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Effect of Gamma-irradiation on Marigold (*Tagetes erecta* l.) Cut Flowers: Changes in Sensory and Physicochemical Properties and Lutein Content

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Abstract

Marigold flowers packaged in low density polyethylene (LDPE) were subjected to gamma irradiation in the dose range of 0.02-2.5 kGy and stored in an environmental chamber (23±2°C, 80% R.H.) along with positive (non-irradiated LDPE packaged flowers) and negative control (non-packaged, non-irradiated flowers) sets for evaluation of their shelf-lives. Assays of sensory and physicochemical parameters of senescence revealed that flowers irradiated at 2.3 kGy had the highest shelf-life of 11 days, with a lead of 5 and 7 days vis-à-vis positive and negative controls, respectively. This study established for the first time the phenomenon of carotenogenesis (synthesis of 'lutein') in both irradiated and non-irradiated marigold flowers, with highest production and preservation of lutein in 2.3 kGy irradiated flowers. Thus gamma irradiation can boost the export potential of this flower, both for its ornamental and therapeutic value.

INTRODUCTION

African marigold (*Tageteserecta* L.), a native plant from Mexico (Andrea, 2008) belonging to the Asteraceae family is one of the most important commercial flower crops cultivated globally. In India too, it accounts for more than half of the total flower production (Desai et al., 2012). World trade in marigold flowers in 2011 has been estimated to be Rs. 5 billion, while India's share in the export has been to the tune of Rs. 2.2 billion (Krishnakumar, 2014). Besides ornamental appeal, marigold is also valued for its wide applications in processed foods, poultry and confectionary and has significant usage as pharmaceuticals in preventing the onset of several diseases such as cataract, age related macular degeneration and cancer (Eid et al., 2011; Alejandro and Enrique, 2008). The therapeutic efficacy of marigold flowers is principally due to the presence of its major bioactive pigment 'lutein' (Vargas and Lopez, 1996), which is a natural colorant (Halagur et al., 2013) possessing antioxidant activity (Wang et al., 2006).

Despite these medicinal properties of marigold flowers, its high perishability leads to quality deterioration and significant post-harvest loss (Raina et al., 2011; Chu et al., 2015). This necessitates implementation of effective preservation techniques for marigold flowers, for effective utilization of their ornamental and therapeutic values.

One of the conventional techniques for preserving marigold flowers is air drying; however, there is a risk of degradation of lutein by this technique (Tiwari et al., 2013). Using preservative solutions such as sugar (glucose, fructose and ribose) solutions also have their own limitations, since it necessitates disinfection of these solutions with various non-GRAS (Generally Regarded As Safe) biocides [such as hydroxyquinoline (HQ) compounds, chlorine and bromine compounds] to prevent microbial infestation of flowers (Edrisi et al., 2012). These techniques are therefore not only cumbersome, but also non-environment-friendly and relatively expensive (Emily and Sharma, 2007).

Hence, to circumvent the limitations of the above techniques for preservation of marigold flowers, we utilized a less explored, environment-friendly, non-thermal green technique of preservation, i.e., gamma irradiation (γ-irradiation). In this study, gamma irradiation has been used in combination with low density polyethylene (LDPE) packaging to enhance the shelf-life of marigold cut flowers while preserving its lutein content. The present investigation is an augmentation of our previous study on extension of shelf-life of marigold flowers by different packaging material. We found that LDPE pack (of dimensions 0.18 m × 0.25 m for 20 g cut flowers) contributed to higher shelf life of marigold flowers over other composite packaging designs (such as bags and boxes made of polyethylene terephthalate, cellophane paper and glassine paper) at 23±2ºC and 80% RH (Pal et al., 2015). These packaging materials have been characterized for their chemical and mechanical properties at the National Test House (NTH),

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Kolkata, India. These data have been provided as supplementary material as Table S1.

The objective of this investigation, therefore, was to determine the shelf-life of LDPE packaged-γ-irradiated marigold flowers by evaluation of sensory parameters of senescence. This study also aims at selection of the appropriate gamma dose which could best preserve lutein and delay senescence in marigold cut flowers.

MATERIALS AND METHODS

Raw Materials

African marigold (*Tagetes erecta* L.) flowers were procured from Barasat area, Kolkata, from a registered farmer and authenticated by West Bengal Food Processing and Horticulture Development Corporation Limited, Kolkata. The plants were cultivated in well-drained, sandy loam soil of pH 7.0-7.5 (Gupta et al., 2002) in mild climate (14.5°C-28.6°C) under high light intensity (AU, 2015). Low density polyethylene (LDPE) packs (thickness: 0.06 mm) with dimensions 0.18 m × 0.25 m were purchased from M/s Prince plastic pvt. Ltd., Barabazar, Kolkata, India. 1, 1-diphenyl-2-Picrylhydrazyl (DPPH), thiobarbituric acid (TBA), malondialdehyde and lutein (xanthophyll, X6250) were procured from Sigma, St. Louis, MO, USA. Folin Ciocalteu's reagent, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxyl toluene (BHT), ascorbic acid, 30% H_2O_2 and all the other chemicals were purchased from M/s Merck, India. 2,2,5,5-tetramethyl-4-piperidin-1-oxyl (TEMPO) was kindly donated by M/s Summit Technodyne Pvt. Ltd., Mumbai, India. All chemicals used in this work were of AR grade.

