Original Article

Toxicological Effects of a Post Emergent Herbicide on *Spirodela polyrhiza* as a Model Macrophyte: A Comparison of the Effects of Pure and Nano-capsulated Form of the Herbicide

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Received: 08.10.2017 *Accepted: 12.11.2017*

ABSTRACT

Background: One of the main reasons of environmental contaminations is the broad application of herbicides. Controlled release technologies such as encapsulation of herbicides are as an effective tool to reduce environmental contaminations. The aim of the present study was successful nanocapsulation of Gallant Super (GS), its characterization and compare the physiological responses of *Spirodela polyrhiza* L. upon exposure to GS and its encapsulated form.

Methods: Nanocapsulation of GS in the poly (methyl methacrylate) (PMMA) was performed in the Department of Nanotechnology, Faculty of Sciences and biological effects of the contaminants on *S. polyrhiza* was investigated in Biotechnology Research Center, both in Urmia University, Urmia, Iran in 2016. The surface morphology of PMMA/GS nanocapsules was studied by SEM and TEM and their chemical characterization was determined by FT-IR spectroscopy. For assessment of the effects of the encapsulated Gallant Super (ECGS) and GS on *S. polyrhiza,* some plant physiological parameters were investigated.

Results: Direct treatment of GS had more and notable negative effects on the plant growth when compared with ECGS treatments. Moreover, different examined concentrations of the two contaminant groups led to the remarkable induction of the activities of the antioxidant enzymes such as SOD. Even though the enhancement of the antioxidant enzymes activities when the plant was treated with GS was notably more than the effects of ECGS.

Conclusion: ECGS caused to the fewer changes in the plant physiological parameters and negative effects of the treatment of ECGs were less than when the plant had direct contact with GS.

Keywords: Aryloxy-Phenoxy Propionate, Environmental Pollution, Herbicide Encapsulation, Phytotoxicity, *Spirodela Polyrhiza*.

IJT 2018 (2): 45-54

INTRODUCTION

Aquatic environment is exposed to various organic pollutions such as different classes of pesticides. One of the major causes of environmental contaminations is the wide application of herbicides and their continuous discharge to the aquatic environments via surface runoff [1]. This group of contaminants can pose an important threat and stress factor to the aquatic environment and endanger human and ecosystems' health [2].

Encapsulation of herbicides as a controlled release technology can be an effective tool for reduction of environmental contaminations [3, 4]. In the last decades, many different techniques by application of different polymers such as chitosan, polyvinyl alcohol (PVA) and poly (methyl methacrylate) (PMMA) have been used for the encapsulation of various herbicides [4-7]. Encapsulation technology can reduce the total amount of used herbicide, protect herbicide against environmental degradation and extent of duration of herbicide activity [4,7,8].

Gallant Super (Methyl (*R*)-2-[4-(3-chloro-5-trifluoromethyl-2 pyridyloxy) phenoxy] propionate) belongs to aryloxy-phenoxy propionate herbicides [9]. It was a post-emergent herbicide used to control

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different grass and broadleaf weeds in various crops [10]. In Iran, it is commonly used in sugar beets, canola, soybean, and onion farms. Gallant Super (GS) prevents fatty acids biosynthesis by inhibiting acetyl-CoA carboxylase (ACCase), causing certain biochemical responses in different plants [10,11].

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 targeted and non- targeted plants [11,12]. Lemnaceae family has received wide attention in ecotoxicological researches and the use of different species of this family such as *Lemna* sp and *Spirodela* sp. in phytotoxicological studies of pesticides has been reported [13-15]. Due to the simple structure and morphology, high and speedy growth rate and the small size of these species, they are considered as a suitable model for ecotoxicological investigations [16-18]. Additionally, their sensitivity to different classes of the pollutants and easy cultivation make them suitable for such investigations. In order to evaluate the toxic effects of the pollutants and their mode of action on plant physiology, different phytotoxicity tests based on duckweed growth inhibition and changes in photosynthetic pigments content and some antioxidant enzymes activity were used in such ecotoxicological researches [19-21].

The specific objectives of the present study were to develop successful controlled release GS formulation and characterization of encapsulated gallant super (ECGS) and compare the physiological responses of *S. polyrhiza* L. upon exposure to GS and its encapsulated form with references to changes in the growth rate, pigment content and the activities of antioxidant enzymes (superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6)).

MATERIALS AND METHODS

Chemicals

All chemicals of the plant culture medium were obtained from Merck and were applied without extra purification. Gallant Super was obtained from Golsam Chemical Co. Iran. Its chemical structure and characteristics are given in Table 1.

