Cytotoxicity, Oxidative Stress and Biochemical Alterations Induced by Traditional and Nanoform of Pendimethalin in Freshwater Alga Chlorella vulgaris

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Abstract

The roles of pendimethalin and its nano-form to induce oxidative stress, osmolytes, biochemical alterations and cytotoxic effect in algal cells of Chlorella vulgaris after exposure to sub-lethal concentrations were carried out. The data indicated that, 96-h EC₅₀ values were 20 and 19 ppb for pendimethalin and its nano-form, respectively. Three concentration levels (0.1EC₅₀, 0.025EC₅₀ and EC₅₀) of the examined herbicide decreased the algal pigments (Chlorophyll a and b), but increased carotenoid contents compared with the control group. Biomolecules such as protein, carbohydrates, proline and sucrose of the algal cells significantly increased for the all treatments compared with the control groups. Similarly, malondialdehyde (MDA), some enzymes such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) showed significant increases for the all treatments. On the other hand, ultrastructural investigation of the herbicide-treated algal cells showed significant changes in their organelles in comparing with the untreated cells through transmission electron microscope (TEM) images. From all findings, it was obtained that the nano-pendimethalin was more potential toxic than the traditional form to induce adverse effects on the freshwater alga. Thus, biosafety procedures must be followed on non-target species before decision for nano-herbicide practices.

Keywords

Pendimethalin, Nano-emulsion, Chlorella vulgaris, TEM, Biochemical alterations

Introduction

Today, environmental problems are multiple and/or complex, especially those associated with the disposal of identification and assessment of the toxicity of such substances. Assessment of human exposure to pesticides and other toxicants through biological monitoring offers one means to evaluate the magnitude of the potential health risk of these chemicals. Herbicides are considered as a necessary factor in agricultural practices. However, its extensive use has elicited more research into herbicide effects on non-target organisms such as algae. They affect when herbicides turn back into the lakes or rivers by irrigation water and/or precipitation. The potential effect of herbicides on the aquatic primary producers is mainly vital, and has to be studied in ecotoxicological research trails. These chemicals can change the structure and function of aquatic communities by altering the species composition for the communities such as algal species. Generally, pesticides may be also metabolized or bioaccumulated by microorganisms. The most of the pesticides studied are herbicides. They are generally high toxic to phototrophic micro-organisms, exhibiting toxicity by disrupting
photosynthesis. In the algal toxicity tests, median effective concentration (EC_{50}) was determined using growth rate as an endpoint. In fact, algal growth rate was assessed using direct cell counts or optical density [1].

Herbicide, pendimethalin with IUPAC name; 3, 4-dimethyl-2, 6-dinitro-N-pentan-3-ylaniline, (CAS: 40487-42-1) is used primarily to destroy or prevent the growth of certain plants like weeds. It is also used on crops such as fruits, grapes, vegetables, oil seeds, cereals, tobacco and ornamentals. It is used and applied in various forms including liquid, solid and granules. Pendimethalin dispenses in the environment through binding to soil microbially-mediated metabolism and volatilization. It is essentially immobile in soil. It is slightly to moderately persistent in aerobic soil environments. Extensive use of this compound may adversely affect endangered species of terrestrial and semi-aquatic plant, aquatic plants and invertebrates including mollusks, fish and birds. The risk to non-target terrestrial and semi-aquatic plant is expected to be moderate [2]. It’s mode of action is as the inhibitor for cell division and cell elongation [3, 4].

Algae have a vital role in the primary production for aquatic ecosystem. They are considered as bio-indicators of the bioactivity of industrial wastes and others as well as they vary in their response to a variety of toxicants [5]. For example, Chlorella vulgaris is used as an indicator in many research trials to establish the impact of chemicals released into aquatic environment in dependence on its highly sensitivity to pollutant and chemicals [6, 7].

Oxidative stress caused by herbicides generates large amount of reactive oxygen species (ROS). Excessive levels of ROS result in oxidative damage to key-cell components, e.g. nucleic acids damage, oxidation and defect in proteins, lipid and degradation of Chlorophyll pigments [8, 9]. On the other hand, cytotoxicity of pesticides on micro-organisms represents different techniques e.g. ultrastructural pattern, flow cytometry, genomic and proteomic profiles. Transmission electron microscope (TEM) visualizes the interaction of xenobiotics with cell organelles and impacted disorganization.

