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Nanocapsulation of herbicide Haloxyfop-R-methyl in poly(methyl methacrylate): phytotoxicological effects of pure herbicide and its nanocapsulated form on duckweed as a model macrophyte

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Abstract: In the present study the nanocapsulation of Haloxyfop-R-methyl in poly(methyl methacrylate) was successfully performed. Poly(methyl methacrylate)/Haloxyfop-R-methyl nanocapsules were synthesized using a miniemulsion method and their surface morphology was studied by scanning electron microscopy and transition electron microscopy. The chemical characterization of nanocapsules was done by FT-IR spectroscopy. The herbicide loading and encapsulation efficiency were also analyzed for the herbicide-loaded nanocapsules. In order to evaluate the toxic effects of nanocapsulated herbicide and pure herbicide on *Lemna minor* L., some physiological effects of these two compounds were investigated. The plant growth rate, photosynthetic pigment contents, and activities of antioxidant enzymes were evaluated as indices to assess the effects and toxicity of the encapsulated Haloxyfop-R-methyl and its nonencapsulated form on *Lemna minor*. The obtained results confirmed that the negative effects of encapsulated Haloxyfop-R-methyl on the physiological parameters of *Lemna minor* were less than the effects of the pure herbicide. In the case of antioxidant enzymes activities, it was shown that all concentrations of the two examined groups led to the remarkable induction of superoxide dismutase activity as compared with the control sample. Possibly, the enzyme played an important role in the plant's resistance to the existence of the studied contaminants.

Key words: Aryloxy-phenoxy propionate herbicides, duckweed, encapsulation, environmental pollution, phytotoxicity, plant physiological responses

1. Introduction

The widespread use of herbicides and their continuous discharge into aquatic environments via surface runoff is one of the major causes of environmental contaminations[1,2] This group of contaminants can pose an important threat and stress factor to aquatic environments and endanger human and ecosystem health[3,4]

Controlled release technology such as encapsulation can modify an herbicide's behavior and serve as an effective tool to reduce environmental contaminations[5–7] Moreover, encapsulation technology can reduce the total amount of used herbicide and protect the herbicide against environmental degradation and extent of duration of herbicide activity[7–9] According to the literature, many different techniques have been applied for the encapsulation of various herbicides up to now[7,9–12]

Haloxyfop-R-methyl (methyl (*R*)-2-[4-(3-chloro-5-trifluoromethyl-2 pyridyloxy) phenoxy] propionate) belongs to the aryloxy-phenoxy propionate herbicides[13] It was developed as a selective herbicide for the control of

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grass weeds in broadleaf crops[14] In Iran, it is commonly used on sugar beet, canola, soybean, and onion farms. Haloxyfop-R-methyl prevents fatty acid biosynthesis by inhibiting acetyl-CoA carboxylase (ACCase), causing certain biochemical responses in different plants[14–16]

In the last two decades, many comparative studies have been conducted to assess the toxicological effects of different classes of herbicides and their environmental risks on different target and nontarget plants[15,17] Among different plant species, the family Lemnaceae has received broad application in ecotoxicological studies as model organisms and the use of *Lemna* sp. in phytotoxicological investigations of pesticides has been reported by some previous studies[18–21] Due to the simple structure and morphology, high and speedy growth rate, and small size of *Lemna* species, these plants have many applications in ecotoxicological researches[22–24] Their sensitivity to different classes of pollutants and easy cultivation also make them suitable for such investigations. Different phytotoxicity tests such as determination of duckweed growth inhibition rate and changes in some physiological responses were carried out in previous studies for evaluation of the toxic effects of pollutants and their mode of action on plant physiology[25–28]

One of the main objectives of the present study was the successful nanocapsulation of Haloxyfop-R-methyl in the poly(methyl methacrylate) (PMMA). The comparison of the physiological responses of duckweed (*Lemna minor* L.) upon exposure to pure Haloxyfop-R-methyl and encapsulated herbicide (ECH) was another aim of this study. In order to evaluate the effects of these two groups of examined compounds, changes in the plant growth rate, pigment content, and activities of antioxidant enzymes (peroxidase (POD) and superoxide dismutase (SOD)) were studied.

2. Results and discussion

2.1. Characterization of the synthesized nanoparticles

According to Eqs. (1) and (2), the loading efficiency (LE%) and encapsulation efficiency (EE%) of Haloxyfop-R-methyl were determined to be about 25 ± 1.96% and 36 ± 2.37%, respectively. Figure 1a shows the SEM image of nanocapsules and it is clear from Figure 1b that the size of the synthesized nanocapsules is in nanometer range and the major particle size distributions of nanocapsules are in the range of 60–100 nm. As can be seen in TEM image of nanocapsules, their core shell structure was observed clearly (Figure 1c).