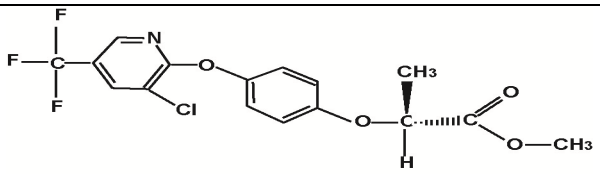
In the case of the compounds for nanoencapsulation of the herbicide, shell-forming monomers, methylmethacrylate, and methylene bis acrylamide were obtained from Merck. Ammonium persulphate and Triton-x-100 were also bought from Merck and used as the surfactant and the initiator, respectively.

Plant Cultivation and Treatments

Plants were gathered from Anzali Lagoon, north of Iran in spring 2016. These plant materials were transferred to the laboratory and washed by distilled water. The plants were adapted for three weeks in a large aquarium containing special nutrient solution [22] and the nutrient solution was replaced every week. This solution contained 0.205 mmol/L $CaCl_2$, 0.0008 mmol/L $CoCl_2$, 0.0012 mmol/L $CuCl_2$, 0.081 mmol/L $FeSO_4$, 0.178 mmol/L H_3BO_3, 0.323 mmol/L K_2HPO_4, 0.202 mmol/L K_2SO_4, 0.739 mmol/L KH_2PO_4, 4.04 mmol/L KNO_3, 0.826 mmol/L $MgSO_4$, 0.047 mmol/L $MnCl_2$, 0.026 mmol/L Na_2-EDTA, 0.00007 mmol/L $(NH_4)_6Mo_7O_{24}$. Plants were kept at 25 °C and photoperiod of 16h/8h (light/dark)).

The plants (about 5 gr) were transferred into 500 mL beakers containing 400 mL of the culture medium with different concentrations of the herbicide and its encapsulated form (0, 1, 10 and 100 ppm). The temperature was kept constant in the incubator (Sanyo, Ogawa Seiki Co., Japan) during the experiments.

Table 1. Structure and characteristics of GS.

Chemical structure	
Synonym	Haloxyfop-R-methyl
CAS No.	072619-32-0
Molecular formula	$C_{16}H_{13}ClF_3NO_4$
Molecular weight (g/mol)	375.72

Preparation and Characterization of PMMA Nanocapsules

Poly (methyl methacrylate) (PMMA) nanocapsules containing the herbicide (GS) were prepared using a miniemulsion method. Before polymerization, 36.6 mL deionized water with 0.8 g of Triton X-100 (surfactant) was mixed for half an hour. Then, 10 mL methylmethacrylate, 4 mL GS, 1.3 g methylene bis acrylamide and 0.1 g of ammonium persulphate 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 ºC for 24 h.

For determination of the GS loading efficiency (LE%) and encapsulation efficiency (EE%), after crushing 0.1 g of the nanocapsule herbicide in a mortar and washing with certain amount of methanol for the complete dissolution of the herbicide, the precipitate was dried under vacuum at 60 ºC for 24 h and weighed again. LE% and EE% were calculated according to Eq. (1) and (2), respectively [5]:

LE%= (mass of the herbicide in nanocapsules/mass of nanocapsules) ×100…….. (1)

EE%= (mass of the herbicide in nanocapsules / initial mass of herbicide) ×100…… (2)

To determine the amount of the herbicide released from ECGS, UV/Visible absorbance of the solution containing the ECGS was determined every day on a T80+ UV/Visible spectrophotometer (China) at 236 nm during the 7 d of the experiment.

SEM analysis was performed by Field emission scanning electron microscopy (FE-SEM) (S-4160, Hitachi, Japan) to obtain the surface morphology of the herbicide loaded nanocapsules and the size of nanocapsules was determined on the SEM images. Transmission electron microscopy (CMC Philips 300 KV) was used for study of structure, shape, and size for 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 postal and mortar and the crushed postal and mortar were washed and rinsed several times with methanol to completely dissolve the loaded herbicide in methanol. Remained shell material was dried under vacuum in oven for one day at 60 ºC. Fourier Transform Infrared (FTIR) spectrophotometer was used (Nexus- 670, Thermo Nicolet Company) for identification of the separated core and shell of nanocapsules.

Toxicological and Physiological Analysis

Calculation of the Plant Growth Rate

The relative growth rate (RGR) parameter was used as the suitable indicators of potential toxicity and applied for the determination of the plant growth rate. RGR was quantified according to the increase in the plant fresh weight (FW) after 10 d of the experiment using Eq. (3) [23]:

RGR (day $^{-1}$) = [(ln (final weight) - ln (initial weight)]/day…….. (3)

Photosynthetic Pigments Content Assay

The content of chlorophyll "*a*" (Chl *a*), chlorophyll "*b*" (Chl *b*) and carotenoids were measured using the method [24]. For this propose, 100 mg of leaves were ground in pure acetone. The mixture was centrifuged at 2000 g for 10 min. A number of pigments was quantified using equations.

