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Comprehensive Phytochemical Analysis and Bioactivity Evaluation of γ-Irradiated *Chlorella vulgaris*

Nahla T. Elazab[1], Sabah Mohamed Ahmed El-Gamal[1], Moussa, H.R.[2]*

[1]Department of Biology, College of Science, Qassim University, Buraidah 51452, Saudi Arabia
[2]Radioisotope Department, Nuclear Research Center, Atomic Energy Authority, Dokki, Giza, Egypt
***Corresponding Author:** helal_moussa@hotmail.com

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ABSTRACT

The use of γ-irradiation to promote phytochemical components in numerous plants, including microalgae, has gained popularity in recent years. The aim of this investigation was to evaluate the phytochemical analysis and bioactivity evaluation of *Chlorella vulgaris* subjected to γ-irradiation at a dose of 200 Gy after 20 days of growth. A significant increase was recorded in the contents of phytochemical constituents of proanthocyanidins, saponins, β-carotene, vitamins (B_1, B_2, B_3, B_6, A, and K), amino acids, fatty acids, and mineral contents (Ca, Na, N, P, K, Fe, and Mg) in γ-irradiated *C. vulgaris* as compared to the control samples. Protein constituents typically consist of 24 standard amino acids. However, *C. vulgaris* had twenty essential amino acids present in sufficient concentrations. The data revealed the presence of twelve identified fatty acids. This article supports the idea of improving health perspective by incorporating *C. vulgaris* into the formulation of a great number of functional foods, medicinal foods, and nutritional supplements, as it offers numerous advantages in the food industry. Therefore, using metabolites of γ-irradiated *C. vulgaris* may unveil its prospects as a promising drug or contribute, either directly or indirectly, to keeping living things healthy since they are natural, safe, cheap, available, and easy to obtain.

INTRODUCTION

The increasing human population has prompted researchers to look for new ecological technologies and sources of food. Microalgae are the principal producers in the food chain and have numerous environments, including marine and freshwater systems **(Udayan *et al.*, 2021)**. There has been a growing interest in applying γ-irradiation in recent years to stimulate biological functions in microalgae **(Tale *et al.*, 2018)**. γ-irradiation is often used to enhance the nutritional and therapeutic properties of the microalgae *C. vulgaris* **(El-Sheekh & Hamouda, 2014)**. Recently, there has been an increasing interest in using low doses of γ-irradiation, which have potent penetrating capability in addition to being more affordable and efficient than other ionizing radiation, to stimulate biological processes in microalgae and to alter the composition and concentration of bioactive compounds, potentially enhancing their antioxidant properties, which are crucial for developing applications in nutrition, pharmaceuticals, and other health-related fields **(Tale *et al.*, 2018; Gabr *et al.*, 2019; Almarashi *et al.*, 2020)**. Microalgae are among the most important natural biochemical contents for food, pharmaceuticals, and cosmetics, in addition to being potential sources of vitamins, amino acids, proteins, lipids, and minerals for humans. Most microalgae can be an untraditional source of these compounds instead of artificial antioxidants **(Rani *et al.*, 2016; Shabana *et al.*, 2017)**. The antioxidant activity of *C. vulgaris* is significant due to its high content of chlorophylls, carotenoids, and polyphenols. Free radicals are neutralized by antioxidants, which reduce oxidative stress and may therefore decrease the risk of chronic diseases **(Lee *et al.*, 2020)**. Recently, an increasing trend has been recorded among scientists concerning the use of natural products as antioxidants with algal origins; this tend is due to their safe nature, low cost, and ease of use in addition to the aim of avoiding the use of industrial products (excessive costs), which have many harmful effects on human health causing numerous diseases **(Lourenço *et al.*, 2019; Abdel-Farid *et al.*, 2020)**. *C. vulgaris* is a type of freshwater seaweed (green algae), being a rich source of protein, lipids, carbohydrates, β-carotenes, polysaccharides, vitamins, carotenoids, minerals, and antioxidants has made *Chlorella* an intriguing kind of algae frequently utilized to make dietary supplements, cosmetics, medicinal treatments, and even to detoxify heavy metals from wastewater **(Rani *et al.*, 2016; Shabana *et al.*, 2017)**.