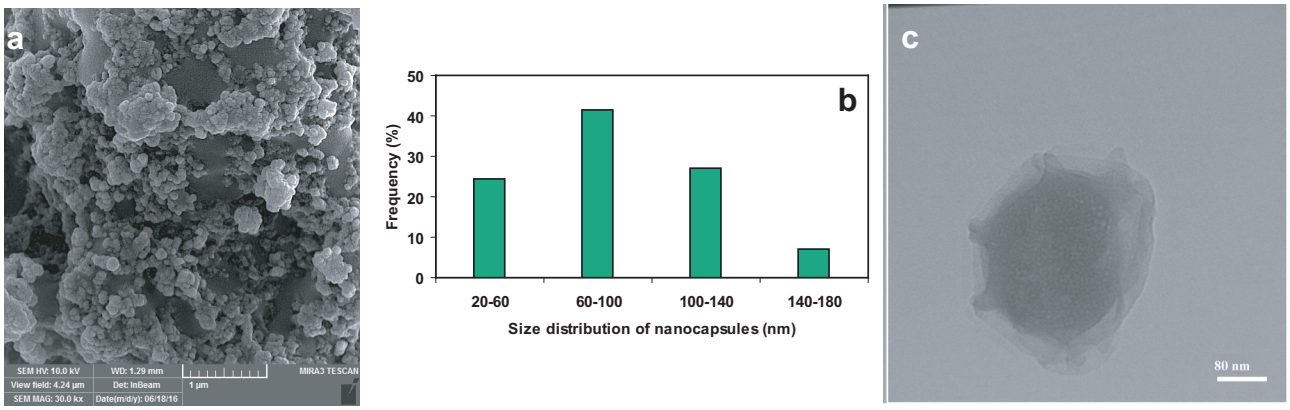

Figure 1. (a) SEM image of the nanocapsules, (b) size distribution of nanocapsules, and (c) TEM image of one synthesized nanocapsule.

Chemical characterization of nanocapsules was carried out using FT-IR spectroscopy. Figures 2a and 2b show the FT-IR spectra of nanocapsules. In Figure 2a, related to uncrushed nanocapsules and the PMMA shell of nanocapsules rinsed with methanol, a peak at 1734 cm^{-1} appeared due to the carbonyl stretching vibration of PMMA. The peak at 1250 cm^{-1} is assigned to the C–O–C stretching of the ester group of PMMA. The peak at 1385 cm^{-1} is related to C–H bending of PMMA. According to Figure 2a, the graphs of uncrushed nanocapsules and methanol-washed shells of nanocapsules are similar. The FT-IR spectra of Haloxyfop-R-methyl and extracted herbicide with methanol are shown in Figure 2b. The peaks at 1729 cm^{-1} and 1218 cm^{-1} correspond to stretching vibrations of C=O and C–O–C in Haloxyfop-R-methyl, respectively. The peaks observed at 1631 cm^{-1} and 1427 cm^{-1} appeared due to the C=C bond of the aromatic ring in the structure of Haloxyfop-R-methyl. The peak at 3449 cm^{-1} corresponds to the stretching vibration of the O–H group in the herbicide. The most obvious peak in Haloxyfop-R-methyl can be seen at 2353 cm^{-1}, which also exists in the extracted phase (methanol solution). The sharp decline in peak intensity in this solution is related to the low concentration of herbicide.

The amount of Haloxyfop-R-methyl released from nanocapsules was determined by measuring the UV absorbance of the plant growth medium containing the herbicide-loaded nanocapsules (Figure 3a). As shown in Figure 3b, the absorbance of the herbicide at 236 nm (λ_{max}) in the plant growth medium was increased up to day 7. The findings showed that by a gradual release of the herbicide from its capsules, the amount of released Haloxyfop-R-methyl was gently increased in the treated solution.

2.2. The effect on the growth of the plant

Figure 4 illustrates the relative growth rate (RGR) of *L. minor* during 10 days in the presence of various concentrations of the herbicide and ECH. According to the results, high concentrations of the herbicide and ECH had notable negative effects on the plant growth.

RGR was significantly reduced by increasing the concentrations of the herbicide and ECH. Between the two groups of treatments (herbicide and ECH), it seemed that direct treatment with Haloxyfop-R-methyl had notable negative effects on the plant growth when compared with ECH treatments and the control. For instance, direct treatment of 0.1 mg/L of the herbicide led to the significant reduction of RGR (up to 8%), as compared with the control ($P < 0.05$), but after 7 days of treatment with 0.1 mg/L of ECH, there was no notable negative effect on RGR ($P > 0.05$) (Figure 4). In another example, RGR was reduced up to 42.8% and 20.8% during 10 days of the treatment by 10 mg/L of the herbicide and ECH, respectively (Figure 4). Less negative effects of ECH on the plants could be related to the slow release of the herbicide from capsules into the plant environment.

Additionally, relative frond number (RFN) was decreased by increasing the concentrations of Haloxyfop-R-methyl and ECH, but the negative effects of direct treatment with the herbicide were notable when compared to the treatments with ECH. For instance, RFN was significantly decreased up to 54.5% and 36.3% after 6 days of exposure to 10 mg/L of the herbicide and ECH, respectively (Table 1).