As stated in the literature, most of herbicides mainly disrupt normal photosynthesis resulting in alterations in lipid and carbohydrates formation [10].

Nano-pesticides or nano plant protection products represent an emerging technological development which could offer a range of benefits: increased efficacy, stability, and reduction in the amounts of active ingredients (a.i) that need to be used. Nanotechnology aims to provide protection of active ingredient (a.i) or enhance its delivery to the site of action. Different formulations have been suggested including emulsions (e.g. nano-emulsions), nano-capsules (e.g. polymers), and products containing engineered nanoparticles (NPs), such as metals, metal oxides, and nano-clays [11].

In fact, there are negative aspects of conventional herbicides on environment under argument. Therefore, use of NPs resolve these problems, where its application with herbicides reduces the amount of the herbicides required for weed eradication. Combined a.i with smart delivery system leads to a.i release in the soil as required [12]. There is a lack concern the impact of nano-herbicides on algae. However, toxicity of poly (ε-caprolactone) nano-capsules containing herbicides, ametryn and atrazine against Pseudokirchneriella subcapitata was studied. The prepared formulations resulted in lower toxicity to the alga as compared to the herbicides alone [13]. The increasing interest in the use of nano-pesticides introduce questions around the environmental risk of these materials, its effect on non-target organisms such as algae and their environmental persistent in comparing with conventional products. Alga C. vulgaris is one of the most commonly used species in microalgal toxicity tests and considered as a good indicator to find answers for these questions.

As stated in the literature, the impact of nano-pesticides already differs from its forms of conventional pesticides on terrestrial or other non-target organisms. Thus, information of the stability of nano-pesticide products and the fate in soil should be examined and analyzed in aquatic fate studies and in ecotoxicological studies. These studies are essential to deliberate the form of the accumulated material (free versus nano-bound a.i), because this provides the implications for the progression of the toxicity over time [14]. Additionally, there is the potential that a.i may be more active in the bound state (nano-a.i complex), where nano-formulation would lead to higher local concentrations even at shorter time points. The components of nano-pesticide mostly protect a.i from degradation, then the uptake and behavior will be more than that of conventional pesticide formulation [11]. The study was designed to 1) evaluate the toxicity of nano-emulsion pendimethalin on the freshwater alga C. vulgaris in comparing with its conventional formulation. 2) Evaluate the cytotoxic and biochemical alterations which may be induced after exposure to sub-lethal concentrations.

Material and Methods

Freshwater alga

Alga Chlorella vulgaris Beyerinck [Beijerinck] was used in this study. It is unicellular freshwater alga. It was obtained from The Institute of the Oceanography and Fishers at Alexandria, Egypt. This organism is classified as follows: Empire: Eukaryota; Kingdom: plantae; Phylum: Chlorophyta; Subphylum: Chlorophyta; Class: Trebouxiophyceae; Order: Chlorellales; Family: Chlorellaceae; Genus: Chlorella and Species: vulgaris.

Herbicide and Chemicals

Herbicide

Pendimethalin (Stomp® 40% EC); IUPAC Name: 3, 4-dimethyl-2, 6-dinitro-N-pentan-3-ylaniline was obtained from Shoura Co. for Agrochemicals, Egypt. On the other hand, active ingredient of pendimethalin (purity 95%) was supplied by Kafr EL-Zayat Co. for pesticides and fertilizers, Egypt. It was used for nano-emulsion preparation.

