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MSM performed the field experiment and prepared first draft manuscript; NUM performed chemical analysis, data analysis, and assisted in writing of the first draft of the manuscript; MoR contributed in design of experiment, chitosan dose preparation, and chemical analysis; MZS, DRG, MH, and MaR wrote and edited the manuscript; MTI conceived and coordinated the project and edited the manuscript; MSM, NUM, and MoR contributed equally

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ORIGINAL RESEARCH PAPER

Chitosan biopolymer improves the fruit quality of litchi (*Litchi chinensis* Sonn.)

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Abstract
Chitosan (CHT) is a natural compound that has been used to control postharvest pathogenic diseases due to its capability of eliciting natural defense responses in plants. The aim of this study was to investigate the effect of foliar CHT application on yield and quality of the litchi fruit. Chitosan was applied by spraying on to fruit and foliage just after fruit set four times at 7-day intervals with four varying doses viz. 100, 250, 500, and 1,000 μg L^{-1} and a control (0 μg L^{-1}). Although the application of CHT had no significant effect on the size of the fruits (length and width), the total contents of phenolics, flavonoids, and ascorbic acid and the antioxidant activity of litchi fruit arils were significantly increased in CHT-treated fruit compared with the untreated control. The highest phenolic, flavonoid, and ascorbic acid contents were 334 μg gallic acid g^{-1}, 881 μg quercetin g^{-1}, and 178 μg g^{-1}, respectively, in fruits treated with 500 μg L^{-1} CHT. However, the highest antioxidant activity (622 μg butylated hydroxytoluene g^{-1}) was recorded in 250 μg L^{-1} CHT-treated fruits. Our findings revealed that the application of low doses of CHT in a litchi orchard might improve fruit quality by increasing the content of antioxidants and antioxidant activities.

Keywords
antioxidant; ascorbic acid; biostimulants; chitosan; flavonoids; phenolics; secondary metabolites

Introduction

Litchi (*Litchi chinensis* Sonn.) is a highly popular table fruit in many countries of the world in tropical and subtropical regions. Litchi belongs to the Sapindaceae family, which originated in southern China and northern Vietnam [1]. It is a rich source of vitamin C and various bioactive compounds particularly antioxidants such as flavonoids and other phenolics [2]. Antioxidants of litchi fruit reduce human blood serum cholesterol, reduce the risk of cardiovascular disease, and scavenge reactive oxygen species (ROS) [3–5]. Therefore, a higher consumption of bioactive secondary metabolites through eating litchi fruits should provide benefit to human health.

Synthetic chemicals and pesticides are used frequently in litchi orchards for higher yield and to protect fruits from insects and diseases. However, indiscriminate use of synthetic chemicals and pesticides has detrimental effects on human health and the environment. Many children of Bangladesh and India suffer from a sudden illness and succumb to death every year due to the exposure of banned pesticides used in litchi orchards [6,7]. In order to minimize pesticide use and prevent pesticide related accidents, it is important to introduce an eco-friendly approach to protect litchi fruits

from various pests and diseases, and improve the quality of litchi fruit. As chitosan (CHT) is a natural plant protection agent, it can be an alternative to hazardous synthetic chemicals for sustainable production of high quality litchi fruits in Bangladesh.

Chitosan is a polycationic polymer of β-1,4, linked D-glucosamine, which is derived from chitin [8]. Chitin is the second largest natural product abundant in the exoskeletons of marine crustaceans such as prawn, shrimp, crab, insect, and the cell walls of fungi. The biopolymer CHT is highly effective in decreasing disease incidence and increasing the quality of strawberries, raspberries, and other crops [9,10]. It is nontoxic to humans and other animals, biodegradable, and environment-friendly for agricultural uses [11]. Chitosan and its derivatives have been reported to elicit natural defense responses in plants and have been used as a natural compound to control postharvest pathogenic diseases [9]. Antimicrobial activities of CHT against various phytopathogens have also been reported [12,13]. Chitosan has been widely used as a coating agent of various fruits mainly for protection from postharvest losses, enhancing the storability and preservation [9,14–18]. A large body of literature indicates that foliar application of CHT increases vegetative growth, yield and induces synthesis of secondary metabolites, such as polyphenolics, lignin, flavonoids, and phytoalexins in plants [19–23]. It also promotes expression of genes involved in hormone metabolism, photosynthesis, the plant immune system, heat-shock proteins, systemic acquired resistance, and reprogramming of protein metabolism with an increment of storage proteins [24,25]. However, scant information is available on the effects of CHT on yield and the beneficial biochemical contents in litchi fruits. The aims of this study were therefore to evaluate the effect of varying doses of CHT on yield, contents of antioxidants such as phenolics, flavonoids, vitamin C, and antioxidant activity of the fruit arils of litchi in an orchard growing environment.

