Research article

Molecular events confirming antimutagenicity to abscisic acid derived from a floral honey establishing its functional relevance

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Keywords: Honey, Antimutagenicity, Abscisic acid, Proteomics, E. coli knockouts

Natural dietary products of health promoting and disease preventive functional relevance are gaining significant prominence. Current investigation was aimed to decipher the underlying molecular mechanism responsible for the antimutagenic action contributing to functional relevance of floral honey (Pongamia pinnata, Karanj honey) derived abscisic acid (ABA) against ethyl methanesulfonate (EMS) induced mutagenesis. Differential expression of proteins under different treatment conditions was ascertained by 2D gel electrophoresis. Selectively up-regulated characterized using MALDI-TOF MS/MS were identified as polyribonucleotide nucleotidyl transferase (PNPase), LPS-assembly lipoprotein (LptE), Outer membrane Usher protein (HtrE), ATP-dependent DNA helicase (RecG), and Phosphomethyl pyrimidine synthase (ThiC). Antimutagenicity exerted by ABA against EMS was ~78% in wild type E. coli MG1655 strain however, in E. coli MG1655 ΔthiC, ΔpnpA, ΔrecG, and ΔhtrE this activity was found to be ~60, 10, 9 and 10%, respectively. Proteomic analysis and antimutagenicity studies using E. coli single gene knockout strains thus indicated about the possible role of thiC, htrE, lptE, recG and pnp in observed antimutagenicity. Cyclic voltametry as well as competition kinetics through pulse radiolysis confirmed lack of antioxidant capacity in abscisic acid apparently ruling out the possibility of scavenging of electrophilic intermediates generated by ethyl methanesulfonate. It is proposed that ABA is exerting antimutagenicity through its involvement at the cellular level leading to physiological adaptation, strengthening of cell wall proteins and up-regulation of the repair proteins. This study provides a novel dimension to the functional role of abscisic acid from its nutraceutical perspective.

1. Introduction

The diet is closely linked to cancer prevention and as per the projection of the World Health Organization around one-third of these deaths are preventable (Eastmond et al., 2009). Besides, in the current COVID-19 pandemic, significant proportion of consumers are opting dietary foods of functional relevance (AlNajrany et al., 2021). Therefore, disease prevention including chemoprevention through diet modulation is being significantly recognized and has subsequently emerged as one of the most promising and potentially cost-effective approach in cancer mitigation strategies (Thun et al., 2010; George et al., 2017; Tao et al., 2018). With this perspective, dietary phytochemicals of antimutagenic relevance from health protective foods could serve as effective means to prevent the harmful effect of mutagens (Shu et al., 2010). Therefore...
identification of broad spectrum natural antimutagens may prove worthy in the development of strategies to mitigate mutation associated diseases and onset of further neoplastic inductions. In this context, exploring compounds with antimutagenic potency and understanding associated mode of molecular action bears profound significance.

Honey is globally recognized as a natural functional food for its broad spectrum nutritional and health protective attributes (Hossen et al., 2017). In COVID-19 honey consumption has been reported to mitigate the disease symptoms (Hossain et al., 2020). In our earlier study a raw and unprocessed mono-floral ‘Karanj’ (Pongamia pinnata) honey was reported to display prominent antimutagenicity and the antimutagenic bioactive was identified as abscisic acid (ABA) based upon HPLC, ESI-MS and H1-NMR analyses (Saxena et al., 2017). ABA is a sesquiterpenoid signaling molecule that is known to have several important functional roles in the physiology of higher plants (Hauser et al., 2011; Olds et al., 2018). As an endogenous immune regulator, it is also involved in mammalian physiology and other functional bioactivities. Besides, its production has also been ascertained in various human cells including granulocytes, β cells of pancreas, and mesenchymal stem cells (MSCs) (Li et al., 2011; Qi et al., 2015).