Chemicals

Ethanol, sodium bicarbonate, sodium phosphate
monobasic, sodium phosphate dibasic, potassium phosphate monobasic, potassium phosphate dibasic, aqueous KOH, sulfuric acid and glacial acetic acid were obtained from J.T. Baker chemical Co., Philippines, N.J.08865. Methanol, chloroform and n-hexane were supplied by BDH laboratory supplies pool, BH 15 1T, England. Riboflavin 99%, ninhydrin 99%, nitro blue tetrazolium (NBT; 99.8%) were supplied by Sigma Chemical Co. P.O. box 14508 St. Louis, Mo 63178 USA. Thiobarbituric acid (TBA), anthrone (C<sub>12</sub>H<sub>10</sub>O) and 5-sulphosalicylic acid were obtained from LOBA CHEMIE Ltd, India. Trichloroacetic acid (TCA) was supplied by SDFCL- CHEM limited, India. Ascorbate and Hexahydrate ferric chloride (FeCl<sub>3</sub>. 6H<sub>2</sub>O) were obtained from Merck, Darmstadt, Germany. Boric acid (H<sub>3</sub>BO<sub>3</sub>) and magnesium sulfate, 7-Hydrate (MgSO<sub>4</sub>. 7H<sub>2</sub>O) were obtained from Sigma, USA. Ferric chloride (FeCl<sub>3</sub>. 6H<sub>2</sub>O) and sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), sodium nitrate (NaNO<sub>3</sub>), magnesium chloride hexahydrate (MgCl<sub>2</sub>·6H<sub>2</sub>O), cobalt (II) chloride hexahydrate (CoCl<sub>2</sub>·6H<sub>2</sub>O), (Na-K tartrate), copper (II) chloride dihydrate (CuCl<sub>2</sub>·2H<sub>2</sub>O), potassium chloride (KClO<sub>3</sub>), ethylene diamine tetra acetic acid (EDTA), calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O) and manganese (II) chloride tetrahydrate (MnCl<sub>2</sub>·4H<sub>2</sub>O) were obtained from BDH chemicals Ltd England.

Synthesis of nano-emulsion

Pendimethalin was prepared as a nano-emulsion form; 40% a.i under high energy mode using sonicatation technique [15]. The technical a.i. (purity 95%) was dissolved in a vegetable oil and employed for dispersion in a liquid (water) with surfactant 10% and co-surfactant (0.5%). The mixture was subjected to ultrasonic machine (Branson, Digital sonifier, Shanghai Co. Ltd, China) at 60-100 Hz for enough time to generate homogenous solution (o/w) as a desirable nano-emulsion 40% a.i of the total volume.

Characterization of nano-emulsion

Thermo-dynamic stability

The prepared nan-form (40% a.i) was examined for different storage conditions of temperature and humidity to assess the emulsion stability according to ICH guidelines Q1A [16]. An aliquot (3 ml) of nano-emulsion was centrifuged at 3500 rpm for 30-min to check any phase separation. Ten ml of formulation was diluted to 100 ml with dis. H<sub>2</sub>O in graduated cylinder. The solution was achieved to shake at 30 times from top to bottom continuously. At the end, the jar was allowed to stand for 10-min and observe any oil separation, creaming or sedimentation.

On the other hand, an aliquot of nano-form of pendimethalin was examined through heating and cooling cycle. Six cycles between refrigerator and oven temperature of 4 and 48 °C were performed for 48 h. So, it was employed to freeze-thaw cycle test. Three cycles were done between -20 and 25 °C.

Transmission Electron Microscope (TEM)

Morphology and structure of the prepared nano-form was examined on transmission electron microscope (TEM) (JOEL 1400 Plus, Japan) at filament 80 Kev to achieve the shape and size of prepared nano-emulsion. An aliquot of this form was diluted with deionized water (1/100) and sonicated for enough time. Aliquot of the diluted solution was dropped on the film grid, dried and examined [17, 18]. A combination of bright-field imaging at increased magnification with diffraction modes was used.

Fourier Transform Infrared (FTIR)

The conventional pendimethalin and its nano-form were achieved on TENSOR 27 Buer, Germany-FTIR L203/12887 instrument. The spectrophotometer ranged from 4000 to 400 cm<sup>-1</sup>. The run was conducted with sensitivity range 50 and absolute threshold level of 6.07.

Acute toxicity test

Green alga C. vulgaris population was exposed to series of concentrations of the examined herbicide for 96 h in a static system according to USEPA protocol [19]. The response of population was measured in term of changes in cell density (cell counts/ml). Probit analysis was used to estimate EC<sub>50</sub>. The average growth rate was calculated according to the following equation [20].

where, μ<sub>i-j</sub> is the average growth rate from moment time i to j, ti is the time for the start of the period (zero time), tj is the time for the end of the period (96 h), Bi is the initial cell number/ml at start of the experiment, and Bj is the cell number/ml after 96 h, respectively.

