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In vitro application of chitosan effects on essential oil content and physiological characteristics of *Dracocephalum kotschyi* Boiss

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Abstract

D. kotschyi* is a source of essential oils with medicinal properties such as antihyperlipidemic, immunomodulatory, antinociceptive and cytotoxic effects. This study aimed to investigate whether the application of chitosan can improve plant growth, increase secondary metabolite production and help antioxidant enzymatic systems in *Dracocephalum kotschyi* under *in vitro* culture. Tow-week-old seedlings of *D. kotschyi* were cultured in MS medium containing 0, 5, 10 and 20 mg.L^{-1} chitosan. Four weeks after treatments, the enzymatic and morphological parameters were measured and the GC/MS analysis was used to evaluate the secondary metabolites. Application of chitosan resulted in changes in physiological and morphological responses. It had a beneficial effect on the main essential oil contents such as thymol, p-cymene and candinol (16.2%, 20.3% and 34% increasing, respectively). In spite of reduction of antioxidant activities and growth, 20 mg/l had a positive effect on the oil components in *D. Kotschyi.

Keywords: Antioxidant enzymes, Chitosan, Essential oil, Morphological parameters, Phenolic compound

Introduction

Dracocephalum kotschyi Boiss, commonly known as Badrandjboie-Dennaie and Zarrin-Giah, is an endemic herbaceous plant of Labiateae family rich in essential oils (Fattahi *et al*., 2011), flavonoid, monoterpene glycosides, trypanocidal terpenoids, rosmarinic acid and linolenic acid (Goli *et al*., 2013). *D. kotschyi* has medicinal properties including antihyperlipidemic, immunomodulatory, antinociceptive and cytotoxic effects (Jahaniani *et al*., 2005). In recent decades, due to the side-effects of chemical drugs, using some of the plant natural products with different pharmacological effects has grown rapidly (Amenu, 2014). However, because of the excessive harvesting of wild plants, limited distribution areas, and a lack of cultivation and domestication, many medicinal plants are listed as endangered plants. Therefore, it is important to develope novel methods for the improvement of secondary metabolite synthesis.

Recently, different strategies under an *in vitro* system with comparatively faster reactions, controlled environment and shorter generation time (Perez-Tornero *et al*., 2009) have been the subject of extensive researches. Researchers have attempted to utilize plant cell biosynthetic capabilities for obtaining medicinal metabolites and for studying the metabolism (Verpoorte *et al*., 2002). Numerous valuable secondary metabolites such as alkaloids, polyphenols and terpenes have been reported from *in vitro* culture studies and reviewed several times (Verpoorte *et al*., 2002; Vanisree and Tsay, 2004).

Biotic or abiotic elicitors can be used as compounds triggering the formation of secondary metabolites. Chitosan, as a biotic elicitor, is being frequently used in a number of plant cell cultures for efficient induction of important secondary metabolites. Chitosan is a natural and safe polysaccharide produced from chitin, the major constituent of arthropods exoskeleton and fungi cell walls and the second renewable carbon source after lignocellulosic biomass (Kurita, 2006). It has various industrial, medicinal, pharmaceutical, and agricultural applications (Krupa-Małkiewicz and Fornal, 2018; Zeng and Luo, 2012; Nge *et al*., 2006; Barka *et al*., 2004). Chitosan treatments have been shown to act as an elicitor in plants, to improve yields and plant growth in numerous crops and enhance secondary metabolite production in cell suspensions and calli of various species (Ohta *et al*., 2004; Kowalski *et al*., 2006; Yu *et al*. 2002; Putalun *et al*. 2007). Chitosan application could improve plantlet quality *in vitro* such as *S. tuberosum* (Asghari-Zakaria *et al.*, 2009) and facilitate the acclimatization of platelets to *ex vitro* conditions (Nge *et al*. 2006). It is thought that this can lead to the induction of plant defense enzymes, and to the synthesis

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of secondary metabolites, such as polyphenolics, lignin, flavonoids, and phytoalexins observed in many plant species treated with chitosan (Malerba and Cerana, 2016).

The aim of this research was to investigate effect of *in vitro* chitosan on improvement of plant growth and secondary metabolite production in *D. kotschyi*.

