



## Article

# Improvement of Physiological Features and Essential Oil Content of *Thymus vulgaris* after Soil Amendment with Chitosan Nanoparticles under Chromium Toxicity

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**Abstract:** An excessive amount of chromium in soil has detrimental effects on plant processes, and impairs food security, and public health. The application of nanoparticles may be a suitable solution and an innovative strategy by which to reduce plant abiotic stresses and pollution in the agricultural ecosystems. This research focuses on the effects of chitosan nanoparticles (CS-NPs) on thyme (*Thymus vulgaris* L.) plants grown in Cr-contaminated soil. The effects of CS-NPs as a soil amendment at four concentrations were investigated on plant nutrient uptake, photosynthesis parameters, antioxidant system, and essential oil (EO) content under soil Cr stress. The results show that chromium stress reduced fresh and dry weight of shoots, the uptake of macro-, and micro-elements, chlorophyll and carotenoids. The application of CS-NPs improved the antioxidant enzyme activity, reduced malondialdehyde, and increased the content of nutrients, EOs, photosynthetic pigments, and chlorophyll fluorescence parameters. The intermediate dose of chitosan nanoparticles (0.1% *w/v*) best valorized the content and yield of thyme EOs under chromium stress. These results are indicative that the application of CS-NPs can represent a supportive approach for plant production in soils contaminated with heavy metals.

**Keywords:** sustainable management; soil pollution; heavy metals; plant stress tolerance; enzyme dynamics

## 1. Introduction

The consumption of medicinal plants in the pharmaceutical industry is on the rise [1], as these plants offer various ecosystem services, such as healthcare, local economic benefits, cultural value, and heritage, particularly in developing countries [2]. The Lamiaceae family is among the most diverse and extensive herbal families that have been investigated [3], including within the pharmaceutical field, due to the significant content of essential oils that the related plant species contain [4]. *Thymus vulgaris* L., commonly known as garden thyme, is an edible Lamiaceae herb grown in central and southern Europe, Africa, and Asia [5]. Thyme contains important components in its essential oils, including thymol, carvacrol, *p*-cymene, and  $\alpha$ -terpinene [6], which have numerous healthy properties, such as

antioxidant, anti-inflammatory, neurological and gastrointestinal properties [7,8]. Aerial parts and volatile components of thyme plants are commonly used in folk medicine to treat coughs, diabetes, colds, and chest infections [1]. This is owing to its recorded antiseptic, antibacterial, antifungal, antispasmodic, antitussive, expectorant, and analgesic characteristics, which are mainly attributed to the presence of the phenolic monoterpenes, thymol and carvacrol [8–10].

For the past 50 years, heavy metal pollution in farmland has become a global issue [11]. This is caused by human activities such as the output of specific industries, mining, and the incorrect use of agricultural products and practices [12,13]. Significant amounts of heavy metals are released particularly when, for example, using wastewater for irrigation, utilizing factory liquid waste, or when adding chemical fertilizers, pesticides, and sewage sludge [1,14,15]. These create unsuitable soil conditions, making agricultural activities difficult [16], and have the potential to kill microorganisms living in the soil [1,12]. Compounds containing chromium are commonly utilized in electroplating, metal finishing, the leather tanning industry, and the production of pigments [11]. There are two common oxidation states of chromium: Cr (III) and Cr (VI). Cr (VI) is more toxic due to its ability to oxidize other species and negatively impact human lungs, liver, and kidneys [17]. Furthermore, the existence of Cr can be extremely harmful to plants and can negatively impact their growth and development [16]. Excess amounts of Cr in plant tissues can cause a severe abiotic stress, resulting in various toxic effects on plant physiology, biochemistry, and morphology [18,19]. It can harm enzyme and pigment activity, hinder absorption, decrease assimilation, generate ROS, and cause membrane peroxidation [17,20]. Cr toxicity negatively influences plant growth and development, including growth inhibition, crop yield reduction, biochemical damage, enzyme changes, and decreased chlorophyll synthesis [11,16,21]. Zaheer et al. [19] have found that a high concentration of chromium leads to increased levels of malondialdehyde, hydrogen peroxide ( $H_2O_2$ ) and electrolyte leakage (EL), which results in oxidative damage to the roots and leaves of *S. oleracea*. Meanwhile, Kumar et al. [17] have reported that exposure to Cr (VI) affects the guard and epidermal cell structure, and photosynthetic pigments in *Helianthus annuus* L. Similarly, Barzin et al. [20] have observed that Cr stress negatively affects pot marigold plants, resulting in reduced photosynthetic pigments, soluble sugars, starch, mineral nutrients, height, and biomass.

Nanotechnology holds promise in addressing numerous agricultural and environmental challenges, while also serving as a valuable tool for managing energy and resource limitations, promoting sustainable resource utilization, mitigating urbanization impacts, and optimizing fertilizer management [16]. With the global population continuing to grow, there is an imperative for increased crop yields and more efficient agricultural practices. Hence, the utilization of nanomaterials in agriculture is gaining traction [1]. Nanoparticle (NP) compounds, in particular, show potential in advancing sustainable agriculture and precision farming by enhancing crop yields and reducing input requirements such as fertilizers, pesticides, and herbicides [17]. This is achieved through precise control of environmental parameters and effective management practices [16]. The unique properties of NPs, stemming from their small size and increased surface area, result in heightened solubility and surface reactivity when compared with bulk materials [1,17].

Several methods for removing heavy metals from high-salinity brine include reverse osmosis, ultrafiltration, coprecipitation, and ion exchange resins [18,22,23]. However, implementing these strategies is expensive [22,24]; therefore, researchers have been exploring more cost-effective and efficient ways to pre-concentrate and eliminate heavy metals to address this issue [25,26]. A cost-effective process for heavy metal remediation uses chitosan, an ordinary polysaccharide made up of D-glucosamine [23,27], which is a naturally occurring compound found in the cell walls of various organisms, including fungi, crabs, shrimp and insect exoskeletons [28]. In previous research [29], this compound was seen to recover the morpho-physiological appearances in agriculture and lessen the harmful effects of abiotic stresses across the stress pathway of transduction. Additionally, the utilization of chitosan as a bio-stimulant has been shown to have numerous benefits, enhancing photo-

synthesis and leaf biomass, boosting growth, and increasing the number of flowers and fruits, as well as the flowering time in diverse plant species. These findings demonstrate the potential of chitosan as a valuable tool for improving plant growth and development [26,30], based on its ability to immobilize metals in soil and reduce heavy metal levels by chelating specific ions [22,26,28]. Chitosan has hydroxyl and amino groups that allow it to make complexes with non-essential element ions, such as heavy metals [20,23]. Qu et al. [28] have found that chitosan prevents reactive oxygen species production, inhibits the degradation of antioxidant enzymes, and improves chlorophyll content and photosynthesis in maize plants under chromium stress. According to Shaheen et al. [27], chitosan exerts a significant reduction of the mobility and water-soluble concentrations of Ni and Zn in soil, which results in a noteworthy decrease of metals absorption by rapeseed.

However, most studies have focused on the application of chitosan through foliar spraying, and more research is needed that focuses on the effects of chitosan soil amendment on heavy metal deposit and tolerance in plants. Additionally, there is no information about the impact of chitosan nanoparticles on the nutrient absorption and essential oil content of thyme under chromium toxicity. Therefore, this study aimed to evaluate the effects of chitosan on nutrient uptake, the antioxidant defense system, the secondary metabolism, and Cr absorption and deposit in thyme plants supplemented with chromium.

## 2. Materials and Methods

### 2.1. Plant Materials, Growth Conditions, and Experimental Protocol

Research was carried out on common thyme (*T. vulgaris*), with plants provided by Pakan Bazr, Isfahan, Iran, in a controlled environment at the Research Greenhouse of the Faculty of Agriculture, Maragheh University, Iran, in 2022–2023. The experimental protocol was based on the factorial combination of four chitosan nanoparticle (NP) concentrations (0%, 0.05%, 0.1%, 0.2% *w/v*) and four concentrations of chromium in soil (0, 10, 20, and 40 mg Cr kg<sup>−1</sup> DM soil), using a split plot design with three repetitions. The thyme plants were grown in a clay–loam soil (Table 1), at 25 °C, 65% RH and a photoperiod of 8 h.

**Table 1.** Soil physicochemical features at 0–20 cm depth.

| Soil Texture | Sand (%) | Silt (%) | Clay (%) | Organic Matter (g kg <sup>−1</sup> ) | EC (ds m <sup>−1</sup> ) | pH   | Field Capacity (%) | Permanent Wilting Point (%) | Exchangeable Potassium (mg kg <sup>−1</sup> ) | Cation Exchange Capacity (Cmolc kg <sup>−1</sup> ) | Available Phosphorus (mg kg <sup>−1</sup> ) | Total Nitrogen (g kg <sup>−1</sup> ) |
|--------------|----------|----------|----------|--------------------------------------|--------------------------|------|--------------------|-----------------------------|-----------------------------------------------|----------------------------------------------------|---------------------------------------------|--------------------------------------|
| clay–loam    | 27       | 20       | 53       | 8.1                                  | 1.17                     | 7.43 | 27.1               | 13.7                        | 563.85                                        | 26.5                                               | 9.7                                         | 0.87                                 |

After sieving, the soil was transferred to a greenhouse, supplied with the four planned concentrations of chromium (0, 10, 20, and 40 mg Cr kg<sup>−1</sup> DM soil) and left for sixty days in incubation at 28 °C, with regular mixing to ensure the complete uniformity [31]. Then, the chitosan NPs were applied at four different concentrations (0%, 0.05%, 0.1%, and 0.2% *w/v*) into the soil mix in a plastic container, which was set aside for 15 days to make it stable, watered to 75% of field capacity, left in a dark room, and incubated at 25 °C for the specified duration. Homogenous thyme plants, 20–25 cm high, were then planted in 5 L pots, grown for two months, and fertigated daily with half-strength Hoagland’s nutrient solution during the initial growth stage, maintaining 80% of its field capacity. Weeds were removed manually.

