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Antagonistic effects of Zingerone, a phenolic alkanone against radiation-induced cytotoxicity, genotoxicity, apoptosis and oxidative stress in Chinese hamster lung fibroblast cells growing *in vitro*

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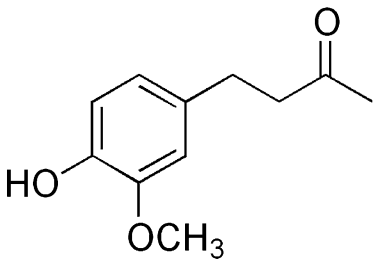
Zingerone (ZO), a dietary phenolic compound was investigated for its ability to protect against radiation-induced oxidative stress and DNA damage in Chinese hamster fibroblast cells (V79). Cells treated with optimal dose of ZO (25 μg/ml), 1 h prior radiation exposure resulted in a significant ($P < 0.01$) elevation of cell survival and decreased the genotoxicity (micronuclei and comet assays). Further, pretreatment with ZO significantly reduced radiation-induced oxidative stress as indicated by decreased reactive oxygen species levels and inhibition of mitochondrial depolarisation. The experiments conducted to evaluate the intracellular antioxidant activity in ZO-pretreated cells demonstrated a significant ($P < 0.01$) increase in the various antioxidants like glutathione, gluthione-*S*-transferase, superoxide dismutase, catalase and a significant ($P < 0.01$) decrease in malondialdehyde levels versus irradiation alone. Further, ZO scavenged various free radicals generated *in vitro* ($OH^{\cdot}$, $O_2^{\cdot}$, $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $NO^{\cdot}$) in a dose-dependent manner. The anti-apoptotic effect of ZO pretreatment was by the inhibition of the activation of capase-3, by upregulating Bcl-2 and down-regulating Bax proteins. Our study demonstrates the antagonistic effect of ZO against radiation-induced cytotoxicity. Further, ZO rendered anti-genotoxic, anti-apoptotic and anti-lipid peroxidative potency, plausibly ascribable to its antioxidant/free radical scavenging ability and also by the suppression of radiation-induced oxidative stress.

Introduction

Ionising radiation induces a variety of damages in cellular DNA, either by energy deposition on DNA (direct effect) or by reacting with diffusible water radicals (indirect effect) (1) leading to cell death or neoplastic transformation. In the presence of oxygen, ionising radiation leads to formation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and singlet oxygen (2). Hydroxyl radicals produced during radiolysis of water can trigger oxidation of lipids, amino acids and saccharides leading to formation of various secondary free radicals and reactive nitrogen intermediates. All these toxic products may further chemically modify DNA, proteins and lipids, causing cellular damage. Apart from the lipid peroxidation (LPO), ROS can also alter the balance of endogenous protective systems such as glutathione (GSH) and enzymatic antioxidant [superoxide dismutase (SOD) and catalase] defence systems (3). The endogenous antioxidant defences are inadequate to reduce the radiation-induced free radical changes. Antioxidants are regarded as compounds that are able to delay, retard or to prevent oxidation processes (4). Appropriate antioxidant intervention seems to inhibit or reduce free radical toxicity and thus offers protection against radiation. Antioxidants scavenge free radicals and hence are associated with reduced risk of cancer and cardiovascular diseases (5).

Phenolic antioxidants play an important role in the oxidation process by being preferentially oxidised by the attacking radicals. Because of their high redox potential, they can act as reducing agents, hydrogen donors, singlet oxygen quenchers and as metal chelating agents giving intrinsic antioxidant properties (6). To reduce ROS-mediated disorders, considerable research has been directed towards the discovery of novel antioxidants. In view of this, natural phenolic antioxidants with strong pharmacological action and less cytotoxic properties are highly acceptable. Herbal medicine has been used in Asia to improve the well-being of people for thousands of years and is regaining interest in health care (7). Apart from this, a number of dietary antioxidants have been reported to decrease free radical attack on biomolecules (8).

The rhizome of *Zingiber officinale*, commonly known as ginger, is known to contain oxymethyl phenols such as zingerone (ZO), gingerol, shogaol, paradol etc. in different proportions (9) and pharmacological properties. Most of these compounds have been reported to possess antioxidant and free radical scavenging activities (10), are known to increase intracellular antioxidant enzymes (11) and have an anti-lipid peroxidative effect (12).



Structure of Zingerone

(4-(4-hydroxy-3-methoxyphenyl)-2-butanone)

ZO, one of the active components, isolated from *Z.officinale* is a phenolic alkanone, containing a vanilloid (3-methoxy-4-hydroxy benzene) group in its structure. ZO exerts several

biological effects, inhibited ultraviolet (UV) radiation-induced mutation in *Escherichia coli* (13), scavenged superoxide anions and peroxynitrate radicals *in vitro* (14). Besides, ZO has also been demonstrated to exhibit potent radioprotective effects against radiation-induced toxicity in Swiss albino mice (15) and also inhibited liver microsomal LPO at higher concentrations (16). In present study, we document detailed effects of ZO to protect normal Chinese hamster lung fibroblast (V79) cells from the cellular injury induced by gamma radiation. We also characterize its mechanisms of action, by assaying antioxidant, DNA damage and apoptotic death.

Materials and methods

Chemicals

ZO, Trypsin (0.1%), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], trypan blue (0.1%), foetal calf serum (FCS), minimum essential medium (MEM), L-glutamine, penicillin, streptomycin, 2,2-diphenyl-2-picryl hydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), thiobarbutyric acid, phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT) and trichloroacetic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). Acridine orange (AO) was purchased from BDH Chemicals Ltd, Poole, England. All the other chemicals such as absolute alcohol, dimethyl sulphoxide (DMSO), EDTA, sodium bicarbonate, sodium chloride, disodium hydrogen phosphate, potassium chloride and hydrochloric acid were purchased from Qualigens Fine Chemicals (A Division of GlaxoSmithKline Pharmaceuticals), Mumbai, India.

Cell line and culture

The Chinese hamster lung fibroblast (V79) cells were obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown exponentially in 25 cm^2 T-flasks (Nunc, Roskilde, Denmark) with MEM supplemented with 10% FCS, L-glutamine (2 mM), 100 U/ml of penicillin and 100 μg/ml of streptomycin and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell irradiation procedure

Exponentially growing V79 cells were treated with or without ZO prior exposure to gamma radiation from ^{60}Co gamma teletherapy facility (Theratron; Atomic Energy Agency, Ontario, Canada) at the Shirdi Saibaba Cancer Hospital, Manipal, at a dose rate of 1 Gy/min and source-to-surface distance of 73 cm. The different radiation doses were used to assess the radioprotective potential and radiation doses selected were on the basis of earlier studies (17).

Preparation of ZO solution

ZO was dissolved in 0.01% absolute alcohol at a concentration of 1 mg/ml and diluted with MEM immediately before use.

Free radical scavenging by ZO

The scavenging activity of ZO on various radicals generated *in vitro* was measured by using hydroxyl (OH·) according to Halliwell *et al.* (18), superoxide $O_2^{\cdot}$ as described by Hyland *et al.* (19), $ABTS^{\cdot+}$ (2,2′-azinobis (3-ethyl benzothiazoline-6-sulphonic acid diammonium salt) radical scavenging assay described by Miller *et al.* (20) reduction of DPPH free radical according to the method of Mensor *et al.* (21) and nitric oxide scavenging potential by the method of Sreejayan and Rao (22) was carried out using spectrophotometer (UV-260; Shimadzu Corp, Tokyo, Japan). The inhibitory concentration (IC_{50}) was estimated and calculated as described by Kroyer (23). The modified methodologies for all these assays were described in detail in an earlier paper (24).

