Insights into the synergistic radioprotection by secondary metabolites of Pterocarpus santalinus L. aqueous extract

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Abstract

Radioprotective mechanism of chloroform and hydroalcoholic extracts of *Pterocarpus santalinus* on spleen lymphocytes and sub-cellular systems were previously studied. In the present study, we have attempted a comprehensive assessment of the synergistic radioprotective efficacy of *Pterocarpus santalinus* aqueous extract (PSAE). All the studied models were gamma-irradiated with prior treatment with PSAE. First, the content of total phenols (4.061 µg mg^{-1} Gallic acid equivalents), flavonoids (6.616 µg mg^{-1} Quercetin equivalents), and anthocyanins (0.008 mg Cyn3-glu/g) were determined spectrophotometrically. Second, UHPLC-HRMS analysis was performed to identify the possible radioprotectors. Of those, Santalins A & B are known for their usage as natural color in foods and alcoholic beverages identified in PSAE. Treatment was well tolerated with no side effects from PSAE. Later, it was shown that radiation-induced lethality significantly amended in PSAE-treated spleen lymphocytes as evidenced by reduced elevated levels of ROS and lipid peroxidation, restored total thiols and GSH: GSSG, inhibited DNA DSBs and apoptosis. Furthermore, an immunomodulation study was carried out, because radiation exposure induces an inflammatory response. Our study shows that PSAE suppressed concanavalin A-induced T-cell proliferation as evidenced by CFSE dye dilution and CD69 antibody staining methods. Taken together, the current study explored the protective efficacy of PSAE from gamma radiation-inflicted injuries and hence we recommend PSAE as a synergistic radioprotective formulation.

Introduction

Cancer is an important barrier to mounting life expectancy and ranks as the foremost cause of death in the world [1]. In 2023, around 1,958,310 new cancer cases and an estimated 609,820 cancer deaths are projected in the United States [2]. Radiotherapy is one of the highly used treatment strategies which has become a cornerstone of the therapeutic modalities in modern oncology. Based on the size of the tumor, type of cancer, tumor's location, and various other factors, the type of radiation therapy and dose limit of gamma rays vary. Gamma rays are produced following the spontaneous decay of radioactive materials like cobalt-60 and cesium-137, of which, gamma radiations from cobalt-60 can penetrate deeply into the human body. Hence, they are widely used in cancer radiotherapy.

Gamma irradiation-induced elevation in reactive oxygen species (ROS) in cells may trigger cellular derangements such as metabolic stress and DNA lesions [3], damage to lipids by reacting with polyunsaturated fatty acids present in the lipid layer of cell membranes [4], cell death, and cell proliferation [5] probably by exhausting cellular GSH levels that are prominently considered as antioxidant stores [6]. Therefore, it is very important to identify an ideal radioprotector, with fewer side effects and non-toxicity, which can significantly and selectively protect normal cells from the above-mentioned gamma radiation-induced derangements. The most protective radioprotectors developed so far are aminothiols and their derivatives such as aminoethanisothiuronium bromide hydrobromide, cysteamine, and amifostine (WR-2721). Amifostine is the only chemical-based radioprotector approved by the US Food and Drug Administration (US-FDA) [7]. However, report has been made on the adverse

effects of amifostine that lead to its discontinuation [8]. Therefore, the development of effective and ideal radioprotectors has remained an unsolved problem for decades, and the use of herbal radioprotectors possessing pharmacological abilities thus put into effect due to their minimal toxicity.

Around 35% of total drugs in the USA and 80% of drugs used in fast-developing countries like India and China are derivatives of phytoextracts [9]. Many botanicals have been evaluated for their radioprotective efficacy [10]. Of those, *Pterocarpus santalinus* (*P. santalinus*) is a most sought-after plant belonging to Fabaceae family that is widely distributed in tropical regions of Andhra Pradesh in Southern India [11]. In the Indian system of Ayurveda, a wide range of medicinal properties of the heartwood of *P. santalinus* are described [12]. However, the current study is aimed to investigate the protective efficacy of *P. santalinus* aqueous extract (PSAE) against the lethal radiation-induced damage to spleen lymphocytes and sub-cellular systems. The present study also demonstrates the effect of PSAE on redox homeostasis, DNA damage and cell death, and immunomodulatory activity in spleen lymphocytes.

Materials and methods

Chemicals

Trifluoroacetic acid (TFA), oxidized glutathione (GSSG), reduced glutathione (GSH), propidium iodide, and ethidium bromide were purchased from Sigma-Aldrich, Bangalore, India. Carboxyfluorescein succinimidyl ester (CFSE) was procured from Molecular Probes, Netherlands. Concanavalin-A (Con A), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Calbiochem, USA,); antibiotics, RPMI-1640, Fetal Bovine Serum, were obtained from Hi-Media Pvt. Limited. CD-69 monoclonal antibody was procured from BD Pharmingen (USA). All other chemicals were bought from local manufacturers.

Animal maintenance and isolation of splenic lymphocytes

Male Balb/c mice of 10–12 weeks old, weighing 20–25 g, were obtained from the institute and maintained under standard temperature (23 ± 2°C), humidity with a regular food and water supply ad libitum. Spleen lymphocytes were isolated by squeezing the spleen through a nylon mesh (100 μm) into a container with RPMI media followed by a brief hypotonic shock to lyse the RBC [13]. After RBC lysis, cells were washed with ice-cold PBS twice and the cell viability was determined by trypan blue dye exclusion assay. The viable spleen lymphocytes thus obtained were directly used for various cellular assays.

Exposure to gamma radiation

All the radioprotective assays were performed by exposure of samples to gamma radiation (in the range of 4–50 Gy) in the ^{60}Cobalt (^{60}Co) gamma chamber, at a dose rate of 1 Gy min^{-1}, at Bhabha Atomic Research Center (BARC), Mumbai, India.

Intracellular GSH:GSSG ratio

GSH/GSSG ratio was assessed by conventional enzyme cycling method, namely Ellman's reagent [5,5'-dithiobis-2-nitrobenzoic acid (DTNB)], in the presence of NADPH and glutathione reductase (GR) as described by Patwardhan *et al*. [13] Briefly, 2 x 10^6 viable spleen lymphocytes were seeded in a 24 well plate with different concentrations of α-ketoglutaric acid (αKGA) and PSAE (50 µg ml^{-1}), and incubated in a CO_2 incubator (95% relative humidity, 5% CO_2 atmosphere) for 24 h post-irradiation. After incubation, cells were harvested at 14,000 rpm for 2 min, and the collected pellet was re-suspended in ice-cold extraction buffer which was followed by sonication and vortexing for 30 s repeatedly for three times. Then, cells were thawed in liquid N_2 at 37°C for 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant thus obtained was split into two sets, one each for reduced (GSH) and oxidized glutathione (GSSG) estimations. For GSH estimation, DTNB:GR (1:1) was added followed by the addition of 60 µl of NADPH to each well. Similarly, for GSSG estimation, 2 µl of vinyl pyridine, 6 µl of triethanolamine, DTNB:GR (1:1), and NADPH were added. Finally, the absorbance was taken at 412 nm spectrophotometrically using Synergy Bio-Tek microplate reader.