The purpose of this study was to assess the phytochemical analysis and to determine the bioactivity evaluation of the contents of minerals (potassium, calcium, magnesium, iron, sodium, nitrogen and phosphorus), composition of fatty acids, amino acids, vitamins (B_1, B_2, B_3, B_6, A, and K), mineral contents (Na, N, P, K, Fe, Ca, and Mg), phytochemical constituents of proanthocyanidins, saponins, and β-carotene in *C. vulgaris* subjected to γ-irradiation at a dose of 200 Gy after 20 days of growth.

MATERIALS AND METHODS

Growth medium and growth conditions

The algae used in this study, *C. vulgaris*, were obtained from the National Institute of Oceanography and Fisheries, hydrobiology laboratory, Qanater branch, Egypt, and cultured in BG-11 media **(Al-Habeeb *et al.*, 2024)**. The culture medium was autoclaved for 20 minutes at 121°C before inoculation, and the required illumination was provided by sunlight. The solution was continually mixed by an aerator at a temperature of 30±2°C; the pH was adjusted to 7.5, and the photoperiod was 16/8h of a day/night cycle. The harvested biomass was allowed to precipitate before being filtered using 0.45-mm pore-size Whatman GF/C filter paper to get a concentrated- algae paste **(Hamid *et al.*, 2016)**.

Table 1. Chemical composition of BG-11 media used

Chemical (g/L)	BG-11 media
$NaNO_3$	1.6
K_2HPO_4	3.050
$MgSO_4.7H_2O$	7.500
$CaCl_2.2H_2O$	3.600
Citric acid. $1H_2O$	0.600
Ammonium ferric citrate	0.600
EDTA (disodium salt)	0.100
Na_2CO_3	0.020
Trace metal	1 ml
H_3BO_3	2.860
$MnCl_2.4H_2O$	1.810
$ZnSO_4.7H_2O$	0.223
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.078
$Co(NO_3)_2.6H_2O$	0.049
Distilled water	1.0 L
pH	7.5±0.2

Previous studies have shown that the dose of 200 Gy is the best in terms of increasing the growth and productivity of the algae and proliferating the production of many important and effective biological substances for the algae **(Helal *et al.*, 2023; Al-Habeeb *et al.*, 2024)**. In a preliminary experiment, volumes of 500ml of algal batches, having the same concentration of cells (50 × 106 cells/ml) for *C. vulgaris* culture grown for 4 days, were subjected to γ-irradiation at a dose of 200 Gy. The exposure rate was 0.85 Gy/min utilizing Co^{60} as a gamma-ray source at the Egyptian Atomic Energy Authority **(Moussa & Jaleel, 2010; Al-Habeeb *et al.*, 2024)**.

Following irradiation, we used a certain volume of the overnight dark-adapted *C. vulgaris* cells to inoculate 750ml of BG-11 media into 1 L Erlenmeyer flasks. Under sterile control conditions, the culture medium was placed in an autoclave at 121°C for 25 minutes before inoculation using an autoclave (STERIF0W-1362), and the required illumination was supplied by a white fluorescent lamp (110 µmol photons $m^{-2}s^{-1}$). The solution was continually mixed by an aerator at a rate of 0.5 L/min (Heidolph MR Hei-Mix S magnetic stirrer, Germany); the photoperiod was 16/ 8h of a day/night cycle; temperature was set at 30±2°C, and the pH was 8.5. The harvested biomass after 20 days was allowed to precipitate before being filtered using 0.45mm pore-size Whatman cellulose filter papers to get concentrated- algae paste **(Hamid *et al.*, 2016)**. Samples were taken from the flasks for the physiological and biochemical investigations, and these were either immediately used or fixed in liquid nitrogen for further examination.

Phytochemical analysis of *C. vulgaris*

Proanthocyanidins (condensed tannin) content was evaluated by the method of **Tyler (1994)**. Saponin content was determined by the reported method of **Obadoni and Ochuka (2001)**. β-carotene was estimated according to the protocol of **Craft and Soares (1992)**. Quantitative estimation of amino acids was done by using an automated analyzer for amino acids (Dionex ICS-3000) through the procedure of **Abugrara *et al.* (2020)**. Fatty acid compositions were determined by a standard capillary gas chromatographic method **(Diraman *et al.*, 2009)**. The estimation of vitamins (B_1, B_2, B_3, B_6, A, and K) were conducted according to the study of **Amidzic *et al.* (2005)**. While, the determination of mineral contents was performed by spectrometry using the atomic absorption spectrophotometer (GFASHIMADZU-AA–6800), applying the procedure of **Moussa (2001)** and **Shreadah *et al.* (2018)**.

