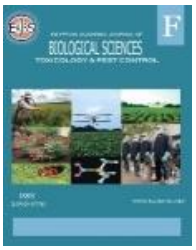
Egypt. Acad. J. Biolog. Sci., 12(1): 45-57 (2020)

Egyptian Academic Journal of Biological Sciences
F. Toxicology & Pest Control
ISSN: 2090 - 0791
http://eajbsf.journals.ekb.eg/



Toxicity and Disruptive Impacts of Fenhexamid Fungicide Against the Green Alga, *Chlorella vulgaris*.

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ARTICLE INFO
Article History
Received:9/3/2020
Accepted:30/3/2020
Keywords:
Toxicology, *Chlore vulgaris*, Fenhexan Antioxidant

ABSTRACT

Fenhexamid is one of the most extensively used fungicides against several plant pathogens. The green alga *Chlorella vulgaris* can be used as a model organism for toxicological tests in aquatic environments. The toxicity of fenhexamid was evaluated and the sub-lethal concentrations (LC_{10}, LC_{25}, and LC_{50}) were determined after 24, 48, and 96h of exposure. The physiological responses of *C. vulgaris* to fenhexamid exposure including growth, photosynthetic efficiency, as well as antioxidant activities were used to investigate its impacts on aquatic organisms. The results showed a high reduction in cell number of *C. vulgaris* in a concentration-dependent manner, with maximum reduction up to 62.9% after 96h exposure to 120 µg. L^{-1} fenhexamid. Also, a remarkable decrease in the concentration of chlorophyll *a*, *b* and total chlorophyll with 2.04, 1.06, and 2.3-fold time, respectively. The content of antioxidant enzymes elevated after exposure to fenhexamid**,** indicating the oxidative damage in the algal cell after treatment. The activity of GSH, MDA, and SOD enzymes elevated by 41.3%, 82.8%, 65.3% respectively, after 96h exposure to 120 µg. L^{-1} fenhexamid, While the content of POD and CAT enzymes increased by 56.1% and 58%, respectively after 24h of exposure. Examination by electron microscopy shows high damage in the cytomembrane and the cell wall in addition to increasing in size and number of the starch granules which indicate high stress associated with exposure to the tested fungicide. Therefore, preventing or at least reducing the broad use of fenhexamid as a fungicide becomes an urgent necessity to protect aquatic organisms.

INTRODUCTION

The problem of starvation increases every day forcing humankind to rearrange its relationship with nature in a world where agricultural areas continue to decrease at the same time population increment cannot be controlled (Bedil and Cetin, 2017). A lot of chemicals were started to be used in agriculture, to provide control from pests and microbes in order to save the quality of the agricultural crops and increase its quantity.

Fenhexamid, a hydroxyanilide derivative, is one of the most extensively used fungicides against the grey mould disease which affects many crops and caused by *Botrytis cinereal* (Suty *et al.*, 1997; Mosleh *et al.*, 2014a, b; Mosleh and Mofeed 2014b). It is also showed high activity against other fungal plant pathogens such as *Monilinia* spp, and *Scleorotinia sclerottioum* (Ziogas *et al.*, 2002; Mosleh *et al.*, 2005).

As a result of watershed drainage and accidental spillage, aquatic environments are exposed to contamination with such pesticides including fungicides, with concentrations ranging from sub-lethal to lethal levels (Khun and Streit, 1994; Mofeed and Mosleh, 2013). Phytoplankton is one from a large number of non-target organisms exposed to toxic circumstances and subjected to the risk of pesticide influence (Chen *et al.*, 2016). Algae represent one of the most important components of aquatic ecosystems. They are the major source of the dissolved oxygen via photosynthesis and they comprise the basic nutrition of different organisms in aquatic environments (Mosleh and Mofeed, 2014a; Mofeed, 2015).

The alga *Chlorella vulgaris* is commonly found in freshwater, due to its high chlorophyll content, it is considered a powerful oxygen source in aquatic habitat (Kong *et al.*, 2014). The high growth rate of *C. vulgaris* may take culture to autotrophy conditions. *Chlorella vulgaris* frequently used as a model organism for toxicological tests in aquatic environments (Geiger *et al.*, 2016; Borecka *et al.*, 2016).

There is limited information about the effect of fenhexamid fungicide against the growth of algae, especially the common distributed alga *Chlorella vulgaris*. As a result, this study aimed to evaluate the effect of fenhexamid against the growth and antioxidant activities of *C. vulgaris* to provide a good resource for future studies about fungicides and environmental control.

MATERIALS AND METHODS

Algal Strain and Growth Condition:

Chlorella vulgaris used in the present study was obtained from the algological collection of the National Institute of Oceanography and Fisheries. The algal strain was stored under the reference number NIOF-108. The alga was maintained in batch cultures containing 100 mL of Bold`s Basal Medium (BBM) with a final pH 6.3 (Archibald and Bold, 1970), continuous aeration and incubated on an orbital shaker (130 rpm) under continuous illumination of 63 mmol PAR/m^2/S provided by white fluorescent lamps (Toshiba 38W).