Cellular ROS and total thiols status

1 x 10^6 viable spleen lymphocytes were washed with PBS and incubated with dichlorofluorescein diacetate (DCF-DA, 20 µM) for 30 min in dark at 37°C. After incubation, these cells, obtained at 1h post-experimentation (treatment with 25, 50 µg ml^{-1} of PSAE), were exposed to 4 Gy. Change in the fluorescence due to oxidation of H_2DCF to DCF was measured using Synergy Bio-Tek microplate reader (Ex/Em: 480/ 530 nm) as per the procedure described by Patwardhan *et al*. [13] In parallel, cellular thiol levels were also measured using the monochlorobimane (MCB) fluorometric method after 24 h post experimentation (treatment with 50 µg ml^{-1} of PSAE) [14]. Getting monochlorobimane (40 µM) dye into cells and their reaction results in the formation of GSH-monochlorobimane adduct and was measured fluorometrically (Ex/Em: 390/470 nm) using Synergy Bio-Tek microplate reader.

Assessment of radioprotective property of PSAE

Measurement of lipid peroxidation

Estimation of lipid peroxidation in the liver mitochondrial membrane was performed as per the method of Maurya and Devasagayam [15]. Briefly, liver isolated was homogenized in lysis buffer (0.2% sodium deoxycholate, 50 mM Tris–HCl, 1% NP-40, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA). Mitochondrial membrane fraction (300 µl), isolated as per the procedure described by Shetty *et al*. [16] in potassium phosphate buffer (pH 7.4) was mixed with PSAE (25, 50 µg ml^{-1}), and allowed for 30 min at room temperature and exposed to 50 Gy. The assay mixture obtained at 15 min post-exposure was mixed with 900 µl of thiobarbituric acid (TBA) reagent followed by incubation at 95°C for 20 min. After incubation and cooling, the assay mixture was centrifuged at 12,000 g for 5 min. Fluorescence of the supernatant was taken (Ex/Em: 530/590 nm) against the blank. Quantity of lipid peroxidation was expressed in terms of nmoles of TBARS per mg protein using 1,1,3,3-tetra methoxy propane as standard.

Similarly, estimation of lipid peroxidation in splenic lymphocytes was measured according to the procedure of Sharma *et al*. [17] Around 20 x 10^6 viable spleen lymphocytes were treated for 1 h with PSAE (50 µg ml^{-1}) at 37°C followed by exposure to 20 Gy. The cell pellet was suspended in 300 µl RPMI medium post-exposure, and a TBARS assay was performed as described in the previous section.

Measurement of plasmid DNA damage

Plasmid pBR322 DNA (250–300 ng) in 50 mM potassium phosphate buffer (pH 7.4) was exposed to 50 Gy in the presence and absence of PSAE (2.5, 5, 10, 25, 50 µg ml^{-1}). Radiation-induced plasmid DNA damage was identified by electrophoresis in 1% agarose gel in TBE buffer (89 mM, pH 8.3). Ethidium bromide-stained supercoiled and open circular forms of DNA were separated based on different treatments, and their images were captured using a Syngene gel documentation system [18].

DNA fragmentation analysis

DNA fragmentation analysis is a qualitative technique convenient to visualize the ladder pattern generated due to DNA cleavage by the activation of nuclear endonuclease in agarose gel electrophoresis. Briefly, 1 x 10^6 viable spleen lymphocytes 24 h post experimentation (PSAE treatment, 50 µg ml^{-1}), were incubated in a dry bath in lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 25 mM EDTA, 1% sarcosyl], for 1 h at 50°C followed by the addition of RNaseA (10 µl from 1 mg/ml stock) and incubation for 1 h at 50°C. The activity of RNase A was inactivated by further incubation of the reaction mixture at 65°C for 5 min. Afterward, all the samples were added by 6X DNA loading dye. DNA samples were electrophoresed in a 1.8% agarose gel electrophoresis in TBE (Tris-borate-EDTA) buffer (pH 8.0) and the image of ladder pattern of DNA fragmentation was captured using a Syngene gel documentation system [19].

Estimation of DNA strand breaks

DNA double-strand breaks were evaluated in treated and control murine splenocytes by neutral comet assay as described earlier [20]. Briefly, cells were pretreated with PSAE (50 µg ml^{-1}) and irradiated as described in the previous section. After irradiation, control and irradiated cells were suspended in 0.9% low melting agarose and were layered onto frosted microscopic slides (Gold Coin, Mumbai), processed and images were captured with a high-performance color video camera. Using CASP software, comet parameters like tail DNA percent, tail length, tail moment, and Olive tail moment were measured [21].

Measurement of cell death

Propidium iodide (PI) mediated flow cytometric assay is widely used for the estimation of cell death and is based on the identification of fragmented DNA and, consequently, loss of nuclear DNA content in different groups by using a fluorochrome (PI), capable of binding and labeling DNA. 2 x 10^6 live spleen lymphocytes were treated with PSAE (25, 50 µg ml^{-1}) and incubated for 1 h at 37°C. After incubation, cells were exposed to 4 Gy and cultured for 24 h. Afterward, cells were harvested and incubated with PI staining solution (0.5 µg/ml propidium iodide, 10 µg/ml ribonuclease A, 0.1% sodium citrate and 0.1%

Triton X-100) overnight. Using Partec PAS III flow cytometer, percent cell death was monitored and the data were analyzed using Flowjo software [22].

Proliferation assay

2 x 10^6 viable spleen lymphocytes were stained with CFSE (20 µM, 10 min, 37°C) and washed three times with ice-cold complete medium (RPMI medium containing 10% FBS, 100 U/ml penicillin, and 100 µg ml^{-1} streptomycin). After staining, cells were treated with PSAE (25, 50, 100 µg ml^{-1}) and were stimulated with Con A (2.5 µg ml^{-1}) for 72 h at 37°C. Vehicle-treated cells served as control and the samples were acquired by a flow cytometer (Partec PAS III). Percent daughter cells were calculated using FlowJo software based on the decrease in the CFSE fluorescence intensity [23].

