Effect of Tinospora cordifolia on the Reduction of Ultraviolet Radiation-induced Cytotoxicity and DNA Damage in PC12 Cells

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Short title: Effect of Tinospora cordifolia on UV

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ABSTRACT

The safety of Tinospora cordifolia and its potential to protect against ultraviolet radiation-induced cytotoxicity and DNA damage in PC12 cells were investigated. To evaluate the safety of T. cordifolia, cell viability, and agarose gel electrophoresis were carried out using PC12 cells treated with 0 to 100 µg mL^{-1} of methanol extract of T. cordifolia. T. cordifolia extracts did not show cytotoxicity ranging 0 to 100 µg mL^{-1}. In addition, T. cordifolia extracts significantly increased cell viability at 1 ng, 10 ng, and 1 µg mL^{-1} concentrations in serum-deprived medium compared to control. To confirm the protective role against UV-induced damage, PC12 cells alone or in presence of 10 ng, 100 ng, or 1 µg mL^{-1} of T. cordifolia extract were exposed to 250, 270, and 290 nm of UV radiation, which corresponded to doses of 120, 150 and 300 mJ cm^{-2}, respectively. Treatment with T. cordifolia extracts significantly increased the cell survival rate irradiated at 290 nm. In addition, T. cordifolia extracts significantly reduced cyclobutane pyrimidine dimer formation induced by UV irradiation at all wavelengths. In conclusion, T. cordifolia is not toxic and safe for cells. Our findings can support its application as phototherapy in the medical sector.

Key words: UV radiation; cyclobutane pyrimidine dimer; DNA damage; Cell viability

INTRODUCTION

The study of human safety related to medicinal plant extracts has been gaining immense interest. Plants that have traditionally provided cures for various human ailments have been a major source of medicine. According to the WHO, up to 80% of people rely on medicinal plants for their ailments.[1] Tinospora cordifolia Miers which belongs to the Menispermaceae family, is a large, glabrous, deciduous, succulent, climbing shrub, which is distributed throughout the tropical regions of Indian subcontinent and China. The T. cordifolia extract has been reported to be rich in several bioactive components such as alkaloids; diterpenoid lactones; glycosides; steroids; sesquiterpenoid, phenolic, and aliphatic compounds; and polysaccharides.[2] Methanol extracts of T. cordifolia leaves are abundance of phenolic and flavonoid contents that exhibit anti-oxidant as well as superoxide radical scavenging activity.[3] Antioxidant saponarin, characterized as α-glucosidase inhibitor has been isolated from T. cordifolia methanol extracts show anti-diabetic property.[4] The therapeutic value of T. cordifolia is varied. It has been used in several indigenous drug preparations and exhibits anti-allergic,[5] anti-inflammatory,[6] and hepatoprotective activity.[7] Furthermore, alcoholic extracts of the plants has shown immunomodulatory, antitumor, and anti-neoplastic activity, and has been found to increase bone marrow precursor cells in tumor-bearing hosts.[8] However, little information exists on the effects of T. cordifolia extract on apoptosis or DNA damage.

Because T. cordifolia contains different kinds of bioactive components, the evaluation of its protective effect against photo-induced DNA damage is of great importance. Exposure to ultraviolet (UV) radiation present in sunlight is a major risk factor for the development of skin diseases such as erythema, edema, sunburn, hyperpigmentation, melanoma, and

non-melanoma forms of skin cancer.[9,10] UV radiation-subdivided into UVA (315-400 nm), UVB (280-315 nm), and UVC (200-280 nm)-is directly absorbed by DNA, and damages it.[11] The UV-induced damages are associated with cell-cycle arrest, cell death, mutation, neoplastic transformation, immunosuppression, and finally, photo aging and photocarcinogenesis.[12] Most of the mutagenic and carcinogenic effects of UV radiation have long been attributed to the shorter wavelengths of UVB and UVC.[13] Many plant products rich in bioactive molecules capable of protecting DNA from radiation-induced damage have been investigated.[14] Identification and development of safe, non-toxic, and effective radioprotective compounds are of enormous importance in mitigating the toxic effect of UV radiation-induced DNA damage.

