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# *Allomyrina Dichotoma* Larva Extracts Protect Streptozotocin-induced Oxidative Cytotoxicity

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# *Allomyrina Dichotoma* Larva 추출물이 췌장 β-세포에서 streptozotocin에 의해 유도된 산화적 손상에 대한 보호효과

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## 요 약

장수풍뎅이 유충(*Allomyrina dichotoma* larva, ADL)은 중국의 전통 약재로서, 특히 항산화 효과가 우수하여 항당료 제재로 사용되고 있다. 본 연구에서는 이러한 ADL의 추출물을 이용하여 햄스터 췌장의 β-세포(HIT-T15)에서 Streptozotocin에 의해 유발된 산화적 손상에 대한 보호효과 및 그 작용기전을 조사하였다. ADL추출물은 처리농도 의존적으로 Streptozotocin에 의해 유발된 지질과산화 및 세포 내 자유산소종의 양을 억제함으로서 β-세포의 산화적 스트레스에 의한 손상을 보호하였다. 또한 DNA laddering 방법을 사용하여 Streptozotocin에 의해 유발된 DNA 손상을 조사한 결과, ADL추출물 처리농도에 비례하여 Streptozotocin에 의해 유발된 DNA 손상이 감소하였다. 이러한 산화적 손상의 억제능 관련 작용 기전을 조사하기 위해 DPPH free radical 소거능을 실시하였다. 그 결과 ADL추출물 자체가 DPPH 자유 레디컬 소거능이 있음을 확인하였으며, 또한 플라스미드를 이용한 Single-strand break 방법을 통한 DNA 손상 보호능을 측정한 결과도 $Fe^{3+}$ 및 $H_2O_2$에 의해 유발된 DNA 손상이 ADL추출물 처리농도에 비례하여 감소하였다. 이러한 결과들을 종합하여 볼 때, 장수풍뎅이 유충의 추출물들이 자체의 레디컬 소거능 및 산화적 손상에 의한 DNA 손상을 억제함으로써, Streptozotocin에 의해 유발된 산화적 손상을 억제할 수 있을 것이라 사료된다.

**Key words** : *Allomyrina dichotoma* larva, streptozotocin, DNA damage, oxidative stress

## INTRODUCTION

Type 2 diabetes mellitus is characterized by both peripheral insulin resistance and pancreatic β-cell

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failure (DeFronzo 1988). The pancreatic β-cell defect results from a combination of insulin-secretory dysfunction and decreased β-cell mass, which is determined by a balance between β-cell replication and apoptosis (Weir *et al.*, 2001; Robertson, 2006). Streptozotocin (STZ) is a potent DNA methylating agent and acts as a free radical donor in the pancreas where the β-cells are particularly sensitive to damage from free radicals because of a low level of free radical scavenging enzymes (Larsen and Grude, 1978; Newsholme *et al.*, 2007). Normally, the ROS are scavenged by endogenous antioxidants and cellular enzymatic defense mechanisms, mediated by superoxide dismutase, glutathione peroxidase and catalase (Yu, 1994). However, cellular injury can occur when large acute doses of or chronic exposure to toxic substances overpower the cellular antioxidant defense system (Halliwell *et al.*, 2000). Several harmful reactions such as DNA degradation, membrane peroxidation and the destruction of endothelial cells have been attributed to oxygen-derived free radicals (Brown *et al.*, 1995). DNA damage by ROS can initiate in development of cardiovascular complications in diabetes mellitus (Haidara *et al.*, 2006). Generally, exposure of vascular endothelial cells to high concentrations of glucose results in the overproduction of superoxide anions, which interferes with nitric oxide metabolism damaging the endothelial cells (Giugliano *et al.*, 1996), whereas high glucose levels may also damage pancreatic beta cells by increasing the level of intracellular oxygen free radicals (Termini, 2000). It is well established that ROS are regarded as having hyperglycemia potential and antioxidants can block the process of diabetes mellitus (Mooradian, 2006). Oriental natural plants are recently being investigated for their pharmacological properties in the regulation of blood regulation and apoptosis induced by oxidative stress, a process which is crucial in the pathology of diabetes mellitus (Kinloch *et al.*, 1999). Among the oriental natural plants, *Allomyrina dichotoma* larva is one of the well-known insects used in traditional Chinese medicine for anti-hepatofibrosis and anti-diabetic agent (Miyanoshita *et al.*, 1996; Sagisaka *et al.*, 2001). Extract of ADL has been found to contain an antineoplastic agent such as lectin and the substance responsible for this biological activity was located primarily in the beetle larva (Yoshikawa *et al.*, 1999). However, it is not known whether ADLE can prevent or alleviate liver injury induced by oxidative stress and also still lacking unequivocal proof of this assumption. Therefore, we investigated that the protective effect of the extract of ADLE on STZ-induced oxidative cytotoxicity and DNA damage and also elucidated the mechanism (s) underlying its protective effects in HIT-T15 cells.

