp.의 이차 대사산물이다. 반면 Gliotoxin의 자궁경부암 (HeLa) 및 연골육종 (SW1353) 세포에 대한 세포사멸 작용기전은 여전히 밝혀지지 않고 있다. 따라서 본 연구에서는 해양 진균에서 분리한 Gliotoxin에 대한 이화학적 특성과 두 종의 암세포주에 대한 세포독성, flow cytometry 분석 및 DNA fragmentation 등 방법으로 세포사멸 효능을 확인하였으며, 아울러 단백질과 유전자 레벨에서의

세포사멸 관련 인자, caspase-3, caspase-8, caspase-9, Bax, Bcl-2, p53 cytochrome c 등을 Western blot 와 RT-PCR 분석방법으로 작용기전을 규명할 수 있었다. 그 결과 Gliotoxin은 두 세포에서 모두 Bcl-2 발현의 감소와 Bax, cytochrome c, caspase-3, caspase-8, caspase-9 발현을 활성화 또는 증가함으로써 세포사멸을 유도시키는 작용기전을 확인하였다.

I. INTRODUCTION

A. Activation and inhibition of apoptosis

Cells undergo death by two major mechanisms is necrosis or apoptosis (Lee et al., 2011c). Apoptosis, a major form of cell death, is characterized by a series of stereotypic morphological and biochemical features, such as a loss of cell volume, membrane blebbing, nuclear collapse, chromatin clumping, and internucleosomal DNA cleavage (DNA fragmentation) (Susan et al., 2007; Kim et al., 2010). DNA fragmentation in apoptotic cells is the result of the activation of deoxyribonucleases that cleave the genomic DNA at regular intervals. Programmed cell death plays critical roles in a wide variety of physiologic processes during fetal development and in adult tissues (Oikawa et al., 2008). Defects in apoptotic cell death contribute to neoplastic diseases, by preventing or delaying normal cell turnover, thus promoting cell accumulation. Defects in apoptosis also facilitate tumor progression, by rendering cancer cells resistant to death mechanisms relevant to metastasis, hypoxia, growth factor deprivation, chemotherapy, and irradiation. Apoptosis is a distinct form of cell death characterized by nuclear and cytoplasmic condensation, DNA fragmentation and externalization of membrane-associated phosphatidylserine (Park et al., 2008). The evidences were gradually accumulated that many cancer chemothera-peutic agents killed the cancer cell by inducing apoptosis. These mechanisms include factors that lead to perturbation of the mitochondria leakage of cytochrome c or factor that directly activate members of the death receptor family (**Fig.1**). Thus, identifying mode of cell death has been recognized as a novel strategy for the screening of anti-cancer drugs (Karagozlua et al., 2010; Lee et al., 2011b).

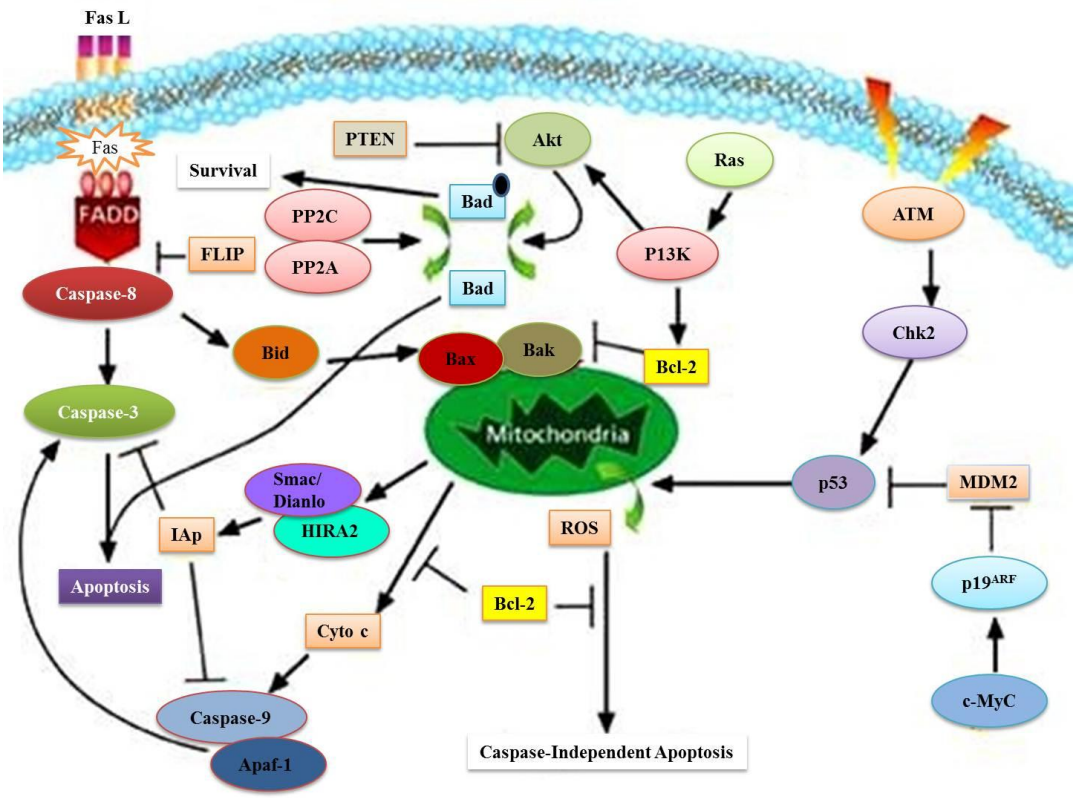


Fig.1. Activation and inhibition of apoptosis (http://www.sigmaaldrich.com)

B. Caspases: the executioners of apoptosis

Classes of cysteine proteases, known as caspases, produce these biochemical and morphological changes by selectively cleaving a number of structural and regulatory proteins at specific aspartate residues. Tumor cells do not undergo apoptosis easily because they have defects in their ability to activate the death-signaling pathway. Thus, one effective cancer therapy is to activate the tumor cell's apoptosis pathway. There are at least two signaling pathways involved in apoptosis. Mitochondria play a decisive role in the apoptotic pathway mediated by certain agonists (Slee et al., 1999). Disruption of the inner, outer mitochondrial membrane and opening of the mitochondrial permeability transition pore, which is regulated by members of the Bcl-2 family as well as the redox and energy state of the cell. Result is a collapse of the mitochondrial membrane potential and in the exit of soluble proteins, such as cytochrome c and apoptosis-inducing factor (Eiichi et al., 2005). The translocation of cyt c from the mitochondrial intermembrane to the cytosol and the subsequent activation of caspases have been identified to be the major steps in the apoptotic process (Bratton et al., 2001). After the induction of apoptosis by many different stimuli, holocytochrome c, which normally resides exclusively in the intermembrane space of mitochondria, is released into the cytosol following the opening of the mitochondrial permeability transition pore. Bcl-2 family proteins are subdivided into three groups, depending on the conservation of Bcl-2 homology (BH) domains and function (**Fig.2**). The multidomain anti-apoptotic Bcl-2 proteins contain BH domains BH1 to BH4 and function to inhibit apoptosis by binding to pro-apoptotic Bcl-2 family members (Basu et al., 1998; Liu et al., 2008). Bax is a pro-apoptotic member of the Bcl-2 family involved in triggering apoptosis via mitochondria (Altznauer et al., 2004). Bax and Bak are the two main members of the multidomain group, containing BH domains BH1 to BH3. These proteins are primarily responsible for the permeabilization of the mitochondrial outer membrane, if

their activity is not suppressed by anti-apoptotic Bcl-2 family members. These proteins function as upstream sensors of signaling pathways and convey to other Bcl-2 family proteins the signals to initiate apoptosis (Mohammed, Cell engineering. Vol.4). Various stimuli, including cytokines, anticancer drugs, growth factor deprivation, radiation damage and so on can cause a cell to undergo apoptotic cell death.

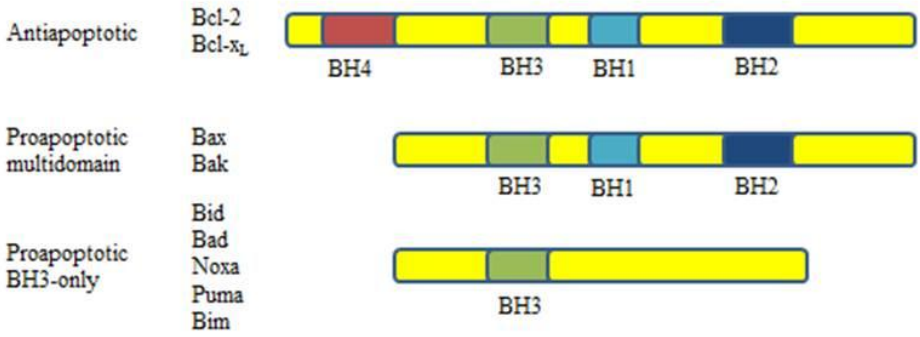


Fig.2. The Bcl-2 family (Cooper et al., 2009)

Caspases, the interleukin-1β-converting enzyme family proteases, are highly homologous to caenorhabditis eleganscell death gene CED-3. Fourteen caspases have been identified so far, all of which share some common properties: they are all aspartate-specific cysteine proteases; their precursors are all zymogens known as procaspases (Pablo et al., 2004; Pop et al., 2009). Based on their homology in amino acid sequences, caspases are divided into three subfamilies, as shown in **Table 1**.

Table 1. Subfamily members of caspase family (Fan et al., 2005)

Subfamily	Role	Members
I	Apoptosis activator	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Apoptosis executioner	Caspase-3
		Caspase-6
		Caspase-7
III	Inflammatory mediator	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

Three-Dimensional structure of sature caspases, X-ray crystal structures have been determined for mature caspase-1 (Walker et al., 1994; Wilson et al 1994; Cohen et al., 1997), caspase-3 (Rotonda et al., 1996; Riedl et al., 2001), caspase-8 (Blanchard et al., 1999; Watt et al., 1999; Xu et al., 2001) and caspase-9 (Renatus et al., 2001), all bound to either synthetic substrate-based peptide inhibitors or natural inhibitors. The overall architecture of caspase-3, caspase-8 and caspase-9 is similar and consists of two heterodimers composed of a large and small subunit (**Fig.3**).

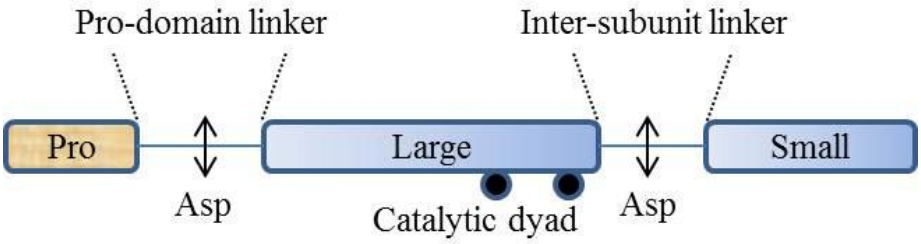


Fig.3. Structure of caspase (Harmeet et al., 2010)

Caspases can cleave other caspases thereby generating an intracellular protease cascade leading to cellular demise. Caspases also cleave a variety of substrates involved in activities that lead to dismantling of the cell such as disruption of organelle function and cytoskeletal and nuclear disassembly, resulting in the typical hallmark features of apoptotic cell death. However, one should also be aware that non-apoptotic and caspase-independent ways of cell death exist (Bari et al., 2003; Fiers et al., 1999; Denecker et al., 2001). Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3. The latter can be activated by caspase-3, caspase-8 and caspase-9. The downstream substrates of caspase-3 include procaspase-3, procaspase-9, inhibitor of caspase-activated deoxyribonuclease (Yang et al., 2006; Park et al., 2008).