Assessment of cell cytotoxicity by MTT assay

The viability of cells under the influence of ZO and radiation alone or in combination of both was measured by MTT assay (25). V79 cells cultured in a 96-well plate were treated with various concentrations (0–100 μg/ml) of ZO alone for 24, 48 and 72 h or in combination with 10 Gy gamma radiation and further incubated for 24 h.

A separate experiment was carried out to study the radioprotective effect of ZO. V79 cells were treated with 25 μg/ml of ZO for 1 h before exposure to different doses (0, 7, 8, 9 or 10 Gy) of gamma radiation and the cells were allowed to grow for 24 h. The modified methodologies for this entire assay were described in detail in an earlier paper (26).

Clonogenic assay

Clonogenicity of cells treated with various agents was measured using colony-forming assay of Puck and Marcus (27). Briefly, a fixed number of cells (10^5) growing in 25-cm^2 culture flasks were pretreated with various concentrations of ZO (5–100 μg/ml) for 1 h before exposure 3 Gy gamma irradiation (IR).

A separate experiment was carried out to study its radioprotective effect in V79 cells. The optimum concentration of 25 μg/ml ZO was selected from previous experiment, cell cultures were divided into Group-I (radiation alone): the cultures of this group were exposed to different doses (2–10 Gy) of gamma radiation. Group-II (ZO + IR): the cultures of this group were treated with 25 μg/ml of ZO 1 h before exposure to different doses (2–10 Gy) of gamma radiation. The dose modifying factor (DMF) (the ratio of the ZO with radiation dose and the radiation dose alone) was estimated. The modified methodologies for this entire assay were described in detail in an earlier paper (26).

Experimental design

A fixed number (5×10^5) of exponentially growing cells were inoculated into several individual culture flasks and allowed to grow. An initial experiment was carried out to select the optimum radioprotective dose of ZO wherein V79 cells were divided into the following groups:

1. Control group: This group of cultures were not treated with ZO/radiation.
2. ZO-alone group: The cultures of this group were treated with various concentrations (0–100 μg/ml) of ZO for 1 h.
3. IR-alone group: The cultures of this group were exposed to 3 Gy of gamma radiation.
4. ZO + IR group: The cells of this group were treated with different concentration (0–100 μg/ml) of ZO for 1 h before exposure to 3 Gy gamma radiation.

The radioprotective potential of ZO was confirmed by dividing the cells into following groups:

1. IR-alone group: The cultures of this group were exposed to different doses (0–4 Gy) of gamma radiation.
2. ZO + IR group: The cultures of this group were treated with optimum concentration of ZO (25 μg/ml) for 1 h before exposure to different doses (0–4 Gy) of gamma radiation.

The cells from above groups were dislodged by mild trypsin EDTA treatment, micronucleus and comet assays were carried out from the same stock of cells.

Micronucleus assay

The micronuclei (MN) assay was carried out according to the method of Fenech and Morley (28). The modified methodologies for this entire assay were described in detail in an earlier paper (26). A minimum of thousand binucleated cells with well-preserved cytoplasm was scored from each culture and the frequency of micronucleated binucleate cells (MNBNC) was determined. The MN identification was done according to the criteria of Fenech and Morley (29). The cytokinesis blocked proliferation index (CBPI) was then calculated according to the method of Surralles *et al.* (30).

Single cell gel electrophoresis for DNA damage

This assay was performed under alkaline conditions according to the procedure of Singh *et al.* (31) with minor modifications of Collins *et al.* (32). To examine DNA repair at different post-incubation times, the cells were treated with or without 25 μg/ml of ZO for 1 h before exposure to 3 Gy gamma IR, cells from both the groups were taken and processed for comet assay at 30, 60, 120 and 180 min of post-IR treatment in an ice bath to stop the DNA repair. The modified methodologies for this entire assay were described in detail in an earlier paper (26).

Measurement of ROS using DCFH-DA and mitochondrial membrane potential

A fixed number (10^6) of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups. Group-I (radiation alone): the cultures of this group were exposed to 4 Gy of gamma radiation. Group-II (ZO + IR): the cultures of this group were treated with optimal concentrations of ZO (25 μg/ml) 1 h before exposure to 4 Gy of gamma radiation and the cells were allowed to grow further 30, 60 and 120 min. Finally, these cells were processed for mitochondrial membrane potential (MMP, $\Delta\psi m$) analysis and intracellular ROS measurement.

The changes in MMP were estimated using the fluorescent cationic dye Rhodamine 123 (33). Fluorescence spectrophotometry was used to measure intracellular ROS using 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) fluorescence assay as described by Bai and Cederbaum (34). The modified methodologies for this assay were described in detail in an earlier paper (26).

Apoptosis assays

A fixed number (10^6) of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into

following groups. Group-I (radiation alone): the cultures of this group were exposed to 10 Gy of gamma radiation. Group-II (ZO + IR): the cultures of this group were treated with different concentrations (0, 10, 25, 50 and 100 μg/ml) of ZO 1 h before exposure to 10 Gy of gamma radiation and the cells were allowed to grow. Twenty-four hours after post-incubation, media and cells were dislodged by trypsin EDTA (0.1%) treatment and the cells were processed for apoptosis assays. On the basis of earlier study (17), the radiation doses of 10 Gy was selected as this dose was induced detectable levels of apoptotic cells both in quantitative (apoptotic index) and qualitative (DNA ladder assay) assays.

Fluorescence microscopic analysis of apoptotic cells

The morphological changes that occur in a cell during apoptosis were also analysed by the differential uptake of fluorescent DNA binding of AO and Ethidium bromide (EtBr) stains as described earlier (35). The modified methodologies for this assay were described in detail in an earlier paper (36).

Detection of DNA fragmentation by agarose gel electrophoresis

The formation of internucleosomal DNA fragmentation (ladder formation) as the qualitative indication of apoptosis was performed according to the protocol described earlier (37), with minor modifications (26).

Measurement of apoptosis by Annexin V

The externalisation of phosphatidylserine, another reliable early apoptotic marker, was detected in V79 cells using Annexin V-FITC Kit (Sigma–Aldrich, St Louis, MO, USA) that differentiates between viable apoptotic and necrotic cells.

Immunoblot analysis

A fixed number (10^6) of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups. Group-I (radiation alone): the cultures of this group were exposed to 10 Gy of gamma radiation. Group-II (ZO + IR): the cultures of this group were treated with optimal concentration of ZO (25 μg/ml) 1 h before exposure to 10 Gy of gamma radiation and the cells were allowed to grow further 24 h. The cells from all the above groups were harvested and washed twice in ice-cold phosphate-buffered saline. Cell pellets were treated with the protein lysis buffer (40 mM HEPES, 500 mM NaCl, 0.1% Nonidet P-40, 10% glycerol and 1 μg/ml protease cocktail inhibitor) for 2 h. The cell lysates were then centrifuged, and the protein content was quantified by the method of Bradford (38). The total proteins (20 μg) were resolved on 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred onto the nitrocellulose membrane and then immunoblotted with the corresponding antibodies. Anti-Bcl_2, anti-Bax, anti-caspase-3 and anti-actin antibodies obtained from Millipore (Temecula, CA, USA). The immobilised proteins were incubated with goat anti-rabbit IgG (Millipore). Protein expression levels were detected using enhanced chemiluminescence western blotting detection reagents (Thermo scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Biochemical analysis

The cells were treated with 25 μg/ml of ZO for 1 h before exposure to 3 Gy gamma radiation and the cells were allowed to grow for further for 24 h. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay as described below. Concentrations of total protein were measured by the method of Bradford (38). The cells were lysed at 4°C for 2 h using 5% w/v metaphosphoric acid (chilled) to extract the cellular GSH by the method of Moron *et al*. (39). Gluthione-*S*-transferase (GST) activity was determined according to the procedure of Habig *et al*. (40). SOD activity was assayed by NBT of Beauchamp and Fridovich (41). The catalase activity was estimated by the method of Abei (42). LPO products were determined by thiobarbituric reactive substance (TBARS) as described by Buege and Aust (43). LPO was expressed as malondialdehyde (MDA) per milligram total protein. The modified methodologies for all these assays were described in detail in an earlier paper (44).