### 2.2. Chitosan Nanoparticles

#### 2.2.1. Materials

Chitosan with a molecular weight of 100 ± 20 kD and a degree of deacetylation of 80 ± 5% was purchased from Sabz Gostaresh Azin Turkan Company, Maragheh, Iran. Analytical acetic acid and sodium tripolyphosphate (TPP) grades were provided by Mojallali Co. (Tehran, Iran) and used without purification.

### 2.2.2. Instruments

The measurement of the hydrodynamic size of chitosan nanoparticles was undertaken using dynamic light scattering (DLS) by diluting the nanoparticle solutions up to 50 times and recording the sizes on DLS/Zeta, Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK. The sample transition electron microscopy (TEM) was recorded by TEM Philips CM10 (Amsterdam, The Netherlands) operating at 60 kV. The chemical structures of chitosan and dried chitosan nanoparticles were identified using Fourier transform infrared (FT-IR) in KBr pellets (Bruker 113V FT-IR, Billerica, MA, USA). In order to understand any changes in the crystallinity of chitosan during nanoparticle formation, the XRD patterns of chitosan and dried chitosan nanoparticles were recorded by the Siemens D-500 X-ray diffractometer (Munich, Germany).

### 2.2.3. Synthesis of Chitosan Nanoparticles

The preparation of chitosan nanoparticles was completed using the gelation method, as previously reported [32]. To study the effect of chitosan nanoparticles, three concentrations were prepared (0.05%, 0.10%, and 0.20%, referring to the weight), based on the used chitosan content. To prepare 2 L of the mentioned concentrations of chitosan nanoparticles, the corresponding weight of chitosan was added into one weight % of the acetic acid solution under continuous stirring until the chitosan was completely dissolved. To reach chitosan nanoparticles through the gelation method, sodium tripolyphosphate (TPP) solution was used as a gelation agent. The amount of TPP was a 1/2.5 ratio of the used chitosan (0.02, 0.04, and 0.08 g dissolved in 5 mL of distilled water for solutions containing 0.05, 0.1, and 0.2 g of chitosan, respectively) [33]. While the chitosan solutions were strongly stirred, TPP solutions were slowly dropped into corresponding chitosan solutions. Solutions that were cloudy, indicating the presence of chitosan nanoparticles, were left to be stirred for 30 min. The resultant nanoparticle solutions were kept and used without any treatment.

### 2.3. Chlorophyll Fluorescence Parameters

A pulse amplitude modulation fluorometer (PAM-2500, Heinz Walz, Effeltrich, Germany) was used to determine the chlorophyll fluorescence parameters of randomly selected, fully developed young leaves. The leaves were adapted in darkness for 20 min prior to the measurements and the obtained data were processed using PamWin-3 software V4.02m [34].

### 2.4. Plant Sampling and Analysis

Plant height, dry (DW) and fresh (FW) weights were measured with three replications, during the harvest stage when the inflorescence was visible. After determining the fresh weight, the dry weight was obtained by drying samples of inflorescences, leaves, and shoots in an oven at 70 °C for 72 h.

### 2.5. Root, Leaf and Soil Cr Content

Leaves and roots of thyme plant samples were collected and washed with deionized water to assess the Cr concentration. They were then dried in an oven at 65 °C for 48 h and ground into a powder. One gram of powdered sample was digested in HNO<sub>3</sub>/HClO<sub>4</sub> (10 mL) at 100 °C and heated in a furnace (550 °C) for 5 h to obtain ash, which was then put in 2 N HCl (10 mL) and filtered. The Cd content was determined using an atomic absorption spectrometer (Shimadzu, AA6300, Tokyo, Japan) [35].

### 2.6. Determination of Photosynthetic Pigments

To measure the Chl a, Chl b, and CARs levels in thyme leaves, 0.5 g of leaves were mixed with 10 mL of 80% acetone, frozen, and centrifuged at 10,000 rpm. The supernatants were then measured at 645, 663, and 470 nm by a spectrophotometer. Finally, the content of photosynthetic pigments was calculated [36].

### 2.7. Elemental Composition

The Kjeldahl method [37] was utilized to measure the N content of thyme leaves, whereas to assess the concentration of P and K, 1 g of dried thyme leaves was placed in a porcelain crucible and heated in a muffle furnace at a gradually increasing temperature of 550 °C. The resulting solution was brought to a volume of 50 mL and left to stand for 30 min. Flame photometry (Jenway PFP7C, London, UK) was used to determine K concentration, whereas P concentration was detected by a spectrophotometer at 470 nm absorption spectra by the ammonium vanadate/molybdate colorimetric method [38]. The atomic absorption spectroscopy (Shimadzu, AA6300, Tokyo, Japan) was employed to assess the contents of Mn, Zn, and Fe [39].

### 2.8. Total Soluble Carbohydrates Content (TSC)

To determine TSC content, 0.2 g of fresh leaves was extracted using 10 mL of 95% ethanol for 1 h at 80 °C and centrifuged at 16,000 rpm for 10 min, with 500 µL of phenol and 5 mL of 98% sulfuric acid added to 1000 µL of the supernatant. The absorption was measured at 483 nm wavelength and TSC content was recorded as mg·g<sup>-1</sup> fresh weight [40].

### 2.9. Total Protein Content (TSP)

The standard bovine serum albumin (BSA) protein was used to determine the TSP, according to the Bradford method [41]. One gram of fresh thyme leaves was extracted with 4 mL of a 50 mM phosphate buffer solution, then the mixture was centrifuged at 12,000 rpm for 15 min. Next, 50 µL of the extract was added to 1000 µL Bradford solution (Merck, Darmstadt, Germany) and the absorbance was measured after 5 min at 595 nanometers.

### 2.10. Essential Oil Content (EOC) and Yield (EOY)

The extraction of essential oil (EO) from fresh thyme plant samples was completed by a Clevenger-type apparatus (British Pharmacopoeia model) for 3 h. During the extraction, the samples were kept in the shade to prevent any alteration of the EOs composition or quality. The extraction continued until the samples reached a constant weight. Once the extraction was complete, the EOs were dehydrated using anhydrous sodium sulfate to remove any remaining water content that might affect their purity or stability. Finally, the EO was stored in a glass vial at a temperature of 4 °C to preserve its quality. The yield (EOY) was measured based on the dry weight of the samples per pot, which was a critical factor in determining the concentration and quality of the EO [42].

$$\text{EOC} = (\text{EO weight/sample DM}) \times 100; \text{EOY} = (\text{Total DM (g pot}^{-1}) \times \text{EOC})/100$$

### 2.11. Malondialdehyde

For MDA determination, 0.5 g of fresh leaf samples was ground with 1500 µL TCA and centrifuged at 12,000 rpm for 10 min at 4 °C. Then, 1000 µL TBA was added to 500 µL supernatant, heated at 95 °C for 30 min, and cooled with ice. The absorbance was measured at 532 nm and 600 nm. MDA content was expressed in nmol g<sup>-1</sup> fresh weight [43].

### 2.12. Electrolyte Leakage (EL)

Leaf discs of 0.5 cm diameter were used and thoroughly washed with deionized water to measure electrolyte leakage. The leaf samples were left at room temperature for 24 h, after which the initial electrical conductivity of the solution (EC1) and the electrical conductivity that resulted from the incubation in a water bath at 95 °C for 20 min (EC2) were measured at 25 °C. The EL was expressed as a percentage of the total conductivity.

### 2.13. Catalase (CAT)

Catalase activity was strictly determined at 25 °C using UV-vis spectrophotometry. The reaction mixture was made up of 1.5 mL of 0.05 M sodium phosphate buffer (pH = 7.8), 1 mL of deionized water, 0.3 mL of 0.1 M H<sub>2</sub>O<sub>2</sub>, and 0.2 mL of enzyme extract. The catalase



activity was precisely calculated by monitoring the absorbance at 240 nm due to  $\text{H}_2\text{O}_2$  consumption and was presented as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein [44].

#### 2.14. Ascorbate Peroxidase (APX) Activity

To determine the APX activity, the reaction mixture was prepared using 100 mM phosphate buffer (100 mM along with EDTA at pH 7.7),  $\text{H}_2\text{O}_2$  (2 mM), sodium ascorbate (0.5 mM) and the extract (50  $\mu\text{L}$ ). The absorbance was measured at 290 nm for 180 s according to the method described by Nakano and Asada [45] and the APX activity was expressed in  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein.

#### 2.15. Peroxidase (POX) Activity

A reaction mixture of phosphate buffer, pyrogallol,  $\text{H}_2\text{O}_2$ , and enzyme extract was incubated to determine POX activity at 25 °C for 5 min. The reaction was stopped with  $\text{H}_2\text{SO}_4$ , the absorbance was measured at 420 nm, and the POX activity was expressed as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein [46].

#### 2.16. Glutathione Reductase (GR) Activity

To track the behavior of glutathione reductase, a mixture was created that contained 200 mM phosphate buffer (pH 7.5), 6.3 mM EDTA, 3 mM 5,50-dithiobis-2-benzoic acid (DTNB), 2 mM NADPH, and 100  $\mu\text{L}$  of the extract. The reaction was initiated by adding glutathione oxide (2 mM), and the absorbance was documented at 412 nm for 120 s and expressed as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein [47].

