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Understanding the effects of electron-beam irradiation of honey on its biochemical properties

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ABSTRACT

The influence of ionizing radiation on the biochemical properties of honey has attracted growing scientific interest. This study investigates the effects of electron-beam irradiation (EBI) on commercially available honey. Samples were treated with 3.5 MeV EBI at doses of 10–40 kGy using a linear pulse accelerator. Key physicochemical and biochemical parameters, including pH, color, refractive index, reducing sugar content, antioxidant activity, total phenolic content (TPC), and chemical changes (via NMR), were analyzed. Irradiation decreased pH, enhancing antimicrobial activity, while refractive index measurements revealed a slight increase in moisture at higher doses, with 30 kGy identified as optimal to avoid exceeding the 20 % moisture limit. Reducing sugar analysis showed the fructose/glucose (F/G) ratio remained above 1, preventing crystallization at room temperature. Irradiation increased phenolic content, antioxidant activity, and amino acid levels, as confirmed by Folin-Ciocalteu, DPPH, and NMR analyses. NMR spectra after irradiation revealed new peaks corresponding to increased levels of phenolics, amino acids, 5-hydroxymethylfurfural (HMF), and minor components such as citric acid. This could be attributed to the radiolysis of water in honey by EB irradiation. Microbiological experiments showed that the antimicrobial activity of EB-sterilized honey was retained even after two years of storage after irradiation. These findings highlight the potential of EB irradiations to enhance the biochemical properties of honey while maintaining its safety and quality.

1. Introduction

The quality of honey is determined by factors such as its floral origin, geographical location, and season, which influence its physicochemical characteristics, bioactive compounds, pollen profile, aroma, and distinct marker compounds (Cimpoiu et al., 2013). Honey is well-known for its various health benefits, including its roles as an energy source, sweetener, nutritional and health supplement, skincare product, cough remedy, wound healer, antiinflammatory, antimicrobial and antioxidant source (Becerril-Sánchez et al., 2021; Chua et al., 2013; Farooqui and Farooqui, 2011; Majtan, 2014; Mandal and Mandal, 2011; Saxena et al., 2010; Tashkandi, 2021). Furthermore, honey-based hydrogels have shown promise in wound healing and controlled drug delivery systems (Chin et al., 2024; El-Kased et al., 2017). The medicinal applications of honey can be attributed to its rich phytochemical contents. Depending on its floral source, honey mainly comprises sugars and water, with minor components such as minerals, vitamins, amino acids, organic acids, flavonoids, enzymes, phenolic compounds, and aroma compounds (Bogdanov et al., 2008; Buba et al., 2013; Tafere, 2021). Natural honey is clean and pure, free from microbial contamination and anthropogenic substances. Its antibacterial properties can be linked to the high osmotic pressure caused by its sugar content, low pH, polyphenols, hydrogen peroxide, 1,2-dicarbonyl compounds, and bee defensin-1 (Almasaudi, 2021; Feknous and Boumendjel, 2022). However, certain microorganisms, including pollen, molds, yeasts, and bacterial spores, have been found in honey (Feás et al., 2010; Fernández et al., 2017). These microbes may originate from primary sources such as pollen, the digestive systems of honeybees, soil, air, and nectar, or secondary sources like animals visiting beehives and human handling during post-harvest processing (Adadi and Obeng, 2017). Of particular concern in post-harvest handling are microorganisms commonly present in honey such as yeasts and spore-forming bacteria, as those indicating sanitary or commercial quality such as coliforms and yeasts, and in addition to this those that may, under certain conditions, pose a risk to human health (Snowdon and Cliver, 1996).

The processing of honey using high-energy gamma rays emitted by

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^{60}Co at low doses (5–40 kGy) has been used for the sterilization of honey, and also honey-based materials for medical applications (Bera et al., 2009; Hussein et al., 2014; Khatun et al., 2022; Klutse et al., 2021; Saxena et al., 2014; Wahyono et al., 2024). These studies suggested that gamma irradiation is effective and non-thermal in eliminating microbial contaminants, including yeasts, molds, and bacterial spores, without deteriorating the natural physicochemical and sensory properties of honey. In general, gamma irradiation preserves bioactive compounds such as antioxidants, enzymes, and phenolic substances, ensuring the retention of honey's nutritional and therapeutic benefits, unlike conventional treatments such as heating, refrigeration, freezing and pasteurization. In addition to these, the gamma irradiation of honey was found to enhance its quality in terms of an increase in antioxidant properties and phenolic contents (Hussein et al., 2011; Khalil et al., 2012). Saxena et al. have found that rheological properties and glass transition temperatures of Indian honey irradiated with 5–15 kGy dose of gamma rays remained unchanged, suggesting physicochemical properties of honey remained preserved (Saxena et al., 2014a,b). Similarly, the rheology of irradiated honey was studied by Sabato, who found no effect of gamma irradiation, and nonirradiated and irradiated honey exhibited a similar Newtonian behaviour (Sabato, 2004).

In several research studies, it has been reported that phenolic contents of food materials (whole grain rice, honey, herbs) increase on gamma irradiation as compared to unirradiated samples (Shao et al., 2013; Khatun et al., 2022; Gumus et al., 2011; Hussein et al., 2011; Khalil et al., 2012). However, the mechanistic explanation for the increase in phenolic content and antioxidant activities on gamma irradiation was not elaborated. It is well known that food materials contain water in varying proportions. The water undergoes radiolysis on irradiation with ionizing radiation, such as gamma rays or electron beams, producing several free radicals as given below (Shrivastava et al., 2020):

$$H_2O \rightarrow \bullet H,\ \bullet OH + e^-_{aq} + H_2O_2$$

During radiolysis of water, both oxidizing species (•OH & H_2O_2) and reducing species (e^-_{aq} & •H) are formed. The hydroxy radicals and H_2O_2 can oxidize large polyphenols or their glycosides, leading to structural rearrangements and enhanced electron-donating (antioxidant) properties. The cleavage of glycosidic bonds would release more bioactive aglycone flavonoids. The reducing species oxidized polyphenols back to more active antioxidant forms. The plausible mechanistic explanation for the enhancement of antioxidant properties and phenolic content is illustrated in Fig. 1.

