Antioxidant and Anti-inflammatory Activities Mediate the Radioprotective Effect of Trianthema Portulacastrum L. Extracts

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Research Article

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Abstract

Ionizing radiation (IR) generates reactive oxygen species (ROS) which leads to oxidative stress and often leads to inflammatory responses in organisms. *Trianthema portulacastrum* L., a plant commonly growing in India, is rich in antioxidant phytochemicals which is responsible for scavenging free radicals, and may provide radio-protective and anti-inflammatory effects in response to ionizing radiation. The effect of *T. portulacastrum* extracts was studied in hepatic cells, which are susceptible to radiation-induced damage, and in macrophages which are the primary inflammatory cells of the body. *T. portulacastrum* stem extracts showed efficient free radical scavenging activity in hepatocytes and reduction of radiation-induced lipid peroxidation in cell and mitochondrial membranes. Treatment of irradiated cells with *T. portulacastrum* stem extracts enhanced cell viability, although at higher concentrations there was reduction in cell viability. Treatment with low concentration of *T. portulacastrum* stem extract also reduced cellular ROS generation and increased cellular concentration of the anti-oxidant glutathione. *T. portulacastrum* extracts also showed a marked anti-inflammatory effect in macrophages activated by the inflammatory agonist bacterial lipopolysaccharide (LPS) by reducing inflammatory gene expression and nitric oxide (NO) production, and increasing glutathione content. LPS treatment lowered expression of Nrf2, a transcription factor involved in regulation of multiple anti-oxidant genes, while treatment with low concentration of *T. portulacastrum* stem extract significantly restored it. Together, these observations demonstrated a potential radioprotective role of *T. portulacastrum* extract mediated by both its antioxidant activity on hepatic epithelial cells and its anti-inflammatory activity on immune cells.

Introduction

Exposure to ionizing radiation (IR) causes cellular damage either by direct impairment of biomolecules or indirectly by generation of free radicals. Water radiolysis leads to generation of reactive oxygen species (ROS) which causes lipid peroxidation in membrane, DNA strand breaks and oxidation of cellular protein (Wang et al. 2018). Radiation not only affects the irradiated cells but also non-irradiated cells and tissues undesirably due to bystander effect through the activation of inflammatory responses (Shemetun and Pilins'ka 2007). IR sensitizes lymphocytes, macrophages, monocytes and other immune cell (Carvalho and Villar 2018). Radiation exposure shows immune-modulatory properties through the production of reactive oxygen and nitrogen species (RONS), and release of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), tumor growth factor-beta (TGF-β), interleukins, nucleoside, high mobility group box-1 molecule (HMGB1) and heat shock proteins (HSPs) (Carvalho and Villar 2018). As ionizing radiation is a mainstay of therapy in many cancers, methods of protection against radiation-induced damage are an important requisite for cancer radiotherapy.

Radioprotectors may be naturally occurring antioxidants that can protect normal cells and tissues from radiation-induced damage. Sulfhydryl agents such as glutathione, cysteine, cystamine and other antioxidants have shown protective activity against the lethal effect of radiation and also increased the survival rate of irradiated mice (Obrador et al. 2020). Sulfhydryl group containing erdosteine protect rats against gamma radiation through antioxidants and anti-inflammatory properties (Elkady and Ibrahim
Sulfhydryl compounds protect cellular DNA through a combination of free radical scavenging, modulation of repair process, and hydrogen donation ability (Kumar et al. 2002).

Natural products are rich in antioxidants and are possible sources of various dietary supplements with therapeutic importance. Phenolic compounds present in plant-based natural products have significant pharmacological properties. They are reported as antioxidants observed by metal chelation and free radicals scavenging activities (Godlewska-Żyłkiewicz et al. 2020) and have significant anti-inflammatory properties (Farhood et al. 2019). Curcumin and epigallocatechin-3-gallate, are well-known natural compounds having both radioprotective as well as anti-inflammatory properties (Azab et al. 2016; Farhood et al. 2019). However, depending on several factors, an antioxidant may act as prooxidants in a concentration-dependent manner. This possibly involves the reduction of transition metal ions from Fe$^{3+}$ and Cu$^{2+}$ to Fe$^{2+}$ and Cu$^{+}$ respectively and inducing Fenton reaction (Maurya and Devasagayam 2010). Therefore, further exploration of naturally occurring compounds in plants with antioxidant properties is warranted.

*Trianthema porulacastrum* L., a well-known medicinal plant from the family of Aizoaceae, is a natural source of antioxidant and phytochemicals and has been used for treatment of numerous disease conditions in Indian and African traditional medicine (Shivhare et al. 2012; Das et al. 2020). *T. portulacastrum* is also well-known for its hepatoprotective activity against chemical-induced toxicity such as carbon tetrachloride (CCl$_4$) (Sarkar et al. 1999), paracetamol and thioacetamide (Kumar et al. 2004).

The hepatoprotective activity of *T. portulacastrum* was marked by enhancement of antioxidant enzymes, suggesting that the protection of liver cells from oxidative damage may be a mode of hepatoprotection by *T. portulacastrum* extract. However, no studies have been performed on the anti-inflammatory role of *T. portulacastrum*, although inflammatory responses are known to be a major contributor to hepatic damage. Therefore, the objective of this study was to evaluate the radioprotective activity of *T. portulacastrum* in hepatocytes and its anti-inflammatory effects using murine macrophages.