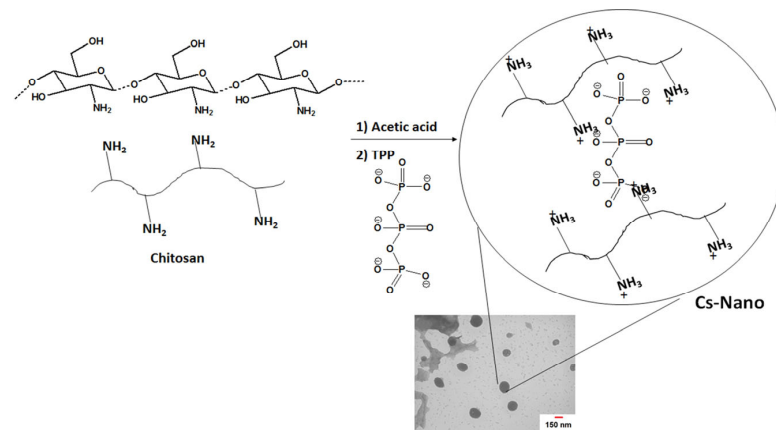
#### 2.17. Data Analysis

The data with three replications were statistically processed by the analysis of variance using the SAS software (version 9.3) and mean separation was performed through the LSD test at 0.05 probability level. Pearson's correlation and the cluster dendrogram heatmap analysis were undertaken by R software (version 4.3.1), Iran (2021) (URL <https://cran.um.ac.ir/>, accessed on 10 September 2023).

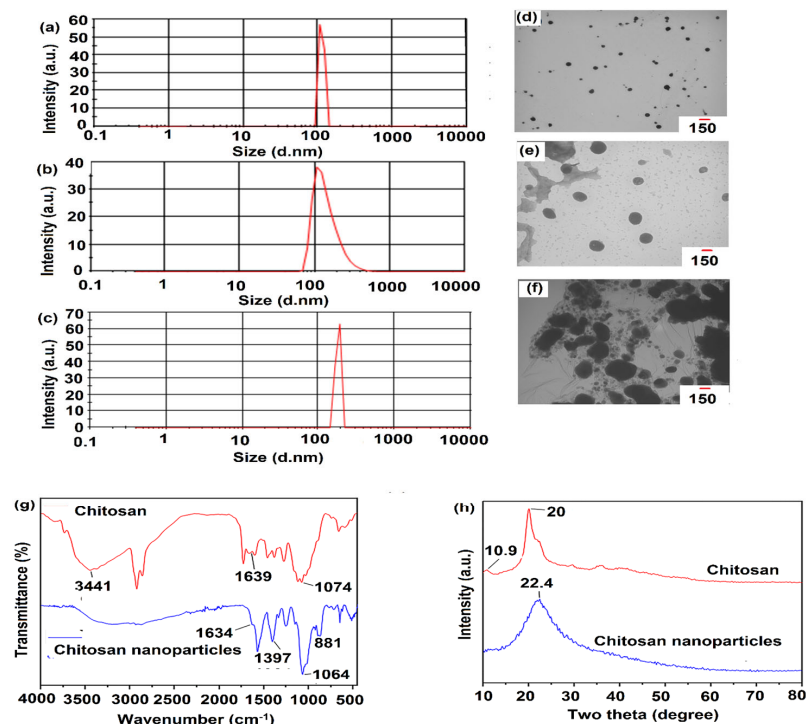
### 3. Results

#### 3.1. Synthesis and Characterization

The procedure used to prepare chitosan nanoparticles is shown in a simple scheme in Figure 1. Dissolving chitosan in an acetic acid solution results in positive  $-\text{NH}_3^+$  free groups on chitosan with the ability to complex with anionic materials. Sodium tripolyphosphate (TPP) is the common ingredient used to obtain chitosan nanoparticles through gelation. TPP consists of five anionic centers that can easily interact electrostatically with  $-\text{NH}_3^+$  pendants on chitosan to obtain chitosan nanoparticles. DLS and TEM techniques were applied to explore the impact of chitosan concentration on the diameters of nanoparticles. Moreover, the chemical structure and crystallinity of chitosan were investigated by FTIR and XRD techniques. The average sizes of chitosan nanoparticles were 114, 149, and 189 nm for CS-Nano1, CS-Nano2, and CS-Nano3, respectively. In fact, by increasing the concentration of chitosan, the size of nanoparticles tends to rise. This was previously reported by Ribeiro et al. [48], who found that the size of chitosan-TPP nanoparticles increases as the chitosan concentration rises. According to the TEM micrographs, the chitosan nanoparticles were spherical at all the used concentrations of chitosan (Figure 2a–h). However, at 0.2% of nanoparticles, the spherical morphology was obtained irregularly, compared with the lower chitosan concentrations. The average diameters of CS-NPs<sub>0.05</sub>, CS-NPs<sub>0.1</sub>, and CS-NPs<sub>20.2</sub> obtained were 95, 135, and 165 nm, respectively. The diameters of nanoparticles differed from DLS results, originating from the interaction between nanoparticles in solution during DLS recording. Similarly, it has been observed in chitosan/hyaluronic acid/ê-carrageenan nanocarriers that the diameter of nanoparticles obtained by DLS is more significant than that obtained by TEM [49].



**Figure 1.** A simple procedure to produce chitosan/TPP nanoparticles.



**Figure 2.** DLS graphs of (a) CS-Nano1, (b) CS-Nano2, and (c) CS-Nano3; TEM images of (d) CS-Nano1, (e) CS-Nano2, and (f) CS-Nano3; (g) FTIR spectra of chitosan and CS-Nano2 nanoparticles; and (h) XRD patterns of chitosan and CS-Nano2 nanoparticles.

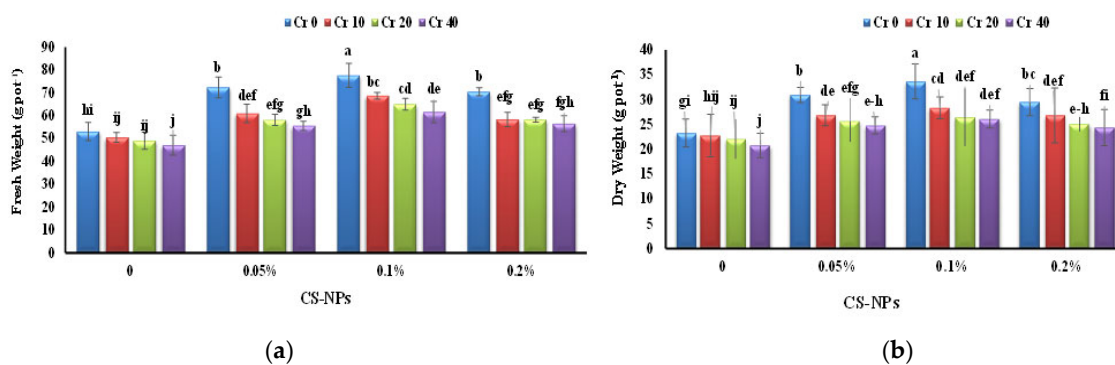
The characterization of the structure of neat chitosan and chitosan nanoparticles (CS-Nano2) was undertaken by FTIR, in order to understand the changes in chitosan structure during its complexing with TPP. A broad band at around 3500 cm<sup>-1</sup> in the FTIR spectra of both chitosan and CS-Nano2 indicates the stretching vibration of the –OH functional group. In the chitosan spectrum, the peak at 1074 cm<sup>-1</sup> can be attributed to the pyranose ring in the chitosan structure, and the peaks at 1639 and 1560 cm<sup>-1</sup> of the C=O stretching (amid I) and N–H bending can be attributed to the –NH<sub>2</sub> groups on the chitosan backbones.

In the spectrum of CS-Nano nanoparticles, a new and sharp peak at 1064 cm<sup>-1</sup> appeared. This is due to the antisymmetric stretching of the P–O–P of the TPP component in the nanoparticles' composition. A shifting in the characteristic bands of chitosan occurred, and this can be assigned to the electrostatic interactions between positively charged chitosan and negatively charged TPP. Similar results in the shifting of frequencies of chitosan functional groups due to the interaction with TPP originating from electrostatic interactions and hydrogen bonding have been reported [50,51]. The XRD and the results relevant

to the effect of introducing TPP on the change of the chitosan crystallinity are shown in Figure 1. In the XRD pattern of chitosan, a sharp peak at  $2\theta = 20^\circ$  is related to the semi-crystalline structure of chitosan [50]. When chitosan was complexed with TPP to produce nanoparticles, the characteristic chitosan peak disappeared, and a broad peak at  $2\theta = 22.4^\circ$  appeared, showing the common peak of amorphous polysaccharides (Figure 2a–h). The crystallinity of chitosan originates from hydrogen bonding between  $-\text{NH}_2$  and  $-\text{OH}$  groups on chitosan backbones. When chitosan is dissolved in acetic acid solution by protonation of amine groups, the hydrogen bonding between chitosan chains is disrupted and this results in dissolution in solution. By introducing TPP to produce chitosan nanoparticles, the closing chitosan backbones that form crystalline points are missed. By introducing TPP into the chitosan solution to produce nanoparticles, the possibility of the chains approaching each other is disrupted, resulting in the formation of an amorphous structure. In fact, with the introduction of TPP between chitosan chains, the possibility of forming chitosan crystal points is greatly reduced. A similar observation has been reported by Soleymannfallah et al. [52], indicating the amorphous structure of chitosan/TPP nanoparticles due to the use of acetic acid and TPP ingredients.

### 3.2. Fresh Weight (FW) and Dry Weight (DW) of Shoots

Shoot FW and DW were significantly affected by CS-NPs, Cr stress, and their interaction (Table 2). In the present study, Cr toxicity had detrimental influence on shoot FW and DW. The lowest values of shoot FW and DW were found to be associated with the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment, which reduced the values of FW by 12.7% (Figure 3a) and DW by 10.6% (Figure 3b) when compared with the control. CS-NPs reduced the adverse effects of Cr toxicity and, with respect to the control plants, the FW and DW of the shoot attained the highest increase by 46.3% and 44.7%, respectively, under CS-NPs<sub>0.1</sub> application with no Cr pollution. The latter treatment increased shoot FW and DW content, compared with CS-NPs<sub>0</sub>-Cr<sub>40</sub>, by 64.8 and 62.1%, respectively (Figure 2).