Electron beam (EB) irradiation-based sterilization of agricultural products is an emerging method as it has better precision, quicker processing, and avoids the use of radioactive materials, which emit radiation continuously (Sridhar and Bhat, 2008). Electron beam processing depends on key variables such as absorbed dose, beam energy, and penetration depth. Due to its unidirectional emission, EB delivers a high energy dose per unit length directly into the material. While similar to gamma radiation in ionizing capability, EB differs in penetration depth and dose rate, and is a low-temperature technique that offers benefits such as uniform irradiation, rapid dose adjustment, minimal product degradation, high processing speed, precise reaction control, energy efficiency, and ease of operation and maintenance (Bansal and Arora, 2024). Thus, EB is ideal for thermosensitive products like honey, which can lose its natural flavour, enzymes, or nutrients under heat. However, EB irradiation of honey has not been studied extensively, especially on its biochemical effects on honey (Baldos et al., 2021; Migdał et al., 2000).

In the present work, the effects of EB on the biochemical properties of honey have been studied with the expectation of improving its medicinal value. Four commercially available honey samples were irradiated with beams of 3.5 MeV electrons at dose rates 10, 20, 30, and 40 kGy using the linear pulse accelerator. These samples were subjected to various tests of their color, pH, refractive index, free radical scavenging activity, sugar analysis, and total phenolic content (TPC), and compared with pristine honey samples. The chemical composition of the irradiated and unirradiated samples were studied by proton NMR.

2. Materials and methods

2.1. Reagents and instruments

All chemicals and reagents used were of AR grade. Ultrapure water

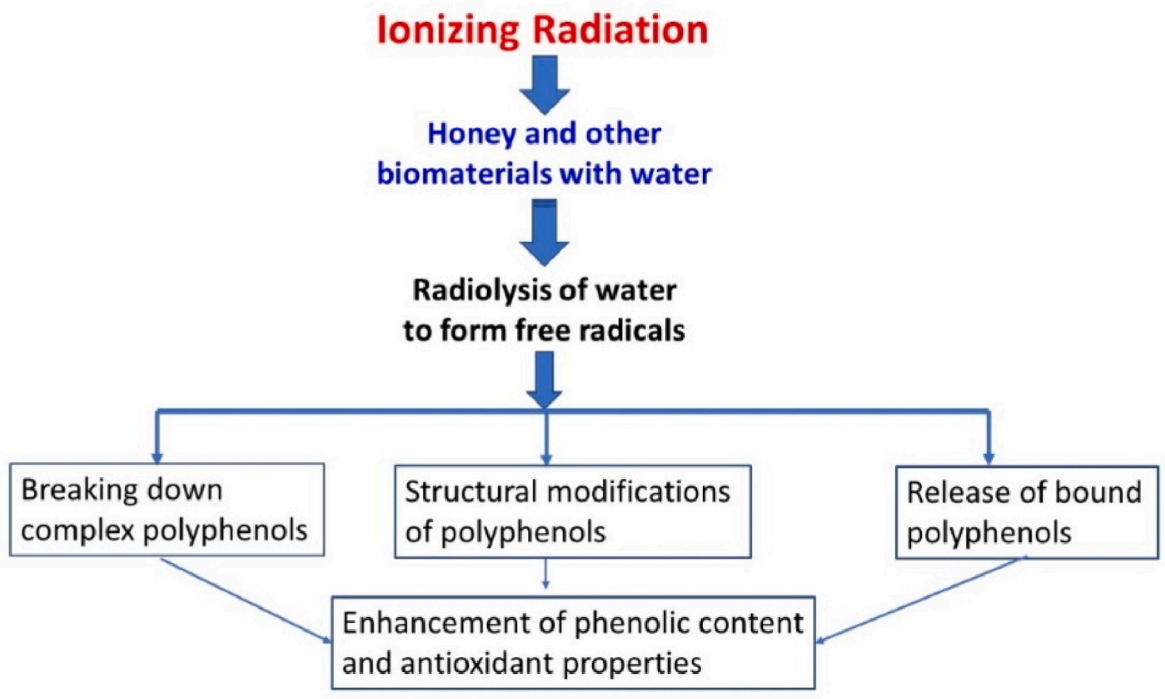

Fig. 1. Illustration of chemical changes produced by ionizing radiation in biomaterials with water content.

from ELGA Purelab was used. Folin-Ciocalteu phenol reagent (2 N) was obtained from Fisher Scientific Pvt. Ltd. Ascorbic acid, gallic acid (3,4,5-trihydroxybenzoic acid), HPLC grade glucose and fructose standards were obtained from Sigma Aldrich. 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95 % purity) was procured from Research Lab Fine Chem Industries. For microbiological experiments, Soya Casein Digest Agar (SCDA), Mueller–Hinton Agar, and cultures of *Staphylococcus aureus* and *Escherichia coli* were prepared in the Department of Microbiology. Gentamicin (30 μg, SD016) was sourced from HiMedia. E-beam irradiation was carried out at the ILU-6 EB accelerator facility, BRIT, BARC, Mumbai. The absorbance measurements were carried out using a UV–visible spectrophotometer (UV-2600, Shimadzu). HPLC experiments were carried out using Agilent HPLC 1200; Model: G1362A.