CD69 antibody staining

For antibody staining of an early activation marker, CD69, 2 x 10^6 spleen lymphocytes were pretreated with PSAE (25, 50 µg ml^{-1}) for 1 h, stimulated with Con A (2.5 µg ml^{-1}), and incubated for 24 h at 37 ^oC. After 24 h, cells were stained with FITC conjugated CD-69 antibody and fixed with 10% formaldehyde after PBS wash. Finally, cells were stained with Hoechst dye (10 µg ml^{-1}) and the frequency of CD69 + cells in each treatment group was determined using Flowjo software [23].

Statistical analysis

All data are presented as mean ± standard deviations (SD). Statistical analyses were applied using the One-way ANOVA with Turkey-Kramer Multiple Comparisons as post-test. Asterisks denote statistical significance (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Statistical analyses were carried out using the KyPlot 6.0.

Results

PSAE restored the cellular redox status

The cellular redox status of splenic lymphocytes was assessed by measuring intracellular ROS levels, GSH/GSSG ratio, and total thiols contents. On exposure to 4 Gy, a significant increase in intracellular ROS levels, a decrease in GSH/ GSSG ratio, and total thiols compared to that in unirradiated cells. PSAE (50 µg/ml, 1h) treated cells experienced a significant rise in restored GSH/ GSSG ratio (Fig. 1a) and total thiols (Fig. 1c) than that of 4 Gy cells alone and thus supported in protection from radiation-induced elevated intracellular ROS levels (Fig. 1b).

PSAE exhibits radioprotective role by ameliorating radiation-induced damages

PSAE decreases radiation-induced peroxidation of lipids

Malondialdehyde and other aldehydes are TBARS that can react with TBA to give a pink-colored chromophore, which can be identified fluorometrically (Ex/Em: 530/590nm). In the present study, levels of TBARS were measured in the sub-cellular model (liver mitochondrial membrane) and cellular model (spleen lymphocytes), respectively. When mitochondrial membrane alone was irradiated to 50 Gy, there is a rise in TBARS by 96%. However, treatment of membrane with PSAE (25, 50 µg/mL, 1h) showed a reduction in TBARS levels by 70% compared to that in 50 Gy samples, indicating the radioprotective property of PSAE (Fig. 2a). Likewise, when spleen membranes alone were exposed to 20 Gy, there is a significant rise in TBARS level compared to control. However, treatment with PSAE (50 µg/ml, 1h) reduced their levels by 43% compared to that in the 20 Gy alone group (Fig. 2b).

PSAE protected DNA against radiation exposure

From Fig. 3a, our study demonstrated that gamma irradiation created strand breaks and formed two different bands, namely the upper open circular band (OC) and the lower supercoiled band (SC). During radiation exposure, the SC band of plasmid DNA has decreased significantly due to strand break and is relaxed to the OC band, compared to that in the cells without irradiation. In the case of PSAE (2.5, 5, 10, 25, 50 µg/ml, 1h) treated plasmid DNA, there was an increase in the SC band and a decrease in the OC band compared with that in 50 Gy alone. In addition, our study demonstrated DNA fragmentation patterns of PSAE pretreated and untreated groups of spleen lymphocytes in the presence and absence of gamma radiation (Fig. 3b). Treatment of cells with 4 Gy induced DNA strand breaks as compared to that in the control group. However, PSAE (10, 25, 50 µg/ml, 1h) pretreated cells (-1h), when exposed to 4 Gy, reduced the ladder pattern of the DNA as shown in Fig. 3b, compared to that in the 4 Gy group, indicating that PSAE protected spleen lymphocytes from gamma radiation-induced DNA damage.

PSAE inhibits DNA double strands breaks in splenic lymphocytes

Our study demonstrates that PSAE (50 µg/ml, 1h) showed significant protection to spleen lymphocytes from gamma radiation-induced DNA DSBs (Fig. 4a). In the 4 Gy treated cells, DNA showed very much damage, as evident from increased levels of % tail DNA, tail length, tail moment, and olive tail moment compared to those in the control group. PSAE treated cells (50 µg ml^{-1}, 1h) significantly decreased % tail DNA, tail length, tail moment, and olive tail moment by 52%, 30%, 82%, and 74% respectively, and were represented in the corresponding bar graphs in Fig. 4b-e.

PSAE protects lymphocytes from cell death

Figure 5 shows the effect of PSAE on cell death as assessed by PI staining in gamma-irradiated and non-irradiated spleen lymphocytes. The bars represent the percent cell death at 24 h post-exposure. Cells exposed to 4 Gy showed significant death as compared to that in un-irradiated control cells. PSAE (50 µg/ml, 1h) treated and irradiated cells showed 83% of radioprotection compared to that in 4 Gy alone

cells, indicating that PSAE significantly protected spleen lymphocytes from gamma radiation-induced cell death.

PSAE inhibits Con A-induced T-cell proliferation

Since exposure to radiation causes tissue injuries and induces inflammation, the immunomodulatory role of PSAE was also studied. The effect of PSAE on Con A-induced lymphocyte proliferation, as assessed from CFSE dye dilution and CD69 antibody staining techniques, is shown in Fig. 6. PSAE (25, 50, 100 µg/ml, 1h) significantly inhibited the Con A-induced proliferation of lymphocytes in a dose-dependent manner. This inhibition is not due to cytotoxicity because PSAE (25 µg ml^{-1} and 50 µg ml^{-1}, 1h) did not show any cell death compared to that in control vehicle-treated cells as estimated by PI staining. This study indicates that PSAE has significant immunosuppressive activity.

Discussion

Gamma rays are one of the ionizing radiations, having much more penetrating capacity, mitigating malignant tumors by cell killing mechanism during radiation therapy [24]. Their usage in radiation therapy is crucial as they offer a quality of life for cancer patients. In addition, they are commonly used for the irradiation of foods to improve their quality and extend shelf life. Also, gamma-ray irradiation is commonly applied in sterilizing medical products due to its simple sterilization without heat generation [25]. A COVID-19 rapid letter stated by Venkatraman *et al*. [26] proposed an idea of how gamma rays are used to breakthrough COVID-19 to manage its outbreak. Despite many applications, gamma rays induce adverse effects via the generation of ROS. Due to their instability, ROS are associated with the principle of oxidative stress that induces damage to cellular components like DNA, lipids, and proteins [27]. Biological effects of ionizing radiation vary based on nature and timing after radiation exposure through direct and indirect mechanisms. Hence, it is crucial to provide awareness to the people dealing with gamma radiation about the radiation risks and a comparison of natural sources to medical procedures. The need for radioprotection is therefore paramount and resulted in the identification of various synthetic compounds, antioxidants, phytocompounds, and vitamins, which are being administered exogenously over decades. Howbeit, to date, there is no potential and safe compound that mitigates gamma radiation-induced cellular injuries. Therefore, there is an advantage in the usage of natural compounds bearing negligible side effects. A review by Mun *et al*. [28] evaluated the radioprotective efficacy of several plant-derived compounds extensively based on previous scientific literature. Our research group has been studying intensively on the heartwood of *Pterocarpus santalinus* as the tree is growing most frequently in uranium-rich regions of the Seshachalam hill ranges of the Eastern Ghats in the state of Andhra Pradesh, situated in the southeastern coastal region of India. From the previous literature, we knew that a compound with antioxidant, immunomodulatory, and free radical scavenging activities may act as a potent radioprotector and is the right choice for the researchers to evaluate its radioprotective property [10]. Hence, the current study has designed in the same line and tried to evaluate the radioprotective efficacy of PSAE.