**Figure 3.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress on shoot fresh weight (a) and dry weight (b) of thyme plants. Cr was applied at the concentrations of 0, 10, 20 and 40 mg Cr kg<sup>-1</sup> soil. Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

**Table 2.** ANOVA related to the effects of chitosan nanoparticles (CS-NPs) and chromium (Cr) stress on fresh weight (FW), dry weight (DW), essential oil content (EOC), essential oil yield (EOY), total protein content (TSP), and total soluble carbohydrates (TSC) of thyme plants.

| S.O.V.      | df | Shoot Fresh Weight | Shoot Dry Weight | Essential Oil Content | Essential Oil Yield | Total Soluble Protein | Total Soluble Carbohydrate |
|-------------|----|--------------------|------------------|-----------------------|---------------------|-----------------------|----------------------------|
| chromium    | 4  | **                 | **               | *                     | **                  | **                    | *                          |
| CS-NPs      | 4  | **                 | **               | *                     | **                  | *                     | *                          |
| Cr × CS-NPs | 16 | **                 | **               | *                     | **                  | **                    | *                          |
| LSD         |    | 2.48               | 4.60             | 0.048                 | 0.022               | 0.186                 | 0.465                      |

\*: significant at  $p < 0.05$ ; \*\*: significant at  $p < 0.01$ .



### 3.3. Chlorophyll Fluorescence Indices

The fluorescence parameters, including  $F_0$ ,  $F_m$ ,  $F_v/F_m$ ,  $F_v$ , and  $F_0/F_v$  ratios, were significantly affected by the Cr pollution and the application of CS-NPs (Table 3). The highest values of  $F_0$  and  $F_0/F_v$  were found under the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment (Table 3), while the highest levels of the  $F_m$ ,  $F_v/F_m$ , and  $F_v$  were related to the CS-NPs<sub>0.1</sub>-Cr<sub>0</sub> treatment.  $F_0$  and  $F_0/F_v$  reached 37.8 and 70.1% with the CS-NPs<sub>0</sub>-Cr<sub>20</sub> treatment, respectively, compared with the control plants (Table 3). The CS-NPs<sub>0.1</sub>-Cr<sub>0</sub> treatment increased  $F_m$ ,  $F_v/F_m$ , and  $F_v$  by 15.9, 2.9, and 20.5%, respectively, compared with control, and by 24.7, 19.1, and 48.5%, respectively, when compared with the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment (Table 3).

**Table 3.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress treatments on the content of photosynthetic pigments and chlorophyll fluorescence indices of thyme plants.

| CS-NPs (%)          | Cr (mg kg <sup>-1</sup> ) | Chl a (mg g <sup>-1</sup> FW) | Chl b (mg g <sup>-1</sup> FW) | Chl T (a + b) (mg g <sup>-1</sup> FW) | CARs (mg g <sup>-1</sup> FW) | $F_0$    | $F_m$   | $F_v/F_m$ | $F_v$   | $F_0/F_v$ |
|---------------------|---------------------------|-------------------------------|-------------------------------|---------------------------------------|------------------------------|----------|---------|-----------|---------|-----------|
| 0                   | 0                         | 36.96 e-h                     | 21.74 efg                     | 58.70 gh                              | 21.25 def                    | 0.651 kl | 3.14 gh | 0.792 c   | 2.49 f  | 0.261 h   |
|                     | 10                        | 33.43 ij                      | 21.03 fgh                     | 54.46 i                               | 20.55 efg                    | 0.728 h  | 3.08 hi | 0.763 e   | 2.35 g  | 0.309 f   |
|                     | 20                        | 32.33 jk                      | 20.36 gh                      | 52.69 ij                              | 19.32 h                      | 0.638 lm | 2.88 k  | 0.724 h   | 2.08 i  | 0.381 c   |
|                     | 40                        | 31.36 k                       | 19.96 h                       | 51.32 j                               | 18.65 h                      | 0.897 a  | 2.92 k  | 0.692 i   | 2.02 i  | 0.444 a   |
| 0.05                | 0                         | 39.62 bc                      | 24.26 bc                      | 63.89 bc                              | 22.62 bcd                    | 0.643 lm | 3.35 e  | 0.808 b   | 2.71 e  | 0.237 i   |
|                     | 10                        | 38.09 c-f                     | 22.36 def                     | 60.46 def                             | 22.22 b-e                    | 0.703 i  | 3.19 g  | 0.779 d   | 2.49 f  | 0.282 g   |
|                     | 20                        | 37.09 e-g                     | 21.83 ef                      | 58.89 efg                             | 21.92 cde                    | 0.674 j  | 2.99 j  | 0.741 g   | 3.22 h  | 0.349 d   |
|                     | 40                        | 35.46 gh                      | 21.43 fg                      | 56.89 h                               | 21.65 c-f                    | 0.859 b  | 3.04 ji | 0.717 h   | 2.18 h  | 0.394 b   |
| 0.1                 | 0                         | 41.86 a                       | 26 a                          | 67.86 a                               | 24.72 a                      | 0.795 e  | 3.64 a  | 0.824 a   | 3 a     | 0.212 j   |
|                     | 10                        | 40.69 ab                      | 24.86 ab                      | 65.56 ab                              | 23.95 bc                     | 0.775 f  | 3.53 c  | 0.809 b   | 2.85 cd | 0.236 i   |
|                     | 20                        | 38.36 c-f                     | 23 cde                        | 61.36 de                              | 23.12 abc                    | 0.759 g  | 3.44 d  | 0.800 d   | 2.68 e  | 0.282 g   |
|                     | 40                        | 38.86 b-e                     | 22.16 def                     | 61.02 def                             | 22.78 bcd                    | 0.827 c  | 3.15 g  | 0.737 g   | 2.32 g  | 0.356 d   |
| 0.2                 | 0                         | 39.16 bcd                     | 23.43 cd                      | 62.59 cd                              | 21.02 d-g                    | 0.636 m  | 3.54 bc | 0.820 a   | 2.91 bc | 0.218 j   |
|                     | 10                        | 37.42 d-g                     | 22.24 def                     | 59.66 efg                             | 20.68 efg                    | 0.661 jk | 3.63 ab | 0.817 a   | 2.96 ab | 0.222 j   |
|                     | 20                        | 36.52 fgh                     | 21.13 fgh                     | 57.66 gh                              | 20.42 e-h                    | 0.740 h  | 3.57 bc | 0.792 c   | 2.83 d  | 0.261 h   |
|                     | 40                        | 35.32 hi                      | 21.81 ef                      | 57.12 h                               | 20.05 fgh                    | 0.809 d  | 3.27 f  | 0.752 f   | 2.46 f  | 0.329 e   |
| LSD                 |                           | 2.01                          | 1.39                          | 2.34                                  | 1.18                         | 0.014    | 0.065   | 0.007     | 0.072   | 0.013     |
| Significance levels |                           |                               |                               |                                       |                              |          |         |           |         |           |
| Cr                  | **                        | **                            | **                            | *                                     | *                            | *        | **      | **        | **      | **        |
| CS-NPs              | *                         | **                            | **                            | **                                    | **                           | *        | **      | **        | **      | *         |
| Cr × CS-NPs         | *                         | **                            | **                            | **                                    | **                           | *        | **      | **        | **      | **        |

CS-NPs: chitosan nanoparticles; Cr: chromium; \*, significant at  $p < 0.05$ , \*\*: significant at  $p < 0.01$ . Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

### 3.4. Root, Leaf and Soil Cr Content

Cr content in soil, roots, and shoots of thyme plants increased under Cr-stress conditions (Table 4). The highest Cr content of underground and aerial parts was related to the 40 mg kg<sup>-1</sup> Cr treatment without chitosan application, which was higher by 234.2 and 169.1%, respectively, compared with the control. However, all concentrations of CS-NPs caused a significant decrease of Cr concentration in the roots and shoots, although the CS-NPs<sub>0.1</sub> treatment was much more effective than other concentrations. In this respect, the CS-NPs<sub>0.1</sub> treatment in the presence of 10, 20, and 40 mg kg<sup>-1</sup> of Cr, compared with the absence of CS-NPs, reduced Cr concentration in the roots by 53.4, 35.8, and 14.7%, and in the shoots by 37.1, 19.3, and 17.8%, respectively (Table 4). In addition, at all three concentrations of Cr (10, 20, and 40 mg kg<sup>-1</sup>), the utilization of CS-NPs significantly reduced Cr content in the soil (Table 4). The highest soil Cr concentration was recorded under the Cr<sub>40</sub> treatment. However, the application of CS-NPs<sub>0.1</sub>, in combination of 10, 20, and 40 mg kg<sup>-1</sup> Cr, reduced the content of Cr by 27.9, 17.6, and 11.3%, respectively, when compared with the untreated control (Table 4).