Materials and methods

This research was conducted in 2017 at Payame Noor University of Isfahan. The seeds of *Dracocephalum kotschyi*, was provided from the oil seed cultivation company (Pakan Bazr, Isfahan, Iran). They were washed with distilled water and then surface sterilization was done by incubating seeds in 70 % ethanol for 20 seconds, followed by transferring them to the 20% sodium hypochlorite solution for 5 mins. Subsequently, the seeds were carefully soaked in 70% sulfuric acid for 12 mins. to remove the external germination inhibitor. Then, they were washed three times with sterile water. Then, 10 disinfected seeds were placed in jars containing 30 ml of in ½ MS-basal medium (Murashige and Skoog, 1962), containing 15 $g.L^{-1}$ sucrose (Merck, Germany), 10 $g.L^{-1}$ agar (Agar-agar powder, Merck, Germany). All cultures were incubated in the growth chamber (16 hrs. under 90 $\mu molm^{-2}s^{-1}$ light / 8 hrs. dark) at 25°C. Vigorous seedlings were obtained after two weeks of culturing. Then, 5 aseptic seedlings were re-cultured directly on fresh medium on MS medium supplemented with 0, 5, 10 and 20 $mg.L^{-1}$ chitosan, respectively and were kept in the growth chamber (16 hrs. under 90 $\mu molm^{-2}s^{-1}$ light / 8 hrs. dark) at 25°C. Four weeks after the treatment, effects of chitosan on total phenolic content, antioxidant enzyme activities and secondary metabolites of roots and leaves were studied.

Growth parameter: Length of shoot and root, dry and fresh weight of shoot and root were measured.

Total phenolic content*:* Total phenolic content was estimated according to Sonald and Laima's method (1999). A portion (0.1 g) of shoots and roots were homogenized in 5 mL of 95% ethanol and then kept for 24-72 hrs. in the dark at 25°C. After homogenizing, 1 mL of extraction was mixed with 3 mL of distilled water, 1 mL of ethanol 95%, 1 mL of 5% aqueous sodium carbonate, and 0.5 mL of 50% folin-ciocalteu reagent. Finally, after one hour, the absorbance of each sample was determined at 725 nm using spectrophotometer. Gallic acid was used for constructing the standard curve. Results were reported as mg /g fresh weight.

Enzyme extraction and assay: For enzyme extraction, 0.1 g of shoots and roots from the treated seedlings were homogenized in 2 mL of 25 mM sodium phosphate buffer (pH=7). To prepare 100 mL sodium phosphate buffer of the desired pH, 84 mL of 25 mM Na_2HPo_4 buffer was added to 16 mL of 25 mM $NaH_2Po_4.2H_2O$ buffer (Sambrook and Russell, 2001). The homogenate was centrifuged at 15,000 × g for 20 mins. at 4°C. The supernatant was collected for measurement of antioxidant enzyme activities.

Catalase activity: Catalase activity (CAT) was assayed according to the rate of H_2O_2 decomposition (extinction coefficient= 36 mM^{-1} cm^{-1}) as measured by the reduction of absorbance at 240 nm, following the procedure of Aebi (1974). The reaction mixture consisted of 2 mL of 25 mM sodium phosphate buffer (pH =7), 50 µL of extraction enzyme and 100 µL of 37% H_2O_2. The activity was expressed as µmol/min/gr FW.

Ascorbate peroxidase activity: Ascorbate peroxidase activity (APX) activity was determined in a 1-mL reaction mixture containing 50 mM sodium phosphate buffer (pH =7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.1 mM H_2O_2 and 100 µL of the enzyme extract. The subsequent decrease in ascorbic acid was determined at 290 nm (E = 2.8 mM^{-1} cm^{-1}) and the activity was expressed as µmol/min/gr FW (Nakano and Asada, 1981).

Determination of essential oils: The gas chromatography–mass spectrometry (GC/MS) was used for the identification of essential oil components in *C. copticum*. A Hewlett-Packard 5890 GC (Hewlett Packard, Waldbronn, Germany), equipped with a flame ionization detector (HP-5970 mass-selective detector) and a 50 m × 0.20 mm HP-5 (cross-linked Phenyl–Methyl Silicon) column with 0.25 µm film thickness, was used for this study. The FID was maintained at 250 ºC. In addition, ionization energy was 70 eV. Temperature program was 100-250°C with changes of 4°C/min. Helium was used as carrier gas, the flow through the column was 1 mL/min, and the split ratio was set to 100:1. Identification was based on sample retention time and mass recorded (Li *et al*., 2009; Davies, 1990).