## MATERIALS AND METHODS

### 1. Materials

Chemicals and cell culture materials were obtained from the following sources: Streptozotocin (STZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitrilotriacetic acid, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, and PCMV-β plasmid DNA from Sigma-Aldrich Chemical Co.; WST-1based colorimetric assay kit from Roche Co.; RPMI 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin solution from Life Technologies; 5-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate ethyl ester (CM -DCFDA) was from Molecular Probes; other chemicals were of the highest commercial grade available.

### 2. Preparation of *Allomyrina dichotoma* larva extract

*Allomyrina dichotoma* larva was obtained from Kachi-maeul Co., chonnam, South Korea. The extract of *Allomyrina dichotoma* larva (ADLE) was prepared using the method described elsewhere and their compositions were previously published (Yoshikawa *et al.*, 1999). Briefly, *Allomyrina dichotoma* larva was extracted twice with ethanol for 2 days at room temperature. After the organic solvents were evaporated, the dried fractions of *Allomyrina dichotoma* larva were dissolved in dimethyl sulfoxide (DMSO)

(final concentration not more than 0.1%) and phosphate buffered saline (PBS, pH=7.0) for following studies.

### 3. Cell culture, treatments, and cell viability assay

The HIT-T15 cell line was obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% $CO_2$ humidified incubator. ADLE was dissolved in ethanol and added directly to the culture media. HIT-T15 cells were pretreated with ADLE for 6 h prior to the addition of 10 mM STZ. Control cells were treated with ethanol, the final concentration of which never exceeded 0.1%, and this concentration did not have any noticeable effect on the assay systems. Cell viability was assessed by WST-1 assay. For the WST-1 assay, 24 h after treatment with STZ, medium containing STZ was replaced with fresh media without STZ, and cellular cytotoxicity was measured using a WST-1based colorimetric assay kit according to the manufacturer's instructions.

### 4. DNA laddering

DNA samples for DNA laddering were extracted from lysates using a total DNA separator kit (Promega, USA) and electrophoretically separated in a 1 % agarose gel, which were treated ethidium bromide and visualized and photographed under ultraviolet light.

### 5. Measurement of lipid peroxidation and intracellular ROS production

Thiobarbituric Acid Reactive Substances (TBARS), the lipid peroxidation product in the cells, was assayed according to a thiobarbituric acid fluorometric method using 1,1,3,3-tetramethoxypropane, as described previously (Choi *et al.*, 2001).

The fluorescent probe, CM-DCFDA, was used to monitor the intracellular generation of reactive oxygen species by STZ, as described previously (Ding *et al.*, 2006).

### 6. Assay of free radical-quenching activity

The free radical-quenching capacity of ADLE was tested by a method involving the bleaching of stable DPPH, as described previously (Lee *et al.*, 2007).

### 7. $Fe^{3+}$-nitrilotriacetic acid preparation

To prepare $Fe^{3+}$-nitrilotriacetic acid (NTA), $FeCl_3$ was dissolved in 20 mM phosphate buffer to a $Fe^{3+}$ to NTA molar ratio of 1 : 1, and the pH was adjusted to 7.4, as described previously (You *et al.*, 2002).

### 8. Assay of oxidative DNA single-strand breaks

DNA single-strand breaks in supercoiled DNA were analyzed after agarose gel electrophoresis. PCMV-β plasmid DNA (0.5 μg) was treated with 100 μM $H_2O_2$, 100 μM $Fe^{3+}$-NTA in 50 mM $KH_2PO_4$-KOH buffer at pH 7.4 with or without ADLE. The reaction solution (30 μL) was mixed with 3 μL of electrophoresis loading buffer and loaded onto a 1.0% agarose gel prepared in TAE buffer (40 mM Tris acetate, 2 mM EDTA). After electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide for 10 min, destained for 30 min, and then visualized under UV light and photographed. To quantitatively analyze DNA single-strand breaks, gel images were captured on a Gel Doc Image Analysis System (Kodak) and the density of the supercoiled (sc) DNA band and the open circle (oc) DNA band were measured by NIH Image software (Bethesda, MD). The degree of DNA cleavage activity was expressed in terms of the percentage of scDNA to ocDNA conversion according to the following equation: DNA cleavage activity=$([\%\ \text{of scDNA}]_{\text{control}} - [\%\ \text{of scDNA}]_{\text{sample}} \times 100)/[\%\ \text{of scDNA}]_{\text{control}}$.