Statistical analysis

Analytical statistics were estimated with the SPSS version 17 statistical software package (SPSS Incorporated Company, Illinois, USA). Data were described as means ± SD (standard deviation). The significance was determined to be statistically different when $P \leq 0.05$ **(Moussa & Galad, 2015; Abdel-Alim *et al.*, 2023)**.

RESULTS AND DISCUSSION

Phytochemical constituents (proanthocyanidins, saponins, and β-carotene) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

Data demonstrated the existence of numerous advantageous natural health substances such as proanthocyanidins (condensed tannins), β-carotene, and saponins that increased in γ-irradiated *C. vulgaris* (200 Gy) compared to the control group after 20 days of growth **(**Fig. 1).

Tannins (proanthocyanidins) exhibit significant and encouraging antioxidant properties, and they may have the potential to protect and preserve biomolecules (DNA, proteins, and lipids) that are subjected to free nitrogen ions, such as peroxynitrite ($ONOO^-$), and free oxygen radicals. Additionally, they have a strong and crucial defense against oxidative/nitrifying stress linked to a variety of dangerous and damaging diseases, including cancer, neurodegeneration, and cardiovascular disease **(Mohesein *et al.*, 2020)**.

Proanthocyanidins have the ability to scavenge free radicals and to improve the body's defense system, thereby reducing oxidative stress in organs like the liver and kidneys by mitigating lipid peroxidation and by preserving antioxidant enzyme activities **(Bagchi *et al.*, 2003)**.

Saponins have been shown to reduce lipid peroxidation, stimulate the body's endogenous antioxidant defenses, reduce oxidative stress by scavenging free radicals, and improve antioxidant enzyme levels **(Mohesein *et al.*, 2023)**. β-carotene supports the maintenance of cellular antioxidant enzymes, thereby stimulating the body's overall antioxidant capacity **(Krinsky & Yeum, 2003)**. β-carotene is a potent antioxidant that reduces oxidative stress by neutralizing free radicals **(Miazek *et al.*, 2022)**.

Countless antioxidants, including polyphenols, saponins, β-carotene, and vitamin E, have been studied in recent years for their potential or actual benefits against oxidative stress **(Pizzino *et al.*, 2017)**. In medicine, saponins are used as potent antioxidants, antibacterial, hyperglycemia, anticancer, hypercholesterolemia, anti-inflammatory, and reduction in weight **(Abdel-Karim *et al.*, 2020)**.