Statistical analysis: The experiment was performed in a completely random design with three replicates. One-way ANOVA was used for treatment assay and Duncan tests were used to compare the mean values.

Results

Results of chitosan influence on the morphological characteristics of *D. kotschyi* are shown in Figure 1. In comparison to the control, application of 20 mg/l of chitosan induced a significant decrease in shoot length (Fig. 1a). However, no significant differences were observed in other levels of chitosan. Moreover, 10 and 20 mg/l chitosan decreased the root length significantly, as compared to the controls (Fig. 1b). The largest reduction of the plant height was observed at the 20 mg/l of chitosan. In comparison to the control, chitosan prompted a significant increase in fresh weight of shoot at 5 and 10 mg/l of chitosan. Moreover, the differences were not significant between the control and 20 mg/l chitosan (Fig. 1c). As shown in figure 1d, chitosan led to noticeable increase in root fresh weight. However, the decrease at 20 mg/l resulted with value which was still significantly higher than the control. The highest value of fresh weight was obtained in culture media

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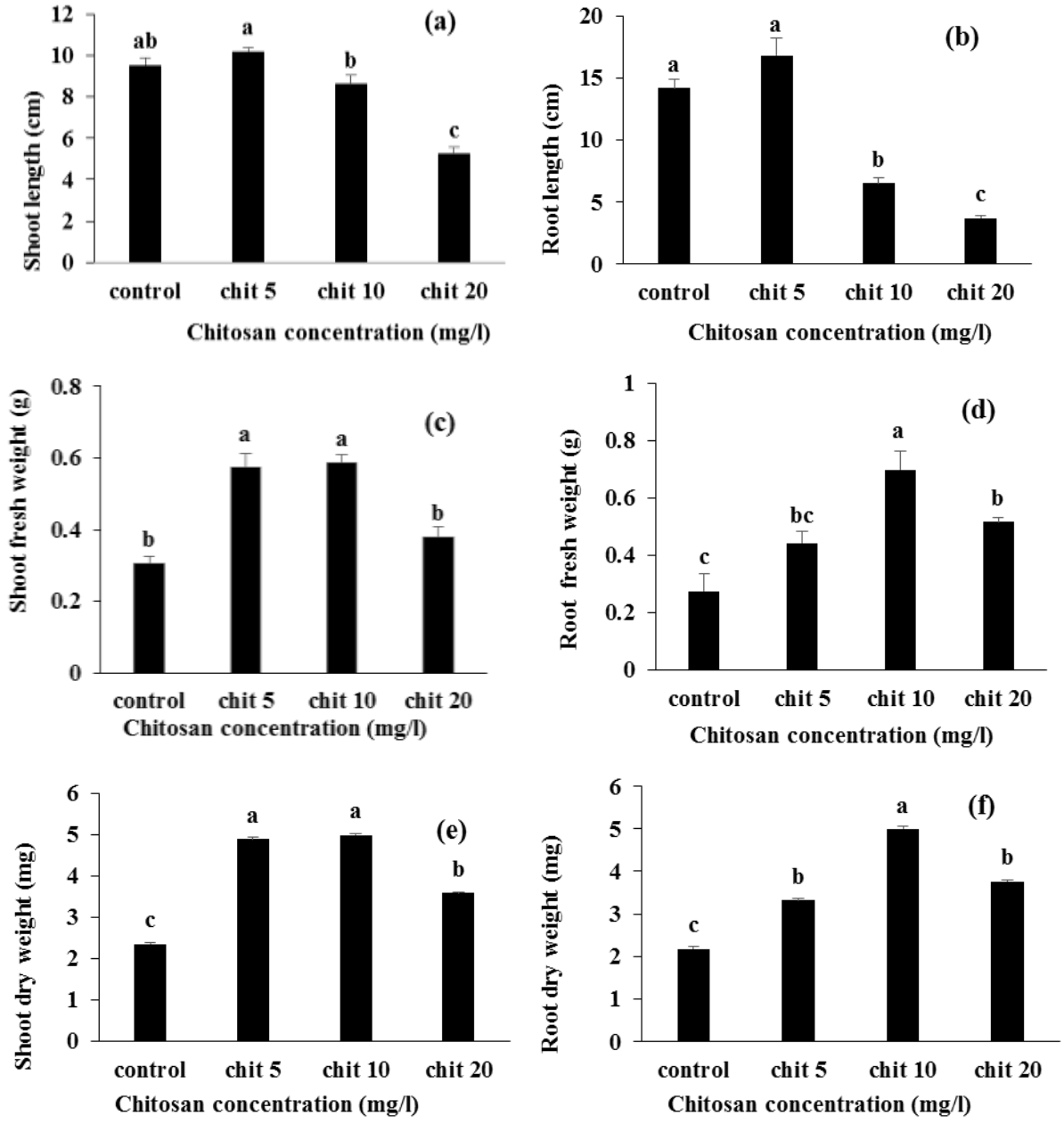