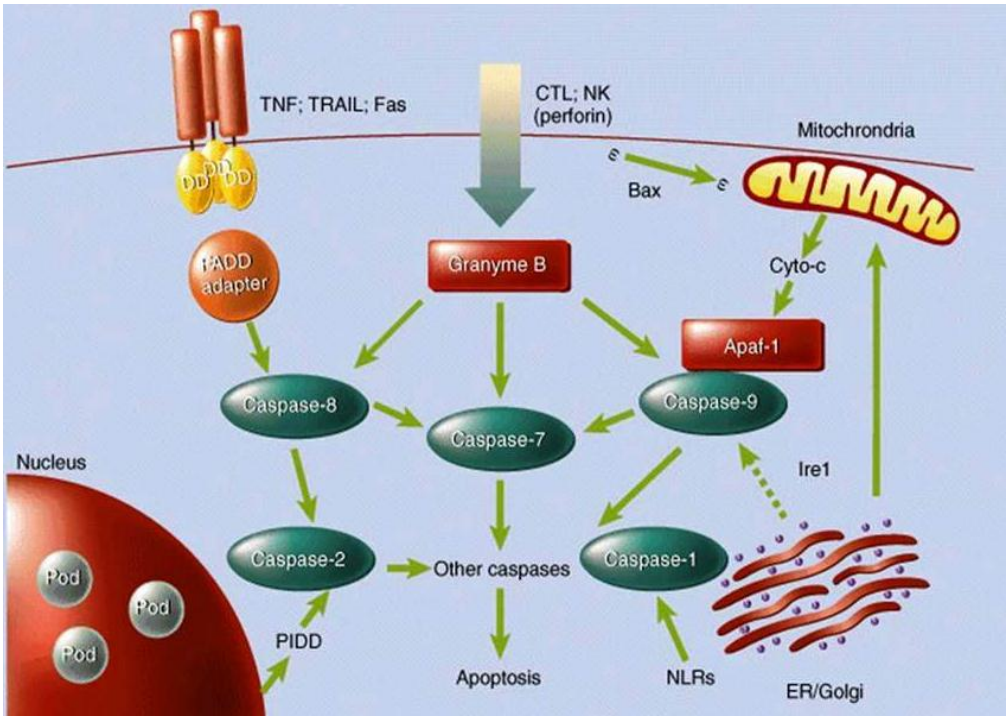


Fig.4. The major pathways for caspase activation in mammalian (John et al., 2010)

In the cytosol, cytochrome *c* binds and activates Apaf-1, allowing it to be associated with and activate procaspase-9 (Garland et al., 1998). Released cytochrome c associates with Apaf-1 and induces its oligomerization, which forms complexes with inactive procaspase-9, inducing caspase-9 activation (Wu et al., 2002; Cain et al., 1999; Adrain et al., 1999). Active caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3 (**Fig.4**). Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3. The tumor suppressor p53 induces apoptosis via several mechanisms (Karagozlua et al., 2010). Bax, the pro-apoptotic member of Bcl2 family, which is found to be upregulated in number of systems during p53-mediated apoptosis, operates the apoptotic machinery under the transcriptional regulation of p53 (Haupt et al., 1995). Translocation of Bax to mitochondria results in the release of cytochrome c into cytosol. p53 is one of the major regulators of the apoptotic process in response to DNA damage and environmental stress and induces cell cycle arrest and apoptosis (Wang et al., 2011; Haupt et al., 2003).

C. *Aspergillus sp.* and gliotosin

Marine-derived fungi have proved to be a promising source of bioactive metabolites and a growing number of marine fungi have been reported to produce novel bioactive secondary metabolites (Wei et al., 2010; Shao et al., 2011). *Aspergillus* species are filamentous saprophytic fungi that can be found in almost all aerobic environments (**Fig.5**). They have been found to produce a wide range of complex metabolites, some of which have important commercial application potentials. Some *Aspergillus* are on the generally recognized as safe list of the Food and Drug Administration (FDA) in the United States (Luo et al., 2011). Several fungal metabolites

isolated from *Aspergillus sp.* have been shown to exhibit antitumor, anti-inflammator, induced cytotoxicity and antibacterial activity (Lee et al., 2011a)

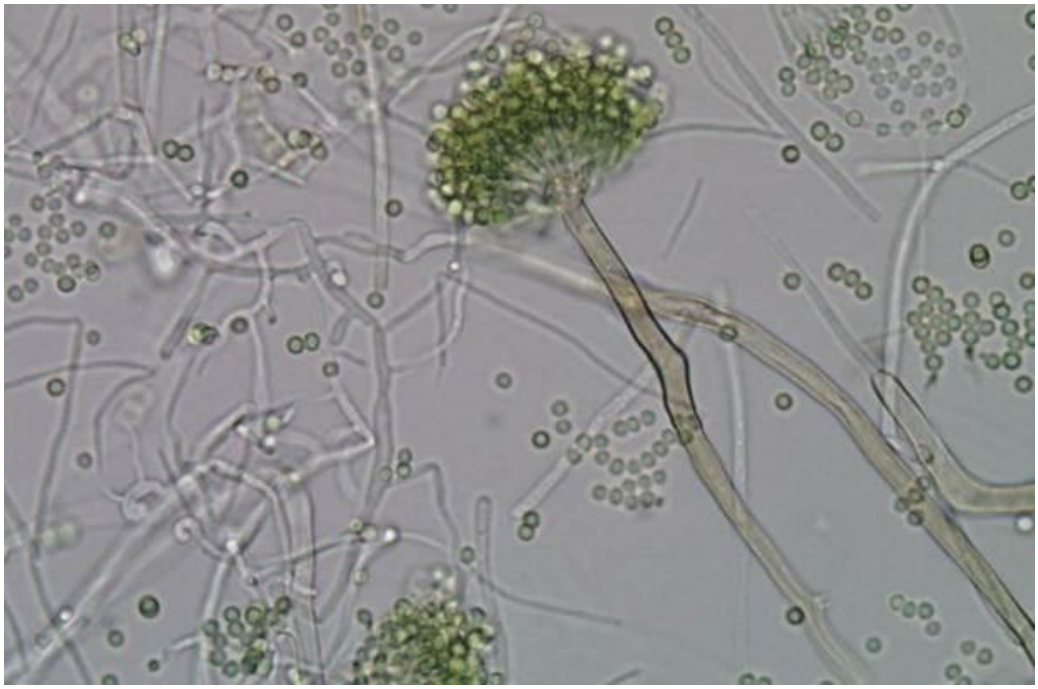


Fig.5. Morphology of *Aspergillus sp.* (http://www.emlab.com)

Gliotoxin is one of the secondary metabolites produced by a number of *Aspergillus*, *Gliocladium* and *Penicillium* species, is a tricyclic alkaloid, a member of the epipoly-thiodioxopiperazine family (Axelsson et al., 2006; Waring et al., 1996; Nieminen et al., 2002a; Comera et al., 2007). Some members of this family of compounds have attracted attention because of their biological properties and role in disease. Sporidesmin is responsible for producing facial eczema in sheep, and gliotoxin has been linked to the pathogenesis of aspergillosis. Gliotoxin is suspected to be a significant actor in aspergillosis pathology for three main reasons. Firstly, it has multiple cellular activities that are mainly immuno-suppressive. Secondly, it has been shown to be produced in situ during animal aspergillosis. Thirdly, it has been shown that gliotoxin could exacerbate experimental aspergillosis in mice. It possesses a spectrum of biological activities including antibacterial and antiviral activities, and it is also a potent immunomodulating agent (Nicholas et al., 2003). Gliotoxin could positively exacerbate aspergillosis by inducing leukocyte apoptosis and inhibiting the respiratory burst during mycelial development, but also by inhibiting phagocytosis either at the initial stage of the infection during spore germination or at a later stage by facilitating conidia dissemination. The immunosuppressive properties of gliotoxin have considerable therapeutic potential, as has been shown in several transplantation models. These applications depend on the observation that different cell types have variable susceptibilities to gliotoxin, and cells of the immune system are particularly sensitive. For the full potential of gliotoxin to be realised, it is important that its mechanisms of action are determined, and any effect of gliotoxin on cellular function could give clues as to these mechanisms. Gliotoxin is also an inducer of apoptotic cell death in a number of cell types (Kweon et al., 2003; Xiaoming et al., 2000; Anselmi et al., 2007). It has been found to be associated with some diseases attributed directly or indirectly to fungal infections.

Here, for the first time, this study demonstrated that gliotoxin actively induced apoptosis and reduced proliferation of HeLa and SW1353 cells. The pathways involved in the activation of caspase-3, caspase-8 and caspase-9. These results are significant in that they provided a mechanistic framework for further exploring the use of gliotoxin as a chemotherapeutics for human cervical cancer cells and human chondrosarcoma cells.

II. MATERIALS AND METHOD

A. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). Hela and SW1353 cells were obtained from American Type of Culture Collection (Manassas, VA, USA). Methanol, dichloromethane, chloroform, acetone, hexane and ethyl acetate (95%, SK chemicals) were used as extract solvents in this study. For NMR spectroscopy (^{1}H 400MHz, ^{13}C 100MHz, JEOL JNM-ECP 400 NMR spectrometer), was used for dissolved compounds. Ribonuclease A Solution (R4642) and proteinase K: (P6556) were purchased from Sigma-Aldrich. Primary and secondary antibodies used for Western blot analysis were caspase-3 (sc-136219), caspase-8 (sc-70501), caspase-9 (sc-81663), Bax (sc-70407), Bcl-2 (sc-7382), cyt c (sc-13561), p53 (sc-56179), β-Actin (sc-130656), goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG1-HRP (sc-2060), and purchased from Santa Cruz Biotechnology, INC (CA, USA). Other chemicals and reagents used were of analytical grade.

B. Method

1. Preparation of *Aspergillus sp*.

The fungal strain (stock no. YL-06) was isolated from the surface of the marine brown alga collected in the Ulsan City, Korea and identified as an *Aspergillus sp.*. The fungal strain was

stored in the 10% glycerol YPG (Yeast extract-peptone-glycerol) medium at -75^{0}C. The further culture for investigation was completed on YPG medium from 10 mL to large scale (1.0 L and 10.0 L).

2. Isolation of gliotoxin

The fungus was cultured (30.0 L) for 30 days at 29^{0}C in YPG medium. The culture broth and mycelium were separated, and the filtered broth was extracted with ethyl acetate to provide the broth extract (1.58 g). The broth extract extracted with ethyl acetate to provide the broth extract (1.58 g), which was fractionated by silica gel chromatography (n-hexane/EtOAc) to generate six fractions. The further purification of the active fractions by ODS column chromatography (H_2O in MeOH), followed by HPLC (YMC ODS-A, MeOH), yielded compounds gliotoxin (23.0 mg).

3. Cell line and cell culture

The human cervical cancer cells (Hela) and human chondrosarcoma cells (SW1353) were purchased from American Type of Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cells were incubated under a fully humidified atmosphere and 5% CO_2 at 37^{0}C.