Material and methods

Experimental site and soil type

This study was conducted in a litchi orchard situated in the Meherpur District of Bangladesh (23.75° N, 88.70° E) under the High Ganges River Floodplain agro-ecological zone of Bangladesh during the summer season (April 24 – May 15, 2017). The soil type is Gangetic Alluvium which ranges from clay loam to sandy loam, being calcareous and not acidic in nature with a pH ranging from 7 to 7.5.

Preparation of chitosan solution and its application

Chitosan biopolymer [$C_{12}H_{24}N_2O_9$, 2-amino-2-deoxy-(1→4)-β-glucopyranan] produced from shrimp shells were purchased from Sigma (Sigma-Aldrich, USA). Five different concentrations 0, 100, 250, 500 and 1,000 μg L^{-1} of CHT solution were prepared by measuring the required amount of product followed by dissolving in 0.1 M HCl acid and diluting in distilled water with the pH adjusted at 6.5 [26]. Freshly prepared doses of CHT solutions were sprayed four times on newly set litchi fruits at 7-day intervals from (April 24 – May 15, 2017). Only water was applied to the nontreated control litchis. In addition, a fungicide treatment was included in the study that represented the usual practice of litchi growers, and applied four times at the recommended rate with similar intervals as chitosan. One hundred litchi fruits were subjected to each treatment. All intercultural operations and fertilization were performed following commercial recommendations.

Harvesting and data collection

The treated litchi fruits were harvested at the ripening stage. Fruit length (cm) and width (cm) were measured using a ruler. Data on fruit weight (g), seed weight (g), and aril weight (g) of litchi were also recorded.

Determination of antioxidant activity in litchi fruit (DPPH radical scavenging assay)

The antioxidant activity of the fruits was determined by the 1,1-diphenyl-2-picryl hydrazyl (DPPH; CalBiochem, Germany) radical scavenging assay. The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical of DPPH has an odd electron, which gives a maximum absorption at a wavelength of 517 nm (purple color). Methanol-extracted supernatant of a fruit aril sample (1 mL) was taken in a test tube, and DPPH solution (0.0788 g of 0.2 mM DPPH in 1 L methanol) added to the test tube. The reaction mixture was incubated at 25°C for 5 min, after which time the absorbance was measured at 517 nm [27]. When the antioxidants react with DPPH, the DPPH is reduced to DPPH-H and, as a consequence, the absorbance decreases. DPPH-H formed in DPPH solution with the same solvent (i.e., without fruit extract) was used as a control. Methanol with the respective fruit extract served as the blank. The DPPH radical scavenging activity of each fruit extract was calculated as the percentage inhibition of radical activity: % *DPPH radical activity* = (*Absorbance of control* − *Absorbance of sample*)/(*Absorbance of control*)×100.

Determination of total phenolic content

The content of total phenolic compounds was determined spectrophotometrically by placing 1 mL methanol extract sample and standards into test tubes. One mL methanol was used instead of the fruit extract as the blank. Half mL 10% (0.2 N) Folin–Ciocalteu reagent was added to each standard and sample test tube. Then, the test tubes were shaken for 10 s and incubated for 15 min at room temperature in the dark. Aqueous 700 mM sodium carbonate (Na_2CO_3) solution (2.5 mL) was added to each test tube followed by vortexing the mixture and incubating at room temperature for 2 h in the dark. The absorbance of the solution in each tube was measured at $\lambda = 765$ nm against the blank [28]. The measurements were compared to a standard curve of gallic acid solutions and expressed as μg g^{-1} fresh weight (FW) gallic acid equivalents.