Figure 1. Effect of chitosan on plant growth of *D. kotschyi*. Shoot length (a), root length (b), shoot fresh weight (c), root fresh weight (d), shoot dry weight (e) and root dry weight (f). Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan**'s test ($P \leq 0.05$).**

supplemented with 10 mg/l chitosan. In spite of reduction of root and shoott length, chitosan had a positive effect on dry weight. Increasing of dry weight was observed in all of the chitosan concentrations, but 10 mg/l of chitosan had increased it more (Fig. 1e and f).

No statistical difference was observed in the shoot phenolic compound among different concentrations of chitosan (Fig. 2a). While, the mean comparison results indicated that chitosan caused a significant decrease in amount of phenolic compound in root of *D. kotschyi*. In the chitosan treated roots, 20 mg/l chitosan decreased the phenolic compounds significantly about 50%, as compared to the control (Fig. 2b).

In this study, it was noticed that increasing concentration of chitosan resulted in significant reduction of CAT activity in the treated shoots (Fig. 3a). While, in the treated roots, the CAT activity in medium containing 5 mg/l chitosan showed almost the same as control did, the activity was increased by enhancing the chitosan concentrations in the culture media, compared to the control (Fig. 3b). The maximum APX activity was observed at 10 mg/l chitosan in the treated shoots and at 5 mg/l chitosan in the treated roots, about 2 times when compared to the control (Fig. 3c and d).

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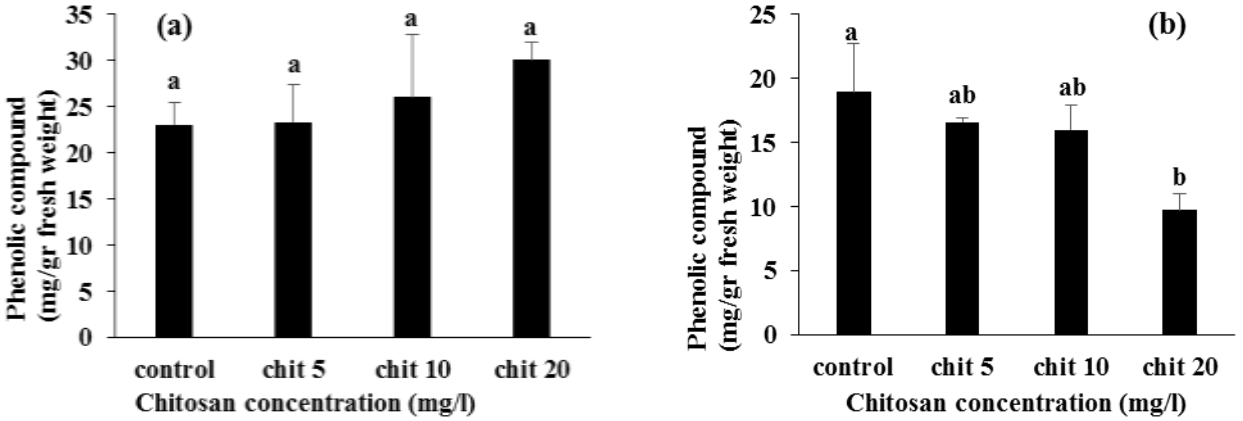