Risk phrases of pendimethalin and its nano-form were done independent on their EC<sub>50</sub> according to legal requirements of EU guidance [21]. The risk phrase categories of EU are R50, R50/53, R51/53. R52/53 and R53 with EC<sub>50</sub> values; < 0.1, 0.1-1, ≥ 1-10, ≥ 10-100, and > 100 mg L<sup>-1</sup>, 96 h, respectively.

Sub-lethal toxicity

Three levels: 0.1, 0.025 and EC<sub>50</sub> were selected to study the impact of pendimethalin and its nano-form on different biochemical parameters. The procedures were done as described above in case of acute toxicity experiment. After 96 h, algal biomass of the treatments and control were harvested for ultrastructural investigation and other quantifications.

Algal pigments

One g of algal biomass was homogenized with 50 ml of 96% methanol for one day [22]. The extract was centrifuged at 2500 rpm for 10-min. The supernatant was taken and the absorbances of Chlorophyll a, Chlorophyll b and Carotenoids were recorded by using spectrophotometer (Spectronic 21D, Milton Roy, USA) at wavelengths: 662, 646 and 470 nm, respectively. Algal pigment content was calculated according to the following equations:

\[ C_a = 15.65 A_{662} - 7.340 A_{646} \]
\[ C_b = 27.05 A_{662} - 11.21 A_{646} \]
\[ C_{car} = 1000 A_{470} - 2.270 C_a - 81.4 C_b \]

where: \( C_a \) = Chlorophyll a, \( C_b \) = Chlorophyll b, and
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Estimation of carbohydrate

Carbohydrate content was determined by using anthrone reagent method [23]. One mg of algal biomass was mixed with 1 ml of 1N NaOH and heated in boiling water bath for 10 min. Each ml of the extract was mixed with 5 ml of reagent A (prepared by adding 1 ml of freshly prepared 1% Na-K tartrate solution containing 0.5% CuSO₄ into 50 ml 2% Na₂CO₃ solution) and incubated at room temperature for 10 min. Then, 5 ml of reagent B (folin reagent) was added, mixed well at the shaker and again incubated at room temperature for 30 min. The absorbance was read at 650 nm against folin reagent as a blank. Carbohydrate content was expressed as mg g⁻¹ dry algal biomass.

Estimation of lipid peroxidation

Lipid peroxidation was quantified through measuring malondialdehyde (MDA) level by using thiobarbituric acid (TBA) method [25]. The absorbance was recorded at 532 nm. MDA content was expressed as mM g⁻¹ algal biomass by using its extinction coefficient (155 mM cm⁻¹).

Antioxidant enzymes assay

The algal cells were harvested by centrifugation and broke by ultrasonication in 1.5 ml of extraction buffer (50 mM Tris HCl; pH 7.8), 1 mM EDTA, 1 mM MgCl₂ and 1% polyvinylpyrrolidone (PVP), but 1 mM ascorbate was used in case of ascorbic peroxidase (APX) assay. The homogenate was centrifuged at 12,000 rpm for 20-min. The supernatant was used as a source to assay enzymes: catalase (CAT), superoxide dismutase (SOD) and APX, respectively [26].

CAT assay

The enzyme activity was assayed according to method of [27]. The absorbance was read on 240 nm for 2-3 min and the activity was expressed as U mg⁻¹ protein. The unit of CAT is expressed as U mg⁻¹  protein.

SOD assay

Enzyme activity was quantified according to method of [28]. The absorbance was recorded at 240 nm for 2-3 min and expressed as U mg⁻¹ protein.

APX assay

The enzyme activity was assayed according to method of [29]. The absorbance was recorded at 290 nm for 2-3 min and the activity was expressed as U mg⁻¹ protein by using an extinction coefficient (2.8 mM).