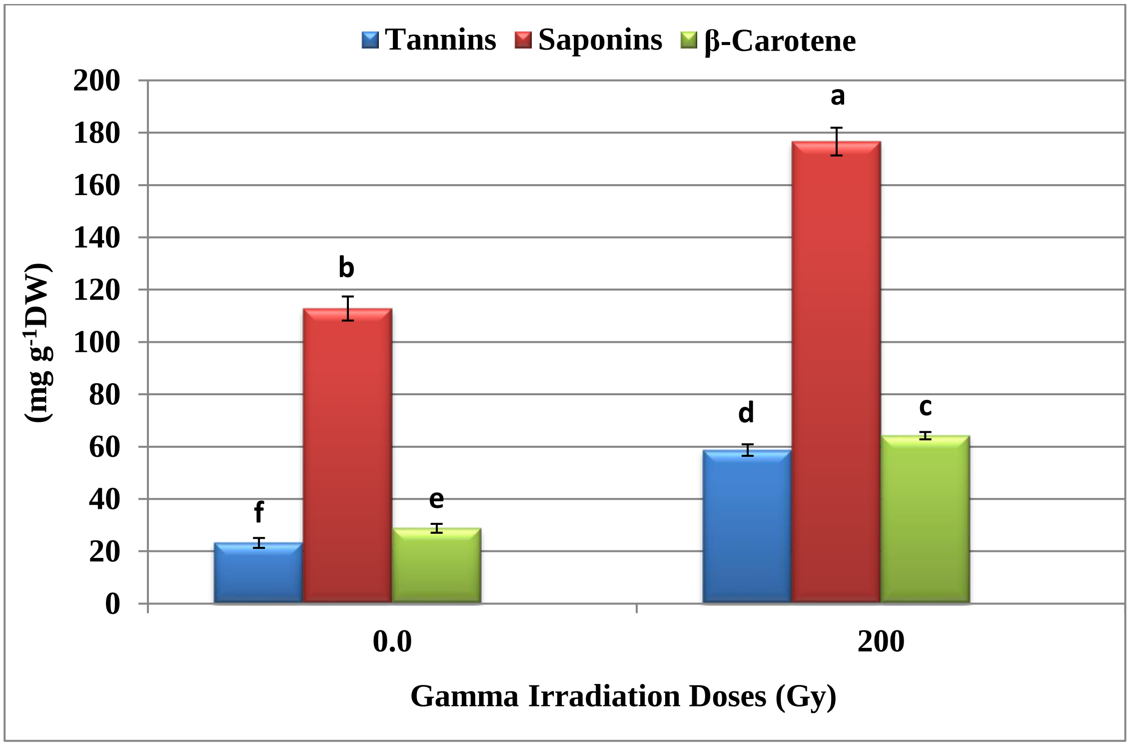

Fig. 1. Quantitative analysis for proanthocyanidins (condensed tannins), saponins, and β-carotene, and protein (mg/gDW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

Data are represented as the mean ± SD of samples in triplicate. Different letters indicate significant differences (*P*< 0.05).

Vitamin contents of B_1, B_2, B_3, B_6, A, and K (mg $100g^{-1}$FW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

The results for vitamin contents of B_1, B_2, B_3, B_6, A, and K in γ-irradiated *C. vulgaris* (200 Gy) are listed in Fig. (2). Results indicated that the various vitamin contents (B_1, B_2, B_3, B_6, A, and K) were significantly increased by the γ-irradiation compared to the control, which agrees with the findings of **Abomohra *et al.* (2016)** and **Shabana *et al.* (2017)**.

The activation of other precursors or related chemicals may be connected to the increase in vitamins. For instance, **Abomohra *et al.* (2016)** found that increased γ-irradiation up to a dose of 2.5 KGy resulted in an increase in carotenoids contents (as provitamin A), which may be related to the increased vitamin A contents. It has been demonstrated that vitamin A regulates bone physiology by means of retinoic acid receptor signaling **(Yee *et al.*, 2021)**. The correlation between the elevated levels of retinol and osteoporosis is intensified in individuals with insufficient amounts of vitamin D **(Mata‑Granados *et al.*, 2013)**. Group B vitamins are a class of water-soluble, structurally diverse compounds that function as cofactors for numerous enzymes involved in human energy metabolism **(Hanna *et al.*, 2022)**. In postmenopausal women, vitamin K administration successfully increases bone mineral density and lowers the incidence of fractures. Furthermore, it amplifies the anti-osteoporotic benefits of supplementing with calcium and vitamin D **(Skalny *et al.*, 2024)**.