Apoptosis is the programmed cell death that occurs as a normal developmental response to a physiological stimulus. Exposure to UV radiation triggers the apoptotic mitochondrial pathway if damage to the DNA is severe.[15] A rat pheochromocytoma (PC12) cell line has been shown to be a useful model for studying the mechanism of induction and inhibition of apoptosis. Furthermore, apoptosis is induced in PC12 cells when they are cultured in a serum-free medium. The PC12 cell system makes it possible to investigate the effect of trace amounts of chemical substances on the apoptotic reaction.[16,17]

The first aim of this study was to investigate the toxic effects of different concentrations of T. cordifolia on cell viability and apoptosis in PC12 cells. The second aim was to elucidate the protective effects of T. cordifolia on UV-induced cell toxicity and DNA damage by using the cell viability and enzyme-linked immunosorbent assay (ELISA) in PC12 cells that had been irradiated with 3 different UV wavelengths (250, 270, and 290 nm).

MATERIALS AND METHODS

Materials

PC12 cells were purchased from the American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was obtained from Hyclone (Rockville, MD, USA); Dulbecco's modified Eagle medium (DMEM), streptavidin-conjugated peroxidase, ribonuclease A (RNase), o-phenylenediamine dihydrochloride (OPD), and ethidium bromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). High Pure PCR Template Preparation Kit and blocking reagent were obtained from Roche Applied Science (Mannheim, Germany). Monoclonal antibody to cyclobutane pyrimidine dimer (CPD) was from Cosmo-Bio Co., Ltd. (Tokyo, Japan). Biotinylated goat anti-mouse immunoglobulin was from Amersham Pharmacia Biotech (Buckinghamshire, England); DNA marker from Promega (Madison, WI, USA); and Trypan blue stain solution (0.5%) from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical reagent grade.

Collection and Preparation of T. cordifolia Methanolic Extract

Dry powdered T. cordifolia was collected from Bangladesh. After air-drying, 10 gm of the material was extracted by macerating it for 24 to 48 h with continuous stirring in 100 mL of 70% methanol. The macerate was centrifuged at 1,500 rpm for 5 min, and the supernatant filtered using Whatman No. 1 filter paper. After the solvent was evaporated at room

temperature, a solid, dark, reddish-brown residue (yield 2.2%) was obtained. It was reconstituted with distilled water at concentrations of 1, 10, and 100 ng mL^{-1} and 1, 10, and 100 µg mL^{-1}, and these solutions were stored under refrigeration.

Cell Culture

The PC12 cells were maintained in DMEM in a humidified incubator at 37°C under 5% CO_2. After incubation in 25-cm^2 flasks overnight, the medium was replaced with a serum-rich or serum-deprived medium containing 0–100 µg of T. cordifolia methanol extract (TCME). Before transferring cells to the serum-deprived medium, they were washed twice with serum-free DMEM.

Cell Viability

Trypan blue exclusion assay was used to evaluate cell viability. After incubation with 0-100 µg TCME in serum-rich or serum-deprived medium for 48 h, the PC12 cells were stained with trypan blue 0.25% in phosphate-buffered saline. The total cells and trypan blue-stained cells were counted with using a hemocytometer (TC10™ automated cell counter, Bio-Rad, Hercules, CA). Cell viability was expressed as a percent ratio of total viable cells (unstained) against total cells (stained and unstained), and each experiment was carried out 3 times.

Isolation and Electrophoresis of Genomic DNA from PC12 cells

After treatment with 0-100 µg of TCME in serum-rich and serum-free medium for 48 h, the cells were washed with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and harvested using a scraper. The genomic DNA from the harvested PC12 cells was isolated using High Pure PCR Template Preparation Kit.