2.2. Electron-beam irradiation

The EB irradiations were carried out at 3.5 MeV energy, 250 mA pulse current, 1 mA average current, and 10Hz pulse repetition rate. A conveyer system was used for sample irradiation with 3 cm/s conveyor speed and 5 kGy dose per pass. The four commercial samples of honey–H1, H2, H3, and H4 were weighed 50 g and placed in glass plates with radiometric B3 films for dose monitoring as shown in Fig. 2. The samples were irradiated with 10, 20, 30, and 40 kGy dose each and then stored in glass bottles at room temperature.

2.3. Measurement of color intensity and pH

To carry out color measurements, 50 % (w/v) honey solutions were prepared using warm ultrapure water. The solutions were sonicated for 5 min, filtered, and analyzed using a UV–visible spectrophotometer by recording absorbance at 450 nm (for chromophoric compounds) and 720 nm (to correct for turbidity and light scattering) (Beretta et al., 2005). The difference in absorption $ABS_{450} - ABS_{720}$ is known as the color intensity and is expressed in milli absorbance units (mAU). For pH measurements, 10 % (w/v) honey solutions were prepared and analyzed using the pH meter (ELICO pH meter, model LI613).

2.4. Measurements of refractive index and reducing sugar content

A BRIX honey refractometer (ERMA RHB-92ATC) was used to analyze the refractive index of honey, which is a measure of the quantity of water and sugars, along with other solids. Honey samples were directly placed onto the glass slide of the refractometer and the moisture content in each sample was noted on the BRIX scale. Triplicate readings were noted and the standard conversion chart was used for converting the average °Bx readings to the refractive index (η) values.

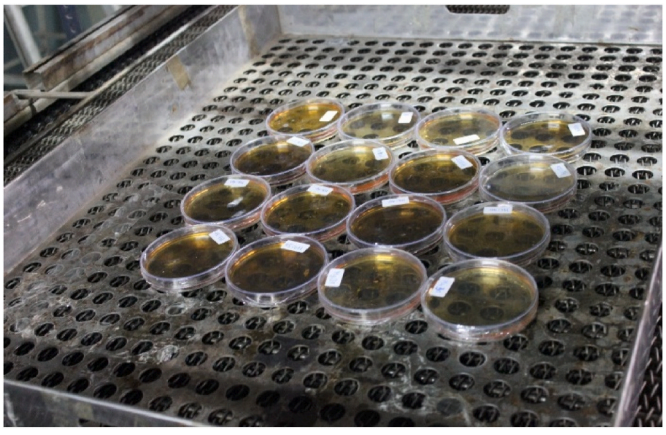

Fig. 2. Honey samples in Petri dishes with dose monitor B3 film for irradiation with an electron beam.

The reducing sugar content in honey (Glucose, Fructose) was analyzed by HPLC (Agilent 1200 with RI detector employing the Zorbax NH_2 (3-aminopropyl triethoxy silane) column. A 1 % solution of honey was prepared in ultrapure water and sonicated for 15 min for complete dissolution. It was passed through a 0.2 μm filter membrane cartridge to remove suspended solids from the sugar solution. The filtrate was then used for analysis of Glucose and Fructose content. In the HPLC column, AcN/Buffer (75/25) was used as the mobile phase at the flow rate of 1 ml/min, and RID temperature of 40 °C.

2.5. Total Phenolic contents (TPC)

TPC assay was carried out using a modified Folin-Ciocalteu (F–C) assay (Hatami et al., 2014; Lawag et al., 2023). In this method, 20 % (w/v) solutions of honey were prepared in ultrapure water and 0.5 mL of each solution was added to tubes containing 2.5 mL of 0.1 N F–C reagent and 2 mL of 1 N Na_2CO_3 solution. The solutions were kept in the dark for 120 min. Absorbance was measured at 760 nm using a UV–visible spectrophotometer. A standard curve of gallic acid was prepared using a range of concentrations from 20 to 140 mg/L. The total phenolic counts were expressed in terms of Gallic Acid Equivalents (GAE) in milligrams per 100 g of the sample (mg/100 g).

2.6. Antioxidant activity by DPPH assay

The free radical scavenging activity of honey was quantified using the 2,2′-diphenylpicrylhydrazyl (DPPH) method (Brand-Williams et al., 1995). All the solutions were prepared in AR-grade ethanol. 0.2 mM DPPH solution was freshly prepared in a 100 mL standard flask and kept in the dark for 1 h. Three concentrations of ethanolic honey solutions – 30, 50, and 80 mg/mL were prepared for each of the pristine and irradiated samples. 1 mL of the prepared DPPH solution was added to the test tubes containing 1.5 mL of honey solution and 1 mL of acetate buffer solution (pH 5.5). After mixing appropriately, the solutions were kept in the dark for 60 min. The negative control was composed of the same amounts of DPPH and buffer but with 1.5 mL of ethanol instead of honey solution. Ascorbic acid was used as a positive control and ethanol as a blank. Photometric measurements were carried out at 517 nm on a UV–visible spectrophotometer. The radical scavenging activity was calculated based on published literature (Barbosa-Pereira et al., 2013; Chua et al., 2013) as:

$$RSA\ (\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100 \quad (1)$$

2.7. Proton NMR

The 1D ^{1}H-Nuclear Magnetic Resonance (NMR) analyses on all samples were performed at 25 °C using a Bruker Advance Neo 800 MHz NMR spectrometer. Instruments were calibrated and maintained according to standard practice. All spectra were acquired using a 5 mm TXI triple resonance inverse probe. The NMR spectra were acquired using the following parameters: 16K data points, 12 ppm spectral width, 11.69 μs pulse width corresponding to 90° transmitter pulse, 0.85 s acquisition time, 128 scans and a relaxation delay of 3 s. Water suppression was achieved using the excitation sculpting pulse program of the Topspin 4.1.1 software provided by Bruker.