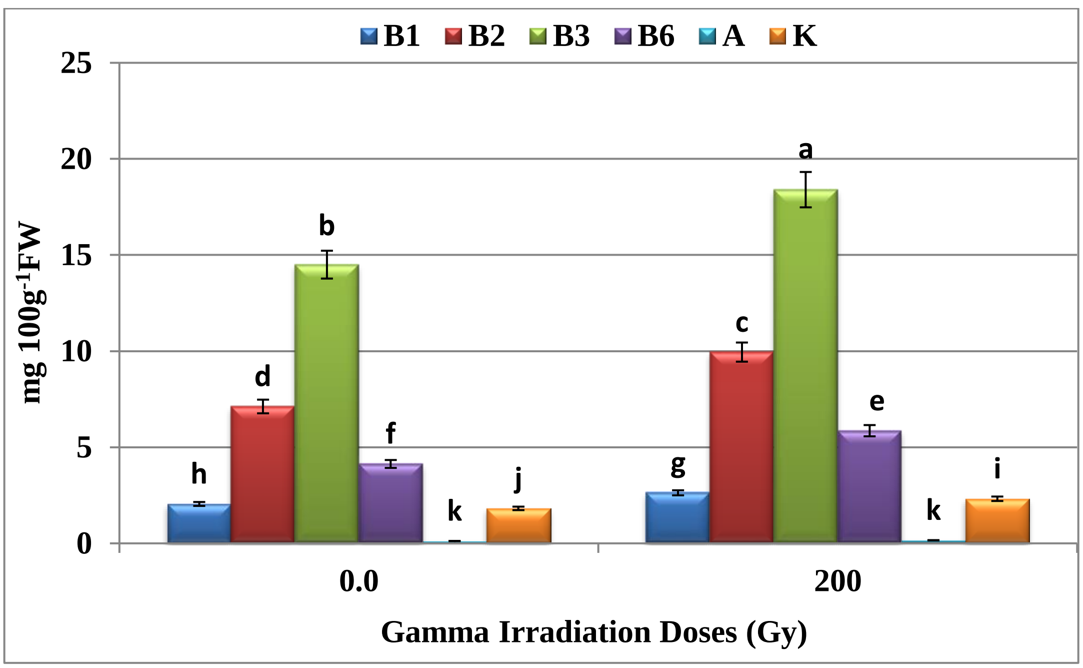

Fig. 2. Vitamin contents of B_1, B_2, B_3, B_6, A, and K (mg $100g^{-1}$FW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

The values are the means of at least three replicates ± standard deviation (SD). Different letters indicate significant differences (*P*<0.05).

Amino acid contents in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

Findings regarding the amino acid composition of γ-irradiated *C. vulgaris* (200 Gy) after 20 days of growth are listed in Fig. (3). The amino acid composition of γ-irradiated *C. vulgaris* increased significantly compared to the control samples. There are typically 24 standard amino acids found as constituents of proteins. *C. vulgaris* had twenty amino acids. **Farhi *et al.* (2008)** illustrated that even at low radiation doses, the pool of free amino acids increased in Chlorophyceae green microalga. An important function that protein content played in the DNA repair pathway was linked to the increase in amino acid concentration **(Yu *et al.*, 2016)**. γ-irradiation treatments increased the amino acid contents in *C. vulgaris* compared to the control samples, with results similar to those of **Moussa *et al.* (2015)** and **Shabana *et al.* (2017)**.

In both the animal and human bodies, amino acids are essential for cellular assembly and metabolism to produce proteins, which are then utilized to construct various bodily tissues **(Debnath *et al.*, 2019)**.

