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Nanochitosan-encapsulated melatonin: an eco-friendly strategy to delay petal senescence in cut gerbera flowers

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Abstract

Background The preservation of cut flowers, particularly *Gerbera jamesonii*, is crucial for maintaining their aesthetic value and extending vase life in the floriculture industry. To address this challenge, this study investigated the effects of melatonin (Mel) and encapsulated melatonin with nanochitosan (nCS-Mel) as preservative solutions on cut *Gerbera jamesonii* cv. 'Terra kalina' flowers. In research, we examined various physiological and biochemical parameters, including relative water content, membrane stability index, carbohydrate content, and antioxidant enzyme activities, to evaluate the efficacy of these treatments in prolonging the vase life and quality of cut gerbera flowers under controlled environmental conditions.

Results Our results demonstrated that cut *Gerbera jamesonii* flowers maintained in vase solutions containing 0.1 and 0.5 mM nCS-Mel exhibited enhanced preservation of cell membrane integrity and anthocyanin content, while also maintaining higher levels of carbohydrates and total flavonoids in petals at the conclusion of their vase life. A decline in petal relative water content and protein levels was observed concomitantly with petal senescence, whereas total phenolic compounds showed an increase. The hydrogen peroxide (H_2O_2) content in petals exhibited an upward trend during vase life in control specimens, but this effect was mitigated in treatments containing melatonin. Although malondialdehyde (MDA) content generally increased throughout the vase life period, flowers subjected to either Mel or nCS-Mel treatments displayed reduced MDA accumulation. The activity of catalase (CAT) demonstrated an increasing trend during vase life, with the maximum activity observed in Gerbera flowers treated with 0.1 mM nCS-Mel. A similar upward trend was noted for superoxide dismutase (SOD) activity, with flowers in 0.5 mM nCS-Mel treatment exhibiting peak SOD values on day 12 relative to control and other treatments. Peroxidase (POD) activity also increased across all treatments, with particularly pronounced effects in vase solutions containing 0.1 mM Mel and nCS-Mel. Notably, flowers placed in vase solutions containing 0.1 mM nCS-Mel, followed by 0.5 mM nCS-Mel and 0.1 mM Mel, exhibited the most prolonged vase life, extending up to 12, 10.66, and 10.33 days, respectively, under room temperature conditions.

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Conclusions The application of nanoencapsulated melatonin as a vase solution for cut *Gerbera jamesonii* flowers demonstrates significant potential in extending vase life and maintaining flower quality through enhanced preservation of cellular integrity, antioxidant activity, and biochemical parameters. This innovative approach not only outperforms conventional treatments but also presents a more environmentally friendly alternative to traditional antimicrobial preservatives and sugars, offering a promising solution for the floriculture industry to improve cut flower longevity and reduce ecological impact.

Keywords Antimicrobial properties, Petal water balance, Preservative solution, Vase life, Encapsulated melatonin

Background

Gerbera jamesonii L., a member of the Asteraceae family indigenous to South Africa's Transvaal region, holds significant economic importance as the fifth most valuable cut flower globally, following rose, chrysanthemum, tulip, and lilium [1]. Its prominence in the floriculture industry is attributed to its aesthetic appeal, diverse color palette, high yield potential, and short harvest intervals [2]. Despite their popularity among consumers, gerbera cut flowers exhibit a relatively brief vase life of 5–8 days at ambient temperatures post-harvest [3], primarily due to stem bending and petal wilting, which compromise their post-harvest quality and longevity [4]. Especially, vase life is a critical determinant of the commercial value of cut flowers. While visual attributes such as appearance, form, and color influence initial consumer decisions, vase life is the principal factor driving repeat purchases [5]. Petal senescence is characterized by a reduction in protein content, accompanied by the degradation of other macromolecules including nucleic acids and lipids. Conversely, the accumulation of proline serves as an osmolyte, offering protection to enzymes and macromolecules against senescence and environmental stressors [6]. Consequently, treatments aimed at maintaining petal water content play a crucial role in mitigating senescence. The primary cause of vascular obstruction in cut flowers is microbial activity, including bacteria, yeasts, and fungi, which proliferate in vase solutions utilized throughout the supply chain [7]. These microorganisms can induce vascular blockage in cut stems, release toxic metabolites or deleterious enzymes, enhance ethylene production, and trigger programmed cell death [8]. Furthermore, microbial growth in vase solutions leads to decreased hydraulic conductivity in flower stems. Thus, antimicrobial treatments are commonly employed to extend the vase life of cut flowers [9].

To prolong the post-harvest longevity and quality of cut flowers, various chemical compounds are introduced into vase solutions. These additives encompass carbohydrates, germicides, anti-ethylene agents, pH regulators, and growth regulators. Among these, melatonin has recently garnered attention for its preservative properties, sharing biosynthetic pathways and physiological actions with the auxin IAA [10]. Melatonin functions as both an antioxidant and a plant growth regulator [11], exhibiting structural similarities to auxin and demonstrating parallel growth-promoting effects [11]. Its antioxidant capacity mitigates free radical accumulation, while its ability to enhance water relations and maintain plant pigment stability contributes to delayed senescence and extended vase life in cut flowers [12]. Additionally, melatonin has been shown to reduce chilling injury in Anthurium cut flowers [13], potentially through the neutralization of excess reactive oxygen species, thereby preserving chlorophyll and delaying leaf senescence [12]. Chitosan, renowned for its broad-spectrum antimicrobial properties against bacteria, viruses, and fungi [14], enhances plant resistance to environmental stressors by activating enzymes such as phytotoxins and chitinases. Its capacity to scavenge hydroxyl and superoxide radicals provides protection to DNA under adverse conditions [15]. Among various polymers employed in nanoparticle formulations, chitosan has gained prominence due to its biodegradability, biocompatibility, FDA approval for food and pharmaceutical applications, non-toxicity, sustained release capabilities, potential for surface modification, and targeted delivery to specific organs or cells [14]. The antimicrobial efficacy of chitosan is attributed to the presence of positively charged amine groups at C2, which interact with negatively charged microbial cell membranes, leading to cellular content leakage [16]. The antimicrobial properties of chitosan render it an effective component in preservative solutions for cut flowers, as demonstrated in carnations [17], roses [18, 19], and orchids [20]. Moreover, nanoencapsulation with chitosan not only enhances absorption but also improves the stability of encapsulated compounds against environmental and physicochemical factors [21]. Encapsulation, a process of containing solid, liquid, or gaseous components within small capsules, allows for controlled release under specific conditions [21]. The encapsulation of materials with biopolymers such as chitosan [19] enhances their biodistribution and bioavailability, consequently extending product shelf life.

In light of the advantages listed above, the primary objectives of this study were to investigate the efficacy of melatonin and nano chitosan-encapsulated melatonin (nCS-Mel) in prolonging the vase life of cut gerbera flowers by (i) assessing their impact on physiological and biochemical parameters associated with petal senescence,

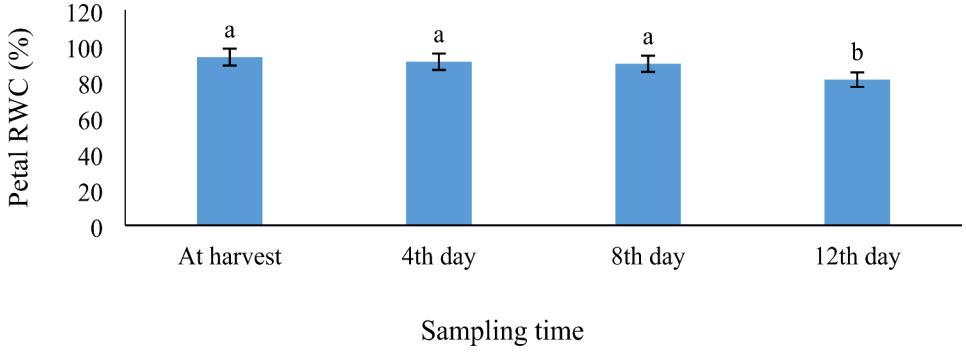

Fig. 1 Effect of different sampling times on gerbera petal's RWC. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

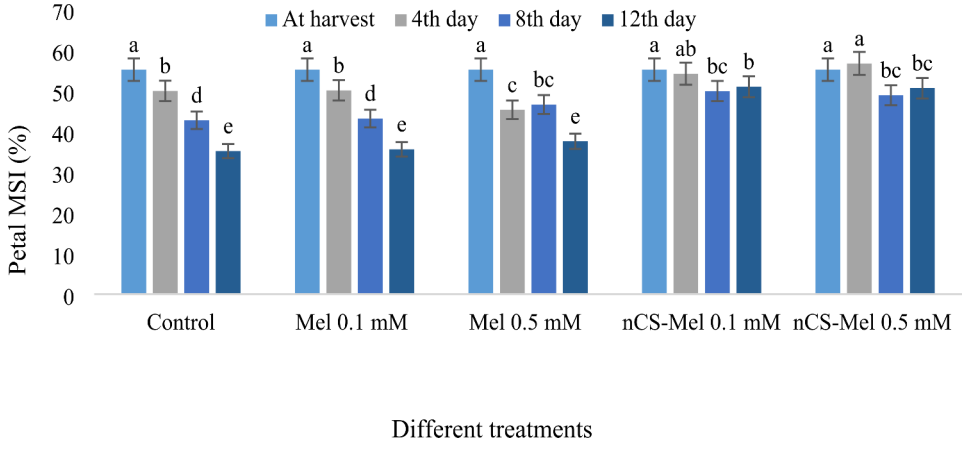

Fig. 2 Effect of different treatments on gerbera petal's MSI during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

(ii) evaluating their capacity to maintain petal water balance, and (iii) exploring their potential as an environmentally sustainable alternative to conventional antimicrobial preservatives and sugars in the floriculture industry.

Results and discussion

The experimental results depicted in Fig. 1 reveal a notable decline in petal Relative Water Content (RWC) of *Gerbera jamesonii* flowers by day 12, with a reduction of up to 13% compared to control specimens. Interestingly, the various treatments applied did not yield statistically significant differences in petal RWC, despite previous research demonstrating melatonin's capacity to reduce osmotic potential and enhance water absorption [22]. RWC, a key indicator of tissue hydration status, exhibited peak values at harvest and subsequently decreased across all treatments throughout the vase life period. This gradual decline in RWC can be attributed to the natural progression of senescence, during which cellular membranes lose integrity, leading to reduced water retention capacity. The lack of significant differences among treatments suggests that factors beyond osmotic regulation, such as vascular occlusion or changes in aquaporin activity, may play a more dominant role in determining water status in cut Gerbera flowers. In this regard, present findings show the complex interplay of physiological processes involved in maintaining water balance during the post-harvest life of cut flowers and highlight the need for further investigation into the mechanisms by which preservative treatments influence water relations in floral tissues.