Ultrastructural investigation

After sub-lethal toxicity experiments, algal cells of the treatments and control were collected through centrifugation at 3000 rpm for 5 min. The supernatant was discarded and the pellets were washed with 10 ml of deionized H₂O. Fastly, washed pellets were fixed with 2 ml of 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.2), and stored at 4 °C until used. These fixative samples were washed with physiological saline or 0.1 M phosphate buffer pH 7.2. The pellets were put into 1% osmium tetra oxide (OSO₄) for 1-2 h at 4 °C and rinsed in buffer for 2-min. The samples were dehydrated in a series of increasing concentrations of acetone (25, 50, 75 and 100%) for 5-min. When dehydrgation in 100% acetone solution is finished, the tissues were infiltrated using propylene oxide. Epon araldite was used to embed the specimen for 48 h under heating. Capsulated samples were sectioned by using Ultratome machine at 20-30 nm thickness. The sections were stained with uranyl acetate (4%) for 5-min and then rinsed in series in four beakers of pure water. Then, the grids were stained with lead acetate (1%) for 5-min, rinsed again in water and stored in a grid box until observed [32].

Statistical analysis

The EC₅₀ values and the regression equations were calculated according to method of [33] using LdPLine® software. The data were cited as mean ± SE. The analysis of data was done by using COSTAT program (Costate User Manual), version 3. Cohort Tucson, Arizona, USA [34].

Results

Characterization of nano-emulsion

The prepared nano-emulsion of herbicide, pendimethalin was stable during freeze-thaw cycle’s storage. No creaming or floating phases were made up. In addition, no separation phase was formed after centrifugation or shaken processes.

As observed from TEM images, the particles of nano-form appeared in spherical shape and the size was mainly in the range from 26 to 109 nm (Figure 1A).
Cytotoxicity, Oxidative Stress and Biochemical Alterations Induced by Traditional and Nanoform of Pendimethalin in Freshwater Alga Chlorella vulgaris

Noaman et al.

FTIR pattern of pendimethalin in its traditional formula was formed in nearly similar profile to prepared nano-pendimethalin (Figure 1B). The traditional formula obtained aromatic ring stretching at 1460 cm$^{-1}$, but the nano-emulsion obtained shifting peaks at 1570 cm$^{-1}$. The chemical structure of pendimethalin mainly depends on active groups NH and NO$_2$. Strongly overlapped peak obtained at 3440 cm$^{-1}$ for NH stretching in traditional formula, but slightly overlapped peak was formed in nano-emulsion. Double-bonded nitrogen groups exhibited absorption attributed to carbonyl (C=O) and alkene (C=C) double bond stretching region for aromatic nitro compounds absorption were formed at 1300–1350 cm$^{-1}$ [35].

Acute toxicity

Herbicide, pendimethalin and its nano-form were assayed on the freshwater alga C. vulgaris to obtain 96-h toxicities (Table 1). Nano-form of herbicide exhibited the greatest toxic effect with EC$_{50}$: 19 ppb and EC$_{90}$ 120 ppb. Traditional pendimethalin exhibited EC$_{50}$: 20 ppb

Based on the magnitude of EC$_{50}$ values, toxicity rating and estimated risk phrases, the decreasing order of aquatic risk was nano-pendimethalin ≥ pendimethalin with risk categories R50 according to EU guidance. The values indicate that, both herbicides were very toxic on the tested aquatic organism.

Biochemical responses

Algal pigments

The levels of pigments in the treated C. vulgaris with herbicide showed that, Chlorophyll a content decreased after exposure to the examined herbicide compared with the control (Figure 2). Each treatment exhibited decrease in Chlorophyll a content compared with the control which did not exceed 410 μg g$^{-1}$. Pendimethalin exhibited decrease values: 264, 266 and 322 μg g$^{-1}$ biomass for EC$_{50}$, 0.1 EC$_{50}$ and 0.025 EC$_{50}$ treatments, respectively, with mean value (284 μg g$^{-1}$ biomass). Nano-form of pendimethalin exhibited the values: 349, 369 and 392 μg g$^{-1}$ algal biomass for all treatments, with mean value (370 μg g$^{-1}$ algal biomass). Regarding Chlorophyll b, the treatments caused the decrease of algal pigment content compared to the control (234 μg g$^{-1}$ algal biomass) (Figure 2). Pendimethalin exhibited decrease in Chlorophyll b levels accounting for 82.70, 121 and 173 μg g$^{-1}$ algal biomass for EC$_{50}$ 0.1 and 0.025 EC$_{50}$ treatments, respectively, with mean value (125 μg g$^{-1}$ biomass). Nano-form of pendimethalin exhibited the values: 143, 195 and 294 μg g$^{-1}$ algal biomass. Regarding carotenoid, the all treatments significantly caused the increase of algal cell content compared with the control, which did not exceed 87 μg g$^{-1}$ algal biomass (Figure 2). Pendimethalin exhibited increase