2.8. Microbiology experiments

The total plate count (TPC) of pristine and irradiated honey samples, aged two years post-irradiation, was evaluated using Soya Casein Digest Agar (SCDA) media. A 100 μL aliquot of each honey sample was evenly distributed beneath the surface of the SCDA plates, which were then incubated at 37 °C for 24 h. Following incubation, colonies were

counted on each plate, and the TPC was calculated as colony-forming units (cfu) per mL, taking dilution factors into account.

The antimicrobial activity of honey samples was evaluated against *Staphylococcus aureus* and *Escherichia coli* using the disc diffusion method. Test inoculums were prepared by suspending portions of the microbial strains in sterile normal saline, adjusted to a density of 0.1 OD, and evenly spread on Mueller–Hinton agar plates. Wells with a diameter of 6 mm and a depth of 4 mm were created in the agar at 2 cm intervals using a sterile cork borer. Each well was filled with 100 μL of the respective honey sample. The plates were incubated at 37 °C for 24 h, after which the mean diameters of the inhibition zones were measured in millimetres. Gentamycin (30 μg) was used as a positive control, while sterile distilled water served as the negative control.

All experiments were performed in triplicate to ensure reproducibility, and all graphs and data presentations show mean values with corresponding standard deviations. However, the experiments could not be done for multiple irradiations due to limited access to the electron-beam facility.

3. Results and discussion

The four honey samples studied in the present work are Indian honey. H1, H2, and H3 are of multifloral origin, and H4 is monofloral tulsi (*Ocimum tenuiflorum* L.) honey from the foothills of the Himalayan mountains. H1 honey is forest honey. Therefore, the honey samples studied in the present work represent a wide range of botanical and geographical origins, which is well-suited for the present work.

3.1. Color intensity and pH

The darkening of different samples of honey as a function of EB dose is shown in Fig. 3. It is seen that color intensity of sample H4, calculated as described in the experimental section, does not vary significantly as a function of doses. However, other samples show that the color intensity increases with doses after 10 kGy. The darkening of honey could be attributed to Maillard reactions, caramelization, and polyphenol oxidation, which are induced by EB irradiation. The darkening of honey was also observed in the case of gamma irradiation by several researchers (Hussein et al., 2014; Khalil et al., 2012; Khatun et al., 2022; Migdał et al., 2000).

The pH of honey may change due to EB irradiation-induced radiolysis of water present in honey. The pH of honey, typically ranging from 3.4 to 6.1, plays an important role in its antimicrobial properties, stability, and shelf life. Its natural acidity inhibits the growth of bacteria, fungi, and yeast, preventing spoilage and ensuring long-term preservation without refrigeration. This acidic environment also influences enzymatic activity (e.g., invertase, glucose oxidase) requisite for maintaining the quality and health benefits of honey. Therefore, the pH of honey samples was monitored after EB irradiation, and compared with pristine samples, see Fig. 4. The pH values of all pristine samples were within 4–4.5. It was observed that the pH of honey samples decreased after irradiation in all the samples, as seen in Fig. 4. The decrease in the pH of honey after EB irradiation could be explained by the radiolysis of water present in honey and the subsequent chemical reactions. Honey generally has 17–20 % water content, and EB irradiation interacts with this water which may undergo radiolysis to produce free radical species or products of these species such as hydroxyl radicals (•OH), hydrated electron (e^-), hydrogen atoms (•H), hydrogen peroxide (H_2O_2) and hydronium ions (H_3O^+) (Buxton et al., 1988). The combined effects of radiolytic byproducts and their interactions with the complex chemical matrix of honey to produce organic acids such as formic acid, and acetic acid would lead to the acidification process (Fan, 2012; Ramírez-Cahero and Valdivia-López, 2018). The generation of hydronium ions directly contributes to the pH decrease. The decrease in pH shown in Fig. 4 appears to be almost linear, and varies sample to sample depending upon the water contents in the honey sample. Several studies have reported a slight decrease in the pH of honey upon irradiation doses ranging from 10 to 40 kGy (D.A. et al., 2022; Bera A. et al., 2009; Khatun, Mst.A.et al., 2022).

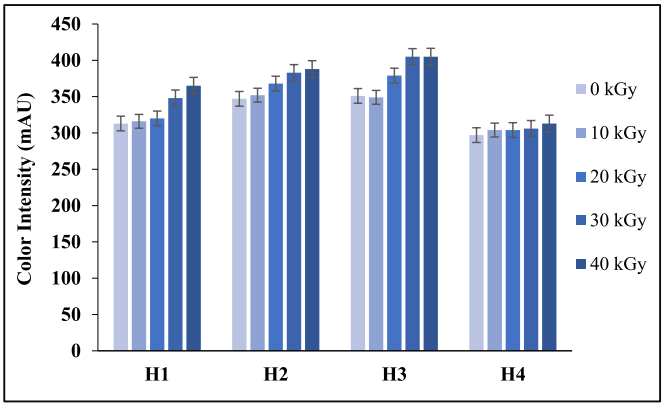

Fig. 3. Variations of color intensity of honey samples as a function of EB irradiation doses with respect to the pristine sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Refractive index and reducing sugar content

