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RESEARCH PAPER

Electron Paramagnetic Resonance Spectrometry-based Assessment of Free Radicals Scavenging Potential of N-acetyl Tryptophan Glucoside

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ABSTRACT

Gamma radiation generates free radicals in biological system by inducing cellular water radiolysis. If not neutralised, free radicals oxidise vital bio-macromolecules causing structural and functional impairment and contribute to cell death. In present study, free radical scavenging activities of a novel bacterial secondary metabolite, N-acetyl tryptophan glucoside (NATG) was assessed against gamma-radiation induced damage *in vitro* and *in vivo* models. Effect of irradiated NATG (UV and gamma radiation 8 Gy and 20 Gy) on its free radical (DPPH radicals) and SOD-like activity was evaluated using EPR spectrometry. To assess the effect of NATG irradiation on its antioxidant potential, EPR based ascorbate, PBN and NO radicals scavenging activities were evaluated in blood and spleen tissue of strain A male mice. Results of the study indicated significant ($p < 0.05$) increase in DPPH radicals scavenging ability of irradiated NATG as compared to un-irradiated NATG. Similarly, irradiated NATG exhibited significant ($p < 0.05$) elevation in SOD-like activity as compared to control. Subsequently, NATG treatment displayed enhanced antioxidant activity as evident by significant ($p < 0.05$) decline in ascorbate, PBN and NO radicals at 1 h and 2h in blood and spleen tissues homogenate of treated mice as compared to control group. In conclusion, NATG possesses significant free radicals scavenging and radio protective capabilities against gamma radiation induced oxidative stress.

Keywords: Electron paramagnetic resonance spectrometry; Bacterial metabolite; Radiation protection; Oxidative stress; Free radicals

1. INTRODUCTION

Ionising radiation exposure causes oxidative damage to the living tissues and creates oxidative stress in the biological systems that may lead to death[1,2]. With the increasing use of nuclear radiation in daily life particularly in the field of research, energy generation, disease diagnosis, radiotherapy of cancer patients, space applications and persistent threat of unplanned radiation eventuality due to accidental or deliberate radiation exposure due to atomic weapon detonation, safety of humankind is of utmost significance. Therefore, it is necessary to develop radioprotective agents with low toxicity to combat planned and unplanned radiation emergency. Most of the radiation-induced damage to biomolecules in cellular aqueous media is caused by the free radicals generated by water radiolysis. Antioxidant agents are able to prevent the occurrence of such deleterious processes, mainly due to their free radical scavenging properties[3]. Antioxidants convert the free radicals into stable products and thereby prevent them to react with other molecules in the vicinity and thus block the free radicals mediated chain reactions like interactions with hydrogen peroxide to form ferryl, perferryl species, which can initiate lipid peroxidation[4].

The capability of radioresistant bacteria to produce radioprotective molecule(s) of human interest has been extensively reviewed[5]. Various prokaryotic organisms including *Deinococcus radiodurans, Rubrobacter radiodurans, Micrococcus radiodurans, Thermococcus gammatolerans* and a gigantic group of *Bacillus* sp. are known to resist against extreme environments such as extreme gamma-radiation, desiccation, high temperature, oxidative stress, etc[6]. Radioresistant bacteria possibly adapted against oxidative stress induced by desiccation or supra-lethal doses of gamma-radiation. Therefore, hypothetically these microbes might be a rich source of antioxidants and radioprotective agents. One of the several hypotheses of radioresistance development in the microbes can be explained by their ability to synthesise specific anti-radiation/antioxidant biomolecules to neutralise the free radicals generated by irradiation in their own cellular environment. In view of above, novel strategies adopted by radioresistant bacteria to combat oxidative stress induced by gamma irradiation may open-up new avenues to explore them as novel source of radioprotective drug for human applications against radiation induced lethality[5,7].

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In view of above background in the present study, N-acetyl tryptophan glucoside (NATG), a secondary metabolite, initially isolated from radioresistant bacterium *Bacillus sp*. INM-1 was evaluated in irradiated and un-irradiated form for its free radicals and antioxidant activities as determined via DPPH- and SOD-like scavenging activity *in vitro* using electron paramagnetic resonance spectrometry. Animal studies were undertaken to elucidate the role of NATG in the modulation of radiation-induced hematopoietic damage. This assessment comprised of free radicals scavenging efficacy of gamma irradiated and untreated NATG in spleen and blood of mice.

2. MATERIAL AND METHODS

2.1 Electron Paramagnetic Resonance Spectrometric Analysis

For all electron paramagnetic resonance (EPR) measurements X-band EMX micro EPR spectrometer (Bruker, Germany) equipped with a standard resonator was used. Standard quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (3 mm i.d., 150 mm length, 0.1 mm wall thickness) that placed in the EPR cavity. All EPR experiments were performed in triplicate at room temperature (18 °C to 23 °C). Spectral processing (g-value calculation) was performed with Bruker WIN-EPR and SimFonia software.

2.2 Direct EPR Spectrometric Analysis of NATG

Direct EPR spectrometric analysis of NATG in powdered and aqueous solution before and after UV irradiation. For all EPR measurements, X-band EMX micro EPR spectrometer (Bruker, Germany) equipped with standard resonator was used. Experiments were performed in triplicate at room temperature (18 °C - 23 °C) and relative humidity 40 per cent. Spectral processing (g-value calculation) was performed using Bruker WIN-EPR and SimFonia software. Spectra of NATG in powder and aqueous solution were recorded using following EPR settings.

2.3 EPR Analysis in Dry Powdered Form

Gain 2 x 10^3, microwave power 0.645 mW, centre field 3514 G, time constant 327.680 ms, sweep time 61.440 s, modulation amplitude, 12.00 G, 1 scan per sample.