In accordance with the obtained results, RGR and RFN reduction by increasing the concentrations of some herbicides was reported in previous studies[15,29,30] For instance, it was reported that in the presence of a high concentration of Propanil, significant reduction of growth of *L. minor* was observed[29] Moreover, the growth of *L. minor* was inhibited up to 50% after 48 h of treatment with 3.6 μg/L of flumioxazin[27] Indirect decrease or inhibition of photosynthesis by Haloxyfop-R-methyl could possibly be one of the important reasons for the delayed growth[15]

Table 1. The effect of four concentrations of the herbicide and ECH (0, 0.1, 1, 10, and 100 mg/L) on relative frond number (RFN) of *L. minor* (mean ± SD, n = 4).

Day	RFN									
	Pure herbicide concentration (mg/L)					ECH concentration (mg/L)				
	0	0.1	1	10	100	0	0.1	1	10	100
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.15 ± 0.02	0.10 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.00	0.15 ± 0.02	0.20 ± 0.03	0.05 ± 0.02	0.00	0.00
4	0.4 5± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.01	0.15 ± 0.03	0.45 ± 0.02	0.30 ± 0.02	0.25 ± 0.01	0.15 ± 0.02	0.15 ± 0.01
6	0.55 ± 0.03	0.35 ± 0.02	0.30 ± 0.02	0.25 ± 0.03	0.20 ± 0.02	0.55 ± 0.03	0.40 ± 0.03	0.35 ± 0.02	0.35 ± 0.02	0.30 ± 0.03
8	0.70 ± 0.02	0.40 ± 0.01	0.50 ± 0.03	0.35 ± 0.02	0.30 ± 0.01	0.70 ± 0.02	0.55 ± 0.03	0.50 ± 0.02	0.40 ± 0.02	0.35 ± 0.02
10	0.80 ± 0.01	0.55 ± 0.02	0.55 ± 0.01	0.45 ± 0.04	0.40 ± 0.02	0.80 ± 0.01	0.70 ± 0.03	0.60 ± 0.01	0.50 ± 0.01	0.45 ± 0.03

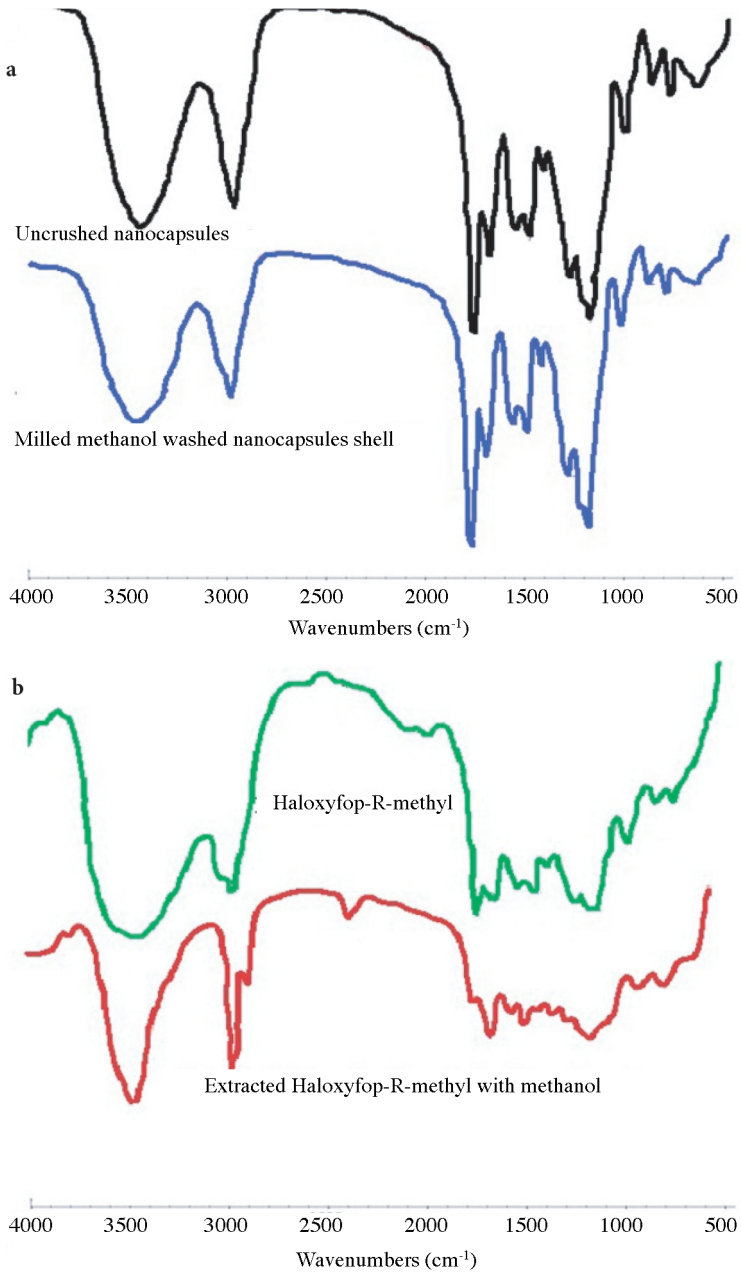

Figure 2. FT-IR spectroscopy of (a) uncrushed and milled methanol-washed nanocapsules and (b) pure Haloxyfop-R-methyl and extracted Haloxyfop-R-methyl with methanol.