Sample preparation, irradiation treatment and storage

Marigold flowers of fixed batch size (20±1 g), optimized through preliminary trials, were packed in LDPE packages (providing sufficient free space for each flower) and sealed by a heat sealer (DELTA SEAL V2, M/s Sevana Traders and Services Pvt. Ltd., Cochin, India). The packs were subjected to γ-irradiation at different doses using ^{60}Co in a GC-5000 irradiation chamber [(Serial No. GIC 038), constructed by Board of Radiation and Isotope Technology (BRIT), Vashi, Navi Mumbai, India] in the Food Irradiation Laboratory at NIL (National Instruments Laboratory) campus of Jadavpur University, Kolkata. The dose rate certification of this gamma chamber has been conducted by BRIT in their laboratory using standard Fricke's Dosimeter and the uncertainty in the measured dose was recorded as ±5% during calibration of the GC-5000 unit. The packaged flowers were irradiated with thirteen individual doses *viz*. 0.02, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.3 and 2.5 kGy at a dose rate of 5.73 kGy/h. These irradiation treatments were conducted by keeping each sample at the geometric centre of the sample chamber in stirring/rotating mode which ensured uniform dose distribution within each sample (IAEA, 2002). The temperature of the ^{60}Co chamber during irradiation was 34±4°C. These sets of flowers were considered as 'test' (irradiated) sample sets. The second set of flowers designated as 'positive control', were similarly packed in LDPE packages but not irradiated; and the third set i.e., the 'negative control', consisted of non-packaged and non-irradiated flowers kept in a wire basket. All the three sets were then placed in a humidity chamber (M/s Prime Instruments, Ahmedabad, India) at ambient/room temperature (23±2°C), 80% R.H and allowed to senesce.

Selection of two γ-irradiation doses by sensory evaluation

Marigold flowers irradiated with 13 above mentioned doses, along with positive and negative controls sets, were subjected to sensory evaluation (by visual observation) using standard 9-point hedonic scale ratings (1-dislike extremely, 9-like extremely), by a thirty-member semi-trained panel. The panellists consisted of University faculty and research scholars in the age group of 20-45 (15 men and 15 women). They were trained to recognize and score the quality attributes of marigold flowers prior to the test. The attributes of flowers evaluated were overall appearance, color, petal texture (shrinkage), firmness and fungal infestation (adjudged visually) (Ghosh et al., 2015).

Sensory evaluation of each set of flowers was carried out from day 0 up to the end of their corresponding shelf-lives at an interval of two days. Based on these sensory scores, two doses of irradiation were shortlisted which conferred high shelf lives (days 11 and days 8) to the LDPE packed flowers. This study was repeated thrice and the same result was obtained in each trial. Subsequently, to ascertain the appropriate dose of irradiation between the selected doses, the two irradiated flower sets along with positive and negative control sets were subjected to assays of physicochemical parameters of senescence and for estimation of their lutein content. The remaining other irradiated flower sets were sensorically rejected and therefore not considered for further analyses.

Selection of the best γ-irradiation dose by physicochemical assays and lutein content

20±1 g of fresh marigold flowers were packaged in LDPE and subjected to γ-irradiation at the two selected doses and stored at 23±2°C, 80% R.H, in an environmental chamber, along with the positive and negative control sets. This time all the four sets (2.3 kGy irradiated, 2.5 kGy irradiated, positive control and negative control) of flowers were withdrawn at an interval of three days up to day 8, i.e., on days 0, 4, 8 and finally on day 11 (the end of shelf-life of flowers having highest sensory scores) to perform the physicochemical assays. All assays were performed by utilizing either petals *per se* or petal extracts (as per respective methodologies). Lutein content alone was determined on days 0, 3, 6, 9 and 12 (which includes the days after end of shelf life of irradiated marigold flowers. These days were included to understand the effect of storage period on this major bioactive pigment of marigold). The assays conducted were as follows-

Leakage of ions from the flower petals

Leakage of ions from the petals of both irradiated as well as control setswere determined w.r.t. storage, using an electrical PC 510 conductivity meter (M/s Eutech Instruments, Malaysia) in accordance with the method reported by Chakrabarty et al. (2009) and was expressed as %MSI.

Moisture content of flower petals

Moisture content was determined with 0.1 g sample of raw petals using an IR moisture analyzer (Citizen MB 200 C) at 90°C until constant weight (Latorre et al., 2010).

Hydrogen peroxide (H_2O_2) content of flower petals

Hydrogen peroxide content of the petals was measured spectrophotometrically (at 317 nm, obtained by λ_{max}scanning) in accordance to the method reported by Chakrabarty et al. (2009). The amount of H_2O_2 was determined from a standard curve prepared with known concentration of 30% H_2O_2.

Measurement of lipid peroxidation in flower petals

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by thiobarbituric acid (TBA) reaction. The procedure of Ghosh and Bhattacharjee (2013) was adopted with few modifications. The petals (5 g) of marigold flowers were homogenized in 15 ml of 0.2 M sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 3500×g for 20 min and the supernatant was analyzed. To 0.5 ml of this sample solution, 1 ml TCA (10%), 1 ml TBA (0.1 M), and minimum quantity of BHT were added and vortexed. The mixture was then heated in a boiling water bath for 30 min. The samples were centrifuged at 1000×g for 15 min and the absorbance was read at 535 nm in an UV-Vis spectrophotometer (U-2000, M/s Hitachi, Tokyo, Japan). The amount of malondialdehyde formed was determined from its standard curve. The malondialdehyde content was expressed as mmolemalondialdehyde/100 g fresh weight (F.W) of petal.

Ascorbate peroxidase (APX) activity of flower petals

Preparation of enzyme extract from petals.