Our experimental data presented in Fig. 2 demonstrate a significant reduction in petal electrolyte leakage for *Gerbera jamesonii* flowers treated with nCS-Mel, indicating enhanced membrane stability compared to control and non-encapsulated Mel treatments. This observation aligns with the general trend of decreasing Membrane Stability Index (MSI) throughout the vase life, with the highest MSI observed at harvest. Notably, petals treated with 0.1 and 0.5 mM nCS-Mel exhibited minimal electrolyte leakage (approximately 8% reduction from harvest) by day 12, in stark contrast to control and Mel-treated flowers, which showed a substantial increase in leakage (about 35%). This divergence in membrane integrity can be attributed to the protective effects of nCS-Mel against senescence-induced oxidative stress. The observed membrane stabilization in nCS-Mel treatments may be

explained by several physiological mechanisms. Firstly, the encapsulation of melatonin with nanochitosan likely enhanced its bioavailability and sustained release, prolonging its antioxidant effects. Secondly, the combination of melatonin's free radical scavenging properties with chitosan's ability to form a protective barrier may synergistically mitigate lipid peroxidation and preserve membrane phospholipids [23]. On the other hand, while the treatments did not significantly affect RWC, the improved membrane stability in nCS-Mel treated flowers suggests that water loss may be more influenced by factors such as vascular occlusion or changes in aquaporin activity rather than membrane leakage alone. The discrepancy between membrane stability and RWC highlights the complex interplay of factors governing water relations in cut flowers. Indeed, these findings corroborate previous research by Wang et al. [12], emphasizing the efficacy of nCS-Mel in maintaining cellular integrity and delaying senescence in cut flowers. The enhanced membrane stability observed in this study may be attributed to both direct scavenging of reactive oxygen species by nCS-Mel and its potential role in upregulating endogenous antioxidant systems, thereby indirectly preventing oxidative damage.

Our results revealed a complex interplay between various preservative treatments and petal carbohydrate content in *Gerbera jamesonii* flowers during their vase life (Fig. 3). The observed decline in total carbohydrates in control and 0.5 mM Mel treatments aligns with the established understanding of petal senescence, which typically involves the depletion of carbohydrates and reducing sugars [24]. Interestingly, the preservative solutions containing nCS-Mel, particularly at 0.1 mM concentration, demonstrated superior efficacy in maintaining petal carbohydrate levels. This observation suggests a potential synergistic effect between melatonin and chitosan nanoparticles in preserving cellular metabolites. The enhanced performance of nCS-Mel compared to non-encapsulated melatonin may be attributed to improved bioavailability and sustained release of melatonin, facilitated by the nanoencapsulation process. The divergent responses to different concentrations of Mel and nCS-Mel highlight the importance of dosage optimization in floral preservatives. The superior performance of 0.1 mM treatments compared to 0.5 mM suggests a potential hormetic effect, where lower concentrations elicit more favorable physiological responses. The maintenance of higher carbohydrate levels in nCS-Mel treated flowers could be explained by multiple physiological mechanisms. Firstly, melatonin has been shown to modulate amino acid and carbohydrate metabolism under stress conditions, potentially enhancing the synthesis of reducing sugars and secondary metabolites [25]. This metabolic shift may contribute to increased stress resistance and delayed senescence. Secondly, chitosan's inherent properties, including its ability to form a protective barrier and stimulate plant defense responses [20], may complement melatonin's effects in preserving cellular integrity and metabolic function. Furthermore, the nano-scale dimensions of the chitosan particles likely enhance their permeability across cell membranes, potentially facilitating greater intracellular efficacy of the encapsulated melatonin [26]. This increased cellular penetration may allow for more efficient regulation of senescence-related metabolic processes, resulting in better preservation of carbohydrate reserves. The absence of significant differences in protein content among treatments, despite the observed variations in carbohydrate levels, indicates the complexity of senescence-related biochemical changes. This discrepancy suggests that carbohydrate metabolism may be more responsive to the applied treatments than protein metabolism during the vase life of cut Gerbera flowers. On the other hand, while the results demonstrate the potential of nCS-Mel in maintaining petal carbohydrate

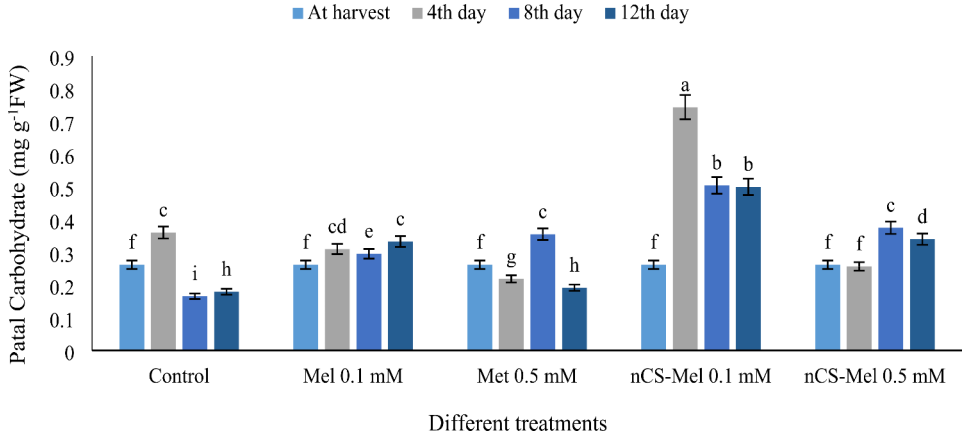

Fig. 3 Effect of different treatments on gerbera petal's carbohydrates during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

content and delaying senescence in cut Gerbera flowers, they also highlight the need for further research to elucidate the precise mechanisms underlying these effects.

The data presented in Fig. 4 demonstrate a substantial reduction in petal protein content, with a decrease of up to 27% observed by the 12th day of vase life compared to initial harvest levels. This decline in protein content is consistent with the established understanding of petal senescence biochemistry. In addition, the findings align with established literature regarding the biochemical changes associated with petal senescence, particularly the observed loss of carbohydrates and reducing sugars [24]. This depletion of energy reserves is a hallmark of the senescence process in cut flowers, reflecting the metabolic shifts that occur as tissues transition from maintenance to degradation. The differential response to melatonin treatments, especially the enhanced carbohydrate retention in flowers treated with nCS-Mel, can be interpreted through the lens of melatonin's known physiological effects. Melatonin has been shown to modulate plant metabolism under stress conditions, potentially increasing the synthesis of reducing sugars and secondary metabolites [25]. This metabolic reprogramming may contribute to improved stress resistance and delayed senescence in treated flowers. The absence of a similar effect in flowers treated with 0.5 mM non-encapsulated melatonin suggests that the nanoencapsulation process may play a crucial role in enhancing melatonin's efficacy. Chitosan, as a nanocarrier, likely contributes to this enhanced effect through its own bioactive properties [20] and by improving the cellular uptake and sustained release of melatonin. The nano-scale dimensions of the chitosan particles may facilitate greater permeability across cell membranes, potentially leading to improved intracellular performance of the encapsulated melatonin [26]. The observed increase in petal carbohydrate content in nCS-Mel treated flowers could be attributed to a combination of factors: (i) enhanced stress tolerance induced by melatonin. (ii) Improved cellular uptake and sustained release of melatonin due to nanoencapsulation. (iii) Potential synergistic effects between melatonin and chitosan in preserving cellular integrity and metabolic function. The lack of significant differences in protein content among treatments, despite variations in carbohydrate levels, highlights the complex nature of senescence-related biochemical changes. This discrepancy suggests that carbohydrate metabolism may be more responsive to the applied treatments than protein metabolism during the vase life of cut Gerbera flowers. Further research is, therefore, needed to elucidate the precise mechanisms underlying these differential responses and to optimize treatment formulations for maximal efficacy in preserving cut flower quality.

The results depicted in Fig. 5 reveal a notable increase in total flavonoid content (TFC) of *Gerbera jamesonii* petals, particularly pronounced on the 4th day of vase life. This trend was most significant in flowers treated with nCS-Mel at both 0.1 and 0.5 mM concentrations, showing a threefold increase in TFC compared to controls by the 12th day. Non-encapsulated melatonin treatments also demonstrated efficacy, albeit to a lesser extent, with a twofold increase in TFC. In contrast, control flowers exhibited a decline in petal TFC by the 12th day. These observations align with previous studies on the role of melatonin in plant stress responses and secondary metabolite production [27–31]. The observed increase in total flavonoid content (TFC) in *Gerbera jamesonii* petals treated with Mel and nCS-Mel can be attributed to several interconnected mechanisms. Melatonin's known ability to enhance antioxidant systems in plants [27] may have triggered an upregulation of flavonoid production as a defense against senescence-associated

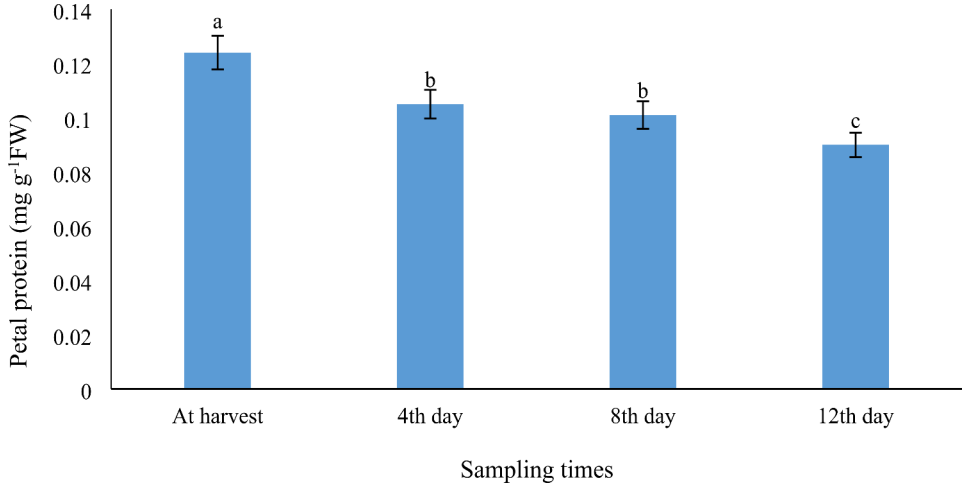

Fig. 4 Effect of different sampling times on gerbera petal's protein content. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