Determination of total flavonoid content

The content of total flavonoid compounds was determined spectrophotometrically according to aluminum chloride colorimetric assay. One mL methanol extract sample and standards were placed in test tubes; the blank reaction tube had 1 mL methanol instead of the fruit extract. Then, 0.4 mL 5% sodium nitrate was added to each standard and sample test tube. After 5 min, 0.6 mL of 10% $AlCl_3 \cdot 6H_2O$ was added to the mixture. Then, 1.0 M NaOH was added followed by shaking the tubes thoroughly. The absorbance of the solution in the tubes was measured at $\lambda = 510$ nm against a blank sample [29]. The measurements were compared to a standard curve of quercetin solutions and expressed as μg g^{-1} FW quercetin equivalents.

Determination of total vitamin C (L-ascorbic acid)

The content of vitamin C was determined spectrophotometrically by taking 1 g of the fruit sample in 10 mL 4% trichloroacetic acid (TCA). The sample was homogenized followed by centrifugation at 2,000 rpm for 10 min. The supernatant was transferred to a new tube and was treated with a pinch of activated charcoal. The contents in the tube were then shaken vigorously using a cyclometer and incubated on the bench top for 5 min. The charcoal particles were then removed by centrifugation, and aliquots were used for the estimation of vitamin C. One mL of supernatant was transferred to a test tube and the volume was increased up to 2.0 mL with 4% TCA. 2,4-Dinitrophenylhydrazine (DNPH) reagent (0.5 mL) was added to the tube followed by two drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 h which resulted in the formation of osazone crystals. These crystals were dissolved in 2.5 mL of 85% sulfuric acid in the cold. For the blank reaction, DNPH reagent and thiourea were added after

the addition of sulfuric acid. The tubes were cooled in ice, and the absorbance was read at λ = 520 nm on a spectrophotometer [30]. A standard curve was prepared by taking varying known concentrations of ascorbic acid solution in tubes and measuring the absorbance following the method mentioned above. The absorbance of fruit samples was then compared to the standard curve of the ascorbic acid solution and expressed as μg g^{-1} L-ascorbic acid equivalents. All the above-mentioned experiments for determining antioxidants were repeated at least three times.

Statistical analysis

The SPSS 25.0 software package was used for analysis of variance of the replicated data. Treatment means of data were separated using Fisher's protected LSD test ($p \leq 0.05$).

Results

Effects of chitosan on fruit size and weight of litchi

Application of CHT slightly increased the width of litchi fruits. However, the increment was significant only at 100 μg L^{-1} CHT. Compared to the control, 100 μg L^{-1} CHT resulted in 12% increase in fruit width which was similar to the fungicide treatment. There was no significant effect of any treatment on fruit length. Fungicide application significantly improved total, seed, and aril weights compared to the nontreated control. Chitosan had no significant influence on the total weight but seed and aril weights were significantly improved by CHT application. Chitosan concentrations which improved seed weight were 500 and 1,000 μg L^{-1}, whereas aril weight was significantly enhanced by 500 μg L^{-1} compared to the nontreated control (Tab. 1, Fig. 1).

Enhancement of antioxidant contents and total antioxidant activities of fruits by chitosan

Total flavonoids and phenolics contents. The total flavonoid content of the fruit varied significantly with the application rate of CHT compared to the nontreated control and fungicide treatment. Plants treated with 500 μg L^{-1} CHT showed the highest total flavonoid content (880.73 μg quercetin g^{-1} fruit) in fruit arils, followed by 678 μg quercetin g^{-1} in the nontreated control. The lowest total flavonoid content (538.64 μg quercetin g^{-1} fruit) was recorded for the 1,000 μg L^{-1} treatment (Fig. 2A). Similar to total flavonoids, total phenolics content in fruits was also significantly enhanced by the application of CHT compared to the nontreated control and fungicide. The highest total phenolics content (334.41 μg gallic acid g^{-1} fruit) was recorded in the 500 μg L^{-1} CHT treatment followed by the 250 μg L^{-1} CHT (293 μg gallic acid g^{-1} fruit) treatment (Fig. 2B). Total phenolics contents of 206 μg gallic acid g^{-1} and 158 μg gallic acid g^{-1} fruit were recorded in fruits produced in the nontreated control and fungicide-treatment, respectively.

Total antioxidant activity. Total antioxidant activity of fresh fruits was highest in the 250 μg L^{-1} CHT treatment (622 μg BHT g^{-1} fruit), followed by the 500 μg L^{-1} CHT (400 μg BHT g^{-1} fruit) spray on the foliage, which was significantly different from all other treatments. The lowest antioxidant activity was found in fruits of nontreated control plants (135 μg BHT g^{-1} fruit) (Fig. 2C). Our results indicate that antioxidant activity in litchi fruits was increased up to nearly fivefold and twofold by the application of CHT and fungicide, respectively, compared to the untreated control.