Enzyme supernatant was prepared according to the procedure reported by Prochazkova et al. (2001).

Determination of APX activity.

The activity of APX (EC 1.11.1.11) enzyme was determined in accordance with the method described by Prochazkova et al. (2001). The enzyme activity was expressed in enzyme unit/mg protein. [One unit of enzyme was the amount necessary to decompose 1 µmol of ascorbate per minute at room temperature].

Guaiacol peroxidase (GPOD) activity of flower petals

Preparation of enzyme extract from petals. The enzyme extract was prepared in accordance with the procedure described by Sedghi et al. (2012), with few modifications. 5 g of petals were homogenized in a pre-chilled mortar-pestle with 15 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mMdithiothreitol and 2% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000×g for 20 min and the supernatant, i.e., the enzyme extract was subjected to GPOD assay.

Determination of GPOD activity. The activity of GPOD (EC 1.11.1.7) was determined by the method described by Sedghi et al. (2012). [GPOD activity has been expressed as nmols of guaiacol oxidized to tetraguaiacol by one unit of enzyme per min].

Protein content of flower petals

Protein content of the petals was determined using Lowry's method (Lowry et al., 1951). Bovine serum albumin (BSA, Sigma) was used as standard and a standard curve of the same was prepared.

TPC and DPPH radical scavenging activity of flower petals

Preparation of petal extract by solvent extraction method.

The extracts were prepared in accordance with method described by Li et al. (2007) with few modifications. 3-5 g of petals were homogenized in ethanol (petal to solvent ratio = 1:10) in a mortar-pestle and subjected to shake-flask extraction (at 110 rpm) in an incubator-shaker (M/s Incon, India) overnight (i.e., 12 h) at 23±2ºC. The homogenate was then filtered through a Whatman no 1 filter paper. The filtrate was collected and evaporated to dryness in a rotary vacuum evaporator (Rotavac system M/s Eyela Corp., Japan) under reduced pressure (50 mbar Hg) at 40°C and the extracts obtained were stored in amber-colored screw capped glass vials at 4°C for further analyses.

Determination of TPC. TPC was determined spectrophotometrically by Folin-Ciocalteu colorimetric method as described by Spanos and Wrolstad (1990). A standard curve of gallic acid was used to calculate the TPC in the extracts, which was expressed as gallic acid equivalent i.e., mg GAE/100 g F.W. of petals.

Determination of DPPH radical scavenging activity. The antioxidant activity of the petal extract was determined by measuring the radical scavenging activity of DPPH (Aiyegoro and Okoh, 2010) and expressed as IC_{50} values (mg/mL).

Lutein content of flower petals

Preparation of lutein rich extracts (solvent extract).

5 g dry flowers were subjected to shake flask extraction (at 110 rpm) in an incubator-shaker (M/s Incon, India) using dichloromethane (DCM, b.p. 39.6°C) as solvent (petal:solvent = 1:10) at 23±2ºC overnight (i.e., 12 h). The petal-solvent mixture was then cooled to room temperature and centrifuged at 3000×gfor 15 min. The supernatants were collected and evaporated to dryness in a rotary vacuum evaporator under reduced pressure (50 mbar Hg) at 30°C, and the residues (extracts) obtained were stored in nitrogen flushed screw-capped amber-colored glass vials at 4°C. For analysis, the extracts were dissolved in DCM. The DCM extracts of treated (LDPE packaged-non irradiated; 2.3 kGy irradiated-LDPE packaged; 2.5 kGy irradiated-LDPE packaged) and untreated flowers were then saponified (discussed later) to obtain 'free lutein'.

Saponification of extracts to obtain free lutein.

1 g of marigold flower extract was added to alcoholic KOH (0.6 g of KOH dissolved in 10 ml of ethanol) in a flask. The flask was then allowed to shake at 150 rpm and 50°C for 4 h. 50 ml ethanol was added into this saponified mixture, and the entire volume was transferred to a separatory funnel. To this, 100 ml of 5% Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were added. All components were mixed well and then allowed to separate into two phases.

The upper phase (ether fraction) was collected as solution of free lutein, while the lower phase was drained off (Boonnoun et al., 2012). For complete recovery of lutein, the extraction process was repeated until the water phase was colorless. The ether fraction was then evaporated to dryness under vacuum to obtain the extract, weighed and successively dissolved in minimum quantity of DCM. This constituted the stock solution of 'free lutein'.

Quantification of lutein by high performance liquid chromatography (HPLC) analyses.

Lutein content of the extracts was analyzed by HPLC, in accordance with the method reported by Bhattacharyya et al. (2010) with slight modifications. 20 µL of the stock solution of 'free lutein' was filtered through a 0.45 µm syringe filter and the filtrate was injected into JASCO HPLC (LC-Net-2/ADC, PU-2080 Plus HPLC pump, DG-2080-54 degasser, MD-2015 Plus detector) system. A C18 reversed phase column (l = 250 mm and i.d = 4.6 mm) was used for the separation. The mobile phase was acetonitrile: methanol: ethyl acetate (9:1:2) and its flow rate was maintained at 1 mL/min. The eluents were continuously monitored in a PDA detector at 447 nm (i.e., at λ_{max} of lutein). Peak identification was based on the retention time of standard lutein (M/s Sigma, St. Louis, MO, USA) (Figure 11).

