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Original Article

EFFECT OF GAMMA IRRADIATION ON ANTIOXIDANT POTENTIAL AND BIOACTIVES OF A COSMECEUTICALLY SIGNIFICANT *CHLORELLA EMERSONII KJ725233*

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ABSTRACT

Objective: Gamma radiation induces free radicals with a corresponding alteration in the cell's antioxidant defense system. The present study thus aimed at determining the role of gamma irradiation in improving the cosmeceutical potential of *CEK* in terms of antioxidants.

Methods: *C. emersonii* KJ725233 (CEK) was subjected to low (100 Gy) and high (1000 Gy) doses of gamma irradiation. The effect of such gamma radiation doses on the chlorophyll content was evaluated. The quantitative alterations in the antioxidant content of *CEK* were evaluated by phosphomolybdenum assay (TAC), ferric reducing antioxidant potential (FRAP), 2, 2-Dipheny-1-picryl hydrazyl radical scavenging assay (DPPH), total phenolic (TPC) and total flavonoid content (TFC). Also, the corresponding qualitative alterations in the bioactives of *CEK* were determined by GC-HRMS analysis.

Results: A 179.57±2.55% increase in the total chlorophyll content along with a 71.76±2.96%, 32.08±2.16%, 11.67±0.89%, 42.85±8.0% and 31.37±3.18% increase was observed in the TAC, FRAP, DPPH radical scavenging, TPC and TFC respectively in *CEK* irradiated at 1000 Gy. GC-HRMS analysis revealed the induction of Vitamin E on irradiation at both the doses with a corresponding decrease in the phytol content, whereas 100 Gy stimulated the induction of phytosterols.

Conclusion: The potent intrinsic antioxidant activity of cosmeceuticals significant *CEK* can be elevated with the induction of the most sought after antioxidant in cosmetology–Vitamin E as a response to irradiation.

Keywords: Gamma irradiation, *C. emersonii* KJ725233, Antioxidant, Vitamin E, Cosmeceutical

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INTRODUCTION

Cultural milieus such as the temperature, light intensity, photoperiod, irradiation and pH are known to impact the growth and subsequently the biochemical composition of microalgae [1]. Under such constraints, microalgae are reported to produce a maximal amount of varied bioactives as compared to normal conditions due to the stressed altered metabolic pathways. The induction or overproduction of these bioactives is primarily a survival technique to overcome the inconsiderate growth environments [2]. It is thus, necessary to manipulate the growth conditions in order to stimulate the synthesis of commercially significant microalgal bioactives [3].

Water that makes up 70-90% of the cell contents is the chief target of ionizing radiations. Water molecules are ionized into $H_2O^{\cdot+}$alongwith $H^{\cdot}$ and. OH which further generate the peroxyl and superoxide radicals [4, 5]. As a result, the cell's antioxidant defense systems have to be modified to balance the detrimental effects of the free radicals generated [6, 7]. Gamma irradiation with Co_{60} (1.33 MeV) has an ability to induce cytological, anatomical, biochemical, morphological and physiological alterations in cells and tissues [8-11].

The cosmetic industry has been leveraging on algae as a source of bio-sustainable ingredients as they are rich in biologically active components that are cost-effective. Such compounds meet consumer demands of being "natural" as well as "healthy" as compared to their synthetic counterparts. Some of the bioactives associated with skincare are the algal proteins, lipids, vitamins and secondary metabolites such as phenolics, hydrocarbons, pigments etc [12].

Effect of gamma radiations on *Chlorella* has been reported in terms of changes in the rates of respiration and photosynthesis, colony-forming ability, modulation of cell division and DNA synthesis [13]. The sensitivity of *Chlorella* to ionizing radiations depends on several factors, including the DNA content per cell and the stage of the cell cycle [14].

