Protective Effect of Triphlorethol-A from *Ecklonia cava* against Ionizing Radiation *in vitro*

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Radiation protection/Oxidative stress/Reactive oxygen species.

We studied the cytoprotective effect of triphlorethol-A against γ-ray radiation- induced oxidative stress. In this study, hydrogen peroxide, which is a reactive oxygen species (ROS), was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) assay. Triphlorethol-A reduced intracellular hydrogen peroxide generated by γ-ray radiation. This compound provided protection against radiation-induced membrane lipid peroxidation and cellular DNA damage which are the main targets of radiation-induced damage. Triphlorethol-A protected the cell viability damaged by the radiation through inhibition of apoptosis. Triphlorethol-A reduced the expression of bax and activated caspase 3 induced by radiation, but recovered the expression of bcl-2 decreased by radiation. Taken together, the results suggest that triphlorethol-A protects cells against oxidative damage induced by radiation through reducing ROS.

INTRODUCTION

Radiation induces cell damage through the formation of ROS.[1] Sulfhydryl agents such as cysteine, glutathione, β-mercaptoethylamine (cysteamine), and other antioxidants have been shown to protect mice against the lethal effects of radiation. Increased understanding of the interrelationship between the effects of oxygen and radiation exposure leads to the rational application of naturally occurring antioxidants.[2] Recently, we reported that triphlorethol-A, derived from *Ecklonia cava*, decreased H_2O_2-induced cell damage through activation of an antioxidant system.[3] *Ecklonia cava* is a brown alga (Laminariaceae) that is abundant in the subtidal regions of Jeju Island, Korea. It has been reported that *Ecklonia* species exhibit radical scavenging activity,[4,5] antiplasmin inhibiting activity,[6–8] antimutagenic activity,[9–11] bactericidal activity,[12] HIV-1 reverse transcriptase and pro-tease inhibiting activity,[13] and tyrosinase inhibitory activity.[14,15] Phlorotannins (polymers of phloroglucinol) have been identified as being responsible for the biological activities of *Ecklonia*. Triphlorethol-A, an open-chain trimer of phloroglucinol, is a phlorotannin component that is isolated from *E. cava*.

In this study, we investigated the protective effects of triphlorethol-A on cell damage induced by γ-ray radiation and the possible mechanism of the cytoprotection.

MATERIALS AND METHODS

Preparation of Triphlorethol-A

Dried *Ecklonia cava* (4 kg), collected from Jeju Island, Korea, was immersed in 80% methanol at room temperature for 2 days. The aqueous methanol was removed *in vaccuo* to produce a brown extract (1 kg), which was partitioned between ethyl acetate and water. The ethyl acetate fraction (230 g) was mixed with celite. The mixed celite was dried and packed into a glass column, and eluted in following order, with hexane, methylene chloride, diethyl ether, and methanol. The diethyl ether fraction (14 g) was subjected to Sephadex LH-20 chromatography using $CHCl_3$-MeOH gradient solvent (2/1→0/1). Triphlorethol-A (220 mg) was retrieved from these fractions and was identified according to a previously reported method (Fig. 1).[8] The purity of triphlorethol-A, which was assessed by HPLC, was > 90%. Triphlorethol-A was then dissolved in dimethylsulfoxide (DMSO); the final concentration of which did not exceed 0.1%.

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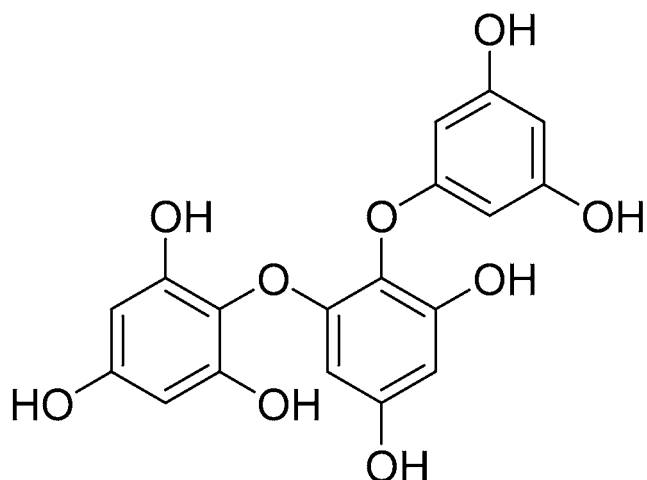
Fig. 1. Chemical structure of triphlorethol-A.