### Materials And Methods

#### Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2',7'-dichlorofluorescin diacetate (H$_2$DCFDA), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS), monochlorobimane (MCB), Lipopolysaccharides (LPS) and primers from Sigma; DMEM and Fetal bovine serum (FBS) from HiMedia; FBS from Invitrogen; RPMI-1640, L-Glutamine, oligo-(dT) primer, M-MLV reverse transcriptase from Thermo Fisher Scientific and all other chemicals of AR grade were procured from SRL India Ltd and Merck India LTD.

#### Plant materials

*T. portulacastrum* L. plants were collected from fields in Kalyani, Dist. Nadia, West Bengal, India and were authenticated from the Department of Botany, University of Kalyani, Kalyani, Nadia (Voucher No. UD-101).
Preparation of TP extracts: Dried powder of different parts of *T. portulacastrum* such as leaves, stem and whole plant (100 g) was extracted with 500 ml petroleum ether for 24 h with constant shaking and filtered. This process was repeated twice. Ethyl acetate, acetone and ethanol solvent were used twice sequentially followed by petroleum ether. All the solvents were evaporated and dried. Further studies were carried out with ethanolic fractions.

**Antioxidant capacity study**

Antioxidant capacity of the different extracts of the TP was measured using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) (Maurya and Devasagayam 2010) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Maurya and Devasagayam 2010) radical scavenging assays, whereas metal reducing power was evaluated using ferric reducing power assay (FRPA) (Maurya and Devasagayam 2010) and molybdenum reduction assay (MRA) (Saxena et al. 2016).

**Cell Lines:**

Human hepatic cells (WRL 68) were cultured in Dulbecco's modified Eagle's Medium (DMEM) and RAW 264.7 cells in RPMI-1640 medium with L-Glutamine. For culturing both the cell line media were supplemented with 10% FBS (Thermo Fisher Scientific, 10500064) and 1% Pen-Strep (Thermo Fisher Scientific, 15140-122).

**Evaluation of radioprotective property of *T. portulacastrum* extract**

Radioprotective property of *T. portulacastrum* extract was evaluated using sub-cellular and cellular model systems. For sub-cellular assay, we have used murine mitochondrial membrane whereas for cellular assay we have employed human hepatic cells (WRL 68) as a model system.

**Evaluation IR-induced lipid peroxidation**

For evaluation of lipid peroxidation mouse mitochondrial membrane and human hepatic cells were used. Mitochondrial membrane fractions were isolated from the liver of male Swiss mice as described (Checker et al. 2010). Damage to the mitochondrial membrane fraction after radiation exposure was assessed in terms of lipid peroxidation (Maurya and Devasagayam 2010). Mitochondrial membrane fraction (a protein equivalent of 300 µg) was suspended in 300 µl of 10 mM potassium phosphate buffer, pH 7.4, and exposed to 50 Gy radiations in the absence and presence of different concentrations of TP extracts (pre-treated for 30 min at 37°C). After treatment, 900 µl TBA reagent (0.375% TBA, 0.25 M HCl, 15% trichloroacetic acid (TCA) and 6 mM Na$_2$-EDTA) was added. The reaction mixture was incubated at 95°C for 20 min, cooled to ambient temperature and centrifuged at 12,000 g for 5 min at 25°C. Malondialdehyde (MDA) equivalents in the supernatant were estimated by measuring the fluorescence (as fluorescence provide more sensitivity) with excitation at 530 nm and emission at 590 nm using a microplate reader.
WRL 68 \((2\times10^6)\) cells were collected and treated with different \textit{T. portulacastrum} extracts for 1 h at 37\textdegree C for IR-induced cellular lipid peroxidation study inhuman hepatic cells. Subsequently cells were exposed to 8 Gy of IR. IR-induced cell membrane damage was assessed in terms of lipid peroxidation as described above ([Maurya and Devasagayam 2010]).

**MTT assay**

To study the cytotoxicity and proliferation of cells after IR exposure, MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-difenyl-tetrazolium bromide) was used. In brief, \(15\times10^3\) WRL 68 cells were seeded in 96-well plate one day prior to addition of extract. Next day cells were treated with different concentration of the extracts and incubated for 1 h. These extract treated cells were exposed to 4 Gy of IR. After completion of incubation period, MTT assay was performed by adding 10 µl MTT solution (10 mg/ml) to each well. Formazan crystals formed inside the cells were dissolved by adding 100 µl of solubilizing buffer (0.01 N HCl in 10% SDS) and incubated for overnight at 37\textdegree C. The absorbance was measured at 570 nm using Synergy Bio-Tek (USA) microplate reader ([Maurya et al. 2011]).

**Clonogenic assay**

The clonogenic assay was used to evaluate the radioprotective efficiency of TP extract using WRL 68 cells. In brief, \(2\times10^3\) exponentially growing cells were plated in a 6-well plate for overnight. Next day, cells were treated with different concentration of \textit{T. portulacastrum} extracts 1h before 4 Gy IR-exposure. After irradiation, cells were cultured for 12 days at 37\textdegree C in CO\textsubscript{2} incubator for the development of macroscopic colonies. The colonies were fixed with methanol, stained with 0.5% crystal violet and counted using a colony counter ([Oxford Optronix, UK]) ([Jayakumar et al. 2015]).

**Evaluation Of The Cellular Redox Status**

To study the mechanism of the \textit{T. portulacastrum} extracts for radioprotection, cellular redox study was carried out. For this cellular ROS and intracellular thiol (GSH) levels were monitored using H\textsubscript{2}DCFDA and monochlorobimane (MCB) fluorescence dye respectively.