C. emersonii KJ725233 is a novel, non-fastidious microalga which is an antioxidant reserve isolated from the western regions of Maharashtra. The alga has been identified both morphologically as well as by 18s rDNA sequencing [15, 16]. The alga contains a plethora of compounds such as phytol, its isomer 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, Vitamins and hydrocarbons with anti-aging (anti-elastase, anti-collagenase, anti-hyaluronidase), antioxidant and anti-inflammatory properties thus exemplifying its significance as a source of cosmeceuticals [17]. The present study was designed to evaluate the effect of gamma radiation on the cosmeceutical potential of *C. emersonii* KJ725233 in terms of its pigment and antioxidant content.

MATERIALS AND METHODS

Chemicals and reagents

AR grade sodium hydroxide, hydrochloric acid, sulphuric acid, methanol, dimethyl sulpoxide Coomassie brilliant blue G-250, ethanol, o-phosphoric acid, bovine serum albumin, sodium dihydrogen phosphate, disodium hydrogen phosphate, ferric chloride, trichloroacetic acid, ascorbic acid, gallic acid, sodium carbonate, potassium ferricyanide, aluminium trichloride hydrate, quercetin and ammonium molybdate were obtained from SD Fine-Chem Ltd, Mumbai. The plant grade chemicals required for BG-11 medium along with DPPH, folin-ciocalteau reagent were procured from HiMedia, Mumbai.

Chlorella emersonii KJ725233 culturing and irradiation conditions

CEK was isolated from the western regions of Maharashtra and identified morphologically as well as by 18s rDNA sequencing [16]. *CEK* was grown in 1L BG-11 and incubated at 34±1 °C with a lux intensity of 1000X for a period of 15 d. At the end of incubation, this culture was equally distributed into three flasks, which were exposed to Co_{60} at a dose rate of 37.51 Gy min^{-1} for doses 100 Gy and

1000 Gy in a GC5000 (BRIT) at the Department of Atomic Energy, BARC, Mumbai, India.

Determination of chlorophyll content

After irradiation, 10 ml of theses cultures were drawn into preweighed centrifuge tubes. These were centrifuged at 5000 rpm for 20 min and the supernatant was discarded. To the biomass, 10 ml of methanol was added and chlorophyll extraction was carried out in an electric water bath at 60 °C for 2 h. At the end of incubation, the absorbance of the supernatant was measured at 665 and 652 nm. The chlorophyll content was determined by the following formulae [18, 19]–

*Chlorophyll a (µg ml-1) = 16.72 * Absorbance (665)–9.16 * Absorbance (652)*

*Chlorophyll b (µg ml-1) = 34.09 * Absorbance (652)–15.28 * Absorbance (665)*

Preparation of *CEK* extracts for determination of the antioxidant potential

The dried biomass was suspended in absolute methanol at a concentration of 0.1 g $ml^{-1}$and sonicated for 30 min. These suspensions were centrifuged at 5000 rpm for 20 min and the supernatants were transferred to three preweighed crucibles. The extraction was repeated thrice and the supernatants were pooled together. 1 µl of these pooled supernatants were subjected to GC-HRMS analysis for the identification of bioactives. The remaining supernatants were dried at 32±1 °C for 24 h. The dried extracts were reconstituted in dimethyl sulfoxide and used for antioxidant studies.

Antioxidant potential

Total antioxidant capacity by phosphomolybdenum method [TAC]

The alterations in the total antioxidant capacity of CEK were determined by the phosphomolybdenum method as described earlier by Prieto et al. [20]. To 300 µl of the extracts, 3 ml of the total antioxidant capacity reagent (0.6 mmol sulphuric acid, 28 mmol sodium phosphate and 4 mmol ammonium molybdate) was added and the reaction mixtures were incubated at 95 °C for 90 min. At the end of incubation, the absorbance was measured at 695 nm. Ascorbic acid was used as a standard and the total antioxidant capacity was expressed as mg ascorbic acid equivalence per g dry weight (mg AAE g-1 DW).

Ferric reducing antioxidant potential [FRAP]

The reducing potential was determined by using potassium ferricyanide by modifying the procedure as described earlier by Hemlatha *et al.* 21]. 125 µl of 1% potassium ferricyanide was added to 50 µl of the extract and incubated at 50 °C for 20 min. After incubation, 125 µl of 10% trichloroacetic acid was added and 100 µl of this reaction mixture was transferred to fresh wells with an equal volume of distilled water. Finally, 20 µl of 0.1% ferric chloride was added and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard and both the total antioxidant capacity (TAC) as well as the reducing potential (FRAP) was expressed as mg ascorbic acid equivalent per g dry weight (mg AAE g^{-1} DW).