Cell Culture
    Chinese hamster lung fibroblasts (V79-4) cells from the American Type Culture Collection were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Irradiation
    Cells were exposed to γ-ray from a ^{60}Co γ-ray source (MDS Nordion C-188 standard source, located in Cheju
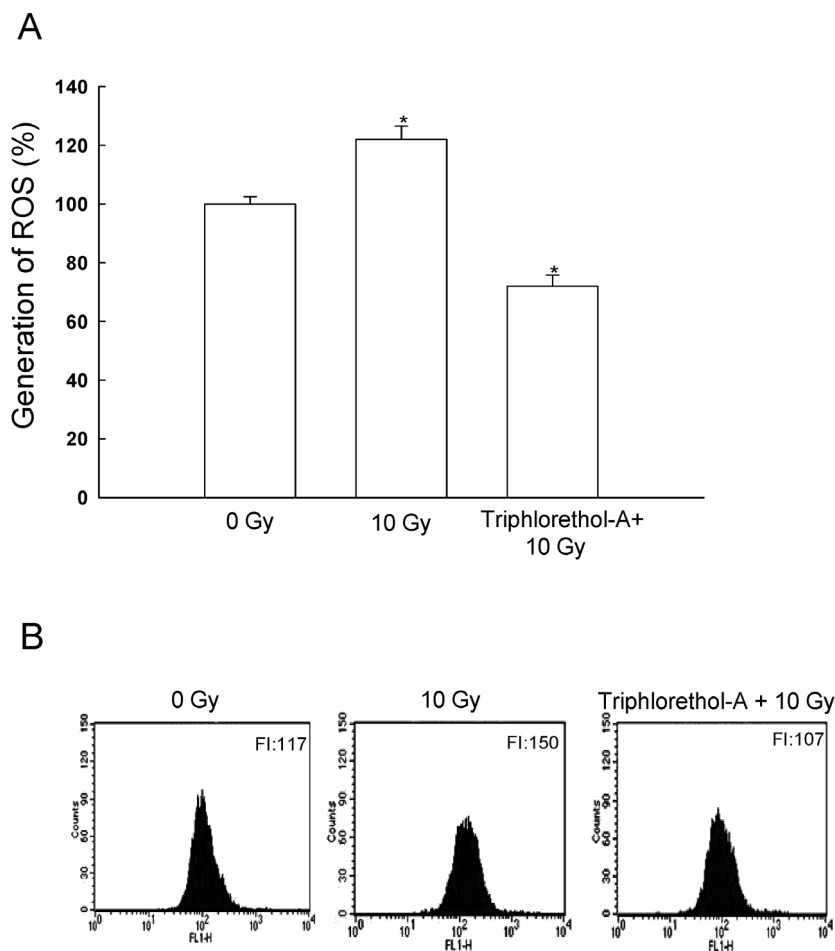

Fig. 2. Effect of triphlorethol-A on scavenging intracellular ROS generated by γ-ray radiation. The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The intracellular ROS was detected using fluorescence spectrophotometer (A) and flow cytometry (B) after DCF-DA staining. The measurements were made in triplicate and values are expressed as means ± S.E. FI means fluorescence intensity. *Significantly different from 0 Gy ($p < 0.05$).

National University, Jeju, Korea) at a dose rate of 1.5 Gy/min.