**Measurement of cellular ROS level**

2',7'-dichlorofluorescin diacetate (H\textsubscript{2}DCFDA) is a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. \(4\times10^6\) WRL-68 cells were incubated with 10 µM H\textsubscript{2}DCFDA in serum-free medium by incubating at 37\textdegree C for 45 min. After incubation, cells were washed to remove the excess dye and suspended in the phosphate-buffered saline (PBS, pH = 7.4). For studying inhibition of radiation-induced ROS formation, cells were exposed to IR in presence of different concentrations of the \textit{T. portulacastrum} extracts and incubated at 37\textdegree C for 1 h. The fluorescence intensity of the oxidized probe was read using a microplate reader (excitation/emission wavelength, 485/ 520 nm) ([Maurya and Devasagayam 2010]).
Intracellular GSH

Monochlorobimane (MCB) is a fluorescence dye which has a high affinity for GSH. In this study, MCB was used to measure cellular thiol (GSH) levels. WRL-68 cells were incubated with the different concentrations of *T. portulacastrum* extracts for 1 h at 37°C. Monochlorobimane (40 µM, 30 min at 37°C) was used to measure the level of the GSH in these cells. Fluorescence emission (excitation/emission wavelength, 380/460) from cellular sulfhydryl-reacted monochlorobimane was measured using a microplate reader (Checker et al. 2010).

Evaluation Of Anti-inflammatory Property Of Tp Extract

The anti-inflammatory effects of *T. portulacastrum* extract in LPS-stimulated RAW 264.7 macrophages were evaluated by nitric oxide (NO) assays and quantitative real-time reverse transcription-polymerase chain reaction analysis of expression of inflammatory genes. For studying the cytotoxicity in RAW 264.7 activated with LPS and treated with the *T. portulacastrum* extract, MTT assay was performed as described previously. GSH concentration was measured through Ellman's reagent and calculated from standard curve using pure GSH (Moron et al. 1979).

Nitric oxide (NO) assay

RAW 264.7 cells (0.5×10⁵ cells/well) were seeded into 96 well plates for 24 h. Next day, cells were pre-incubated with different concentrations of *T. portulacastrum* extracts (0, 31.25, 62.5, 125 µg/ml) for 1 h and further stimulated with 500 ng/ml of LPS. The culture supernatants were collected 24 h after the LPS stimulation, and the concentrations of NO were measured using Griess reagent. 100 µl of culture supernatant was mixed with 100 µl of Griess reagent (sulfanilamide 1%, 2% phosphoric acid and 0.1% NEDD in water) and the mixture was incubated at room temperature for 10 min before measuring the absorbance at 550 nm. In all experiments, fresh culture medium was used as the blank and sodium nitrite was used as the standard (Kacem et al. 2015).

Semi-quantitative PCR: For semi-quantitative PCR, RAW 264.7 cells were pretreated with *T. portulacastrum* extract for 1 h followed by LPS treatment. Total cellular RNA was extracted using Trizol following manufacturer's protocol. cDNA was synthesized from RNA using oligo-(dT) primer by M-MLV reverse transcriptase (Thermo Fisher, 28025-013). Specific primers for TNF-α, Nrf-2 and iNOS were used for PCR reactions and then run on 1.5% Agarose gel. GAPDH primers were used for normalization of mRNA quantity respectively (Ahuja et al. 2016). The following primers were used for semi-quantitative PCR: iNOS (forward 5'-TTCTTTCAGCTCAAGAGCCAGAAA-3'; reverse 5'-GGGATTGCATTTCGCTGTCT-3'), Nrf2 (forward 5'-CCCGAATTACAGTCTTAATACCG-3'; reverse 5'-AGGTGGGATTTGAGTCTAAGGA-3'), TNF-α (forward 5'-ATGGCCTCCCTCTCATCAGT-3'; reverse 5'-GGGAGTAGACAAGGTACAACC-3'), GAPDH (forward 5'-TGATGACATCAAGAGTGGAAG-3'; reverse 5'-TCCTTGAGCCATGTGGGCCC-3').
Statistical analysis

All experiments with *T. portulacastrum* extracts were performed in triplicate and mean ± standard error (SE) of each triplicate result was considered for statistical analysis. Analysis of results was performed using the Statistical Package for Social Science, version 23 (SPSS, Chicago, Illinois) software. Significant differences were assessed through the one-way analysis of variance (ANOVA), followed by the Tukey test for individual differences. A value of $P < 0.05$ was used to evaluate statistical significance.

Results

*T. portulacastrum* extracts show antioxidant activities by free radical scavenging and transient metal reduction

The major form of oxidants in cells are oxidative free radicals such as hydroxyl and peroxide and transition metal ions such as Fe$^{2+}$ and Cu$^+$, both of which oxidize a variety of biomolecules and cellular components and cause oxidative damage. Therefore, we investigated the free radical scavenging and transition metal ion reducing activities of *T. portulacastrum* extracts. Extracts of leaf, stem and whole plant of *T. portulacastrum* exhibited concentration-dependent scavenging activity in model free radicals such as DPPH and ABTS radicals (ABTS•$^+$), in free radical scavenging assays (Fig. 1). The half inhibition concentration [IC$_{50}$] of stem extract (SE) was found to be the lowest (245.04 and 290.79 in the ABTS and DPPH assays respectively) in free radical scavenging activity (Table 1). We also determined the transition metal ion reducing activity of these extracts by FRPA and MRA assays based on reduction of iron and molybdenum ions respectively. All the *T. portulacastrum* extracts showed reducing capacity in a dose-dependent manner, with the highest activity being shown by leaf extracts (LE) (Fig. 1). Together, these observations showed significant antioxidant capacity of *T. portulacastrum*, with different parts of the plant contributing to the same. Further assays were therefore carried out with stem extracts (SE).