DPPH radical scavenging potential

The radical scavenging activity of the extracts was evaluated by DPPH (2, 2-Dipheny-1-picryl hydrazyl radical) assay by modifying the earlier reported procedure [22]. Methanol was used as a blank and ascorbic acid as a positive control. 150 µl of 2% aluminium trichloride was added to an equal volume of varying concentrations of the extracts (2-10 mg ml^{-1}) and incubated in the dark at 32±1 °C for 30 min. After incubation the absorbance was read at 517 nm. The free radical scavenging activity was then calculated as % inhibition by the following formula: $[(A_{(blank)}-A_{(test)})/A_{(blank)}] * 100$, where $A_{(test)}$ is the absorbance of the extracts and $A_{(blank)}$ is the absorbance of the blank. IC50 for the extracts was determined by plotting a graph of percentage inhibition against extract concentration. The tests were carried out in triplicates and the values were expressed as mean±SD.

Total phenol content [TPC]

The total phenolic content was determined by modifying the Folin's Ciocalteau method, as reported by Wu *et al.* [23]. To 25 µl of the extracts, 50 µl of 1 N Folin's Ciocalteau reagent was added followed with 125 µl of 20% sodium carbonate was added. The reaction mixtures were incubated at 32±1 °C in the dark for 30 min. The absorbance was read at 765 nm. Gallic acid was used as a standard and the total phenolic content was expressed as mg gallic acid equivalence per g dry weight (mg GAE g^{-1}DW).

Total flavonoid content [TFC]

To 150 µl of the extracts, an equal volume of 2% aluminium trichloride was added and incubated in the dark at 32±1 °C for 30 min. At the end of incubation, the absorbance was measured at 415 nm. Quercetin was used as a standard and the total flavonoid content was expressed as mg quercetin equivalence per g dry weight (mg QE g^{-1} DW) [24].

Determination of radiation-induced alterations in bioactives of *CEK* by gas chromatography–high resolution mass spectrometry

GC-HRMS analysis was carried out using Agilent Technologies GC equipped with Accutoff MS. Bioactives were separated on HP-5 MS capillary column having 5% phenyl polysiloxane as stationary phase, column length 30 m, internal diameter 0.32 mm and film thickness 0.25 µm. 1 µl of the sample was injected in the split ratio of 10:1, the injector and transfer line temperature was 250 °C and 260 °C while the ion source temperature was 200 °C. Oven temperature programmed from 80 to 280 °C at 10 °C min-1; flow rate of carrier gas helium was 1 ml min^{-1}. Essential compounds were identified comparing their retention times and mass fragmentation patterns with the data of standards at the National Institute of Standards and Technology (NIST) library [22].

RESULTS AND DISCUSSION

Effect of gamma radiation on the chlorophyll content of *CEK*

The quantitative shift in the total chlorophyll content in *CEK* on irradiation was determined spectrophotometrically and an increase of 179.57±2.55% was observed in *CEK* irradiated at 1000 Gy (fig. 1). An alteration in the chlorophyll content is one of the preliminary indicators of the mutagenic effects of gamma irradiation [25]. Gamma radiation is reported to induce photoluminescence [26] which can be due to a corresponding increase in chlorophyll content. In addition to an increase in the total chlorophyll content, an alteration in the chlorophyll a: chlorophyll b ratio from 1:2 in the control to 1:1 was observed on irradiation. This shift in the chlorophyll a: chlorophyll b ratio on radiation might be due to a reduction in chlorophyll b synthesis at a higher radiation dose [4].