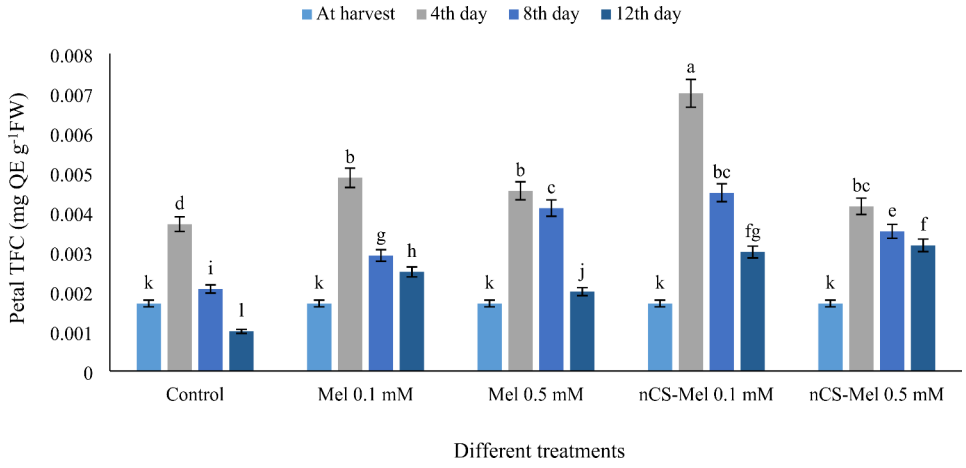

Fig. 5 Effect of different treatments on gerbera petal's total flavonoid content (TFC) during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

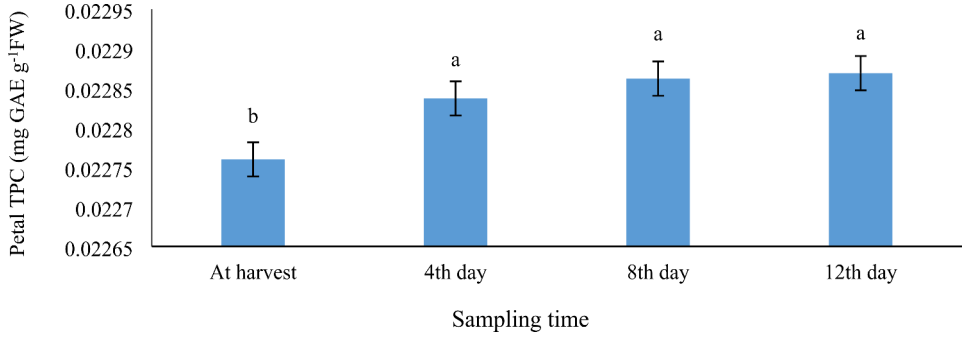

Fig. 6 Effect of different sampling times on gerbera petal's total phenolic content (TPC). Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

oxidative stress. This effect is likely mediated through transcriptional regulation, as melatonin has been shown to modulate gene expression related to flavonoid biosynthesis pathways [28]. Key enzymes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) may have been activated, leading to increased flavonoid synthesis. Furthermore, melatonin's interaction with other plant hormones, including jasmonic acid and salicylic acid, which are involved in stress signaling and secondary metabolite production [29], may have contributed to the enhanced flavonoid accumulation through complex hormonal crosstalk. The superior performance of nCS-Mel compared to non-encapsulated melatonin suggests that the nanocarrier system plays a crucial role in enhancing melatonin's efficacy. This may be due to improved bioavailability and sustained release of melatonin, leading to more pronounced and prolonged effects on flavonoid metabolism [30]. Additionally, the chitosan component of the nanocarrier system may have contributed synergistically to the observed effects, as chitosan itself has been reported to elicit defense responses in plants, including the production of secondary metabolites [31]. The combination of chitosan and melatonin in the nCS-Mel formulation may have resulted in a synergistic enhancement of flavonoid biosynthesis. The decline in TFC observed in control flowers by the 12th day aligns with the general pattern of metabolic degradation associated with petal senescence. In contrast, the ability of melatonin treatments, particularly in nanoencapsulated form, to not only prevent this decline but to significantly increase TFC levels shows the potency of this intervention in modulating the senescence process.

The results depicted in Figs. 6 and 7 reveal interesting trends in the total phenolic content (TPC) and anthocyanin levels of *Gerbera jamesonii* petals during their vase life. The observed increase in TPC throughout the vase life of gerbera petals, regardless of treatment, is a noteworthy phenomenon. This trend could be interpreted as a stress response mechanism, where phenolic compounds are synthesized as part of the plant's defense against oxidative stress associated with senescence. The lack of significant differences among treatments suggests that this increase in TPC may be a fundamental physiological response to post-harvest conditions, rather than a treatment-specific effect. In contrast, the anthocyanin content exhibited a more nuanced response to the

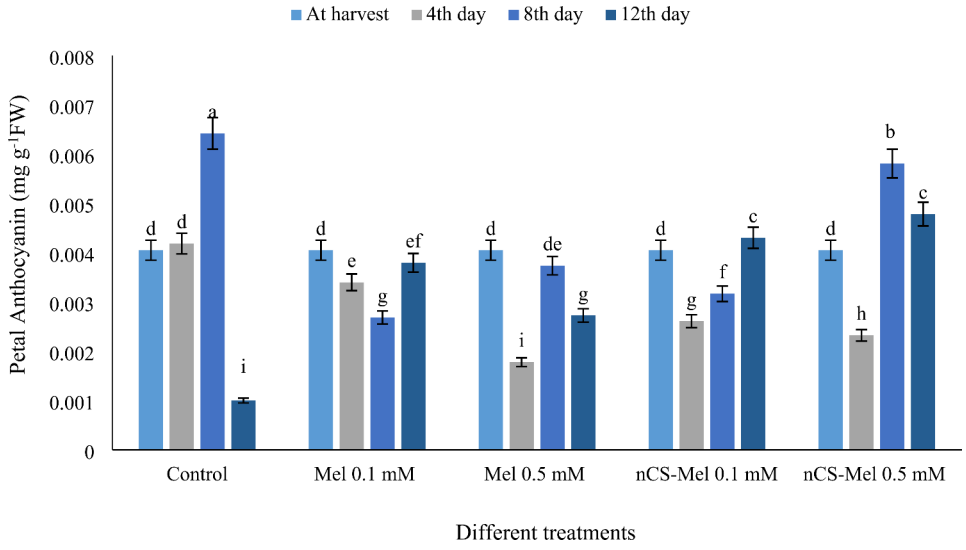

Fig. 7 Effect of different treatments on gerbera petal's anthocyanin during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

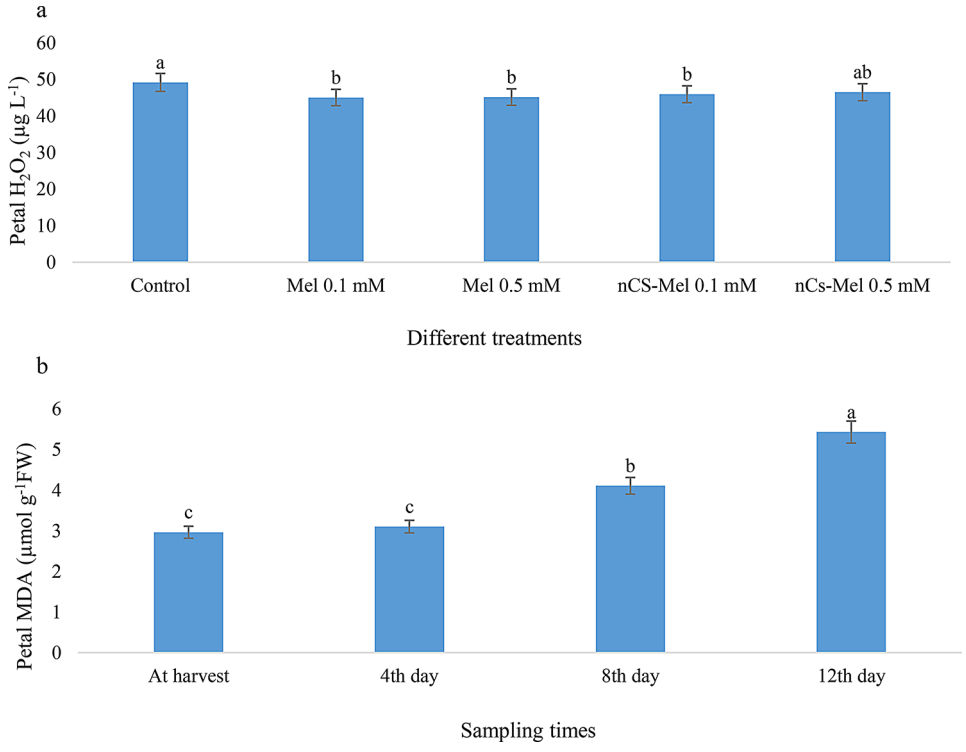

Fig. 8 Effect of (**a**) different treatments on gerbera petal's H_2O_2 and (**b**) different sampling times on gerbera petal's MDA. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

various treatments. The decline in anthocyanin levels observed in control flowers and those treated with non-encapsulated melatonin (0.1 and 0.5 mM Mel) aligns with the typical degradation patterns associated with petal senescence. Anthocyanins, being vacuolar pigments, are susceptible to degradation as cellular integrity diminishes during senescence [32]. The superior performance of nCS-Mel treatments in maintaining and even increasing anthocyanin levels by the end of the vase life is particularly intriguing. This effect could be attributed to several potential mechanisms. Enhanced antioxidant protection may have played a role, as melatonin's potent antioxidant properties could have provided better protection against oxidative degradation of anthocyanins [33]. Additionally, the combination of melatonin and chitosan in nanoparticle form may have contributed to better preservation

of cellular and vacuolar integrity, thereby stabilizing cellular membranes and protecting anthocyanins from degradative enzymes [34]. Melatonin has also been shown to influence the expression of genes involved in anthocyanin biosynthesis, and the sustained release of melatonin from nanoparticles may have prolonged this effect throughout the vase life [35]. Furthermore, chitosan's buffering capacity might have helped maintain an optimal pH for anthocyanin stability within the cellular environment [36]. The combination of melatonin's physiological effects with chitosan's protective properties in a nanoencapsulated form may have created a synergistic environment conducive to anthocyanin preservation and possibly continued synthesis. The contrasting responses of TPC and anthocyanins to the treatments highlight the complex nature of secondary metabolite dynamics during petal senescence. While the general increase in TPC across all treatments suggests a broad stress response, the specific preservation of anthocyanins by nCS-Mel treatments indicates a more targeted effect on this class of phenolic compounds.