2.3. The effect on photosynthetic pigment contents

The pigment content was determined after 7 days of exposure of the plant to 0.1, 1, 10, and 100 mg/L of the herbicide and its encapsulated form. According to Table 2, after 24 h (day 1) of exposure of the plants to different concentrations of the herbicide, the amounts of chlorophylls and carotenoids were notably increased ($P < 0.001$). For example, treatment with 1 mg/L of the herbicide led to the enhancement of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids at 1.9-, 1.3-, and 1.6-fold, respectively, as compared with the control.

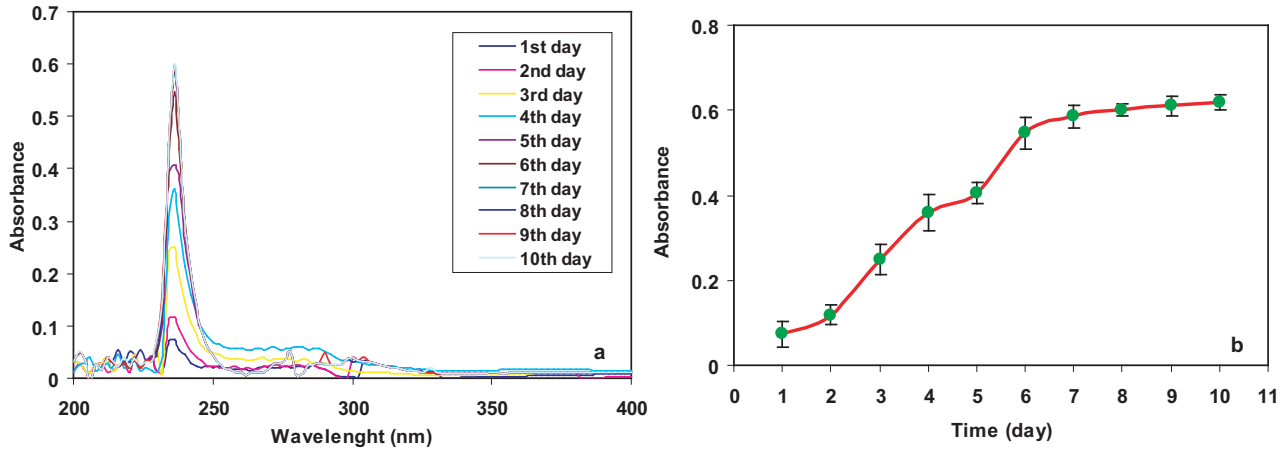

Figure 3. (a) UV/Visible spectra of the encapsulated Haloxyfop-R-methyl (100 mg/L) during 10 days and (b) absorbance of Haloxyfop R-methyl at 236 nm released from ECH to the plant growth medium during 10 days.

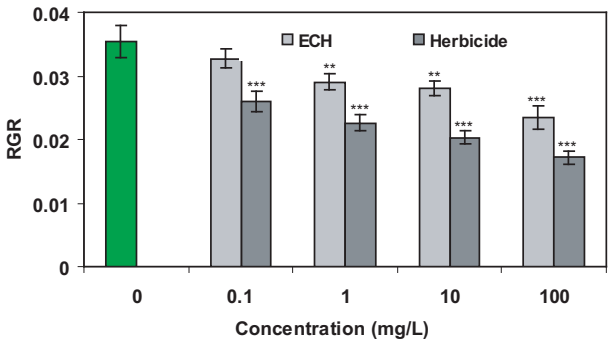

Figure 4. The effect of four concentrations of the herbicide and ECH (0, 0.1, 1, 10, and 100 mg/L) on the relative growth rate (RGR) of *L. minor* (mean ± SD, n = 4, *significant difference at $P < 0.05$, **significant difference at $P < 0.01$, ***significant difference at $P < 0.001$).

This increase in pigments content was continued up to day 4 ($P < 0.001$), but the measurement of pigment contents on day 4 showed that the enhancement was less than that on day 1 for all types of pigments. Finally, after 7 days of treatment with different concentrations of the herbicide, the content of Chl a and carotenoids was notably decreased. Presumably, direct contact with a high amount of the herbicide for a long time finally caused the negative effect on the plant pigment contents (Table 2). A similar trend was previously reported in the case of plant species treated with the aryloxy-phenoxy propionate group of herbicides, such as Quizalofop-p-ethyl and Fluazifop-p-buthyl[14,15,31] For instance, increase of Quizalofop-p-ethyl concentration was led to the reduction of the Chl a/b ratio in *L. minor* and *L. gibba*. Additionally, strong negative correlations were reported between carotenoid content of *L. gibba* and the herbicide concentration[15] According to the literature, this group of herbicides can indirectly disrupt fatty acid biosynthesis via the inhibition of ACCase[15,16,32] Therefore, Haloxyfop-R-methyl, which belongs to this group, can inhibit the formation of thylakoid membranes and finally reduce the pigment contents, thereby causing chlorosis. Moreover, the reduction of pigment content in *Lemna* species after treatment with other classes of herbicides was also reported. It was shown that the contents of Chl a and b in *L. minor* were notably decreased by increasing Roundup Ultra 360 SL concentrations[17]

Table 2. Contents of chlorophyll a, chlorophyll b, and total carotenoids (mg g^{-1} fresh weight (FW)) in control *L. minor* plants and plants exposed to 0.1, 1, 10, and 100 mg/L of the herbicide for 7 days (mean ± SD, n = 4, **significant difference from the control at $P < 0.01$, ***significant difference from the control at $P < 0.001$).