Quantitative effect of gamma radiation on the antioxidant content of *CEK*

The quantitative alterations in the antioxidant potential of *CEK* as a response to gamma radiation were determined in terms of total antioxidant capacity, ferric reducing antioxidant potential and DPPH radical scavenging potential together with modulations in the total phenolic and total flavonoid content. A 71.76±2.96%, 32.08±2.16%, 11.67±0.89%, 42.85±8.0% and 31.37±3.18% increase was observed in the total antioxidant capacity, ferric reducing antioxidant potential, radical scavenging potential (IC_{50}), total phenol and flavonoid content respectively in 1000 Gy irradiated *CEK* as compared to non-irradiated *CEK* (fig. 2a).

Gamma rays are known to interact with the cellular molecules thereby inducing the generation of free radicals such as ROS and RNS. This oxidative stress is counteracted by cellular enzymatic and non-enzymatic antioxidant defense systems [27, 28]. Literature also suggests a positive correlation between FRAP and Phosphomolybdenum assay with superoxide dismutase (SOD), catalase (CAT) and peroxidase activity (POD); wherein an increase in the antioxidant capacity of *Monodora myristica* was observed with a corresponding increase in the antioxidant enzyme activity [29]. The increase in antioxidant potential obtained in the present study thus could be due to enhanced expression of these antioxidant enzymes since gamma radiation is reported to stimulate antioxidant enzymes such as SOD, CAT, POD and polyphenol oxidase (PPO). Besides these enzymatic antioxidants, the phosphomolybdenum

method measures non-enzymatic antioxidants such as ascorbic acid, phenols, α-tocopherols, carotenoids and reduced glutathione [30].

Although dramatic induction of total antioxidant capacity was observed, an increase in antioxidant potential as measured by FRAP was modest but significant as seen in fig. 2a. Both 100 Gy and 1000 Gy caused an increase in the range of 30% and it possibly indicates saturation in the antioxidant species as measured by FRAP. Radical scavenging ability is seen in fig. 2b does not show significant alterations in response to radiation, while total phenolics increased marginally under similar treatment conditions. Only water-soluble antioxidants are measured by FRAP [31] therefore, an increase in reducing power obtained in the present study can thus be mainly attributed to a corresponding increase in the hydrophilic antioxidants. Also, the reducing power obtained at 1000 Gy i.e. 15.86±0.7 mg AAE g^{-1}DW was more than twenty and sixteen folds higher than that reported for the methanolic extract of *Chlorella marina* and *Chlorella salina* respectively [21, 32].

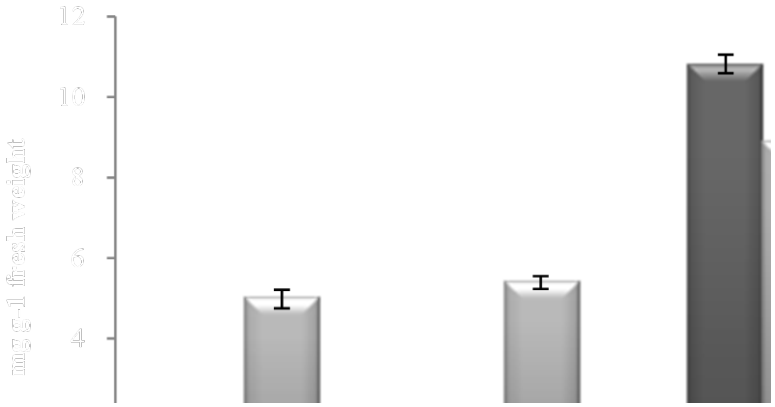

Fig. 1: Effect of gamma radiation doses (100 Gy and 1000 Gy) on the chlorophyll content of *CEK*. The data is expressed as mean±SD (n=3)