Statistical analysis

The significance of the differences between treatments and respective controls was analysed using the Student's *t*-test and one-way analysis of variance and with Bonferroni's post hoc test using GraphPAD InStat (La Jolla, CA, USA). All the data were expressed as mean ± SEM.

Results

Free radical scavenging assays

DPPH radical scavenging effect. In the present investigation, ZO at different doses demonstrated significant DPPH scavenging activity indicating their abilities to act as radical scavengers. ZO at the concentration of 75 μg/ml exhibited 68.18% inhibition, whereas a standard compound α-tocopherol (Vitamin E) at a concentration of 100 μg/ml exhibited 81.53% of inhibition. The IC_{50} value of ZO was found to be 61.4 μg/ml, whereas the IC_{50} value of Vitamin E was found to be 53.5 μg/ml (Figure 1A).

Nitric oxide radical scavenging effect. Nitric oxide (NO) is an important chemical mediator when it reacts with oxygen it generates nitrite and peroxynitrite anions, which act as free radicals. In the present study, ZO competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. The IC_{50} value of ZO was found to be 58.7 μg/ml, whereas the IC_{50} value of standard compound curcumin was found to be 15.97 μg/ml (Figure 1B).

Superoxide anion radical scavenging effect. Superoxides are produced from molecular oxygen via non-enzymatic reactions such as autoxidation. In the present study the superoxide scavenging effect of ZO and curcumin on the PMS/NADH–NBT system was studied. ZO at concentration of 10–100 μg/ml inhibited the production of superoxide anion radicals by 10.3–63.45%. The IC_{50} value of ZO on superoxide radical scavenging activity was found to be 29.27 μg/ml and for curcumin it was 8.9 μg/ml (Figure 1C).

Hydroxyl radical scavenging effect. Hydroxyl radicals are the major active species causing lipid oxidation. In the present study, we tested the scavenging activity of ZO along with ascorbic acid as positive control. The concentration of ZO needed for 50% inhibition was found to be 58.1 μg/ml. Ascorbic acid used as a standard was highly effective in inhibiting the oxidative DNA damage, showing an IC_{50} = 10.6 μg/ml (Figure 1D).

Total antioxidant status. The total antioxidant activity was measured using ABTS assay and the inhibition of $ABTS^{\cdot+}$ radicals showed a dose-dependent scavenging up to 100 μg/ml. The IC_{50} value of ZO was found to be 43.09 μg/ml, whereas the IC_{50} value of Vitamin E was found to be 21.27 μg/ml (Figure 1E).

MTT assay. Exposure of V79 cells to different concentrations of ZO did not alter the cell viability at 24, 48 and 72 h significantly, whereas radiation exposure decreased the cell viability as expected (Figure 2A). Treatment of V79 cells with various concentrations of ZO before exposure to 10 Gy gamma IR resulted in a gradual increase in the cell viability (MTT formazan formation) and a maximum viability of 69.6, 52.4 or 42.2% was observed at a concentration of 25 μg/ml ZO at 24-, 48- and 72-h post-IR treatment time (Figure 2A). Exposure of V79 cells to different doses of gamma radiation resulted in a dose-dependent reduction in the cell viability (Figure 2B). Treatment of V79 cells with ZO (25 μg/ml) before exposure to different doses of gamma radiation inhibited the radiation-induced decline in the cell viability. The increase in cell viability was significantly greater in ZO + IR group when compared to IR-alone-treated group (Figure 2B). A significant elevation in cell viability of 1.10-, 1.12-, 1.13- and 1.16-fold was observed in ZO-pretreated groups in comparison with 7, 8, 9 and 10 Gy gamma radiation-alone-treated group (Figure 2B).

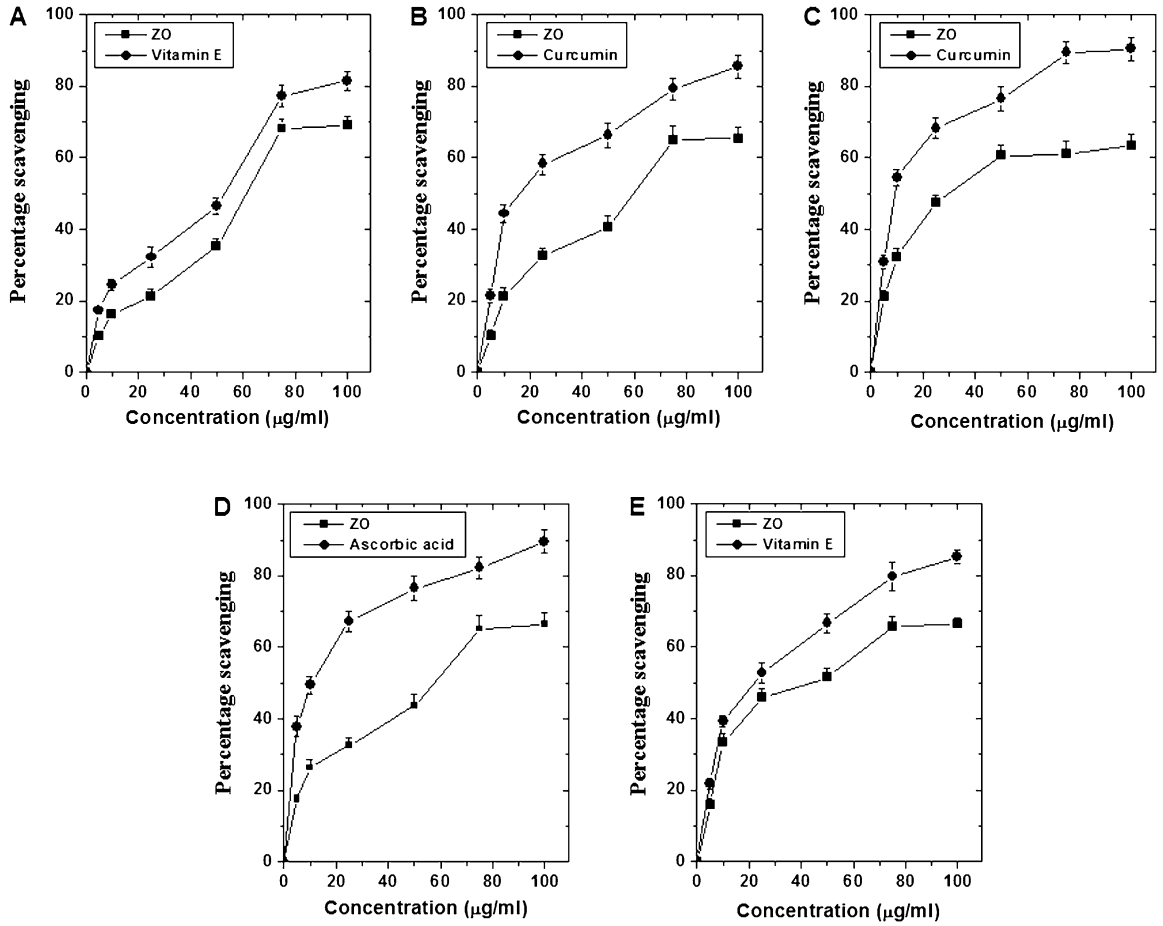


Fig. 1. Effect of various concentrations of ZO on the scavenging of free radicals generated *in vitro* systems. (**A**) DPPH·, (**B**) Nitric oxide (NO·), (**C**) Super oxide (O_2·), (**D**) Hydroxyl (OH·), (**E**) ABTS·$^+$.