2.4 EPR Analysis Insolution Form

Gain 1 x 10^5, microwave power 6.494 mW, center field 3514 G, time constant 163.840 ms, sweep time 16.384 s, modulation amplitude 12.00 G, 1 scan per sample. The EPR analysis parameter settings for the UV-irradiated NATG samples were same as mentioned above except reduced modulation amplitude to 1.00 G.

2.5 Sample Preparation

Sample preparation for determination of DPPH radical scavenging activity of NATG. NATG powdered samples were UV irradiated using a UV–VIS Transilluminator-4000 (Stratagene, USA) using wavelength range from 290 nm - 320 nm for 2 h in the dark. While, in another group, NATG was irradiated with 8 Gy (dose rate of 0.574 Gy/min) and 20 Gy (dose rate of 1.11 kGy/h) gamma radiation. 1.0 mg UV-irradiated and gamma irradiated (20 Gy and 8 Gy) as well as untreated control NATG powdered samples were stirred with 1.0 ml of distilled water for 30 minutes at room temperature. Solutions with different concentrations of NATG (0.1- 0.012 per cent) were sonicated for 2 min (Sonicator Water bath Elmasonic PH750 EL). Homogeneous NATG solution (0.1 per cent - 0.0125 per cent) was mixed with 250µl of ethanolic solution of DPPH (200 µM). DPPH free radicals scavenging activity of NATG was determined using EPR spectrometry method as follows.

2.6 Evaluation of DPPH Radicals Scavenging Activity of NATG

Radical scavenging activity of NATG was determined according to Bernardo[8], *et al*. with slight modifications as described by Zheleva[9], *et al*. Briefly, 250 µl of DPPH (200 µM) alcoholic solution was added to 10 µg - 60 µg samples of NATG stock (1 mg/ml aqueous stock) solution. After 2 min incubation in the dark, the mixture was transferred to a quartz capillary tube. The control sample contained 250 µl ethanol solution of DPPH plus 10 µl of distilled water was also prepared simultaneously. Time-dependent DPPH radicals scavenging activity was determined by adding 60 µg samples of NATG (1 mg/ml stock) to 250 µl ethanol solution of DPPH (200 µM) and EPR spectra was recorded after completion of 10 min - 30 min incubation period. The percent of the DPPH radicals scavenged by the NATG was calculated according to the equation:

$$S\textit{cavenged DPPH radicals}\ (\textit{per cent}) = [(I_o\text{-}I)/I_o] \times 100\ \%$$

where $-I_o$ was integral intensity of the DPPH radical signal of the control sample and I was the integral intensity of the DPPH radical signal after addition of radical scavenger NATG to the control sample.

2.7 Determination of DPPH Radicals Scavenging Activity of NAT

DPPH radicals scavenging activity of NATG was determined according to the Brand-Williams[10], *et al*. with some modifications. 0.3 ml of NATG (stock 1 mg/ml in H_2O) before and after UV/ gamma irradiation was added to 0.1 ml 1M Tris-HCl buffer (pH 7.9) and mixed with 0.6 ml of 80 µM DPPH ethanolic solution. The reaction mixture was incubated for 10 minutes at room temperature. After incubation completion, absorbance of the reduced DPPH was recorded at 517 nm. The experiments were carried out in triplicate. Percent of the DPPH radicals scavenged by NATG was calculated according to the equation:

$$\textit{Scavenged DPPH radical}\ (\textit{per cent}) = [(A_0 - A_S)/A_0] \times 100$$

where $A_{0;}$ absorption of the control samples while, $A_{S;}$ absorption of the test samples at 517 nm.

2.8 Determination of Sod-Like Activity of NATG

Superoxide dismutase activity (Cu/Zn-SOD) was determined in the erythrocyte lysate by the method described by Sun[11], *et al*. and modified by Gadjeva[12], *et al*. The hypoxanthine/xanthine oxidase system was used to generate superoxide anion. 0.2 ml hypoxanthine, 0.02 ml EDTA, 0.4 ml NBT, 0.88 ml PBS-buffer was mixed with 1 mg/ml of NATG. Reaction mixtures were incubated for 20 min at 37 °C and then chilled in ice bath to stop the reaction. Superoxide anion reduces nitrobluetetrazolium (NBT) into soluble formazan which was estimated spectrophotometrically at 560 nm. One unit of superoxide dismutase like activity is defined as the amount of enzyme that produces 50 per cent inhibition of reduction to formazan.

2.9 EPR Studies to Evaluate *In Vivo* Oxidative Stress

Electron Paramagnetic resonance studies conducted to evaluate *in vivo* oxidative stress in un-irradiated and irradiated NATG treated mice. For EPR measurements an X-band EMXmicro, EPR spectrometer (Bruker, Germany) equipped with standard resonator was used. Spectral processing was performed using Bruker WIN-EPR and SimFonia software. To evaluate the role of NATG in overcoming oxidative stress induced by chemical oxidants, levels of ascorbate, NO radicals and other ROS were calculated by double integration of the corresponding EPR spectra registered with blood and spleen tissue homogenates.

3. SAMPLE PREPARATION

Male strain A'mice (n=6/ group) were injected (-2h) with un-irradiated and (8Gy and 20Gy) irradiated NATG (40-80 mg/kg b.wt, i.p). After completion of incubation period (1-2h) animals were dissected. Blood and spleen tissue were collected and homogenized in cold PBS buffer and direct as well as spin trapping EPR spectrometric analysis was performed. Results were compared to those of non-treated control mice.