Apart from augmenting immunity in Apis mellifera, ABA also leads to improved colony health and adaptation to cold stress (Negri et al., 2015; Ramirez et al., 2017). As an anti-cancer compound earlier evidences have proved that ABA prominently blocks tumor cell proliferation and also leads to the reduction of cancerous to normal cells (US patent no. 8,389, 02432; Tan et al., 2006). In a latest report, it inhibited expression of proliferation marker Ki-67 in the prostate cancer cells eventually leading to their reduced proliferation as well as relatively increased percentage of cells arrested in Go phase for prolonged duration (Jung et al., 2021). It has also been reported to ameliorate influenza-virus-induced pathology and in a malarial mouse model oral supplementation led to reduced load of malarial parasites coupled with decreased disease severity as well as transmission of parasites (Hontecillas et al., 2013; Glennon et al., 2018).

However, there are very limited reports on the functionality of honey in terms of antimutagenicity. Therefore the current investigation was undertaken as an extension of our earlier study to further decipher the underlying molecular mechanism responsible for the antimutagenic action of floral honey derived abscisic acid through analytical and proteomic approaches to provide the scientific basis contributing to the health promoting attributes of honey. This study will also provide a new dimension to the functionality of abscisic acid from the prospective nutraceutical point of view.

2. Material and methods

2.1. Purification of bioactive from Pongamia pinnata (‘Karanj’) honey displaying significant antimutagenicity

Extraction of the bioactive compound from raw unprocessed Pongamia pinnata (‘Karanj’ honey) was done as per the method detailed in our earlier report (Saxena et al., 2017).

Phenolics extraction was performed through amberlite (XAD-2) resin and phenolics enriched fraction was eluted with methanol and further subjected to vacuum drying. This fraction was subjected to reverse-phase HPLC (UltiMate 3000 Dionex Corporation, CA, USA). Methanol (A) and 2% glacial acetic acid (B) comprised the mobile phase with linear gradient as following: 10% A in B (at start), 60% A in B (for 28 min); 90% A in B (for 30 min) with 1 mL/min flow rate. Chromatogram monitoring was at 280 nm and the peak fraction that displayed prominently higher antimutagenicity (Rt 27.9 min) was further collected and subjected to vacuum drying for further assays (Saxena et al., 2017).

2.2. Bacterial strains

The Escherichia coli strain MG1655 (F- ιhG- rfp-50 rph-1) was provided by Prof. M.Z. Humayun, UMDNJ, USA. The single gene knock out strains of E. coli were procured from Keio collection, National Bio-Resource Project, National Institute of Genetics, Japan.

2.3. Proteomics analysis

To investigate the underlying molecular basis contributing to the observed antimutagenicity by ABA purified from ‘Pongamia pinnata’ (‘Karanj’) honey, proteomics analysis was also performed employing two dimensional (2-D) protein gel electrophoresis. The experimental conditions included the following sets: control (not subjected to mutagen or ABA), treatment 1 (with EMS only), and treatment 2 (EMS and ABA). Overnight grown E. coli culture (2 mL) corresponding to different experimental sets was centrifuged (7500xg, 7 min), washed with chilled PBS (Phosphate buffered saline, 10 mM, pH 7.4) followed by cell lysis. Later, chloroforom (200 μL) was added, allowed to stand for 5 min at 25 °C and centrifuged (12000xg at 4 °C for 15 min). The pellet was suspended in ethanol (300 μL) and was later centrifuged (5000xg at 4 °C for 5 min). Further, isopropanol (1.5 μL) was added to the supernatant, allowed to stand for 20 min at 25 °C and centrifuged (12000xg at 4 °C). Pellet was washed (with 95% ethanol), air dried and rehydration buffer (200 μL) was added to dissolve the protein as per the manufacturer’s guidelines (Bio-Rad Laboratories, USA). The protein concentration was evaluated through Bradford’s method.

From each of the treatment conditions, protein sample (90 μg) was loaded on the IPG strip (7 cm; pH gradient: 4–7) and further left for absorption. This was followed by addition of mineral oil (1 mL) and it was left at ambient temperature for 12–16 h. Isoelectric focusing was performed using an IEF tray wherein the IPG strip was covered with the fresh mineral oil and lid was placed onto the tray. The protocol was programmed to attain 8000 V-h (pH gradient 4–7) and electrophoresis was initiated. After completion, the IPG strip was transferred (gel side up) into a new clean and dry rehydration tray. Prior to the second dimension run, the IPG strip was stored at -70 °C overnight, later placed on the SDS-PAGE gel (12%) and run at 200 V. Upon run completion, the gel was subjected to silver staining as per the standard protocol till the spots were developed followed by washing of the gel. The gel was immediately transferred to the stopping solution and documentation of the resolved protein spots was done and the comparison was performed utilizing the PDQuest software, Bio-Rad Laboratories, USA.