Enzymatic Assay

For determination, the effect of the compounds on antioxidant enzymes activities, of control plant sample and different concentrations of GS and its encapsulated form were prepared in the nutrient solution (1, 10 and 100 ppm). 0.25 g of fresh plant tissues were 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 ºC and the resulting supernatants were used to measure the activities of antioxidant enzymes and the protein content.

The SOD activity was assayed by barring photoreduction of nitroblue tetrazolium (NBT) [25]. The absorbance of the solution was recorded at 560 nm. The amount of the enzyme catalyzing 50% inhibition of NBT photochemical reduction was considered a unit of SOD. The control assay was done in the absence of plant extract to prevent possible auto-oxidation of the substrates.

The POD activity was calculated following the polymerization of guaiacol to tetraguaiacol according to the method of Chance and Maehly [26]. The reaction mixture contained citrate-phosphate-borate buffer (0.1 mol/L, pH 7.5), 25 µL enzyme extract, 15 mmol/L guaiacol and 3.3 mmol/L H_2O_2. One unit of POD activity was considered as the amount of enzyme that can produce one 1 mol L^{-1} tetraguaiacol min^{-1} [ε= 26.6 $(mmol/L)^{-1}$ cm^{-1}].

CAT activity was determined by the consumption of H_2O_2 in 3 min by measuring the absorbance

decline at 240 nm [ε = 39.4 $(mol/L)^{-1}$ cm^{-1}] [27]. One unit of CAT activity was the amount of enzyme needed for reduction of 1 µmol of H_2O_2 per minute. The method was used for measurement of protein content [28] using bovine serum albumin (BSA, Sigma Aldrich) as a standard protein.

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey- Kramer multiple comparisons test by GraphPad software (GraphPad Software, Inc. USA) was used for analyzing data with four replicates. The results were described as mean ± standard deviation (SD). A significant difference was reported when the probability was less than 0.05.

RESULTS

Characterization of the Synthesized Nanocapsules

SEM image of ECGS is shown in Fig. 1. Accordingly, the synthesized ECGS samples were of nanometer size and their capsules size was approximately between 20-200 nm. In TEM images of nanocapsules, core-shell structure of nanocapsules was observed clearly (Fig. 2). Another important point seen in the right image is the existence of some pores in polymeric shell. This observation is very useful to explain loading and encapsulation efficiency.

FT-IR spectroscopy was used for chemical characterization of PMMA/GS nanocapsules. Fig. 3 shows the FT-IR spectra of nanocapsules. In Fig. 3a related to uncrushed nanocapsules and PMMA shell of nanocapsules rinsed with methanol, peak at 1734cm^{-1} is appeared due to carbonyl stretching vibration of PMMA. The peak at 1250 cm^{-1} is allocated to the C-O-C stretching of the ester group of PMMA. The peak at 1384 cm^{-1} is related to C–H bending of PMMA. Graphs are completely similar together. FTIR spectrum of GS and extracted herbicide with methanol were shown in Fig. 3b. The peaks at 1728 cm^{-1} and 1218 cm^{-1} correspond to stretching vibration of C=O and C-O-C in GS respectively. The peaks observed at 1631 cm^{-1} and 1427 cm^{-1} is appeared due to C=C bond of aromatic ring in the structure of GS. The peak at 3449 cm^{-1} correlates with stretching vibration of O-H group in herbicide. The most obvious peak in GS can be seen at 2353 cm^{-1} which also exist in extracted phase (methanol solution). The sharp decline in peak intensity in this solution is related to low concentration of herbicide. The absence of any peak in range of 2261 cm^{-1} to 2135 cm^{-1} in Fig. 3a indicates that herbicide is completely covered with PMMA shell.

In addition, LE% and EE% of GS were determined to be about 25±1.96% and 36±2.37%, respectively, according to Eq. 1 and 2. The release behavior of GS from its nanocapsules was determined by measuring the UV absorbance of the plant growth medium containing GS loaded nanocapsules. The absorbance of the herbicide at 236 nm in the plant growth medium was increased during the experiment (7 d) (Fig. 4). The findings showed that by a gradual release of the herbicide from its nanocapsules, the amount of released GS was gently increased in the treated solution during 7 d.