UHPLC-ESI-HRMS/MS studies identified eleven compounds, of which most of them belong to polyphenols. Exposure to ionizing radiation elevates ROS levels and, to combat it, cells have an inbuilt antioxidant system. A previous review stated that antioxidants are efficiently used to prevent oxygen poising and radiation injury caused by ROS [29]. Polyphenols, the main compounds present in plants with good antioxidant properties, facilitate the donation or acceptance of electrons due to the presence of aromatic rings and a hydroxyl group. The total phenol content varies from plant to plant depending on the plant species, developmental stage, environmental factors, and the plant tissue [30, 31]. The findings in our previous and present studies have demonstrated that on changing the solvent system employed in the preparation of extract, there is a variation in estimated amounts of phytoconstituents. Our previous study used chloroform as a solvent system and showed that *Pterocarpus santalinus* Chloroform Extract (PSCE) possesses phenols (404µg/mg Gallic acid equivalents), flavonoids (22.6 µg/mg Quercetin equivalents), anthocyanins (0.066 mg Cyn3-glu/g), and tannins (12.477 g/L) [32]. In line with this report, the present study showed that PSAE contains phenols (4.061µg/mg Gallic acid equivalents), flavonoids (6.616 µg/mg Quercetin equivalents), and anthocyanins (0.008 mg Cyn3-glu/g). Furthermore, PSAE significantly reduced molybdenum (VI) to molybdenum (V) and Fe (III) to Fe (II) in a concentration-dependent manner. In congruence with an earlier report by Gupta *et al*. [33], this finding indicated that the polyphenols from the extract of heartwood of *Pterocarpus marsupium* showed an increased total antioxidant capacity in a concentration-dependent manner. Chang *et al*. [34] also showed that the antioxidant property of phenolics of heartwood extract of *Acacia confusa* is effective.

Radioactive source, Co-60, emits gamma rays and plays a prominent role in medical, commercial, and research facilities for sterilization purposes. However, it is well known that radiation-induced oxidative stress generates ROS and disturbs redox homeostasis, which is considered as one of the primary causes of chronic diseases such as chronic inflammation atherosclerosis, myocardial infarction, diabetes, rheumatoid arthritis, stroke, cancer, and other degenerative diseases in humans [35, 36]. GSH is known to be involved in the natural detoxification of gamma radiation-induced radiolytic products. It donates hydrogen to highly reactive ROS and plays a key role in their conversion to more stable compounds [37]. PSAE supported spleen lymphocytes by reducing radiation-induced intracellular ROS levels by restoring total thiols content and GSH/ GSSG ratio. This study is in consonance with an earlier report of Chang *et al*. [34] on heartwood extract of *A. confusa*, stating the reduced levels of intracellular ROS in HL-60 cells. Gamma radiation-induced excessive ROS levels can cause an elevation in lipid peroxidation levels; thus, the function and structure of biomolecules alter, leading to tissue damage [38]. PSAE (10 µg/ml, 1h) treated membrane samples showed a downfall in TBARS levels by 70% and 43% in sub-cellular and spleen lymphocytes compared to that in the 50 Gy and 20 Gy alone groups, respectively. In line with this report, a study by Patil *et al*. [39] reported that TBARS levels were significantly diminished with an increase in the aqueous extract concentration than that of methanolic extract. Our studies are also in accordance with the results reported by Mansour and Hafez [40]. Several conflicting results of US-FDA-approved radioprotector, amifostine, lead to a need to identify an effective radioprotector [41]. In this view, herbal extracts hold promising effects due to their less toxicity, easy accessibility, acceptability, and economical [7]. In the present study, PSAE has shown non-toxic nature towards spleen lymphocytes and

RAW264.7 macrophage cell lines. It was non-toxic until 100 µg/ml and started slight toxicity from 200 µg/ml.

Ionizing radiation directly causes DNA structure effects, mainly by inducing DNA DSBs [42], which is the most perilous state of DNA induced by radiation exposure due to erroneous DNA repair or loss of genetic information [43]. Excessive production of radiation-induced intracellular ROS can also cause tissue injury through DNA DSBs [44]. Recent studies reported an increase in DNA DSBs after exposure to gamma radiation [45, 46]. In the present study, we identified that PSAE protected plasmid DNA from strand breaks and restored the original DNA confirmation, i.e., SC form. In a previous study, it was reported that pretreatment with green tea Catechin, EGCG, reduced DNA strand breaks on exposure to gamma radiation; thus, there is an increase in the S.C. form of DNA [47]. Meanwhile, PSAE treated cells (50 µg/ml, 1h) have been shown a significant reduction in % tail DNA, tail length, tail moment, and olive tail moment by 52%, 30%, 82%, and 74%, respectively. Irradiated cells pretreated with PSAE showed 83% protection of spleen lymphocytes from gamma radiation-induced cell death and a decrease in ladder formation, as evident from DNA fragmentation assay and PI staining. A study by Mansour and Hafez [48] showed that pretreatment with *Withania somnifera* before gamma irradiation significantly reduced DNA fragmentation. A previous *in vitro* study demonstrated the protective role of extract of *Mesua ferrea* on gamma radiation-induced cell death in spleen lymphocytes [49]. Since exposure to radiation causes tissue injuries and induces inflammation, there is a need to study its immunomodulatory property. In response to T cell mitogen, Con A, spleen lymphocytes showed significant proliferation. However, PSAE significantly inhibited the Con A (10 µg/ml)-induced proliferation of lymphocytes by 73%, as evident from CFSE dye dilution and CD69 antibody staining. It is mainly due to the augmentation of protection of spleen lymphocytes from cell death, as observed in our current study with PI staining. A study by Checker *et al*. [22] demonstrated the immunomodulatory and radioprotective effects of lignans and is in line with our present findings. This finding has also shown in accordance with the report of Murthuza *et al*. [49] that on treatment with the extracts of *Mesua ferrea*, Con A-induced T-cell proliferation was inhibited significantly in a dose-dependent manner. A study by Zimmermann-Klemd *et al*. [50] has also demonstrated that treatment of lymphocytes with *Artemisia argyi* extract significantly lowered CD69 expressions.