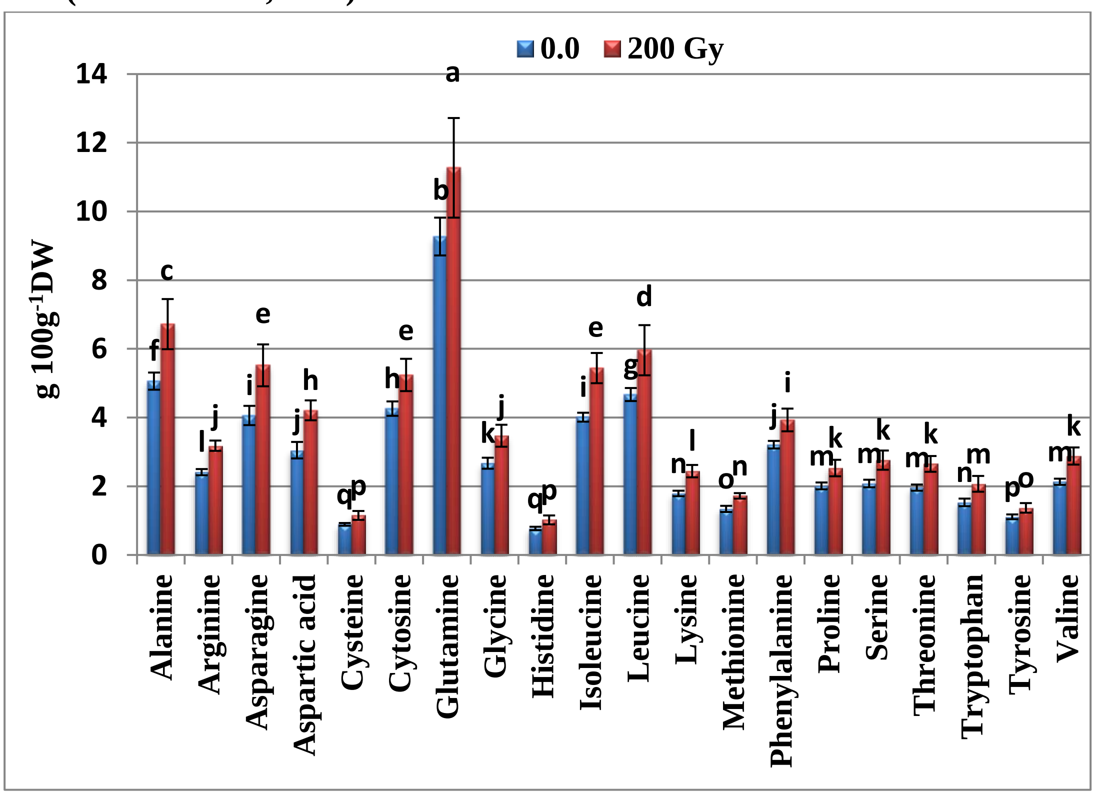

Fig. 3. Amino acid contents (g 100g^{-1}DW) of *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

The values are the means of at least three replicates ± standard deviation (SD). Different letters indicate significant differences (*P*< 0.05).

Fatty acids composition (mg $100g^{-1}$DW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

Information about the lipid profiles of γ-irradiated *C. vulgaris* (200 Gy) after 20 days of growth are depicted in Fig. (4). The data revealed the presence of twelve identified fatty acids. Moreover, fatty acid methyl esters (FAMEs) mostly have both saturated and unsaturated fatty acids, with carbon chains ranging from C12 to C24 in *C. vulgaris,* in agreement with the findings of **El-Sheekh and Alaa (2009)**.

Exposure of microalgae to UV-B and γ-irradiation increases the contents of saturated fatty acids and monounsaturated fatty acids **(Tale *et al.,* 2018; Abo-State *et al.,* 2019; Oliver *et al.,* 2020)**.

In addition, the authors hypothesized that the lipid biosynthesis pathway may be upregulated as a result of the observed ROS buildup and higher lipid levels in response to γ-irradiation. **Tale *et al.* (2018)** suggested that this could be a potential method by which lipid accumulation could be brought on by γ-irradiation. In their study, they postulated that under γ-irradiation, there are changes in the expression of some important genes for lipid metabolism, such as Acetyl-CoA carboxylase and diacylglycerol acyl transferase. Additionally, they proved that these two genes are upregulated following gamma irradiation, sustaining a possible mechanism by which γ-irradiation could induce increased lipid biosynthesis in *Chlorella sorokiniana*.

In their investigation, **Tale *et al.* (2018)** demonstrated that γ-irradiation causes alterations in the expression of several key genes involved in lipid metabolism, including diacylglycerol acyl transferase and Acetyl-CoA carboxylase. They demonstrated that these two genes are elevated after gamma irradiation, supporting a potential mechanism by which *Chlorella sorokiniana* could experience an increase in lipid production as a result of γ-irradiation. γ-irradiation increases the lipid accumulation of *C. vulgaris* **(Abo-State *et al.,* 2019)**.