Vitamin C content in fruit

Vitamin C contents of fruits in the CHT treatment, nontreated control and fungicide-treated plants were estimated from total L-ascorbic acid activities of fresh litchi fruits.

Fig. 1 Photographs of harvested litchi fruits treated with varying doses of chitosan: (**A**) control (treated with water only); (**B**) 100 µg L^{-1}; (**C**) 250 µg L^{-1}; (**D**) 500 µg L^{-1}; (**E**) 1,000 µg L^{-1} chitosan; (**F**) commercial fungicide-treated litchi.

Tab. 1 Effect of chitosan on fruit length, fruit width, fruit weight, seed, and aril of litchi.

Treatment	Length (cm)	Width (cm)	Total weight (g)	Seed weight (g)	Aril weight (g)
Control	38.20 ±0.33 ^{a}	28.70 ±0.73 ^{c}	16.00 ±0.99 ^{b}	2.91 ±0.14 ^{d}	11.500 ±0.30 ^{bc}
100 µg L^{-1}	36.90 ±0.81 ^{a}	32.05 ±0.63 ^{a}	16.35 ±0.84 ^{b}	2.82 ±0.10 ^{d}	11.000 ±0.22 ^{c}
250 µg L^{-1}	38.60 ±0.52 ^{a}	29.80 ±0.42 ^{bc}	16.25 ±0.51 ^{b}	2.85 ±0.05 ^{d}	12.170 ±0.24 ^{ab}
500 µg L^{-1}	39.05 ±0.63 ^{a}	28.85 ±0.49 ^{c}	16.40 ±0.56 ^{b}	3.83 ±0.08 ^{b}	12.570 ±0.10 ^{a}
1,000 µg L^{-1}	36.55 ±0.34 ^{a}	29.60 ±0.15 ^{bc}	15.35 ±0.38 ^{b}	3.33 ±0.08 ^{c}	9.000 ±0.24 ^{d}
Fungicide	36.90 ±1.29 ^{a}	31.30 ±0.42 ^{ab}	19.40 ±0.98 ^{a}	4.47 ±0.05 ^{a}	13.000 ±0.27 ^{a}

Mean values within a column followed by the same letter do not differ significantly by Fisher's protected LSD test at $p \leq 0.05$. Data are presented as mean ± *SE* ($n = 100$).

Plants treated with 500 µg L^{-1} CHT showed the highest L-ascorbic acid content (178 µg g^{-1} fruit) followed by 250 µg L^{-1} (168 µg g^{-1} fruit), and the lowest (123 µg g^{-1} fruit) was recorded in the nontreated control (Fig. 2D). The highest antioxidant activity was obtained by the application of 500 pm CHT, which was 1.5-fold higher than the untreated control.

Discussion

The use of synthetic plant growth promoting and pest protection chemicals is increasing day by day in agricultural production systems, which is hazardous for both the environment and human health. Alternative solutions should be explored for sustainable and environment-friendly crop production worldwide. Chitosan is a biopolymer derived from naturally abundant chitin [8]. During the past decades, use of biostimulants has gone up and is increasing steadily worldwide because of their remarkable effects on both enhancing crop yield and quality. Finding an effective biostimulant for better quality fruit production is an important task for the horticulturists and plant biotechnologists. Chitosan is an environment-friendly biorational product that can promote plant growth, induce disease resistance and improve fruit quality [31]. Several lines of evidence suggest