The observed increases in flavonoids (Fig. 5), phenols (Fig. 6), and anthocyanins (Fig. 7) during the vase life of gerbera flowers across all treatments, particularly in those containing Mel and nCS-Mel, present an intriguing area for discussion. These secondary metabolites, known for their antioxidant properties and roles in flower pigmentation, exhibited dynamic changes that warrant further exploration. The significant effect of Mel and nCS-Mel on total flavonoid content (TFC) in gerbera petals aligns with the hypothesis that these compounds enhance antioxidant accumulation. This observation is consistent with previous research on cut roses [27], which reported an increase in total phenolic compounds during flower development and senescence. The physiological basis for this increase could be attributed to the plant's stress response mechanisms, as cut flowers undergo various stresses during postharvest handling and vase life. Chitosan's involvement in the signal transduction pathway of phenolic compound biosynthesis [28] offers a plausible explanation for the observed increases in phenolic content. It is conceivable that chitosan triggers defense-related pathways, leading to enhanced production of phenolic compounds as part of the plant's adaptive response to postharvest conditions. The accumulation of anthocyanins in vacuoles of petal epidermal cells contributes significantly to flower coloration. The preservation and potential enhancement of anthocyanin content by nCS-Mel treatments suggest a multifaceted effect of melatonin on the biochemical and physiological attributes influencing postharvest quality. This finding corroborates our previous work on rose cut flowers [19], highlighting the potential of nCS-Mel in improving overall flower color retention. A physiological hypothesis to consider is the role of protein synthesis in petal senescence. As petal pigment degradation in senescent petals requires the synthesis of specific proteins, chitosan's potential role as an inhibitor of harmful protein synthesis [29] could explain its effectiveness in preventing pigment degradation. This mechanism could work in synergy with melatonin's known antioxidant properties to preserve petal color and integrity. Moreover, the observed effects of Mel on pigment preservation could be attributed to its potential influence on gene expression related to anthocyanin biosynthesis and stability. Melatonin has been shown to modulate various physiological processes in plants, and its sustained release from nanoparticles might prolong these effects throughout the vase life of cut flowers. The complex interplay between these various mechanisms – antioxidant protection, signal transduction, protein synthesis inhibition, and gene expression modulation – indicates the need for further research to elucidate the precise physiological pathways involved in the observed effects of nCS-Mel on cut flower preservation.

The observed changes in hydrogen peroxide (H_2O_2) levels, malondialdehyde (MDA) content, and catalase (CAT) activity in gerbera flowers during vase life presented a complex picture of oxidative stress dynamics and antioxidant responses. These findings indicate a comprehensive discussion of the underlying physiological mechanisms and their implications for cut flower senescence. In this regard, the decrease in H_2O_2 levels in treated flowers, particularly those exposed to Mel and nCS-Mel treatments (Fig. 8a), suggests an enhanced capacity to mitigate oxidative stress. This effect could be attributed to melatonin's well-documented antioxidant properties [12]. The lower H_2O_2 content in treated flowers indicates a potential delay in senescence-associated oxidative damage, which is crucial for maintaining petal integrity and overall flower quality. Conversely, the increase in MDA content throughout the vase life, with a notable 1.8-fold increase by the end (Fig. 8b), points to progressive lipid peroxidation. This observation aligns with the understanding that membrane lipid peroxidation is a key indicator of tissue senescence, often triggered by reactive oxygen species (ROS) or lipid-oxidizing enzymes such as lipoxygenase [30]. The accumulation of MDA, a lipid oxidation product and biomarker for lipid peroxidation, suggests ongoing cellular damage despite the treatments. A physiological hypothesis to consider is that while the treatments effectively reduced H_2O_2 levels, they may not completely prevent the cascade of lipid peroxidation once initiated. The automatic decomposition of lipid hydroperoxides can lead to the production of oxygen free radicals, potentially triggering further lipid peroxidation cascades [30]. This could explain the continued increase in MDA content even in treated flowers. The contrasting trends of H_2O_2 and MDA levels raise interesting questions about

the specificity of antioxidant responses. It's possible that while melatonin effectively scavenges H_2O_2, it may be less effective against other ROS or unable to completely halt ongoing lipid peroxidation processes once they've begun. This hypothesis is supported by the observation that all treatments except for controls decreased H_2O_2 content in petals, suggesting a direct effect of both Mel and nCS-Mel on H_2O_2 levels. The increase in CAT activity, particularly in flowers treated with nCS-Mel 0.1 mM (Fig. 9a), and the overall rise in CAT activity during vase life (Fig. 9b), indicate an upregulation of enzymatic antioxidant defenses. This upregulation could be a response to the increasing oxidative stress signaled by rising MDA levels. The 3-fold increase in CAT activity by the end of vase life compared to harvest day suggests a significant mobilization of antioxidant resources to combat accumulating oxidative damage. These findings align with previous research on post-harvest flower physiology, which has shown that melatonin can improve water relations and maintain plant pigment stability, thereby delaying senescence processes and extending vase life [12]. The observed effects on H_2O_2 levels and CAT activity in this study provide further evidence for melatonin's role in modulating antioxidant responses in cut flowers. However, the continued increase in MDA content despite reduced H_2O_2 levels and increased CAT activity presents a complex picture of oxidative stress management in cut flowers. It suggests that while certain aspects of oxidative stress (such as H_2O_2 accumulation) can be effectively mitigated by treatments, other processes (like lipid peroxidation) may continue to progress, possibly due to factors beyond direct ROS scavenging. These results indicate that future research could focus on elucidating the specific pathways through which nCS-Mel enhances antioxidant responses, particularly in relation to different ROS species and lipid peroxidation cascades.

The observed trends in superoxide dismutase (SOD) and peroxidase (POD) activities in gerbera flowers during vase life presented an intriguing picture of the antioxidant defense mechanisms at play in response to various treatments. The increasing trend in SOD activity, particularly in flowers treated with nCS-Mel 0.5 mM and Mel 0.5 mM (Fig. 10), suggested an enhanced capacity to convert superoxide radicals into hydrogen peroxide.

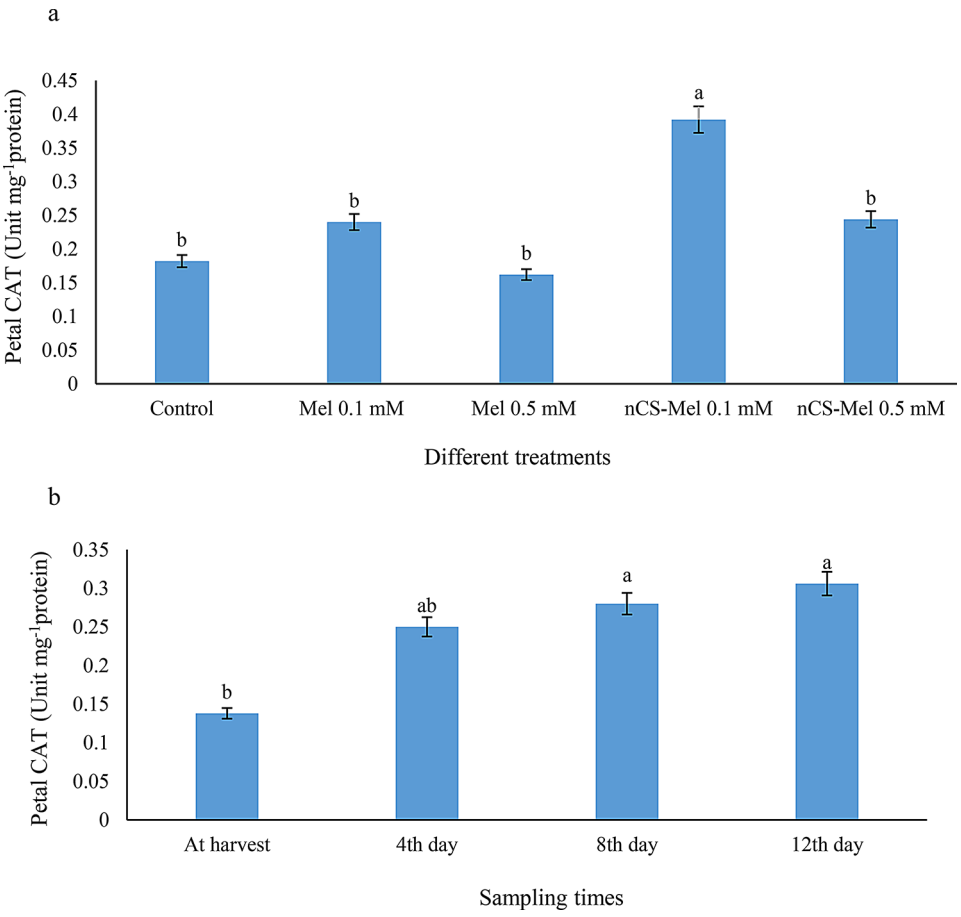

Fig. 9 Effect of (**a**) different treatments and (**b**) different sampling times on gerbera petal's CAT activity. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

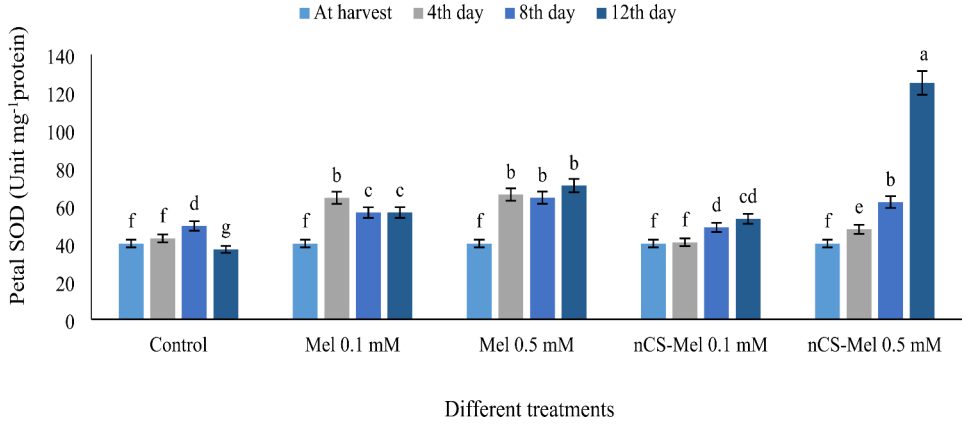

Fig. 10 Effect of different treatments on gerbera petal's SOD activity during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