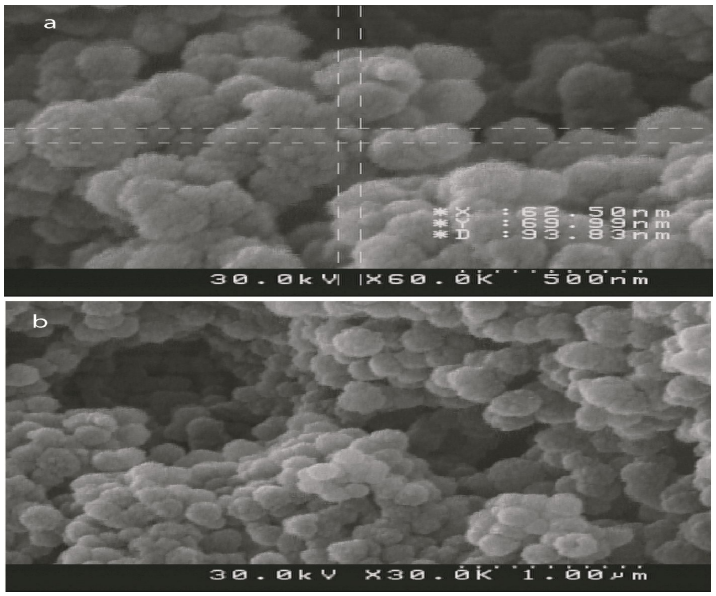


Figure 1. SEM images of the encapsulated gallant super (ECGS) with two different magnifications. (a ana b)

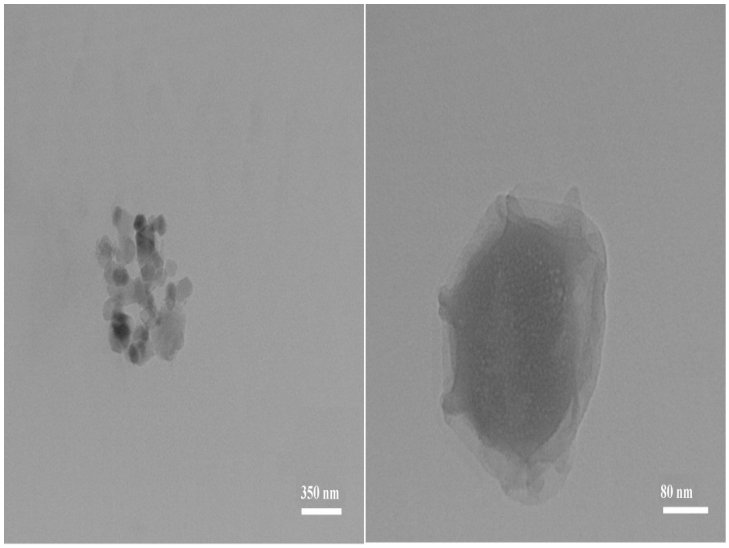


Figure 2. TEM images of the encapsulated gallant super (ECGS) with two different magnifications.

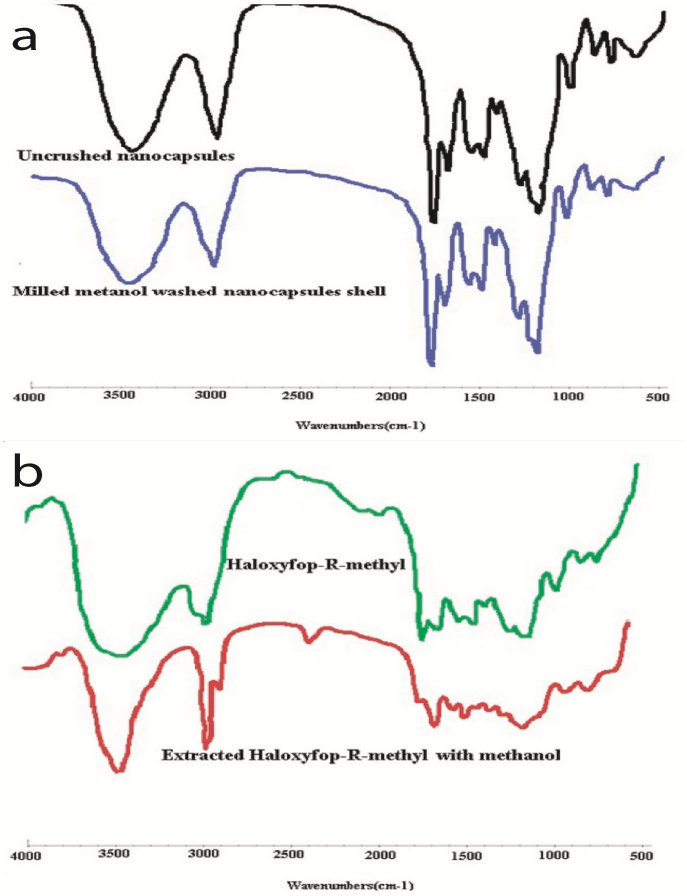


Figure 3. FT-IR spectroscopy of (a) uncrushed and milled methanol washed nanocapsules and (b) pure GS and extracted GS with methanol.