The pattern of genomic DNA was analyzed by agarose gel electrophoresis by using 5 µg of the isolated DNA on 1.5% agarose gel. The separated material was visualized with ethidium bromide stain under UV illumination.

UV-irradiation

PC12 cells from 35-mm tissue culture dishes were cultured for 24 h in serum media containing 0, 10 ng, 100 ng, or 1 µg mL^{-1} of TCME. The cells were washed twice with 2 mL of phosphate buffer saline, and the medium was changed by adding 2 mL of the same buffer solution. A xenon-lamp light source (MAX-301, Asahi Spectra, Tokyo, Japan), equipped with bandpass filters that isolate specific wavelength regions, was used for the UV irradiation.

Before exposure to each wavelength, the dose of UV-radiation was measured at the position of the target cells by using a radiometer (IL 1400 A, International Light Technologies, Peabody, MA) connected to a silicon-photodiode detector (SEL033, International Light

Technologies). The doses were determined according to the following formula: dose (mJ cm^{-2}) = exposure time (s) × irradiance (mW cm^{-2}). The cells in the dish were exposed to UV radiation in bandwidths of approximately 10 nm at 3 different wavelengths: 250, 270, and 290 nm at their corresponding median lethal doses (120, 150, and 300 mJ cm^{-2}) as previously reported.[18] Immediately after UV irradiation, the medium was changed with 10% FBS at different concentrations of TCME and incubated for 24 h.

Quantification of the Formation of CPD by using ELISA

Genomic DNA was extracted from the UV-irradiated PC12 cell (described above), and then 1× TBE was added to adjust the DNA concentration to 1 µg DNA μL^{-1}. DNA was denatured at 100°C for 10 min and then chilled immediately on ice for 15 min. After denaturation, 4 µL of the DNA and 46 µL of 1× TBE were added to each well in a titer plate (2 wells for each sample), and the DNA solutions were dried completely overnight at 37°C.

The CPDs were quantified using ELISA as previously described.[18] In briefly, after the overnight incubation, the titer plate was washed 3 times with a 40 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. To block unbounded areas in the wells, 100 µL of 2% blocking reagent in the same buffer was added and the solution was incubated for 30 min at 37°C. First antibody to the CPDs (diluted 1:1000 in the same buffer) was immobilized by incubating them for 1 h at 37°C. The second antibody, a biotinylated anti-mouse immunoglobulin (diluted 1:500 in the same buffer) was added, and the plate was incubated for 1 h at 37°C. Finally, after washing of wells streptavidin-conjugated peroxidase (diluted 1:400 with the same buffer) was added. The plate was incubated for 30 min at 37°C. To detect the activity of

binding enzyme, 0.1% OPD in 50 mM phosphate-citrate buffer (pH 5.0) containing 0.03% sodium perborate was added. Five to 10 min later, 6N HCl was added to stop the enzyme reaction. The absorbance at 495 nm was measured with a Microplate Reader (model 450; Bio-Rad).

Statistical Analysis

Each value is expressed as mean ± SEM. The statistical analysis was performed with the unpaired *t*-test. A difference was considered significant when $p < 0.05$. Each experiment was carried out 3–4 times.

RESULTS AND DISCUSSION

The present study demonstrated that in PC12 cells cultured in serum-deprived medium, TCME treatment at low concentrations (1 ng-1 µg mL^{-1}) enhanced the cell survival rate and was not itself cytotoxic to PC12 cells cultured in a serum medium (Figs. 1A and B). T. cordifolia treatment is known to ameliorate the leukopenic effects of cyclophosphamide by increasing neutrophils and white blood cells.[19] Other studies suggest that treatment with T. cordifolia increases red blood cells in anemic conditions.[20]