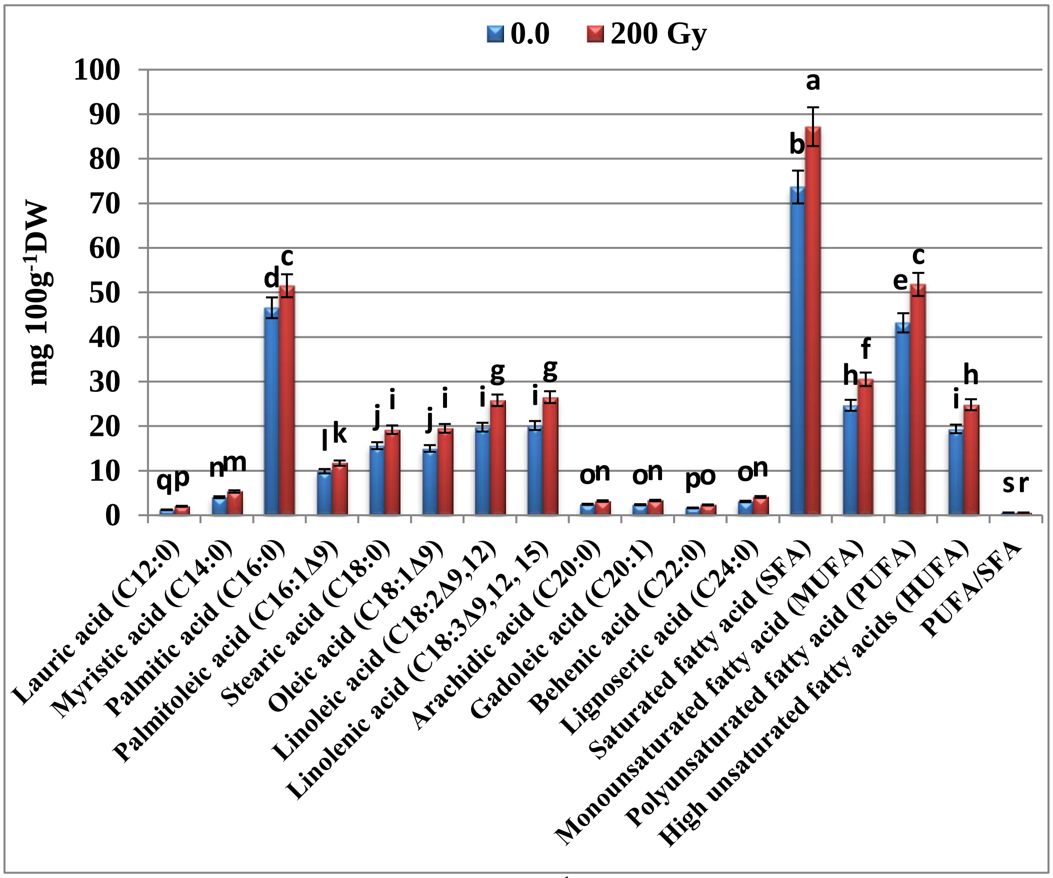

Fig. 4. Fatty acid compositions (mg 100g^{-1}DW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

Data are represented as the mean ± SD of samples in triplicate. Different letters indicate significant differences (*P*<0.05).

Mineral contents of Na, N, P, K, Mg, Ca, and Fe (mg^{-1}DW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

The data for mineral levels of Na, N, P, K, Mg, Ca, and Fe of γ-irradiated *C. vulgaris* (200 Gy) after 20 days of growth are assessed in Fig. (5).

Compared to the control samples, γ-irradiation treatments increased the mineral contents of *C. vulgaris*, which agrees with the findings of **Abomohra *et al.* (2016)** and **Shabana *et al.* (2017)**.

Algae are rich in various minerals that have been shown to possess antimicrobial properties and contribute to overall health **(Franceschi *et al.*, 2014)**. Nitrogen assimilation occurs when nitrogen is integrated into the most significant structural and functional macromolecules in organisms such as amino acids. The most significant element is nitrogen, which makes up a large portion of the cellular components of algal cells, providing roughly 10% of the dry weight of cyanobacterium cells **(Perez-Garcia *et al.,* 2011)**. Nucleic acids and proteins make up 15% of the nitrogen **(Inokuchi *et al.,* 2002)**.

Calcium involved in protein phosphorylation has been linked to some hormonal and environmental reactions as a second messenger, and may have a significant role in controlling the actions of several different enzymes **(Shabana *et al.,* 2017)**. While iron is essential for both humans and bacteria, controlled supplementation through dietary sources like algae ensures that iron availability supports the host's immune function without excessively promoting bacterial proliferation **(Suleria *et al.,* 2015)**.

Magnesium is crucial for various biological processes including immune function. Sufficient levels of magnesium improve the body's capacity to develop a powerful defense against bacterial infections **(Suleria *et al.,* 2015)**. Algae are known to be a rich source of various minerals, and their combined effects can support overall health and can enhance the body's defense **(Mohamed *et al.,* 2012)**. Nitrogen is a key component of amino acids and proteins. Adequate protein intake is necessary for maintaining bone health; however, consuming too much protein, particularly from animal sources, might increase the excretion of calcium **(Zittermann *et al.,* 2023)**.

The conversion of vitamin D to its active form, which is necessary for calcium absorption and bone re-modelling, may be influenced by potassium **(Han *et al.,* 2015)**. In addition to being engaged in protein phosphorylation and implicated as a second messenger in several hormonal and environmental reactions, calcium may also play a significant role in controlling the activity of several different enzymes **(Shabana *et al.,* 2017)**.

Magnesium is crucial for various biological processes including immune function. Sufficient levels of magnesium improve the body's capacity to produce a successful immunological response **(Suleria *et al.,* 2015)**. Additionally, iron is crucial for the creation of collagen and the metabolism of vitamin D **(Yang *et al.,* 2023)**.