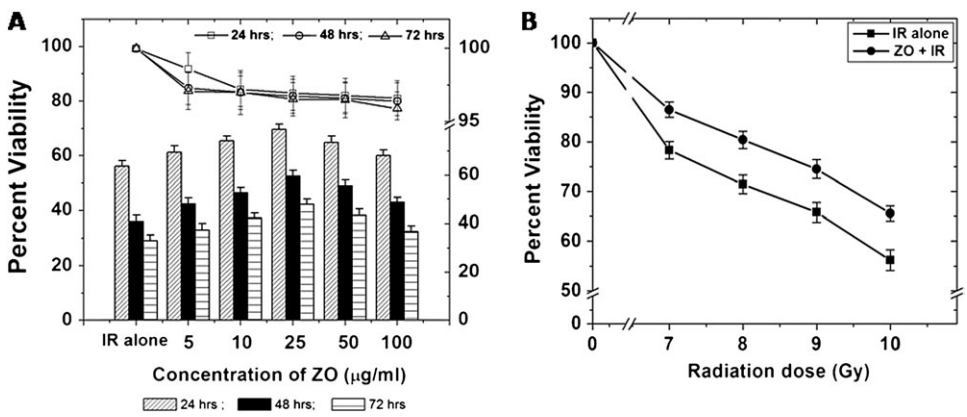


Fig. 2. (**A**) Percent viability assessed by MTT assay, V79 cells treated with various concentrations of ZO alone for 24, 48 or 72 h and V79 cells pretreated with different concentrations of ZO before exposure to 10 Gy of gamma radiation and further incubated for 24, 48 or 72 h. (**B**) V79 cells following 1-h treatment with 25 μg/ml ZO before exposed to different doses of gamma radiation and further incubated for 24 h. All the results are shown as mean ± standard error of the mean from the data of a minimum of three separate experiments.

Clonogenic assay

In the present study, clonogenic survival assay was performed with 3 Gy of gamma radiation alone or combination with increasing concentration of ZO (5–100 μg/ml) to assess the radioprotective potential of ZO. As shown in Figure 3A, clonogenic survival of IR-treated V79 cells was significantly reduced. The treatment of cells with various doses of ZO before IR significantly ($P < 0.01$) increased clonogenic survival and a maximum increase was observed at a concentration of 25 μg/ml ZO (Figure 3A).

Treatment of V79 cells with different doses (2–10 Gy) of gamma radiation resulted in a dose-dependent decline in the cell viability as evidenced by the significant reduction in surviving fraction. However, treatment of V79 cells with 25 μg/ml ZO before exposure to different doses of gamma radiation resulted in a significant increase in the cell viability

when compared with the radiation-alone group. Radioprotective effect of ZO increased with increasing doses of radiation in ZO + IR group when compared with the concurrent IR treatment alone with the DMF of 1.24 (Figure 3B).

MN assay

The protection against radiation-induced genotoxicity by ZO was studied by the micronucleus assay. ZO treatment did not alter the spontaneous frequency of MN in binucleate cells. However, a marginal but non-significant decline in MNBNC was observed up to a concentration of 100 μg/ml after ZO treatment alone (Figure 4A). Treatment of V79 cells with 3 Gy gamma radiation resulted in a significant elevation in the MNBNC. Exposure of V79 cells to different concentrations of ZO before exposure to 3 Gy radiation caused a significant decline in the radiation-induced MN formation and a maximum decrease in MNBNC was observed for 25 μg/ml ZO. This oncentration was considered as the optimum protective concentration as the frequency of MN was minimal at this concentration when compared to other concentrations of ZO. Therefore, further studies were carried out using this ZO concentration. CBPI was significantly reduced in the IR group,

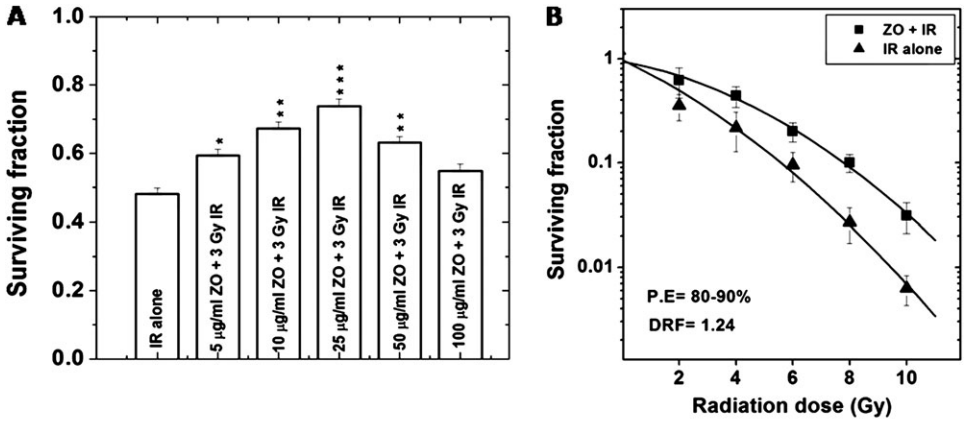


Fig. 3. (**A**) Changes in V79 cell survival after treatment with different concentrations of ZO prior exposure to 3 Gy gamma radiation. (**B**) Changes in V79 cell survival after treatment with 25 μg/ml ZO for 1 h before exposure to different doses of gamma radiation. All the values were shown as mean ± standard error of the mean from the data of a minimum of three separate experiments. The significant levels $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and no symbol = non-significant, when compared with radiation-alone group.