Figure 2. Effect of chitosan on phenolic content of *D. kotschyi*. The phenolic content of leaf (a) and root (b). Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan**'s test (P ≤ 0.05).**

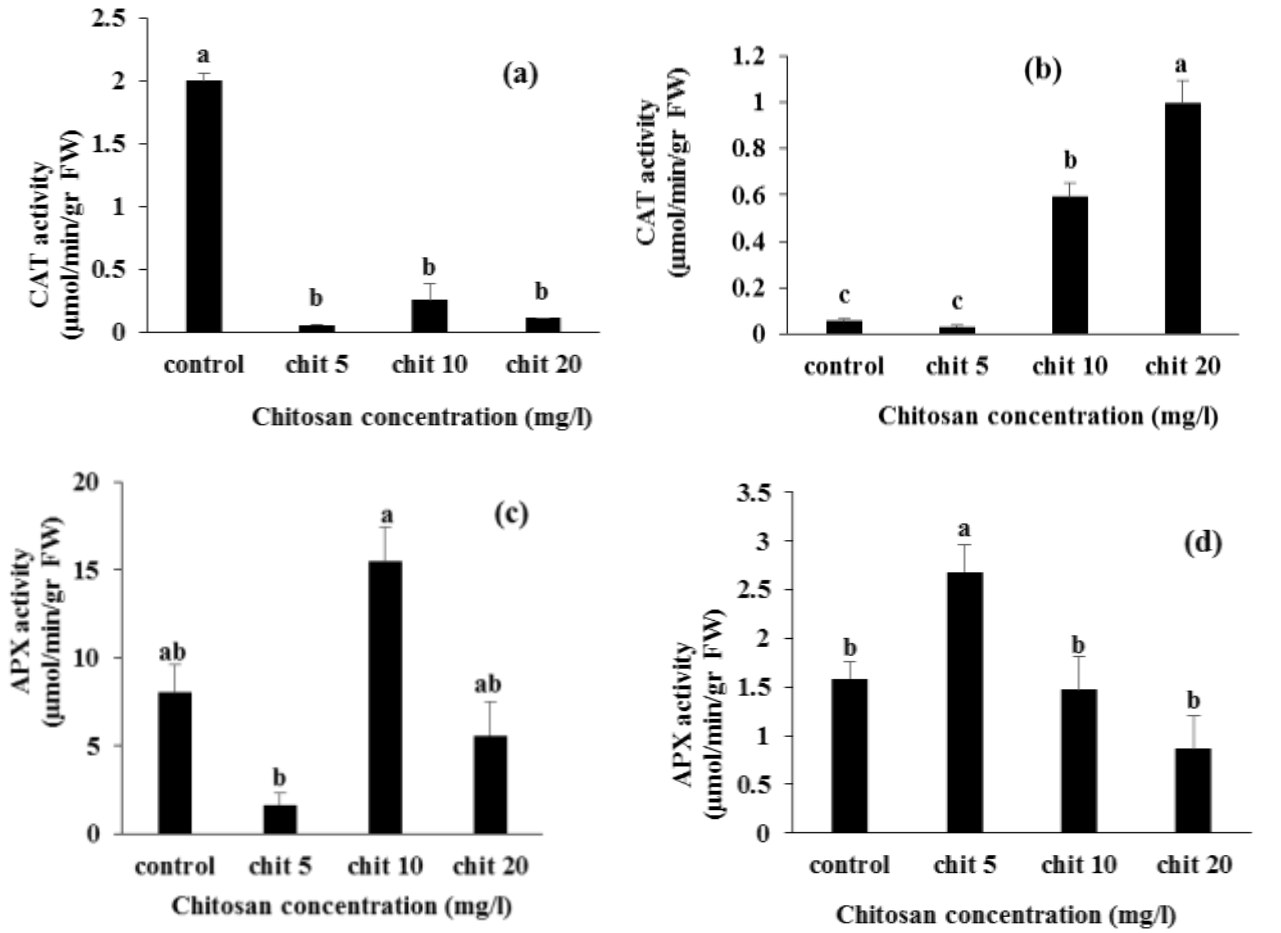

Figure 3. Effect of chitosan on antioxidant enzymes activity of *D. kotschyi*. Catalase activity in leaf (a), root (b), ascorbate peroxidase activity in leaf (c) and root (d). Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan**'s test (P ≤ 0.05).**