2.4. Identification of the protein spots

2.4.1. In-gel digestion

Differentially expressed protein spots were manually excised from gel and transferred to microtube tubes. An aliquot (30 μL) of freshly prepared K3[Fe(CN)6] and Na2S2O2 (1:1; v/v) in water was added and these gel pieces were further incubated for 30 min for degrading. The gel was subjected to washing with water and ammonium bicarbonate (50 mM)/ acetoniitrile (1:1; v/v) for 15 min followed by the addition of acetoniitrile to cover the gel. After drying in a vacuum concentrator, the gel was further swelled in 10 mL dithirotiolate/50 mM NH4HCO3 (45 min; 56 °C). The liquid was removed and a fresh solution of iodoacetamide (55 mM in 50 mM NH4CO3) was added and incubated for 30 min in dark at ambient temperature. The liquid was removed and washed twice using NH4CO3

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Table 1. Physico-biochemical attributes of Pongamia pinnata honey.

| Attribute            | Pongamia pinnata |
|----------------------|------------------|
| **Moisture content (%)** | 18.9 ± 0.1       |
| **Viscosity (cP)**    | 6450.0 ± 78.0    |
| **TS5 (Brix)**        | 79.6 ± 0.01      |
| **HMF (mg/kg)**       | 3.2 ± 0.2        |
| **Phenolic (mgGAE/100g)** | 45.0 ± 1.8      |
| **Flavonoid (mgCE/100g)** | 4.6 ± 0.4     |
| **Proline (mg/kg)**   | 355.0 ± 3.0      |
and acetonitrile. The gel piece was again subjected to acetonitrile treatment which was later removed. This was vacuum centrifuged for drying and then subjected to in-gel digestion. The resulting peptide was extracted using 100 μL of 0.1% TFA, 0.1% TFA in 50% acetonitrile, and acetonitrile, sequentially, pooled and vacuum dried.

2.4.2. MALDI-TOF MS

The peptides were mixed with α-cyano-4-hydroxycinnamic acid (5 mg/mL) and 0.1% TFA & 50% acetonitrile (1:1) in 1:2 ratio and then subjected to the matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis. For this, spotting of 2 μL aliquot was done on a MALDI plate. The analysis was done employing a mass spectrometer (ULTRAFLEX III, Bruker Daltonics, Germany) having laser beam (100 μJ at 337 nm) and operating at 200 Hz. The instrument was calibrated using PEPMMIX mixture of peptides with masses varying from 1046-3147 Da. The data was analyzed through the FLEX analysis software (version 3.3) and for identification purpose the submission of the masses was done in the protein database for Mascot search.

2.5. Knockout studies using relevant E. coli gene knock-out strains

The assessment of antimutagenicity conferred by honey derived ABA was further performed in the relevant E. coli knock-out strains (JW 3627, JW 3958, JW 0135, JW 5851) procured from Keio collection, Japan (elaborated in Table 4). The assessment of antimutagenic potential was done employing the rpoB-RifR rifampicin based antimutagenicity assay as described earlier (Saxena et al., 2017). This was conducted to ascertain the functionality of certain up-regulated proteins(s) in the observed antimutagenicity due to the presence of bioactive compound ABA.

2.6. Molecular analysis of antioxidant capacity of ABA

In order to further assess whether antioxidant capacity of ABA is also contributing to the observed antimutagenicity, different analytical approaches were also employed.

2.6.1. Pulse radiolysis

The pulse radiolysis technique is based upon a linear accelerator and associated fast detection set-up. The high energy (7Mev) electron pulses from the linear accelerator are allowed to fall on the sample solution and the dose delivered per pulse is estimated using a thiocyanate dosimeter consisting of aqueous solution of potassium isothiocyanate saturated with N2O. Upon irradiation, ⋅OH radical is formed which reacts with thiocyanate ion to form the radical (SCN)2+/C14 having absorption maxima at 480 nm.