4. Cell viability assay

Cell viability was determined by a colorimetric MTT assay, as previously described (Lee et al., 2011c). Briefly, 24 hours after exponentially growing cells were seeded at 1×10^4 cells/well in a 96-well plate. The culture medium was changed to the experimental medium supplemented with

gliotoxin at different concentrations. After 36 hours, cells were incubated with 50 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (1 mg/mL) reagent for 4 hours. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO and the optical density was measured at 540 nm by using a microplate reader. Relative cell viability was calculated compared to the non-treated blank group. The data were expressed as means of at least three independent experiments.

5. Nuclear staining with DAPI

Cells seeded in 24-well plate were treated with different concentrations gliotoxin. After 36 hours incubation, the cells were harvested, washed in ice-cold phosphate-buffered saline (PBS) and fixed with 1 mL methanol solution at room temperature for 10 min. The fixed cells were washed with PBS and stained with a 4,6-diamidi-no-2-phenylindile (DAPI: VECTASHIELD® H-1200) solution at room temperature in the dark. The nuclear morphology of the cells was examined by fluorescent microscopy (Carl Zeiss MicroImaging GmbH, Germany).

6. Annexin-FITC V and propidium iodide staining apoptosis tests

Apoptosis tests were performed as follows to the manufacturer's manual provided with the Annexin V-FITC kit (BD Pharmingen™). Briefly, cells samples (1×10^5/ml) were washed with PBS and then resuspend cells in binding buffer (0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$). The cell pellet was incubated by FITC Annexin V and propidium iodide (100 µL binding buffer, 5 µL Annexin, 5 µL PI) at room temperature in the dark for 15 min. After add 400 µL binding buffer, the sample was measured by flow cytometry (BD FACS Calibar, Germany).

7. DNA fragmentation assay

Cells (2×10^5 per sample) were harvested, washed with phosphate buffered saline (PBS), lysed with 100 µL RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA (pH 8.0), and 0.1% SDS) supplemented with proteinase K (10 mg/mL) and digested for 1 hour with RNase A (10 mg/mL). After two extractions with phenol and one extraction with phenol/chloroform/isoamylacohol (25:24:1), the DNA was precipitated with 0.1 volumes of 3 M ammonium acetate and 2 volumes of absolute ethanol at -20^0C overnight, and then washed with ice-cold 70% ethanol, air-dried, and resolved in 50 µL TE buffer (10 mM Tris, 1mM EDTA-Na2, pH 8.0). Ten µg of DNA was separated in a 1.5% agarose gel and visualized by ethidium bromide staining under ultra-violet light (Qu et al., 2004).

8. RNA extraction

Total RNA was extracted from Hela and SW1353 cells for 24 hours treated with and without gliotoxin. Cells in 6 cm^2 dishes were lysed with 200 µL of TRIzol® reagent for each dish and the lysate was passed through a pipette several times. Cell lysates were transferred to eppendorfs and incubated 5 min at room temperature. Incubation was followed by adding 100 µL of chloroform to each eppendorf and vortexing. Eppendorfs were centrifuged at 13,000×g for 15 min at 4^0C. After centrifugation, colorless supernatant phase was transferred to a new microtube carefully without mixing with lower protein phase. The RNA in the aqueous phase was precipitated by mixing with isopropanol at the ratio of 1:1, incubation for 10 min at room temperature and centrifugation at 13,000×g for 10 min at 4^0C. Supernatant was discarded and RNA pellet was washed with 700 µL of 75% ethanol, followed by centrifugation at 13,000×g for 15 min at 4^0C. Following removal of ethanol, RNA pellet was suspended in DEPC-treated water and incubation at

55^0C for 5 min. Dissolved RNA pellet was kept at -20^0C for further experiments.

9. Reverse-transcriptase polymerase chain reaction

RT-PCR was performed to check specific mRNA expression in differentiated cells. 1 µg of total RNA was mixed DEPC-treated water to reach the total volume of 19 µL in Maxime PreMix Kit, was reverse transcription reaction 45^0C for 30 min and inactivation of RTase 94^0C for 5 min. Resulted mixture containing complementary DNA (cDNA) was kept at 4^0C for further experiments. cDNA synthesized from RT-PCR was used as a template for normal PCR. PCR reaction mixture was prepared by mixing the chemicals and incubation conditions by a thermal cycler. Sequences of the gene specific primers used in these reactions were shown in **Table 2** (Lee et al., 2011c; Gupta et al., 2001). PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining under ultra-violet light.

Table 2. Sequences of the gene specific primers

Target	Sequence	
Caspase-3	Forward	5’-CCCAGGCCGTGAGGAGTTAGC-3’
	Reverse	5’-CAGCATCACTGTAACTTGCTAATC-3’
Caspase-8	Forward	5’-CACTAGAAAGGAGGAGATGGAAAG-3’
	Reverse	5’-CTATCCTGTTCTCTTGGAGAGTCC-3’
Caspase-9	Forward	5’-GCTCTTCCTTTGTTCATCTCC-3’
	Reverse	5’-CATCTG-GCTCGGGGTTACTGC-3’
Cyt c	Forward	5’-GGAGGCAAGCATAAGACTGG-3’
	Reverse	5’-GTCTGCCCTTTCTCCCTTCT-3’
Bax	Forward	5’-TGCCAGCAAACTGGTGCTCA-3’
	Reverse	5’-GCACTCCCGCCACAAAGATG-3’
Bcl-2	Forward	5’-CGCATCAGGAAGGCTAGAGT-3’
	Reverse	5’-AGCTTCCAGACATTCGGAGA-3’
p53	Forward	5’-GCGCACAGAGGAAGAGAATC-3’
	Reverse	5’-CTCTCGGAACATCTCGAAGC-3’
GAPDH	Forward	5’-GAAGGTCGGAGTCAACGGATTT-3’
	Reverse	5’-ATGGGTGGAATCATATTGGAAC-3’

10. Western blot analysis

Hela and SW1353 cells were treated with different concentrations of gliotoxin. Cells in 6 cm^2 dishes were then washed with PBS and lysed. After 13,000×g centrifugation at 4^0C for 15 min, protein concentration was determined by BCA method. Fifty micrograms of total cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) (12% for caspase-3, caspase-8, caspase-9 and p53; 15% for Bcl-2, Bax, and cyt c), transferred onto a PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 5% skim milk in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) for 2 hours and then incubated with primary antibody (1:500 dilution) in blocking agent at 4^0C overnight. After washing with TBS-T buffer, the membrane was incubated with secondary antibody (1:5,000 dilution) for 1 hour at room temperature. Bands were visualized by enhanced chemiluminescence and LAS-4000 imaging system (FUJIFILM, Japan).

11. Measurement of total protein concentration

Total protein of cell lysate was estimated to the BCA™ Protein Assay. Protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA).

12. Statistical analysis

The results are presented as the mean ± standard deviation. Significant differences among the groups were determined using the unpaired Student's t-test. The differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

A. Extraction of gliotoxin from *Aspergillus sp.*

The fungal strain culture for investigation was completed on YPG medium from 10 mL to large scale (1.0 L and 10.0 L) for 30 days at 29^0C. The culture broth and mycelium were separated, and the filtered broth was extracted with ethyl acetate to provide the broth extract (1.58 g), which was fractionated by silica gel chromatography (n-hexane/EtOAc) to generate six fractions. The further purification of the active fractions by ODS column chromatography (H_2O in MeOH), followed by HPLC (YMC ODS-A, MeOH), yielded compounds gliotoxin (23.0 mg) (**Fig.6**).