Conclusion

We explored the feasibility of PSAE as a radioprotective agent for the treatment of gamma ray-induced ailments. Our preliminary spectral studies showed the presence of polyphenols majorly. Flow cytometry results proved that PSAE is non-toxic to spleen lymphocytes and cytotoxicity study showed that PSAE is safe to RAW264.7 macrophages. In addition, PSAE mitigated the radiation-inflicted ROS, lipid peroxidation, DNA DSBs, and cell death in spleen lymphocytes. Furthermore, PSAE significantly modulated T-cell proliferation in a dose-dependent manner. These findings together conclude that the radioprotective role of PSAE is a combination of its antioxidant, redox modifying, and immunomodulatory efficacies exhibited synergistically by its phytoconstituents. Hence, we recommend

further research on PSAE towards isolation of pure compounds from PSAE and identify radiation countermeasures in the future.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Experiments were performed and manuscript was drafted by **Ghali E N Hanuma Kumar.** UHPLC-HRMS and related analysis were performed by **Sravan Kumar Sandopu.** Supervision of gamma irradiation studies, and data interpretation, and corrections of the manuscript were performed by **Dharmendra Kumar Maurya.** Complete supervision of the work and corrections in the manuscript were performed by **Meriga Balaji**. Permission for cell culture experiments in Radiation Biology & Health Sciences Division (RB&HSD), BARC, Mumbai, was given by **Sandur Santosh Kumar**. Help in acquiring in flow cytometry was done by **Binita Kumar**.

Data availability

The data of these findings will be made available at reasonable request to the corresponding author.

Ethics approval

The study design strictly adhered to the guidelines approved by the Institutional Animals Ethics Committee (IAEC) of Bhabha Atomic Research Centre (BARC), Mumbai (Reference No. BAEC/17/16, dated 18th October 2016).

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Figures

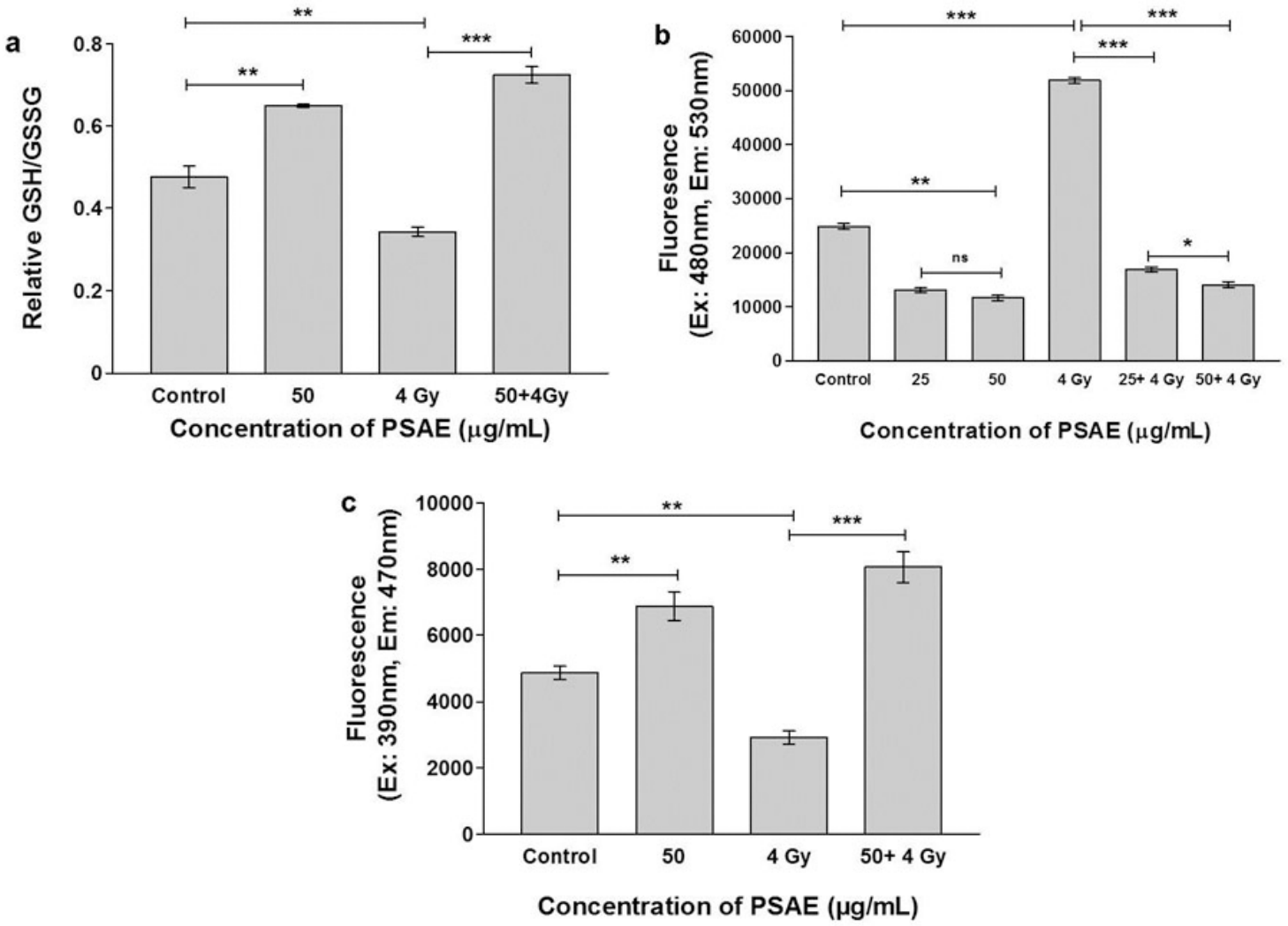

Figure 1

Effect of PSAE on redox homeostasis (a) Relative GSH/GSSG ratio in spleen lymphocytes. (b) Intracellular ROS scavenging activity. (c) Intracellular total thiols by Monochlorobimane fluorometric method. Data represent mean ± SD and experiment was repeated twice. Statistical differences were compared between various experimental groups and a value of $P \leq 0.5$ is considered statistically significant (ns, non-significant, *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$)