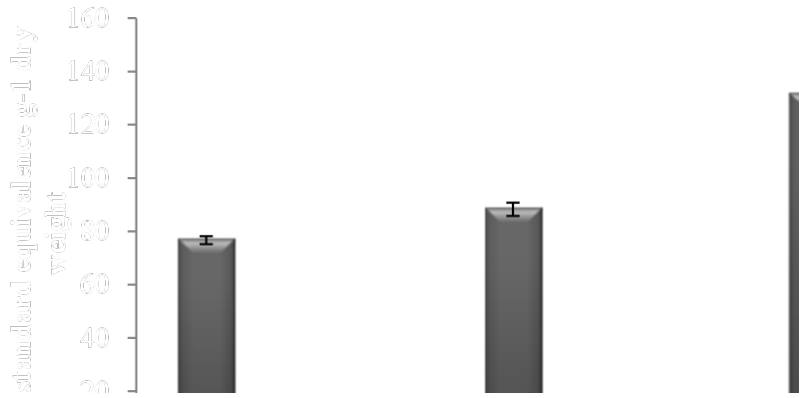

Fig. 2a: Antioxidant potential of non-irradiated *CEK* (control); 100 Gy and 1000 Gy irradiated *CEK* in terms of TAC, FRAP and TPC. The data is expressed as mean±SD (n=3)

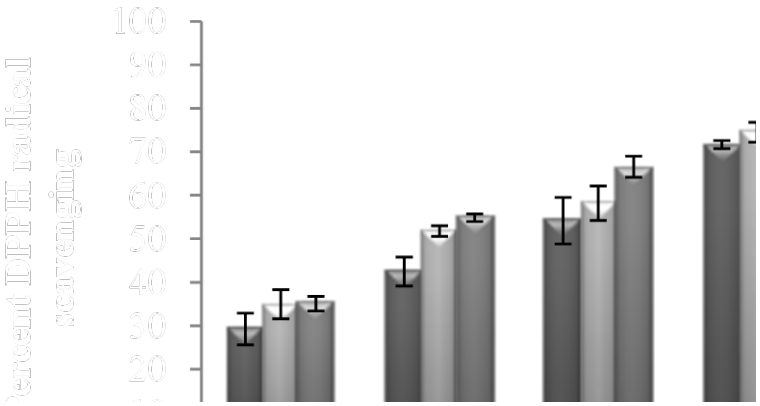

Fig. 2b: Radical scavenging potential of non-irradiated *CEK* (control), 100 Gy and 1000 Gy irradiated *CEK*. The data is expressed as mean±SD (n=3)

Phenolic content was determined by Folin's Ciocalteau method which measures only the hydrophilic phenols [21]. The improved phenolic content on irradiation may be attributed to the increase in the activity of phenylalanine ammonia-lyase an enzyme directly involved in the phenol synthesis [33]. Since Vitamin C used as a standard was a commercially purified product, the radical scavenging activity (IC_{50}) for irradiated as well as non-irradiated *CEK* both were found to be lower than Vitamin C i.e. 6.47±0.89 µg ml^{-1} (data not represented). However, the IC_{50} for 100 Gy irradiated alga was lower than that reported for the commercially available tablets of *Chlorella pyrenoidosa* (Sun Chlorella) which is 9.62 mg ml^{-1} [34]. Though an increase in the phenolic content was observed, no significant change was detected in the radical scavenging activity for the given radiation doses. DPPH has been widely used for measuring the antioxidant status of phenolic compounds; however, only lipophilic antioxidants are measured by DPPH whereas the method used in the present study to detect the phenolic content evaluated only the hydrophilic antioxidants [21].

Qualitative alterations in the bioactives of *CEK* as a response to gamma radiation

GC-HRMS analysis was performed to identify the pattern of metabolite induction as a response to low and high doses of gamma radiation that could have eventually influenced the antioxidant potential in *CEK*. The compounds identified along with their percentage areas were shown in table 1. The GC-HRMS analysis revealed a spectrum of ten different compounds belonging to the varying classes predominantly in the category of alcohols, hydrocarbons, sterols and fat-soluble vitamin.