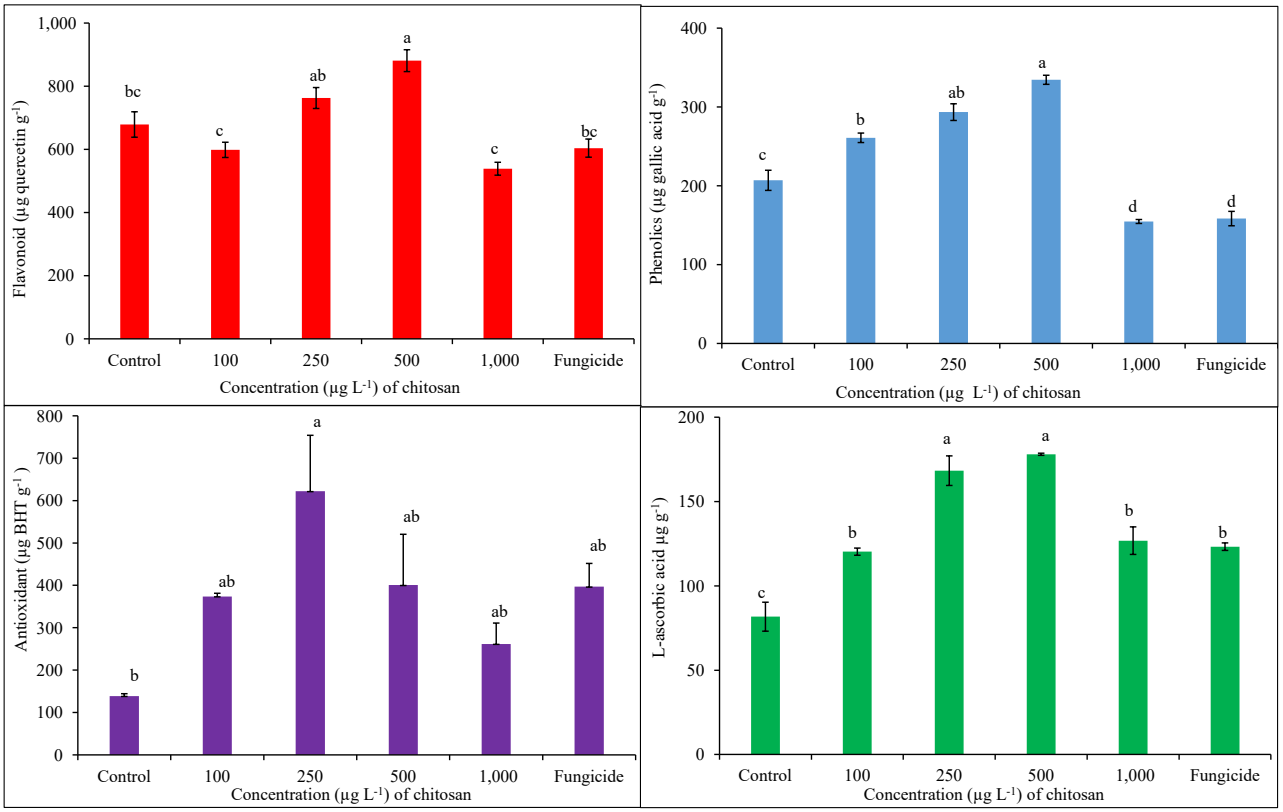

Fig. 2 Enhancement of (**A**) total flavonoids (µg quercetin equivalent g^{-1} FW), (**B**) phenolics (µg gallic acid equivalent g^{-1} FW) contents, (**C**) antioxidant activity (µg BHT equivalent g^{-1} FW), and (**D**) vitamin C content (µg ascorbic acid g^{-1} FW) of fresh litchi fruit by the treatment of varying doses of chitosan. One-way ANOVA was performed for analysis of the data and mean values in the bars followed by the same letter(s) are not significantly different as assessed by Fisher's protected LSD (least significance difference) at $p \leq 0.05$.

that CHT can induce numerous biological responses in plants, which are dependent on its structure and concentration as well as the species and developmental stage of plants [8,19–21]. Our study demonstrated for the first time that application of low doses (at µg L^{-1} level) of CHT significantly improved the contents of various antioxidants and total antioxidant activity of litchi fruits under field conditions. These are among the key indicators of litchi fruit quality. Improvement of fruit quality and protection from postharvest rots of various fruits by CHT has also been reported.