Table 1: Relative toxicities and risk phrases of the examined herbicide on C. vulgaris.

| Herbicide       | EC$_{50}$ Lower limit | Upper limit | 1  | 2  | Index % | folds | slope | EC$_{90}$ | Risk Phrases |
|-----------------|-----------------------|-------------|----|----|---------|-------|-------|-----------|--------------|
| Nano-form of pendimethalin | 19 | 16 | 22 | * | * | 100 | 1 | 1.6 | 120 | R50 |
| pendimethalin   | 20 | 17 | 23 | * | * | 96 | -0.97 | 1.8 | 106 | R50 |

- The folds were estimated depending on nano-pendimethalin as a highest toxic compound.
- The used concentrations were corrected for active ingredient percent in each formulation.
- R-phrase value indicates that, the examined herbicides were very toxic on the tested aquatic organism.
Cytotoxicity, Oxidative Stress and Biochemical Alterations Induced by Traditional and Nanoform of Pendimethalin in Freshwater Alga Chlorella vulgaris

Noaman et al.

levels: 1567, 1097 and 795 μg g⁻¹ algal biomass for EC₅₀, 0.1 and 0.025 treatments, respectively, with mean value (1153 μg g⁻¹ algal biomass). Nano-form of pendimethalin exhibited the levels: 1211, 1083 and 649 μg g⁻¹ algal biomass for the same treatments with mean value (981 μg g⁻¹ algal biomass).

Protein content

Content of total protein in algal biomass of treated C. vulgaris observed significant decrease compared with the control group (277 mg g⁻¹ algal biomass) (Figure 3). EC₅₀ treatment showed decline in protein content as follows: 89 and 179 mg g⁻¹ algal biomass for pendimethalin and nano-pendimethalin, respectively. The same manner was observed in case of 0.1 and 0.025 EC₅₀ treatments with values: 152, 210 mg g⁻¹ and 175, 236 mg g⁻¹. The mean values were 139 and 209 mg g⁻¹ for pendimethalin and its nano-form, respectively.

Carbohydrates

The examined herbicide for 96 h exhibited significant increase in carbohydrate content in biomass of C. vulgaris compared with the control (188 mg g⁻¹ dry weight) (Figure 3). EC₅₀ treatment showed the following order: 207, and 263 mg g⁻¹ dry weight for nano-form and pendimethalin, respectively. Other treatments (0.1 and 0.025 EC₅₀) were in the same manner as follows: 200, 228 mg g⁻¹ dry weight and 194, 222 mg g⁻¹ dry weight. The mean values were 200 and 237 mg g⁻¹ dry weight for nano-form and pendimethalin, respectively.

Lipid peroxidation (LPO)

The all treatments exhibited increase in MDA level greater than the control (0.01 mM g⁻¹ dry weight) (Figure 3). EC₅₀ treatment induced increase in the following order: 0.06, and 0.75 mM g⁻¹ dry weight for nano-form and pendimethalin, respectively. At the same manner, 0.1 and 0.025 EC₅₀ treatments induced increase as follows: 0.03, 0.59 mM g⁻¹ dry weight and 0.02, 0.32 mM g⁻¹ dry weight.

Antioxidant enzymes

CAT

The enzyme activities in alga C. vulgaris exposed to the examined herbicide increased greater than the control (Figure 4). EC₅₀ treatment increased enzyme activity in the following order: 64.4 and 12.1 U mg⁻¹ protein for pendimethalin and its nano-form, respectively. Also, 0.1 and 0.025 EC₅₀ treatments increased enzyme activity as follows: 18.3, 6.5 U mg⁻¹ protein and 10.3, 6.3 U mg⁻¹ protein. The mean values were 31.0 and 8.3 U mg⁻¹ protein for the treatments as described above.