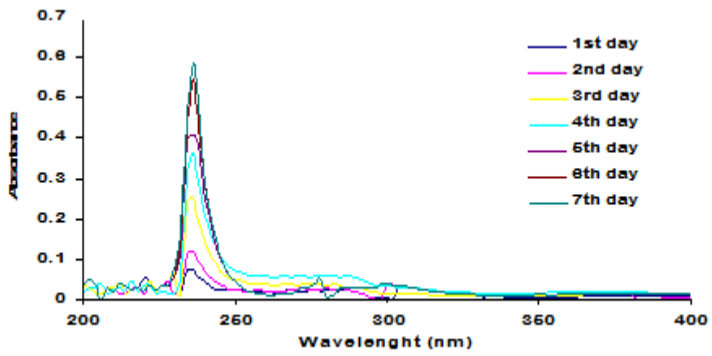


Figure 4. UV/Visible spectra of ECGS (100 ppm) during 7 d.

The Effect on the Growth of the Plant

Fig. 5 illustrates the RGR of *S. polyrhiza* during 10 d in the presence of various concentrations of the GS and ECGS. Accordingly, direct treatment of 10 ppm of GS led to the significant reduction of RGR (up to 42.9%), as compared with the control (P<0.01), but after 10 d treatment of the plant by 10 ppm of ECGS, there was no significant negative effect on RGR (P>0.05) (Fig. 5). Moreover, RGR was reduced up to 60.8% and 21.5% during 10 d of the treatment by 100 ppm of GS and ECGS, respectively (Fig. 5).

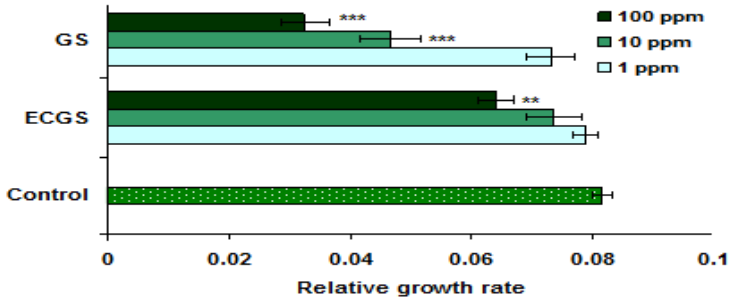


Figure 5. The effect of three concentrations of the herbicide and ECGS (1, 10 and 100 ppm) on relative growth rate (RGR) of *S. polyrhiza* (The error bars represent standard deviation of the mean, n = 4 replicates, *Significant difference at P<0.05, **Significant difference at P<0.01, ***Significant difference at P<0.001, Significant differences were shown compared to the control sample).

Assessment of Photosynthetic Pigments Content

The photosynthetic pigments content of the plant was assessed at 4th and 7th d of exposure to 1, 10 and 100 ppm of GS and ECGS. According to Fig. 6a, after 4 d treatment of the plants by 10 and 100 ppm of the herbicide, the content of Chl *a* was significantly increased compared with the control samples (P<0.001). In contrast, there was no notable change in the amount of Chl *a* after 4 d treatment with different concentrations of ECGS (Fig. 6a). Moreover, after 7 d treatment of the plant samples by different concentrations of GS, the amount of Chl *a* was remarkably decreased by 10 and 100 ppm of the GS (P<0.001) but 7 d treatment of 10 and 100 ppm of the ECGS was led to the enhancement of the Chl *a* content (Fig. 6b). Treatment of 1 ppm of GS and ECGS had no notable effect on Chl *a* after 4 and 7 d exposure.

High concentration of GS was led to the significant reduction of Chl *b* up to 15.1% and 41.1% after 4 and 7 d treatment of the plant samples, respectively, as compared with the control (Fig. 6c and 6d). It is while, after 7 d exposure of the plant with high concentration of ECGS, Chl *b* content was enhanced (Fig 6d). In addition, the content of total carotenoids was significantly decreased after 4 and 7 d treatment of the plant with 1, 10 and 100 ppm of GS and 100 ppm of the ECGS also had the same effect on the carotenoids content (Fig 6e and 6f).

The content of Chl *a* and Chl *b* pigments was notably enhanced up to 1.4 and 1.5-fold, respectively after 7 d of treatment with 10 and 100 ppm of ECGS. Whereas, 4 d treatment of the different concentration of ECGS had no notable effect on Chl *a* and *b* contents.