**Table 4.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) concentration on plant and soil Cr content.

| CS-NPs (%)          | Cr (mg kg <sup>-1</sup> Soil) | Soil Cr Content after Harvest (mg kg <sup>-1</sup> Soil) | Root Cr Content (mg kg <sup>-1</sup> DW) | Shoot Cr Content (mg kg <sup>-1</sup> DW) |
|---------------------|-------------------------------|----------------------------------------------------------|------------------------------------------|-------------------------------------------|
| 0                   | 0                             | 1.53 j                                                   | 0.76 hi                                  | 1.23 hi                                   |
|                     | 10                            | 8.52 g                                                   | 1.89 e                                   | 2.53 de                                   |
|                     | 20                            | 17.41 e                                                  | 2.37 b                                   | 2.9 bc                                    |
|                     | 40                            | 36.23 a                                                  | 2.54 a                                   | 3.31 a                                    |
| 0.05                | 0                             | 1.483 j                                                  | 0.71 ij                                  | 1.19 hij                                  |
|                     | 10                            | 5.48 hi                                                  | 1.17 g                                   | 1.68 g                                    |
|                     | 20                            | 15.72 e                                                  | 2.07 d                                   | 2.64 cde                                  |
|                     | 40                            | 24.63 c                                                  | 2.32 bc                                  | 3.08 ab                                   |
| 0.1                 | 0                             | 1.01 j                                                   | 0.58 j                                   | 0.86 j                                    |
|                     | 10                            | 4.35 i                                                   | 0.88 h                                   | 1.59 g                                    |
|                     | 20                            | 11.89 f                                                  | 1.52 f                                   | 2.34 ef                                   |
|                     | 40                            | 21.25 d                                                  | 2.18 cd                                  | 2.72 cd                                   |
| 0.2                 | 0                             | 1.19 j                                                   | 0.63 ij                                  | 1.08 ij                                   |
|                     | 10                            | 7.22 gh                                                  | 1.07 g                                   | 1.48 gh                                   |
|                     | 20                            | 12.98 f                                                  | 1.66 f                                   | 2.07 f                                    |
|                     | 40                            | 29.88 b                                                  | 2.26 bc                                  | 2.73 cd                                   |
| LSD                 |                               | 2.40                                                     | 0.15                                     | 0.33                                      |
| Significance levels |                               |                                                          |                                          |                                           |
| Cr                  |                               | **                                                       | **                                       | **                                        |
| CS-NPs              |                               | **                                                       | **                                       | **                                        |
| Cr × CS-NPs         |                               | **                                                       | **                                       | **                                        |

CS-NPs: chitosan nanoparticles; Cr: chromium; \*\*: significant at  $p < 0.01$ . Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

### 3.5. Photosynthetic Pigment Content

Chl a, b and total, as well as CARs, were significantly influenced by CS-NPs, Cr and their interaction (Table 3). Cr toxicity decreased the photosynthetic pigments content, with the lowest recorded in the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment, which reduced the content of chlorophyll a, b, total, and CARs by 15.1%, 8.1%, 12.5%, and 12%, respectively, when compared with the control (Table 3). The application of CS-NPs considerably increased the content of Chl a, b, total, and CARs, which reached the highest values under the CS-NPs<sub>0.1</sub>-Cr<sub>0</sub> treatment (Table 3). In addition, CS-NPs<sub>0.1</sub>-Cr<sub>0</sub> enhanced the content of Chl a, b, total, and CARs by 34, 30, 33 and 32%, respectively, when compared with CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment, and by 13.2, 19.5, 17.6, and 16.3%, respectively, when compared with control (Table 3).

### 3.6. Shoot and Root Macro- and Micronutrient Content

The CS-NPs and Cr pollution significantly impacted N, P, and K content in shoots and roots (Table 5). Indeed, the content of N, P, and K in the shoots was reduced as levels of Cr increased, with the lowest concentrations (−19.6, −21.4, and −15.3%, respectively, compared with the untreated control) under the treatment of 40 mg kg<sup>-1</sup> Cr with no CS-NPs application. The highest concentrations of N, P, and K were recorded under the treatment CS-NPs<sub>1</sub> without Cr pollution (+15.8, +34.2, and +18.1%, respectively, compared with the untreated control), and the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment increased the contents of N, P, and K by 44.2, 72.6, and 39.5%, respectively (Table 5).

**Table 5.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress treatment on the content macro- and micro-nutrient content in thyme plants.

| CS-NPs (%)  | Cr (mg kg <sup>-1</sup> ) | Shoot N (%) | Root N (%) | Shoot P (%) | Root P (%) | Shoot K (%) | Root K (%) | Shoot Fe mg g <sup>-1</sup> DM | Root Fe mg g <sup>-1</sup> DM | Shoot Zn mg g <sup>-1</sup> DM | Root Zn mg g <sup>-1</sup> DM | Shoot Mn mg g <sup>-1</sup> DM | Root Mn mg g <sup>-1</sup> DM |
|-------------|---------------------------|-------------|------------|-------------|------------|-------------|------------|--------------------------------|-------------------------------|--------------------------------|-------------------------------|--------------------------------|-------------------------------|
| 0           | 0                         | 2.34 cde    | 2.66 def   | 0.606 g–i   | 0.891 b–f  | 1.326 ef    | 1.334 cd   | 0.339 gh                       | 0.691 e–i                     | 0.230 d–g                      | 0.258 fg                      | 0.124 def                      | 0.137 fg                      |
|             | 10                        | 2.21 efg    | 2.28 hi    | 0.546 jk    | 0.643 gh   | 1.227 gh    | 1.083 ef   | 0.320 ij                       | 0.600 ijk                     | 0.211 ij                       | 0.240 hi                      | 0.118 efg                      | 0.124 hi                      |
|             | 20                        | 2.06 gh     | 2.31 hi    | 0.536 k     | 0.626 gh   | 1.146 ij    | 1.033 f    | 0.310 jk                       | 0.547 jk                      | 0.198 k                        | 0.232 i                       | 0.104 hi                       | 0.117 ij                      |
|             | 40                        | 1.88 i      | 2.11 i     | 0.476 L     | 0.611 h    | 1.123 j     | 1.031 f    | 0.301 k                        | 0.497 k                       | 0.191 k                        | 0.212 j                       | 0.098 i                        | 0.111 j                       |
| 0.05        | 0                         | 2.51 bc     | 3.06 bc    | 0.756 b     | 1.01 bc    | 1.473 bc    | 1.467 bc   | 0.384 bc                       | 0.841 abc                     | 0.245 bc                       | 0.280 abc                     | 0.143 b                        | 0.157 bc                      |
|             | 10                        | 2.33 cde    | 2.71 de    | 0.661 def   | 0.901 b–e  | 1.36 de     | 1.396 bcd  | 0.367 de                       | 0.757 c–g                     | 0.234 def                      | 0.273 cde                     | 0.129 cde                      | 0.141 def                     |
|             | 20                        | 2.26 def    | 2.56 efg   | 0.593 g–j   | 0.749 e–h  | 1.301 efg   | 1.303 cd   | 0.351 fg                       | 0.701 d–i                     | 0.223 gh                       | 0.266 def                     | 0.121 efg                      | 0.134 fg                      |
|             | 40                        | 2.11 fgh    | 2.43 fgh   | 0.556 ijk   | 0.861 c–f  | 0.121 hi    | 1.251 de   | 0.342 gh                       | 0.634 hij                     | 0.219 hgi                      | 0.249 gh                      | 0.113 fgh                      | 0.130 gh                      |
| 0.1         | 0                         | 2.71 a      | 3.45 a     | 0.813 a     | 1.18 a     | 1.566 a     | 1.701 a    | 0.410 a                        | 0.903 a                       | 0.258 a                        | 0.291 a                       | 0.159 a                        | 0.172 a                       |
|             | 10                        | 2.53 b      | 3.21 b     | 0.710 bcd   | 1.04 ab    | 1.517 ab    | 1.556 ab   | 0.396 ab                       | 0.884 ab                      | 0.251 ab                       | 0.285 ab                      | 0.145 ab                       | 0.159 b                       |
|             | 20                        | 2.42 bcd    | 2.96 bc    | 0.673 cde   | 0.851 e–f  | 1.431 cd    | 1.471 bc   | 0.374 cd                       | 0.789 b–e                     | 0.237 cde                      | 0.278 bcd                     | 0.134 bcd                      | 0.148 cde                     |
|             | 40                        | 2.30 de     | 2.71 de    | 0.683 cde   | 0.933 bcd  | 1.316 ef    | 1.384 bcd  | 0.358 ef                       | 0.776 b–f                     | 0.228 efg                      | 0.271 cde                     | 0.128 cde                      | 0.143 def                     |
| 0.2         | 0                         | 2.32 de     | 2.85 cd    | 0.726 bc    | 1.03 bc    | 1.426 cd    | 1.426 bcd  | 0.369 de                       | 0.810 bcd                     | 0.242 bcd                      | 0.274 b–e                     | 0.137 bc                       | 0.151 bcd                     |
|             | 10                        | 2.25 def    | 2.51 e–h   | 0.641 efg   | 0.832 def  | 1.341 ef    | 1.350 cd   | 0.361 def                      | 0.724 d–h                     | 0.225 fgh                      | 0.265 ef                      | 0.125 c–f                      | 0.139 efg                     |
|             | 20                        | 2.17 efg    | 2.41 gh    | 0.613 fgh   | 0.783 d–g  | 1.269 fgh   | 1.267 d    | 0.349 fg                       | 0.654 g–i                     | 0.215 hi                       | 0.259 fg                      | 0.123 def                      | 0.135 fg                      |
|             | 40                        | 1.97 hi     | 2.45 fgh   | 0.571 h–k   | 0.730 fgh  | 1.231 gh    | 1.276 d    | 0.334 hi                       | 0.667 f–i                     | 0.201 jk                       | 0.252 gh                      | 0.110 ghi                      | 0.131 gh                      |
| LSD         |                           | 0.174       | 0.241      | 0.053       | 0.165      | 0.078       | 0.183      | 0.014                          | 0.111                         | 0.011                          | 0.12                          | 0.013                          | 0.010                         |
| Cr          |                           | **          | **         | *           | *          | *           | **         | **                             | **                            | **                             | *                             | *                              | **                            |
| CS-NPs      |                           | **          | **         | **          | **         | *           | *          | **                             | **                            | *                              | **                            | *                              | **                            |
| Cr × CS-NPs |                           | **          | **         | **          | **         | *           | **         | **                             | **                            | *                              | **                            | *                              | **                            |

CS-NPs: Chitosan nanoparticles; Cr: chromium; \*: significant at  $p < 0.05$ ; \*\*: significant at  $p < 0.01$ . Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

In addition, the concentration of N, P, and K in the roots decreased significantly under Cr toxicity, but their content was augmented with the use of CS-NPs (Table 5). The highest contents of N, P, and K were recorded with the application of CS-NPs<sub>0.1</sub> without Cr stress (+29.7, +30.2, and +65%, respectively, compared with the untreated control). The lowest concentrations of the mentioned parameters were observed at 40 mg kg<sup>-1</sup> Cr with no CS-NPs (−20.7%, −31.4%, and −22.8%, respectively, in comparison with the untreated control) (Table 5).