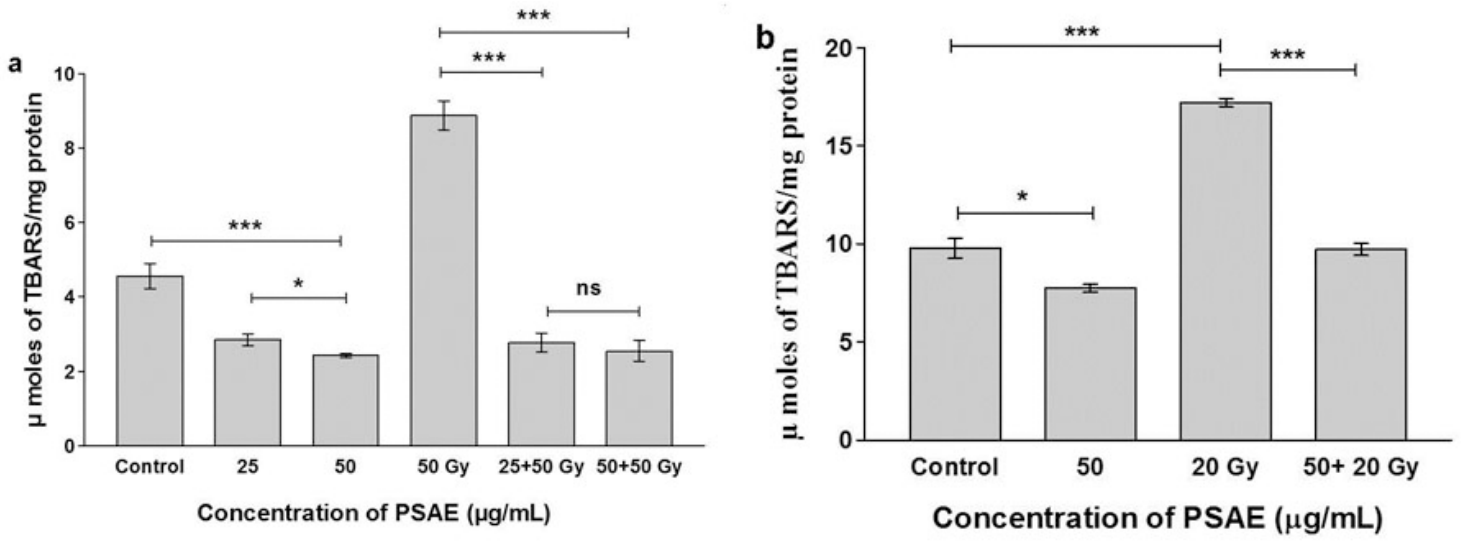

Figure 2

Effect of PSAE on lipid peroxidation by TBARS assay. (a) Protection in the mitochondrial membrane of Balb/c mice liver by PSAE. (b) Protection of spleen lymphocytes by PSAE in spleen lymphocyte membrane. Data represent mean ± SD and experiment was repeated twice. Statistical differences were compared between various experimental groups and a value of $P \leq 0.5$ is considered statistically significant (ns, non-significant, *$P \leq 0.05$ and ***$P \leq 0.001$)

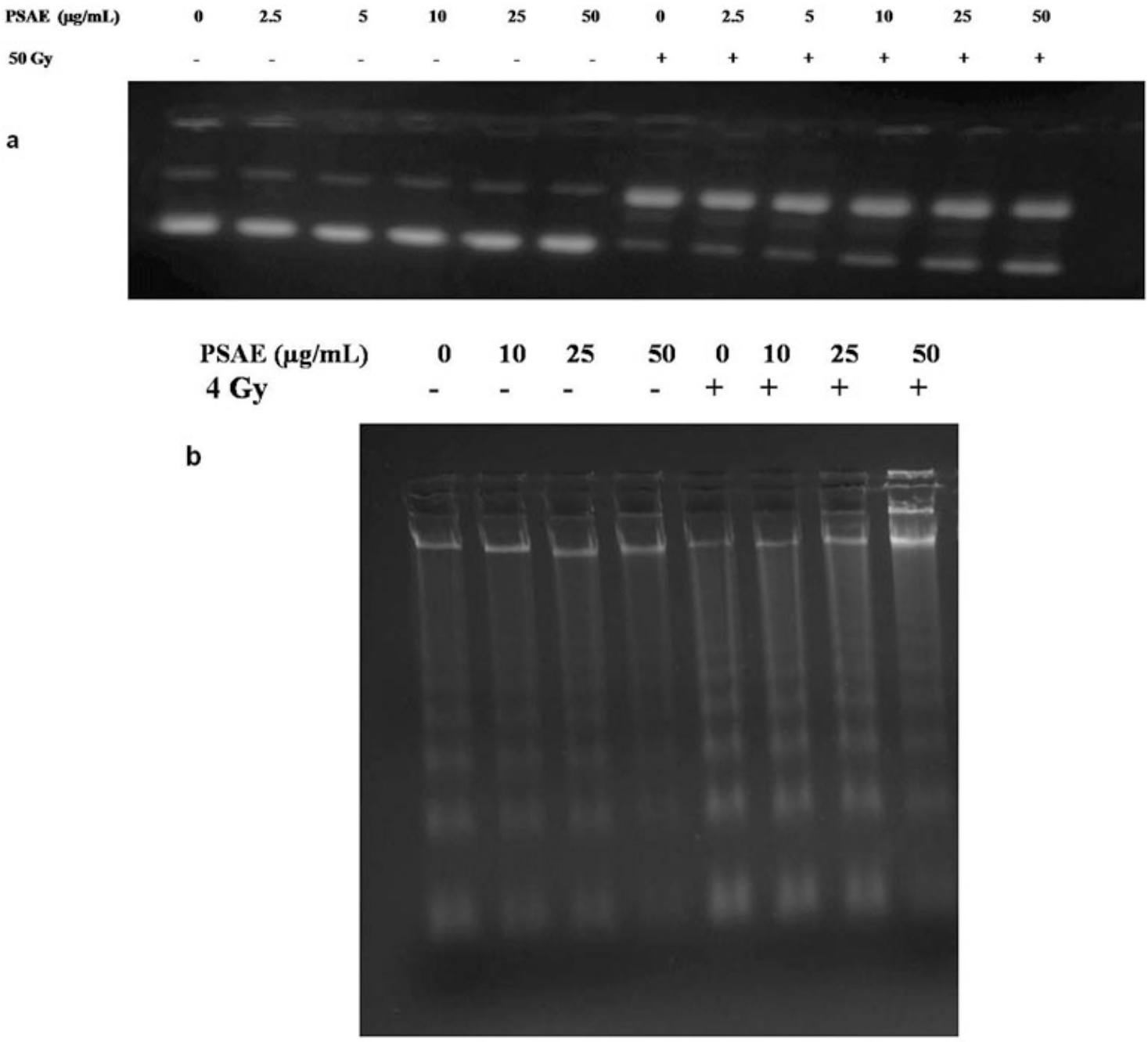

Figure 3

Effect of PSAE on DNA damage. (a) Measurement of plasmid DNA damage by plasmid relaxation assay. (b) DNA fragmentation assay showing Agarose gel with DNA ladders in different groups showing radioprotection of PSAE against γ-radiation-induced DNA breaks-apoptosis