Intracellular Reactive Oxygen Species Measurement

The DCF-DA method was used to detect the intracellular hydrogen peroxide level.[16] DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichlorofluorescein. The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The cells were incubated for an additional 24 h at 37°C. After the addition of 25 μM of DCF-DA solution, the fluorescence of 2',7'-dichlorofluorescein was detected using a Perkin Elmer LS-5B spectrofluorometer and using flow cytometry (Becton Dickinson, Mountain View, CA, USA), respectively.
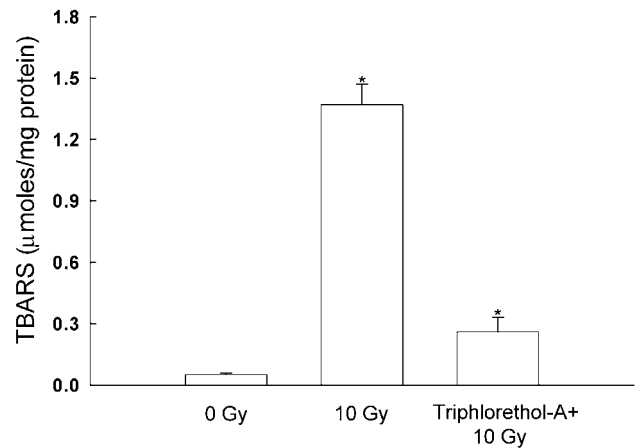

Fig. 3. Protective effect of triphlorethol-A upon γ-ray radiation-induced lipid membrane damage of V79-4 cells. Lipid peroxidation was assayed by measuring the amount of TBARS. The measurement was made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy ($p < 0.05$).
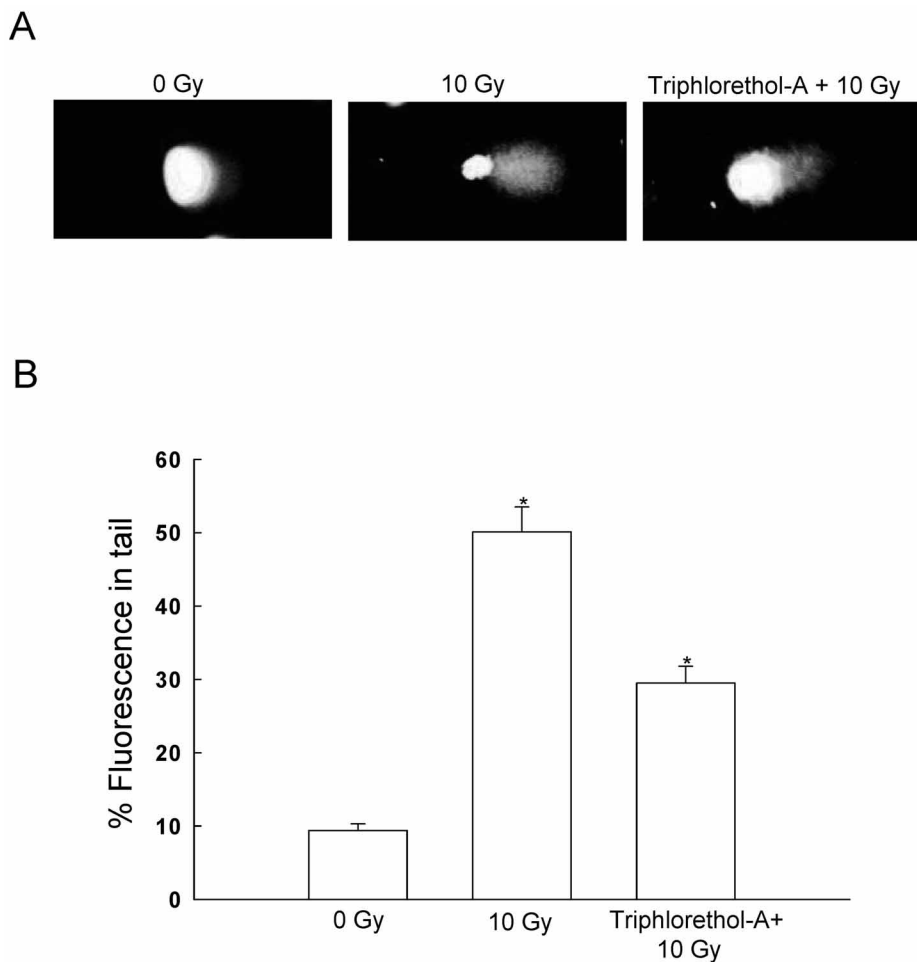

Fig. 4. Protective effect of triphlorethol-A upon γ-ray radiation-induced cellular DNA damage of V79-4 cells. The cellular DNA damage was detected by an alkaline comet assay. The measurement was made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy ($p < 0.05$).