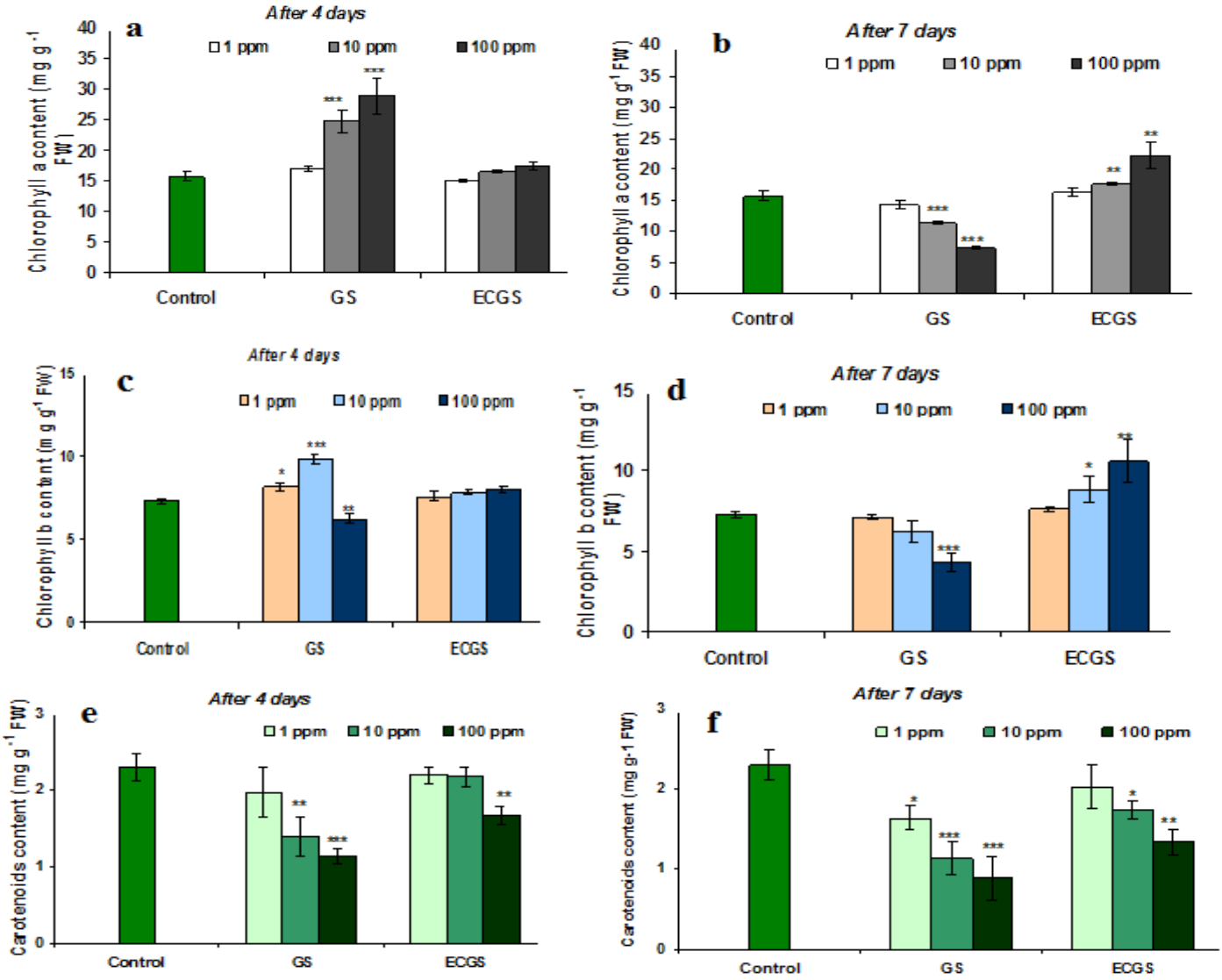


Figure 6. Contents of chlorophyll *a* (a and b), chlorophyll *b* (c and d) and total carotenoids (e and f) (mg g^{-1} Fresh Weight (FW)) in control *S. polyrhiza* plants and plants exposed to 1, 10 and 100 ppm of GS and ECGS for 4 and 7 d (The error bars represent standard deviation of the mean, n=4 replicates, *Significant difference at $P<0.05$, **Significant difference with the control at $P<0.01$, ***Significant difference with the control at $P<0.001$, Significant differences were shown compared to the control sample).

Enzymatic Analysis

The activities of SOD, POD, and CAT were assessed after 4 and 7 d treatment of different concentrations of GS and ECGS and illustrated in Fig. 7. SOD activity was increased up to 1.4, 2.8 and 3.5-fold after 4 d treatment with 1, 10 and 100 ppm of GS, respectively, as compared to the control ($P<0.001$) (Fig. 7a). Increasing in SOD activity of the treated plant samples with 1 and 10 ppm of GS was continued up to day 7 but the treatment of 100 ppm of GS for 7 d was caused by notable reduction of SOD activity. In contrast, there was no notable change in SOD activity when the plant samples were treated with different concentrations of ECGS after 4 days. Whereas, after 7 d exposure by 1, 10 and 100 ppm of ECGS, SOD activity was enhanced up to 1.1, 1.9 and 2.3-fold, respectively, as compared to the control sample (Fig. 7a).