Cell Viability and DNA Electrophoresis

To determine whether the TCME itself was toxic, cell viability was measured by staining PC12 cells with trypan blue after exposing them to concentrations from 1 ng to 100 µg mL^{-1} TCME for 48 h. As shown in Figure 1A and Figure 2, no significant difference was found between the control cells in serum-rich medium without TCME and those cultured with TCME. TCME did not show cytotoxicity in PC12 cells cultured in a serum-containing medium. On the other hand, the cell viability of PC12 cells in serum-deprived medium was significantly higher than that of control cells following treatment with 1 ng, 10 ng, and 1 µg mL^{-1} of TCME (Fig. 1B). Apoptosis is induced in PC12 cells when they are cultured in serum-deprived medium. In the apoptotic situation, the cell viability was recovered by addition of TCME (Fig. 1 B). From the results, TCME reduced apoptosis induced by serum deprivation.

Viability of UV-irradiated Cells

To determine the effect of TCME on UV-induced toxicity, PC12 cells alone or treated with 10 ng, 100 ng, or 1 µg mL^{-1} TCME were irradiated at 250, 270, and 290 nm, corresponding to 120, 150, and 300 mJ cm^{-2}, and the cell viability was evaluated. The irradiation reduced the PC12 cell viability from 40% to 60%. The effect of TCME on PC12 cell survival rate after exposure to 3 different wavelengths of radiation was examined. At every concentration tested, TCME significantly increased the viability of the cells irradiated at 290 nm (Fig. 3).

Plants that exhibit anti-inflammatory, antimicrobial, immunomodulatory, anti-stress, lipid peroxidation inhibitory, and free-radical scavenging activity may also be radioprotectors.[21] Premanath and Lakshmidevi [3] report that leaf extracts of T. cordifolia inhibit lipid peroxidation. Methanol extracts of T. cordifolia exhibit antibacterial properties against Staphylococcus aureus, as reported by Rose et al..[22] On the other hand, (1,4)-α-D-glucan isolated from T. cordifolia activates the immune system by activating macrophages.[23] While a number of different therapeutic properties of TCME are already known, this study, for the first time, demonstrates its effects on UV-induced cytotoxicity and DNA damage in the PC12 cell system. TCME increased the cell survival rate at all the UV wavelengths tested, although significant protection against cytotoxicity was seen at 290 nm (Fig. 3). Cell death from UV light is caused by high amounts DNA damage, damage that is greatest at shorter wavelengths.[24] T. cordifolia has been shown to extend the life span of mice with Ehrlich ascites carcinoma exposed to 6 Gy of hemi-body gamma radiation. It reduced the mortality of Swiss albino mice from ^{60}CO-gamma radiation.[25] Another study demonstrated that in gamma-irradiated control mice, pre-irradiation treatment with a stem extract of *T. cordifolia* reduced mortality to 76.3% compared to the 100% mortality normally seen.[26]

Quantification of CPD Formation by ELISA

The formation of CPD in irradiated PC12 cells, with and without TCME treatment, was analyzed using ELISA. UV irradiation at 250, 270, and 290 nm enhanced CPD formation, compared to control cells that were not irradiated (data not shown). However, when the cells were subjected to TCME treatment before irradiation, the concentration of CPD was reduced

significantly. At all 3 radiation wavelengths, and at every concentration tested, TCME treatment significantly diminished the CPD formed at 250 nm (Fig. 4A). On the other hand, TCME at concentrations of 10 ng and 100 ng mL^{-1} induced a significant reduction in irradiation-induced CPD formation at 270 and 290 nm (Figs. 4B and C).