Determination of TEMPO radical scavenging activity through Electron Paramagnetic Resonance (EPR) spectroscopy

Antioxidant activity was determined for the lutein-rich extract of the 2.3 kGy irradiated set and the negative control set on day 9 of storage, since the lutein content was found to be the maximum on that day (discussed earlier). 100 µl (1 mg/mL) test

sample was added to 100 µL of TEMPO (100 mM) solution. After shaking vigorously for 10 s, the solution was transferred into a capillary quartz tube and inserted into the microwave cavity of the EPR spectrometer (M/s Magnet Tech MS400 GMBH, Germany). The EPR spectrum was recorded after 40 s. The conditions of the EPR spectrometer were as follows: room temperature, power: 1 mW, magnetic field: 336.0±2 mT, field modulation width: 0.5 mT, sweep time: 30 s and time constant: 0.03 s. The radical scavenging activity was calculated by the following equation:

$$\%\text{TEMPO scavenging} = [(I_0 - I)/I_0] \times 100 \quad (1)$$

where: I_0 =intensity of TEMPO signal and I = integral intensity of the TEMPO signal after addition of the extract (Wasek et al., 2001).

Statistical analysis

All physicochemical assays were performed for three independent experimental sets. Duncan's multiple range tests were carried out to analyze the effects of different treatments (2.3 kGy irradiation + packaging, 2.5 kGy irradiation + packaging, packaging without irradiation) and storage time, on the above-mentioned physicochemical and phytochemical parameters of senescence. The differences among the mean values were determined at a confidence level of $P < 0.01$ in IBM SPSS Statistics Software, Version 20 (IBM, USA).

RESULTS AND DISCUSSION

Sensory evaluation of flowers irradiated with thirteen different γ doses

The 9-point hedonic scale scores of the irradiated and non-irradiated marigold flowers are presented in Figure 1 and the shelf-lives of these six set of flowers are presented in Table 1. From Figure 1, it can be seen that the negative control set always scored less w.r.t. the treated samples and among the treated samples, no significant changes were observed among the positive control set and the 0.02-0.8 kGy irradiated flower sets. However, a clear distinction in all the sensory parameters was observed among the positive control set and those irradiated at medium dose i.e., 1.0-2.0 kGy, 2.3 kGy and 2.5 kGy. Positive control set always obtained lower scores than flowers irradiated at medium dose (1.0-2.5 kGy), during both early (day 0–day 4) and late (day 4 onwards up to day 11) senescence. Therefore, we concluded that low dose irradiation does not have any significant effect on the positive control set. However, medium dose γ-irradiation was found to contribute significantly ($P < 0.01$) in extending the shelf-lives of LDPE packaged flowers (Table 1). Among the medium doses, flowers irradiated at 2.3 and 2.5 kGy scored higher than those irradiated at 1.0-2.0 kGy.

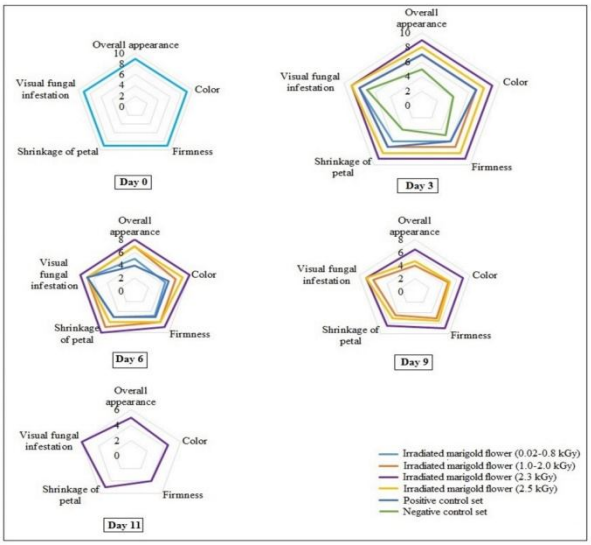
Therefore, 2.3 and 2.5 kGy irradiated flowers along with the positive and negative control sets were subjected to further analyses of senescence parameters (physicochemical assays) along with periodic evaluation of lutein content (by HPLC).

Table 1: Shelf life of treated and untreated marigold flowers

Irradiation doses (kGy)	Shelf-life (Days)[c]
0.02-0.8	6
1.0-2.0	7
2.3	11
2.5	8
Positive control[a]	6
Negative control[b]	4

** [a]LDPE packaged non-irradiated flowers; [b]non-packaged, non-irradiated flowers [c]Evaluation of shelf-lives of these set of flowers was conducted by sensory, physicochemical and phytochemical analyses. These assays were repeated thrice and each replication gave similar results.

Figure 1: Radar plot of hedonic scores of sensory evaluation of marigold flowers. The single line of day '0' represents both irradiated as well as non-irradiated flowers, since all flowers scored the same on day '0'.



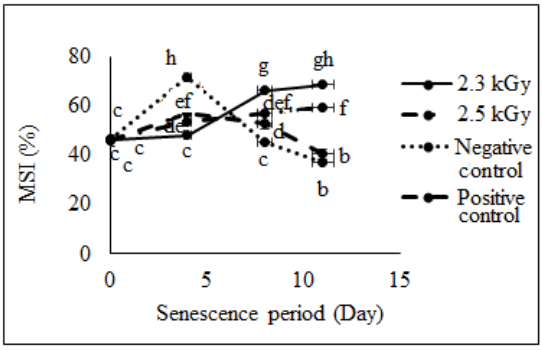
Physicochemical assays of senescence

Leakage of ions

Membrane stability was found to increase significantly (P<0.01) during early senescence (up to day 4) in both treated and untreated marigold flowers; however, during late senescence (day 4 onwards) stability, decreased significantly (P<0.01) in both positive and negative control sets, indicating a gradual loss in membrane integrity of the flowers with time. On the contrary, irradiated flowers continued to show a positive increase in membrane stability during late senescence. The positive control set showed significantly higher %MSI (81.89 on day 8 and 83.5 on day 11) than that of the negative control (%MSI: 63.1 on day 8 and 41.32 on day 11).