3.1 Determination of Reactive Oxygen Species

The level of reactive oxygen species (ROS) generation in the blood and spleen of the mice was studied according to Shi[13], *et al*. with some modifications by Zheleva[9], *et al*.. Following experimental groups were formed, Gp 1: untreated control mice (n=6), Gp 2: mice (n=6) treated with normal NATG (40 mg/kg b.wt), Gp 3: mice (n=6) treated with normal NATG (80 mg/kg b.wt), Gp 4: mice (n=6) treated with irradiated (8Gy) NATG (80mg/kg b.wt), Gp 5: mice (n=6) treated with irradiated (20 Gy) NATG (80 mg/kg b.wt). Briefly, about 0.1gm of spleen sample was homogenised with 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. ROS level was monitored using following EPR settings: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain $1x10^6$; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s, 5 scans/ sample. Data obtained was plotted and compared with control groups. Each experiment was performed three consecutive times. The results obtained were compared between all experimental groups for radical levels. Data was averaged after integration of the double integrated plate and interpreted in terms of arbitrary units (a.u = DI/N).

3.2 Determination of Ascorbate Radicals

The ascorbate levels in spleen homogenate were analysed according to Buettner and Jurkiewicz[14] with slight modifications. Experimental groups taken for the study were same as mentioned above. Spleen tissue was collected in cold saline and processed immediately. Tissue samples were weighed, homogenised in DMSO (10 per cent w/v) and centrifuged at 4000 xg, at 4 °C temperature for 10 minutes. Supernatant was collected and level of ascorbate radicals determined using EPR spectrometry. EPR settings used were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 10^4; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1scans/ sample. The experiments were repeated thrice and results presented as an average after integration of the double integrated plate and interpreted as arbitrary units (a.u = DI/N).

3.3 Determination of Nitric Oxide Radicals

The levels of NO• radicals were studied according to the methods of Yoshioka[15], *et al.* and Yokoyama[16], *et al*. with some modifications. Experimental groups were same as mentioned above. Briefly, 50 µM solution of Carboxy PTIO.K was dissolved in a mixture of 50 mM Tris (pH 7.5) and DMSO in a ratio of 9:1. 100 µl tissue homogenate was added to 900 µl of Tris buffer (pH 7.5) dissolved in DMSO (9:1). The mixture was centrifuged at 4000 rpm for 10 minutes at 4 °C temperature. 100 µl of spleen homogenate and 100 µl of 50 mM Carboxy PTIO were mixed and EPR spectrum of the spin adduct formed between Carboxy PTIO spin trap and generated NO• radicals was recorded. The EPR settings used to captured •NO radicals EPR signals were as follows: 3505 G centerfield, 6.42 mW microwave power, 5G modulation amplitude, 75 G sweep width, $2.5x10^2$ gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample. The results obtained after three repetitions were averaged and compared after integration of the double integrated plate and plotted in terms of arbitrary units (a.u = DI/N).

4. RESULTS

4.1 Analysis of Radiation-induced Effects

Results from direct EPR spectrometric analysis of N-acetyl tryptophan glucopyrranoside powder and aqueous form before and after irradiation are presented in Figs. 1(a), 1(b), and 1(c).

EPR spectra of NATG were recorded after irradiation with UV radiation in powder and solution phase. Results

of the study demonstrated almost similar spectral pattern in terms of shape and intensities with both solid and aqueous solution phase NATG. The EPR spectra recorded with UV irradiated NATG demonstrated similar shape but different peak intensities. Intensity of irradiated NATG radical (Figure 1a,b,c) decreased (three fold) significantly as compared to un-irradiated NATG. Present observations suggested that NATG radical becomes unstable after irradiation and acquired electrondonating ability and thus act as antioxidant by undergoing radiation mediated oxidation.