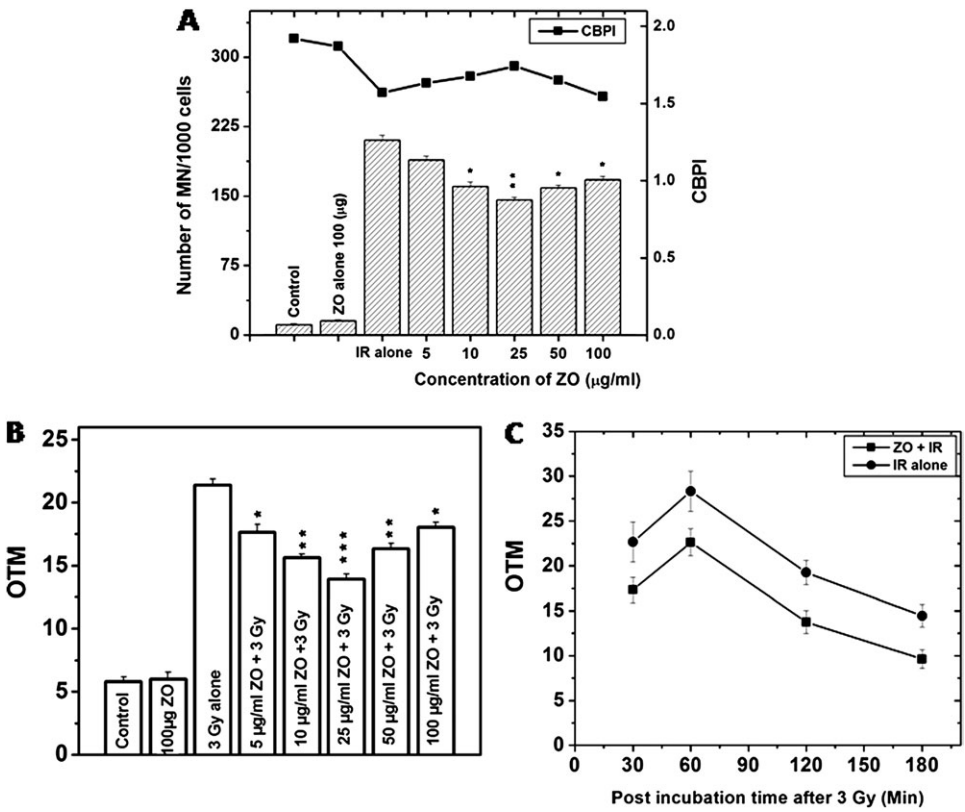


Fig. 4. (**A**) Frequency of MN and CBPI values of different concentrations of ZO prior treatment for 1 h against 3 Gy IR-induced MN in V79 cells. (**B**) Effect of different concentration of ZO on the DNA damage induced by IR in V79 cells. (**C**) Modulation in IR-induced DNA damage by 25 μg/ml ZO at various post-IR treatment times. All the results are shown as mean ± standard error of the mean from the data of a minimum of three separate experiments. The significant levels $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and no symbol = non-significant, when compared with control group.

when compared to untreated control group (Figure 4A) indicating the radiation-induced delay in the cell proliferation. Pretreatment with the different concentrations of ZO resulted in normalised CBPI when compared to IR-alone treatment (Figure 4A).

Radioprotective effect of ZO

The V79 cells exposed to different doses of radiation-induced a dose-dependent linear ($R^2 = 0.9914$) increase in the MN counts (Table I). Pretreatment of V79 cells with 25 μg/ml ZO before exposure to different doses of radiation resulted in a significant ($P < 0.0001$) decline in the frequency of MNBNC when compared with the concurrent radiation-alone group. The frequency of MNBNC with one, two and multiple MN are presented separately. The frequency of binucleate cells bearing one, two and multiple MN increased in a concentration-dependent manner and the highest number of MN was observed for 4 Gy IR. ZO pretreatment resulted in a significant decline in the induction of cells with one, two and multiple MN. The frequencies of two and multiple MNBNC were always lower in the ZO + IR group when compared with the concurrent radiation-alone group (Table I).

Comet assay

Cytotoxicity of radiation is related to its capacity to induce DNA damage. In view of this, we investigated whether ZO will modulate the genotoxic effect of radiation as assessed by DNA strand break analysis using single cell gel electrophoresis. The results are expressed as mean tail DNA and olive tail moment (OTM) in Figure 4B.

Baseline DNA damage did not change significantly with assay time (Figure 4B). In all cases, the DNA damage of control cells was constant, indicating that preparation and subsequent processing of the V79 cells did not introduce significant damage to cellular DNA. V79 cells exposed to 3 Gy gamma radiation caused a significant increase in DNA damage as evident by increased migration of DNA into the comet tails. Whereas, treatment of V79 cells to different concentrations of ZO before exposure to 3 Gy radiation caused a significant ($P < 0.001$) decrease in the percent tail DNA and OTM at 25 μg/ml ZO (Figure 4B).

A maximum DNA damage was observed at 60 min post-IR treatment time that showed a subsequent decline in the radiation-induced DNA damage with assay time that was reflected in increased tail DNA and OTM. The decline in OTM indicated repair of DNA damage that was maximum up to 60 min post-IR treatment time and subsequently decreased up to 180 min post-IR treatment time (Figure 4C). Treatment of V79 cells with 25 μg/ml ZO before exposure to 3 Gy radiation caused a significant reduction in the DNA strand breaks at all post-IR treatment times in ZO + IR group when compared to IR-alone group. ZO treatment allowed early repair of DNA damage when compared to IR treatment alone. The OTM at 120 min of ZO pretreated group was equal to that of 180 min IR treatment alone, indicating rapid repair in the former than latter group (Figure 4C).

A separate experiment was conducted to study the influence of ZO on the DNA damage induced by different doses of gamma radiation. Treatment of V79 cells with different doses of gamma radiation caused a dose-dependent elevation in the DNA damage measured as tail DNA and OTM (Table II). Treatment of V79 cells with 25 μg/ml ZO before exposure to different doses of gamma radiation showed a dose-dependent reduction in the DNA damage in ZO + IR group when compared with IR treatment alone. The reduction in the DNA damage by ZO was statistically significant ($P < 0.001$) at all concentrations of IR, when compared with the respective IR treatment-alone group (Table II).

Effect of ZO on radiation-induced apoptosis

Fluorescence microscopic analysis. Microscopic AO/EtBr dual staining was used to detect morphological evidence of apoptosis on the ZO and gamma-irradiated cells. As shown in Table III, the irradiated group showed a significant increase in the apoptotic index, which was minimised by ZO pretreatment.

DNA fragmentation by agarose electrophoresis. Analysis of DNA from apoptotic cells by agarose electrophoresis produced a characteristic DNA ladder pattern, which is regarded as a biochemical hallmark of apoptosis. Cells exposed to 10 Gy gamma radiation showed the typical DNA ladder pattern (Figure 5A, lane 4). ZO (25 μg/ml) (Figure 5A, lane 6) pretreatment inhibited this radiation-induced DNA fragmentation when compared with other ZO + IR groups (Figure 5A).

FITC–Annexin V staining. To assess the type of cell death induced by gamma radiation, V79 cells treated with or without ZO/radiation were assessed with a flow cytometry by a double staining with annexin V and propidium iodide (PI). The annexin V-FITC-positive population of the cells (apoptotic cells; lower right quadrant) was not significantly increased in the control and ZO-alone-treated cells [Figure 5B (a and b)]. However, the apoptotic cell population was increased to 18.62%, and also the annexin V-FITC-positive/PI-positive population (necrosis and late apoptotic cells; upper right quadrant) was increased to 11.33% in the radiation-alone group

Table I. Radioprotective effect of optimum concentration of ZO (25 μg/ml) for 1 h on radiation-induced MN in V79 cells

IR dose (Gy)	Frequency of BNC cells bearing MN ± SEM							
	One		Two		Multiple (three or more)		Total	
	DDW + IR	ZO + IR	DDW + IR	ZO + IR	DDW + IR	ZO + IR	DDW + IR	ZO + IR
0	9.50 ± 1.21	10.75 ± 1.47	1.5 ± 0.56	1.75 ± 0.21	0 ± 0	0 ± 0	11.0 ± 0.64	12.50 ± 1.78
1	86.25 ± 1.32	54.06 ± 0.91	5.75 ± 1.08	2.0 ± 0.64	1.25 ± 0.64	0.75 ± 0.25	93.25 ± 1.93	56.81 ± 1.25****
2	164.75 ± 1.08	116.49 ± 1.24	7.5 ± 1.47	3.5 ± 1.04	2.75 ± 1.21	1.25 ± 0.40	175.0 ± 2.47	121.24 ± 2.70****
3	198.51 ± 1.21	133.75 ± 3.54	8.5 ± 0.56	9.75 ± 1.49	3.24 ± 0.64	2.50 ± 1.21	210.25 ± 5.15	146.02 ± 3.07****
4	308.0 ± 1.47	250.23 ± 1.68	14.25 ± 1.21	11.02 ± 1.84	5.0 ± 1.29	3.5 ± 0.95	327.25 ± 4.95	264.75 ± 3.24****

All results are shown as mean ± SEM from the data of three independent experiments. The significant levels ****, $P < 0.0001$, when compared with group respective radiation-alone group. DDW, double-distilled water.