| IC 50 (µg/ml) | Leaf Ethanol (LE) | Stem Ethanol (SE) | Plant Ethanol (PE) |
|--------------|-------------------|-------------------|--------------------|
| ABTS         | 279.62            | 245.04            | 307.84             |
| DPPH         | 326.15            | 290.79            | 392.78             |

T. portulacastrum stem extracts protect against lipid peroxidation and cell death consequent to radiation exposure

Radiation exposure is known to damage the structure of cell membranes through degradation of lipids, mainly mediated via lipid peroxidation (LPO) of WRL68 hepatocyte cell and mouse mitochondrial membranes were observed after exposure to 8 Gy and 50 Gy radiation respectively which increased the formation of malondialdehyde (MDA). In both cases, addition of *T. portulacastrum* stem extracts (SE)
mitigated the radiation-induced lipid peroxidation in a dose-dependent manner, showing the protective activity against radiation-induced membrane lipid peroxidation (Fig. 2).

For further evaluation of radioprotective activity of the extract, cell viability of irradiated cells, untreated or treated with *T. portulacastrum* stem extracts, was estimated using MTT and clonogenic assays. Exposure to 12 Gy radiation significantly reduced cell viability after 72 h and cells treated with *T. portulacastrum* extract showed enhanced cell viability compared to irradiated cells in the MTT assay (Fig. 3a). However, only 31.25 µg/ml concentration of the extract demonstrated significant enhancement of cell viability and increased concentrations of the extracts did not show significant difference in cell viability in comparison to the irradiated cells.

In the clonogenic assay, exposure to 4 Gy radiation resulted in a 66% reduction in colony-forming ability of the cells (Fig. 3b). Treatment with high concentrations (to125 µg/ml) of *T. portulacastrum* extracts alone did not show any reduction in colony formation (Fig. 3c). However, on addition of *T. portulacastrum* stem extracts to irradiated cells, significant rescue in colony formation was only observed in cells treated with 31.25 µg/ml extract (Fig. 3d). Together these observations suggest a radioprotective activity of *T. portulacastrum* stem extract, although at a higher concentration the level of protection may decrease due to the presence of compounds with non-specific toxic effect on radiation-induce damaged cells.

*T. portulacastrum* **stem extracts reduces cellular ROS and enhances GSH**

To explore the mechanism of radioprotection by *T. portulacastrum* stem extract, we evaluated the cellular redox status by measuring reactive oxygen species (ROS) and reduced Glutathione (GSH) which are important markers of the redox status of cells. Determination of cellular ROS level by DCFDA fluorescence intensity showed that 4 Gy of radiation increased the cellular ROS level by nearly 2-fold, whereas, pretreatment with the *T. portulacastrum* stem extract reduced the ROS level in a dose-dependent manner (Fig. 4a). Similarly, exposure to 4 Gy of radiation significantly reduced the concentration of GSH, one of the most important cellular antioxidant molecules, whereas treatment with the lowest concentration of *T. portulacastrum* stem extract significantly upregulated the GSH content (Fig. 4b). Higher concentrations of *T. portulacastrum* extract failed to significantly enhance the GSH content, most likely by inhibition of GSH biosynthesis by high concentration of some non-specific molecules. Thus, these observations demonstrated that the observed radioprotection by *T. portulacastrum* extract is due to modification of cellular redox status.

*T. portulacastrum* **stem extract exerts anti-inflammatory effects on LPS-activated macrophages**

Another arm of the radiation induced tissue damage is mediated by secondary inflammatory responses, caused by the activation of inflammatory cells such as neutrophils and macrophages. Therefore, we also investigated the effect of *T. portulacastrum* stem extract in mitigating the inflammatory response by evaluating its effect on mouse macrophage cells (RAW264.7) activated by the inflammatory agonist LPS. RAW264.7 cells were stimulated with 500 ng/ml LPS with and without 1 h pretreatment with *T. portulacastrum* extracts. *T. portulacastrum* stem extract did not show any autonomous cytotoxicity in
RAW264.7 cells at the highest concentration (125 µg/ml). However, MTT assays showed that treatment with TP stem extract reduced the LPS-stimulated proliferation of RAW 264.7 cells in a dose-dependent manner (Fig. 5a).

One of the major inflammatory mediators released by macrophages after LPS stimulation is nitric oxide (NO) generated by upregulation of inducible nitric oxide synthase (iNOS). We therefore determined NO generation by LPS-stimulated RAW264.7 cells in presence and absence of treatment with T. portulacastrum stem extract. LPS stimulation increased the secreted NO level by 4.6 fold while treatment with T. portulacastrum stem extract dose-dependently reduced the secreted NO level (Fig. 5b). LPS induced activation of macrophages not only elevates NO level but also downregulated GSH concentration. Treatment with T. portulacastrum stem extract also enhanced GSH level in LPS-treated RAW264.7 cells, but the highest enhancement was observed at the lowest concentration of T. portulacastrum stem extract as in the case of hepatocytes (Fig. 5c).