About 38 components were recognized in the essential oil of seedlings of *D. kotschyi* under control and 20 mg/l chitosan treatment. The results obtained in our study indicated that the major compositions of *D. kotschyi* oil content of thymol by 16.2%, p-cymene by 20.3%, were thymol, γ-terpinene, p-cymene, β-pinene, limonene, candinol, linalool, geranyl format, comphene, carvacrol, ocymene (Table 1). Among these main constituents, in contrast with the controls, the candinol by 34% and comphene by 10.5% were

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Table 1. Constituents of essential oil of D. kotschyi using GC MS

	Pic. No	RI	control	20 mg/l chitosan
Pyridine	1	924	0.9	1.3
α-Thujune	2	936	1.5	1
α-Pinene	3	947	1.3	1.8
Comphene	4	955	1.9	2.1
Sabinene	5	971	1.1	0.9
β-Pinene	6	984	6.5	4.4
Myrcene	7	999	1.2	0.8
2-Carene	8	1007	0.8	1.1
Beta -Ocymen	9	1017	1.4	0.9
α-Phellandrene	10	1023	1	1.5
Linalool epoxide	11	1030	0.9	1
α-Terpinene	12	1039	1.5	1.7
p-cymene	13	1046	6.9	8.3
Limonene	14	1055	5.8	4.5
γ-Terpinene	15	1066	14.1	11.2
Ocymene	16	1073	1.7	1
Sabinene hydrate	17	1080	0.8	--
Terpinolene	18	1096	1.3	1.5
Linalool	19	1135	2.1	1.7
Pinocarvone	20	1154	--	0.9
Myrtenal	21	1197	1.1	1.3
Verbenone	22	1249	1	1.5
Geranyol	23	1266	6.1	7.4
Borneol	24	1282	0.9	1.1
Thymol	25	1297	17.3	20.1
Terpinen-4-ol	26	1310	1.5	0.8
Carvacrol	27	1333	1.8	1.4
Candinol	28	1341	4.7	6.3
α-cubenen	29	1354	0.8	1.2
β-Caryophyllene	30	1365	1.1	0.8
Heptyl acetate	31	1381	0.9	1.4
α-Hummulene	32	1408	1.3	0.9
γ-Cadinene	33	1463	1	1.1
Geranyl format	34	1492	2.6	2
Hexyl valerat	35	1503	0.9	1
Germacrene D	36	1516	1.4	0.8
α-Bourbonene	37	1528	1.3	1.1
Caryophyllen epoxide	38	1540	0.8	1.4

increased; while γ-terpinene by 20. 6%, limonene by 22.4%, carvacrol by 22.2% and linalool by 19% were decreased in the chitosan-treated seedling in comparison with controls. In addition, pinocarvone was only detected in seedlings grown under chitosan treatment and sabinene hydrate were only detected in controls (Table 1).

Discussion

In this study, application of 10 and 20 mg/l of chitosan decreased the shoot and root length, but its lower concentrations did not significantly affect these traits. Moreover, all of the chitosan concentrations showed a positive effect on shoot fresh and dry weight, the effect of 10 mg/l was more significant. The increase of the fresh and dry weight may be due to a significant increase in the diameter of the stem and root under chitosan treatment. Reports suggest that chitosan has been used as a supplement in order to promote plant growth (Cho *et al*., 2008, Nahar *et al*., 2012, Tantasawat *et al.*, 2010). However, different species respond in different ways to the presence of chitosan that is, addition of higher concentration of chitosan to MS medium, inhibits the growth whereas, lower concentration of chitosan was found beneficial. Similarly, higher concentration of chitosan showed inhibitory effect on the growth of hairy root cultures of *Artemisia annua* (Putalun *et al*., 2007) and *Brugmansia candida* (Pitta-Alvarez and Giulietti, 1999).