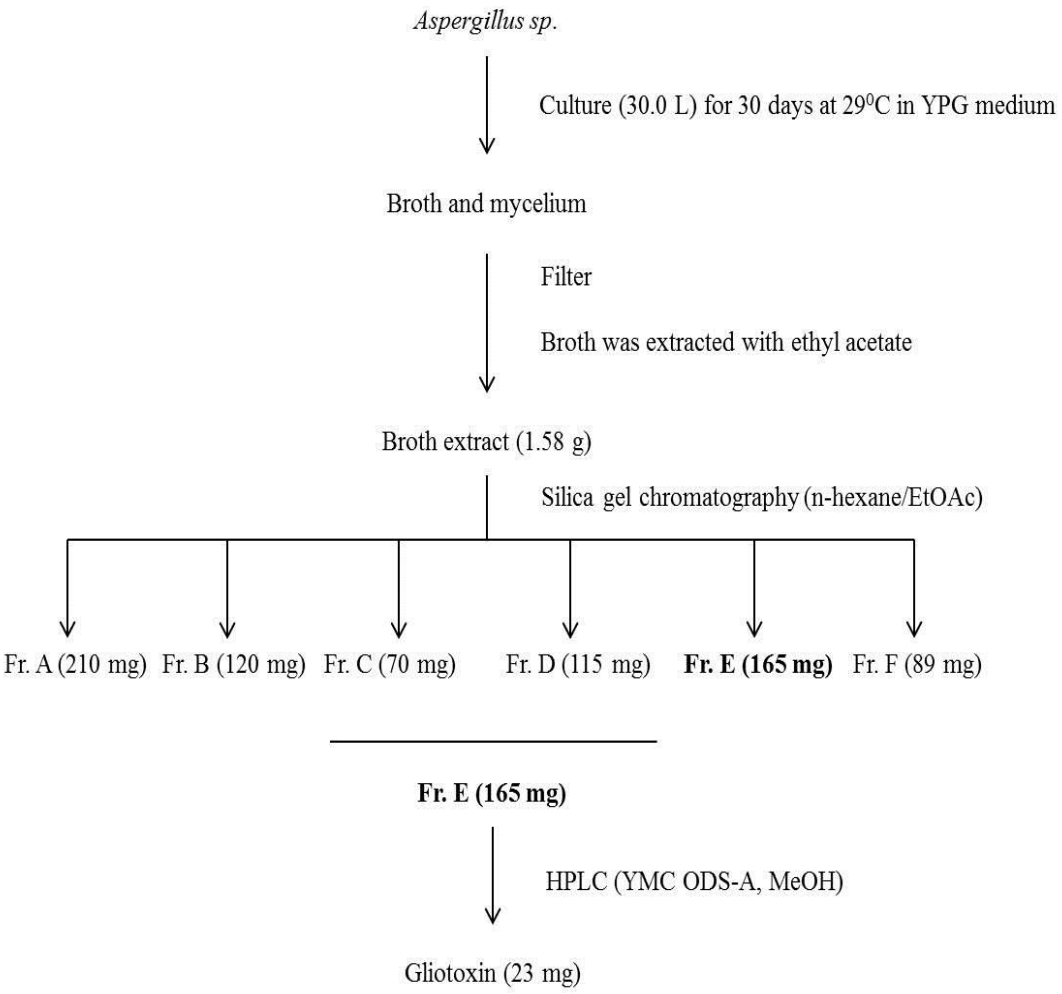


Fig.6. Isolation of gliotoxin from *Aspergillus sp.* extract

B. Elucidation of chemical structure of gliotoxin compound

Gliotoxin was obtained as white needle crystals. The UV spectrum of gliotoxin have λ_{max} at 210 and 266 nm, and with white powder for EI-MS m/z $[M]^+$ 326.0395. $C_{13}H_{14}N_2O_4S_2$ requires 326.39 (Lee et al., 2001; Nicholas et al., 2003). It exhibited negative ESI-MS ion at m/z 325 indicating a molecular weight of 326. The collision-induced dissociation MS-MS spectrum of the gliotoxin standard shows a major fragment ion at *m/z* 262, which is presumably formed by loss of S_2 from the protonated molecule (**Fig.7**). The other main fragment is seen at *m/z* 217 and 233. The ^{1}H-NMR spectrum of gliotoxin showed two sets of quartets between δ 3.44 (1H, dd, *J*=4.8) and δ 4.28 (1H, dd, *J*=9.9) which was assigned to the methylene protons of the primary alcohol fragment. Further, the doublets at δ2.96 (1H, *J*=18) and δ 3.73 (1H, *J*=18.1) were assigned to the endocyclic methylene protons at C-10. The coupling between these protons in compound gliotoxin is due to the trans-diaxial orientation. The remaining signals was assigned to the N-methyl signal at δ 3.20 and the characteristic 1,2,3-substituted aromatic ring signals at δ 5.78 (1H, d, *J*=9.9), δ 5.95 (1H, m) and δ 6.00 (1H, m) (**Fig.8**A and **Fig.8**B). The ^{13}C-NMR and DEPT spectra of gliotoxin showed 13 carbon signals including two sets of methylenes at δ 36.6 and δ 60.5, one N-methyl at δ 27.5 and two carbonyl carbons at δ 165.2 and δ 166.0 (**Fig.9**). The protonnated carbons and their bonded peotons were determined unambiguously by the HSQC expreiment. In the HMBC and HMQC spectrum of gliotoxin, the methylene protons (δ 3.44 and δ 4.28) of the primary alcohol were correlated with δ 77.2 and δ 165.2, which were assigned to the signals of C-3 and C-4, respectively (**Fig.10**A and **Fig.10**B). Other methylene protons at δ 2.96 and δ 3.73 were correlated with δ 73.1 and δ 166.0, which were assigned to the C-10a and C-1, respectively. The doublet signal at δ 4.39 was assigned to the H-5a due to the correlation with δ 75.6 (C-6) and δ 120.2(C-9a). Another methine proton of H-6 was correlated with δ 75.6 (C-5a) and δ 129.9 (C- 7). In the analysis of

gliotoxin from building materials, the atmospheric pressure chemical ionization mass spectrum of gliotoxin shows a protonated molecule at *m/z* 327 and no major fragment ions (Nieminen et al., 2002b). Thus, the structure of gliotoxin was determined as in **Fig.11**.

^{1}H-NMR (400 MHz, $CDCl_3$): 2.96 (1H, d, *J*=18.1, H-10A), 3.20 (3H, s), 3.44 (1H, dd, *J*=4.8, H-3aA), 3.73 (1H, d, *J*=18.1, H-10B), 4.28 (1H, dd, *J*=9.9, H-3aB), 4.39 (1H, dd, *J*=6.8, H-5a), 4.84 (1H, m, H-6), 5.78 (1H, d, *J*=9.9, H-7), 5.95 (1H, m, H-8), 6.00 (1H, m, H-9); ^{13}C-NMR (100 MHz, $CDCl_3$): 27.5 (C-11), 36.6 (C-10), 60.5 (C-3a), 69.8 (C-5a), 73.1 (C-10a), 75.6 (C-6), 77.2 (C-3), 120.2 (C-9), 123.4 (C-8), 129.9 (C-7), 130.7 (C-9a), 165.2 (C-4), 166.0 (C-1).

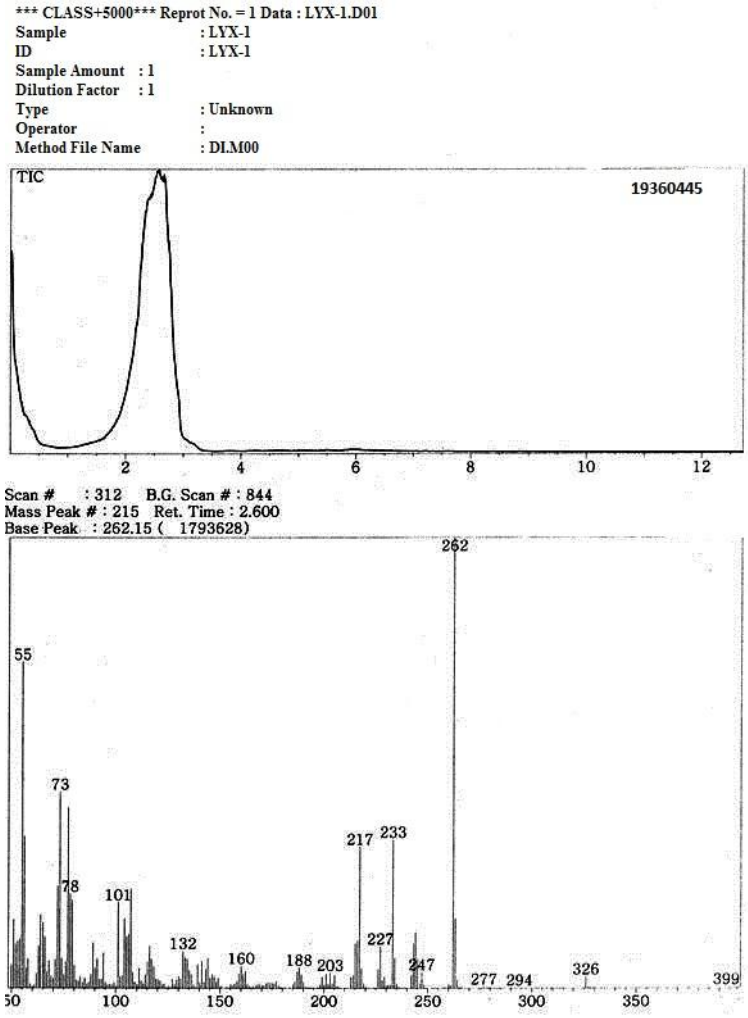
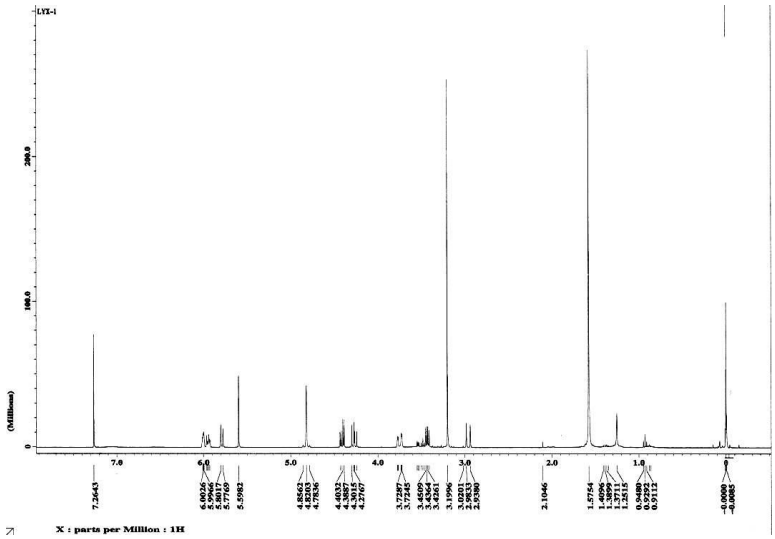


Fig.7. MS-MS spectra of gliotoxin

(A)



(B)

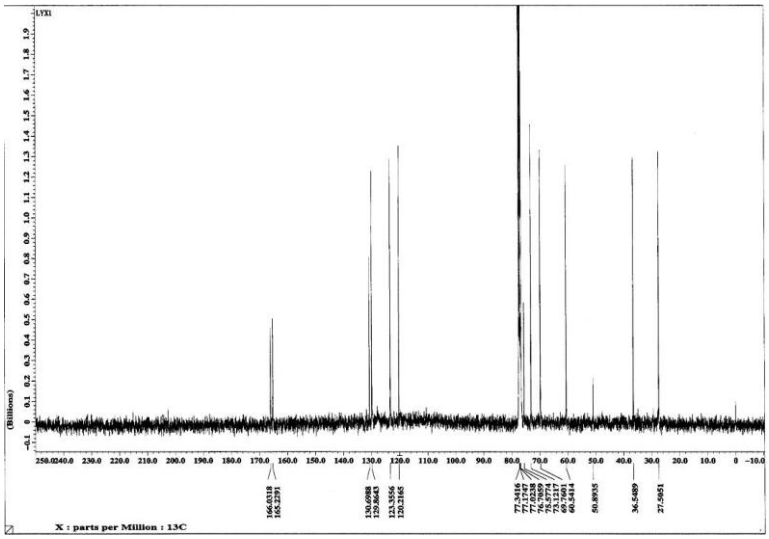


Fig.8. ^{1}H-NMR (400 MHz, $CDCl_3$) (A) and ^{13}C-NMR (100 MHz, $CDCl_3$) (B) spectrum of gliotoxin

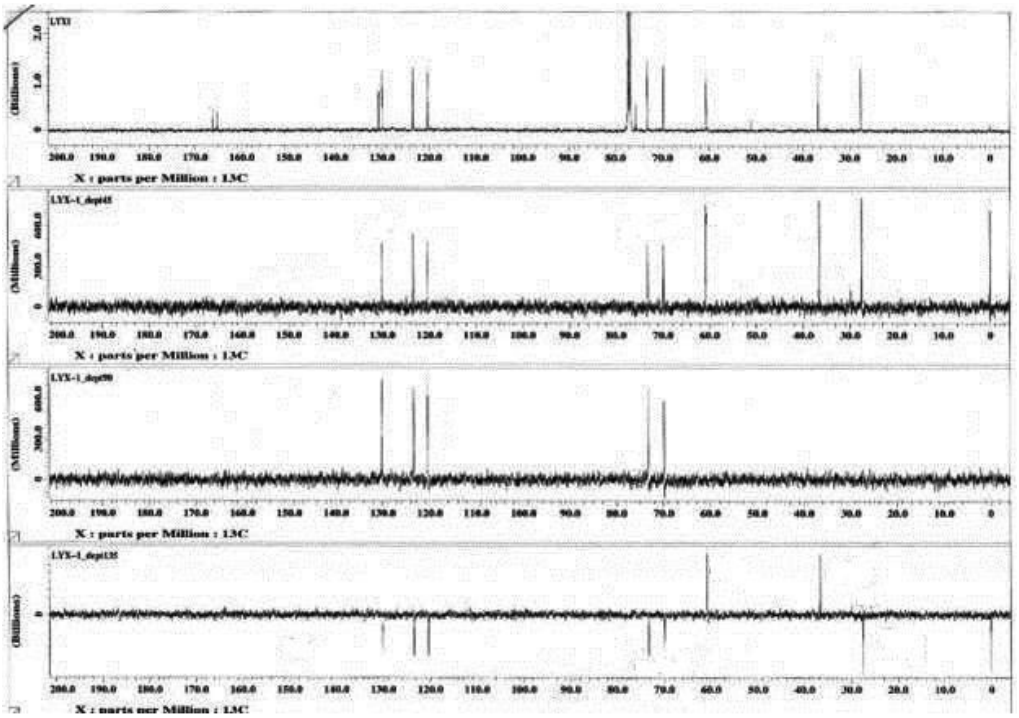
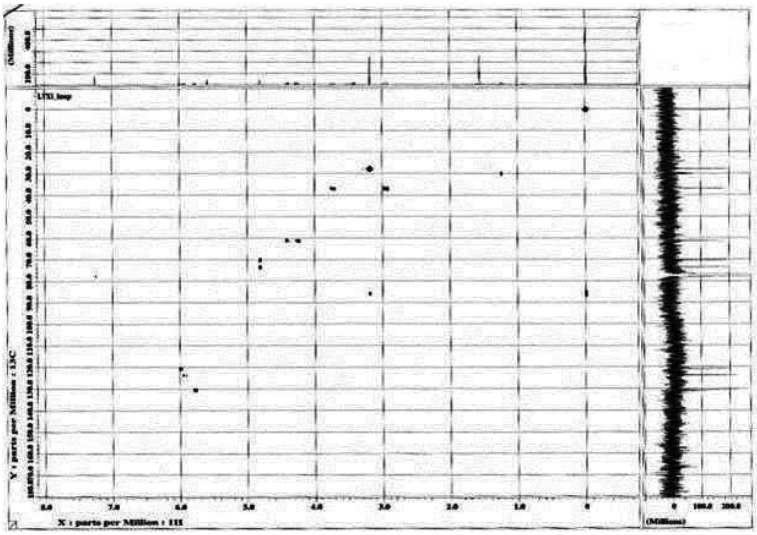