Zn, Fe, and Mn in thyme shoots and roots were significantly influenced by Cr pollution and CS-NP application (Table 5). In this respect, Cr stress considerably decreased the Fe, Zn and Mn contents in the shoots, while the application of CS-NPs increased the amount of the mentioned nutrients by reducing the undesirable impacts of chromium stress (Table 5). The lowest concentrations of Fe, Zn and Mn in the aerial parts of thyme plants were found under 40 mg kg<sup>-1</sup> of Cr stress without CS-NP treatment, whereas the highest were found with CS-NPs<sub>0.1</sub> treatment under no Cr stress (Table 5). Applying CS-NPs<sub>0.1</sub> enhanced the content of Fe, Zn and Mn in the shoots by 21.1%, 17.3% and 27.5%, respectively, compared with those without nanoparticles (Table 5).

Furthermore, the highest Fe, Zn and Mn concentrations in thyme roots were recorded in the absence of Cr with CS-NPs<sub>0.1</sub> (Table 5) and the lowest under severe Cr toxicity without CS-NPs fertilization. In addition, compared with the control plants, severe Cr toxicity decreased the Fe, Zn and Mn concentrations by 28%, 17.8% and 18.9%, respectively. Contrastingly, applying CS-NPs with no Cr enhanced the concentration of the mentioned elements by 30.6%, 12.8% and 25.5% when compared with the control, and a respective 81.7%, 37.2% and 54.9% when compared with the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment (Table 5).

### 3.7. Essential Oil Content (EOC)

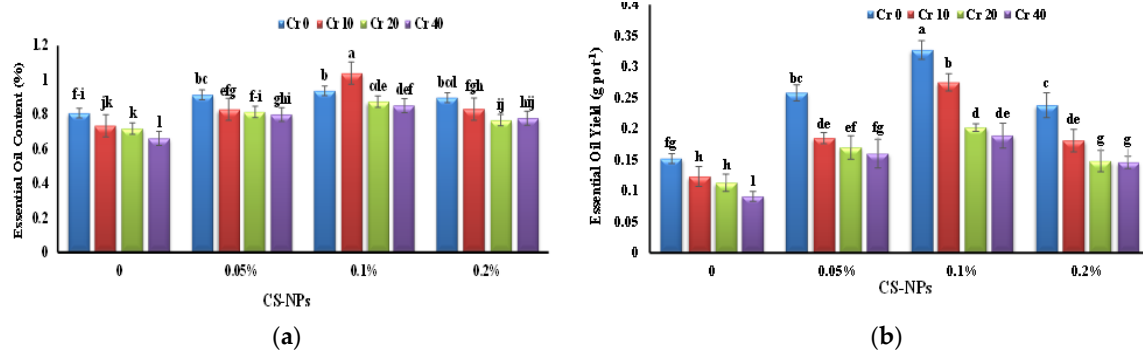
EOC in thyme plants was significantly influenced by Cr stress and CS-NPs application (Table 2). The EOC was reduced by high levels of Cr (20 and 40 mg kg<sup>-1</sup>), compared with the untreated control, and showed the lowest value under the treatment of 40 mg kg<sup>-1</sup> Cr without CS-NPs (−18.2%, compared with the control). All three CS-NP levels significantly increased EOC content in non-stressed conditions (Figure 3a). The application of CS-NPs<sub>1</sub> with no Cr enhanced the content of thyme EOC by 41.8%, compared with the treatment of 40 mg kg<sup>-1</sup> of Cr without CS-NPs<sub>0.1</sub> (Figure 3a).

### 3.8. Essential Oil Yield (EOY)

EOY was significantly affected by CS-NPs, Cr and their interaction (Table 2). Cr toxicity reduced the EOY, so that the lowest EOY was recorded under the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment, 67.0% lower than the untreated control. In the latter condition (Cr-stress 40 mg kg<sup>-1</sup>), the application of CS-NPs<sub>1</sub> enhanced the yield of essential oil by 73.0%, compared with the absence of CS-NPs (Figure 3b). Moreover, the maximum EOY was obtained using CS-NPs in the absence of Cr, which was 115.1% higher than that in the untreated plants (Figure 3b).

### 3.9. Total Protein Content (TSP) and Total Soluble Carbohydrates Content (TSC)

Cr toxicity reduced TSP and TSC, while CS-NPs increased these parameters (Table 2). The maximum content of TPC and TSC was recorded under the CS-NPs<sub>0.1</sub> treatment and non-stress conditions, while the lowest was recorded with the highest Cr stress conditions without CS-NPs (Figure 4). The TPC and TSC content in thyme plants under 40 mg kg<sup>-1</sup> Cr decreased by 12.4% and 32.6%, respectively, compared with the untreated control. CS-NPs<sub>1</sub>-Cr<sub>0</sub> increased TPC and TSC content by a respective 11% and 7.1%, compared with the control plants, and by a respective 26.7% and 59.1% compared with the Cr<sub>40</sub> treatment without CS-NPs (Figure 4).



**Figure 4.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress on the essential oil content (a) and essential oil yield (b) of thyme. Cr was applied at the concentrations of 0, 10, 20 and 40 mg Cr kg<sup>-1</sup> soil. Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

### 3.10. MDA and EL

The EL and MDA concentrations in thyme plants were influenced by the interaction between Cr and CS-NPs (Table 6). Chromium toxicity increased the contents of MDA and EL, which were also positively affected by CS-NPs (Table 6). The highest amounts of EL and MDA were observed under the stress condition of 40 mg kg<sup>-1</sup> chromium without CS-NPs, and the lowest amount was recorded with the use of CS-NPs<sub>0.1</sub> Cr<sub>0</sub>. In addition, the CS-NPs<sub>0</sub>-Cr<sub>40</sub> treatment increased the mentioned parameters by 40% and 97.5%, respectively, compared with the control (Table 6). The use of the CS-NPs<sub>0.1</sub> treatment in combination with 40 mg kg<sup>-1</sup> chromium reduced EL and MDA contents by 10.6 and 12.8%, respectively, when compared with the absence of CS-NPs (Table 6).

**Table 6.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress on antioxidant enzyme activity: catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), malondialdehyde (MDA), percentage of electrolyte leakage (EL) in thyme.

| CS-NPs (%)   | Cr (mg kg <sup>-1</sup> ) | CAT (μmol min <sup>-1</sup> mg <sup>-1</sup> of Protein) | APX (μmol min <sup>-1</sup> mg <sup>-1</sup> of Protein) | GR (μmol min <sup>-1</sup> mg <sup>-1</sup> of Protein) | POX (μmol min <sup>-1</sup> mg <sup>-1</sup> of Protein) | MDA (nmol g <sup>-1</sup> FW) | EL (%)    |
|--------------|---------------------------|----------------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------|----------------------------------------------------------|-------------------------------|-----------|
| 0            | 0                         | 1.34 l                                                   | 0.503 l                                                  | 0.391 i                                                 | 0.383 k                                                  | 2.49 jk                       | 25.08 ij  |
|              | 10                        | 2.45 fg                                                  | 0.851 fg                                                 | 0.623 de                                                | 0.507 i                                                  | 3.24 fg                       | 28.58 gf  |
|              | 20                        | 3.17 cd                                                  | 1.01 cd                                                  | 0.670 bc                                                | 0.788 cd                                                 | 4.03 cd                       | 30.55 de  |
|              | 40                        | 1.92 ij                                                  | 0.750 hi                                                 | 0.503 h                                                 | 0.677 ef                                                 | 4.92 a                        | 35.10 a   |
| 0.05         | 0                         | 1.63 k                                                   | 0.590 k                                                  | 0.443 i                                                 | 0.395 k                                                  | 2.17 k                        | 20.06 l   |
|              | 10                        | 3.06 de                                                  | 0.926 de                                                 | 0.646 cd                                                | 0.576 h                                                  | 2.94 ghi                      | 26.48 hi  |
|              | 20                        | 3.46 ab                                                  | 1.06 bc                                                  | 0.679 ab                                                | 0.837 b                                                  | 3.82 de                       | 30 def    |
|              | 40                        | 2.28 gh                                                  | 0.794 gh                                                 | 0.601 e                                                 | 0.658 ef                                                 | 4.66 ab                       | 32.23 bc  |
| 0.1          | 0                         | 1.78 jk                                                  | 0.636 jk                                                 | 0.412 i                                                 | 0.448 j                                                  | 2.43 jk                       | 22.98 k   |
|              | 10                        | 2.89 e                                                   | 0.933 e                                                  | 0.629 de                                                | 0.642 fg                                                 | 2.74 hij                      | 25.80 ij  |
|              | 20                        | 3.64 a                                                   | 1.21 a                                                   | 0.708 a                                                 | 0.886 a                                                  | 3.25 fg                       | 29.28 efg |
|              | 40                        | 2.14 hi                                                  | 0.824 fgh                                                | 0.571 f                                                 | 0.720 e                                                  | 4.29 bc                       | 31.36 cd  |
| 0.2          | 0                         | 1.59 kl                                                  | 0.684 ij                                                 | 0.468 h                                                 | 0.413 jk                                                 | 2.62 ij                       | 24.22 jk  |
|              | 10                        | 2.59 f                                                   | 0.886 ef                                                 | 0.638 d                                                 | 0.603 gh                                                 | 3.09 gh                       | 27.87 gh  |
|              | 20                        | 3.34 bc                                                  | 1.10 b                                                   | 0.691 ab                                                | 0.821 bc                                                 | 3.51 ef                       | 29.62 ef  |
|              | 40                        | 2.02 hij                                                 | 0.715 i                                                  | 0.541 f                                                 | 0.756 d                                                  | 4.48 b                        | 33.23 b   |
| LSD          |                           | 0.268                                                    | 0.076                                                    | 0.029                                                   | 0.045                                                    | 0.407                         | 1.64      |
| Significance |                           | Significance levels                                      |                                                          |                                                         |                                                          |                               |           |
| Cr           |                           | **                                                       | *                                                        | **                                                      | **                                                       | **                            | *         |
| CS-NPs       |                           | **                                                       | **                                                       | *                                                       | *                                                        | **                            | *         |
| Cr × CS-NPs  |                           | **                                                       | **                                                       | **                                                      | **                                                       | **                            | *         |