A previous report proposes that wavelengths shorter than 310 nm induce DNA damage through dimer formation.[27] Plant extracts rich in antioxidants evidently protect against the effects of UVB-induced DNA damage.[28] An earlier study by Russo et al.[29] affirms that methanol extracts of Picrorhiza kurroa, Celastrus paniculatus, and Withania somnifera exert a protective potential against DNA cleavage induced by H_2O_2-UV photolysis by scavenging free radicals. Rhodiola imbricata, a high-altitude plant also rich in antioxidants, exhibits both *in vitro* and *in vivo* radioprotection via a superoxide ion scavenging process.[30] Recently, Huang et al.[28] found that extracts of the leaves of Nelumbo nucifera Gaertn, because of its antioxidant effects, protects animal models against UVB-induced phototoxicity. Polyphenols (mainly phenols and flavonoids) are the major components of plants that showed antioxidant activity.[31] Chandrashekhar et al.[32] suggested that methanol extracts of *T. cordifolia*, found to contain copious amounts of polyphenol, protected human peripheral lymphocytes from oxidatively generated DNA damage and offered a 64% protection against Fe- and As-induced DNA fragmentation.

CONCLUSION

Doses of TCME significantly reduced the cytotoxicity and apoptosis induced by serum deprivation. In addition, TCME exerts significant protection against UVB- and

UVC-mediated DNA damage. DNA damage due to CPD formation was minimized by TCME treatment. These results suggest the possibility of using T. cordifolia plant extract as a photoprotective agent in clinical settings. However, to verify this probability, the exact molecular mechanism underlying the protective effect of T. cordifolia will be investigated.

DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This research was partially supported by Grants-in-Aid from the Japan Society for the Promotion of Science (No. 24350077 for Kurasaki). The authors also appreciated the technical assistance of Ms. Miyako Komori.

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FIGURES AND FIGURE CAPTIONS

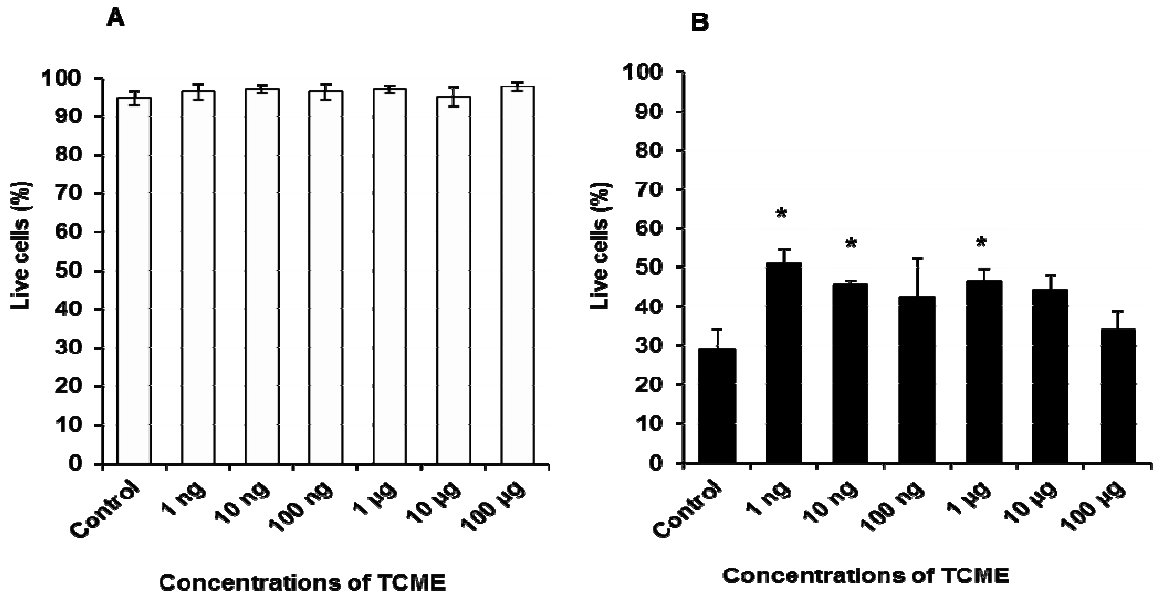

Fig. 1 Viability of PC12 cells treated for 48 h with 0–100 µg mL^{-1} TCME measured by trypan blue staining. PC12 cells cultured in a (A) serum-rich medium and a (B) serum-deprived medium. Error bars indicate SEM (n = 3). Asterisks denote values that are significantly different (*$p < 0.05$) from the control value.