[Figure 5B (c)]. As shown in Figure 5B, treatment with various concentrations of ZO (10–100 μg/ml) prior to radiation reduced both apoptotic and necrosis cells in ZO + IR-treated groups [Figure 5B (d–g)], the maximum reduction in apoptotic percentage was observed at 25 μg/ml of ZO [Figure 5B (e)] when compared with all other ZO-treated groups.

Table II. Effect of various doses of radiation on V79 cells pretreated with optimum concentration of ZO (25 μg/ml)

IR (Gy)	% Tail DNA (mean ± SEM)		OTM (mean ± SEM)	
	IR alone	ZO + IR	IR alone	ZO + IR
0	4.71 ± 0.49	5.01 ± 0.51	2.17 ± 0.19	2.25 ± 0.75
1	13.69 ± 1.01	7.04 ± 0.73***	9.93 ± 1.06	5.12 ± 0.68**
2	18.35 ± 1.03	10.82 ± 1.26***	15.20 ± 1.02	8.04 ± 0.85***
3	24.58 ± 1.31	16.15 ± 0.75***	21.39 ± 1.47	13.92 ± 1.40**
4	29.35 ± 1.55	22.35 ± 1.52**	26.41 ± 1.13	19.41 ± 1.62**

All the results are shown as mean ± SEM from the data of a minimum of three separate experiments. The significant levels **, $P < 0.01$, ***, $P < 0.001$ and no symbol, non-significant, when compared with respective IR-alone group.

Table III. Results of apoptotic index by AO/EtBr staining of V79 cells treated with different concentrations of ZO for 1 h before exposure to 10 Gy gamma radiation

Concentration (μg/ml)	Apoptotic index ± SEM	
	ZO alone	ZO + IR
0	4.25 ± 0.51	27.52 ± 2.25
10	4.51 ± 1.03	22.05 ± 2.08
25	4.82 ± 0.95	15.83 ± 1.84*
50	4.48 ± 0.72	19.62 ± 1.75
100	4.65 ± 0.81	21.05 ± 2.16

All the results are shown as mean ± SEM from the data of a minimum of three separate experiments. The significant levels *, $P < 0.05$ and no symbol, non-significant, when compared with IR-alone group.

IR-induced cellular ROS production and inhibition by ZO

Radiation-induced ROS in V79 cells was determined by measuring fluorescence after loading with DCHF-DA, a dye that is oxidised into a highly fluorescent form in the presence of peroxides. To specify the active oxygen species that were responsible for oxidation of DCHF, we examined whether ZO prevents the oxidation of DCHF. IR alone caused a significant time-dependent increase in ROS generation in the V79 cells, beginning at 30 min (the earliest time point measured) resulting in levels up to 3.0-fold and which increased to 3.76- and 3.83-fold after 60 and 120 min of post-incubation as compared with that of control basal level. However, addition of ZO inhibited the increase in DCHF oxidation as measured at 30, 60 and 120 min of post-IR when compared to IR-alone levels. A known reactive oxygen intermediate scavenger, *N*-acetyl cysteine (1 mM), was used as a positive control, shown to be capable of significantly time-dependent decrease in ROS generation in the V79 cells when compared with other treatment groups (Figure 6A).

Measurement of MMP

To examine IR-induced loss of the MMP, V79 cells exposed to 4 Gy gamma radiation showed a significant loss of the MMP as measured by the uptake of fluorescent cationic dye Rhodamine 123 at 60 min post-incubation time compared to the control. Pretreatment with ZO for 1 h significantly delayed the IR-induced collapse of MMP but was not able to maintain MMP at control levels (Figure 6B).

Enzymatic antioxidant activity (biochemical estimations)

In the present study, the major antioxidants, GSH, GST, SOD and catalase, were analyzed to determine the potential involvement of various types of ROS scavengers in irradiated V79 cells. Our results show significant ($P < 0.01$) decrease in GSH, GST, SOD and catalase activity in the irradiated V79 cells (3 Gy) when compared to the untreated control group. Our results demonstrate 25-μg ZO treatment significantly ($P < 0.01$)

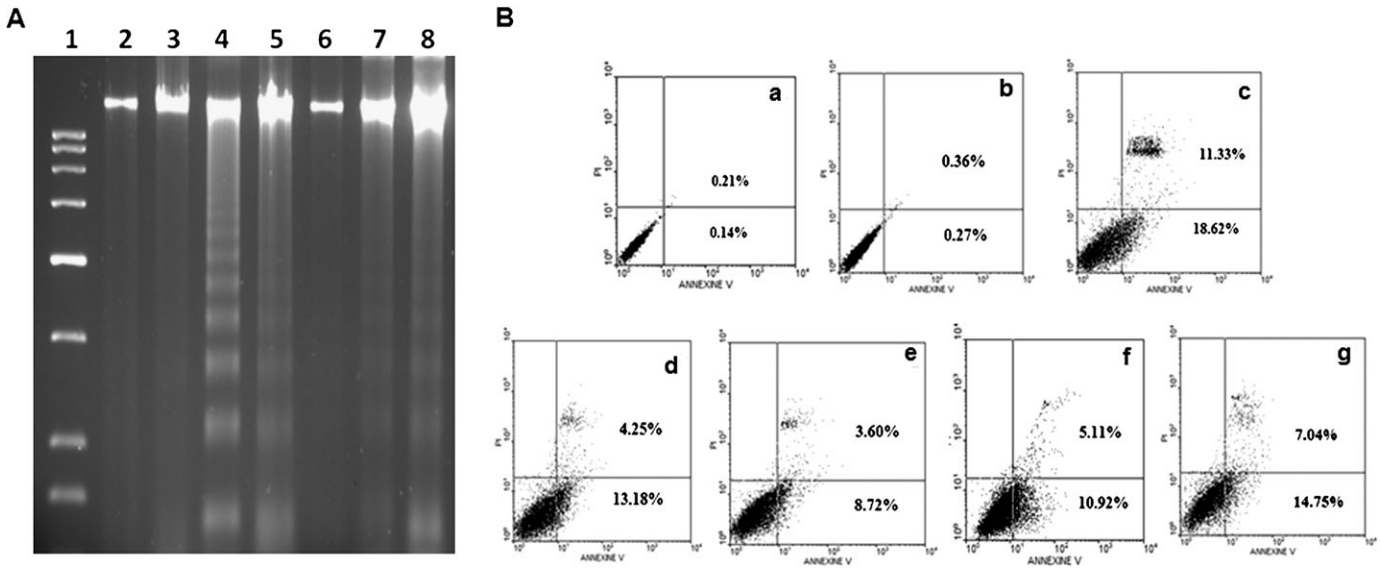


Fig. 5. (**A**) Effect of ZO on radiation-induced DNA fragmentation in the V79 cells. Lane 1, 3-kb ladder marker; lane 2, Control V79 cells (Untreated); lane 3, ZO (50 μg) alone; lane 4, 10 Gy (IR) alone; lane 5, ZO (10 μg) + IR; lane 6, ZO (25 μg) + IR; lane 7, ZO (50 μg) + IR and lane 8, ZO (100 μg) + IR. (**B**) Inhibitory effect of ZO on radiation-induced cell apoptosis assessed by a flow cytometry. Cells were incubated with various concentrations of ZO (10–100 μg/ml) and irradiated with 10 Gy. Early-apoptotic cells, which are the annexin V-FITC-positive/PI-negative population of cells, are reported in the lower right quadrant. Necrotic and late apoptotic cells, which are the annexin V-FITC-positive/PI-positive population of cells, are reported in the upper right quadrant; (a) Control V79 cells, (b) ZO (100 μg) alone, (c) 10 Gy (IR) alone, (d) ZO (10 μg) + IR, (e) ZO (25 μg) + IR, (f) ZO (50 μg) + IR and (g) ZO (100 μg) + IR.

increased GSH, GST, SOD and catalase level when compared to the IR-alone group (Figure 7A and B). The levels, however, could not be bought back all the way to control levels.