We then investigated the expression of some of the genes responsible for the synthesis of the major inflammatory mediators in LPS stimulated macrophages in presence and absence of treatment with T. portulacastrum extract. LPS stimulation increased the mRNA level of iNOS, the enzyme responsible for NO synthesis, by 1.5 fold while treatment with T. portulacastrum stem extract brought down the iNOS mRNA to basal level of expression (Fig. 6a). iNOS expression is mediated through activation of TNF-α, a pro-inflammatory cytokine. TNF-α mRNA level was increased significantly after 24 h of LPS treatment and while treatment with T. portulacastrum stem extract also significantly decreased the TNF-α level in a dose-dependent manner (Fig. 6b).

We also checked the level of Nrf2 mRNA as Nrf2 is a well-known transcription factor which regulates a number of antioxidant genes in cells, including GSH, and is involved in protection against oxidative stress and inflammation. Treatment of the cells with LPS lowers the Nrf2 mRNA level while treatment with T. portulacastrum stem extract significantly restored it although the mRNA level reduced with increasing concentration of the T. portulacastrum extract (Fig. 6c), reflecting the effect on GSH level as observed before.

Together, these observations demonstrate a potential radioprotective role of T. portulacastrum extract mediated by both its antioxidant activity on epithelial cells and its anti-inflammatory activity on immune cells.

**Discussion**

Exposure of cells to ionizing radiation induces reactive oxygen species (ROS) and nitrogen species (RNS) together with alteration in cellular antioxidant status and resulting cell damage (Reisz et al. 2014). Supplements of antioxidants during radiotherapy have been shown to decrease the damaging effects (Singh et al. 2018). Plants are one of the main natural sources of antioxidants. Radioprotective activity has been observed from plant antioxidants such as shigoka extract, green tea polyphenols and curcumins (Seong et al. 2015; Clarke et al. 2016; Shirazi et al. 2012). T. portulacastrum has been shown
to exhibit hepatoprotective activity against chemical-induced toxicity (Yamaki et al. 2016). Therefore, in this study we have demonstrated the radioprotective role of TP extract on hepatocytes, mediated via its activity of reducing oxidative damage to cells. Moreover, we have demonstrated an anti-inflammatory role of *T. portulacastrum* extract in the case of activated macrophages, which is likely to enhance its radioprotective function by mitigating the inflammatory response induced by radiation damage.

A major mode of antioxidant function is via scavenging of oxidative free radicals. Radical scavenging capacity of an antioxidant lies on its proton donating or accepting ability (Singha et al. 2020). *T. portulacastrum* extracts showed efficient free radical scavenging activity in both DPPH and ABTS•+ assays which are based on electron transfer ability used to measure antioxidant capacity. Moreover, *T. portulacastrum* extracts showed efficient reducing ability to reduce Mo (VI) to Mo (V) and Fe (III) to Fe (II) which is a marker of its antioxidant activity.

Exposure to radiation causes damage to different biomolecules such as lipids, proteins and nucleic acids. Cellular membranes are one of the major targets of the oxidative free radicals, generated due to radiation exposure. Damage of the lipids present in the cell membranes changes its fluidity status and also activates several critical signaling pathways (Nicolson and Ash 2014). ROS is responsible for thiol oxidation which initiates lipid peroxidation. Radiation induced free radicals react with macromolecules and damage membranes of intracellular organelles (Singha and Das 2015). It has been reported that 50 Gy of gamma radiation impairs mitochondrial membrane function by damaging complex I (NADH dehydrogenase) and III (cytochrome c reductase) (Pearce et al. 2001). In our study *T. portulacastrum* stem extract showed efficient protection against lipid peroxidation by ionizing radiation in a MDA formation assay, further substantiating its role in radioprotection.

The final effect to check in case of radioprotection is the reduction of cell death in response to radiation exposure. Treatment with *T. portulacastrum* extract showed a survival advantage of irradiated cells in both short term (MTT) and long term (colony formation) cell viability assays. As it has been reported that lower radiation dose gives false-positive result in MTT as high formazan is deposited in irradiated cells compared to control (Rai et al. 2018), the clonogenic assay is a good measure to study the ability of *T. portulacastrum* extract to protect against radiation-induced cell damage. However, interestingly, higher concentration of *T. portulacastrum* extract failed to show this survival advantage and whether this is due to a pro-oxidant activity demonstrated at a higher concentration as reported for other natural products (Banerjee et al. 2008; Sotler et al. 2019) or due to an unknown effect of other compounds present in the extract remains to be investigated. A similar concentration-dependent effect was observed in the case of the effect of *T. portulacastrum* extract on GSH concentration of cells, which suggests that this effect might be mediated by the influence of *T. portulacastrum* extract on the level of GSH, one of the primary anti-oxidant molecules in the cell.

Radiation-induced cellular damage is not only due to the alteration of the redox and antioxidant balance but also due to the activation of inflammatory responses (Sachaue and McBririd 2015). Ionizing radiation-induced activation of the immune system results in inflammation through enhancing the release of
growth factors and pro-inflammatory cytokines (Mun et al. 2018). Changes in the cellular oxidative stress level play a pivotal role in inflammation (Han et al. 2019). However, the effect of natural products on radiation-induced inflammation has not been explored sufficiently. Therefore, we investigated the effect of TP extract on the inflammatory response in LPS-treated macrophages, one of the major immune cells involved in radiation-induced inflammation. Treatment with *T. portulacastrum* extract was able to reduce the secretion of NO from these cells, as well as reduce the expression of inducible nitric oxide synthase (iNOS) gene, which are important mediators of the inflammatory response (Cao et al. 2019). Excess NO induces inflammation and nitrosative stress (Calabrese et al. 2004). These results showed that *T. portulacastrum* extract may protect from radiation damage by modulating the inflammatory response in the body.