Phytol was one of the two compounds which presented quantitative changes which decreased from a concentration of 71.7% to 27.18% at 100 Gy and then increased to 34.17% at 1000 Gy. Both phytol and its isomer 3,7,11,15–tetramethyl–2-hexadecen–1–ol are known to possess antimicrobial, antioxidant and anti-inflammatory activities. These have been used in the production of synthetic forms of Vitamin E and Vitamin K1 [35-37]. Phosphorylation of phytol to phytyl-phosphate and phytyl-diphosphate further feeds into the tocopherol synthesis pathway [38]. It might be the result of channelization of phytol into tocopherol synthesis that led to reduced phytol levels in the irradiated *CEK* as compared to the non-irradiated. As seen in table 2, Vitamin E a known free radical scavenger exhibited quantitative induction on irradiation at 100 and 1000 Gy. Naturally occurring Vitamin E consists of both saturated forms (tocopherols) as well as unsaturated forms (tocotrienols). Tocopherols are reported to be one of the two best chain-breaking phenolic antioxidant groups whereas tocotrienols are known to be potent antioxidants, antibacterial, anti-inflammatory, anticancer, hepatoprotective and hypoglycemic [39-41]. Vitamin E along with Vitamin C is one of the most sought after antioxidants in dermatology with most of the over-the-counter anti-aging creams containing 0.5-1% Vitamin E [42].

The increased antioxidant potential of *CEK* on irradiation can also be attributed to a shift from a C_{10} compound (1-Iodo-2-methyl nonane) to C_{16} compound (Hexadecane); hexadecane is also reported to be an antioxidant as well as an antibacterial [43].

Table 1: Alterations in bioactives of *CEK* as a response to gamma radiation

S. No.	Retention time (min)	Non-irradiated	100 Gy	1000 Gy
1	18.76	1-Iodo-2-methylnonane (5.70%)	Hexadecane (1.17%)	Hexadecane (2.11%)
2	21.26	-	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.67%)	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.39%)
	21.27	Cis-13,15-docasadienoic acid, methyl ester (1.72%)	-	-
3	22.00	-	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.54%)	-
4	22.01	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (4.88%)	-	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2.24%)
5	26.85	-	Phytol (27.18%)	-
6	26.86	-	-	Phytol (34.17%)
7	26.88	Phytol (71.50%)	-	-
8	29.34	-	Cholesta-6,22,24-triene-4,4-dimethyl (9.87%)	-
9	30.51	-	Stigmastan-3,5-diene (30.64%)	-
10	31.61	-	Tert-Hexadecanethiol (5.06%)	-
11	31.62	1,2-Octadecanethiol (15.11%)	-	-
12	31.63	-	-	1-Docosene (8.12%)
13	32.23	-	Campesterol (2.54%)	-
14	32.74	-	Vitamin E (17.97%)	-
15	32.84	-	-	Vitamin E (46.67%)

Irradiation also induced the synthesis of sterols such as Cholesta-6,22,24-triene-4,4-dimethyl, stigmastan-3,5-diene and campesterol at low dose as seen in table 1. The induction of sterols might be a protective response of the alga to resist the harmful effects of radiation. Similar results have been reported [44] in *Cryptococcus neoformans*. However, at higher radiation doses there was a marked absence of sterols. Higher gamma radiation doses have reported to repress the genes coding for enzymes involved in isoprenoid and sterol synthesis in *Trypanosoma cruzi* [45]. The percent increase in the antioxidant activity of 100 Gy and 1000 Gy irradiated alga could, therefore, be related to the induction of Vitamin E as well as hexadecane as identified in the corresponding extracts as observed in table 1.

CONCLUSION

The present study clearly indicated that gamma radiation causes significant qualitative as well as quantitative alterations in the composition of bioactive compounds which subsequently modulates the biological activities of the alga. Manipulation of the physical environment of *C. emersonii* KJ725233 induced the synthesis of cosmeceutically significant Vitamin E. By far chemical synthesis and extraction from vegetable oils are the only commercial sources of Vitamin E. Hence its elevated synthesis by the alga in response to irradiation makes it a potential approach for scale-up of such an essential anti-aging compound and opens up the possibility of its use as a novel cost-effective source of cosmeceuticals.

AUTHORS CONTRIBUTIONS

All authors have contributed equally. Sneha Sawant and Reema Devi Singh have performed the experiments, Sneha Sawant has drafted the manuscript. Sukhendu Ghosh and Varsha Kelkar Mane have contributed in experiment designing and manuscript editing.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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