Highest % MSI was observed in flowers irradiated at 2.3 kGy among all treated flowers (Figure 2). These findings attest 2.3 kGy dose to be the best dose to maintain optimum membrane integrity during senescence. These results clearly indicate the positive impact of medium dose γ-irradiation, even at the later stage of senescence. These results are in agreement with Grover and Khan (2014) who had also observed a higher % MSI in flag leaves, obtained from 1, 0.15, 0.155, 0.165 and 0.17 kGy irradiated wheat seeds, compared to their non-irradiated counterparts.

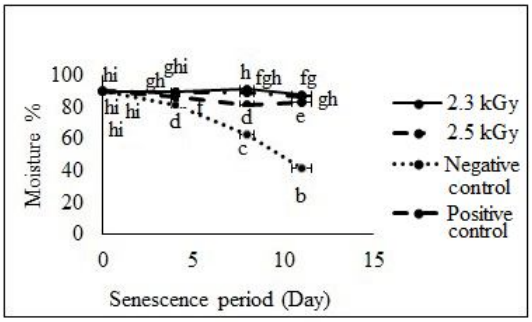
Figure 2:%MSI changes in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at P< 0.01



Moisture content

A significant difference (P< 0.01) in %moisture among all treated and negative control sets was observed from day 4 onwards, up to day 11 (Figure 3) i.e., during late senescence periods. The loss in %moisture was the highest in case of negative control followed by positive control and irradiated LDPE packaged flowers respectively. Hence, it clearly indicates that retention of moisture is significantly higher (P< 0.01) in treated flowers, attesting the superiority of the combined effect of irradiation and packaging, over packaging alone. Between 2.3 and 2.5 kGy irradiated flowers, the former dose was found to be more effective in preventing moisture loss in marigold flowers.

Figure 3:Changes in %moisture of 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at P< 0.01



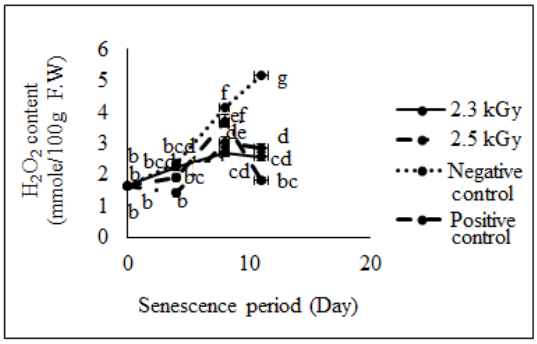
Similar trends in reduction in moisture loss in irradiated mushrooms have been reported by Thomas and Diehl (1988). The high moisture barrier properties of LDPE (water vapor permeability of 0.06 mg/cm^2/24 h/mm, Table S1), could have reduced moisture loss of flowers. This finding was in agreement with Lin and Zhao (2007), who had stated the positive impact of edible films of high moisture barrier properties in preventing moisture loss of fresh and minimally processed fruits and vegetables. Therefore, it could be concluded that both packaging and γ-irradiation at appropriate dose can help in preventing moisture loss, thereby reducing early drying of the marigold.

Hydrogen peroxide (H_2O_2) content

The effect of increasing storage period on formation of H_2O_2 in both irradiated and non-irradiated flowers in this present study, suggest a gradual increase in the formation of H_2O_2, with progression of senescence (Figure 4). On day 4 of storage, it can be seen that there was negligible difference in H_2O_2 content among the irradiated, non-irradiated positive control and untreated negative control sets (Figure 4). However with the onset of late senescence, a significantly higher (P< 0.01) H_2O_2 content was observed in the negative

control set compared to to those obtained for 2.3 and 2.5 kGy irradiated flowers (Figure 4).

Figure 4: Changes in hydrogen peroxide content in the petals of 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.

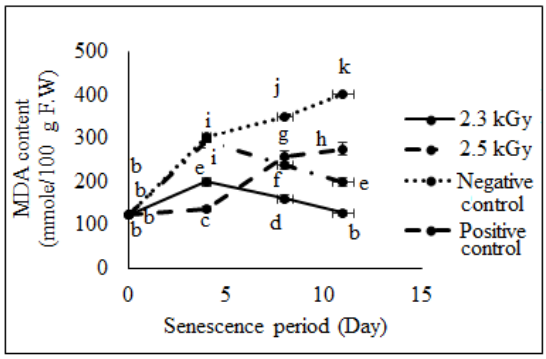


Although negligible difference was found between the peroxide content of 2.3 kGy and 2.5 kGy irradiated flower sets, during early senescence, a considerable less peroxide was formed in 2.3 kGy irradiated flowers than in 2.5 kGy irradiated set during late senescence. This established 2.3 kGy to be the best dose in reducing oxidative stress, which occur due to the overproduction of reactive oxygen species (ROS) and lead to oxidative damage of cellular components (Sreedhar et al., 2013). This incidence of accumulation of minimum H_2O_2 in the treated flowers might be attributed to their enhanced peroxidase (POD) activity (discussed later), induced by γ-irradiation (which reduces H_2O_2 into water and oxygen) thus preventing formation of excess peroxide radicals in cells. Similar observation has been reported by Jan et al. (2012), who also found that the accumulation of H_2O_2 in irradiated pumpkin leaves and petioles was concomitant with POD activity, where H_2O_2 content was reduced in the cells due to enhanced activity of this enzyme.