Herbicide concentration (mg/L)	Chl a			Chl b			Carotenoids		
	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7
0 (control)	9.42 ± 0.15	9.50 ± 0.03	9.52 ± 0.05	3.33 ± 0.06	3.40 ± 0.08	3.44 ± 0.02	3.22 ± 0.02	3.26 ± 0.13	3.3 ± 0.05
0.1	12.96 ± 0.76 ***	11.40 ± 0.19 ***	8.42 ± 0.23 **	5.79 ± 0.31 ***	5.78 ± 0.24 ***	3.50 ± 0.27	4.70 ± 0.16 ***	4.30 ± 0.19 ***	1.30 ± 0.11 ***
1	18.72 ± 0.50 ***	12.40 ± 0.21 ***	8.73 ± 0.11 **	8.05 ± 0.08 ***	5.44 ± 0.12 ***	3.67 ± 0.17	5.33 ± 0.11 ***	4.80 ± 0.20 ***	1.40 ± 0.05 ***
10	12.18 ± 0.19 ***	11.69 ± 0.55 ***	8.35 ± 0.31 **	7.80 ± 0.14 ***	5.48 ± 0.24 ***	4.22 ± 0.41 *	4.18 ± 0.21 ***	4.08 ± 0.22 **	1.45 ± 0.11 ***
100	13.61 ± 0.33 ***	12.57 ± 0.38 ***	7.58 ± 0.21 ***	7.99 ± 0.35 ***	5.88 ± 0.17 ***	4.59 ± 0.24 **	4.62 ± 0.30 ***	4.31 ± 0.19 ***	1.57 ± 0.08 ***

The amounts of photosynthetic pigments were not significantly changed after 1 day of treatment with different concentrations of ECH as compared with the control ($P > 0.05$) (Table 3). In contrast, the content of all types of pigments was notably enhanced after 4 days of treatment with different concentrations of ECH. For instance, after 4 days of treatment with 1 mg/L of ECH, the amounts of Chl a, Chl b, and carotenoids were increased up to 1.3-, 1.5-, and 1.3-fold, respectively, compared to the control. These increases continued up to day 7 (Table 3) for Chl a and b. This could be due to the slow and gradual release of the herbicide from its encapsulated form during the experiment (7 days). The amounts of carotenoids were significantly decreased after 7 days of treatment with all examined concentrations of ECH ($P < 0.001$).

According to the obtained results, the effects of direct herbicide treatments occurred earlier and with greater intensity as compared to treatments with ECH at the end of the experiment and led to negative effects on the pigment contents. Therefore, it could be concluded that because of the slow release of the herbicide from its encapsulated form, the negative effects of the treatments with different concentrations of ECH on the plant pigment contents were less than those of the direct treatment with Haloxyfop-R-methyl.

2.4. Enzymatic analysis

In the presence of different classes of contaminants such as salinity, heavy metals, and herbicides, reactive oxygen species (ROS) production is increased, damaging cellular components by oxidizing important macromolecules like lipids, proteins, and nucleic acids[33,34] In response, plants are equipped with an efficient antioxidative system that protects them against such situations. For instance, SOD neutralizes reactive superoxide radicals to less destructive hydrogen peroxides, which are then detoxified by other antioxidative enzymes such as POD. These enzyme activities have already been used to evaluate the toxicity of the contaminants[27,35]

The effects of different concentrations of Haloxyfop-R-methyl on POD and SOD activities are illustrated in Figures 5a and 5b, respectively. According to Figure 5a, POD activity was decreased up to 33.3%, 37.5%, 47.6%, and 54.1% after 4 days of treatment with 0.1, 1, 10, and 100 mg/L of the herbicide, respectively, as compared to the control ($P < 0.001$). It seemed that direct contact of the plant with different concentrations of the herbicide up to day 4 led to the notable negative effects on POD activity. After 4 days, it is possible that the plant gradually adapted to existing conditions and POD activity was increased gently (day 7) in response to the ongoing contact with the herbicide. Transient inhibition and induction of POD activity when exposure to herbicide exceeded 48 h was also reported in duckweed treated with Diuron and Propanil[29,36] In contrast to POD activity, the plant SOD activity was notably increased during treatment with all concentrations of the herbicide (Figure 5b). For example, after 4 days of treatment of the plants with 0.1, 1, 10, and 100 mg/L of the herbicide, SOD activity was increased up to 8.5-, 10.8-, 9.3-, and 5.7-fold, respectively. The high enhancement in SOD activity continued up to day 7 (Figure 5b). SOD activity assay after 7 days of treatment of the plant with all examined concentrations showed that the activities were more than the assayed activities for the control sample and for all treatments on day 4. Some previous studies have also reported the induction of SOD activity in response to the increase of the concentrations of the applied herbicides in the environment of different *Lemna* species[15,21] It was previously reported that treatment with 250 mg/L of Quizalofop-p-ethyl led to 2.29- and 2.16-fold induction of SOD activity in *L. minor* and *L. gibba*, respectively.