Sodium increases the excretion of calcium in the urine and, with current calcium intakes, causes compensatory responses that could result in greater bone re-modelling and bone loss. Sodium is primarily found in sodium chloride. Salt-induced volume expansion, which increased glomerular filtration rate (GFR) of the kidneys, and the competition between sodium and calcium ions in the renal tubule are both contributing factors to calciuria **(Heaney, 2006)**.

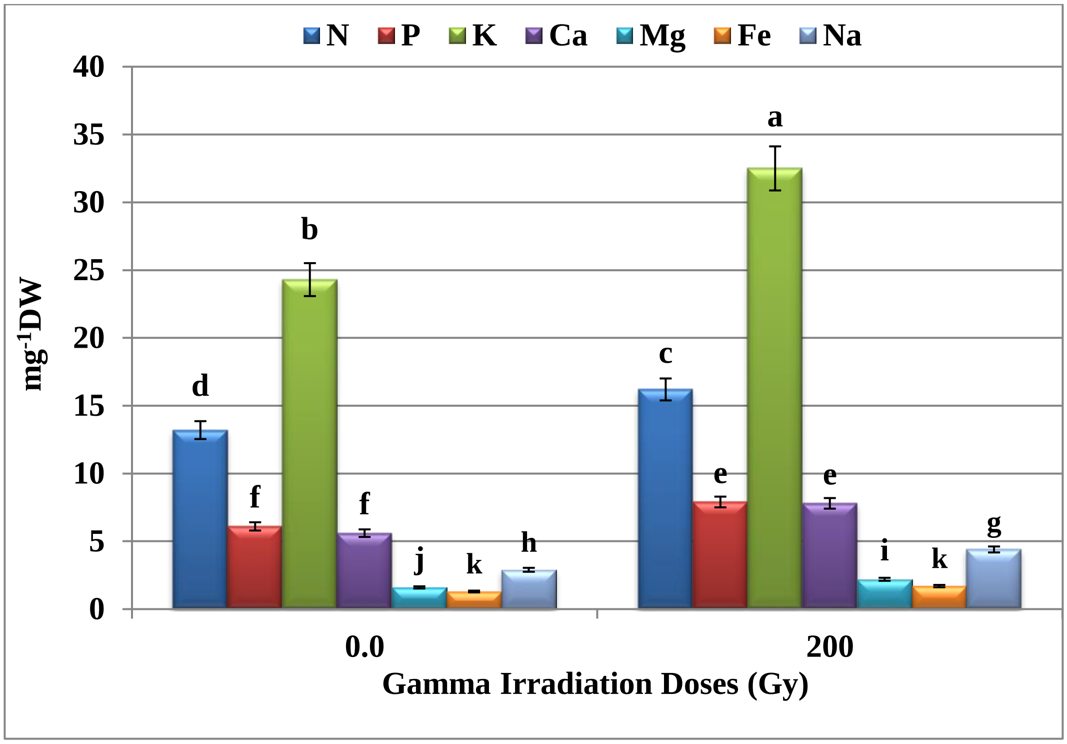

Fig. 5. Mineral contents of Na, N, P, K, Mg, Ca, and Fe (mg^{-1}DW) in *C. vulgaris* treated with and without γ-irradiation (200 Gy) after 20 days of growth

The values are the means of at least three replicates ± standard deviation (SD). Different letters indicate significant differences (*P*<0.05).

CONCLUSION

According to the findings of this investigation, γ-irradiation (200 Gy) has significantly increased the contents of proanthocyanidins, saponins, β-carotene, amino acids, fatty acids and mineral contents in *C. vulgaris* as compared to the control samples. Since *C. vulgaris* has many benefits for the food business, this article advocates using it to improve health perspectives in the development of several therapeutic foods, functional foods, and nutritional supplements. Given that metabolites of γ-irradiated *C. vulgaris* are inexpensive, readily available, safe, and natural, their use may therefore contribute directly or indirectly to life-rejuvenating.

Conflicts of interest

We have no conflicts of interest to disclose.

ABBREVIATION

FRAP: Ferric-reducing antioxidant power

ROS: Reactive oxygen species

DPPH$^{\bullet}$: 2, 2-diphenyl-1-picrylhydrazyl

ABTS$^{\bullet+}$: 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

TAC: Total antioxidant capacity

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