In cells LPS binds with Toll-like receptor 4 (TLR-4) which further activates pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 and IL-1β which further activates an inflammatory signaling mechanism (Cao et al. 2019). In our study, LPS stimulation up regulated the level of TNF-α but TP extract downregulated the TNF-α expression level and thus reduced the TNF-α mediated inflammatory signaling cascade.

The nuclear factor erythroid 2-related factor (Nrf2) is a potent antioxidant marker responsible for the reduction of oxidative stress (Han et al. 2019). Nrf2 protects cell from oxidative stress after dissociation from Keap1 and binds with antioxidant-response elements (AREs) which ultimately promotes the expression of several genes including GSH and GSH dependent antioxidant enzymes (Harvey et al. 2009; Smith et al. 2016). GSH, a well-known antioxidant regulates redox status and signaling, death and cell proliferation (Harvey et al. 2009). LPS stimulation of macrophages decreased the Nrf2 mRNA level as well as the concentration of GSH but treatment with *T. portulacastrum* extract markedly upregulated the mRNA expression of Nrf2 and GSH concentration, suggesting a molecular mechanism for upregulation of GSH in cells treated with TP extract. Interestingly, the same effect of higher dose of *T. portulacastrum* extract failing to enhance Nrf2 expression and GSH concentration was noted, reflecting the similarity with radiation induced GSH concentration, and cell viability in hepatocytes. This warrants further investigation into the concentration dependent effect of *T. portulacastrum* extract, and its bioactive molecules, on regulation of expression of anti-oxidant genes in cells.

**Conclusion**

This study for the first time shows a radioprotective activity of extracts from the plant *Trianthema portulacastrum*, mediated via its dual effect in modifying the redox status of irradiated cells and the inflammatory response of immune cells activated by the inflammatory agonist LPS. Both these effects together may strongly support the role of *T. portulacastrum* extract as a natural product with significant radioprotective ability.

**Declarations**

**Conflict of Interest Statement:**
There is no conflict of Interest

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References

1. Wang Js, Wang Hj, Qian, Hl (2018) Biological effects of radiation on cancer cells. Military Med Res 5:20. https://doi.org/10.1186/s40779-018-0167-4

2. Shemetun OV, Pilins’ka MA (2007) Radiation-induced “bystander” effect. Cytol Genet 41:251–255. https://doi.org/10.3103/S0095452707040111

3. Carvalho HA, Villar RC (2018) Radiotherapy and immune response: the systemic effects of a local treatment. Clinics (Sao Paulo) 73:e557s. https://doi:10.6061/clinics/2018/e557s

4. Obrador E, Salvador R, Villaescusa JI et al (2020) Radioprotection and Radiomitigation: From the Bench to Clinical Practice. Biomedicines 8:461. https://doi:10.3390/biomedicines8110461

5. Elkady AA, Ibrahim IM (2016) Protective effects of erdosteine against nephrotoxicity caused by gamma radiation in male albino rats. Hum Exp Toxicol 35:21–8. https://doi:10.1177/0960327115574919

6. Kumar KS, Srinivasan V, Toles R et al (2002) Nutritional approaches to radioprotection: vitamin E. Mil Med 167:57–59

7. Godlewskas-Żykiewicz B, Świsłocka R, Kalinowska M et al (2020) Biologically Active Compounds of Plants: Structure-Related Antioxidant, Microbiological and Cytotoxic Activity of Selected Carboxylic Acids. Materials (Basel) 13:4454. https://doi:10.3390/ma13194454

8. Farhood B, Mortezaee K, Goradel NH et al (2019) Curcumin as an anti-inflammatory agent: Implications to radiotherapy and chemotherapy. J Cell Physiol 234:5728–5740. https://doi:10.1002/jcp.27442

9. Azab A, Nassar A, Azab AN (2016) Anti-Inflammatory Activity of Natural Products. Molecules 21:1321. https://doi:10.3390/molecules21101321

10. Maurya DK, Devasagayam TP (2010) Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. Food Chem Toxicol 48:3369–3373. https://doi:10.1016/j.fct.2010.09.006

11. Shivhare MK, Singour PK, Chaurasiya PK et al (2012) Trianthema portulacastrum Linn. (Bishkhapsra). Pharmacogn Rev 6:132–140. https://doi:10.4103/0973-7847.99947

12. Das U, Saha T, Ghosh R et al (2020) Trianthema portulacastrum L.: Traditional medicine in healthcare and biology. Ind J Biochem Biophy 57:127–145
13. Sarkar A, Pradhan S, Mukhopadhyay I et al (1999) Inhibition of early DNA-damage and chromosomal aberrations by Trianthema portulacastrum L. In carbon tetrachloride-induced mouse liver damage. Cell Biol Int 23:703–708. https://doi:10.1006/cbir.1999.0439

14. Kumar G, Banu GS, Pappa PV et al (2004) Hepatoprotective activity of Trianthema portulacastrum L. against paracetamol and thioacetamide intoxication in albino rats. J Ethnopharmacol 92:37–40. https://doi:10.1016/j.jep.2003.12.009