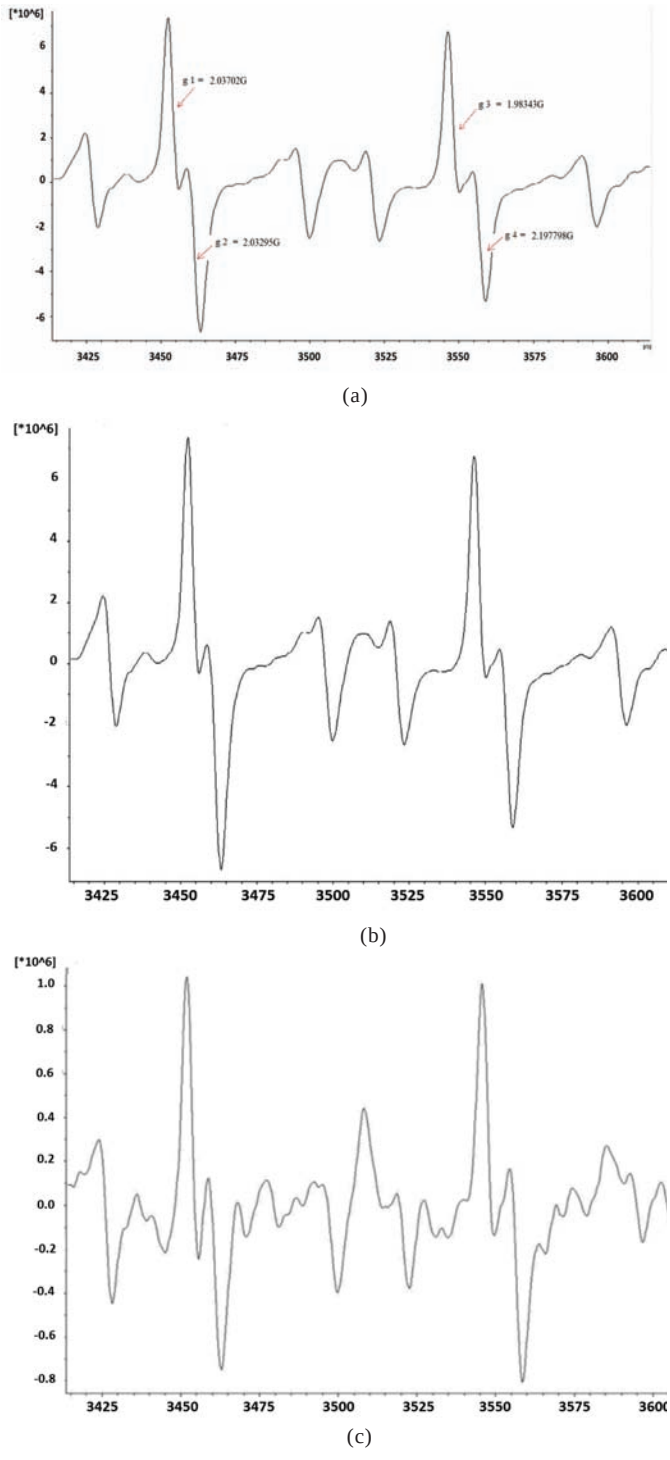

Figure 1. Direct EPR spectrometry of N acetyl tryptophan glucopyrranoside (NATG) recorded in a solid powder form (a) and in aqueous solution, (b) form before UV-irradiation and after irradiation, and (c) The values are average of three consecutive measurements.

4.2 DPPH Radicals Scavenging Activity

To determine the DPPH radicals scavenging properties of NATG via EPR spectrometry, increasing concentration (10 µg - 60 µg) of NATG (1 mg/ml stock) were added to a fixed volume (1ml) of DPPH solution. The neutralisation of DPPH radicals was observed using EPR spectrometer. Decreasing concentration of DPPH radicals was represented by decreasing EPR signals intensity at standard EPR conditions. A significant NATG concentration (10 µg - 60µg) dependent decrease in EPR signal intensity of DPPH radicals was observed (Figs. 2(a) and 2(b). Approximately, 50 per cent reduction in DPPH radical's EPR signals intensity was observed immediately after mixing 60 µg (stock 1mg/ml) of NATG to DPPH solution (Figs. 2(a) 2(b) and 2(c)). However, relatively lower concentrations i.e 10 µg and 30 µg of NATG (1mg/ml stock) were found to neutralise about 14.28 per cent and 41.11 per cent of DPPH radicals, respectively (Figs. 2(b) and (c)).

4.3 Effect of Incubation Time on DPPH Radicals Scavenging Activity of NATG

The effect of incubation time on DPPH radicals scavenging potential of NATG was evaluated using similar EPR spectrometer settings (refer material and method section). As compared to immediate DPPH radicals scavenging (i.e. 50.51 per cent; Figure 2(c)) by NATG (60 µg), 10 min incubation did not enhance NATG radicals scavenging (57.08 per cent) activity significantly (Figs. 2(c) and 2(d)). However, 30 min incubation of NATG with DPPH radicals demonstrated a mild increase (64.99 per cent) in its radical scavenging capacity (Figs. 2(c) and 2(d)).

4.4 Effect of UV and Gamma Irradiation on DPPH Radicals Neutralising Activity of NATG

A dose dependent increase in DPPH radicals scavenging activity of both UV and gamma-irradiated NATG (10 µg – 60 µg) was observed (Fig. 3(a). UV-treated and gamma-irradiated (20Gy) NATG was found more efficient in DPPH radicals scavenging (88.9 ±0.00 %, 91.54 ±0.026 per cent) as compared to un-irradiated NATG (50.51 ±0.04 % at 60 µg). Interestingly, even at lower concentration (i.e. 10 µg of stock 1mg/ml), UV-treated and gamma-irradiated (20Gy) NATG was observed (Fig. 3(a) to neutralise DPPH radicals more efficiently (51.2 ±0.03 % and 80.41 ±0.09 % for UV and gamma irradiated NATG respectively) as compared to un-irradiated NATG (14.28 ±0.0 per cent). Therefore, present observations suggested that UV-and gamma-irradiation enhanced radicals scavenging activity of NATG.