TBARS is one well-accepted index of LPO. In the present study, LPO levels were significantly ($P < 0.01$) higher in the irradiated group when compared to the control or ZO-treated groups (Figure 7B). Pretreatment with ZO (25 μg/ml) significantly ($P < 0.01$) reduced LPO when compared to the IR-alone group (Figure 7B).

Effect of ZO on radiation-induced caspase-3, Bcl-2 and Bax expression

Exposure of V79 cells to gamma radiation showed increase in the caspase-3 activation that executes the apoptosis. Cells pretreated with or without ZO (25 μg/ml) significantly inhibited the activation of capase-3 indicating inhibition of apoptosis. Bcl-2 and Bax are apoptosis-related proteins and play an important role in keeping mitochondrial membrane integrity and permeability. We examined the level of these proteins in 10-Gy irradiated cells with or without ZO (25 μg/ml) pretreatment. Down-regulated Bcl-2 and upregulated Bax were found in 10-Gy irradiated V79 cells, while pretreatment with ZO partly reversed this tendency (Figure 8).

Discussion

IR with gamma rays brings about a range of functional changes in cells. The effects of gamma IR appear due to their aptitude to interact with the different cell organelles. A significant part of the initial damage done to cells by ionising radiation is due to the formation of free radicals, which reacts with almost all cellular components to induce oxidative damage to DNA, LPO and protein oxidation that lead to the destruction of cells damaged beyond their repair capabilities (45). There is considerable literature to suggest that free radical scavengers can be used to prevent oxidative damage and cell death, including apoptosis caused by ionising radiation. Phenolic compounds are known to exhibit antioxidant effects and cytoprotective effects against radiation-induced oxidative stress (46). In the present study, due to presence of a phenolic group with an aromatic conjugation in ZO, an attempt was made to evaluate its protective potential against radiation-induced cytotoxicity, genotoxicity and apoptotic effect and oxidative stress.

Earlier many of our studies have reported that radiation exhibits mutagenic and cytotoxic effects in V79 cells (15,24,44). As anticipated, V79 cells exposed to gamma radiation resulted in elevation in cytotoxicity as evident by the continuous decline of viable cells with increasing dose of IR.

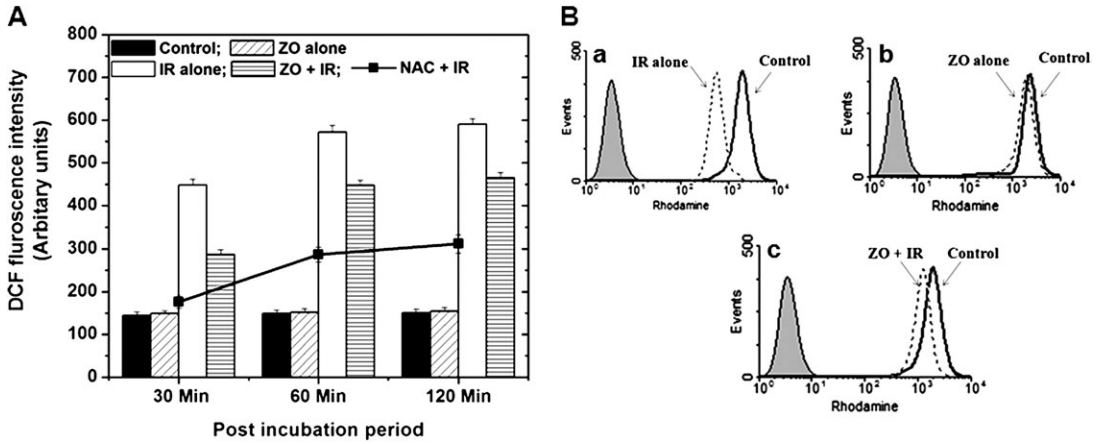


Fig. 6. (**A**) Effect of optimum dose of ZO (25 μg) treatment on radiation (4 Gy)-induced ROS levels in V79 cells and post-incubation for different time periods. (**B**) Effect of ZO (25 μg/ml) on radiation-induced (4 Gy) MMP. The MMP was evaluated by the capacity of V79 cells to take up the fluorescent cationic dye Rhodamine 123; (a) Control, (b) ZO (25 μg/ml) alone and (c) ZO + IR.

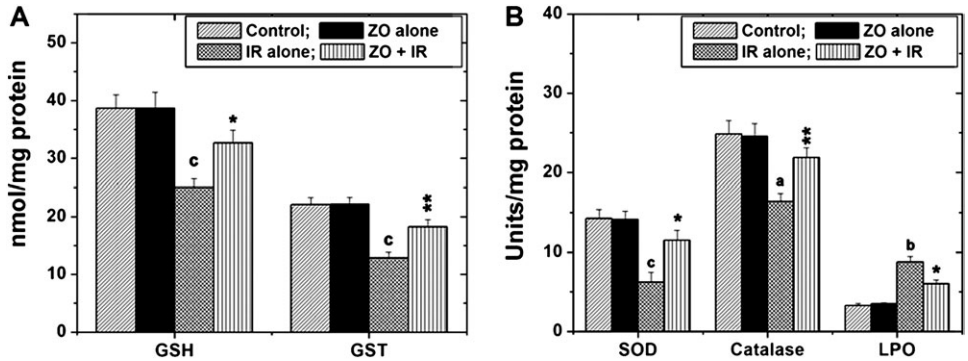


Fig. 7. The effect of ZO (25 μg/ml) on the radiation-induced (3 Gy) changes in the activities of (**A**) GSH and GST, (**B**) SOD, catalase and LPO levels in V79 cells. All the results are shown as mean ± standard error of the mean from the data of a minimum of three separate experiments. The significant levels *, $P < 0.05$; **, $P < 0.01$ and no symbol, non-significant, when compared with respective IR-alone group. Whereas, a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ and no symbol, non-significant, when compared with control (untreated).

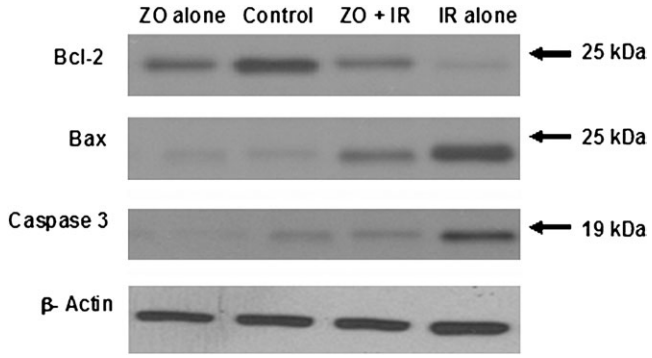


Fig. 8. Effect of ZO on radiation-induced apoptosis in V79 cells. Cells were pretreated with optimum dose of ZO (25 μg/ml) for 1 h, followed by 10 Gy gamma radiation. Changes in Bcl-2, Bax and caspase-3 protein levels were detected using western blotting.

This is in agreement with the earlier studies wherein a similar effect has been reported (17,44). Interestingly, ZO treatment caused a significant decline in the IR-induced cytotoxic effect, as evident by increase in survival of cells receiving ZO before IR treatment.