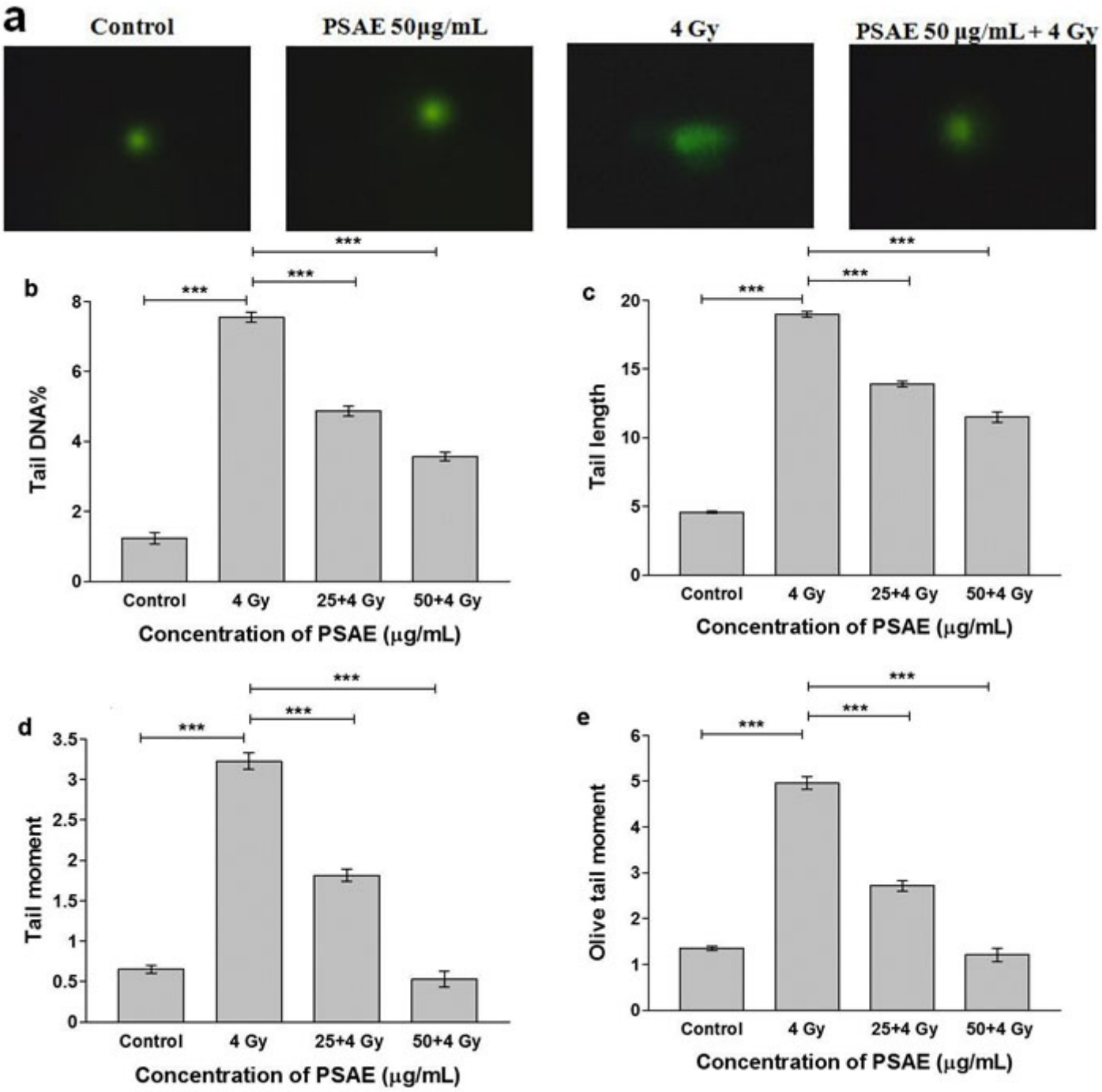

Figure 4

Radioprotective effect of PSAE on DNA DSBs in the presence of γ-radiation. (a) Images of different treatment groups of Comet assay. (b) Tail DNA (%). (c) Tail length. (d) Tail moment. (e) Olive tail moment. Data represent mean ± SD and experiment was repeated twice. Statistical differences were compared between various experimental groups and a value of $P \leq 0.5$ is considered statistically significant (***$P \leq 0.001$)