Fig.9. DEPT (45^0, 90^0, 135^0) spectra of gliotoxin

(A)



(B)

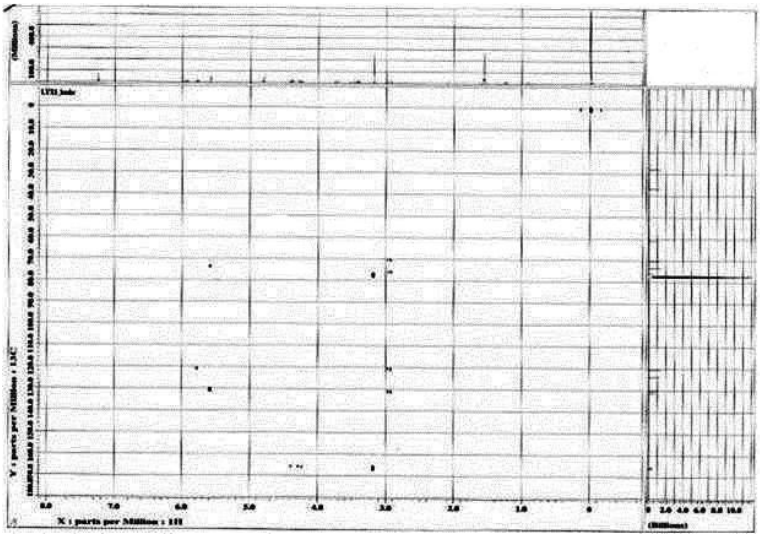


Fig.10. HMBC and HMQC spectrum in $CDCl_3$ of gliotoxin

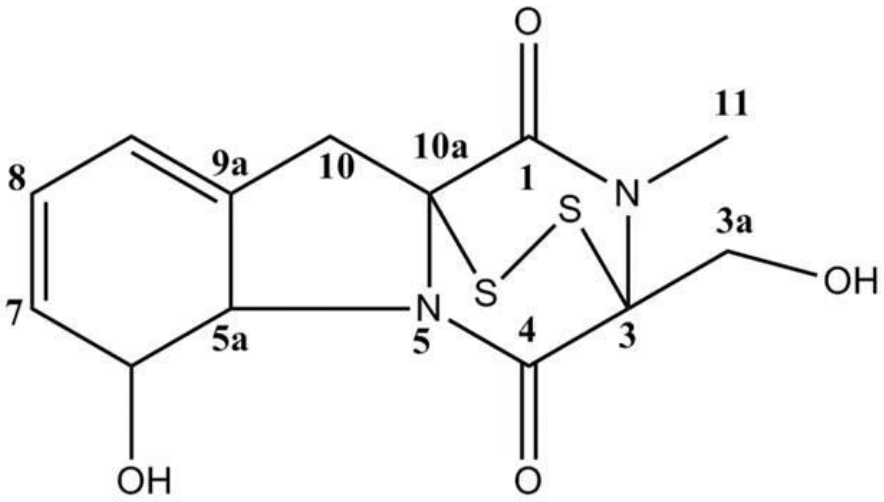


Fig.11. Chemical structure of gliotoxin from *Aspergillus sp.*

Table 3. ^{1}H and ^{13}C NMR spectral data for gliotoxin in $CDCl_3$.

^{1}H and ^{13}C NMR spectral data for gliotoxin in $CDCl_3$. [a]		
Position	δ_H	δ_C
1		166.0 (s)
3		77.2 (s)
3a	3.44 (1H, dd, *J*=4.8), 4.28 (1H, dd,*J*=9.9)	60.5(t)
4		165.2 (s)
5a	4.39 (1H, dd, *J*=6.8)	69.8 (d)
6	4.84 (1H, m)	75.6(d)
7	5.78(1H, d, *J*=9.9)	129.9 (d)
8	5.95 (1H, m)	123.4 (d)
9	6.00 (1H, m)	120.2(d)
9a		130.7(s)
10	2.96 (1H, d, *J*=18.1), 3.73(1H, d, *J*=18.1)	36.6 (t)
10a		73.1 (s)
11	3.20 (3H, s)	27.5 (q)
[a]Recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C and ^{13}C DEPT.		

C. Cell viability of gliotoxin

The effects of gliotoxin on Hela and SW1353 cells were examined using the MTT assay. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials. Those agents would stimulate the inhibition of cell viability and growth. The Hela and SW1353 cells were treated with different concentrations of gliotoxin for 36 hours. As shown in **Fig.12**, gliotoxin treatment inhibits cell growth of Hela (73%) and SW1353 cells (59%). The gliotoxin showed strong anti-proliferative activity in a dose-dependent manner, by presenting relative HeLa cell viabilities of 94%, 74%, 54%, and 27%, and SW1353 viabilities of 83%, 69%, 56%, and 47% at concentrations of 10, 30, 50, and 90 μM, respectively, compared to the control group. Here, cytotoxicity effect on Hela cells was higher than that on SW1353 cells treatment with gliotoxin at 90 μM of concentration.

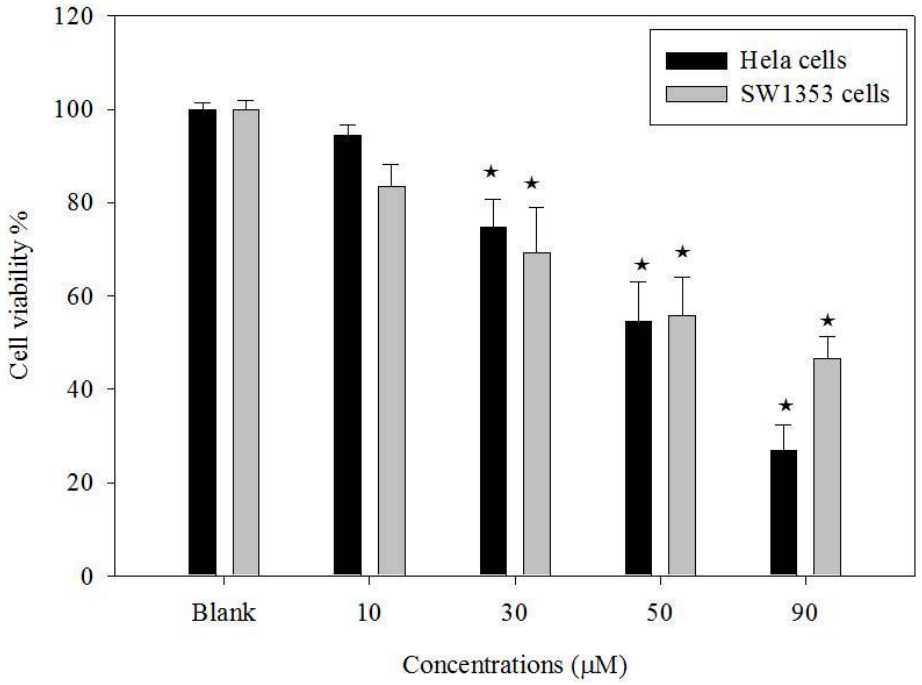


Fig.12. Cell viability of gliotoxin on Hela and SW1353 cells. The Hela and SW1353 cells were treated with various concentrations of gliotoxin for 36 hours. Values represent means±SE (n=3) (★P<0.05).

D. DAPI staining of Hela and SW1353 cells with gliotoxin.

To confirm that gliotoxin may induce apoptotic death in HeLa and SW1353 cells. A microscope following DAPI staining observed morphological changes with treatment of gliotoxin for 36 hours. Apoptosis is a highly conserved phenomenon that plays an important role in the regulation of the cellular activities of eukaryotes (Susan et al., 2007; Qu et al., 2004). As shown in **Fig.13** and **Fig.14**, in order to determine whether the anti-proliferative effects of gliotoxin on HeLa and SW1353 cells were due to apoptotic cell death, the cells were stained with DAPI dye and observed under a fluorescence microscope. Viable cells (control group) with intact DNA and no morphological changes were negative to DAPI or just slightly activated in the fluorescence microscope image. Furthermore, DAPI positive cells and their intensities increased in a gliotoxin dose-dependent manner. This indicates that most of the cells underwent cell death occurring primarily through apoptosis by the treatment of gliotoxin. Thus, the evaluation of internucleosomal DNA fragmentation has been widely accepted as one of the best-characterized biochemical markers for apoptosis. DNA fragmentation is a key feature of apoptosis and is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments.