Lipid peroxidation

During early senescence (up to day 4), MDA (indicator of lipid peroxidation) content was found to increase in both treated flowers and in the negative control set. This gradual increase in lipid degradation continued till late senescence for positive and negative control sets. This enhanced lipid peroxidation could possibly be attributed to the gradual increase in H_2O_2, consequent to oxidative stress leading to senescence (discussed earlier) of the flowers. However, a significant decrease in MDA content was observed in irradiated flowers from day 4 onwards upto day 11 (Figure 5). Flowers irradiated at 2.3 kGy had least MDA content compared to the rest, confirming the effect of γ-irradiation in delaying senescence. These results were in agreement with Moussa (2011), who reported that low dose γ-irradiation (20 Gy) decreased MDA content in drought stressed soybean by 13%, compared to its non-irradiated counterpart.

Figure 5:Changes in lipid peroxidation in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.



APX activity

To overcome oxidative damage, plants have evolved enzymes such as peroxidase which contribute to the inhibition of ROS (Sreedhar et al., 2013; Hamad et al., 2012). This may explain the significant increase (*P*< 0.01) in APX activity in irradiated (2.3 kGy) flowers during storage (up to day 8 in this study), to avert oxidation (Figure 6). However, in case of 2.5 kGy irradiated flowers, no significant increase in activity was found (Figure 6) up to day 8, after which the activity started to decline. This data showed that 2.5 kGy affected the synthesis of APX to a greater extent compared to 2.3 kGy. Positive control showed increased APX activity only during early senescence (i.e., until day 4), but not in late senescence. This possibly indicates the superiority of the combined treatment of packaging and γ-irradiation (at 2.3 kGy) in enhancing APX activity to a greater extent to combat oxidative stress in the flowers. Similar results also have been reported by Wi et al. (2007), who suggested that the activities of scavenging enzymes including APX are generally increased in various plant species by γ-irradiation at appropriate doses. In his study, the significant increase in APX activity in irradiated flowers therefore, shows the influence of γ-irradiation at appropriate dose in

limiting oxidative damage in marigold flowers. In addition, the phenomenon of enhanced APX activity during early senescence and reduced activity during late senescence are also in agreement with the findings of Prochazkova et al. (2001), who also obtained similar results for maize leaves during senescence.

Figure 6:Changes in Ascorbate peroxidase (APX) activity in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control ssets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.

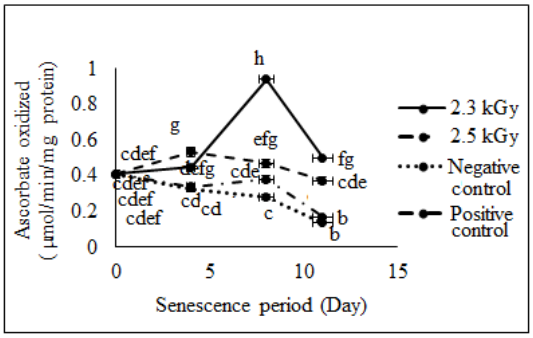
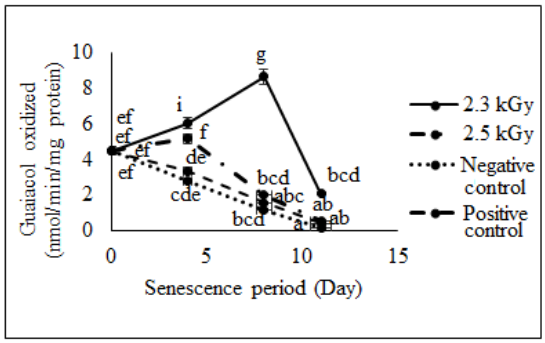


Figure 7:Changes in Guaiacol Peroxidase (GPOD) activity in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01



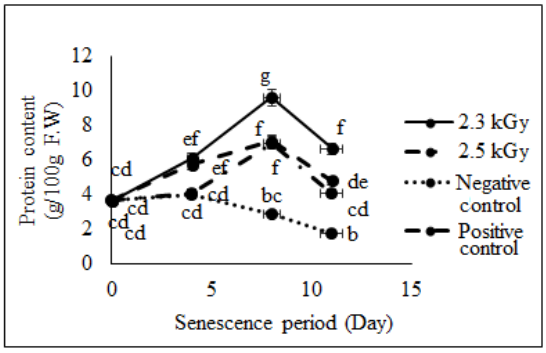
GPOD activity

This assay was meant to exhibit the irradiation induced changes in the activity of guaiacol peroxidase activity w.r.t. increasing storage time. From Figure 7, it could be easily understood that the activity is always higher in irradiated flowers than non-irradiated control sets during the storage period, particularly during late senescence. On day 8, GPOD activity of 2.3 and 2.5 kGy irradiated flowers was 86.5% and 42.07% higher than the negative control; and 81% and 21.78% higher than positive control, respectively. Flowers irradiated at 2.3 kGy showed significantly (*P*< 0.01) higher GPOD activity during both early and late senescence, (day 4-day 11) affirming its effectiveness.