The moisture content of honey is an important parameter that indicates honey quality. It is associated with the geographical location and maturity of the honey. Higher moisture content may decrease the antimicrobial activity of honey and promote the fermentation of sugars by the growth of yeasts like *Saccharomyces cerevisiae*. It may also promote crystallization of honey at room temperature. Thus, the Food Safety and Standards Authority of India (FSSAI) has set the permissible moisture limit in honey, which must be below 20 % (Food Safety and Standards Authority of India, 2020). The moisture content of all four samples was in the range of 15.0 %–19.4 %, with the lowest moisture in sample H1. The BRIX reading and refractive index of honey decreased for all samples after irradiation, due to a marginal increase in the moisture content as seen from Table 1. The representative Fig. 5 (a&b) shows the BRIX reading for H3 sample and the solids and water contents (%) present in pristine honey and its corresponding irradiated sample.

HPLC chromatograms for glucose and fructose content were recorded for all samples, and the representative graph for H3 is shown in Fig. 6. The reducing sugar content in commercial honey was found to be in the expected range of ~30 % glucose and 35–40 % fructose (Codex Alimentarius Commission, 2001) as seen from Table 2. Thus, the Fructose/Glucose (F/G) ratio was found to be greater than unity for all

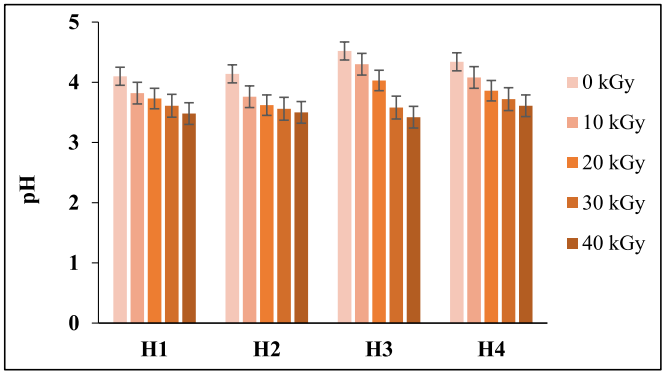

Fig. 4. The variation of pH of honey solutions as a function of EB doses with respect to their respective pristine samples.

Table 1
Average BRIX reading and refractive index of honey samples.

Dose (kGy)	H1			H2			H3			H4		
	Avg. °Bx	Water (%)	RI	Avg. °Bx	Water (%)	RI	Avg. °Bx	Water (%)	RI	Avg. °Bx	Water(%)	RI
0	85.0	15.0	1.4992	81.0	19.0	1.4890	82.0	18.0	1.4915	82.0	18.0	1.4915
10	85.1	14.9	1.4994	79.8	20.2	1.4860	81.9	18.1	1.4912	81.8	18.2	1.4910
20	84.3	15.7	1.4973	79.6	20.4	1.4855	81.3	18.7	1.4897	81.2	18.8	1.4895
30	83.2	16.8	1.4946	79.4	20.6	1.4850	80.8	19.2	1.4885	80.6	19.4	1.4885
40	82.2	17.8	1.4920	79.2	20.8	1.4845	78.5	21.5	1.4827	80.1	19.9	1.4867

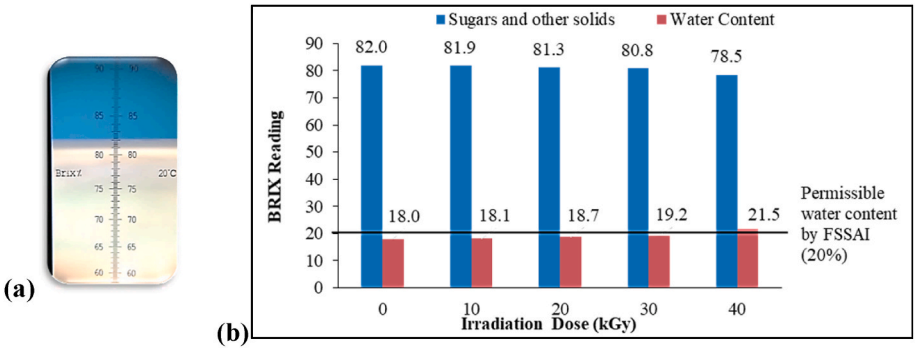

Fig. 5. (a) BRIX reading for sample H3, (b) Sugars/other solids and water content in H3 along with FSSAI limit.

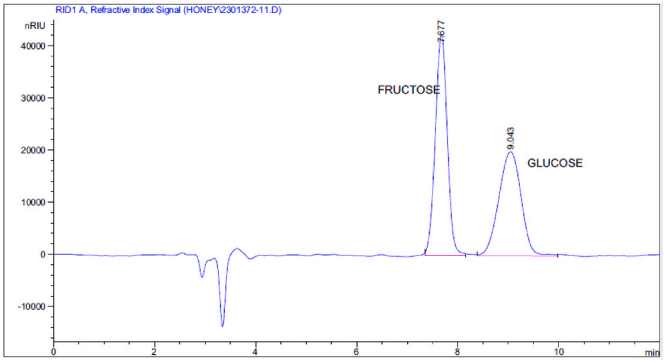

Fig. 6. HPLC chromatogram of H3 sample for reducing sugars (glucose and fructose).

pristine and irradiated samples as seen in Fig. 7. The ratio exhibited minor variation with irradiation, but remained higher than one. The minor decrease in sugar seems to suggest the oxidation of sugars like glucose and fructose to some extent, leading to the formation of organic acids, formic acid, and acetic acid, contributing to a lower pH. However, F/G remained almost constant as there was a similar minor decrease in glucose and fructose content, indicating equilibrium between these sugars.