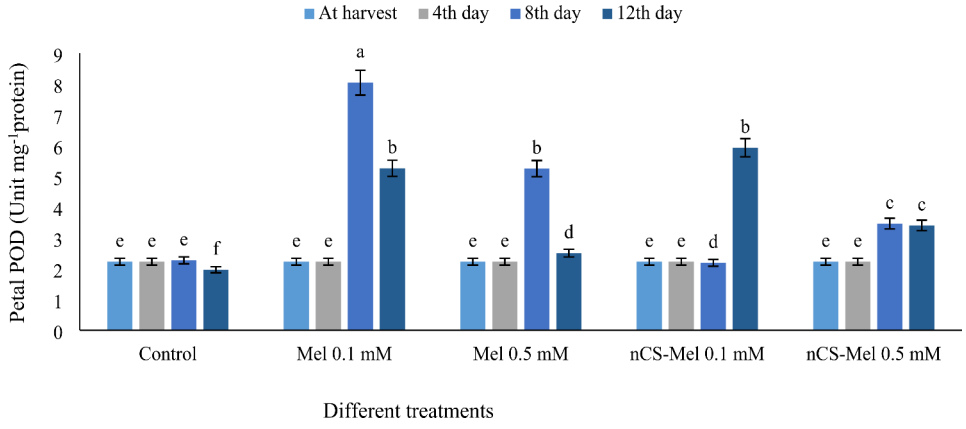

Fig. 11 Effect of different treatments on gerbera petal's POD activity during different sampling times. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

This upregulation of SOD activity is consistent with the plant's response to increasing oxidative stress during senescence. The higher SOD activity in treated flowers compared to controls indicates that both Mel and nCS-Mel treatments may be stimulating the plant's antioxidant defenses more effectively than untreated conditions. Similarly, the increase in POD activity, especially in flowers treated with 0.1 mM Mel and nCS-Mel 0.1 mM (Fig. 11), points to an enhanced capacity for hydrogen peroxide decomposition. The fluctuations observed in POD activity across different treatments and time points suggest a complex, dynamic response to oxidative stress that may be influenced by both treatment type and concentration. These observations align with the understanding that enzymatic antioxidant systems play a crucial role in controlling ROS production and regulating lipid peroxidation [13]. The coordinated increase in activities of multiple antioxidant enzymes (SOD, CAT, POD) observed in this study suggests a comprehensive defense mechanism against oxidative stress, which could contribute to delayed senescence and extended vase life.

A physiological hypothesis to consider is that melatonin, known for its potent antioxidant properties [32], may be acting both directly as an antioxidant and indirectly by stimulating the plant's endogenous antioxidant systems. The higher efficacy of melatonin compared to vitamins E, K, and C in penetrating cells [32] could explain its pronounced effect on antioxidant enzyme activities. Furthermore, the encapsulation of melatonin with nanochitosan may provide additional benefits through controlled release and enhanced stability of the compound [19, 35]. However, the role of chitosan in these processes should not be overlooked. Chitosan has been shown to enhance growth and development through signaling pathways related to auxin biosynthesis [20]. Its potential to increase antioxidant enzyme activity, promote lignification, and induce secondary metabolite production [20] could contribute to the observed effects on enzyme activities and overall flower longevity. The increased activity of antioxidant enzymes may also be linked to increased total protein content in petals, as suggested by previous research [34]. This relationship between enzyme activity

and protein content indicates the complex interplay of biochemical processes involved in delaying senescence. While the specific mechanisms of nCS-Mel action are not fully elucidated, the observed effects can be attributed to a combination of nano-chitosan properties [20, 28] and the advantages of encapsulation [19, 35] in controlling the release of compounds over time. This controlled release could provide sustained stimulation of antioxidant defenses throughout the vase life of the flowers. These findings align with previous research on other cut flowers, such as Anthurium, where melatonin application increased the activity of multiple antioxidant enzymes [13]. The consistency across different flower species suggests a conserved mechanism of melatonin action in enhancing antioxidant defenses in cut flowers.

The findings presented in Fig. 12a and b, demonstrating the extended vase life of gerbera flowers treated with nCS-Mel and Mel, offer intriguing insights into the physiological mechanisms underlying flower senescence and preservation. The marked extension of vase life observed in flowers treated with nCS-Mel 0.1 mM (12 days) compared to controls (5.33 days) suggests a significant impact of this treatment on the physiological processes governing flower senescence. The intermediate effects observed with nCS-Mel 0.5 mM and Mel 0.1 mM treatments (approximately 10 days vase life) further indicate a concentration-dependent response to these compounds. One physiological hypothesis to explain these observations relates to the regulation of ethylene production. Although ethylene levels were not directly measured in this study, the findings align with previous research on melatonin's role in balancing ethylene production and delaying fruit ripening in pears [36]. The potential limitation of ethylene production by melatonin could be a key

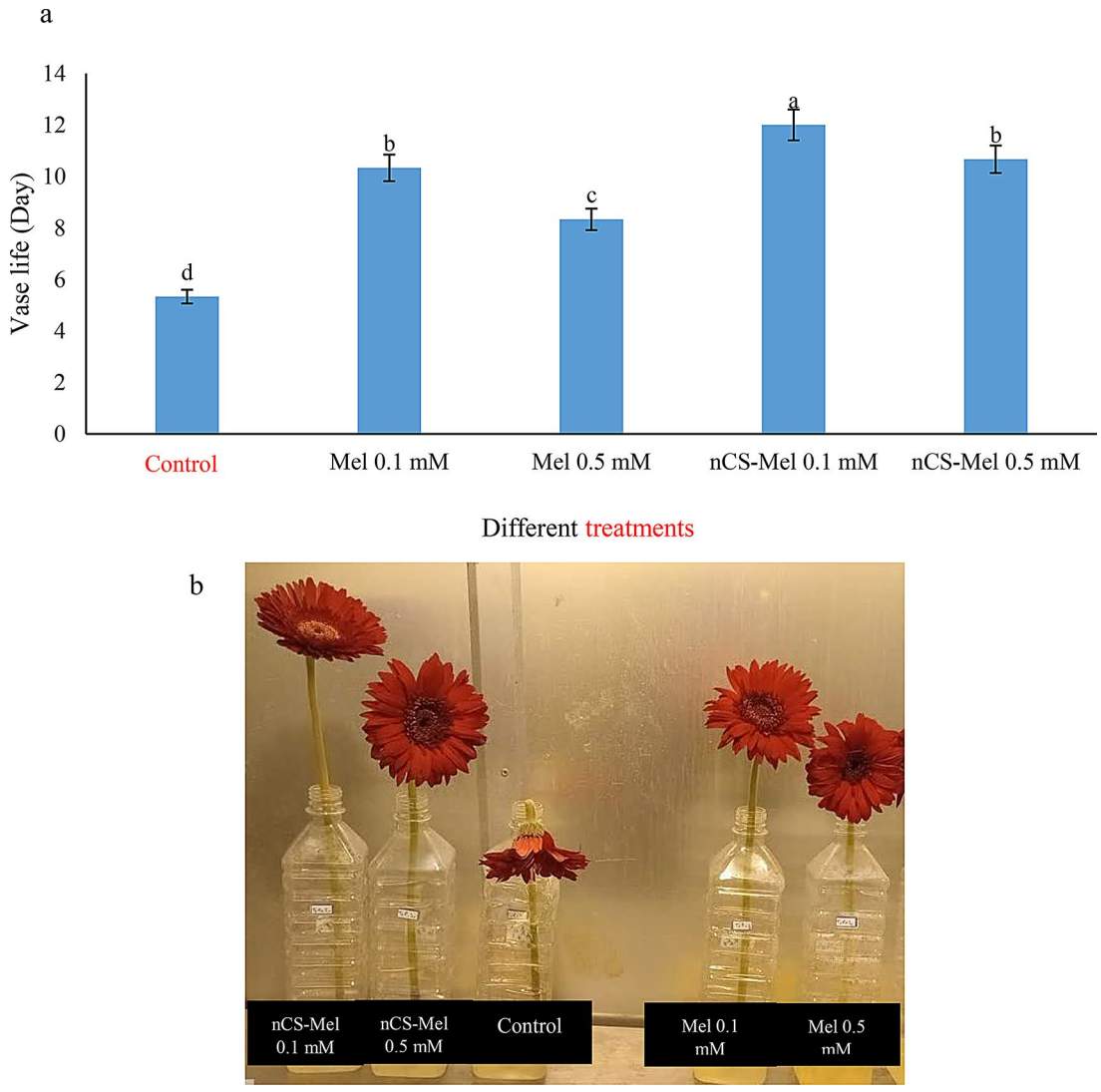

Fig. 12 Effect of different treatments on (**a**) gerbera vase life and (**b**) visual quality of gerbera in nCS-Mel 0.1 mM, nCS-Mel 0.5 mM, control, Mel 0.1 mM and Mel 0.5 mM at 12th day. Different letters indicate significant differences in each trait according to Duncan's test at $p < 0.05$

factor in delaying petal senescence, as ethylene is a well-known promoter of senescence in many flowers. This hypothesis is supported by the observed increase in peroxidase (POD) activity (Fig. 11), particularly in flowers treated with 0.1 mM Mel, 0.1 mM nCS-Mel, and 0.5 mM nCS-Mel on day 12 of vase life. Given that POD activity is known to be enhanced by ethylene [37], the sustained elevation of POD activity in these treatments could indicate a complex interplay between melatonin, ethylene signaling, and antioxidant responses. Another physiological aspect to consider is the role of carbohydrate metabolism in flower longevity. The extended vase life observed in treated flowers could be partly attributed to improved carbohydrate status. Photosynthetic products, particularly carbohydrates, play a crucial role in maintaining water uptake, cell development, and turgor pressure [38]. The treatments may be enhancing the flower's ability to maintain or utilize carbohydrate reserves more efficiently, thereby sustaining cellular functions and delaying senescence. The superior performance of nCS-Mel compared to Mel alone suggests additional benefits conferred by nanoencapsulation. This could be attributed to several factors. Firstly, nanoencapsulation may allow for a more sustained and controlled release of melatonin over time, providing continuous support to the flower's physiological processes. Secondly, the nano formulation might facilitate better uptake and distribution of melatonin within the flower tissues, enhancing its efficacy. Lastly, the combination of chitosan and melatonin in nanoform may create synergistic effects, potentially enhancing the individual benefits of each component. These factors collectively contribute to the enhanced effectiveness of nCS-Mel in extending the vase life of cut flowers compared to melatonin alone. The concentration-dependent effects observed between 0.1 mM and 0.5 mM treatments highlight the importance of dosage in achieving optimal results. The lower efficacy of the higher concentration (0.5 mM) in some cases suggests that there may be an upper limit to the beneficial effects, beyond which additional melatonin does not provide further advantages or may even become counterproductive. These findings align with the broader understanding of melatonin's multifaceted roles in plant physiology, including its functions as an antioxidant, a modulator of gene expression, and a regulator of various physiological processes [12, 32]. The observed effects on vase life likely result from a combination of these functions, working in concert to delay the onset and progression of senescence-related processes.