### 9. Statistical analyses

All data were expressed as the mean±SD from three independent experiments, performed in tripli-

cate. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the student 't' test, using $p<0.05$ as the level of significance.

## RESULTS

ADLE at the tested concentrations did not significantly affect the cytotoxicity, except at 800 μg/mL which showed 95% cell viability (Data not shown).

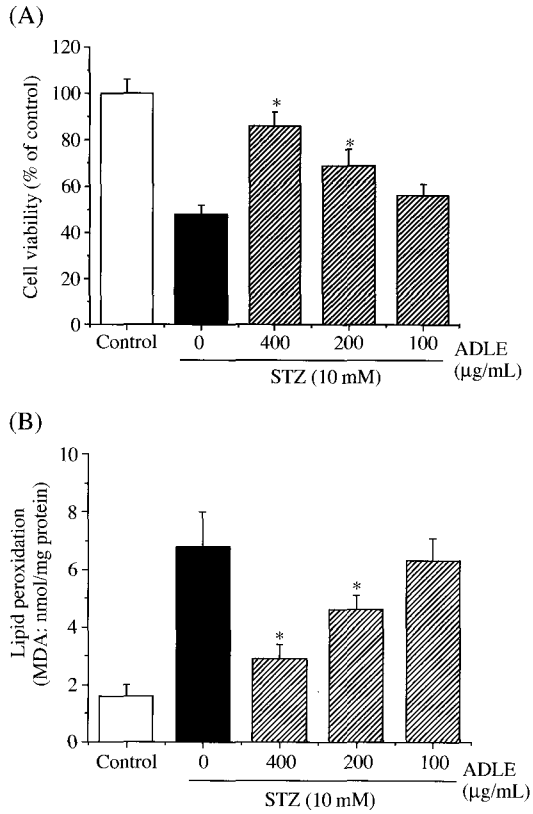


**Fig. 1.** Effects of ADLE on STZ-induced cytotoxicity in HIT-T15 cell. The cells were plated in 24-well plates and various concentration of ADLE was treated with STZ (10 mM) for 24 h. (A) Cell viability were estimated by WST-1 assay. (B) Lipid peroxidation was evaluated by malondialdehyde formation as described in the Materials and Methods. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from control at $p<0.05$.

Thus, we treated cells with 0, 100, 200, 400 μg/mL ADLE in the subsequent experiments. The protective effects of ADLE against the STZ-induced cytotoxicity in HIT-T15 cells were quantified by WST-1 assay. The ADLE, which is nontoxic even at a high concentration (400 μg/mL), afforded full protection from cell injury by WST-1 assay (Fig. 1A). To explore the consequences of STZ-induced oxidative damage to cellular macromolecules and to determine the possible effects of ADLE, we further analyzed the formation of malondialdehyde (MDA: a marker for membrane lipid peroxidation) and intraceullular reactive oxygen species (ROS). The ADLE alone did not change the degree of MDA formation compared to the untreated controls. Whereas, exposure to STZ alone for 24 h increased the amount of cell-associated MDA in HIT-T15 cells and the presence of ADLE significantly prevented STZ-induced the MDA production dose-dependent manner (Fig. 1B). Consistent with cytotoxicity data, apoptosis cells was also either markedly prevented ADLE in a does-dependent man-

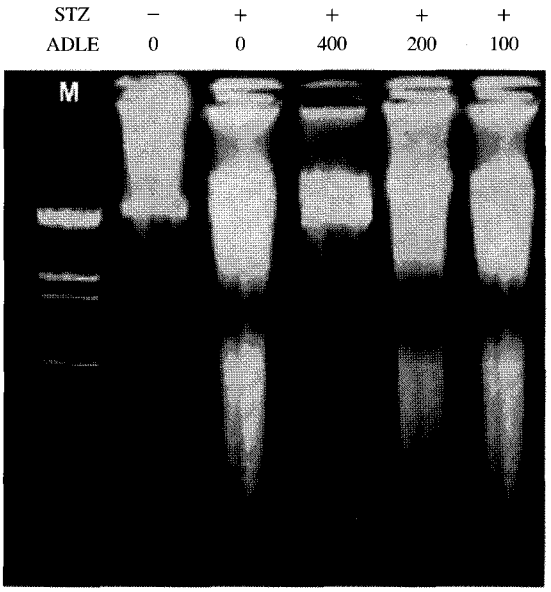


**Fig. 2.** Effects of ADLE on STZ-induced oxidative DNA damage. Cells were treated with ADL for 6 h and then were added STZ (10 mM) for 24 h. Cells were evaluated DNA damage using DNA laddering as described in the Materials and Methods.

nner, based on DNA laddering (Fig. 2).