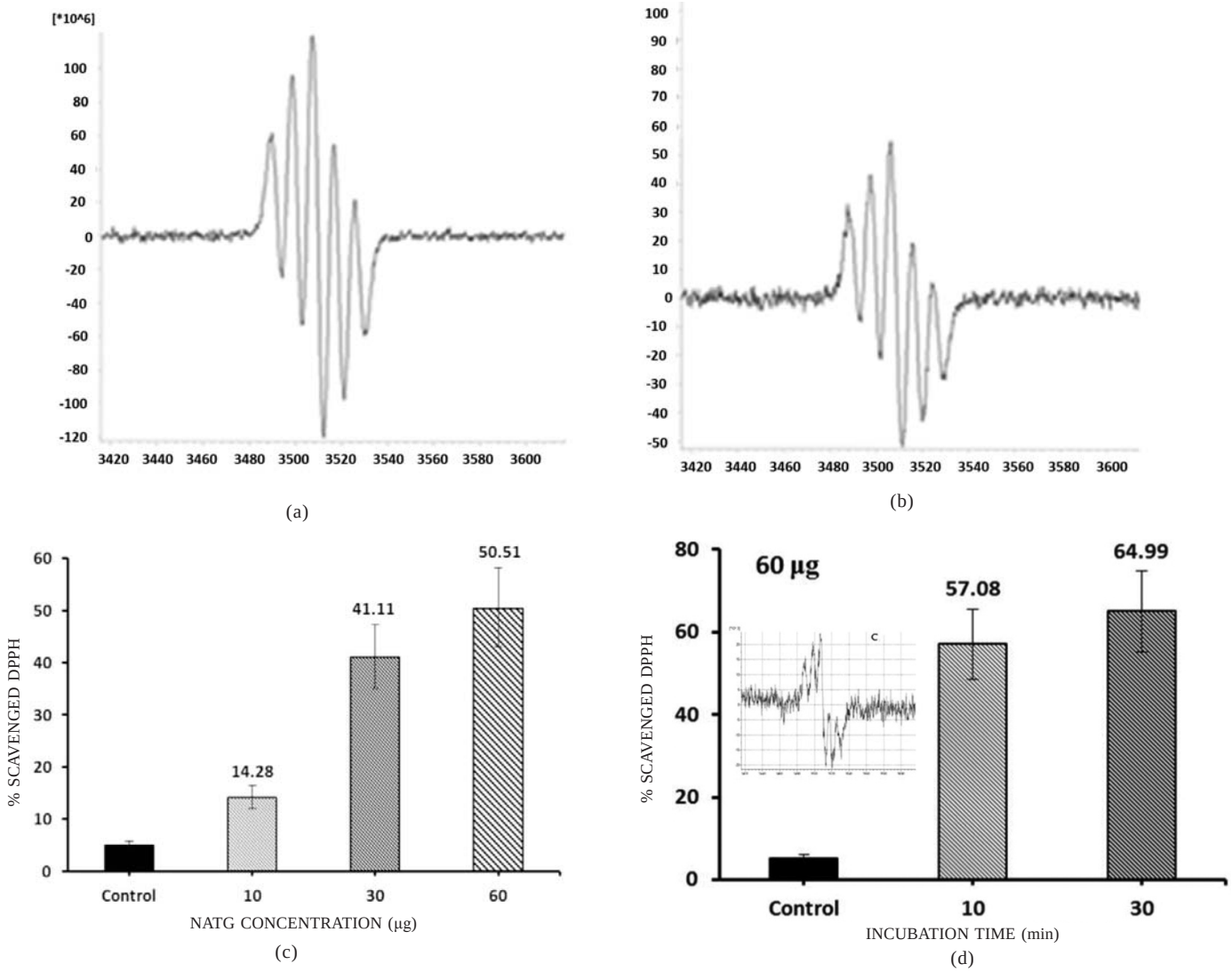

Figure 2. EPR spectrum of the ethanolic solution of DPPH (200 µM) after NATG (1 mg/ml stock) treatment: (a) EPR spectrum of the DPPH and 60 µg NATG mixture, (b) EPR spectrum of DPPH and NATG after 30 min of incubation, (c) Concentration dependent DPPH radicals scavenging activity of NATG, and (d) EPR spectrometric measurements of DPPH radicals neutralising activity of NATG as the function of reaction incubation time.

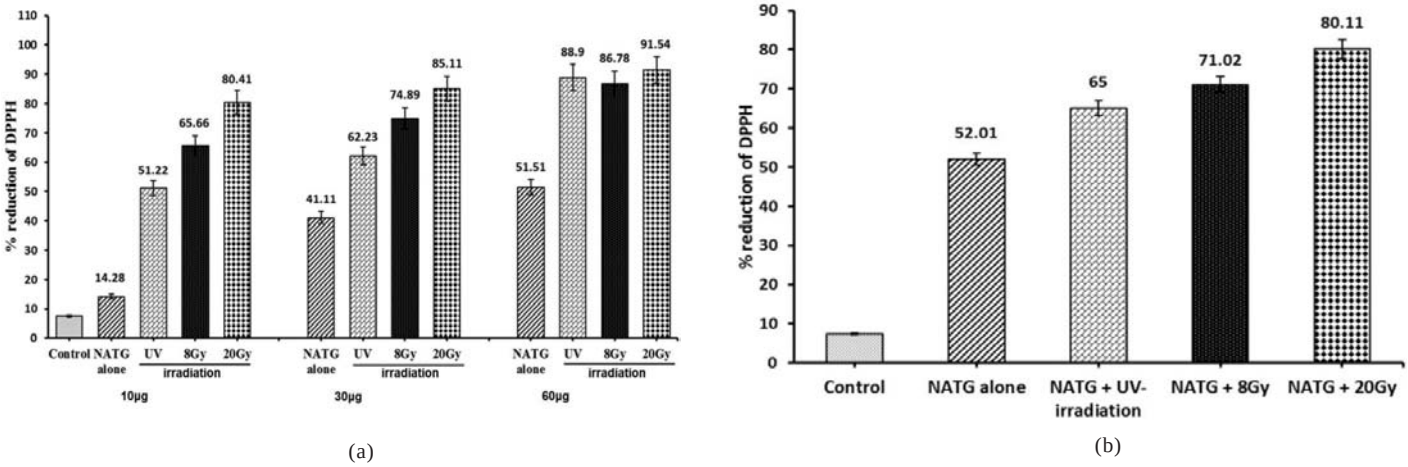

Figure 3. (a) EPR spectrometric measurements of DPPH radicals scavenging activity of NATG upon UV- treatment and gamma irradiation and (b) UV-Vis spectrometric estimation of DPPH radicals scavenging activity of UV treated and gamma-irradiated NATG.

4.5 Effect of Ultraviolet and Gamma Radiation on DPPH Radicals Scavenging Activity of NATG

With complete agreement to EPR based analysis (Fig. 3(a)), UV-Vis spectrometric analysis also demonstrated that UV and gamma-radiation exposure to NATG significantly enhanced [65.0 ±0.71 %, 80.11 ±0.04 % for UV irradiation and gamma irradiation (20Gy) respectively] its DPPH radicals neutralising activity (Fig. 3(b)) as compared to un-irradiated control group (52.01 ±0.09 per cent). Further, radical scavenging activity of NATG was found to be approximately similar whether it was estimated by EPR or UV-Vis spectrometer.