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\begin{align*}
\text{OH}^+ + \text{SCN}^- &\rightarrow \text{OH}^- + \text{SCN}^2-
\end{align*}
\]

Antioxidant present in the solution scavenges the ⋅OH radical and the extent of reduction indicates its antioxidant potential (Jacob et al., 2011). The absorption by the ABA solution was monitored with a collimated light beam from a pulsed 450W Xenon arc lamp.

Further technical operating specifications of the kinetic spectrophotometric detection system were as following: λ (300–800 nm); optical path length (1.0 cm); electronpulse width: 500 ns. Absorption of the radicals was done in UV-visible region both with respect to time and wavelength for getting kinetic as well as absorption attributes.

2.6.2. Cyclic voltametry

The redox property of the honey purified ABA was studied using potentiostat/galvanostat system (model: Autolab PGSTAT 30). The measurements were performed using a solution comprising 0.1 M LiClO4 as a supporting electrolyte and 1 mM solution of purified bioactive ABA in methanol. For the control set, experiments were performed utilizing only the electrolyte solution. The potential was cycled between -1 V and 1.5 V at a scan rate of 0.05 V s⁻¹ using three electrodes system: Pt-coated FTO as working and counter electrodes, and Ag/AgCl as a reference electrode.


### Table 3. Fold change in expression of E. coli proteins in 2-D gel electrophoresis and their identity based on MALDI-TOF-MS/MS.

| Protein spot | Gene Function                                      | Fold change (± up- or Down-regulated) |
|--------------|----------------------------------------------------|--------------------------------------|
| P1 PnpA      | Polyribonucleotide nucleotidyl transferase         | ↓6.5                                 |
| P2 ßlptE     | LPS-assembly lipoprotein LptE (Selective permeation barrier; Physiological adaptation in LPS structure) | ↓8.2, ↑11.2                          |
| P3 htrE      | Outer membrane usher protein HtrE (Fimbrial biogenesis) | ↓7.5, ↑15.3                          |
| P4 RecG      | ATP-dependent DNA helicase RecG (Recombination and DNA repair) | 0, ↑19.1                            |
| P5 thiC      | Phosphomethyl pyrimidine synthase (Thiamine biosynthesis) | ↓6.5, ↑7.7                          |

### 2.7. Statistical analysis

The rpoB-Rif<sup>6</sup> rifampicin based antimutagenicity assessment assay with E. coli knock-out strains was done in two independent sets each having 10 replicates. Similarly, the proteomic analysis was also done in 2 independent sets, each having two replicates. Other experiments were conducted in three independent sets, each having three replicates. Data were analyzed and expressed in terms of means and standard deviations. Statistical analyses were performed using BioStat 2009 Professional 5.8.0.0 software (AnalystSoft Inc.) using one-way ANOVA (p < 0.05).

### 3. Results and discussion

In our earlier published report, abscisic acid purified from ‘Karani’ (Pongamia pinnata) honey was found to be the bioactive contributing to antimutagenicity against EMS induced mutagenesis in diverse models (Saxena et al., 2017). The physico-biochemical attributes of Pongamia pinnata honey is described in Table 1. Different approaches were further employed to elucidate the possible mechanism of ABA mediated antimutagenicity.

### 3.1. Differential expression of proteins from E. coli culture subjected to different treatment conditions

Around 56 proteins were found to be differentially expressed upon EMS treatment with respect to untreated E. coli cells (control) (Figure 1 a, b). Among these, 42 proteins were observed to be up-regulated whereas 14 proteins were down-regulated upon EMS treatment with respect to control cells. When ABA and EMS were administered together, 37 proteins were found to be differentially expressed compared to EMS treated cells (Table 2). Protein expression profile obtained in case of co-treatment with ABA and EMS was found to be quite similar to that of control set. This was attributed to similar kind of up-regulation and down-regulation as observed in the case of control set. 5 proteins were significantly up-regulated in treated E. coli cells compared to EMS alone treated E. coli cells as well as untreated control cells. These protein spots were further characterized using MALDI-TOF MS/MS and were identified as polyribonucleotide nucleotidyl transferase (PnPase), LPS-assembly lipoprotein (LptE), Outer membrane Usher protein (HtrE), ATP-dependent DNA helicase (RecG), and phosphomethyl pyrimidine synthase (ThiC) (Table 3).