It is well established that antioxidants can block the process of diabetes mellitus (Mooradian, 2006). In addition, the β-cells, have a low level of scavenging enzymes, are particularly sensitive to damage from free radicals (Newsholme *et al.*, 2007). Thus, we firstly measured whether ADLE could quench the DPPH free radicals. As shown in Fig. 3A, ADLE was able to quench the DPPH free radicals in a dose-dependent manner. To confirm that ADLE reduces STZ-induced oxidative stress in HIT-T15 cells, the intracellular ROS production was assessed by monitoring CM-DCFDA fluorescence. Rapid increases in intracellular oxidant levels were noted in the cells after STZ treatment, but the oxidant burden after STZ exposure decreased in the presence of ADLE in a dose-dependent manner (Fig. 3B). These results demonstrate ADLE was able to inhibit the membrane lipid peroxidation via suppression of intraceulluar ROS triggered by the injurious peroxy-radicals generated from STZ. Moreover, the incubation of plasmid DNA with $Fe^{3+}$-NTA/$H_2O_2$ resulted in the increased formation of ocDNA, indicating that DNA single-strand breaks can be induced by $Fe^{3+}$-NTA/$H_2O_2$. The addition of ADLE to $Fe^{3+}$-NTA/$H_2O_2$ resulted in an inhibition of the conversion of scDNA to the open circle form in a dose-dependent manner, indicating that ADLE is a potent scavenger of the hydroxyl radical and is able to protect against $Fe^{3+}$-NTA/$H_2O_2$-

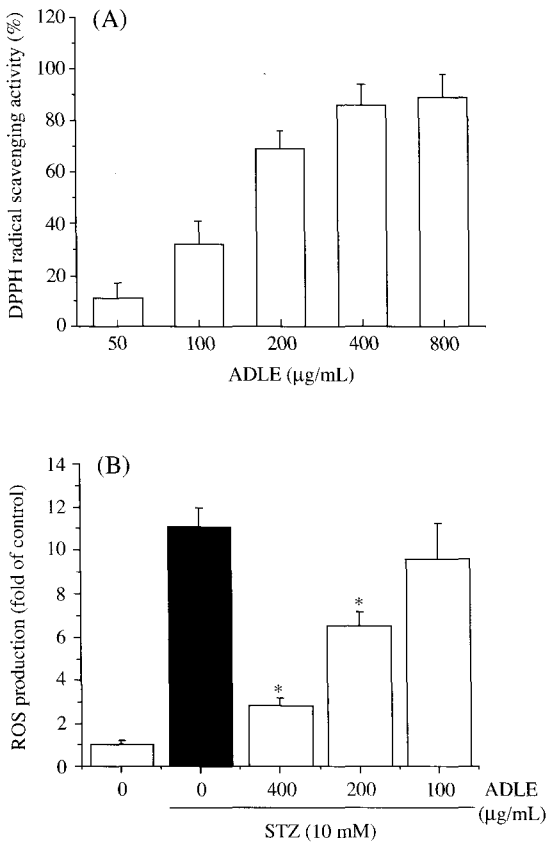


**Fig. 3.** Effects of ADLE on STZ-induced intracellular ROS, and DPPH free radical scavenging activity. Cells were treated with ADLE for 6 h and then were added STZ (10 mM) for 24 h. (A) The intracellular ROS were measured by monitoring fluorescence increases for 30 min. * $p<0.05$, significantly different from STZ alone. (B) DPPH free radical scavenging activity of ADLE. Each bar represents the mean± SD calculated from three independent experiments. *Significantly different from control at $p<0.05$.