Cell Viability

The effect of triphlorethol-A on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells.[17] The V79-4 cells were treated with triphlorethol-A and γ-ray radiation, and the cells were incubated for 48 h at 37°C. Fifty μl of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of DMSO and the A_{540} was read on a scanning multi-well spectrophotometer.

Lipid Peroxidation Inhibitory Activity

Lipid peroxidation was assayed by a thiobarbituric acid reaction.[18] The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The cells were incubated for 24 h at 37°C. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. One hundred μl of the cell lysates was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA). Distilled water was added to the mixture to a final volume of 4 ml and heated to 95°C for 2 h. After cooling to room temperature, 5 ml of an *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample, and the mixture was shaken. After centrifugation at 1000 × g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

Flow Cytometry Analysis

Flow cytometry was performed in order to determine the apoptotic sub-G_1 hypodiploid cells.[19] The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The cells were then incubated for an additional 48 h at 37°C, subsequently harvested, and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37°C in 1 ml of PBS containing 100 μg propidium iodide and 100 μg RNase A. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Sub-G_1 hypodiploid cells were assessed based on the histograms generated using the computer programs, Cell Quest and Mod-Fit.
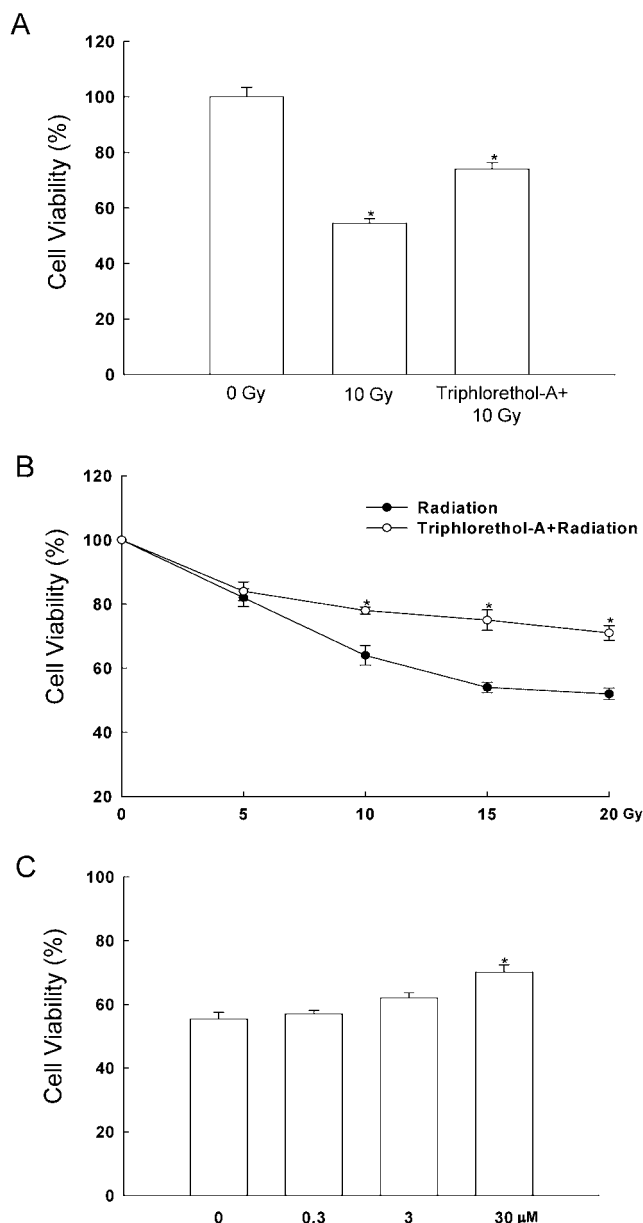
Nuclear Staining with Hoechst 33342

The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The cells were then incubated for 48 h at 37°C. 1.5 μl of Hoechst 33342 (stock 10 mg/ml), a DNA-specific fluorescent dye, was added to each well (1.5 ml) and incubated for 10 min at 37°C. The stained cells were observed under a fluorescent microscope, which was equipped with a Cool-SNAP-Pro color digital camera to order to examine the degree of nuclear condensation.