POD activity was assayed in the treated plant samples and the control samples in order to determine the effect of different concentrations of two groups of examined compounds on its activity. Treatment of 100 ppm of GS and ECGS was led to the significant enhancement of POD activity after 4 d treatment with GS and after 4 and 7 d treatment with ECSG (Fig. 7b). Moreover, after 7 d exposure to the plant to 100 ppm of GS, POD activity was reduced up to 30.1%. POD activity was not significantly promoted during the treatment with 1 ppm of GS and ECGS (Fig. 7b).

The treatment of 100 ppm of GS and ECGS for 4 and 7 d was led to increasing of the CAT activity

but in the case of the treatment of 10 ppm of the examined contaminants, the enhancement in CAT activity was observed just in the plant samples treating by 10 ppm of GS (Fig. 7c). In contrast, there was no significant change in CAT activity when the plant samples were treated with 1 ppm of the GS and ECGS.

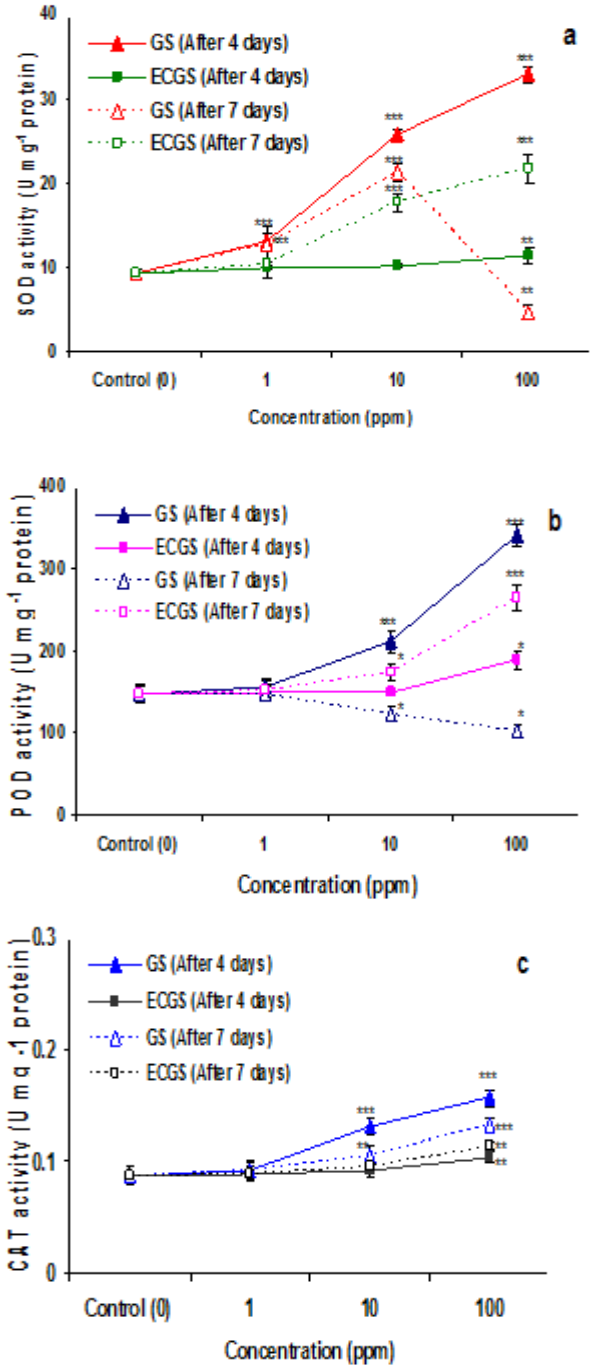


Figure 7. The activities of (a) SOD, (b) POD and (c) CAT in control *S. polyrhiza* plants and plants exposed to different concentrations of GS and ECGS (The error bars represent standard deviation of the mean, n=4 replicates, *Significant difference at $P<0.05$, **Significant difference at $P<0.01$, ***Significant difference at $P<0.001$, Significant differences were shown compared to the control sample).

DISCUSSION

These days wide application of different classes of pesticides such as herbicides and their continuous entrance to the aquatic environment is one of the main and major causes of environmental threat. Therefore, identification and utilization of new technologies for modification of herbicide behavior and reduction of environmental contamination seems to be indispensable. Nanoencapsulation of herbicides is one of the effective strategies for controlled release of the herbicides. In the present study, nanoencapsulation of GS was successfully done by miniemulsion method. The nanocapsules had core-shell structures and were approximately between 20-200 nm. Assessment of toxicological effects of different classes of herbicides and their environmental risks on different targeted and non- targeted plants was one of the main concerns of the studies in last two decades. Hence, in order to determine the toxicological effects of ECGS and GS on the *S. Polyrhiza*, changes in the growth rate, pigment content and the activities of some antioxidant enzymes as indices for toxicological effects were studied.