3.3. Total Phenolic Contents (TPC)

Polyphenolic compounds are associated with functional properties of honey, like antioxidant activity and are important indicators of floral origin (Yayinie et al., 2022). The total phenolic content (TPC) of honey was analyzed using the Folin-Ciocalteu (F–C) method (Al-Farsi et al., 2018; Lawag et al., 2023) in which the F–C reagent is reduced by phenolic groups present in the compound under alkaline conditions. The reagent consists of phosphomolybdic and phosphotungstic acids (Pérez et al., 2023) along with Li salts that minimize turbidity (Blainski et al., 2013). In a positive reaction, the phenolic compound is oxidized by the yellow-colored F–C reagent, which in turn gets reduced to produce blue coloration. This change was analyzed and quantified spectrophotometrically at 760 nm.

TPC values were calculated using the standard curve of Gallic Acid (R^2 = 0.9905) and its linear equation as shown in Fig. 8. The total phenolic content of all honey samples increased with higher doses of irradiation, as seen from Table 3. Maximum phenolic content was found in H4, with 93.65 ± 2.21 mg Gallic acid equivalents (GAE)/100 g at 0 kGy and 126.15 ± 2.04 mg GAE/100 g at 40 kGy. This gradual increase in phenolic components could be attributed to certain chemical changes occurring due to EB irradiation. The increase in total phenolic content (TPC) observed in honey following EB irradiation could be influenced by several factors. For example, there is the possibility of the disruption of bonds between phenolic compounds and macromolecules like carbohydrates and proteins, which frees bound phenolics (Hussein et al., 2011) and makes them more detectable. Another contributing factor is the breakdown of complex organic molecules, such as lignin and

Table 2
Reducing sugar content and fructose to glucose (F/G) ratio in honey samples.

Dose (kGy)	H1			H2			H3			H4		
	G (%)	F (%)	F/G	G (%)	F (%)	F/G	G (%)	F (%)	F/G	G (%)	F (%)	F/G
0	29.93	36.32	1.21	32.02	35.28	1.10	31.07	38.18	1.23	30.09	36.47	1.21
10	29.07	36.03	1.24	30.05	32.06	1.07	29.98	37.48	1.25	30.57	37.08	1.21
20	30.83	37.67	1.22	32.69	34.97	1.07	28.95	36.44	1.26	30.07	36.26	1.21
30	28.63	35.23	1.23	32.38	34.06	1.05	28.8	35.8	1.24	30.83	36.88	1.20
40	30.3	36.41	1.20	30.93	31.98	1.03	27.87	34.83	1.25	29.42	35.47	1.21

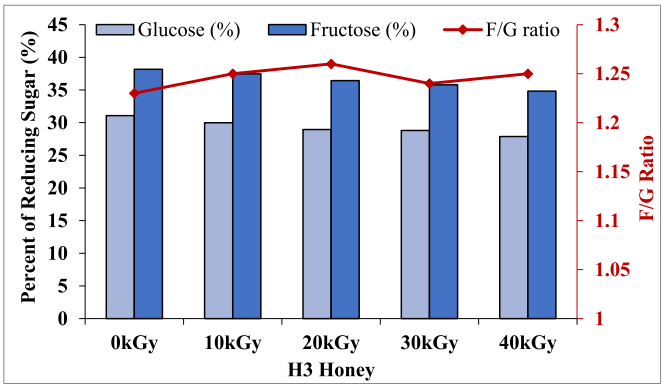

Fig. 7. Percentage of reducing sugars and F/G ratio in the honey samples irradiated with different EB doses with respect to the corresponding pristine samples.

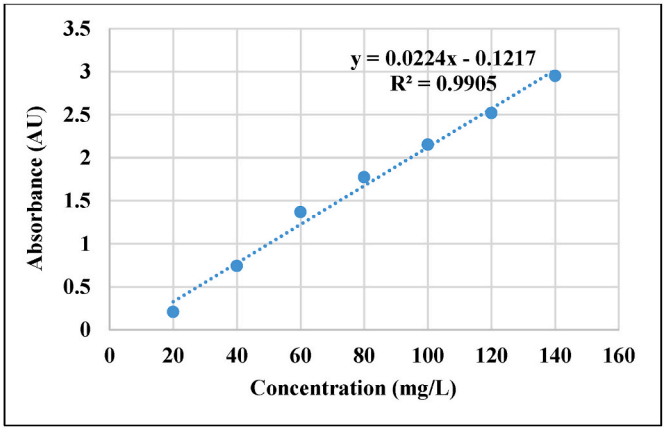

Fig. 8. Gallic acid standard calibration curve.

glycosides, into simpler phenolic compounds during the irradiation process (Mladenova et al., 2022; Aly et al., 2021). In addition to this, EB irradiation triggers the production of reactive oxygen species (ROS) within the honey matrix. These ROS facilitate the oxidation of precursor molecules, transforming them into phenolic compounds and thereby enhancing the TPC. Additionally, structural alterations in honey components, particularly sugars, caused by EB irradiation may lead to the formation or release of phenolic-like substances. It is also likely that EB irradiation would deactivate enzymes that could degrade phenolic compounds (Ahmad et al., 2013), leading to their stability in the honey.