Determination of Sub-Lethal Concentrations:

To determine the sub-lethal concentrations (LC_{10}, LC_{25}, and LC_{50}) of fenhexamid against *C.vulgaris*, a stock solution of fenhexamid fungicide (100 mg. L^{-1} of the active ingredient) was prepared to reach final concentrations of 12.5, 25, 50 and 100 mg. L^{-1}. The toxicity of the fungicide was evaluated in microplates, which were incubated at 25ºC under continuous light (63mmol PAR/m^2/S). Three replicates without fenhexamid were used as control. After incubation (for 24, 48 and 96 h), the LC_{10}, LC_{25}, and LC_{50} values were determined according to Finney (1971).

Determination of Algal Growth:

The growth of *C. vulgaris* was detected by counting cell numbers using *Rafter* Counting Chamber after 24, 48 and 96 h of exposure to fenhexamid concentrations.

Analysis of Chlorophyll Contents:

Both the control and the treated *C. vulgaris* suspensions were centrifuged after 24, 48 and 96 h. Chlorophyll *a*, *b* and total chlorophyll were determined according to Jeffrey and Humphrey (1975).

Enzymes Assay:

After different exposure times, the cultures were collected and the enzyme extracts were analyzed to determine the enzymatic activates of the antioxidant enzymes, where the activity of malondialdehyde (MDA) was assayed as described by Janero (1990), using MDA assay kit. While total glutathione content (GSH) was determined as described by Griffith (1980) and Catalase (CAT) activity was determined spectrophotometrically by following the consumption of H_2O_2 at 240 nm for 1 min at 25 ^oC, (Aebi, 1984). Glutathione S-trans-ferase (GST) activity in the *C. vulgaris* cells was measured according to Regoli *et al.* (1997). The SOD activity was analyzed using assaying kits, where the inhibiting rate of the enzyme to O_2 produced by the xanthine morpholine with xanthine oxidase. The POD activity was also assayed using the POD assay kit according to the change in absorbance at 420nm by catalyzing H_2O_2.

Cellular and Subcellular Structure Observation:

After 96 h, *Chlorella vulgar* cells in both the control and the maximum concentration of Fenhexamid were harvest and then the sample was fixed using 2.5% glutaraldehyde at 4^oC for 12 h. The fixed cells were washed and dehydrated using a graded series of ethanol (35, 50, 70, 90 and 100%) and then embedded in epoxy resin, following the method described by Song *et al.* (2017), then observed using a JEOL JSM 1400- plus model transmission electron microscope (TEM) and JEOL JSM 6510 model scanning electron microscope (SEM).

Quantification of Fungicide Residues:

Chlorella vulgaris was incubated with different concentrations of fenhexamid for 24, 48, and 96 h, the fungicide was analyzed directly with high-performance liquid chromatography (HPLC) without pre-concentration. After incubation, an aliquot (10 mL) of media of each concentration was taken and then analyzed directly using HPLC by a reversed-phase column (Kromasil, C18, 100 A, 5, 250, 3 mm, CIL-Cluzeau). The fenhexamid was eluted isocratically using 100% acetonitrile (Frimpon *et al.*, 2012). The Fenhexamid concentrations were detected by monitoring the UV absorbance (200–340 nm) with a diode array detector (Gynkotek UVD 340S).

RESULTS

Sub-Lethal Concentrations of Fenhexamid and Growth Rates of *Chlorella vulgaris*:

Toxicity of fenhexamid to *C. vulgaris* elevated with the increasing in exposure time and fungicide concentrations. The LC_{25} values were (475.85, 439.35 and 402.36 µg. L^{-1}) and LC_{10} values (312.52, 208.69 and 198.84 µg. L^{-1}) after 24, 48 and 96 h of exposure, are shown in table 1.

Exposition of *C. vulgaris* to sub-lethal concentrations of fenhexamid showed the inhibition in growth after different times of exposure. As shown in figure 1, the fenhexamid inhibits the growth of *C. vulgaris* in a concentrations-dependent manner. The maximum reduction of cell number (62.9%) comparing to control was obtained after 96h exposure to fenhexamid to 120 µg. L^{-1}, while, the minimum reduction (24.7%) was observed after 24h of exposure to 15 µg. L^{-1}.

Table 1. Toxicity of fenhexamid (µg L^{-1}) against C. *vulgaris* after different time of exposure.

Toxicity	Time (h)		
	24	48	96
LC_{10}	312.52 ± 10.25	208.69 ± 14.25	198.84 ± 15.3
LC_{25}	475.85 ± 12.02	439.35 ± 32.35	402.36 ± 13.32
LC_{50}	658.36 ± 21.58	603.25 ± 35.25	569.65 ± 18.99