Principal component analysis, pearson correlation and dendrogram clustering

The principal component analysis (PCA) and hierarchical clustering analysis of the 12 biomass, physiological, and biochemical traits provided valuable insights into the complex interactions and responses of gerbera flowers to nCS-Mel treatments over the vase life period. The PCA results, as illustrated in Fig. 13, revealed that 59.9% of the total variance can be explained by two principal components (PC1 and PC2), with PC1 accounting for the larger proportion (36.30%) of the variance. The loading of oxidative markers (MDA and H_2O_2), antioxidant enzymes (CAT, SOD, and POD), and total phenolic content (TPC) on the positive side of PC1, particularly for untreated gerbera plants at the end of the storage period (12th day), suggested a strong association between these parameters and the progression of senescence. This aligns with previous findings that indicate an increase in oxidative stress and antioxidant enzyme activities during flower senescence [13, 30, 31].

The Pearson correlation analysis (Fig. 14) reveals interesting relationships between various parameters. The positive correlation between anthocyanins (ANs), protein content, and MDA levels could indicate a complex interplay between pigment stability, protein metabolism, and lipid peroxidation during senescence. This observation is consistent with the multifaceted effects of oxidative stress on cellular components during flower aging [30]. The negative correlation between RWC and parameters such as protein content, MDA, and H_2O_2 levels indicates the critical role of water relations in maintaining flower quality. This relationship aligns with previous research highlighting the importance of water status in delaying senescence and extending vase life [38]. The observed correlations suggest that treatments that maintain higher RWC may effectively delay the onset of oxidative stress and associated senescence processes.

The hierarchical cluster analysis (Fig. 15) provided a visual representation of the treatment effects, segregating the gerbera plants into three distinct clades based on nCS-Mel treatment and sampling time. The separation of nCS-Mel 0.5 mM treated flowers at day 12 into a distinct clade (Clade I) showed a unique physiological state induced by this treatment concentration. This observation aligns with the concentration-dependent effects of melatonin and nCS-Mel noted in previous studies on other plant species [12, 36]. The grouping of nCS-Mel 0.1 mM treated and control flowers at day 12 in Clade II, separate from other treatments, indicated that while the lower concentration of nCS-Mel induced some changes, they were not as pronounced as those observed with the higher concentration. This finding indicates the importance of optimizing treatment concentrations for maximal efficacy, as discussed in relation to melatonin's effects on pear ripening [36, 39]. The clustering of all other treatments in Clade III suggested that the earlier time points and lower concentrations of treatments may not induce as dramatic physiological changes as those observed in Clades I and II. This temporal and

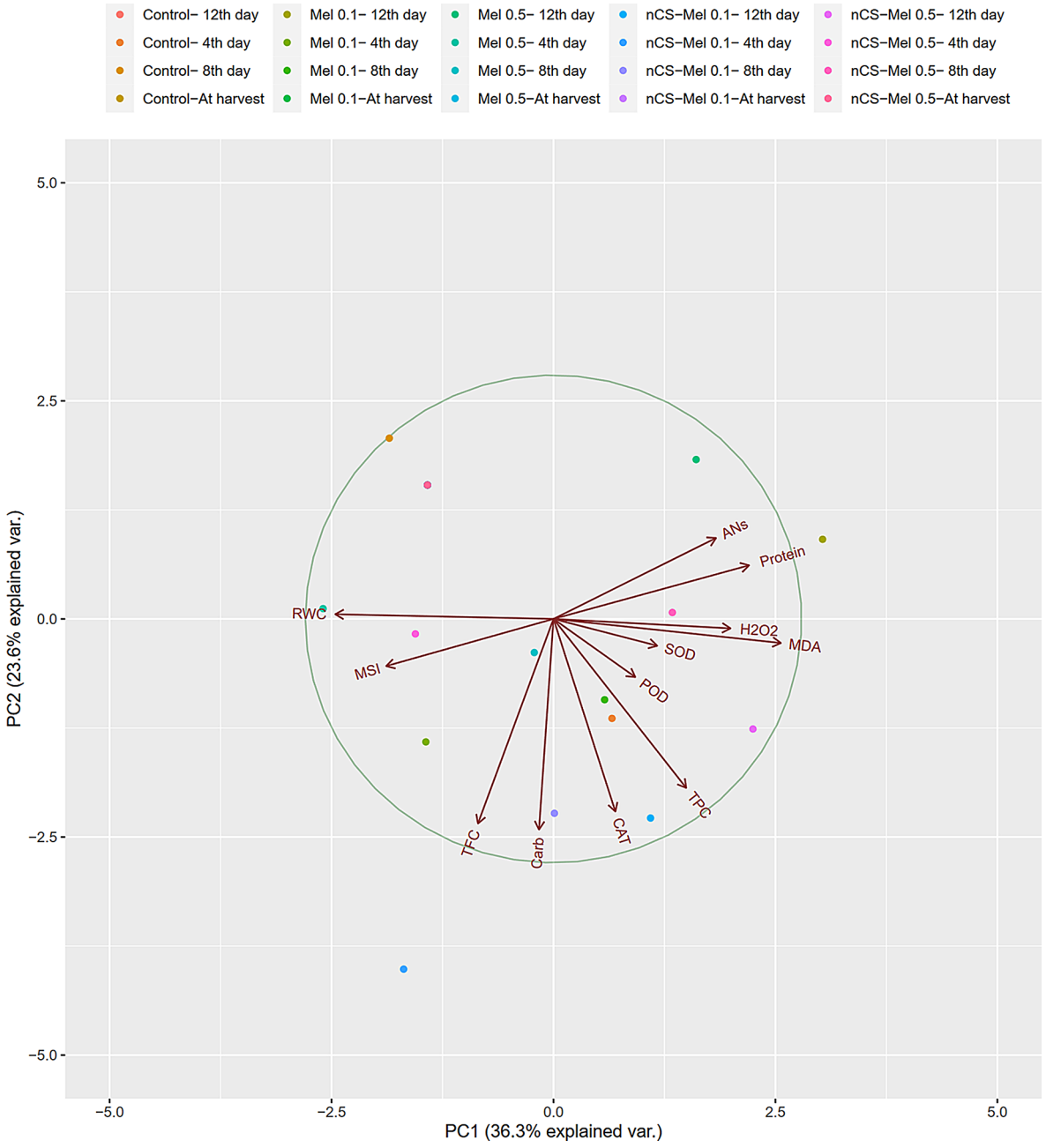

Fig. 13 Principal component analysis (PCA) of nCS-Mel treatment and variable trait relationship in gerbera plants grown at different sampling times. PCA biplot of the treatment-variable association where the lines originating from the center indicate positive or negative correlations of different variables. The tested variables included relative water content, RWC; membrane stability index, MSI; total flavonoid contents, TFC; carbohydrates, Carb; anthocyanins, ANs; total phenolic content, TPC; malondialdehyde, MDA, hydrogen peroxide, H_2O_2; catalase, CAT; superoxide dismutase, SOD; Peroxidase, POD

concentration-dependent response is consistent with the gradual progression of senescence processes and the time-dependent effects of antioxidant treatments observed in previous studies [13, 19]. These multivariate analyses collectively provided a comprehensive view of the complex physiological responses of gerbera flowers to nCS-Mel treatments. The results support the hypothesis that nCS-Mel, particularly at higher concentrations, can significantly alter the physiological and biochemical parameters associated with flower senescence. The observed effects are likely mediated through a combination of enhanced antioxidant defenses, modulation of water relations, and potential interactions with ethylene signaling pathways, as suggested by the elevated POD activities noted earlier [37].

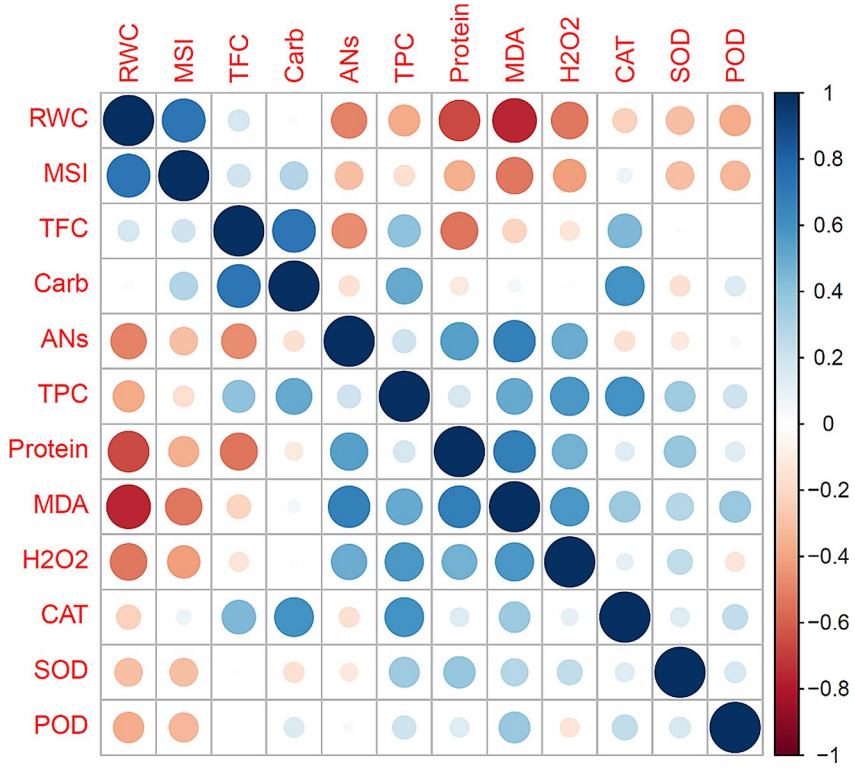

Fig. 14 Pearson correlation analysis of nCS-Mel treatment and variable trait relationship in gerbera plants grown at different sampling times. Heatmap of Pearson correlation coefficient (*r*) values of variable traits, where the colored scale indicates the positive (blue) or negative (red) correlation and the '*r*' coefficient values (*r* = -1.0 to 1.0). The tested variables included relative water content, RWC; membrane stability index, MSI; total flavonoid contents, TFC; carbohydrates, Carb; anthocyanins, ANs; total phenolic content, TPC; malondialdehyde, MDA; hydrogen peroxide, H_2O_2; catalase, CAT; superoxide dismutase, SOD; Peroxidase, POD