According to the results, about the effects of different concentrations of GS and ECGS on the plant RGR, RGR correlated with concentrations of two groups of treated compounds and increasing the concentration was led to remarkable reduction of RGR compared to control samples. Moreover, direct treatment of GS had more and notable negative effects on the plant growth when compared with ECGS treatments and the control. Less negative effects of ECGS on the plants could be related to the slow release of herbicide from nanocapsules to the plant environment.

Reduction of RGR *in Lemna gibba*, *L. minor* and *Allium cepa* has been reported by increasing the concentrations of some herbicides such as Propanil and Quizalofop-p-ethyl in the previous studies [11,23,29]. Possibly, indirect decrease or inhibition of photosynthesis by GS could be one of the important reasons for the delayed growth [11].

About the effects of different concentrations of GS and ECGS on the photosynthetic pigments content, direct contact with a high amount of GS for a long time could finally cause the negative effect on the plant pigment contents. A similar trend has been previously reported in the case of the plant species treated with aryloxyphenoxy propionate

group of herbicides such as Quizalofop-p-ethyl and fluazifop-p-buthyl [10,11,30]. This group of herbicides can indirectly disrupt fatty acid biosynthesis via the inhibition of ACCase [11,31,32]. Therefore, GS, which belongs to this group, can inhibit the formation of thylakoids membranes and finally reduce the pigments content, thereby causing chlorosis.

In fact, the effects of direct GS treatments occurred earlier after 4 d treatment and direct contact with a high amount of GS for a long time (7 d) could finally cause the negative effects on the plant pigment contents. Therefore, because of the slow release of the GS from its encapsulated form, the negative effect of ECGS treatments was observed just for carotenoids content when the plant exposure to 100 ppm of the ECGS during the experiment.

In different unsuitable environmental conditions such as the presence of various classes of contaminants, the assembly of reactive oxygen species (ROS) is raised in the plants and led to damage cellular components by oxidizing important macromolecules like proteins, lipids and nucleic acids [33,34]. In response, ROS are uninterruptedly removed with a complex and efficient antioxidative system in the plants that protect them against such situations. For instance, SOD neutralizes reactive superoxide radicals to hydrogen peroxides and these are then detoxified by other antioxidative enzymes such as POD. These enzymes activities have already been used to evaluate the toxicity of the contaminants [20,35]. According to the results of SOD activity assay after treatment of the plant with different concentrations of GS and ECGS, SOD activity was increased after 4 d treatment with different concentrations of GS. The induction of SOD activity was reported in response to the increase of the concentrations of the treated herbicide in the environment of different duckweed species [11,15]. In contrast, the treatment of high concentration of GS for long time (7 d) was led to the remarkable reduction of the enzyme activity. It might be because of the abundant formation of reactive oxygen species after 7 d exposure of the plant samples with high concentration of GS that leads destruction of tissues and subsequently a decrease in SOD activity.

Moreover, treatment of high concentration of GS and ECGS was led to the significant enhancement of POD activity after 4 d treatment with GS and after 4 and 7 d treatment with ECSG. This increscent in POD activity could be the result of high ROS production in response to existence of the two groups of the examined contaminants in the plant environment even though the enhancement of POD activity when the plant was treated with GS was notably more than the effects of ECGS. It seems that prolonged direct contact with the plant (7 d) with high concentration of GS eventually leading to the notable negative effect on POD activity because, after this treatment of GS, POD activity was notably reduced. The plant direct treatments with GS had more induction effects on antioxidant enzymes activities, as compared to the treatments with ECGS. Although the treatment of high concentration of GS, for a long time was finally caused by the notable negative effects on their activities.

CONCLUSION

PMMA nanocapsules containing gallant super is successfully prepared and SEM analysis indicated that prepared capsules were of nanometer size and their capsules size was approximately less than 100 nm. The negative effects of ECGS on the growth of *S. polyrhiza* were less than those of the herbicide after 10 d treatment of the plant samples with different concentrations of GS and ECGS. The effects of direct GS treatments occurred earlier and direct contact with a high amount of GS for a long time (7 d) could finally cause the negative effects on the plant pigment contents. In contrast, because of the slow release of the GS from its encapsulated form, the negative effect of ECGS treatments was observed just for carotenoids content and after exposure of the plant to 100 ppm of the ECGS. Moreover, different examined concentrations of the two contaminant groups led to the remarkable induction of the activities of the antioxidant enzymes even though prolonged direct contact with the plant two examined high concentrations of GS eventually leading to the notable negative effects on antioxidant enzymes activities.

ACKNOWLEDGEMENTS

The authors thank the Urmia University, Iran for providing all kinds of support during the study. The authors declare that there is no conflict of interest.

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