Data presented are means ± standard deviation

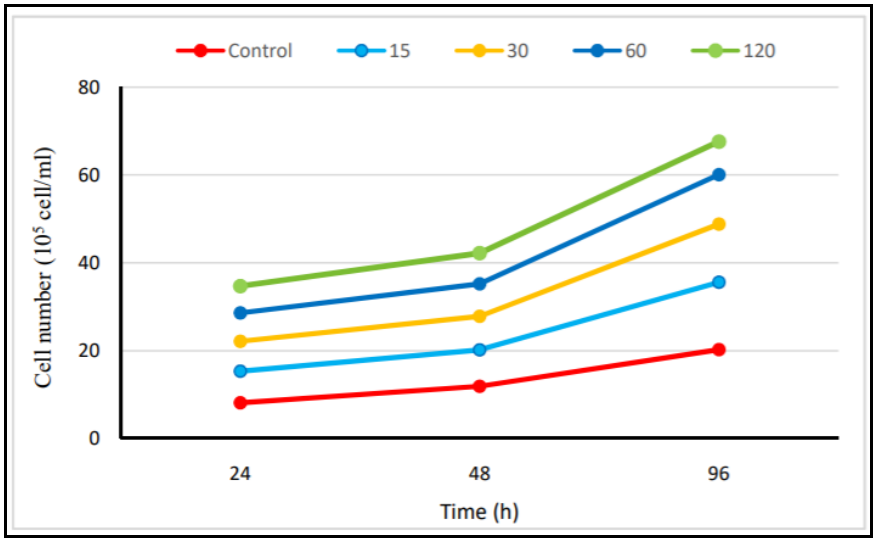

Fig. 1: Effect of sublethal concentrations of fenhexamid on growth (as Cell number) of *Chlorella vulgaris* after different times of exposure.

Cellular and Subcellular Structure of *Chlorella vulgaris*:

Chlorella vulgaris cells were examined using SEM and TEM after exposure to the highest concentration (120 µg. L^{-1}) of fenhexamid for 96h. The resulted SEM and TEM images showed changes in the treated *C. vulgaris*. The control cells were intact and plump while, the treated cells were shrunken, proving that the fenhexamid may affect the permeability of the membrane and cause irreversible lesions on the cell wall and cytoplasmic membrane (Figs. 2 and 3). Also, the TEM images of the treated *C. vulgaris* cells showed increases in the number and size of starch granules comparing to control. The exposure to the fenhexamid also showed the appearance of nipples and protrusions on the outer surface of the treated cell wall. In addition, the cell wall had a noticeable increase in the thickness of and it's appeared in multi-layer (Fig. 3b).

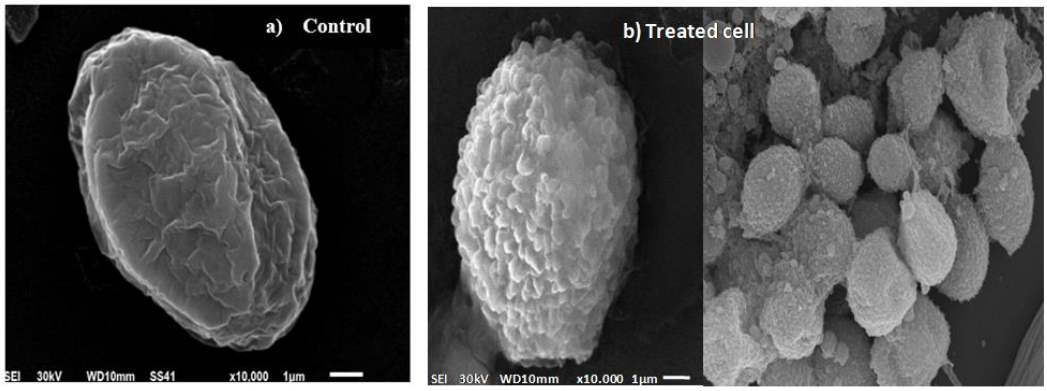

Fig.2: Cellular structure of *Chlorella vulgaris* using SEM (a) control (b) after exposed to 120 µg. L^{-1} of fenhexamid for 96 h

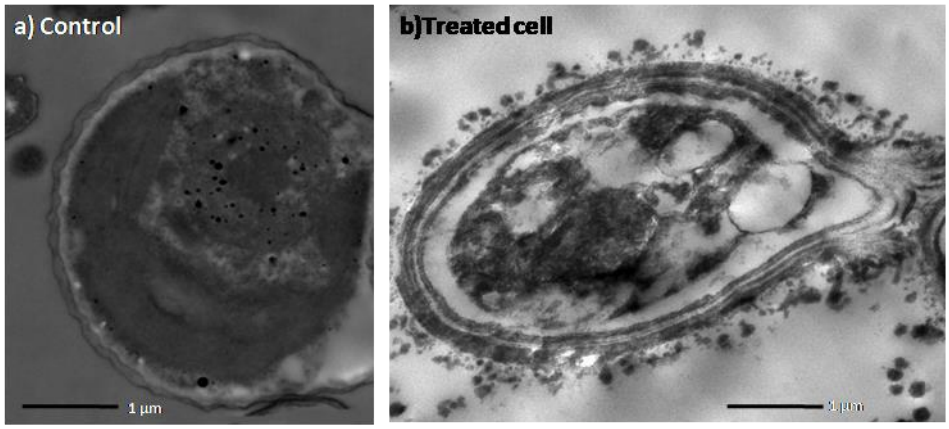

Fig. 3: Subcellular structure of *Chlorella vulgaris* using TEM (a) control (b) after exposed to 120 µg. L^{-1} of fenhexamid for 96 h.

Effect of Fenhexamid on Photosynthetic Pigments of *Chlorella vulgaris*:

Photosynthetic pigments that are responsible for absorbing light energy could be used as an indicator of any environmental stress. In the present study the content of chlorophyll a, b and total chlorophyll showed decreases when compared to control. Chlorophyll a content showed a decrease with 1.34, 1.8, and 2.4-fold time comparing to control after 24, 48, 96 h exposure to 120 µg. L^{-1}fenhexamid, respectively (Fig. 4). In the same line, chlorophyll b content exhibited a decrease of content with 1.01, 1.05, and 1.06-fold time comparing to control and after the same time of exposure (Fig.5). Consequently, total chlorophyll concentration decreased with 1.25, 1.5, and 2.3-fold time during the same period of exposure to 120 µg. L^{-1} fenhexamid (Fig.6).