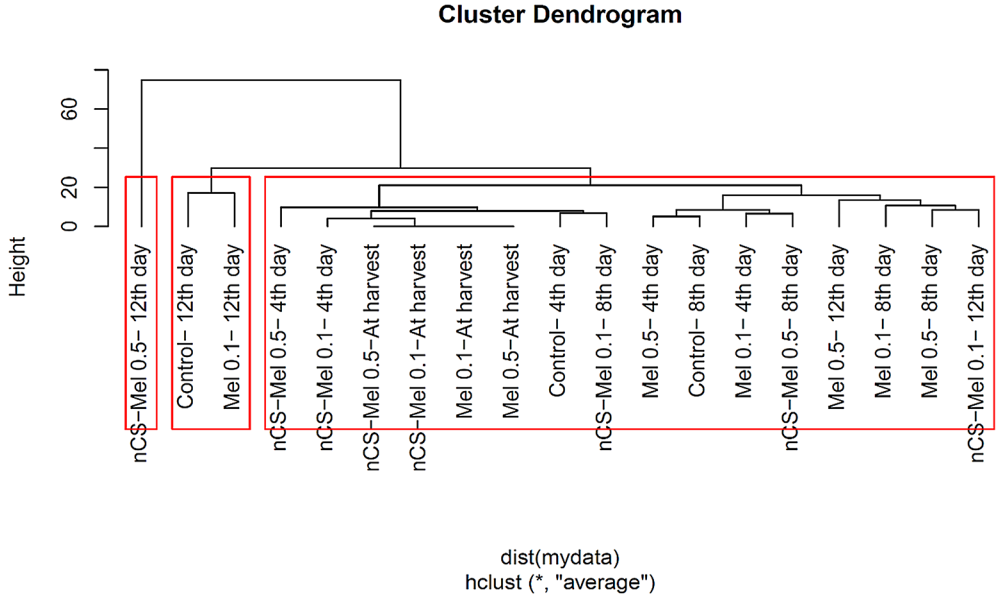

Fig. 15 Dendrogram clustering of nCS-Mel treatment and variable trait relationship in gerbera plants grown at different sampling times. Chitosan nanoparticle; nCS; Melatonin, Mel

Conclusion

The results obtained from the application of melatonin, particularly when encapsulated by nCS-Mel, demonstrated an increase in the activity of antioxidant enzymes, a reduction in electrolyte leakage, and a decrease in H_2O_2 production. Melatonin posed no harmful environmental effects, making it an attractive alternative to conventional preservative compounds used in the cut flower industry for prolonging vase life. According to the results, and in conjunction with previous studies, melatonin at lower concentrations, such as 0.1 mM, exhibited more favorable effects on gerbera quality and longevity compared to higher concentrations like 0.5 mM. However, it was noteworthy that 0.5 mM melatonin encapsulated with nanochitosan exhibited a comparable effect to the 0.1 mM non-encapsulated melatonin treatment. Furthermore, the encapsulation of melatonin with nanochitosan contributed to its stability and controlled release in the preservative solution, potentially enhancing its efficiency during the preservation of gerbera. Consequently, no discernible difference was observed between the two concentrations of melatonin encapsulated with nanochitosan. Moreover, the use of a preservative solution based on chitosan likely eliminated the need for sucrose as demonstrated in our previous work on rose cut flower, a significant advantage considering the environmental concerns associated with conventional preservatives. Further investigations could explore the optimal concentration and encapsulation method for melatonin and nanochitosan to maximize their efficacy in prolonging the vase life of various cut flower species, while minimizing any potential negative impacts. Considering the promising results obtained with the nanochitosan-encapsulated melatonin, future research could focus on developing and commercializing this formulation as an environmentally-friendly preservative solution for the cut flower industry, potentially replacing conventional preservatives with negative environmental consequences.

Materials and methods

Plant material

The experimental design was structured to ensure robust statistical analysis and comprehensive evaluation of treatment effects on *Gerbera jamesonii* cv. 'Terra kalina' over time. Cut flowers were sourced from a greenhouse in Tehran, Iran, adhering to established gerbera maturity indices (opening of 4 rows of radial florets). The stems were standardized to 35 cm length and subjected to various preservative solutions: melatonin (Mel) at 0.1 and 0.5 mM, and nanoencapsulated melatonin with chitosan (nCS-Mel) at 0.1 and 0.5 mM, with distilled water serving as a control. The experiment employed a rigorous replication protocol comprising three independent replications, each with four distinct time points (at harvest, 4th, 8th, and 12th days of vase life), represented by separate vases containing three cut flowers each. This arrangement resulted in 36 cut flowers per treatment (3 replications × 4 time points × 3 flowers per vase). Throughout the experiment, flowers were maintained under controlled conditions (22±2 °C, 65±5% RH, 12-h light period at 15 μM m^{-2} s^{-1}). Physiological and biochemical traits were evaluated at 0 (at harvest), 4, 8, and 12 days after harvest to assess the impact of the preservative solutions.

Treatment materials

Chitosan with low molecular weight of 165 kD, degree of deacetylation of 80% and purity of 99% was purchased from Sabz Gostaresh Azin Turkan, Maragheh, Iran. Sodium tripolyphosphate (TPP) with analytical grade was received from Sigma-Aldrich company.

Synthesis of melatonin-loaded chitosan nanoparticles

The volumes of melatonin-loaded chitosan nanoparticles were 1500 mL. First, 1.5 g of chitosan was poured into 150 mL of distillated water and allowed to disperse. By adding 1.5 mL of acetic acid, it was allowed to dissolve chitosan until obtain a clear solution. The obtained chitosan solution was diluted up to 1500 mL by adding 1350 mL of distillated water and allowed to stir for 30 min. Two different concentrations of melatonin-loaded chitosan nanoparticles were prepared. The concentrations of melatonin were chosen 0.1 and 0.5 mM. Thus, 0.035 g of melatonin in 5 mL of ethanol (0.1 mM of melatonin) and 0.174 g of melatonin in 10 mL ethanol (0.5 mM of melatonin) were dissolved and added to the two separately 1500 mL of chitosan solution. To reach melatonin-loaded chitosan nanoparticles, sodium *tripolyphosphate* as gelling agent through electrostatic interaction with cationic chitosan was used. The content of TPP was according to our previous works. Thus, 0.6 g of TPP was dissolved into 10 mL of distillated water. The melatonin-loaded chitosan solutions were allowed to stir with a rate of 1500 rpm. TPP solution was added dropwise into chitosan solutions and finally, a cloudy solution indicating the produce of melatonin-loaded chitosan nanoparticles was achieved. The as-obtained solutions were used as prepared. The nanoparticle solutions were named as nCS-Mel 1 and nCS-Mel 2 according to the concentration of melatonin 0.1 and 0.5 mM, respectively.

Instruments

The melatonin-loaded chitosan nanoparticles were dried under freeze-drying method and the SEM image was recorded on scanning electron microscopy (SEM; MIRA3 TESCAN, Czech Republic). In order to measure the hydrodynamic size of melatonin-loaded chitosan nanoparticles using dynamic light scattering (DLS), the as-obtained nanoparticles were diluted up

to 50 times and recorded the sizes on DLS/Zeta, Zetasizer Nano ZS90, Malvern Instruments, UK. The transition electron microscopy (TEM) of nanoparticles was recorded on TEM, Philips CM10 operating at 60 kV. In order to understand any changes in the crystallinity of chitosan during nanoparticle formation, the XRD patterns of chitosan and dried melatonin-loaded chitosan nanoparticles were recorded on the Siemens D-500 X-ray diffractometer.

Characterization of melatonin-loaded chitosan nanoparticles

To analyze melatonin-loaded chitosan nanoparticles, the nCS-Mel sample was chosen, and the results are related to the corresponding sample. XRD patterns were used to analyze the crystallinity structure of chitosan before and after nanoparticle formation. From Fig. 16a, the XRD pattern of neat chitosan indicated two characteristic diffraction peaks at $2\theta = 10.1^{\circ}$ and 19.18°, exhibiting high crystalline structure of chitosan. The corresponding peaks originated from hydrogen bonding between $-NH_2$ and $-OH$ functional groups of chitosan. After nanoparticle formation, the characteristic peak at $2\theta = 10.1^{\circ}$ of chitosan disappeared. The other peak at $2\theta = 19.18^{\circ}$ not only is shifted to $2\theta = 21^{\circ}$, but also tends to broaden. This phenomenon originated due to the interactions between TPP and protonated amine groups on chitosan, leading to prevent the closing of chitosan backbones to form crystalline points through hydrogen bonding. Similar observation reporting chitosan nanoparticles by using TPP has been reported by Wu et al. [40]. The morphology of melatonin-loaded chitosan nanoparticles was investigated by using TEM technique. As can be seen from Fig. 16b, spherical morphology of chitosan nanoparticles