In the case of the effect of ECH treatments on the plant antioxidant enzyme activities, as shown in Figure 6a, only the treatment of 10 mg/L ECH led to significant enhancement of POD activity as compared with the control ($P < 0.001$) and treatments with low concentrations of ECH had no notable effects on its activity ($P > 0.05$). In contrast, POD activity was decreased by 24.6% after 7 days of exposure to 100 mg/L of ECH as

Table 3. Contents of chlorophyll a, chlorophyll b, and total carotenoids (mg g^{-1} fresh weight (FW)) in control *L. minor* plants and plants exposed to 0.1, 1, 10, and 100 mg/L of ECH for 7 days (mean ± SD, n = 4, *significant difference from the control at $P < 0.05$, **significant difference from the control at $P < 0.01$, ***significant difference from the control at $P < 0.001$).

ECH concentration (mg/L)	Chl a			Chl b			Carotenoids		
	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7
0 (control)	9.42 ± 0.15	9.50 ± 0.03	9.52 ± 0.05	3.33 ± 0.06	3.40 ± 0.08	3.44 ± 0.02	3.22 ± 0.02	3.26 ± 0.13	3.3 ± 0.05
0.1	10.11 ± 0.59	14.20 ± 0.69 ***	14.99 ± 0.41 ***	4.12 ± 0.23	5.02 ± 0.25 ***	4.60 ± 0.25 ***	4.19 ± 0.47	4.50 ± 0.29 ***	1.20 ± 0.09 ***
1	10.03 ± 0.44	12.50 ± 0.24 ***	12.99 ± 0.15 ***	3.27 ± 0.19	5.28 ± 0.13 ***	5.20 ± 0.27 ***	3.65 ± 0.18	4.26 ± 0.10 ***	1.20 ± 0.04 ***
10	10.20 ± 0.66	11.90 ± 0.59 ***	12.60 ± 0.22 ***	3.30 ± 0.22	5.00 ± 0.24 ***	5.10 ± 0.16 ***	3.57 ± 0.12	3.70 ± 0.06 *	1.20 ± 0.06 ***
100	10.33 ± 0.79	11.50 ± 0.27 ***	11.10 ± 0.20 ***	3.37 ± 0.14	4.90 ± 0.38 ***	5.50 ± 0.27 ***	3.53 ± 0.10	3.80 ± 0.13 **	1.60 ± 0.44 ***

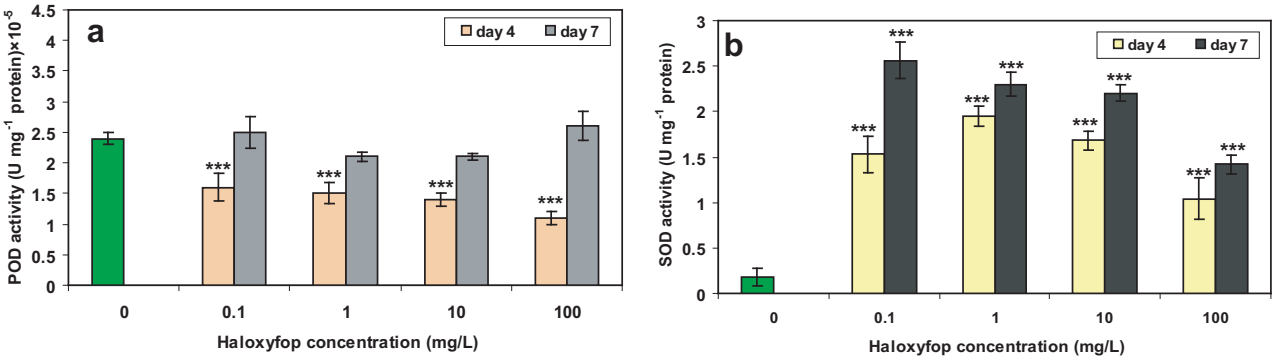

Figure 5. The activities of (a) POD and (b) SOD in control *L. minor* plants and plants exposed to different concentrations of the herbicide (mean ± SD, n = 4, *significant difference at $P < 0.05$, **significant difference at $P < 0.01$, ***significant difference at $P < 0.001$).

compared to the control (Figure 6a). Perhaps treatment of the plants with a high concentration of ECH led to the negative effects on POD activity. SOD activity after treatments of the plants with various concentrations of ECH showed a pattern similar to the activity of SOD in the plants having direct contact with the pure herbicide. For instance, SOD activity was increased up to 3.4-, 3.2-, 2.6-, and 2.4-fold as compared to the control sample after 4 days of the treatment of the plant with 0.1, 1, 10, and 100 mg/L of ECH, respectively (Figure 6b). According to the obtained results, the direct treatments with Haloxyfop-R-methyl had more induction effects on SOD activity as compared to the treatments with ECH.