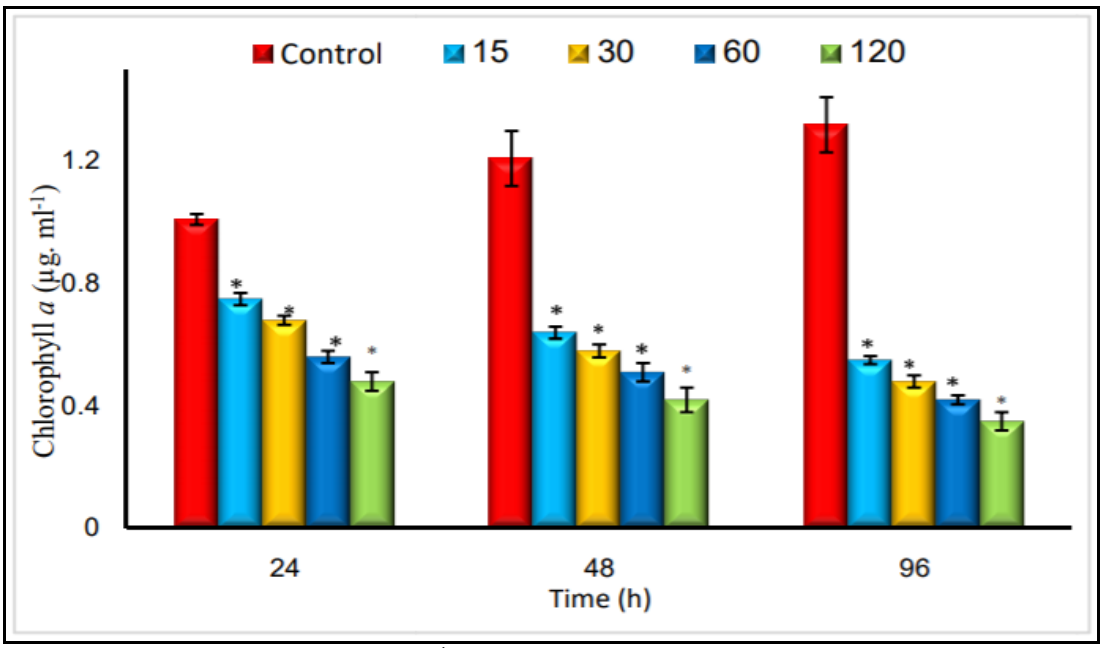

Fig. 4: Effect of fenhexamid (µg. L^{-1}) on chlorophyll *a* content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*significantly different from control at P ≤0.05).

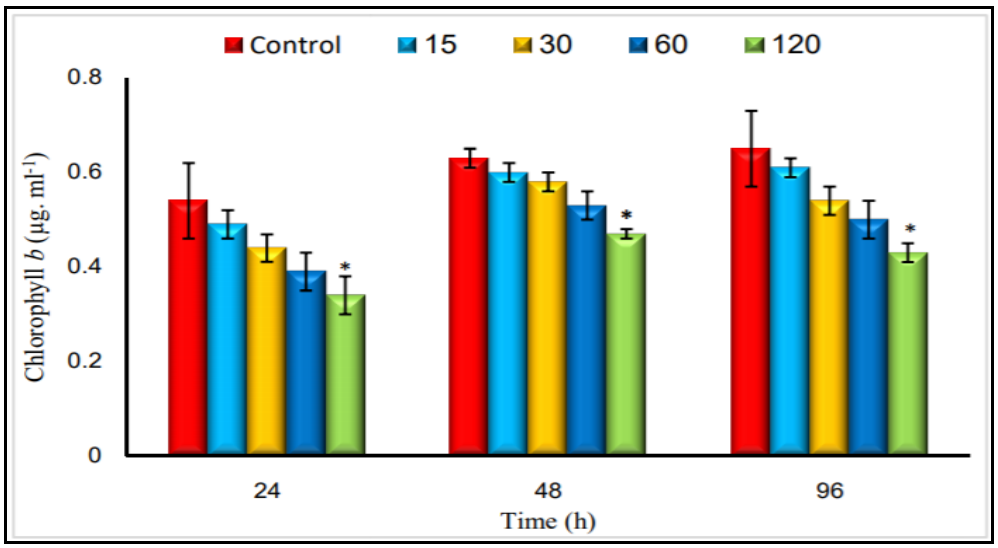

Fig. 5: Effect of fenhexamid (µg. L^{-1}) on chlorophyll *b* content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