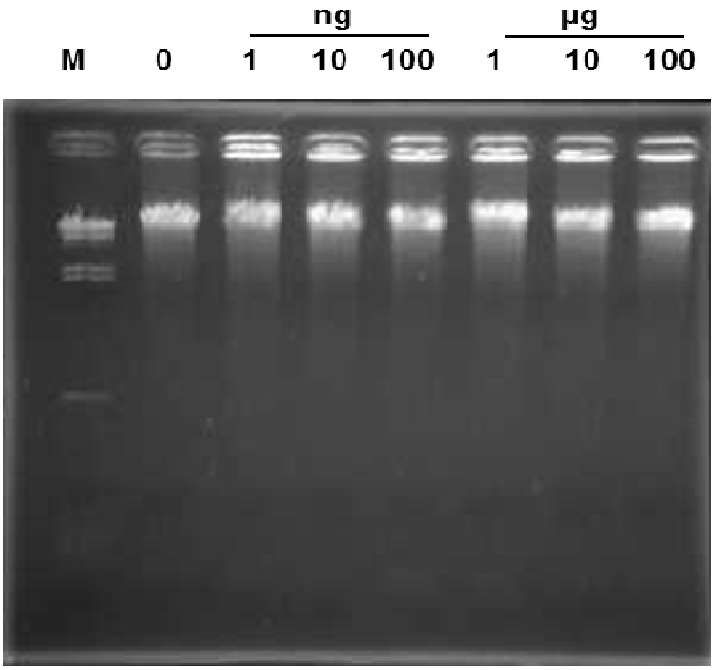

Fig. 2 Agarose gel electrophoresis of DNA extracted from PC12 cells. Cells treated with 0–100 μg mL^{-1} TCME and cultured for 2 days in a serum-rich medium. M indicates λDNA digested with HindIII as a DNA marker.

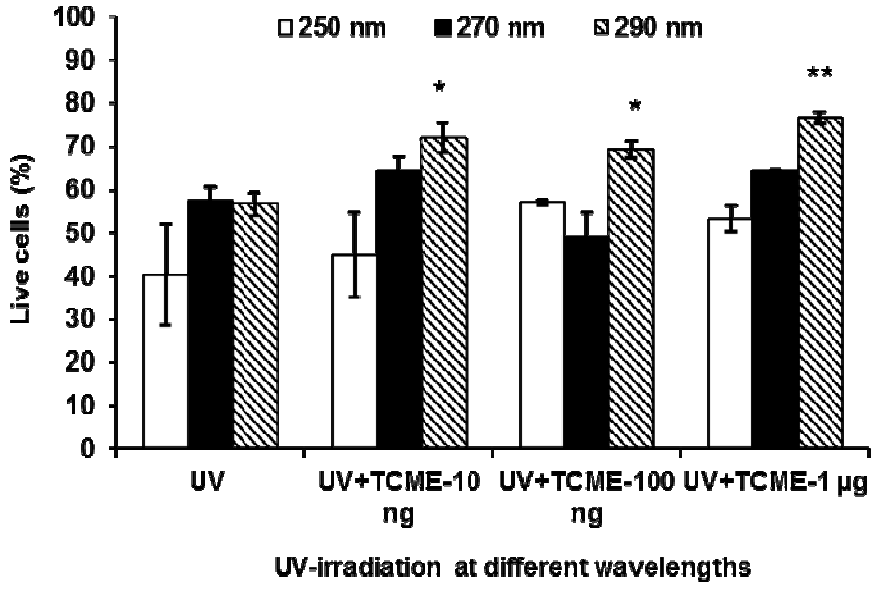

Fig. 3 Effect of TCME (10 ng, 100 ng, and 1 µg mL^{-1}) on viability of PC12 cells after UV irradiation with 250, 270, and 290 nm wavelengths, corresponding to doses of 120, 150, and 300 mJ cm^{-2}. Error bars indicate SEM (n = 3). *$p < 0.05$, **$p < 0.01$ indicate significant differences from the control PC12 cells irradiated without TCME.