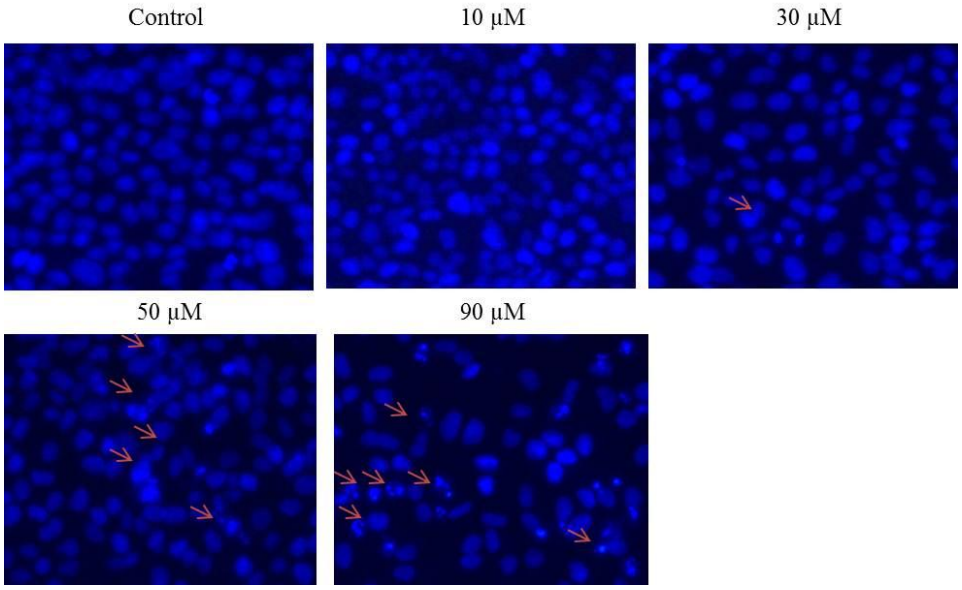


Fig.13. Nuclear staining with DAPI of gliotoxin treated Hela cells

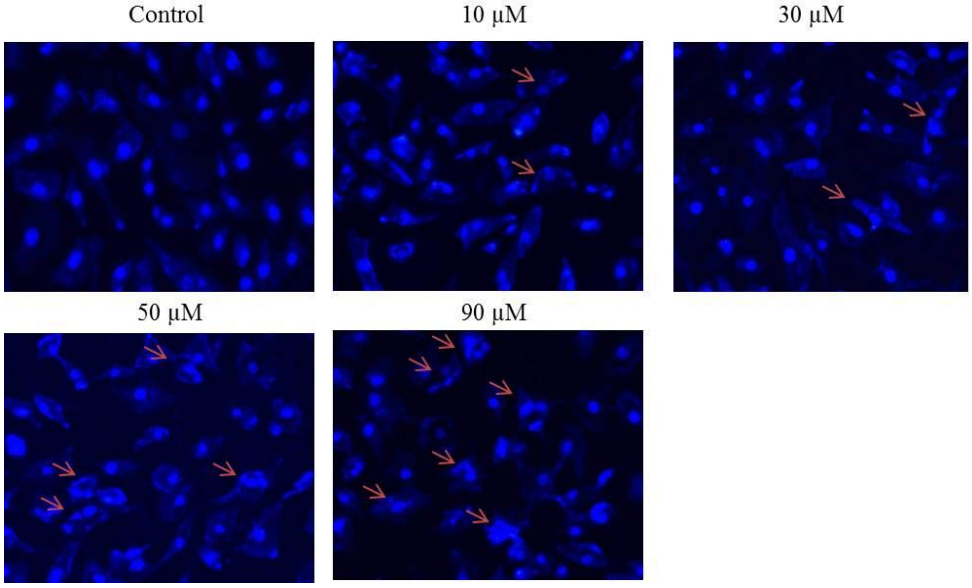


Fig.14. Nuclear staining with DAPI of gliotoxin treated SW1353 cells

E. Annexin-FITC V and propidium iodide staining apoptosis tests

To investigate the effects of gliotoxin on apoptosis cell, HeLa and SW1353 cells were treated with various concentrations of gliotoxin. In apoptosis cells, the membrane phospholipid phosphatidyserine (PS) is translocated form the inner to the outer leaflet of the plasma membrane, and is redistributed to the extracellular surface (Park et al., 2008; Lee et al., 2011c). Surface exposure of phosphatidylserine by apoptotic cells was measured by adding Annexin V-FITC (Vermes et al., 1995). Propidium iodide has been used to identify dead or late apoptotic cells because the membranes of damaged and dead cells are permeable to PI. Viable cells are negative to Annexin V and PI. However, early apoptotic cells are Annexin V positive and PI negative, and apoptotic or already dead cells are positive to both Annexin V and PI. As shown in **Fig.15** and **Fig.16**, upper left (Q1) quadrant of the cytograms shows the dead cells population increases, which positive to PI and Annexin V negative. Here, dead cells effect on Hela cells was higher than that on SW1353 cells treatment with gliotoxin at 90 μM of concentration. The lower left (Q4) quadrant of the cytograms shows the viable cells, which exclude PI and are negative for FITC-Annexin V binding. In the upper right (Q2) quadrants of the dead cells (Annexin V^+ and PI^+) population increases, and at the concentrations of 10, 30, 50, and 90 μM there is a slight increase of the cell population in the lower right (Q3) quadrant (Annexin V^- and PI^+).

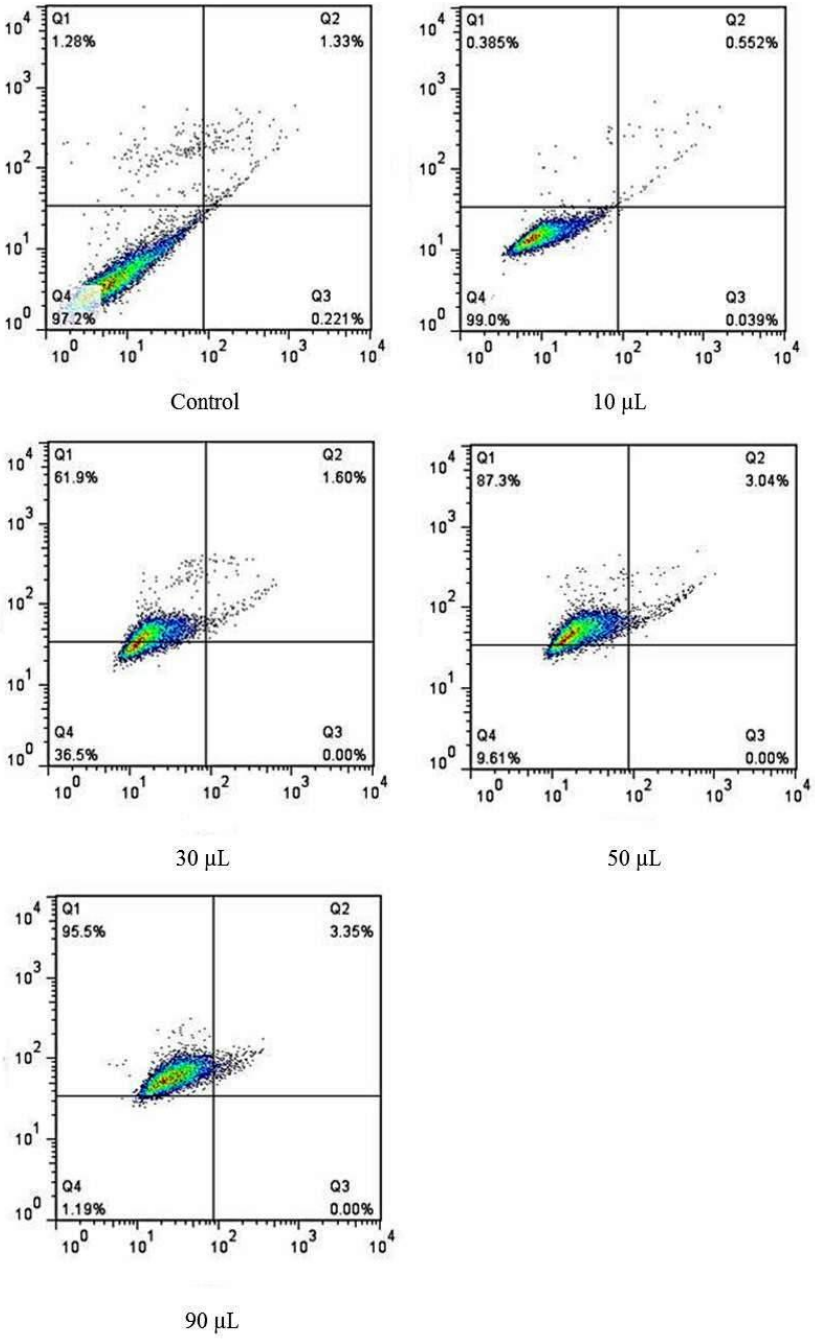


Fig.15. Detection of Annexin V-FITC and PI staining by FACS in gliotoxin treated Hela cells

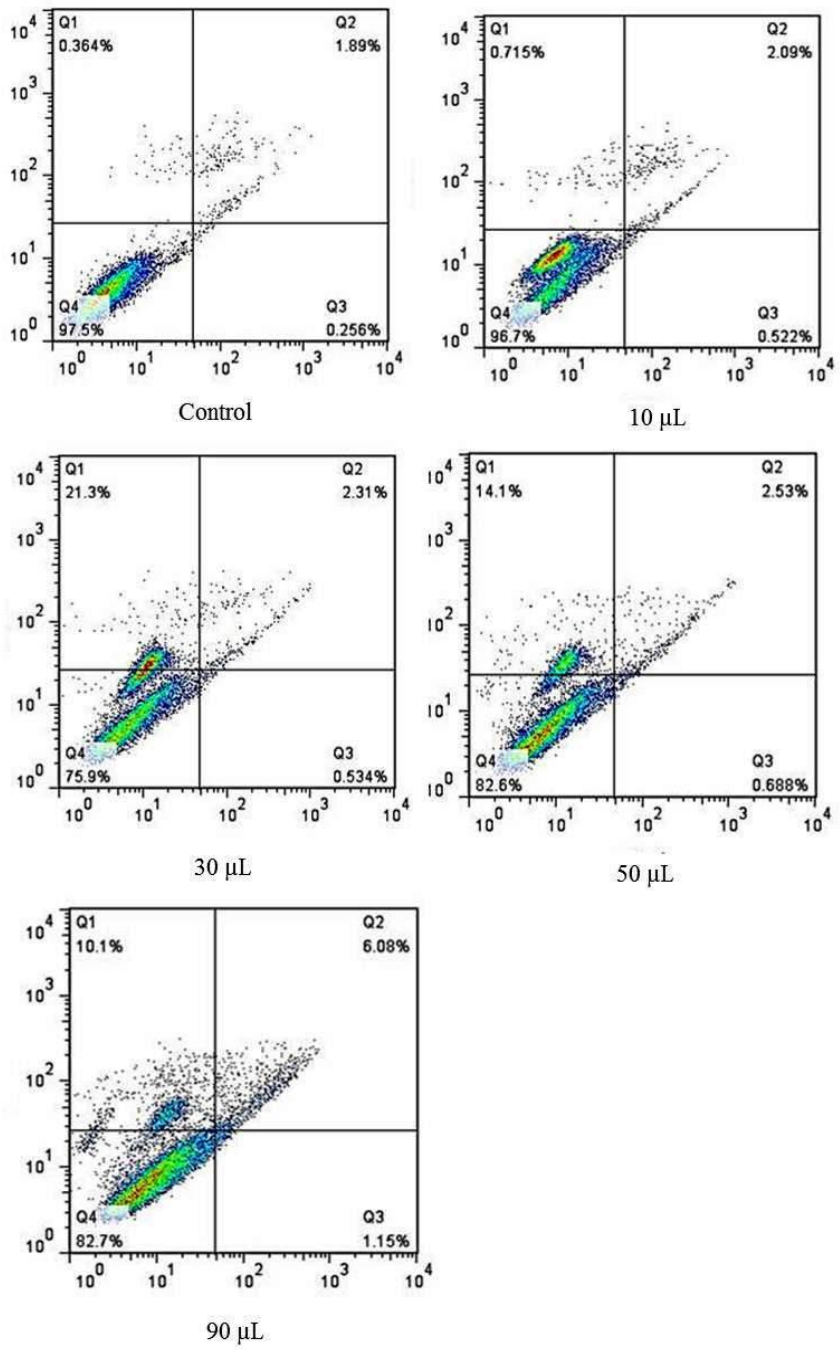


Fig.16. Detection of Annexin V-FITC and PI staining by FACS in gliotoxin treated SW1353 cells

F. DNA fragmentation

The major biochemical hallmark of apoptotic cell death is the cleavage of chromosomal DNA at internucleosomal sites into fragments or multiples of about 200 bp (Qu et al., 2004). Further confirmation that the occurrence of gliotoxin-induced apoptosis, a dose-dependent manner cellular DNA fragmentation was studied by gel electrophoresis. Paul et al (1988) reported that measurement of the molecular weights of the fragments is consistent with internucleosome cleavage characteristic of apoptosis. As shown in **Fig.17** a ladder-like pattern, typical character of DNA cleavage between nucleosomes was visible during 36 hours after incubation with 10, 30, 50, and 90 µM of gliotoxin. The intensity of banding was more prominent at 50 and 90 µM.