Single Cell Gel Electrophoresis (Comet Assay)

Comet assay was performed to determine the oxidative DNA damage.[20,21] The cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA) at 39°C and spread on a fully frosted microscopic slide that was pre-coated with 200 μl of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 μl of 0.5% LMA and then immersed in a lysis solution
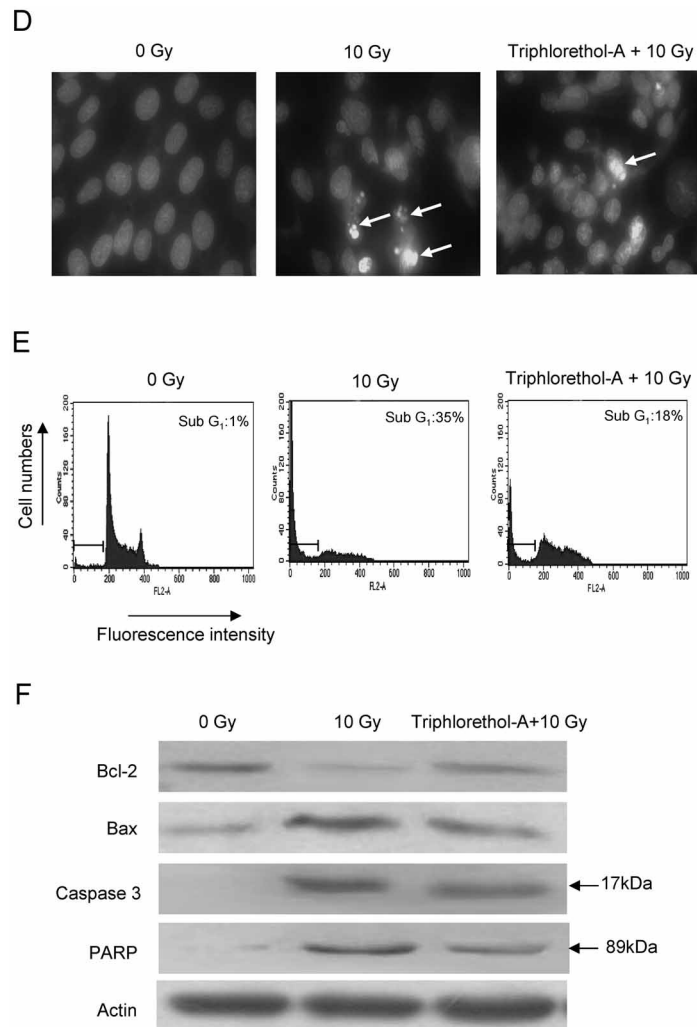

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Fig. 5. Protective effect of triphlorethol-A upon γ-ray radiation-induced cell damage of V79-4 cells. The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy (A) or indicated radiation doses (B) was applied to the cells. Or V79-4 cells were treated with triphlorethol-A at indicated concentration (C) and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The viability of V79-4 cells upon radiation was determined by MTT assay. The measurements were made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy (A) and irradiated cells at indicated radiation doses (B) and 10 Gy (C) ($p < 0.05$). (D) Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows. (E) Apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. (F) Western blot analysis was performed using anti-bcl-2, -bax, -caspase 3 and -PARP antibodies.

(2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were then placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow DNA unwinding and the expression of the alkali labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4°C to draw negatively charged DNA toward an anode. After electrophoresis, the slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 75 μl of propidium iodide (20 μg/ml). The slides were observed with a fluorescence microscope and image analysis (Kinetic Imaging, Komet 5.5, UK). The percentage of total fluorescence in the tail and the tail length of the 50 cells per slide were recorded.

Western Blot Analysis

The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the

cells. The cells were then incubated for 48 h at 37°C. The cells were harvested, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at $13,000 \times g$ for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with anti-primary rabbit monoclonal bcl-2, bax, caspase 3 and PARP antibodies. The membranes were further incubated with goat anti-rabbit immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, USA).

Statistical Analysis

All measurements were made in triplicate and all values were represented as means ± S.E. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ were considered significantly.