4.6 Determination of SOD-like Activity of NATG

Analysis of SOD-like activity of NATG aqueous solution (60 µg) was performed via spectrophotometric method. Un-irradiated NATG (60 µg) exhibited a significant increase (38.04 ± 0.03 µg/ml) in SOD-like activity as compared to control (Fig. 4). Similarly, UV and gamma irradiated NATG demonstrated higher SOD-like activity (40.05 ± 0.005 µg/ml and 45.44 ± 0.07 µg/ml, respectively) when compared to control. Though, maximum (52.2 ±0.008 µg/ml) increase ($p<0.05$) in SOD-like activity was observed with irradiated (8 Gy) NATG as compared to un-irradiated NATG (Fig. 4). These observations indicated that NATG exhibits SOD-like activity that may enhance further by gamma irradiation.

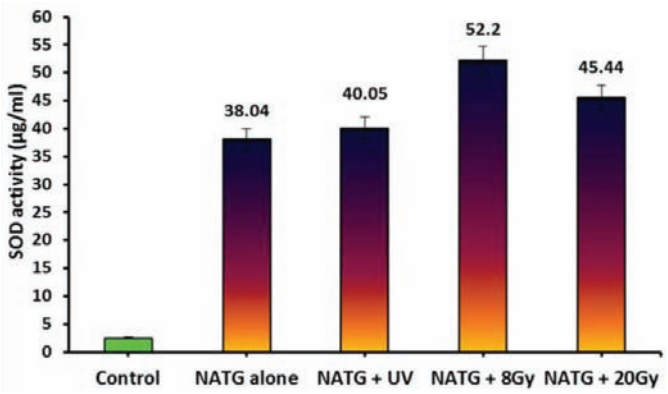

Figure 4. SOD–like activity of NATG (60 µg) before and after UV- and gamma-irradiation.

4.7 *In vivo* Free Radicals Scavenging Properties of Unirradiated and Irradiated (8 Gy, 20 Gy) NATG

Antioxidant status and free radicals scavenging activity of NATG was evaluated in the spleen and blood samples of the mice (Strain A male mice) using EPR spectrometric analysis. Results of the study indicated a significant ($p<0.05$ per cent) reduction in ascorbate radicals concentration in the spleen of NATG treated mice at both the tested concentrations (i.e. 40 mg/kg, 80 mg/kg b.wt.; 1h-2h) as compared to untreated control group of mice. Whereas, no significant modulation in ascorbate radicals concentration was observed in blood samples of NATG treated mice as compared to untreated control mice (Fig. 5(a)).

Significant ($p<0.05$) decline in N-tert-butyl-alpha-phenyl-nitrone (PBN) adducts was observed in the spleen of NATG administered mice at both (40 mg/kg and 80 mg/kg b.wt) concentrations and time points (1 h - 2 h) as compared to untreated control mice (Fig. 5(b)). Though, no such reduction in PBN radicals was observed in the blood of the mice administered NATG. However,

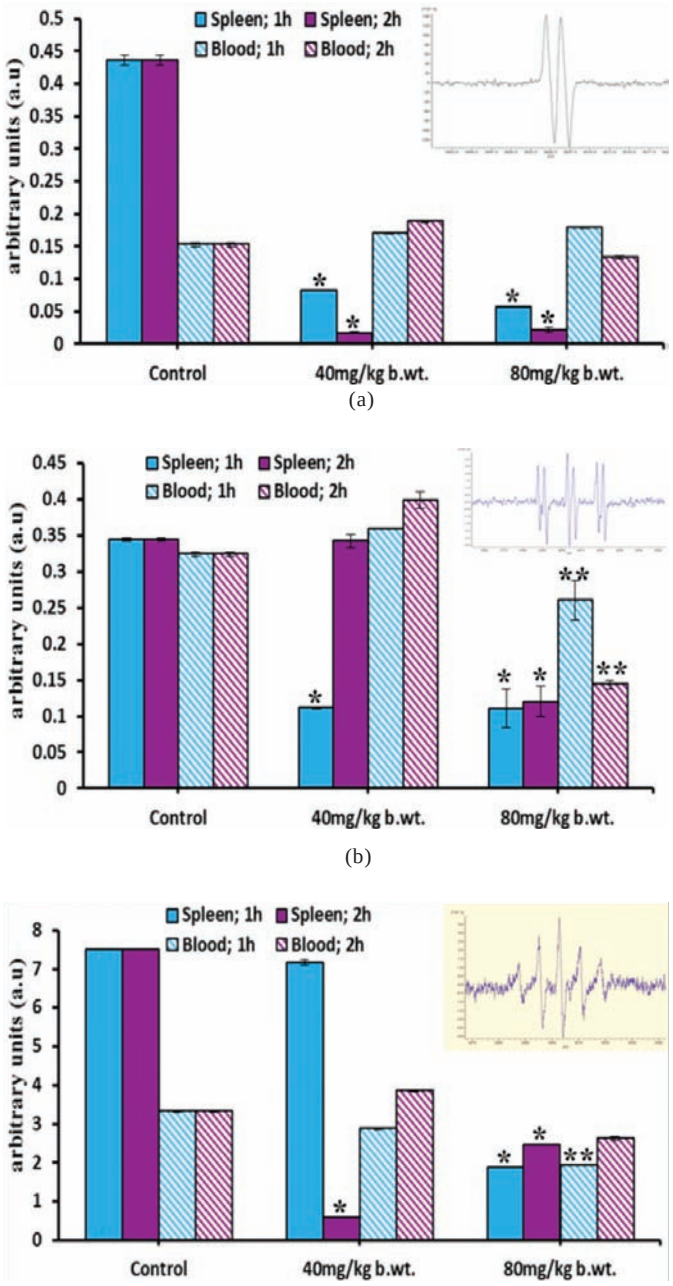

Figure 5. EPR spectrometric analysis of free radical scavenging activity of NATG in spleen and blood tissue of the mice: (a) Ascorbate radical analysis, (b) ROS radical scavenging estimation using PBN adducts, and (c) NO radical scavenging analysis.