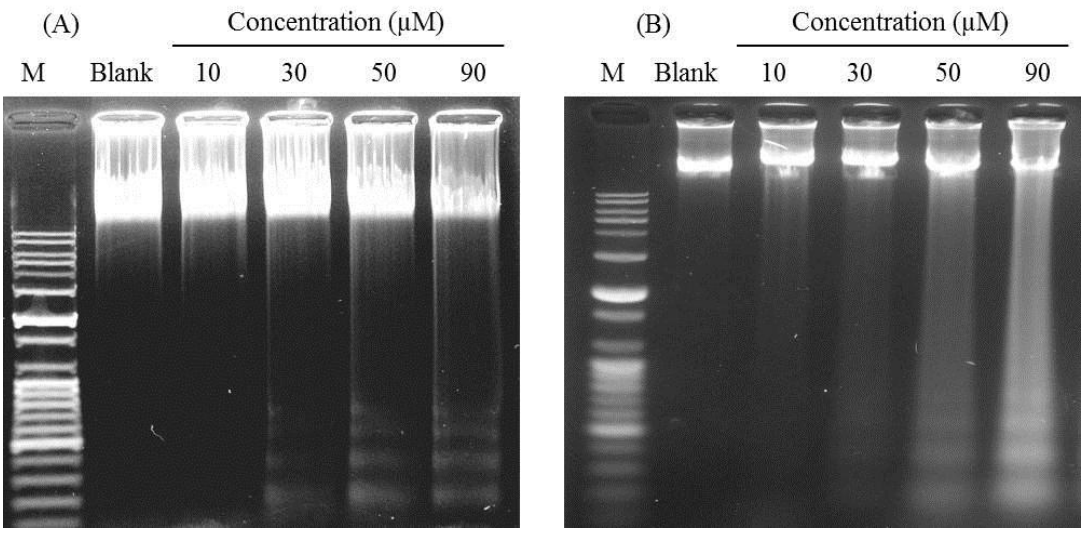


Fig.17. Detection of DNA fragmentation in SW1353 (A) and Hela (B) cells treated with gliotoxin. The genomic DNA was extracted, electrophoresed in a 1.2% agarose gel and and visualized by ethidium bromide staining under ultra-violet light. Lane M is a 100 bp plus DNA ladder.

G. Apoptotic effect in protein and gene expression levels

To determine whether cyt c, Bax, and Bcl-2 was involved in modulating apoptosis induced by gliotoxin, we investigated the effects of gliotoxin on mRNA and protein expressions of cyt c, Bax, and Bcl-2 in HeLa and SW1353 cells. Different concentrations of gliotoxin were added to HeLa and SW1353 cells for 24 hours, and RT-PCR were carried out. In the RT-PCR results, gliotoxin treatment promoted the mRNA expression of cyt c, down-regulated Bcl-2 and up-regulated Bax expressions in a dose-dependent manner (**Fig.18** and **Fig.19**). Apoptotic pathways can be divided into two major groups, such as extrinsic or intrinsic death (Li et al., 2009). Bcl-2 family members have important roles inregulating mitochondrial integrity and mitochondria-initiated caspase activation. The mitochondrial pathway is thought to be important in both events, and is mediated by Bcl-2 family proteins: the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax (Basu et al., 1998). Over-expression of Bcl-2 has been reported to protect tumor cells from apoptosis, whereas increased Bax expression promotes apoptosis via mitochondria (Hockenbery et al., 1990; Altznauer et al., 2004). Bax translocates to the mitochondria where it forms oligomers that are inserted into the outer mitochondrial membrane, and mitochondrial Bcl-2 decreases. Collectively, these results suggest that gliotoxin induced apoptosis by activating expressions of Bax, release of cyt c into cytosol and inhibiting Bcl-2. Several papers have also reported the importance of gliotoxin as induction of MPT, cyt c release and caspase-3 activation (Paul et al., 1988; Kweon et al., 2003; Axelsson et al., 2006).

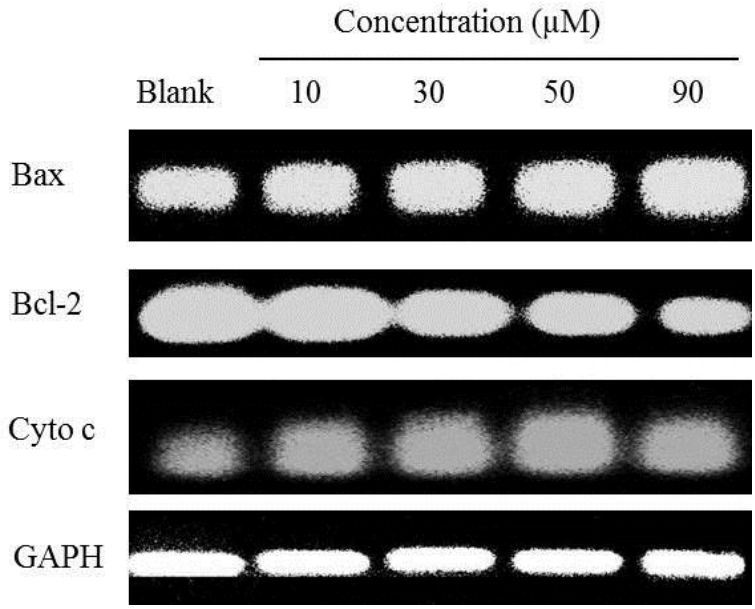


Fig.18. RT-PCR of Bax, Bcl-2, cyt c for gliotoxin treated in Hela cells

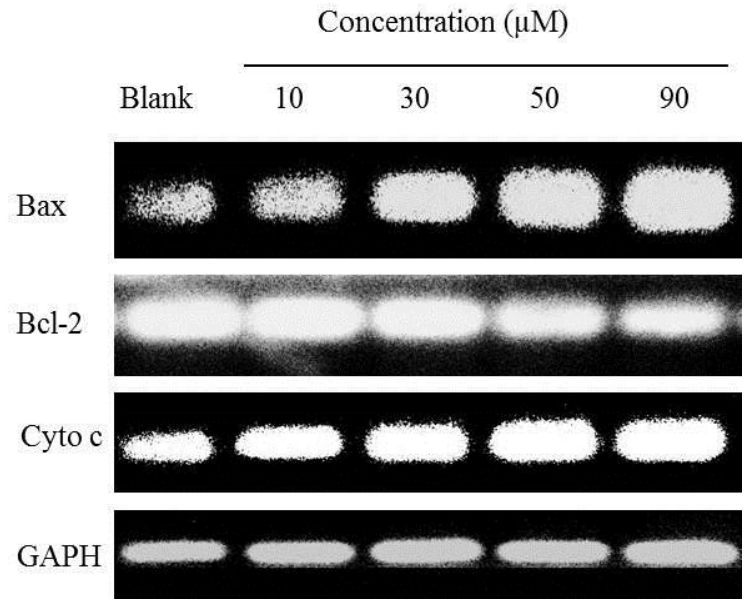


Fig.19. RT-PCR of Bax, Bcl-2, cyt c for gliotoxin treated in SW1353 cells

Caspases are expressed in almost all cell types as inactive proenzymes. Much evidence suggests that activation of caspases triggers the apoptotic process in various cell types (Qu et al., 2004). Activated caspase-3 has important roles in the occurrence of typical biochemical and morphological changes in apoptotic cells (Cohen et al., 1997). In addition, gliotoxin-mediated activation of caspase-3 and induction of apoptosis have been reported for several cell lines (Axelsson et al., 2006; Kweon et al., 2003). To elucidate the possible mechanisms of apoptosis in HeLa and SW1353 cells, we investigated the effects of gliotoxin with respect to mRNA expression by RT-PCR. Different concentrations of gliotoxin were added to HeLa and SW1353 cells for 24 hours. As shown in **Fig.20** and **Fig.21**, the expression levels of caspase-3, caspase-8, and caspase-9 increased at the concentrations of 10, 30, 50, and 90 μM compared to the untreated group, and continuously increased dose-dependently. However, they did not the expression levels of p53 increased.

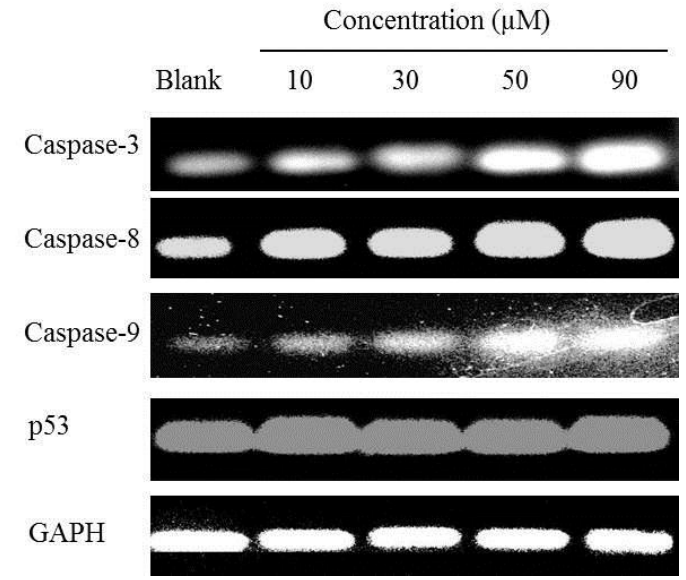


Fig.20. RT-PCR of caspase-3, caspase-8, caspase-9, p53 for gliotoxin treated in Hela cells

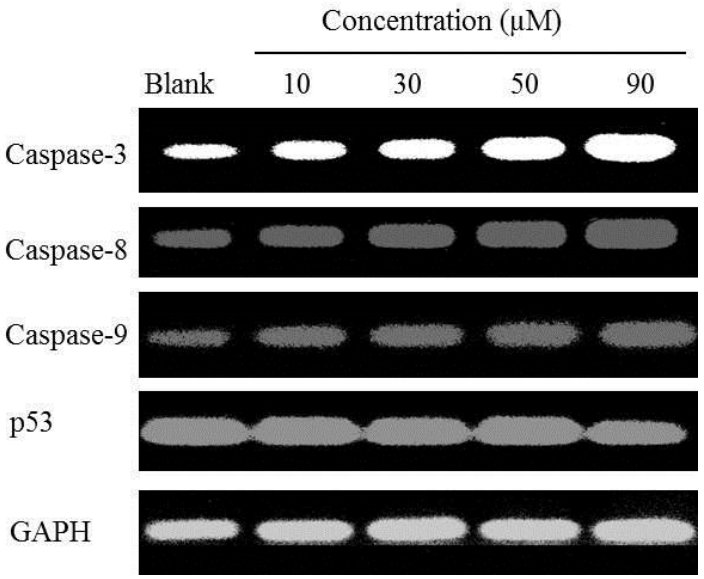


Fig.21. RT-PCR of caspase-3, caspase-8, caspase-9, p53 for gliotoxin treated in SW1353 cells

Moreover, Western blotting analysis used to investigate the effects of gliotoxin on cyt c, Bax, and Bcl-2 protein expression. Cyt c is localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. Release of cyt c from the intermembrane spaces of the mitochondria into the cytosol is a key event in apoptosis (Eiichi et al., 2005; Li et al., 2007). To examine this step in the apoptotic cell death pathway initiated by gliotoxin, the measurement of cyt c content in cytosol of HeLa and SW1353 cells treated with 10, 30, 50, and 90 μM of gliotoxin. As shown in **Fig.22** and **Fig.23**, the release of cyt c was initiated as early as 10 and 30 μM of gliotoxin treatment. The release of cyt c preceding the activation of caspase-3 and caspase-9, suggesting that it might be important in the execution of gliotoxin-induced apoptosis. The release of cyt c from mitochondria is tightly regulated by a variety of factors. Among them, Bcl-2 family proteins, including Bcl-2 (anti-apoptotic members) and Bax (pro-apoptotic members), play a pivotal role (Park et al., 2008). To elucidate further the possible mechanism underlying the gliotoxin-induced apoptosis, the expression of Bcl-2 and Bax in HeLa and SW1353 cells were examined after gliotoxin treatment. As shown in **Fig.22** and **Fig.23**, exposure of HeLa cells to 10, 30, 50, and 90 μM of gliotoxin led to an obvious decrease of Bcl-2 protein expression, but a drastic increase of Bax protein expression in a dose-dependent manner.