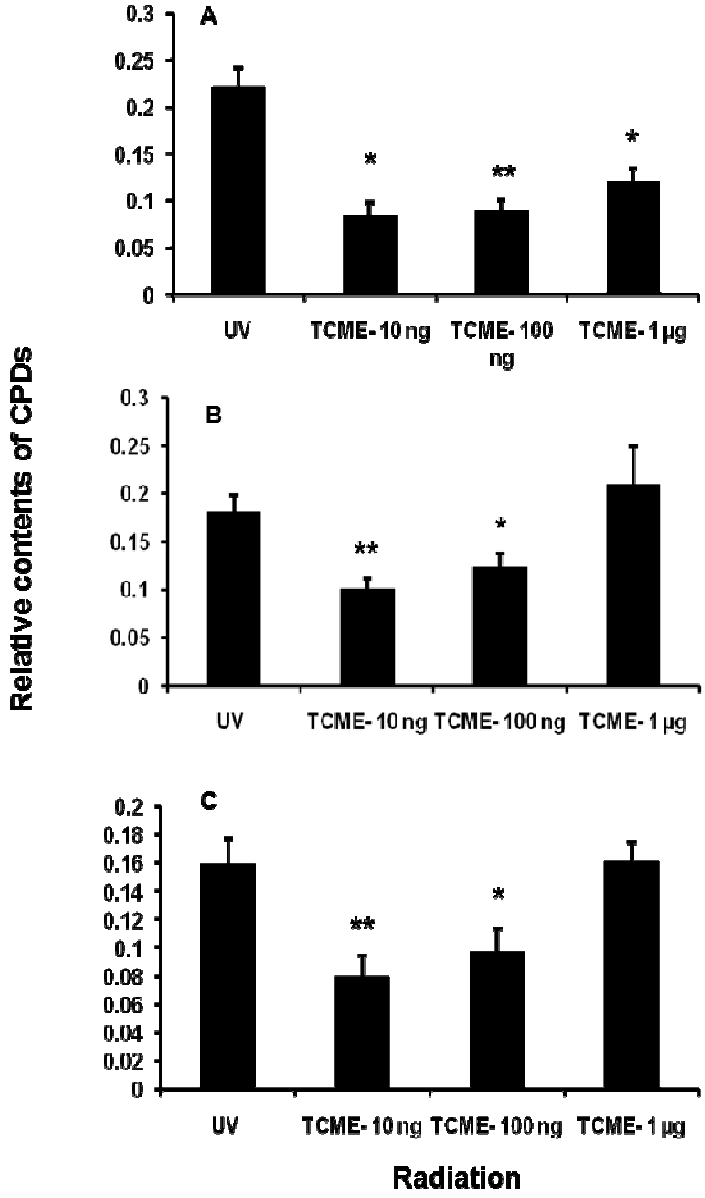

Fig. 4 Effect of TCME on the formation of CPDs 24 h after UV irradiation. After preincubation for 24 h with 0–1 µg mL^{-1} of TCME in 10% FBS containing serum medium, the cells were exposed to UV irradiation at 250, 270 and 290 nm, equivalent to exposures of 120, 150, and 300 mJ cm^{-2}. They were incubated for 24 h with TCME. The amount of CPD formed after (A) 250 nm, (B) 270 nm, and (C) 290 nm. Each column shows mean ± SEM (n = 4). Asterisks denote results that are significantly different (*$p < 0.05$, **$p < 0.01$) from those of cells irradiated without TCME.