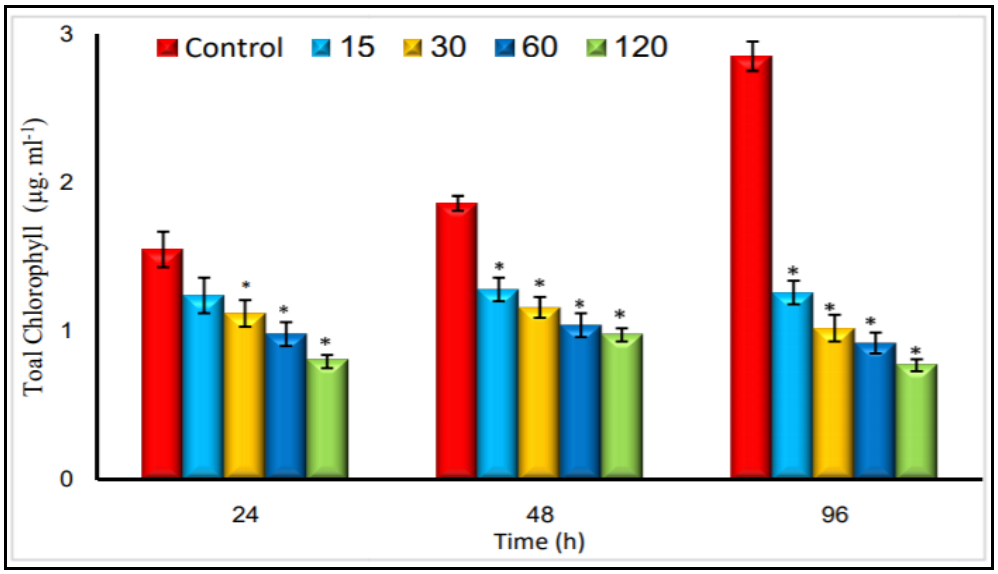

Fig. 6: Effect of fenhexamid (µg. L^{-1}) on total chlorophyll content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

Effect of Fenhexamid on the Antioxidant Enzymes of *Chlorella vulgaris*:

The GSH, GST, and MDA contents of *Chlorella vulgaris* were determined, as well as the activities of SOD, POD, and CAT. The GSH content increased by 35.9, 40.1, and 41.3 % compared to the control after exposure to 120 µg. L^{-1}fenhexamid for 24, 48, and 96 h, respectively (Fig.7). After 96h, The GSH concentration of the control sample was 540±10.31 mg protein/g and significantly increased with the increasing of fenhexamid concentration and the highest GSH content was recorded 920±15.23 mg protein/g. Unlike the GSH, the concentration of GST showed decreasing by 38.1%, 42.6%, and 54.1% compared to control during the same period of exposure to the sub-lethal concentrations of fenhexamid (Fig. 8). The GST content of the control sample recorded 105±1.08 nmol. $Protein^{-1}.min^{-1}$ after 24h and decreased with the increasing of fenhexamid concentration and the lowest one recorded was 65±0.99 nmol. $Protein^{-1}.min^{-1}$

after exposure to 120 µg L^{-1} fenhexamid, while after 48h and 96h, the increase in enzyme activities was mostly non-significant. MDA which measures the lipid peroxidation level in *C. vulgaris* showed an increase during the investigation period (Fig. 9). After 96h, the concentration in control sample was 0.25±0.023 nmol/mg protein and increased gradually with the increase of fenhexamid concentration with the highest concentration was 1.45±0.095 nmol/mg protein. The SOD activity showed an increase by 50.8, 52.1, and 65.2% after 24, 48, and 96h, respectively (Fig. 10). Unlike the previous enzymes, the CAT and POD content displayed a maximum increase after 24h exposure to 120 µg. L^{-1} fenhexamid. The highest concentration of CAT and POD was 198±6.65 nmol. $Protein^{-1}.min^{-1}$ and 3.78±0.24 µl/mg protein respectively, was recorded after 96h exposure to 120 µg L^{-1} fenhexamid (Figs.11 and 12).

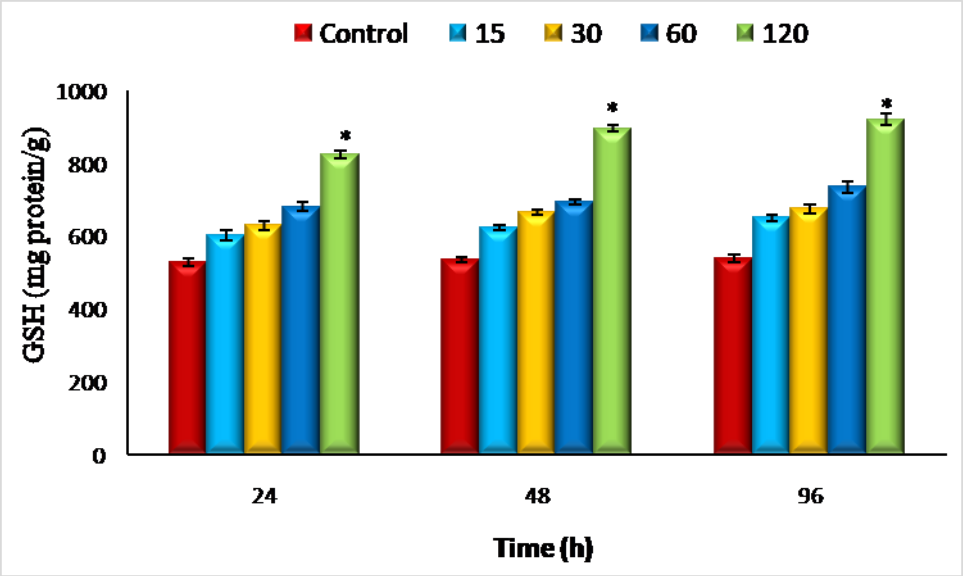

Fig. 7: Effect of fenhexamid (µg. L^{-1}) on GSH content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