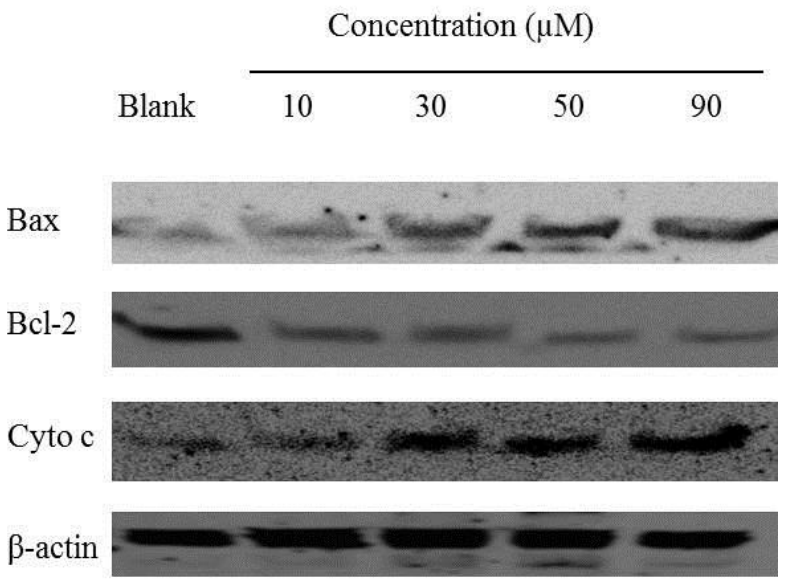


Fig.22. Western blot of Bax, Bcl-2, cyt c for gliotoxin treated in Hela cells

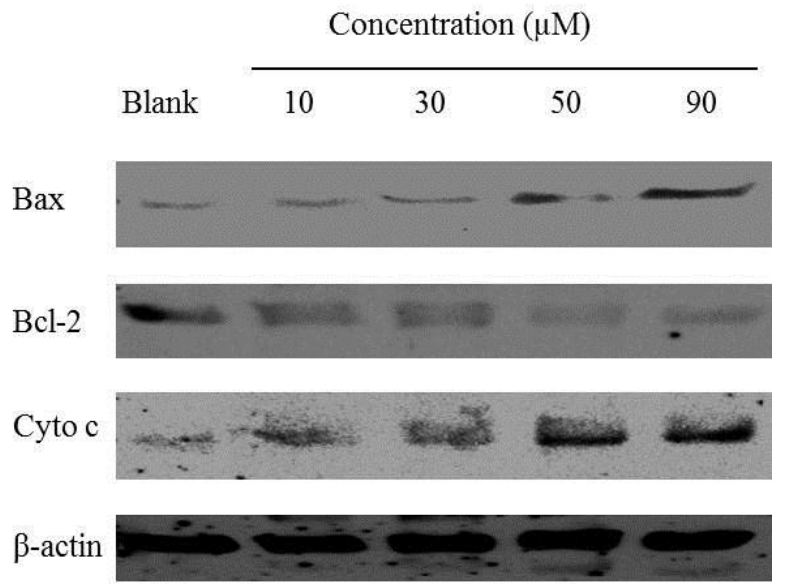


Fig.23. Western blot of Bax, Bcl-2, cyt c for gliotoxin treated in SW1353 cells

Furthermore, the mechanism by which gliotoxin induces apoptosis is being actively investigated. Since a variety of toxins and chemicals induce cytotoxicity via caspase (Anselmi et al., 2007; Axelsson et al., 2006). Western blotting analysis used to investigate the effects of gliotoxin on caspase family protein expression in Hela and SW1353 cells. Gliotoxin treatment promoted caspase-3, caspase-8, and caspase-9 protein expressions dose-dependently in the HeLa and SW1353 cells (**Fig.24** and **Fig.25**). However, it was interesting that gliotoxin almost had no effect on p53 protein expressions. Gliotoxin shows a number of broad toxic and inhibitory effects directed towards immune cells and the induction of apoptosis, which requires the extracellular presence of the epipolythiodioxopiperazine ring (Paul et al., 1988; Xiaoming et al., 2000). Taken together, these results suggest that the mechanism of gliotoxin-induced apoptosis in HeLa and SW1353 cells involves the activation of caspase family expression as caspase-3, caspase-8 and caspase-9.

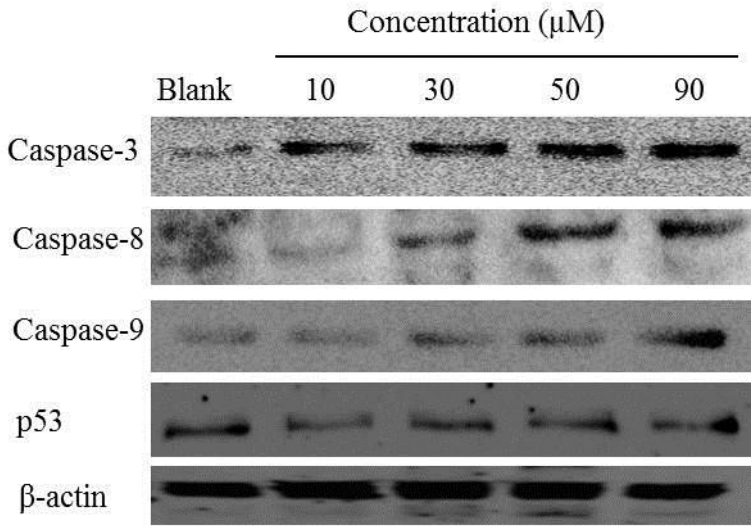


Fig.24. Western blot of caspase-3, caspase-8, caspase-9, p53 for gliotoxin treated in Hela cells

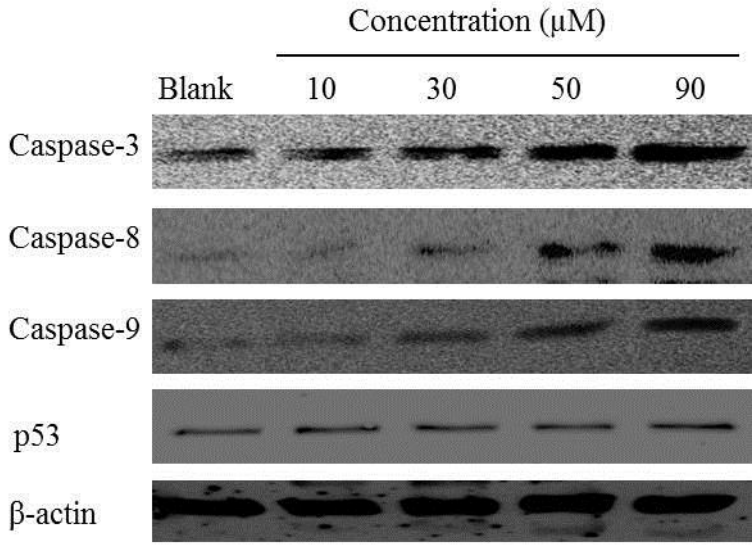


Fig.25. Western blot of caspase-3, caspase-8, caspase-9, p53 for gliotoxin treated in SW1353 cells

In conclusion, the data reveal that HeLa and SW1353 cells are highly sensitive to growth inhibition and apoptosis induction by gliotoxin. These results indicated that gliotoxin induces apoptosis through the recruitment of caspase-8 will be activated and it is able to directly activate caspase-3, an effector protein. The mitochondrial pathways were mediated by down-regulation of Bcl-2 and up-regulation of Bax the release of cytochrome c. Following its formation, the complex will activate caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, which in turn activates caspase-3 to apoptotic mode of cell death. Briefly, the present study examined that gliotoxin induced apoptotic cell death in association with activating expressions of caspase family enzymes followed by cyt c, Bax and Bcl-2 regulation (**Fig.26**).

IV. SUMMARY

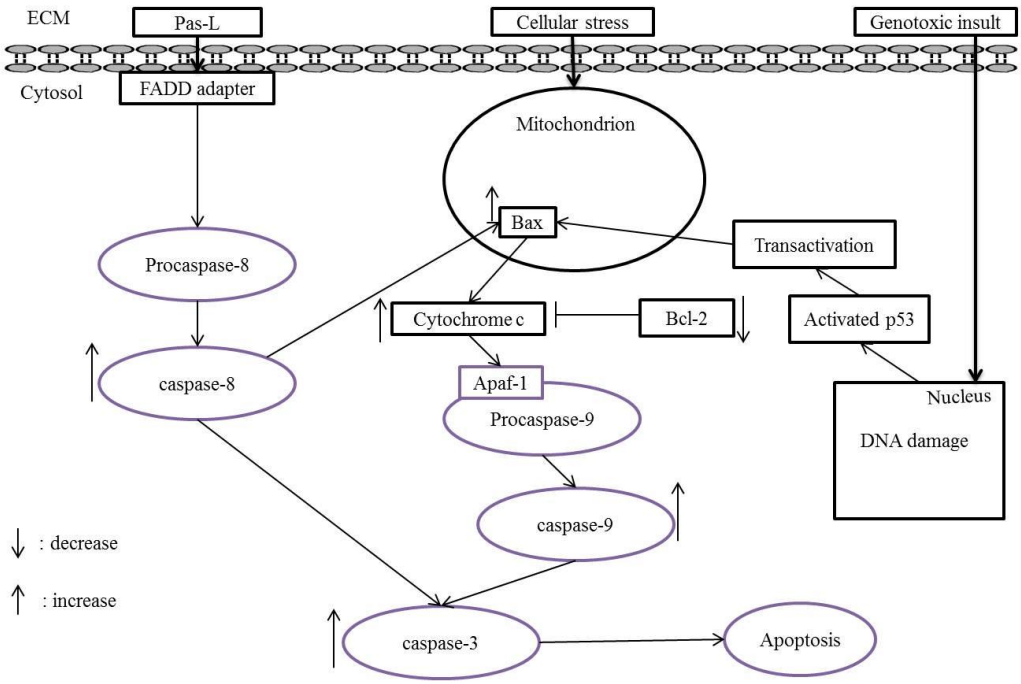


Fig.26. Scheme of apoptosis pathways activated by gliotoxin

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논문제목	**한글:** 해양진균 Aspergillus sp. 로부터 분리된 gliotoxin 의 인간 자궁경부암 및 연골육종 세포에서의 세포사멸 효과 **영문:** Effects of gliotoxin isolated from marine fungus *Aspergillus sp.* on apoptosis in human cervical cancer cell and human chondrosarcoma cell				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함

2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.

3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.

4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.

6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음

7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2012 년 8 월 24 일

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