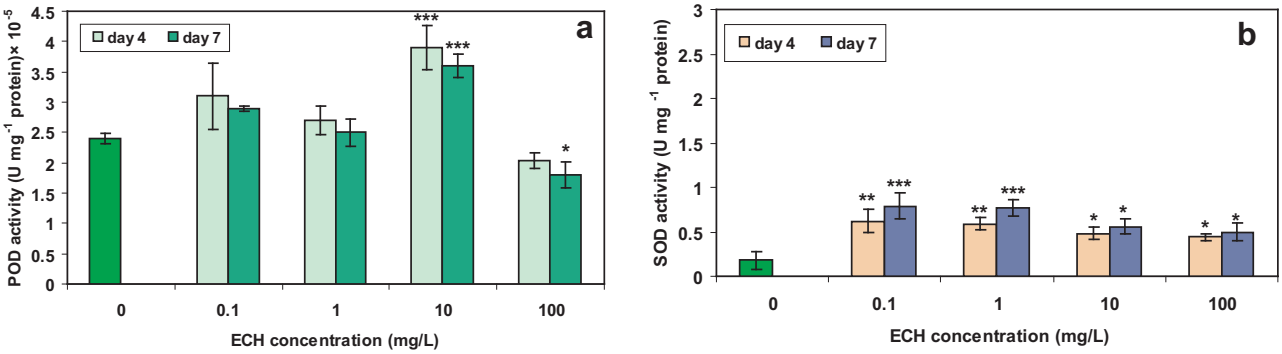

Figure 6. The activities of (a) POD and (b) SOD in control *L. minor* plants and plants exposed to different concentrations of ECH (mean ± SD, n = 4, *significant difference at $P < 0.05$, **significant difference at $P < 0.01$, ***significant difference at $P < 0.001$).

2.5. Conclusions

The obtained results confirmed that the negative effects of ECH on *L. minor* were less than those of the herbicide. RFN and RGR were significantly reduced by increasing the concentrations of ECH and the herbicide but the negative effects of the direct treatments of the herbicide were notable as compared to the treatments of ECH. Moreover, induction effects of the herbicide on the contents of Chl a and Chl b became apparent rapidly as compared to those of ECH. In the case of ECH, enhancement of the pigment contents continued up to the end of the experiment but the direct treatment of the plants by the herbicide led to the decrease in the

amounts of the pigments after 7 days of the exposure. All concentrations of the two examined groups led to the remarkable induction of SOD activity as compared to the control and showed a similar pattern up to the end of the experiment. SOD possibly played an important role in the plant resistance to the existence of the studied contaminants.

3. Experimental

3.1. Chemicals

Methyl methacrylate and methylene bis acrylamide were obtained from Merck (Darmstadt, Germany) and double-distilled before use. Ammonium persulfate and Triton-x-100 were purchased from Merck. The herbicide was purchased from Golsam Chemical (Gorgan, Iran). All chemicals of the plant culture medium and the compounds of enzyme activity assays were obtained from Merck and were applied without extra purification.

3.2. Preparation and characterization of PMMA nanocapsules

PMMA nanocapsules containing Haloxyfop-R-methyl were prepared by an emulsion polymerization method. A reaction flask with 36.6 mL of deionized water and 0.8 g of Triton X-100 (surfactant) was mixed for 30 min before polymerization. Then 10 mL of methyl methacrylate, 4 mL of Haloxyfop-R-methyl, 1.3 g of methylene bis acrylamide, and 0.1 g of ammonium persulfate were added. The resultant mixture was vigorously mixed at 2000 rpm for 15 min. The precipitate was washed with distilled water and methanol and dried under vacuum at 60 °C for 24 h.

In order to determine the Haloxyfop-R-methyl LE% and EE%, after crushing 0.1 g of the nanocapsulated herbicide in a mortar and washing with a certain amount of methanol for the complete dissolution of the herbicide, the precipitate was dried under vacuum at 60 °C for 24 h and weighed again. LE% and EE% were calculated according to Eqs. (1) and (2), respectively:[10]

$$\text{LE\%} = (\text{mass of the herbicide in nanocapsules} \,/\, \text{mass of nanocapsules}) \times 100 \quad (1)$$

$$\text{EE\%} = (\text{mass of the herbicide in nanocapsules} \,/\, \text{initial mass of herbicide}) \times 100 \quad (2)$$

Haloxyfop-R-methyl release from the herbicide-loaded PMMA nanocapsules was investigated in vitro at 25 °C in 250-mL reagent bottles containing 200 mL of the plant culture medium and ECH (100 mg/L). After set time intervals (each 24 h), the UV/Vis absorbance of the sample was determined on a T80+ UV/Visible spectrophotometer (China) at 236 nm during 10 days of the experiment.

Field emission scanning electron microscopy (FE-SEM) was carried out with a Mira microscope (Mira3, Tescan, Czech Republic) to obtain the surface morphology of the herbicide-loaded nanocapsules and the diameters of nanocapsules were measured on the SEM images. Digimizer software was utilized to determine the size distribution of the synthesized nanocapsules[37] Transmission electron microscopy (CMC Philips 300 KV) was used for the study of the structure, shape, and size of prepared loaded nanocapsules.