Genotoxic effect of low linear energy transfer radiations such as γ-radiation is mediated through the formation of free radicals and ROS that causes DNA damage such as single or double strand breaks and damage to bases and sugars, ultimately leading to chromosomal aberrations (47). All the above-mentioned lesions significantly contribute to the increased levels of primary DNA damage that could be detected by the alkaline comet assay, a widely used *in vitro* assay in radiobiological research. Further, MN assay was also performed simultaneously to evaluate the protective effect of ZO against radiation-induced genotoxicity.

MN are acentric fragments or a complete chromosome that fail to attach to the mitotic spindle during mitosis and are excluded from the main nuclei as separate entities. Different mechanisms may be involved in the formation of MN, either from chromosome breakage or damage to the mitotic spindle and increase in its frequency is widely accepted measure of genotoxicity (48,49). Our results of the alkaline comet and CBMN assays indicated that pretreatment of ZO (25 μg/ml) offered better radioprotection in V79 cells in terms of all the comet parameters as well as reduction in MN formation and an increase in CBPI values when compared with other doses of ZO and radiation-alone-treated cells. Even though the protective effect showed a dose-dependent increase, there was an optimum concentration of 25 μg/ml beyond which increase in this effect was non-significant. Further, this is in agreement with our earlier *in vitro* results wherein ZO showed maximum cytoprotection and anti-apoptotic activity in cultured human peripheral blood lymphocytes against gamma radiation at an optimal concentration of 10 μg/ml and further increase in dose did not increase this effect (published elsewhere). A similar observation has been reported by several authors for other antioxidant protectors *in vivo* as well as *in vitro* such as Orientin and Vicenin, 2-mercaptopropionylglycine and DMSO (50–52). These observations clearly substantiate the antigenotoxic potential of ZO, which provides the protective benefits against genomic instability and also must have possibly contributed to the reduction of DNA damage-induced apoptotic cell death.

It is well documented that IR causes a significant increase in ROS, which have the potential to cause cellular damage (2,53). The V79 cells exposed to IR exhibited the distinct features of apoptosis, including nuclear fragmentation and MMP depolarisation. However, the cells pretreated with ZO had significantly normalised the loss of MMP and reduced percentage of apoptotic cells. These results suggest that ZO inhibits IR-induced apoptosis by its ROS scavenging effect. A similar effect has been observed earlier for Eckol in V79-4 lung fibroblast cells against gamma radiation-induced apoptosis (17). ZO has been shown to protect against radiation-induced oxidative damage in *in vivo* model system (15).

In order to further elucidate the molecular mechanism responsible for the protective effect of ZO against radiation-induced apoptosis, we investigated the levels of some critical proteins associated with apoptosis such as Bcl-2 and Bax using western blotting the Bcl-2 family of proteins regulate the release of cytochrome *c* and other inter membrane space proteins. While Bcl-2 is one of the most important anti-apoptotic members in this family, it interacts with Bax, a pro-apoptotic member, thereby preventing the release of cytochrome c and subsequent apoptosis. Earlier studies have suggested that ROS might be associated with Bax activation in apoptosis induced by some stimuli (54,55). Increased expression of Bax can induce apoptosis, while Bcl-2 protects cells from apoptosis (56). In the present study, ZO effectively suppressed programmed cell death by decreasing apoptotic features including caspase activation, increasing anti-apoptotic molecules (Bcl-2) and decreasing pro-apoptotic molecules (Bax), when compared with IR alone. These findings are consistent with a previous report on the crucial regulatory role of Bcl-2 on apoptosis in V79 cells (17). However, much work needs to be done to investigate the source of ROS and how it really regulates the activation of Bax.

Hydroxyl radicals are products of ROS generated in cells due the interaction of radiation with cellular macromolecules, including DNA, proteins and lipids, which may exert in cytotoxic and genotoxic effects (2,57). The antioxidant enzymes are known to play a role in protecting cells against ionising radiation by maintaining the balance between the rate of radical generation and the rate of radical scavenging. Radical oxygen species O_2^- can undergo either spontaneous- or enzyme-catalysed (SOD) dismutation to hydrogen peroxide (H_2O_2) or can react with NO· to form the toxic product peroxynitrite ($ONOO^-$), which could not be detoxified by antioxidant defence enzymes (58). Therefore, high concentrations of NO· or $ONOO^-$ have been shown to oxidise a variety of biological molecules and may produce DNA base modifications, DNA strand breaks and mutations through oxidative mechanisms (59). However, NO· induces biochemical characteristics of apoptosis in several cell types through the activation of apoptotic signaling cascades such as caspases (60) and the mitochondrial cytochrome *c* release (61), the regulator of cell survival or the expression of apoptotic gene (60,62,63). ZO has been reported as a potent $ONOO^-$ scavenger for the protection of the cellular defence activity against peroxynitrite-involved diseases (14). Therefore, it may be possible that ZO can increase the reaction between $\cdot O_2^-$ and NO, whereas the enzyme-catalysed reaction by SOD would weaken. Thus, the SOD can be protected. ZO has been reported to increase SOD activity in the striatal cells of mouse exerting its antioxidant potential. It is reported that hydroxyl radicals can react themselves to form H_2O_2, which could oxidise GSH to

glutathione disulfide (64). The presence of ZO can reduce hydroxyl radical and probably inhibit the formation of H_2O_2 in the reaction chain, which may finally lead to the upholding of intracellular GSH levels. The antioxidant property of ZO may be due to the presence phenolic hydroxyl group and a double bond between C-3 and C-4 (14,65). However, the release of the proton in ZO may be governed by the presence of the methoxy group adjacent to the phenolic hydroxyl group, which may stabilise the release of proton from the hydroxyl group. Further, the presence of the long chain ethyl methyl ketone group may help in increasing the bulk and stabilisation of the ring, presumably because ethyl methyl ketone group could result in an increase in partition coefficient value, which may be one of the factors for increased cell penetration.

Membrane lipids are the major targets of ROS and the free radical chain reaction. The increase in the levels of LPO products such as lipid hydroperoxides, conjugated dienes and MDA are the index of lipid damage (64,66). In view of the fact that membrane phospholipids are major targets of oxidative damage, LPO is often the first parameter analysed for proving the involvement of free radical damage. Thus, the presence of MDA is considered as an indicator of free radical damage through membrane LPO (67). In our study, we observed a significant decrease in the levels of antioxidant and an increase in the levels of MDA in irradiated V79 cells. ZO showed a dose-dependent significant scavenging activity of various free radicals *in vitro*. However, pretreatment of ZO prior to radiation exposure increased the antioxidant status levels and decreased the levels of MDA when compared with radiation-alone-treated V79 cells. These results show that ZO attenuated radiation-induced LPO and that this decrease in LPO with ZO was compatible with our previous *in vivo* studies (15). These findings suggest that ZO treatment exhibited direct antioxidant properties by reducing basal MDA formation and protective antioxidant effects towards ionising radiation-induced cell damage. The decrease in MDA concentration could be due to the ability of ZO to scavenge secondary reactive radicals or to prevent formation of superoxide or hydrogen peroxide in response to radiation treatment.

To conclude, the present *in vitro* investigation on ZO, a phenolic alkanone, exhibited antagonistic effect against radiation-induced cytotoxicity being non-toxic by itself. Further, ZO rendered a significant anti-genotoxic, anti-apoptotic and anti-lipid peroxidative potency plausibly ascribable to its antioxidant/free radical scavenging ability and also by the suppression of radiation-induced oxidative stress.

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