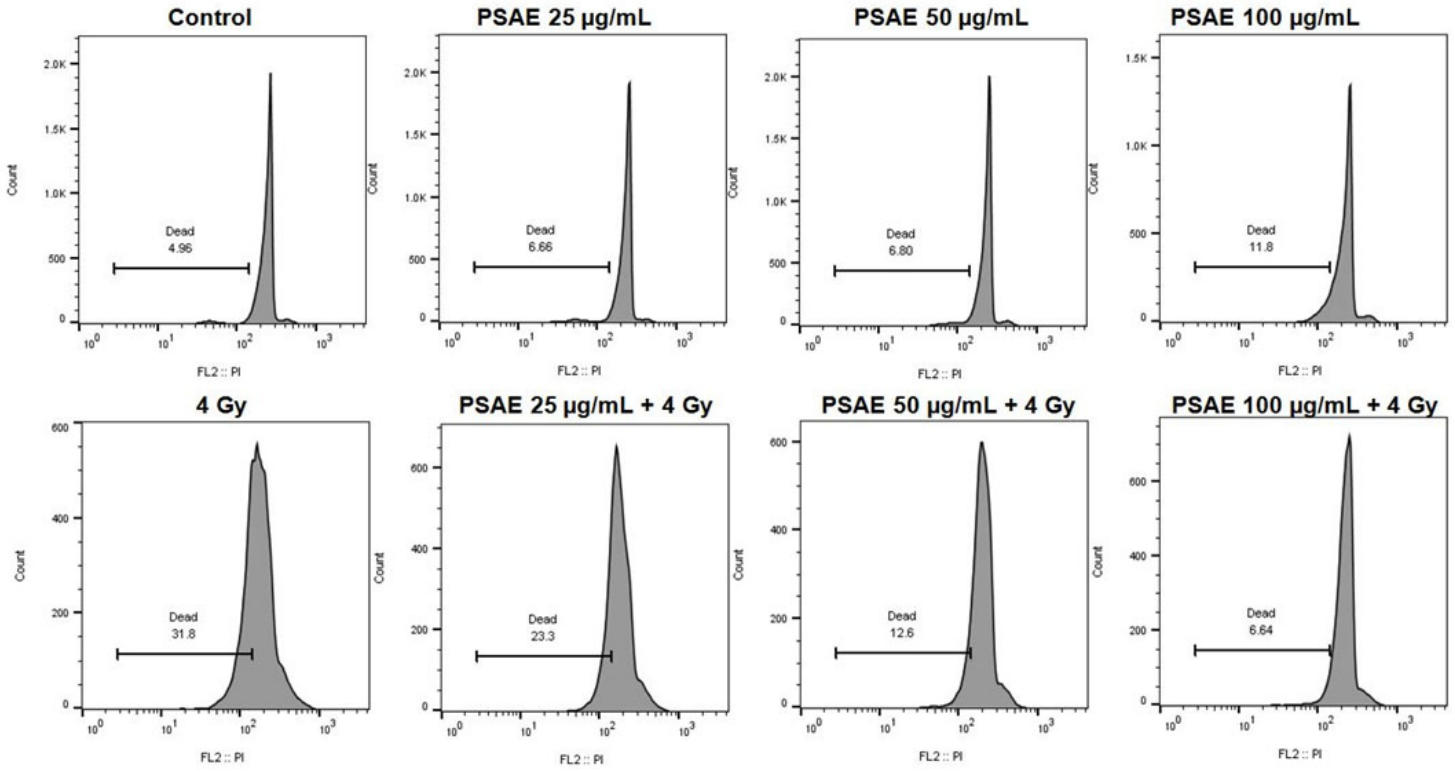

Figure 5

Radioprotective effect of PSAE on cell death. Histograms of Propidium iodide staining of spleen lymphocytes of different treated groups in the presence and absence of radiation exposure (4 Gy)

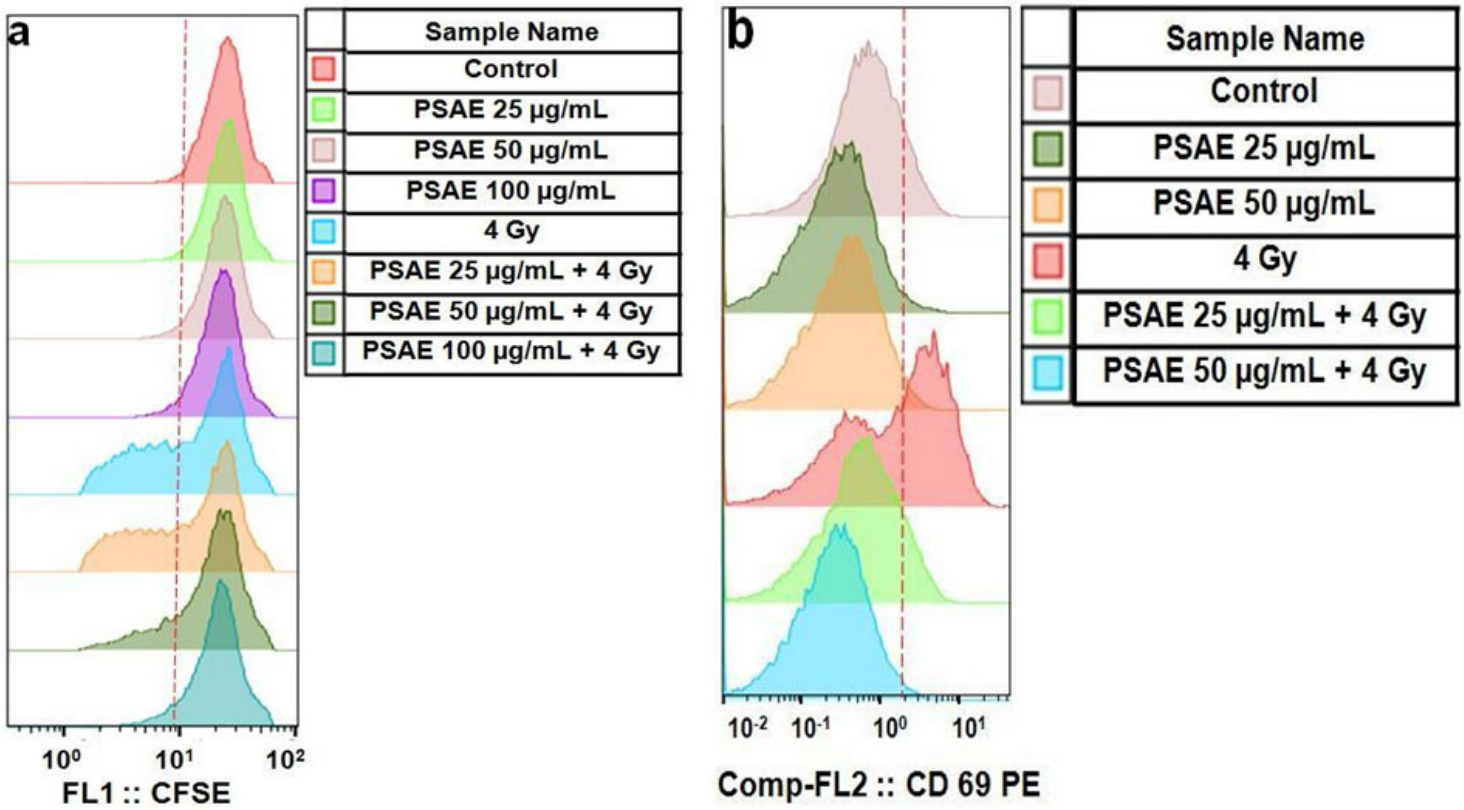

Figure 6

Immunomodulatory efficacy of PSAE. (a) Flow cytometry histogram of CFSE stained spleen lymphocytes followed by Con A stimulation showing immunosuppressive activity of PSAE. (b) Flow cytometry histogram of % $CD69^{+}$ cells in the presence of PSAE by antibody staining technique showing immunosuppressive activity of PSAE

Supplementary Files

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- Supplementarymaterial.docx