3.4. Antioxidant activity

The free radical scavenging activity (RSA) of honey was analyzed using the 2,2′-diphenylpicrylhydrazyl (DPPH) radical assay (Alotibi et al., 2018; Kedare and Singh, 2011). The presence of a delocalized free electron gives DPPH its deep violet color, which turns straw yellow upon reduction with a hydrogen donor. This color change is measured spectrophotometrically at 517 nm. The radical scavenging activity of all four honey samples increased after irradiation, as seen in Fig. 9(a–d). Three concentrations (30, 50, and 80 mg/mL) of each sample were analyzed. H1 and H2 at 80 mg/mL had an RSA of 73 ± 2 % and 92 ± 2 % at 0 kGy and 40 kGy, respectively. H3 was the highest with 85 ± 3 % at 0 kGy and 90 ± 2 % at 40 kGy; H4 was the lowest with 62 ± 3 % at 0 kGy and 86 ± 2 % at 40 kGy. Thus, E-beam irradiation enhances the antioxidant activity of honey. This could be attributed to an increase in polyphenolic compounds, which are major contributors to antioxidant properties. However, it may be noted that there is no correlation between total phenolic content and antioxidant activity of the unirradiated honey sample. Honey also contains enzymes like glucose oxidase, which produces hydrogen peroxide and contributes to its antimicrobial and antioxidant effects, and catalase, which helps maintain oxidative balance by breaking down hydrogen peroxide (Nolan et al., 2019). Vitamins, particularly vitamin C (ascorbic acid), also enhance the free radical scavenging efficacy of honey. In addition to these, honey contains proteins and peptides such as bee defensin-1, which exhibit bioactive properties, as well as organic acids like gluconic acid that enhance its capacity to neutralize free radicals (Bonsignore et al., 2024). These factors would vary the antioxidant activity of the honey depending upon its botanical and geological sources. However, the substantial increase in the antioxidant activity of honey after EB radiation could be solely attributed to the increase in the total phenolic content.

3.5. Proton NMR studies

^{1}H NMR spectra before irradiation showed the presence of valine, proline, citric acid, and a peak of phenylalanine in the amino acid region between 0 and 3 ppm as seen from Fig. 10a. After irradiation, the peaks increased in intensity and were well-defined, as seen from Fig. 10b. New peaks of leucine and alanine were observed. Certain peaks of these compounds overlapped with the sugar region of 3–6 ppm. Detailed analysis of this region was not carried out due to the overlapping peaks as seen from both graphs. The proton peaks of 5-hydroxymethylfurfural (HMF) and phenylalanine were observed between 6 and 10 ppm in the phenolic region. The intensity of these peaks increased after irradiation. Thus, the phenolic and amino acid content increases after irradiation, along with other minor components like citric acid. It has been reported that HMF forms due to ageing and heat treatment, and is toxic to human health above 40 mg/kg according to the Codex Alimentarius Commission (Sajtos et al., 2024). The formation of HMF in the present case could be attributed to the degradation of glucose and fructose with free radicals formed by EB-induced hydrolysis of water, followed by dehydration reactions to form HMF. Fructose is more susceptible to degradation by free radicals to form HMF more readily.

3.6. Microbial studies

In the total plate count analysis, pristine honey samples H1, H2, H3, and H4 exhibited limited bacterial growth. As seen from Table 4, the bacterial colony count decreased significantly with increasing irradiation doses. At 30 kGy and 40 kGy, no bacterial colonies were detected in

Table 3
Total phenolic contents expressed as Gallic Acid Equivalents (GAE) in mg/100 g.

Dose	H1		H2		H3		H4	
	Average TPC	S.D.	Average TPC	S.D.	Average TPC	S.D.	Average TPC	S.D.
0 kGy	85.79	2.15	75.30	2.06	83.47	2.32	93.65	2.21
10 kGy	89.80	2.52	77.44	2.13	86.24	1.85	99.18	2.08
20 kGy	99.18	1.91	83.56	2.55	90.25	2.20	109.45	2.24
30 kGy	109.90	2.40	102.75	2.51	103.65	2.43	121.06	2.15
40 kGy	114.81	1.84	112.31	1.96	111.37	2.18	126.15	2.04

*Average TPC values are based on three readings; S.D. = Standard Deviation.

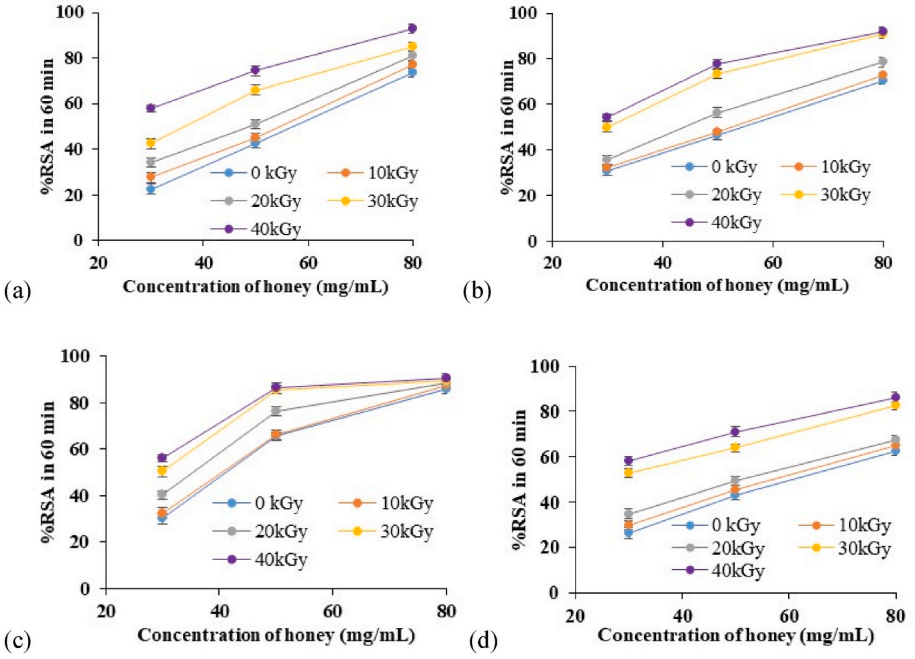

Fig. 9. Variation of Radical Scavenging Activities of (a) H1, (b) H2, (c) H3, and (d) H4 honey samples before and after irradiation with EB at different doses.