After the first report on the eliciting action of chitosan in pea (*Pisum sativum* L.) and tomato (*Solanum lycopersicum* L.) plants (Walker-Simmons *et al*., 1983), chitosan was shown to enhance plant's defense system. It is also one of the most widely used elicitors to increase the secondary metabolites in plants (Karuppusamy, 2009). Plant phenolic is one of the

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major groups acting as primary antioxidants or free radical scavenger (Sanchez-Moreno *et al*., 1998). It has been previously shown that chitosan enhanced the phenolic contents via induction of PAL enzyme (Kim *et al*., 2005; Meng *et al*., 2008). It has been found that the antioxidant substances, such as ascorbic acid, flavonoids, anthocyanin and phenolic compounds were activated by chitosan application in many plants species; barberry (*Berberis vulgaris* L.) (Ozgen *et al*., 2012), broccoli (*Brassica oleracea* L.) (Perez-Balibrea *et al*., 2011), Lamiaceae (*Orthosiphon stamineus*) (Lim *et al*., 2013), sweet basil (*Ocimum basilicum* L.) (Kim *et al*., 2005) and tomato plants (Coqueiro *et al*., 2011). Altough, in this study, chitosan did not show the clear phenolic induction effects on the shoots. Moreovere, addition of chitosan into the culture medium had no significant effects on root phenolic compound with the exception of last concentration (20 mg/l), which decreased phenolic compound. It is thought that chitosan can be a natural molecule that induces numerous physiological responses in plants, dependent on its structure and concentration and on species and developmental stage of the plant.

Another major part of plant antioxidant system is the involvement of antioxidant enzymes. Antioxidant enzymes may reduce the energy of free radicals, stop the free radicals from forming and may also interrupt an oxidizing chain reaction to minimize the damage caused by free radicals (Shull *et al*., 1991). Despite the numerous reports of enhancement of the activity of antioxidant enzymes by chitosan, in this study, CAT activity in the treated shoots decreased with increase of chitosan concentration. However, in the treated roots, the CAT activity increased by enhancing the chitosan concentrations in the culture media. The results of previous researches indicated that chitosan, by increasing the relative water content of the leaves, maintains leaf turgor pressure, protects the cell membrane and decreases the activity of antioxidant enzymes (Yang Feng *et al.*, 2009). Induction of CAT activity could play a protective role against possible oxidative damage (Dorey *et al*., 1998). Consequently, a higher level of CAT activity in roots in response to elicitor may be a pointer to the above observation. Moreover, chitosan increased the APX activity in seedlings of *D. kotschyi*. The maximum of APX activity was observed at 10 mg/l chitosan in the treated shoots and at 5 mg/l chitosan in the treated roots. Increase of anti-oxidant enzymes activity was reported in many species under chitosan treatment (Mandal, 2010; Guan *et al*., 2009; Mahdavi *et al*., 2011).

Recently, more attention has been paid to the chemical composition of *Dracocephalum* L. genus. Essential oil composition of many species of this genus has been reported previously and there is considerable diversites among the major constituents in these oils (Singh *et al*. 2008; Zhang *et al*. 2008). Differences in the volatile oil composition of the plants can be resulted from genetic (genus, species, and ecotype), chemo type, distinct environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, vegetative plant phases, and extraction and quantification methods. The results obtained in our study indicated that the major components of oil of *D. kotschyi* were thymol, γ-terpinene, p-cymene, β-pinene, limonene and candinol. There were many other compounds in minor amounts. Chitosan could have a considerable influence on the content of essential oils and stimulate the biosynthesis of some essential oil compounds in the seedlings of *D. kotschyi.* 20 mg/l significantly increased the amounts of thymol, p-cymene and candinol and reduced the concentration of γ-terpinene, β-pinene and limonene.

Conclusion

In this study, it is confirmed that chitosan can function as a elicitor for *D. kotschyi*. It can be claimed that the chitosan had a significant effect on morphological traits. It can be even said that some concentrations of chitosan caused high inhibition on plant growth. However, chitosan (20 mg/l) is most effective on the main essential oil contents such as thymol, p-cymene and candinol and it could serve as an exciting and promising experience for the pharmaceutical industry. Nevertheless, further biochemical investigations are needed to determine the function of chitosan as a stimulator and further studies in several genotypes are needed to confirm this proposition.

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