RESULT

The radical scavenging effect of triphlorethol-A on the ROS generated by γ-ray radiation was measured. Triphlorethol-A at 30 μM decreased the level of intracellular ROS induced by radiation (Fig. 2A and B). The level of ROS detected using spectrofluorometer (Fig. 2A) showed 72% of ROS level in triphlorethol-A treated irradiated cells compared to 122% of ROS level in irradiated cells. And the level of ROS detected using flow cytometry (Fig. 2B) showed 107 value of fluorescence intensity which was produced from ROS stained by DCF-DA fluorescence dye in triphlorethol-A treated irradiated cells compared to 150 value of fluorescence intensity in irradiated cells. The abilities of triphlorethol-A to inhibit membrane lipid peroxidation and cellular DNA damage in radiated cells were also investigated. Ionizing radiation induced damage to cell membrane and this one of the most important lesions was responsible for loss of viability. The peroxidation of membrane lipids is the major lesion in the membranes. As shown in Fig. 3, V79-4 cells exposed to γ-rays *in vitro* showed an increase in the lipid peroxidation, which was monitored by the generation of thiobarbituric acid reactive substance (TBARS). However, triphlorethol-A prevented the radiation-induced peroxidation of lipids. Damage to cellular DNA induced by γ-radiation exposure was detected using an alkaline comet assay. The exposure of cells to radiation increased comet parameters like tail length and percentage of DNA in the tails of cells.

Treatment with triphlorethol-A before irradiation resulted in a decrease of tail length (Fig. 4A). When the cells were exposed to γ-radiation at 10 Gy, the percent of DNA in the tail was increased 50.1 ± 3.4 as shown in Fig. 4B. Treatment with triphlorethol-A resulted in a decrease to 29.5 ± 2.3, which indicated a protective effect of triphloroethol-A on radiation-induced DNA damage *in vitro*. The protective effect of triphlorethol-A on cell survival in V79-4 cells exposed to radiation was also measured. As demonstrated in Fig. 5A, treatment with triphlorethol-A at 30 μM increased 74% of the cell survival compared to 54% of the cell survival in irradiated cells at 10 Gy. This cytoprotective effect by triphlorethol-A also showed at radiation-dose dependent manner (Fig. 5B) and at concentration of triphlorethol-A dependent manner (Fig. 5C). In order to study the cytoprotective effect of triphlorethol-A on radiation-induced apoptosis, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopy and with propidium iodide for flow cytometric analysis. The microscopic pictures in Fig. 5D demonstrated that the control cells had intact nuclei, and the radiation-exposed cells demonstrated significant nuclear fragmentation, which is characteristic of apoptosis. However, when the cells were treated with triphlorethol-A for 1 h prior to radiation, dramatic decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of triphlorethol-A against apoptosis was confirmed by flow cytometry. As demonstrated in Fig. 5E, an analysis of the DNA content in the radiation-exposed cells revealed an increase of 35% in apoptotic sub-G_1 DNA content, compared to 1% of apoptotic sub-G_1 DNA content in untreated cells. Treatment with 30 μM of triphlorethol-A decreased the apoptotic sub-G_1 DNA content to 18%. These results suggested that triphlorethol-A protects cell viability by inhibiting radiation-induced apoptosis. To understand how triphlorethol-A protects radiation-induced apoptosis, we examined changes of Bcl-2 families and caspases activities by western blot. As shown in Fig. 5F, the Bcl-2, an anti-apoptotic protein, recovered in treatment with triphlorethol-A and radiation compared to irradiated cells and the Bax, a pro-apoptotic protein, decreased in treatment with triphlorethol-A and radiation compared to irradiated cells. It was also observed the decreased activation of caspase 3 (17 kDa), the major effecter caspase of the apoptotic process, and PARP cleavage (89 kDa), one of substrates of activated caspase 3, in combination with triphlorethol-A and radiation compared to irradiated cells. Treatment of triphlorethol-A at 30 μM after irradiation at 10 Gy still showed the protective effect, increasing 77% of the cell survival compared to 58% of the cell survival in irradiated cells (Fig. 6A). In addition, triphlorethol-A reduced intracellular ROS induced by post-irradiation (Fig. 6B).
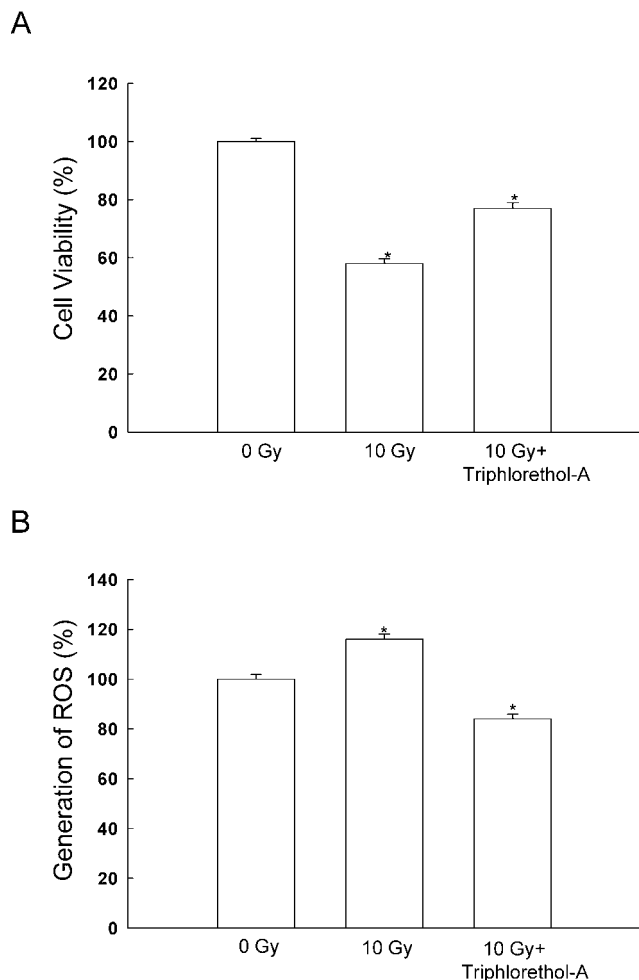
A