Protein content

Protein content increased during early senescence (up to day 4) in both treated as well as in untreated flowers. The increase in treated flowers is continued even during late senescence (up to day 8), and was followed by a significant decline on day 11 (Figure 8). However, in case of negative control set the protein content started to reduce from day 8 up to day 11. Therefore, it can be inferred that LDPE packaging and γ-irradiation (2.3 kGy) in tandem were able to retain more protein in marigold flowers than in untreated flowers, during both early and late senescence. Among the treated flowers, protein content was found to be significantly (*P*< 0.01) greater in 2.3 kGy irradiated flowers, which further affirms the superiority of 2.3 kGy dose. These results could be attributed to the significantly (*P*< 0.01) high APX and GPOD activities in flowers irradiated at 2.3 kGy (discussed earlier). Since these enzymes scavenge H_2O_2, the oxidation of proteins and lipids in plant cells is limited (Wi et al., 2007). The synthesis and degradation of cellular proteins during early and late senescence is in agreement with Wollaston (1997) who have reported similar findings for leaf senescence.

Figure 8:Changes in protein content in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.

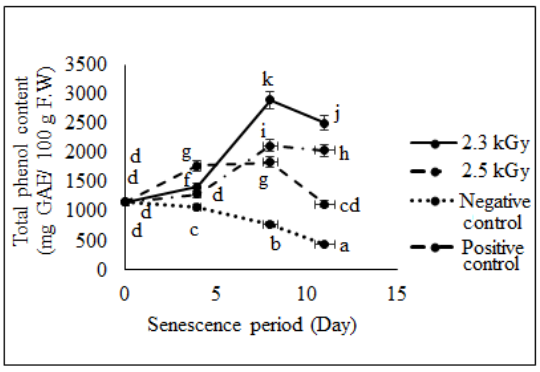


TPC

Total phenol content of the irradiated flower sets showed an increase during both early and late senescence (upto day 8), followed by a gradual decrease on day 11. However a steady reduction in TPC was found in negative control set throughout the storage period. This phenomenon of accumulation and reduction of phenolic compounds in irradiated plants could be attributed to the alterations in plant cellular structure and metabolism during early senescence and oxidative degradation of the same during late senescence as has been reported by Wi et al. (2007).

Moreover, it has been seen that, TPC of positive control set was higher than irradiated flowers during early senescence; however, there was a significant reduction ($P< 0.01$) of it in the former during late senescence, compared to the irradiated flowers (Figure 9).

Figure 9: Changes in TPC in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.

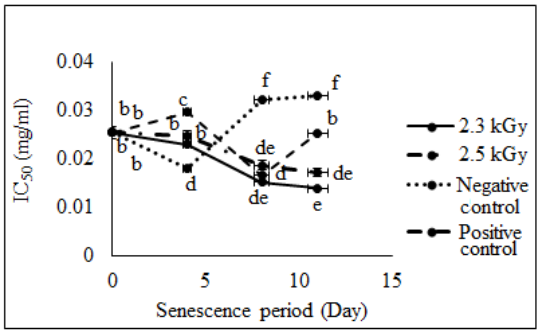


We also observed that at the end of the storage period i.e., on day 11, flowers irradiated at 2.3 kGy had highest TPC (2500 mg GAE/100 g F.W.) followed by those at 2.5 kGy (2033.64 mg GAE/100 g F.W.), positive control (1120.26 mg GAE/100 g F.W.) and negative control (445.31 mg GAE/100 g F.W.). Hence, it is clear from our study that, irradiation at 2.3 kGy in combination with LDPE packaging causes greatest retention of total phenol during stress, hence this dose, was considered to be the best dose for marigold cut flowers.

DPPH radical scavenging activity

The antioxidant activities of flowers irradiated at 2.3 kGy and 2.5 kGy showed an increased with storage. However, the positive control set did not follow a consistent trend. The negative control set showed least antioxidant activity among all sets, during late senescence (on day 8 and 11) (Figure 10). This increase in antioxidant activity of the irradiated flowers could be attributed to the enhanced TPC of the irradiated samples.

Figure 10: Changes in DPPH antioxidant activity in terms of IC_{50} value in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at *P*< 0.01.



Mali et al. (2011) also obtained similar results with pomegranate peels, wherein they have found significant increases in total phenolics and antioxidant activity of 10.0 kGy irradiated peels compared to non-irradiated peels, even after 60 days of irradiation. In our study, 2.3 kGy irradiated flowers showed the maximum antioxidant potency (IC_{50} value of 0.013 mg/mL) even on the last day of storage (day 11), which is significantly higher ($P< 0.01$) than those of flowers irradiated at 2.5 kGy, and the positive and negative control sets. Thus, the antioxidants of marigold flowers were best retained with storage in flowers irradiated at 2.3 kGy.

Lutein content

Figure 11 represents the chromatogram of lutein rich extract (solvent extract) and shows the peak of lutein at around 5.5 min. During storage, lutein content increased. Lutein content increased in the treated flowers (2.3, 2.5 kGy irradiated and positive control) up to day 9 and in negative control set, up to day 6. However, it started to reduce thereafter i.e., on day 12 in case of treated flowers and on days 9 and 12 in case of the negative control set (Figure 12). This observation could be attributed to

carotenogenesis in γ-irradiated marigold flowers. Carotenogenesis is reported for the first time in marigold flowers with storage in this work. Moreover, the amount of lutein preserved is significantly higher ($P< 0.01$) in case of treated flowers compared to that in the negative control set, throughout the aging process.