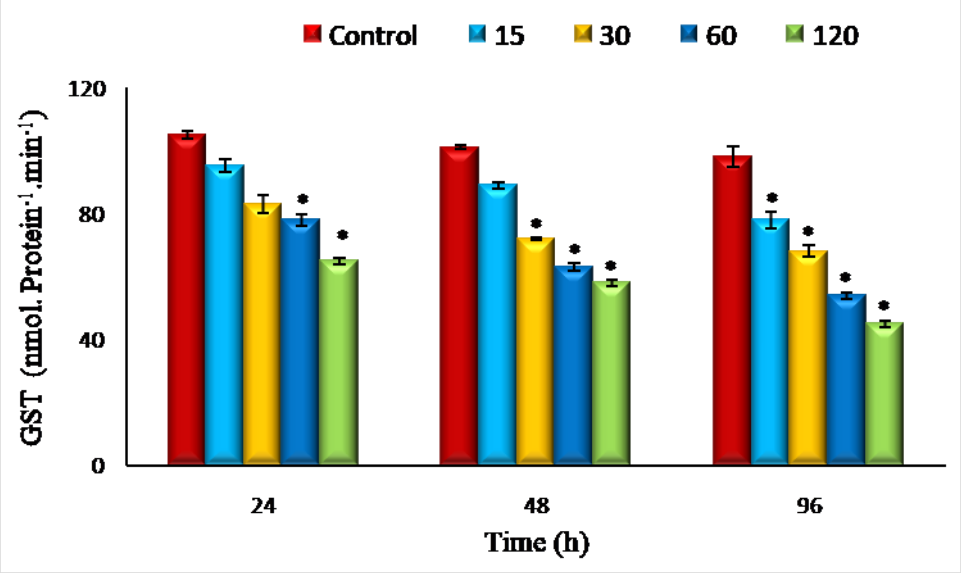

Fig. 8: Effect of fenhexamid (µg. L^{-1}) on GST content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05) .

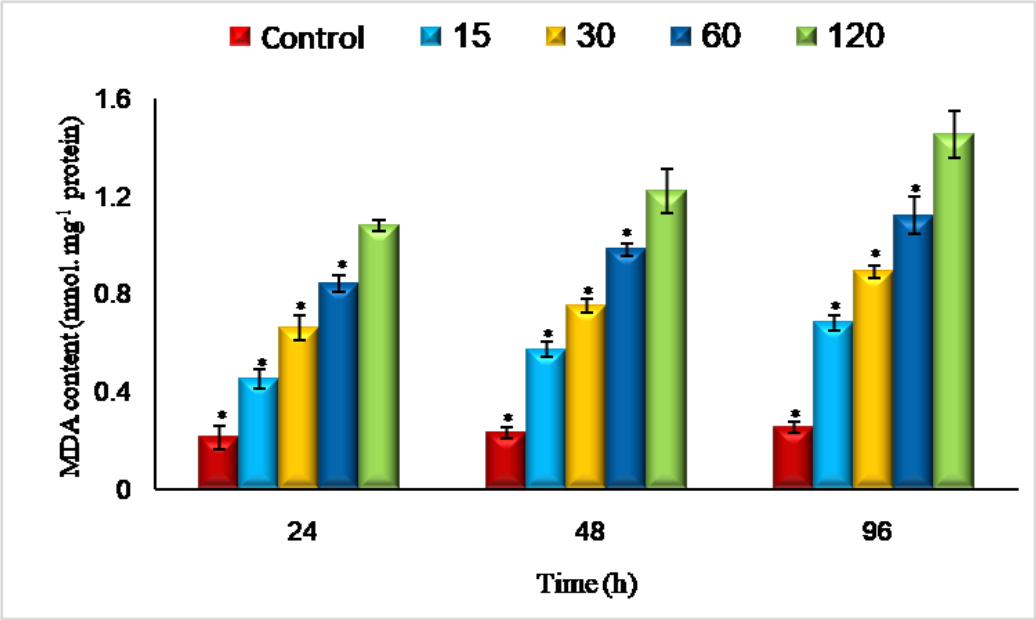

Fig. 9: Effect of fenhexamid (µg. L^{-1}) on MDA content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