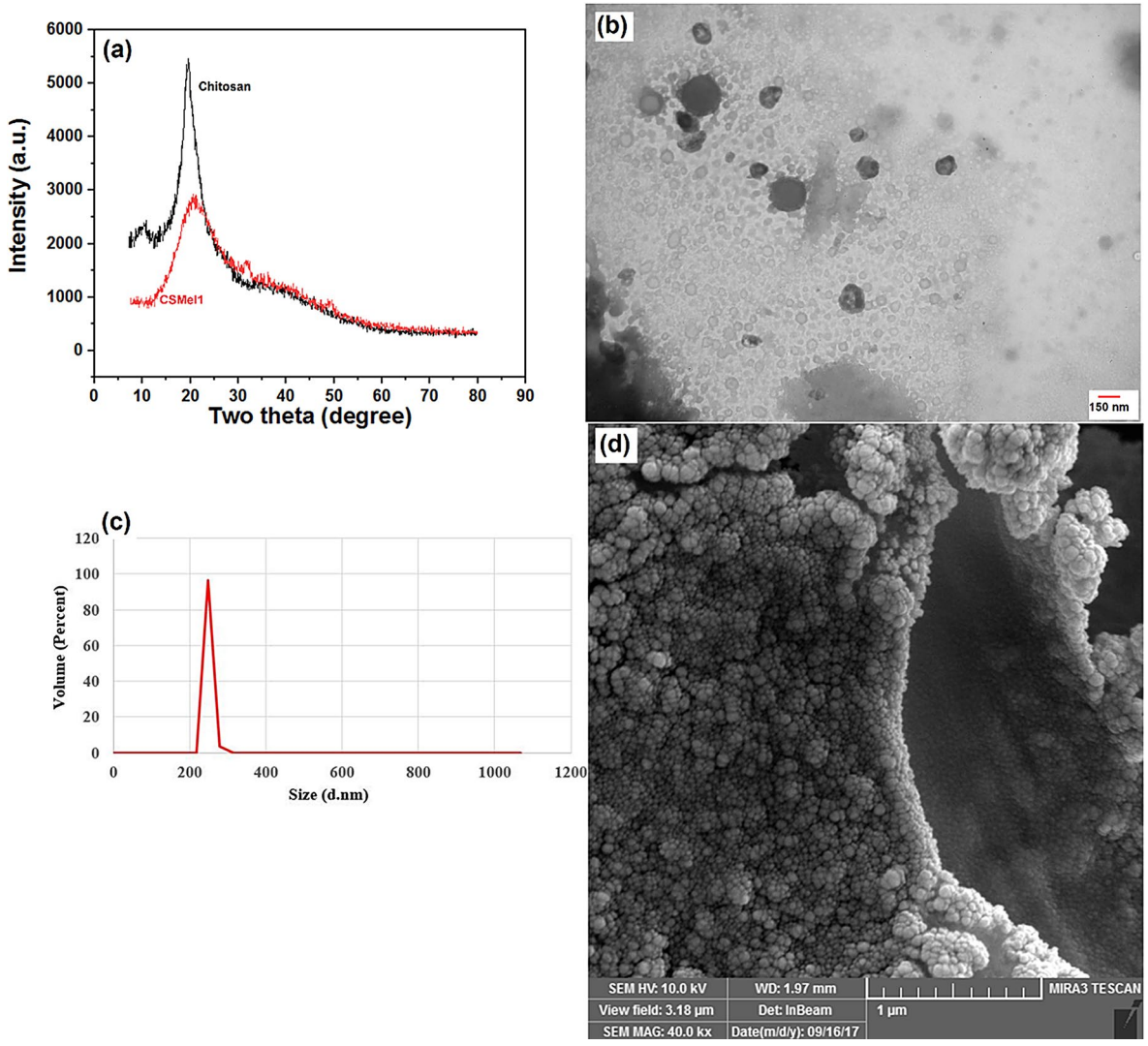

Fig. 16 (**a**) XRD patterns of neat chitosan and CS-Mel nanoparticles; (**b**) TEM image of CS-Mel nanoparticles indicating spherical morphology; (**c**) DLS curve of nCS-Mel solution; and (**d**) SEM image of CS-Mel nanoparticles after freeze-dry process

is obvious. The average diameter of nanoparticles was obtained about 145 nm. However, considering the DLS result, the average diameter of nanoparticles was identified about 220 nm, which was larger than that of TEM result (Fig. 16c). This observation may be attributed to the interactions between nanoparticles in solution leading to observe larger nanoparticles [41]. The SEM image of melatonin-loaded chitosan nanoparticles was studied after the freeze-drying process. Figure 16d illustrates the nanoparticles' spherical shape, consistent with the TEM result. Owing to the aggregation of nanoparticles during freeze-drying process the diameters of nanoparticles is not detectable carefully.

Petal physiological and biochemical traits assay

Petal relative water content (RWC)

Relative water content was assessed using the formula RWC = (FW−DW) / (TW−DW), where FW represents fresh weight, DW is dry weight, and TW is turgid weight. petal discs, were promptly placed in Petri dishes to minimize evaporation. These samples were stored in the dark and weighed to determine the fresh weight (FW). Petal pieces were then submerged in deionized water for 24 h, ensuring inhibition of physiological activity through dark incubation in the fridge. Subsequently, the turgid weight (TW) was determined, and the petal pieces were blotted to dryness, reweighed, and subjected to drying at 70 °C for 3 days for dry weight (DW) determination, following the protocol outlined by He et al. [42].

Petal membrane stability index (MSI)

MSI was assessed to examine the petal membrane's stability. Initially, 0.1 g of petals was weighed and uniformly cut. Subsequently, the cut petals were placed in separate test tubes, each containing 10 mL of distilled water. Two sets of samples were then subjected to measurements of electrical conductivity, with the first set measured after incubation at 40 °C for 30 min (C_1) and the second set after incubation at 100 °C for 15 min (C_2). The electrical conductivity measurements were conducted using EC meters (Jenway model, UK) following the methodology outlined by Almeselmani et al. [43]. The membrane stability index was calculated using the formula (1).

$$\mathrm{MSI}\ (\%) = [1 - (\mathrm{C1} / \mathrm{C2})] \times 100 \quad (1)$$

Petal carbohydrate

Total carbohydrates extraction involved anthrone reagent. Fresh gerbera petals (0.5 g) were ground with 5 mL ethanol, and the extract was centrifuged for 15 min at 4500 rpm. The supernatant was subjected to anthrone reagent (3 mL), and the absorbance was recorded at 625 nm using a spectrophotometer (Shimadzu, Japan) according to the Fales [44] method.

Petal total protein

To begin, 0.1 g of fresh petal tissue was weighed and crushed in phosphate buffer (pH=7.4). It was then centrifuged for 20 min at 10,000 rpm and 4°C. The extract was treated with a bioreagent. The absorbance intensity at 595 nm wavelength was then measured using a spectrophotometer, and different amounts of bovine albumen were used to create a standard curve, and the quantity of protein in mg/g fresh weight was estimated [45].

Petal total flavonoids compounds (TFC)

TFC was measured using the aluminum chloride colorimetric technique [46]. Aluminum chloride (10%), potassium acetate (1 M), and distilled water were added to the methanolic extract for this purpose and left in the dark for 30 min at room temperature. The optical absorbance of the resultant solution was then measured with a spectrophotometer (Shimadzu, Japan) at 415 nm. The standard curve was built using various amounts of Quercetin.

Petal total phenolic compounds (TPC)

TPC were quantified using the Folin-Ciocalteu method with gallic acid as the standard. Total flavonoid compounds (TFC) were determined using a colorimetric assay following the protocol by Shin et al. [47].

Petal anthocyanins (ANs)

Ans were evaluated by measuring 0.1 g of fresh petals which was grounded in 10 mL acidified methanol (1:99 v/v). The solution was centrifuged, and the supernatants were kept overnight in darkness. Absorption was read spectrophotometrically at 550 nm using a spectrophotometer (Shimadzu, Japan). Anthocyanin concentration was calculated using the extinction coefficient (ε=33000 $cm^2\ mol^{-1}$) and the formula A=εbc [48].

Petal hydrogen peroxide (H_2O_2)

H_2O_2 content in gerbera petals was determined as per the procedure by Liu et al. [49]. Specifically, 0.5 g of gerbera petals was ground in liquid nitrogen, and the extracts were centrifuged at 7000 rpm for 25 min at 4 °C. A 100 µL aliquot of the supernatants was combined with 1 mL of xylenol solution, and after 30 min, absorbance was measured at 560 nm using a spectrophotometer (Shimadzu, Japan).

Petal malondialdehyde (MDA)

MDA was measured as a 2-thiobarbituric acid (TBA) reactive metabolite, following the procedure described by Zhang et al. [50]. About 1.5 mL of the extraction was homogenized into 2.5 mL of 5% TBA made in 5%

trichloroacetic acid. The reaction solution was heated at 95 °C for 15 min, cooled rapidly, and the absorbance of the supernatants was read at 532 nm.

Petal CAT, SOD, and POD activity

For measuring antioxidative enzymes, one gram of gerbera petals was homogenized in 5 mL of 50 mM K–phosphate buffer (pH 7.0), supplemented with 5 mM Na–ascorbate and 0.2 mM EDTA from concentrated stocks. The homogenate samples underwent centrifugation at 10,000 rpm for 15 min at 4 °C, and the resulting supernatant was used for enzyme activity measurements at 4 °C. The activity of superoxide dismutase (SOD) and catalase (CAT) was determined following the protocol by Li et al. [51]. Peroxidase (POD) enzyme was extracted using the method by MacAdam et al. [52], and its activity was measured by recording absorbance at 475 nm with a spectrophotometer (Shimadzu, Japan).

Vase life

The lifespan of cut gerbera flowers varies from one to three weeks depending on the variety. One of the obvious signs of the end of life in this flower is the bending of the stem (neck bending>90°) [3], the wilting of the petals by 50% and the change of color of the petals. In this experiment, the lifespan in each treatment was measured and recorded based on the mentioned factors [53].

Statistics

The experiment was designed as a factorial experiment following a completely randomized design with three replications. Statistical analysis of the data was conducted using SAS ver 9.1 software, and mean separations were executed through Duncan's test, considering a significance level of 0.05. Pearson correlation coefficient analysis was carried out using R v3.4.3 (www.r-project.org). Additionally, Pearson correlation and cluster dendrogram heat maps were generated using R foundation for statistical computing (version 4.1.2), Iran (2021).

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Author contributions
H.S.H. conceived and designed the experiments; A.F.C. performed the experiments; S.M.Z and A.M. analyzed the data; G. M. synthesized the nano chitosan and encapsulation of melatonin; H.S.H. and O.K. wrote and proof the final paper. All authors have read and agreed to the published version of the manuscript.

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Data availability
All data will be available on reasonable demand from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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