Litchi is a popular table fruit in most subtropical and tropical countries of the world, which is a rich source of secondary metabolites like polyphenols, flavonoids, and vitamin C [1,32]. These secondary metabolites are largely known for their health benefits due to their antioxidant properties [32]. Besides the antioxidant properties, these metabolites are also responsible for the color-forming pigments of many fruits and vegetables, and protection of plants from diseases and UV light [33,34]. Increased levels of antioxidants have been found to provide protection against various kinds of stresses in many studies. Several studies have indicated that regular consumption of polyphenol-rich fruits, vegetables, and cereals reduces the risk of developing cardiovascular disease and several types of cancer [34]. Our study explored an eco-friendly option for boosting the yield and quality of litchi fruit by the application of CHT and compared its effect with nontreated control and an industry standard fungicide spray program. Our result showed that foliar application of CHT during the fruit setting stage not only significantly increased the fruit size, but also significantly increased various antioxidant contents of litchi fruit compared to an untreated control or synthetic fungicide spray treatment (Tab. 1 and Fig. 2). Among the CHT doses, 500 µg L^{-1} was found the best for increasing the content of secondary metabolites in litchi fruits (Fig. 2). Although plant growth promoting effects of CHT have been demonstrated by many researchers, in our study, however, CHT application after the fruit set of litchi did not remarkably increase fruit size in a dose-dependent manner. Results from similar study indicated that application of CHT on the leaf canopy increased photosynthesis in the plant leading to higher

biomass production [S]. In our case, we applied CHT on to fruits and adjacent foliage but not at an earlier stages for vegetative growth promotion. Further studies are needed to test the efficacy of CHT on the early developmental stage of the plant by spraying on to the leaf canopy to investigate any effects on the fruit size.

Our study showed for the first time that a spray application of CHT biopolymer on to developing fruits significantly increased human health-benefiting antioxidants in litchi fruits without affecting the yield. As there was no pest or disease incidence, we could not evaluate the efficacy of CHT or fungicide for pest management. However, fruit quality improvement due to the enhancement of antioxidant contents was very interesting. A targeted approach is needed to evaluate the effects of CHT on resistance induction against pests and diseases. Chitin from the exoskeleton of shrimps is an abundant bioresource in Bangladesh due to the existence of an export-oriented shrimp industry. Therefore, the findings of the current study should encourage industrial production of CHT from chitin from local shrimp waste. Availability of cheaper chitosan from local sources may help in the production of safe and high-quality litchi production in Bangladesh.

Conclusion

Application of CHT at 500 μg L^{-1} remarkably enhanced various phenolic compounds and antioxidant activities in litchi fruits. A further study is needed to elucidate the mechanism of beneficial effects of CHT on the production of high-quality litchi. Our findings suggest that the biopolymer CHT could be an attractive alternative to harsh synthetic chemicals for the safe and sustainable production of litchi. Both content of secondary metabolites and antioxidant activities of litchi fruits were significantly improved after application of CHT. However, additional research on antimicrobial effects of CHT is needed as related to litchi diseases and postharvest rots. It may also be critical to optimize the actual dose and number of applications that may provide maximum benefit under commercial field conditions.

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Biopolimer chitozan poprawia jakość owoców liczi (*Litchi chinensis* Sonn.)

Streszczenie

Chitozan (CHT) jest naturalnym związkiem wykorzystywanym do zwalczania chorób roślin, ponieważ wykazuje potencjał do wywoływania naturalnej odpowiedzi obronnej w roślinach. Celem niniejszych badań było określenie wpływu dolistnego aplikowania CHT na plonowanie i jakość owoców liczi. Chitozan w stężeniu: 0 (kontrola), 100, 250, 500 lub 1000 µg L^{-1} rozpylano na owoce i liście bezpośrednio po pojawieniu się owoców czterokrotnie w odstępach 7-dniowych. Mimo, że aplikacja CHT nie miała istotnego wpływu na wielkość owoców (długość i szerokość), całkowita zawartość związków fenolowych, flawonoidów i kwasu askorbiowego, jak również aktywność antyoksydacyjna owoców liczi wyraźnie wzrosła po zastosowaniu CHT w porównaniu do owoców nie poddanych działaniu tego związku. Najwyższe stężenia związków fenolowych, flawonoidów i kwasu askorbinowego, wynoszące odpowiednio: ekwiwalent 334 µg kwasu galusowego g^{-1} , ekwiwalent 881 µg kwercytyny g^{-1} i 178 µg g^{-1}, stwierdzono w owocach traktowanych 500 µg L^{-1} CHT. Jednak najwyższą aktywność antyrodnikową (622 µg butylohydroksytoluenu g^{-1}) stwierdzono w owocach traktowanych 250 µg L^{-1} CHT. Uzyskane wyniki wskazują, że zastosowanie niskich stężeń CHT w sadach liczi może poprawić jakość owoców poprzez zwiększenie w nich zawartości antyoksydantów oraz aktywności antyrodnikowej.