Figure 11: HPLC chromatogram of lutein rich extract (solvent extraction) of marigold flower

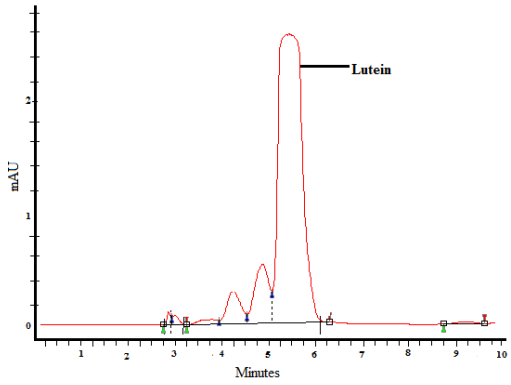
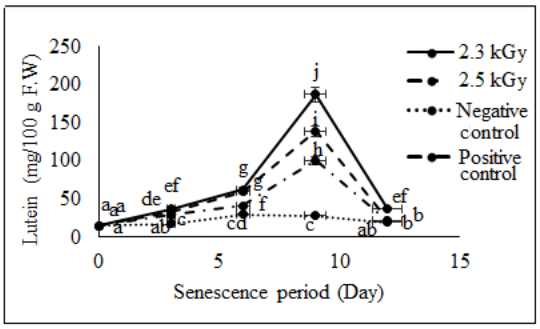


Figure 12: Changes in lutein content in 2.3 kGy, 2.5 kGy irradiated flowers, positive control and negative control sets during different stages of senescence. Each value represents the mean ± SD of three independent experimental data of three batches of marigold flowers. Dissimilar alphabets indicate that the mean values belong to different subsets at $P< 0.01$.

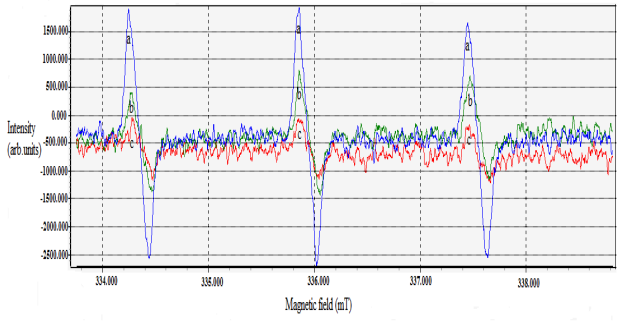


This might be attributed to the preservation efficacies of packaging and γ-irradiation technologies. Similar report on accumulation of lutein was reported in winter squash fruit when stored up to two months at room temperature, which has been attributed to continuous biosynthesis and reduced loss of carotenoids (Zhang et al., 2014). In this study too, among the three treated flowers, 2.3 kGy irradiated flowers was found to possess highest amount (187.27 mg/100 g F.W.) of lutein on day 9 of storage, compared to 2.5 kGy irradiated and positive control sets. Further, on day 12 of storage, when lutein content was significantly ($P< 0.01$) reduced in all flowers, 2.3 kGy irradiated flowers still retained the maximum amount of lutein. Hence, 2.3 kGy was considered to be the best dose of irradiation for preserving lutein in marigold flowers.

%TEMPO scavenging through EPR spectroscopy

The reduction in the intensity of the TEMPO (blank) signal, indicated as 'a' (Figure 13), is due to the radical scavenging activities of the extracts of irradiated as well as non-irradiated flowers. Signal 'b' of negative control, indicates a significant reduction ($P< 0.01$) in peak intensity, compared to that of signal 'a' and signal 'c' of irradiated flowers showed further reduction in the peak intensity when compared to that of signal 'b'. Similar report of reduction in the peak intensity was observed in 235U irradiated $Bi_{12}GeO_{20}$ sample w.r.t its non-irradiated counterpart, when analyzed by EPR spectroscopy (Stefaniuk et al., 2010). From our results, therefore, it can be concluded that 2.3 kGy irradiated flowers were found to be more effective radical scavenger compared to negative control sets, since TEMPO signal almost disappeared in case of the former (Figure 13). For the 2.3 kGy irradiated flowers, the TEMPO radical scavenging activity was found to be 78.66%, while for negative control sets it was 45.12%, indicating 1.74 times higher antioxidant potency of the irradiated flowers. This higher antioxidant potency could possibly be attributed to the greater amount of lutein present in the irradiated flowers, compared to control sets. These findings also strongly indicate that γ-irradiation at appropriate dose could be a feasible technique of preserving phytochemical potency of marigold flowers and possibly of other floriculture products.

Figure 13: EPR spectrum of TEMPO radical, a) blank solution, b) upon addition of the extract of negative control set, c) upon addition of the extract of 2.3 kGy irradiated marigold flowers. Dissimilar alphabets indicate that the values belong to different subsets at $P< 0.01$



CONCLUSION

Sensory and physicochemical analyses of senescence parameters along with lutein content of

LDPE-packaged-γ-irradiated marigold flowers attests 2.3 kGy dose to be the best dose of gamma. The flowers at this dose were found to be most stress (oxidative) tolerant and exhibited highest shelf-life of 11 days, a lead of 5 days and 7 days vis-à-vis positive and negative control sets, respectively. Moreover, the phenomenon of carotenogenesis was also triggered by gamma irradiation since the major bioactive pigment of marigold; 'lutein' was synthesized at the highest level and also best preserved at 2.3 kGy. This study established that the combination of LDPE packaging with γ-irradiation could be effectively used as an alternative technology for preservation of marigold flowers and synthesis of lutein. Utilization of these irradiated flowers as a potential source of lutein for design of lutein-fortified novel nutraceutical foods is underway. We envisage that these findings could boost the export potential of marigold flowers world-wide and also widen the scope of utilization of this ornamental as a source of nutraceutical.

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