To separate and identify core and shell materials of the synthesized nanocapsules, an extraction was carried out with methanol. For this purpose a known weight of nanocapsules was crushed using a pestle and mortar and the crushed samples were washed and rinsed several times by methanol to completely dissolve the loaded herbicide in methanol. Remaining shell material was dried under vacuum in an oven for 1 day at 60 °C. An FT-IR spectrophotometer was used (Nexus-670, Thermo Nicolet Company) for identification of the separated core and shell of nanocapsules.

3.3. Plant cultivation and treatments

Plant materials were gathered from Ali Jan near Bostanabad, in the northwest of Iran, and their surfaces, including shoots and roots, were washed carefully using distilled water. Plants were acclimatized for 1 week in a Steinberg culture medium under laboratory conditions,[38] with a temperature of 25 °C and photoperiod of 16 h/8 h (light/dark). The culture medium contained 1.25 mmol/L $Ca(NO_3)_2$, 3.46 mmol/L KNO_3, 0.66 mmol/L KH_2PO_4, 0.41 mmol/L $MgSO_4$, 0.072 mmol/L K_2HPO_4, 1.94 μmol/L H_3BO_3, 0.63 μmol/L $ZnSO_4$, 2.81 μmol/L $FeCl_3$, 0.18 μmol/L Na_2MoO_4, 0.91 μmol/L $MnCl_2$, and 4.03 μmol/L EDTA (all from Merck).

The plants (about 2 g) were transferred to 250-mL beakers containing 200 mL of the culture medium with different concentrations of the herbicide and its encapsulated form (0, 0.1, 1, 10, and 100 mg/L). The temperature was kept constant in the incubator (Sanyo, Ogawa Seiki Co., Japan) during the experiments.

3.4. Physiological analysis

3.4.1. Growth rate

RFN and RGR were applied as the suitable indicators of potential toxicity and used for the determination of the plant growth rate. RFN was measured using Eq. (3):[29]

$$\text{RFN} = [(\text{frond number on day } n - \text{ frond number on day 0}) \text{ / frond number on day 0}] \quad n = 0, 2, 4, 6, 8, 10. \quad (3)$$

RGR was quantified according to the increase in the plant fresh weight (FW) after 10 days of the experiment using Eq. (4):[39]

$$\text{RGR } (\text{day}^{-1}) = [(\ln (\text{final weight}) - \ln (\text{initial weight})] \text{ / day} \quad (4)$$

3.4.2. Photosynthetic pigments content

An aliquot of 100 mg of freshly sampled leaves was ground in 100% acetone. The content of photosynthetic pigments was measured spectrometrically at 662, 645 and 470 nm for the maximum absorption of Chl a, Chl b and carotenoids, respectively according to the Lichtenthaler method. [40]

3.4.3. Enzyme activity assay

The plants were treated with different concentrations of the herbicide and its encapsulated form in the nutrient solution (0, 0.1, 1, 10, and 100 mg/L) for 7 days to determine the effect of the compounds on antioxidant enzyme activities and compare them with the control. Fresh plant tissue (0.25 g) was homogenized in 3 mL of 0.1 mol/L phosphate buffer solution (pH 7) containing 0.2% polyvinylpyrrolidone (PVP) to get the crude extract. The homogenates were centrifuged at 4000 rpm for 15 min at 4 °C and the resulting supernatants were used to measure the activities of antioxidant enzymes and the protein content.

The SOD activity was assayed by measuring the inhibition of the photochemical reduction rate of nitroblue tetrazolium (NBT) by the plant extract[41] The reaction buffer contained 2.65 mL of the 67 mmol/L potassium phosphate buffer solution (pH 7.8), 0.1 mL of 1.5 mmol/L NBT, 0.2 mL of the 0.1 mmol/L EDTA solution containing 0.3 mmol/L sodium cyanide, 50 mL of 0.12 mmol/L riboflavin, and a suitable aliquot of the enzyme extract. The reaction mixture was illuminated for 15 min at the light intensity of 5000 lx. The absorbance was measured at 560 nm. One unit of SOD was the amount of the enzyme catalyzing 50% inhibition of NBT photochemical reduction. The control assay was done in the absence of plant extract to prevent possible autoxidation of the substrates.

The POD activity was assayed using the method reported by Chance and Maehly[42] The reaction mixture contained 0.1 mol/L citrate-phosphate-borate buffer (pH 7.5), 50 μL of 15 mmol/L guaiacol, 50 μL of 3.3 mmol/L H_2O_2, and 25 μL of enzyme extract. The polymerization of guaiacol was initiated by adding H_2O_2 and an increase in absorbance at 470 nm was recorded for 3 min. The activity was calculated using the extinction coefficient of 26.6 $(mmol/L)^{-1}$ cm^{-1} for guaiacol.

Protein content was determined according to the Bradford method,[43] using bovine serum albumin (Sigma Aldrich) as a standard protein.

3.5. Statistical analysis

Data with four replicates were statistically analyzed by one-way analysis of variance (ANOVA), which was followed by Tukey–Kramer multiple comparison tests using GraphPad software (GraphPad Software, Inc., USA). The results were described as mean ± standard deviation (SD). Significant difference was reported at $P < 0.05$.

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