### 3.2. pnpA, recG and htrE functions are essentially required for the observed antimutagenicity by ABA

The functionality of these up-regulated proteins was further ascertained through antimutagenicity analysis in their respective E. coli single gene knockout strains (Table 4). Antimutagenicity exerted by ABA against EMS was ~78% in wild type E. coli MG1655 strain. However, in E. coli knock-out strains ΔthiC, ΔpnpA, ΔrecG, and ΔhtrE this activity was found to be ~60, 10, 9 and 10%, respectively (Figure 2). Knock-out of E. coli for ΔthiC could not be analyzed as it is required for the viability of the bacterium. Interestingly, mutation frequency in these selected single gene knock out strains were observed to be relatively higher (~2992–5304/10<sup>8</sup> cells) than the wild type strain (2200/10<sup>8</sup> cells) which indicated their possible involvement in preventing induced mutagenesis.

Proteomic analysis and antimutagenicity assessment using E. coli gene knockout indicated possible involvement of thiC, htrE, ßlptE, recG and pnpA in the observed antimutagenicity. The gene thiC encodes for protein (HtrE), ATP-dependent DNA helicase (RecG), and phosphomethyl pyrimidine synthase (ThiC) (Table 3).

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**Table 3. Fold change in expression of E. coli proteins in 2-D gel electrophoresis and their identity based on MALDI-TOF-MS/MS.**

| Protein spot | Gene Function                                      | Fold change (± up- or Down-regulated) |
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| P1 PnpA      | Polyribonucleotide nucleotidyl transferase         | ↓6.5                                 |
| P2 ßlptE     | LPS-assembly lipoprotein LptE (Selective permeation barrier; Physiological adaptation in LPS structure) | ↓8.2, ↑11.2                          |
| P3 htrE      | Outer membrane usher protein HtrE (Fimbrial biogenesis) | ↓7.5, ↑15.3                          |
| P4 RecG      | ATP-dependent DNA helicase RecG (Recombination and DNA repair) | 0, ↑19.1                            |
| P5 thiC      | Phosphomethyl pyrimidine synthase (Thiamine biosynthesis) | ↓6.5, ↑7.7                          |

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**Table 4. E. coli knock out strains (selected based upon proteomic analysis) for the antimutagenicity assessment.**

| Knock out strain | Genotype                                      |
|------------------|-----------------------------------------------|
| JW3627           | Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δλ ph-1 |}
| JW3958           | Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δλ ph-1 |}
| JW0135           | Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δλ ph-1 |}
| JW5851           | Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δλ ph-1 |}

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**Figure 2. Antimutagenicity of ABA in wild type (E. coli MG1655) and knockout strains as observed by rpoB-Rif<sup>6</sup> assay.**
phosphomethylpyrimidine synthase primarily involved in thiamine biosynthesis and to some extent in DNA repair. The htrE gene encodes outer membrane usher protein HtrE that confers adaptation, protection to cope with unique environmental conditions as well as strengthen the attachment between the bacterial cells (Korea et al., 2010). Similarly, lptE encodes a lipoprotein LptE which is an important LPS-assembly lipoprotein essential for cellular viability, physiological adaptation and also restricts the permeability of toxins inside the cells. In bacteria, lipopolysaccharide (LPS) provides cellular protection from challenging conditions and toxic agents (Dong et al., 2014). During the LPS assembly process at the cell surface, proteins LptD and LptE form a two-protein stable complex that further strengthens the cell wall integrity (Chng et al., 2010; Grabowicz et al., 2013). ABA inhibits tumor cell proliferation and also leads to reversal of cancerous cells to normal cells (Ma et al., 2006; Tan et al., 2006). In higher plants, ABA leads to deposition of callose in plasmodesmata which further restricts the movement of virus (Alazem and Lin, 2017). Besides, recG and pnp were also found to be selectively up-regulated in presence of ABA. These encode ATP-dependent DNA helicase RecG and polyribonucleotide nucleotidyltransferase, respectively, which are involved in repair processes (Dixon et al., 2008; Rudolph et al., 2010). Polyribonucleotide nucleotidyltransferase (PNPase) is known to participate both directly and indirectly in recombination, DNA repair, RNA quality control and resistance to genotoxic agents (Andrade et al., 2009; Carzaniga et al., 2017; Cameron et al., 2018). Protective role of human PNPase in reducing level of 8-oxoG in RNA as well as HeLa cells subjected to oxidative stress has been reported earlier. PNPase also plays role in the bacterial response to environmental stresses. Recent report highlighted ABA’s role in controlling RNA stability, turnover, maturation, as well as translation.