B

Fig. 6. Effect of triphlorethol-A on cell viability and scavenging intracellular ROS generated after γ-ray radiation. The V79-4 cells were exposed by γ-ray radiation at 10 Gy and 1 h later, triphlorethol-A at 30 μM was treated to the cells. (A) The viability of V79-4 cells upon radiation was determined by MTT assay and (B) the intracellular ROS was detected using fluorescence spectrophotometer after DCF-DA staining. The measurements were made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy (p < 0.05).

DISCUSSION

Exposure to ionizing radiation causes cell damage through the production of ROS which induces oxidative stress.[22] The role of ROS in radiation injury and the potential of antioxidants to reduce these deleterious effects have been studied for more than 50 years.[2] Radioprotective agents minimize this damage by reducing the generation of free radicals.[23] Increased understandings of the interrelationship between the effects of oxygen and radiation exposure lead to a rational application of naturally occurring antioxidants.[2] Our study demonstrated that in addition to an

antioxidant effect,[3] triphlorethol-A had protective effect against γ-radiation *in vitro*. Triphlorethol-A reduced the ROS, which only hydrogen peroxide was detected in this study, generated by radiation. Radiation-induced cellular lethality occurs through the formation of aqueous free radicals, which attack vital cellular sites, such as DNA and cell membranes. The cellular membrane and DNA are the two main targets of radiation-induced lethality. The formation of lipid peroxides in cells exposed to γ-radiation is one of the markers of the membrane damage. Triphloroethol-A protected the cell membrane lipids from the peroxidation damage induced by radiation. In addition, triphlorethol-A inhibited the increase of radiation-induced comet parameters, which indicated the protection of cellular DNA. These inhibitory effects of triphlorethol-A against lipid and DNA damage resulted in protective effects against radiation- induced cell death. Triphlorethol-A is polymer of phloroglucinol and has a polyphenol structure. The presence of a phenolic group with aromatic conjugation, which exists in the structure of triphlorethol-A, contributes to the scavenging ROS generated by irradiation. Triphlorethol-A increased cell survival via inhibition of radiation-induced apoptosis. This cytoprotective effect induced by triphlorethol-A was associated with the increased bcl-2 expression, the decreased bax expression and caspase 3 activity. Our study demonstrated that triphlorethol-A has a radioprotective effect through scavenging ROS generated by radiation.

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