* $p<0.05$ NATG treated groups Vs untreated control group in spleen tissue of mice;

** $p<0.05$ NATG treated groups Vs untreated control group in blood of mice.

significant ($p<0.05$) reduction in PBN radicals(0.2605 a.u and 0.1439 a.u) was noticed in the blood sample of mice treated with 80 mg/kg b.wt NATG at both time points (1 h - 2 h) as compared to untreated control group of mice.

Results of the present study also indicated a significant reduction in NO* radicals concentration in the spleen of NATG treated mice at 40 mg/kg - 80 mg/kg as compared to untreated control group of mice (Fig. 5(c)). However, no such reduction in NO* radicals was observed in the blood samples of the mice treated with NATG. Though, considerable decrease in NO* radicals concentration was noticed in the blood of mice treated with 80 mg/kg b.wt of NATG at 1h but no such reduction was evident with the mice treated with lower (i.e. 40 mg/kg b.wt) concentration of NATG as compared to untreated control (Fig. 5(c)).

4.8 Effect of Gamma Irradiation of NATG on Free Radicals Scavenging Activity of NATG

Irradiated (8 Gy, 20 Gy) NATG (80 mg/kg b.wt.) was administered to strain A mice and *in vivo* antioxidant status and free radicals scavenging activity was observed in the spleen and blood samples of treated mice using EPR spectrometric analysis. Results of the study indicated a significant reduction in the ascorbate radical concentration in the spleen of mice administered with irradiated (8 Gy, 20 Gy) and normal unirradiated NATG as compared to untreated control group of mice (Fig. 6(a)). Similarly, a significant ($p<0.05$) decline in ascorbate radicals concentration observed in the blood of the mice which were treated with unirradiated and irradiated NATG (0.1734 a.u, 0.0964 a.u and 0.1455a.u for unirradiated NATG, 8 Gy irradiated and 20 Gy irradiated NATG, respectively) as compared to untreated control (0.3468 a.u) group (Fig. 6(a)).

Significant ($p<0.05$) decline(~50 per cent) in N-tert-butyl-alpha-phenyl-nitrone (PBN) adducts was observed in the spleen of the mice administered with irradiated (8 Gy and 20 Gy) NATG and ~75 per cent decline with unirradiated normal NATG as compared to untreated control mice (Fig. 6(b). Additionally, significant ($p<0.05$) reduction in PBN radicals was observed in the blood of mice administered with irradiated (8 Gy, 20 Gy) NATG (0.2766 a.u and 0.2984 a.u, respectively) as compared to untreated control group of mice (0.6317 a.u) (Fig. 6(a)). Therefore, results of the study clearly suggested that irradiation to NATG did not hamper its antioxidant activities *in vivo* models.

A significant ($p<0.05$) reduction in NO* radicals was observed in the blood samples of the mice treated with irradiated NATG (8 Gy and 20 Gy) as compared to untreated control group of mice. In contrast, a significant increase (~3 folds) in NO* radicals concentration was observed in blood samples of mice treated with unirradiated NATG as compared to untreated control group of mice (Fig. 6(c)).

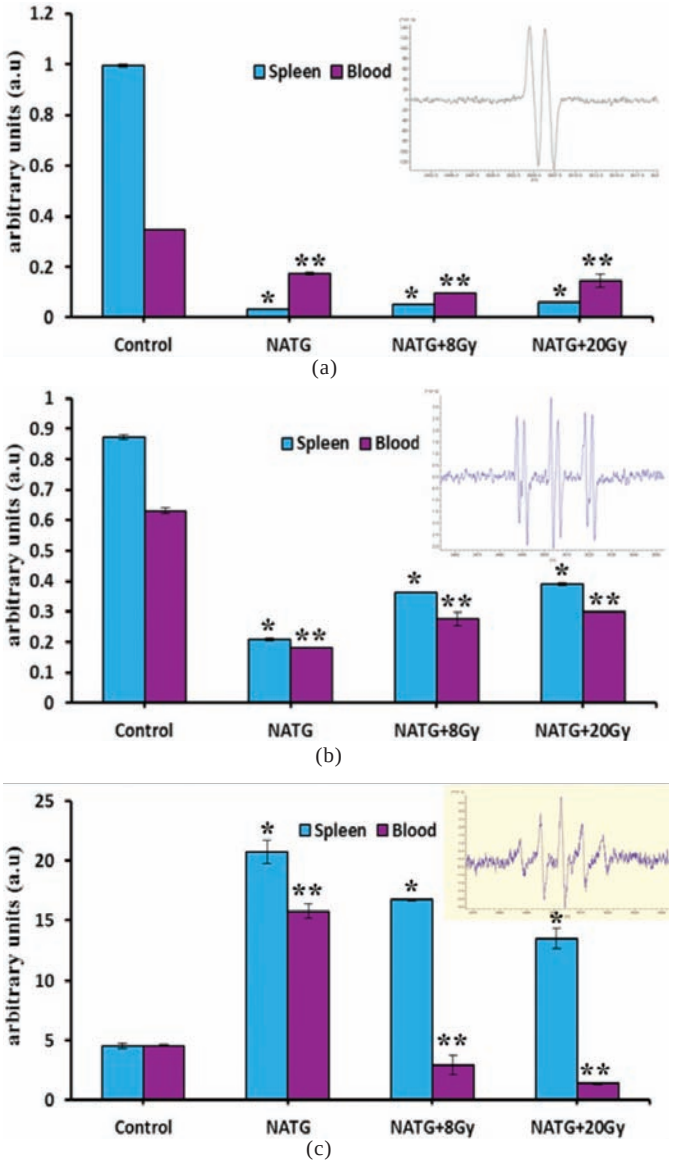

Figure 6. EPR spectrometric analysis of free radical scavenging activity of irradiated NATG in the spleen and blood tissue of the mice: (a) Ascorbate radical analysis, (b) ROS radical scavenging estimation using PBN adducts, and (c) NO radical scavenging analysis.