\*: significant at  $p < 0.05$ ; \*\*: significant at  $p < 0.01$ . Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

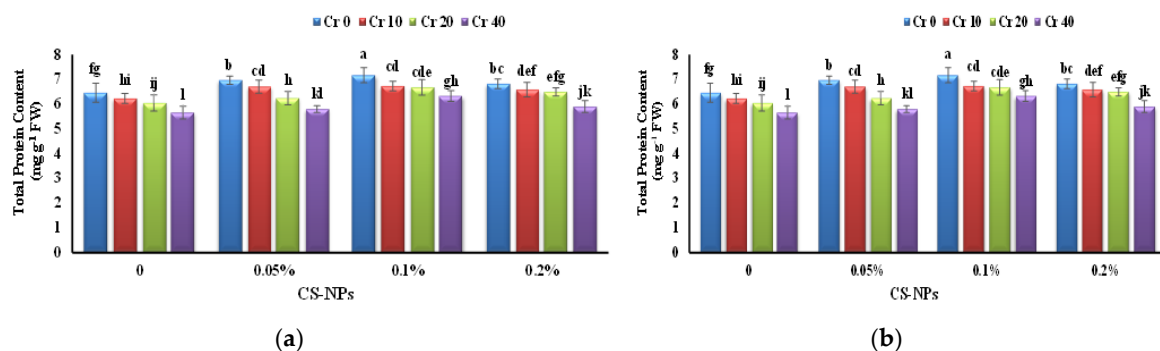


### 3.11. Antioxidant Enzymes Activity (CAT, APX, POD and GR)

Chromium stress, CS-NPs application, and chromium stress  $\times$  CS-NPs significantly affected the thyme antioxidant enzymes (CAT, APX, GR and POX) activity, which increased under the cadmium stress and was effectively regulated by the use of chitosan (Table 6). The maximum capacities of CAT, APX, GR and POX were recorded with 20 mg kg<sup>-1</sup> Cr after applying CS-NPs<sub>0.1</sub>. compared with the control, the latter treatment increased the activity of CAT, APX, GR and PPO enzymes by 171.6, 140.5, 81.1, and 131.3%, respectively (Table 6). At higher concentrations of chromium (40 mg kg<sup>-1</sup>) with nano-chitosan, the activity of CAT, APX, GR and PPO showed decreasing trends, by 38.8, 29.7, 19.3 and 15.6%, respectively, under CS-NPs<sub>0.1</sub>-Cr<sub>20</sub> compared with CS-NPs<sub>0.2</sub>-Cr<sub>40</sub> (Table 6).

### 3.12. Pearson's Correlations and Biplot of Principal Component Analysis (PCA) of Cs-NP-Treated Thyme Plants Grown under Chromium Toxicity

The heat map of Pearson's correlation revealed that Cr content in soil, shoots and roots had a significant positive correlation with  $F_0$ ,  $F_0/F_v$ , MDA, EL and on the activity of the enzymes CAT, APX, GR and POX, but was negatively correlated with shoot FW and DW, EO, EOY, TSC, TSP, N, P, K, Fe, Zn and Mn shoot and root concentrations,  $F_m$ ,  $F_v$ ,  $F_m/F_v$ , as well as content of Chl a, b, a + b and CARs. Moreover, the mentioned parameters positively related to shoot and root FW, shoot and root DW, Chl a, Chl b and carotenoids. Moreover, MDA and EL showed a negative correlation with shoot FW and DW, EO, EOY, TSC, TSP, concentrations of N, P, K, Fe, Zn and Mn in shoots and roots,  $F_m$ ,  $F_v$ ,  $F_m/F_v$ , content of Chl a, b, a + b and CARs (Figure 5a).



**Figure 5.** Interaction between chitosan nanoparticles (CS-NPs) and chromium (Cr) stress on the total soluble carbohydrates (a) and total protein content (b) of thymes. Cr was applied at the concentrations of 0, 10, 20 and 40 mg Cr kg<sup>-1</sup> soil. Different letters indicate significant differences at  $p < 0.05$ , according to the LSD test.

The evaluated variables along with the applied treatments were processed by the principal component and biplot analysis. In this respect, PCA1 caused 71.9% of the observed variance and the PCA2 led to 11.6% of the observed total variance among the evaluated traits. The biplot analysis showed that shoot FW and DW; EO, EOY, and TSP; concentrations of N, P, K, Fe, Zn and Mn in shoots and roots;  $F_m$ ,  $F_v$ ,  $F_m/F_v$ ; content of Chl a, b, a + b and CARs were all considerably associated with Cs-NP<sub>0.1</sub>-Cr<sub>10</sub>, Cs-NP<sub>0.05</sub>-Cr<sub>10</sub>, and Cs-NP<sub>0.2</sub>-Cr<sub>10</sub>. TSC was correlated to Cs-NP<sub>0.2</sub>-Cr<sub>0</sub> and CsNP<sub>0.05</sub>-Cr<sub>10</sub>. Other parameters, such as  $F_0$ ,  $F_0/F_v$ , MDA, EL, POX, APX, CAT, GR, and Cr content in soil, shoots and roots were found to be associated with Cs-NP<sub>0.2</sub>-Cr<sub>20</sub>, Cs-NP<sub>0.05</sub>-Cr<sub>20</sub>, and Cs-NP<sub>0.05</sub>-Cr<sub>40</sub> (Figure 5b). The biplot and PCA analysis determined the Pearson's correlation results (Figure 6).



#### 4. Discussion

The Cr content in roots and shoots is expected to increase under Cr-stress conditions, as demonstrated by other authors [16,51,52]. Chromium is a trace mineral in food that competes with similar minerals for absorption sites, from which it is transported into the cells [52–56]. CS-NP effectiveness in chelating Cr is probably due to the abundance of amino- and hydroxyl groups in its structure, which leads to a decrease in the availability of Cr in soil, thus reducing the uptake of this element in thyme plants [23,57]. Our findings are consistent with previous studies showing that CS-NP can immobilize HM in soil [58]. The toxicity of heavy metals is alleviated as they are adsorbed onto CS-NP surfaces in soil through ion exchange, precipitation, and surface complexation processes [59]. In previous studies [60,61], chitosan soil amendment increased metal phytoremediation efficiency. However, the variations revealed in experimental outcome are directly linked to the soil characteristics and the type of chitosan employed [58].

The present investigation indicated that increasing concentrations of soil-applied Cr caused a significant and progressive reduction of plant growth. It was previously reported that an increased concentration of HMs can negatively impact the structure of root cell membranes and hinder the absorption of water and minerals [62,63]. Cr stress can also lead to various physiological problems, such as reduced transpiration, respiration, and photosynthesis, i.e., metabolism unbalance [64], ultimately resulting in stunted plant growth [15,19,52,65]. However, the outcome of the present experiment suggests that soil amendment with 1% CS-NPs can have a positive impact on plant growth under Cr stress, which represents a useful reference for developing strategies to enhance plant growth and mitigate the negative effects of this heavy metal on crops. It has been reported that the application of chitosan under toxicity caused by heavy metals can have a helpful impact on the performance of thyme plants by reducing accumulation of these elements in the stems and leaves, regulating the antioxidant enzyme activity, decreasing MDA levels, and improving the efficiency of photosynthesis [26,58,60,66]. It was also found that chitosan has the potential to enhance plant growth by stimulating the signaling pathways linked with the biosynthesis of auxin or gibberellin [67]. In plants, CS-NPs can help reduce the uptake, modify the translocation, and decrease Cr mobility in soil, thereby alleviating its toxicity and modifying cell wall membranes to mitigate oxidative stress [58]. These findings are consistent with previous research showing how chitosan can lessen the harmful influences of heavy metal toxicity and lead to improved performance [27,29,67,68].

The determination of photosynthesis and PSII activity, performed through chlorophyll fluorescence, revealed that the exposure of thyme plants to Cr stress caused a reduction of  $F_v$ ,  $F_m$ , and  $F_v/F_m$  values, indicating a reduction in photosynthesis or photoinhibition in stressful circumstances [14]. Other authors have shown that an increase in  $H_2O_2$  can harm the Calvin cycle and lead to a decrease of photosynthesis [69]. CS-NP has the ability to improve photosynthetic parameters through the direct removal of  $OH^-$  and  $O_2^-$  radicals [70], because it contains a significant amount of amino and hydroxyl groups in its particular structure, which react with ROS to generate stable and generally safe molecular radicals [57,59,67]. According to the findings of Faizan et al. [57], using CS-NPs can help preserve photosynthetic pigments in HMs pollution, by counteracting the inhibitory effects of HMs on enzymes involved in the biosynthesis of these pigments. Additionally, CS-NPs can regulate the absorption and relocation of essential elements such as Fe, which acts as a co-factor in many enzymes, including those involved in chlorophyll biosynthesis [71,72]. Other research has suggested that the application of chitosan in stressful conditions can also lead to increased chlorophyll concentration and improved chlorophyll fluorescence parameters, such as the  $F_v/F_m$  ratio [73].