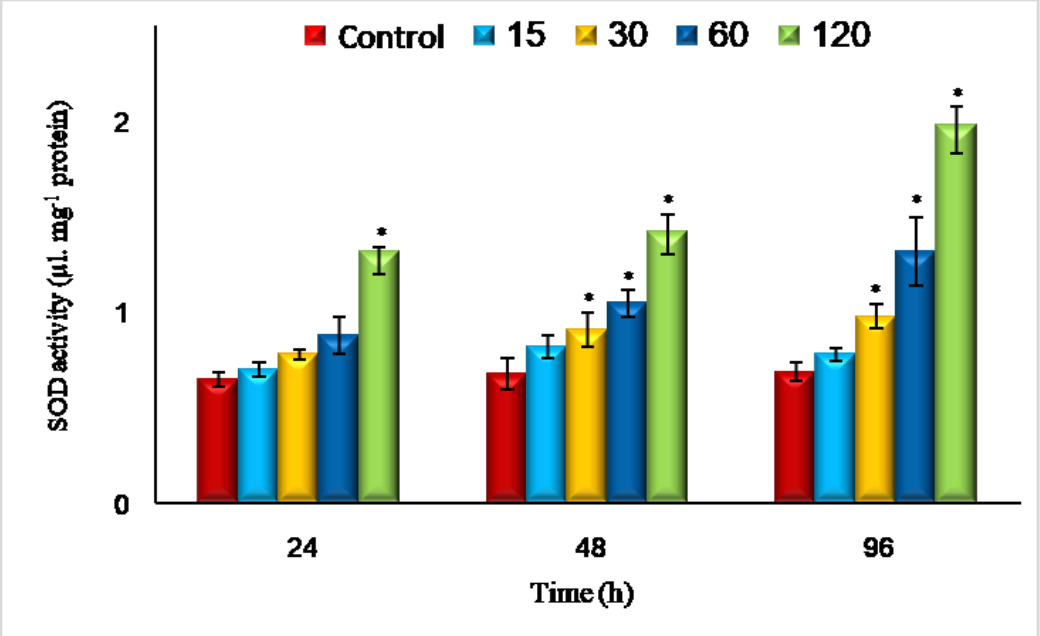

Fig. 10: Effect of fenhexamid (µg. L^{-1}) on SOD content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

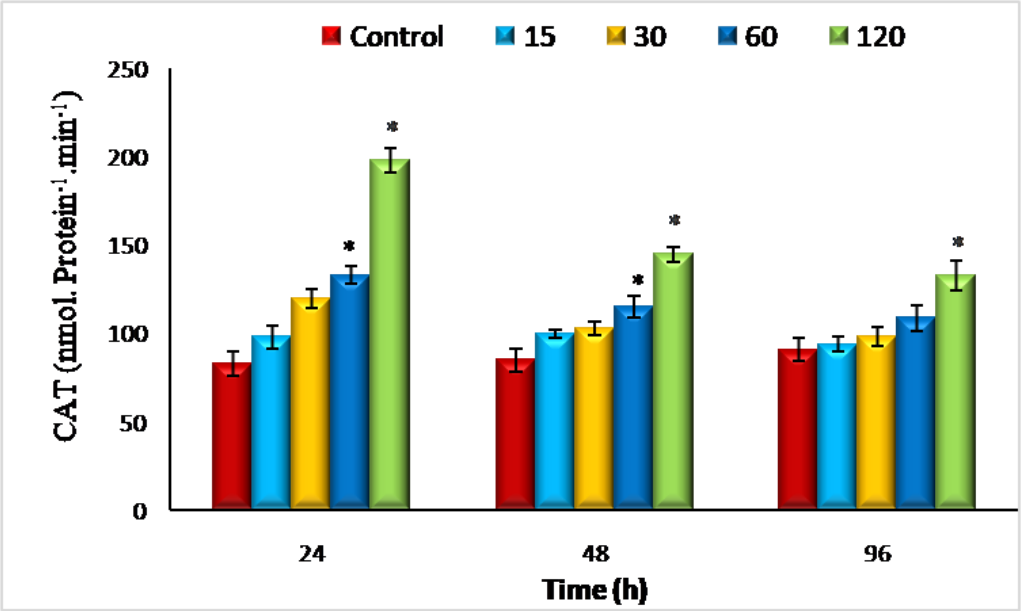

Fig. 11: Effect of fenhexamid (µg. L^{-1}) on CAT content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

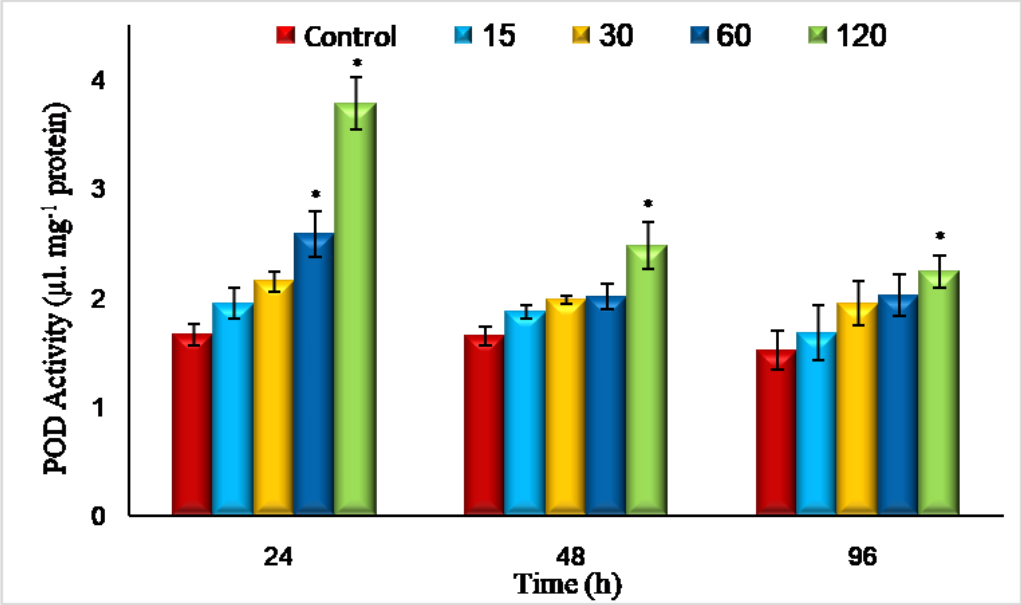

Fig. 12: Effect of fenhexamid (µg. L^{-1}) on POD content of *Chlorella vulgaris* after 24, 48 and 96 h of exposure (*Significantly different from control at P ≤0.05).