15. Saxena S, Verma J, Gautam S (2016) Potential Prophylactic Properties of Apple and Characterization of Potent Bioactive from cv. "Granny Smith" Displaying Strong Antimutagenicity in Models Including Human Lymphoblast TK6(+/−) Cell Line. J Food Sci 81:H508-H518. https://doi:10.1111/1750-3841.13190

16. Checker R, Sharma D, Sandur SK et al (2010) Plumbagin inhibits proliferative and inflammatory responses of T cells independent of ROS generation but by modulating intracellular thiols. J Cell Biochem 110:1082–1093. https://doi:10.1002/jcb.22620

17. Maurya DK, Nandakumar N, Devasagayam TP (2011) Anticancer property of gallic acid in A549, a human lung adenocarcinoma cell line, and possible mechanisms. J Clin Biochem Nutr 48:85–90. https://doi:10.3164/jcbn.11-004FR

18. Jayakumar S, Pal D, Sandur SK (2015) Nrf2 facilitates repair of radiation induced DNA damage through homologous recombination repair pathway in a ROS independent manner in cancer cells. Mutat Res 779:33–45. https://doi:10.1016/j.mrfmmm.2015.06.007

19. Moron MS, Depierre JW, Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 582:67–78. https://doi:10.1016/0304-4165(79)90289-7

20. Kacem M, Simon G, Leschiera R et al (2015) Antioxidant and anti-inflammatory effects of Ruta chalepensis L. extracts on LPS-stimulated RAW 264.7 cells. In Vitro Cell Dev Biol Anim 51:128–141. https://doi:10.1007/s11626-014-9813-7

21. Ahuja D, Goyal A, Ray PS (2016) Interplay between RNA-binding protein HuR and microRNA-125b regulates p53 mRNA translation in response to genotoxic stress. RNA Biol 13:1152–1165. https://doi:10.1080/15476286.2016.1229734

22. Reisz JA, Bansal N, Qian J et al (2014) Effects of ionizing radiation on biological molecules–mechanisms of damage and emerging methods of detection. Antioxid Redox Signal 21:260–292. https://doi:10.1089/ars.2013.5489

23. Singh K, Bhori M, Kasu YA et al (2018) Antioxidants as precision weapons in war against cancer chemotherapy induced toxicity - Exploring the armoury of obscurity. Saudi Pharm J 26:177–190. http://doi:10.1016/j.jsps.2017.12.013

24. Seong KM, Yu M, Lee KS et al (2015) Curcumin mitigates accelerated aging after irradiation in Drosophila by reducing oxidative stress. Biomed Res Int 2015:425380. https://doi:10.1155/2015/425380
25. Clarke KA, Dew TP, Watson RE et al (2016) Green tea catechins and their metabolites in human skin before and after exposure to ultraviolet radiation. J Nutr Biochem 27:203–210. https://doi:10.1016/j.jnutbio.2015.09.001

26. Shirazi A, Mihandoost E, Mahdavi SR et al (2012) Radio-protective role of antioxidant agents. Oncol Rev 6:e16. https://doi:10.4081/oncol.2012.e16

27. Yamaki J, Nagulapalli Venkata KC, Mandal A et al (2016) Health-promoting and disease-preventive potential of Trianthema portulacastrum Linn. (Gadabani) - An Indian medicinal and dietary plant. J Integr Med 14:84–99. https://doi:10.1016/S2095-4964(16)60247-9

28. Singha I, Saxena S, Gautam S et al (2020) Grape extract protect against ionizing radiation-induced DNA damage. Ind J Biochem Biophy 57:219–227

29. Nicolson GL, Ash ME. (2014) Lipid Replacement Therapy: a natural medicine approach to replacing damaged lipids in cellular membranes and organelles and restoring function. Biochim Biophys Acta 1838:1657-79. https://doi:10.1016/j.bbamem.2013.11.010

30. Singha I, Das SK (2015) Grapevine fruit extract protects against radiation-induced oxidative stress and apoptosis in human lymphocyte. Indian J Exp Biol 53:753–761

31. Pearce LL, Epperly MW, Greenberger JS et al (2001) Identification of respiratory complexes I and III as mitochondrial sites of damage following exposure to ionizing radiation and nitric oxide. Nitric Oxide 5:128–136. https://doi:10.1006/niox.2001.0338

32. Rai Y, Pathak R, Kumari N et al (2018) Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. Sci Rep 8:1531. https://doi:10.1038/s41598-018-19930-w

33. Banerjee A, Kunwar A, Mishra B et al (2008) Concentration dependent antioxidant/pro-oxidant activity of curcumin studies from AAPH induced hemolysis of RBCs. Chem Biol Interact 174:134-9. https://doi:10.1016/j.cbi.2008.05.009

34. Sotler R, Poljšak B, Dahmane R et al (2019) Prooxidant activities of antioxidants and their impact on health. Acta Clin Croat 58:726–736. http://doi:10.20471/acc.2019.58.04.20

35. Schaue D, McBride WH (2015) Opportunities and challenges of radiotherapy for treating cancer. Nat Rev Clin Oncol 12:527 − 40. http://doi:10.1038/nrclinonc.2015.120

36. Mun GI, Kim S, Choi E et al (2020) Pharmacology of natural radioprotectors. Arch Pharm Res 41:1033–1050. http://doi:10.1007/s12272-018-1083-6