The level of photosynthetic pigments is an important indicator for detecting heavy metal-induced toxicity symptoms in plants. In this study, it was found that the content of photosynthetic pigments decreased in *T. vulgaris* at all Cr levels. Cr in plants can negatively impact their Chl content, carbon fixation, and photosynthetic activity [74], as it causes stomatal closing; damage to the photosynthetic system, the light-gathering complex,

and photosystems I and II; and a reduction in Fe, which is essential for Chl content, pigment biosynthesis, and the overall photosynthesis process and apparatus [52,75,76]. The application of CS-NP was shown to enhance the amount of chlorophyll in plants [72], maybe due to the high concentration of amino acids in CS-NPs, which can promote the formation of chloroplasts and chlorophyll [77]. Another possible reason may be that chitosan can restore protein pigment complexes, which help protect the photosynthetic apparatus from the oxidative damage caused by proteins and lipids in the chloroplast [78,79]. Previous studies have suggested that chitosan can increase the absorption of minerals such as magnesium and iron, which may also contribute to the improvement of chlorophyll synthesis [68].

In our study, we observed that Cr stress had a complex effect on nutrient absorption, significantly reducing the uptake of micro- and macro-elements, consistent with previous studies showing that Cr pollution can negatively affect the uptake of essential nutrients such as N, P, K, Mg and Fe [80]. The latter phenomenon is due to Cr competition with Fe for binding sites, which hinders Fe absorption and reduces the accumulation required to synthesize chlorophyll and heme [81]. An excessive deposit of heavy metals can cause distortion in the root architecture, which disrupts nutrient absorption [82,83]. Moreover, heavy metals can adversely affect plant metabolic processes, including root membrane dynamics, ATP-ases, and carrier functions [84,85], ultimately hindering plant nutrient uptake and thus slowing growth and respiration. However, CS-NPs significantly influenced the plant nutrient composition and alleviated HMs stress in the current experiment, indicating that chitosan is a potent cell protector against oxidative stress induced by heavy metals [29]. CS-NPs have large surface areas, high degree of deacetylation, and large pore volumes, which make them effective in adsorbing HMs [60,86]. Additionally, CS-NPs have reactive functional groups (e.g., C2-NH<sub>2</sub>, C3-OH, C6-OH) that adsorb heavy metals and reduce plant uptake [52,67,87,88].

The results of this study provide a promising solution by which to mitigate the harmful effects of heavy metal exposure on plants, as it is highly probable that CS-NP and its oligomers, due to their remarkable ability to bind and remove toxic elements, can significantly enhance the uptake of essential minerals [22,57].

The level of secondary metabolites is a crucial factor in delineating the quality of medicinal and spice plants. Our findings show that thyme plants produced the highest concentration of EO when grown under mild Cr-stress conditions. However, it is important to note that severe stress can negatively affect these traits and cause damage [89]. Exposure to high levels of chromium reduces essential oil content by hindering nutrient absorption, lowering photosynthesis potential, and by reducing the chlorophyll content, leaf area, and energy required for essential oil biosynthesis [85,89,90]. It has been demonstrated that exposing plants to heavy metals leads to severe oxidative stress, primarily due to the generation of high levels of reactive oxygen species (ROS), which can damage essential macromolecules in plant cells [31,91,92]. On the other hand, CS-NPs may boost photosynthesis, leading to an improvement in EO and EOY [93,94]. CS-NP positively impact EO, both under normal and Cr-stress conditions, which may be due to increased enzyme activity and substrate availability [31,95]. Razavizadeh et al. [94] have suggested that CS-NPs could modify secondary plant metabolism, leading to an increase in health-promoting compounds. Consistent with our results, previous authors have reported the positive influence of chitosan in improving the percentage and yield of essential oil under heavy metal pollution [31,73,95–97], though the exact mechanism of the latter effect is still unclear and needs further investigation.

In the current experiment, heavy metal stress decreased TSC and TSP in thyme plants. The decrease of plant carbohydrate and protein contents due to Cr contamination may be due to the decreased photosynthesis activity, as leaf pigmentation is closely related to carbohydrate supply [98,99]. Indeed, the carbohydrate accumulation may help plants maintain regular growth rate and prevent tissue death under harsh stress conditions by enhancing osmotic adjustment, preserving biomolecules, and balancing collected ions in vacuoles [100,101]. Similar to our research, Pirsarandib et al. [85] have discovered that the

exposure to high concentrations leads to an evident decrease in both TSP and TSS. The reduction of TSP content can be attributed to the strong attraction of ROS molecules, which leads to the oxidative damage of proteins and, consequently, to a significant reduction of production and function of specific structural proteins, which are crucial for maintaining the integrity and stability of cellular structures [99,102,103]. Contrastingly, CS-NP was found to improve the physiological response and counteract the unfavorable impact of abiotic stress [66,104] through the stress transduction pathway involving secondary messengers [67,105]. Applying CS-NP encourages photosynthesis, triggers the closure of stomata by stimulating ABA biosynthesis, and, remarkably, activates the production of antioxidants, amino acids, organic acids, and sugars through nitric oxide and hydrogen peroxide signaling pathways [106,107]. Indeed, plants cannot survive without these essential metabolites, which are critical in their ability to adjust to changing environments, to signal stress, and to maintain energy metabolism under stressful conditions [28,104]. Additionally, CS-NP can bind with heavy metals and, therefore, is used in soil bioremediation [29,58,88].

The MDA value is a marker of damage to cell membranes and lipids, indicating stress conditions. In thyme plants, both HM and oxidative stress results in MDA content enhancement, which consequently leads to an increase in electrolyte leakage, as reported in Table 4. The Cr stress condition interacts with antioxidant molecules, causing the generation and accumulation of  $H_2O_2$  within cells, inducing lipid peroxidation and the increase in MDA levels [102,108]. A higher content of  $H_2O_2$  encourages lipid peroxidation and MDA increase, contributing to increased EL under HM stress [19]. Under heavy metal toxicity, the antioxidant system greatly reduces the amount of MDA [71] and the ROS balance is altered, thus lowering the related enzymatic activity [52,89,103]. In previous findings, CS-NP reduced the harmful ROS reactions regarding membranes and decreased the levels of  $O^{-2}$ ,  $H_2O_2$ , OH through the activation of ROS scavenging enzymes [31,57,97]. Moreover, the application of CS-Se NPs may decrease MDA and EL and enhance the activity of antioxidant enzymes [72]. Consistent with our findings, other research [28,58,70] has shown CS-NP to have a regulatory effect in reducing Cr-induced oxidative stress in plants by limiting the uptake and changing the transport method of this element within the plant, decreasing its mobility in soil, and altering the cell wall membrane, with a consequent decrease of  $H_2O_2$  and MDA levels under stress situations.

Heavy metal stress, such as that caused by Cr, damages plant cells and triggers the overproduction of ROS, whose concentration is controlled by antioxidant enzymes. Examples of this include CAT, APX, GR and PPO, each with a specific role in overcoming oxidative stress [109]. The latter approach is crucial to help plants reduce heavy-metal-induced stress [110]. The increased concentration of reactive oxygen species (ROS) either inhibits growth or exacerbates the reduction in antioxidant enzyme activity [31,111]. Results of the present experiment indicate that, when the concentration of Cr is low, CAT, APX, GR and PPO activities are increased more than they are in control plants, whereas, at higher concentrations of Cr, the plant reduces its ability to deal with the damaging effects of Cr. These findings suggest the ability of plants to tackle Cr stress by accelerating antioxidant enzyme activities to a certain extent, before it declines as higher concentrations of this metal are reached [31,58]. In addition, CS-NP elicits antioxidant enzymes, potentially raising the antioxidant status to counteract ROS generated by heavy metal exposure [28,31,94]. Chitosan significantly improves antioxidant activity by adjusting cell osmotic pressure, reducing harmful free radical accumulation, and stimulating essential nutrient uptake [60,72]. In this respect, in our experiment increasing antioxidant enzyme activities, particularly upon the application of  $5\text{ mg L}^{-1}$  CS-NP, mitigated the adverse effects of Cr-stress.

## 5. Conclusions

The results of the present research suggest that exposure to Cr stress causes a significant decrease in the production of essential oil content and biomass. The slowed growth of the Cr-stressed plants was attributed to the interaction between Cr and chlorophyll molecules, reduced nutrient availability, and oxidative stress. However, the application of CS-NPs



proved to be an effective strategy by which to mitigate the harmful effects of Cr in thyme plants, resulting in significant improvements of various parameters, such as growth traits, chlorophylls, fluorescence, TSC, TSP, macronutrient and micronutrient content, EOC, and EOY, under both normal and Cr-stress conditions. The outcome of this study shows that plants treated with lower doses of CS-NP demonstrated an increased tolerance to Cr, which is reflected positively in the enhancement of growth attributes, such as height, stem thickness, and leaf size. Furthermore, the CS-NP treatment resulted in a significant increase in the chlorophyll content of the plants, as compared with the untreated control, suggesting that the CS-NP treatment positively impacted the plants ability to efficiently carry out photosynthesis, which is crucial for their growth and overall health. Upon treatment with the CS-NP solution, the plants displayed a significant boost in the production of secondary metabolites, having a wide and positive impact on their vitality, particularly phenological development, as well as to resistance to pests and diseases. Moreover, the treated plants exhibited increased nutrient uptake and growth, and the application of 0.1% *w/w* CS-NP had the best influence under Cr stress, eliciting the highest EOC and EOY.

From this research, it can be inferred that chitosan nanoparticles have the potential to provide a new method for cleaning up polluted soil and developing a plant's ability to withstand metal-induced stress, concurrently helping to meet the growing demand for healthy food while ensuring environmental sustainability. However, further experiments are needed to elucidate the natural mechanism that prevents the absorption and transport of Cr in plants treated with CS-NP.

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