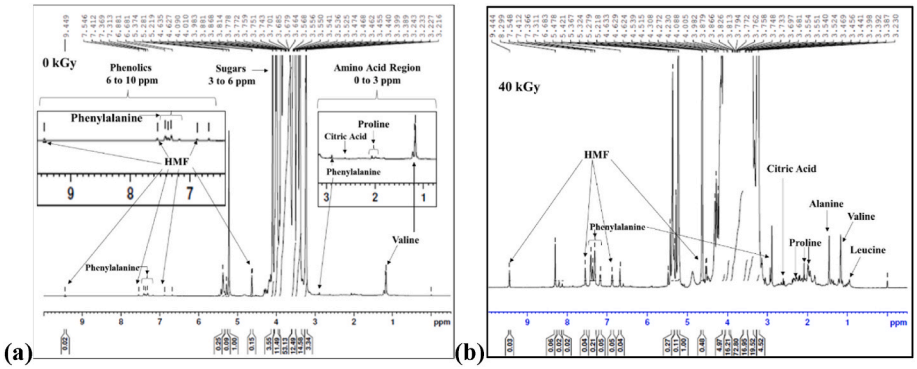

Fig. 10. NMR Spectra of H3 honey: (a) before irradiation and (b) after 40 kGy of EB irradiation.

any of the honey samples, demonstrating complete elimination of viable bacteria in the honey samples due to irradiation, with the effect persisting even after two years post-irradiation.

The antimicrobial activity of pristine and irradiated commercial honey samples against *S. aureus* and *E. coli* was evaluated and compared to the standard antimicrobial activity of Gentamicin (30 μg). As presented in Table 5, irradiated honey samples demonstrated larger zones of inhibition compared to their non-irradiated counterparts. This enhancement in antimicrobial activity following electron beam (EB) irradiation can be attributed to changes in the biochemical properties of honey. Factors such as microbial sterilization, increased levels of phenolic compounds, and a reduction in pH create an environment less favorable for microbial growth. Notably, these effects remained prominent even two years after irradiation.

Table 4
Total Plate Count of honey samples after 24 h of incubation.

Irradiation Dose	Colony Forming Units (cfu) per mL			
	H1	H2	H3	H4
0 kGy	25 ± 2	34 ± 2	19 ± 1	15 ± 2
10 kGy	18 ± 1	30 ± 1	15 ± 1	8 ± 1
20 kGy	No growth	18 ± 1	No growth	5 ± 1
30 kGy		No growth		No growth
40 kGy				

4. Conclusions

Electron-beam irradiation was found to exhibit a promising alternative to traditional sterilization techniques, enhancing not only the physicochemical and biochemical properties of honey but also its shelf life. Irradiation was associated with a reduction in pH, which improves the antimicrobial effectiveness of honey, and a notable increase in phenolic content, as confirmed by TPC and NMR analysis. The increase in HMF content is also noteworthy due to its antioxidant, antimicrobial, and potential therapeutic properties, positioning it as a valuable compound in food science research. However, care should be taken to avoid the formation of the HMF level above the safe limit of human consumption (40 mg/kg). Pristine honey samples had a microbial load of 15–34 ± 2 cfu per mL, which was completely sterilized by 30 kGy of irradiation. The increase in moisture content observed at 40 kGy, along with the optimal antioxidant activity achieved at a dose of 30 kGy, suggests that 30 kGy dose offers the best balance for effective sterilization, extended shelf life, and improved physicochemical properties in honey. The antimicrobial studies revealed that honey possessed

Table 5
Antimicrobial activity of honey samples.

Irradiation Dose	Inhibition Zones (in mm)									
	H1		H2		H3		H4		Gentamicin	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
0 kGy	10.2 ± 0.2	11.5 ± 0.1	8.5 ± 0.1	9.1 ± 0.2	9.1 ± 0.2	11.7 ± 0.3	8.4 ± 0.1	10.8 ± 0.2	**11.5 ± 0.2**	**12.0 ± 0.1**
10 kGy	10.4 ± 0.2	11.7 ± 0.2	8.7 ± 0.2	9.4 ± 0.1	9.5 ± 0.2	12.9 ± 0.2	8.8 ± 0.2	11.5 ± 0.3		
20 kGy	11.9 ± 0.3	12.3 ± 0.2	8.8 ± 0.1	9.9 ± 0.2	9.8 ± 0.2	13.3 ± 0.3	9.2 ± 0.2	12.4 ± 0.2		
30 kGy	12.7 ± 0.2	13.5 ± 0.2	9.5 ± 0.3	10.4 ± 0.1	11.3 ± 0.3	14.7 ± 0.2	9.9 ± 0.3	12.8 ± 0.2		
40 kGy	13.0 ± 0.1	13.9 ± 0.1	9.9 ± 0.2	10.8 ± 0.1	11.5 ± 0.3	14.9 ± 0.1	10.5 ± 0.2	13.7 ± 0.1		

antimicrobial activities even after two years of EB sterilization.

CRediT authorship contribution statement

Asma N. Khan: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Ashok K. Pandey:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Ejazul Haque M. Malik:** Investigation, Formal analysis. **Hemlata K. Bagla:** Writing – review & editing, Resources, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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