*$p<0.05$ NATG treated groups Vs untreated control group in spleen tissue of mice;

**$p<0.05$ NATG treated groups Vs untreated control group in blood of mice.

5. DISCUSSION

Ionising radiation is ubiquitous in nature and released by natural decay of radioactive materials. Besides natural resources, several man-made sources such as nuclear reactors, isotopes used for pharmaceutical purposes, radiotherapy equipments and nuclear weapons tests etc, also account significant amount of ionising radiation. Ionising radiation (IR) exposure induces complex cellular and molecular response in the biological systems[5,17-18]. Ionising radiation activates radiolysis of cellular water and thus increased production of reactive oxygen/nitrogen species (ROS/RNS) such as superoxide radicals (O_2•-),

hydroxyl radical (OH•), hydrogenperoxide (H_2O_2) and singlet oxygen (O). Radiation-induced oxidative stress leads to bio-macromolecular structural damage resulting into functional impairment and cell death[19-20]. Therefore, to combat radiation induced oxidative stress, antioxidant or free radicals scavenging agent need to be investigated. Different methods have been developed for evaluation of antioxidant or free radical scavenging activity of natural or synthetic substances[21-25]. However, EPR spectrometric method possesses advantages over the spectrophotometric assays, because EPR spectrometry involved direct measurement of free radicals in a biological or chemical system. While, optical spectroscopic assessment is an indirect method of free radicals analysis[8,26]. Therefore, observations reported in the present study for DPPH, ascorbate, nitric oxide and superoxide radicals scavenging activity of NATG using EPR spectrometry was considered more explicit than those by spectrophotometry. In the present study, considerably high DPPH radical scavenging activity was observed with UV–irradiated NATG may be explained by formation of radical structures as indicated by EPR spectrometric analysis (Fig. 1). Modifiedradicals may additionally be involved in the reaction with DPPH radicals that suggested radical-radical interaction and thus more efficient DPPH radicals scavenging by NATG (Fig. 1, Fig. 3(a), 3(b)). Time dependent radical neutralisation by natural or synthetic antioxidants is a well-established phenomenon[21]. DPPH scavenging activity of NATG was found to increase with increasing incubation time and the scavenging effect was maximum at 30 minutes (Figs. 2(a), 2(b), 2(d)) provided a gained support to the earlier report[21].

To further confirm free radical scavenging and antioxidant potential of NATG, EPR spectrometric analysis was carried out using blood and spleen tissues homogenate of the mice. From a thermodynamic point of view, ascorbic acid is found at the end of a series of oxidising free radicals. It means all oxidizing species carries high redox potential tend to get reduced and finally convert into ascorbate radicals[27]. Ascorbate radicals are long lived and thus convenient to detect directly by EPR spectrometry[14]. Stability of ascorbate radicals makes them the best non-toxic endogenous marker of oxidative stress in the biological systems[28]. In the present study, the level of ROS products and ascorbate radicals were evaluated in real time scenario using healthy mice before and after treatment with NATG using *ex vivo* EPR spin trapping spectrometry and direct EPR spectrometry (Figs. 5(a)-5(b), 6(a)-6(b)). EPR spectra were recorded to detect the typical PBN spin adducts, consisting of six spectral lines and EPR spectrum of ascorbate radicals consisted of a doublet spectral lines (Figs. 5(a)-5(b), 6(a)-6(b)). Based on the hyperfine splitting constant calculation (G value), radicals trapped by PBN were identified as oxygen-centered lipid radicals (LO)[29]. No significant difference in the level of PBN trapped lipid radicals was observed with irradiated NATG treated or control mice blood and spleen tissue homogenate, suggested that radiated NATG does not provide sufficient shield against lipid peroxidation processes *in vivo* conditions (Fig. 6(b)). Moreover, statistically lower levels of ascorbate radicals were measured in the spleen of NATG treated mice as compared to control, suggested that NATG treatment probably reduces oxidative stress in the spleen (Figs. 5(a), 6(a)).

Angiotensin II, nitric oxide (NO.) and reactive oxygen species (ROS) are important components of the pathologic mechanisms of cardiovascular diseases[30]. It is therefore extremely important to maintain a balance between these three components to maintain homeostasis of the vascular wall. Moreover, decreased vascular nitric oxide concentration promotes Angiotensin II dependent cardiovascular diseases mediated by ROS[31]. Results of the present study revealed significant reduction in nitric oxide (NO) radicals in the blood (Fig. 6(c)) and spleen (Fig. 5(c)) of NATG treated mice, as compared to untreated controls. These observations were aligned with the reduced ROS and ascorbate radical levels with NATG treated mice, further suggested NATG oxidative stress reducing capability.

6. CONCLUSIONS

N-acetyl tryptophan glucoside (NATG), a novel bacterial secondary metabolite, demonstrated efficient DPPH, ascorbate, lipid peroxide and nitric oxide radicals scavenging activity as observed with EPR spectrometry and thus qualifies as potential antioxidant that can be used to manage oxidative stress induced by gamma radiation. Therefore, in conclusion, NATG demonstrated a free radicals scavenging and antioxidant activities and thus can be a potential agent for radioprotector development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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