3.3. ABA purified from honey displayed strong antimutagenicity but lacked antioxidant capacity

The SCN anion (thiocyanate ion) competition kinetics with ABA was investigated to understand the OH scavenging capacity of ABA. In the presence of ABA no reduction in optical density of thiocyanate ion was observed thus indicating that ABA lacks antioxidant capacity in terms of OH scavenging (Figure 3; red curve b; curve e: no absorbance by ABA). However, in the presence of standard antioxidant (ascorbic acid), the absorbance of thiocyanate ion was observed to be significantly reduced (Figure 3; c, d curve). This ascertained that ABA lacks OH radical scavenging.

The antioxidant capacity of honey purified abscisic acid (ABA) was also assessed employing cyclic voltammetry. Upon the application of positive potential, as expected two distinct oxidation peaks were observed in case of standard antioxidant ascorbic acid, whereas abscisic acid did not display any oxidation peak during the scans (Figure 4a, b). Occurrence of peak as close to the base (0.0) indicates that analyte is easily oxidizable. Pulse radiolysis and cyclic voltametry data thus corroborated our earlier observations where during in-vitro radical scavenging activity analyses ABA was found to lack antioxidant activity (Saxena et al., 2017). Therefore apparently the possibility of scavenging of electrophilic intermediates generated by ethyl methanesulfonate (EMS) by ABA is ruled out.

4. Proposed mechanism explaining antimutagenicity of ABA

Based upon the evidence put forth, the proposed molecular mechanism that confers antimutagenic potential to ABA has been proposed. ABA is acting at the cellular level through offering genetic regulation (at the level of t-RNA and DNA) besides strengthening of the cell wall.
(Figure 5). The current hypothesis is being augmented by earlier findings pertaining to clinical and metabolic role of ABA in diverse living systems. However, studies are required to be pursued in higher systems to further establish its antimutagenic relevance.

Functional relevance of ABA in regulation of diseased cells by promoting normal growth and apoptosis induction in cancer cells have been reported earlier (Li et al., 2011). ABA has also been reported to modulate gene expression during metabolic adjustments as well as stress responses (Ramirez et al., 2017). Exogenous ABA treatment in Arabidopsis mutants was found to restore normal cell expansion and growth (Finkelstein 2013). ABA has also been reported to confer adaptive response to UV-B radiation mediated damage in maize plants (Tossi et al., 2012).

5. Conclusions

ABA purified from Pongamia pinnata honey exerted anti-mutagenicity through the possible synergistic regulation of thiC (phosphomethyl pyrimidine synthase), htrE (Outer membrane Usher protein), lptE (LPS-assembly lipoprotein), recG (ATP-dependent DNA helicase) and pnp (polynucleotide nucleotidyl transferase) genes. This is attributed to the ABA mediated physiological adaptation, strengthening of cell wall proteins and up-regulation of the repair proteins such as RecG and Pnp. As ABA was found to lack antioxidant capacity as confirmed by pulse radiolysis and cyclic voltametry, antioxidant capacity and anti-mutagenicity were not found to have any complimentary role particularly in case of ABA.

Declarations

Author contribution statement

Sudhanshu Saxena: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Satyendra Gautam: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

The authors do not have permission to share data.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

Authors thank late Dr. Ravi Joshi, Chemistry Group, BARC for his technical help during the pulse radiolysis experiments. Authors also acknowledge the technical assistance of Smt. Jyoti Tripathi and Ms. Kanchan Deshmukh during the 2D experiments.

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