Quantity of Remaining Fungicide after Treatment:

In the present study, four concentrations were used to estimate the effect of fenhexamid on *C. vulgaris* growth. In this line, we quantified the remaining concentrations of fenhexamid after treatments for 24, 48 and 96h. Generally, the concentrations showed decrease with increasing time of exposure (Table. 2). The maximum decrease of each concentration was remarked after 96h of exposure, the first concentration (15µg.L^{-1}) showed a decrease of 50.7% after 96h. Similarly, the second, third and fourth concentrations were decreased by 53.5, 51.9, and 56.8%, respectively after 96h of treated.

Table 2. Residues of fenhexamid in the *Chlorella vulgaris* culture medium after different times of exposure to sublethal concentrations.

Initial concentration (µg.L^{-1})	Time (h)		
	24	48	96
15	12.585 ± 1.5	9.753 ± 0.95	7.395 ± 0.91
30	26.483 ± 2.31	16.652 ± 1.65	13.952 ± 1.05
60	53.425 ± 4.54	37.36 ± 2.03	28.853 ± 3.09
120	104.337 ± 6.62	73.902 ± 4.25	51.841 ± 4.12

Data presented are means ± standard deviation

DISCUSSION

Accumulations of pesticides affect organisms inhabiting the aquatic environment including algae. Microalgae showed various responses to the pesticides fluctuated from complete growth inhibition to noticeable resistance (Chen *et al.,* 2016). In the present investigation, the toxicity of the fungicide fenhexamid to *C. vulgaris* was evaluated using phytotoxicity tests based on growth inhibition, where the obtained results clarify that, fenhexamid inhibited the growth of *C. vulgaris* in a concentration-dependent manner. The maximum reduction in *C. vulgaris* cells obtained after 96h of exposure to the highest fenhexamid concentration, this result comes consistent with Bedil and Cetin (2017) who reported, treatment of fungicides causes high inhibition to the microalgal growth. Also, Lu *et al.* (2018) reported that, the fungicide azoxytrobin inhibits the growth of *Chlorella pyrenoidosa*.

In this work, we noticed that the lethal concentrations (LC_{10}, LC_{25}, and LC_{50}) of fenhexamid varied with the increase of exposure time. The Commission of European communities, EU-Directive 93/67/EEC (Directive**,** 1996) classified the matters with LC_{50}<1 mg L^{-1} and LC_{50} ranged between 1:10 mg L^{-1} as very toxic and toxic to aquatic organisms, respectively. According to this directive, fenhexamid should be fluctuated between toxic and very toxic matter to *C. vulgaris*.

According to the resulted images by scanning and transmission electron microscope, the treated *C. vulgaris* appeared to be shrunken and also nipples appeared on the outer cell wall surface. These results indicated a very diverse effect on cell structure and membrane permeability. Similar results were observed on *C. vulgar* which treated with trifloxytrobin and azoxytrobin (Liu *et al.,* 2015; Shen *et al.,* 2014). Interestingly, the accumulation of starch grains indicated a stress condition for *C. vulgaris* as it's a natural phenomenon occur in stressed plants and algae (Qian *et al.,* 2008). Photosynthesis considered one of the main evidences that appears when algae suffer from toxic factors (Qian *et al.,* 2016; Xie *et al.,* 2015), accordingly, the concentrations of chlorophyll a, b and total chlorophyll decreased in a dose-dependent manner and with respecting the exposure time.

The antioxidant enzymes activity believed as very sensitive and effective indicators for fungicides pollution on microalgae. In the present work, there was a remarkable increase in the content of GSH as well as the activity of SOD, POD, CAT, and MDA. The SOD, POD, CAT enzymes are considered the first barriers to resist oxidative stress (Ballesteros, 2009), so the obtained increase in levels of these enzymes indicated a response of *C. vulgais* cells against oxidative stress caused by fenhexamid. Bajguz (2010) demonstrated an increase in the activities of *C. vulgaris'* SOD and CAT enzymes when exposed to heavy metals. Lu *et al.* (2018) suggested that the high level of

MDA may due to the disability of antioxidant enzymes to maintain the production of oxidative species and the antioxidant defense in *Chlorella pyrenoidosa*, which causes an increase in lipid peroxidation and damage of the cytomembrane.

CONCLUSION

The present study proved evidence on the highly toxic effect of the widely used fungicide fenhexamid; against one of the most commonly distributed green algae; *Chlorella vulgaris* in aquatic environment. Where, fenhexamid suppressed growth and disturb the production of its antioxidant tools, besides noticeable distortion in cell structure. Therefore, we considered that more studies are required on other aquatic organisms, in order to get a clearer picture of fenhexamid effects and submitting a recommendation for the competent authorities to prevent or at least reduce the broad use of fenhexamid as a fungicide.

ACKNOWLEDGMENT

Authors express appreciation to Doctor Eman Abdelall for the generous donation of the tested alga "*Chlorella vulgaris*" and Professor Doctor Yahia Mosleh for his continuous support.

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