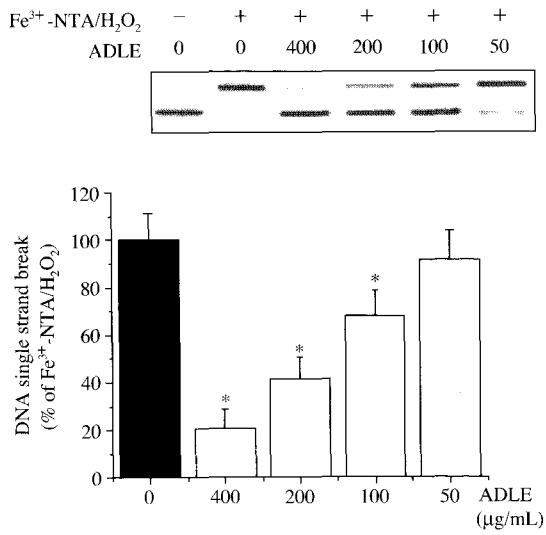


**Fig. 4.** Effect of ADLE on $Fe^{3+}$-NTA/$H_2O_2$-induced DNA strand breaks. pMCV-β plasmid DNA (0.5 μg/lane) was incubated with 100 μM $Fe^{3+}$-NTA and 100 μM $H_2O_2$ in the presence or absence of ADLE for 1 h at 37°C. DNA single-strand breaks were determined as described in Materials and Methods. Each bar represents the mean±SD calculated from three independent experiments. *Significantly different from the $Fe^{3+}$-NTA/$H_2O_2$ at $p<0.05$.

mediated DNA single-strand brakes (Fig. 4).

## DISCUSSION

The aim of this study was to evaluate the ability of ADLE, with its inherent antioxidant activity, to have an effect on the cellular and DNA damage in HIT-T15 cells. Therefore, we examined the protective effect of ADLE on STZ-induced oxidative stress and DNA damege in this study. In the cytotoxicity and lipid peroxidation experiments, ADLE protected the cultured cells effectively from the injury caused by STZ, as reflected in the increased cell viability and the decreased formation of MDA formation. Lipid peroxidation is considered to be an indirect measure of ROS generation in pancreatic β-cells (Spinas *et al.*, 1999). Thus, we determined CM-DCFDA fluorescent changes after STZ treatment for 24 h, as an additional sensitive means of detecting intracellular ROS. It was observed that the levels of ROS in ADLE-treated cells were lower than in control cells after ADLE treatment. Increasing cellular ROS can break DNA strand in the cell (Termini, 2000). DNA damage was assessed by DNA laddering, a procedure that detects DNA breaks in cells by free radical attack (Guetens *et al.*, 2002; Kang *et al.*, 2007). We found that $H_2O_2$ exposure induced a greater level of DNA damage in control cells than in ADLE treated cells. The ROS may subsequently be converted to hydroxyl radicals by further reactions during the cellular metabolic processes (Giugliano *et al.*, 1996). Approximately 90% of cellular DNA damage produced by ionizing radiation is caused by hydroxyl radicals, which generates more than 30 different base adducts as well as various amino acids, proteins, and lipid addition products, strand breaks, and cross-links (Gunther *et al.*, 1995; Halliwell *et al.*, 2000). Consistently, our data suggest that ADLE is effective scavengers of ROS that can protect cellular DNA against oxidative damage. However, the exact mechanisms of the antioxidant effects of the ADLE remain unclear. Thus, we determined whether the protection afforded to cells by ADLE against STZ-induced oxidative cytotoxicity and DNA damage might be a consequence of the free radical-quenching capacity of ADLE.

Induction of a single-strand break into supercoiled plasmid DNA leads to the formation of open circular DNA. DNA is a very sensitive target of hydroxyl radicals and it has been demonstrated that in the presence of $Fe^{3+}$-NTA/$H_2O_2$ is able to cause strand breaks in isolated DNA (Giugliano *et al.*, 1996; Halliwell *et al.*, 2000). Although, the precise nature of the reactive species remains to be chemically defined, a bound hydroxyl radical or its equivalent derived from the reaction between $Fe^{3+}$-NTA/$H_2O_2$ has been suggested to mediate DNA strand single-breaks. In present data, ADLE markedly inhibited DNA strand single-breaks induced by $Fe^{3+}$-NTA/$H_2O_2$ and this result indicating that ADLE is potent scavengers of the hydroxyl radical. Although further study is required to fully understand the antioxidative characteristics of ADLE, the present study provide, for the first time, direct evidence that ADLE can react with hydroxyl radicals to protect DNA damage.

In conclusion, this study demonstrated that ADLE protected STZ-induced oxidative cytotoxicity in HIT-T15 cells. Overall, this study provides biological evidence supporting the use of ADLE for the treatment of diabetic disease in the living system.

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