37. Han S, Gao H, Chen S et al (2019) Procyanidin A1 Alleviates Inflammatory Response induced by LPS through NF-κB, MAPK, and Nrf2/HO-1 Pathways in RAW264.7 cells. Sci Rep 9:15087. https://doi.org/10.1038/s41598-019-51614-x

38. Cao Y, Chen J, Ren G et al (2019) Punicalagin Prevents Inflammation in LPS-Induced RAW264.7 Macrophages by Inhibiting FoxO3a/Autophagy Signaling Pathway. Nutrients 11:2794. https://doi:10.3390/nu11112794

39. Calabrese V, Boyd-Kimball D, Scapagnini G et al (2004) Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: the role of vitagenes. In Vivo 18:245–267
40. Harvey CJ, Thimmulappa RK, Singh A et al (2009) Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. Free Radic Biol Med 46:443 – 53. http://doi:10.1016/j.freeradbiomed.2008.10.040

41. Smith RE, Tran K, Smith CC et al (2016) The Role of the Nrf2/ARE Antioxidant System in Preventing Cardiovascular Diseases. Diseases 4:34. https://doi:10.3390/diseases4040034

**Figures**

**Figure 1**

Scavenging properties of *T. portulacastrum* Free radical scavenging activity and antioxidant capacity from different *T. portulacastrum* extracts through absorption measurement using a) ABTS+• radical scavenging assay at 734nm, b) DPPH radical scavenging assay at 517nm, c) Molybdenum reducing assay at 695nm, and d) Ferric reducing power assay at 700nm. The values are mean ± standard error (SE) of three independent experiments.
Figure 2

Radioprotective activity of T. portulacastrum in cellular and subcellular system T. portulacastrum extract showed protection against lipid peroxidation: LPO activity non-irradiated and irradiated cells treated with increasing concentration of stem extract of T. portulacastrum measured using TBA assay a) in WRL 68 cell, b) in mitochondrial membrane. Values are Mean ± standard error (SE) of three independent experiments. Level of significance: *P<0.05 against control, #P<0.05 against stem extract in ethanol (SE) 125 µg/ml, $P<0.05 against radiation, @P<0.05 against SE 31.25 µg/ml + radiation
Figure 3

Validation of Radioprotective activity Radioprotective property of T. portulacastrum extract in WRL 68 cell system: a) MTT assay of non-irradiated and irradiated cells treated with increasing concentrations of stem extract of T. portulacastrum. OD was read at 595 nm. Clonogenic assay was performed 12 days post irradiation where cells were allowed to form colonies and then counted by crystal violet staining. b) control groups c) different concentration of T. portulacastrum extracts in presence of radiation and e) graphical representation of CFUs from three independent experiments. Values are Mean ± standard error (SE). Level of significance: *P<0.05 against control, #P<0.05 against stem ethanol (SE) 125 µg/ml, $P<0.05 against radiation, @P<0.05 against SE 31.25 µg/ml + radiation
Figure 4

Effect of T. portulacastrum in cellular redox balance Modulation of the cellular redox status by T. portulacastrum extract: a) ROS level of non-irradiated and irradiated cells treated with increasing concentrations of stem extract was measured using DCFDA assay, b) GSH content of non-irradiated and irradiated cells treated with increasing concentrations of stem extract of T. portulacastrum through fluorescent spectra of Monochlorobimane (MCB) at 380/460 excitation/emission wavelength. Values are Mean ± standard error (SE). Level of significance: *P<0.05 against control, #P<0.05 against stem ethanol (SE) 125 µg/ml, $P<0.05 against radiation, @P<0.05 against SE 31.25 µg/ml + radiation, &P<0.05 against SE 62.5 µg/ml + radiation
Figure 5

Anti-inflammatory properties of T. portulacastrum extract upon LPS induced activation of RAW 264.7 cells: a) MTT assay of cells treated with and without LPS in combination of increasing concentrations of stem extract of TP. OD was taken at 595nm. b) NO content of cells treated with and without LPS in combination of increasing concentrations of stem extract using Griess assay. c) GSH content of cells treated with and without LPS in combination of increasing concentrations of stem extract using Ellman's assay. Values are Mean ± standard error (SE). Level of significance: *P<0.05 against control, #P<0.05 against stem ethanol (SE) 125 µg/ml, $P<0.05 against LPS, @P<0.05 against SE 31.25 µg/ml + LPS
Figure 6

Mode of action of T. portulacastrum as anti-inflammatory agent. Semi-quantitative PCR of the total RNA from the cells treated with and without LPS in combination with increasing concentrations of stem extract of T. portulacastrum using a) iNOS primer b) TNF-α and c) Nrf-2. GAPDH was used as internal control. Values are Mean ± standard error (SE). Level of significance: *P<0.05 against control, #P<0.05 against stem ethanol (SE) 125 µg/ml, $P<0.05 against LPS, @P<0.05 against SE 31.25 µg/ml + LPS, &P<0.05 against SE 62.5 µg/ml + LPS.

Figure 7

[Diagram showing radiation, scavenging properties, LPS, and macrophages with reactions involving NO, iNOS, TNF-α, Nrf2, and GSH.]
Schematic representation of radioprotective and anti-inflammatory effect of T. portulacastrum extract
Mode of action of T. portulacastrum extracts exerting radioprotective and anti-inflammatory activity:
Proposed model showing protective effect of stem extract of T. portulacastrum on hepatocytes and
macrophages in response to radiation and inflammatory stimulus. Red arrow indicates the effects of
radiation and LPS; Blue arrow indicates the effects of T. portulacastrum extracts.