SOD

The enzyme activities in alga C. vulgaris exposed to the examined herbicide increased greater than the control (Figure 4). EC₅₀ treatment increased enzyme activity in the following order: 6.1 and 2.2 U mg⁻¹ protein for pendimethalin and its nano-form, respectively. Also, 0.1 and 0.025 EC₅₀ treatments increased enzyme activity as follows: 2.6, 1.3 U mg⁻¹ protein and 2.3, 0.5 U mg⁻¹ protein for the examined herbicide as described above. The mean values were 3.7 and 1.1 U mg⁻¹ protein.

APX

The enzyme activities in alga C. vulgaris exposed to the examined herbicide increased compared with the control (Figure 4). EC₅₀ treatment increased activity in the following order: 134, and 8.8 U mg⁻¹ protein for pendimethalin and its nano-form, respectively. Also, 0.1 and 0.025 EC₅₀ treatments increased enzyme activity as follows: 26.2, 6.8 U mg⁻¹ protein and 19.8, 4.0 U mg⁻¹ protein for the examined herbicide as described above. The mean values of activity were 60.0 and 6.5 U mg⁻¹ protein.

Osmolyte response

Proline

Proline content in the treated alga C. vulgaris with the herbicide for 96 h is cited in table 2. The all treatments induced increase in proline content greater than the control (mean, 0.71 mg g⁻¹ dry weight). EC₅₀ treatment exhibited increases; 0.9,
Cytotoxicity, Oxidative Stress and Biochemical Alterations Induced by Traditional and Nanoform of Pendimethalin in Freshwater Alga Chlorella vulgaris

Table 2: Proline level (mg g⁻¹ dry w) and sucrose (mg g⁻¹ algal biomass) in the tested alga.

| Herbicide                  | Proline (mg g⁻¹ dry w) | Sucrose (mg g⁻¹ algal biomass) |
|----------------------------|------------------------|-------------------------------|
|                            | EC₅₀ | 0.1 EC₅₀ | 0.02 EC₅₀ | Mean | EC₅₀ | 0.1 EC₅₀ | 0.02 EC₅₀ | Mean |
| Pendimethalin              | 1.8  | ± 0.17    | ± 0.10    | ± 0.02 | ± 0.08 | ± 0.52    | ± 0.18    | ± 0.09 | ± 0.26 |
| Nano-form of pendimethalin | 0.9  | ± 0.04    | ± 0.07    | ± 0.08 | ± 0.06 | ± 0.30    | ± 0.20    | ± 0.30 | ± 0.07 |
| Control                    |      |          |          |       | 0.7   | ± 0.09    |           |        | 1.4   |

- Each value is the mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 level.

and 1.8 mg g⁻¹ dry weight for nano-form and pendimethalin, respectively. Also, 0.1 and 0.025 EC₅₀ treatments achieved the values: 0.8, 1.5 mg g⁻¹ dry weight and 0.7, 1.3 mg g⁻¹ dry weight. The mean values were 0.8 and 1.5 mg g⁻¹ dry weight for both treatments.

Sucrose
The all treatments induced increase in sucrose content greater than the control (mean, 1.4 mg g⁻¹ algal biomass). EC₅₀ treatment induced increases in sucrose level: 3.5 and 4.4 mg g⁻¹ algal biomass for nano-form and pendimethalin, respectively. Also, 0.1 and 0.025 EC₅₀ treatments exhibited the values: 2.0, 3.0 mg g⁻¹ algal biomass and 1.7, 2.6 mg g⁻¹ algal biomass. The herbicide and its nano-form induced mean values: 2.2 and 3.3 mg g⁻¹ algal biomass for the all treatments (Table 2).

Ultrastructural investigation
Microscopic examination was performed for the highest toxic concentration (EC₅₀ level) of the tested herbicide in order to visualize the internal damage which may be induced in the cells. TEM images visualized substantial damage due to uptake of herbicide by the examined cells. The untreated cells were observed a compact and round-shaped with its typical characteristic organelles: clear nucleus (N) with clear nuclear envelope, clear cell wall and starch-pyrenoid complex (SPC) (Figure 5). The nucleus showed normal distributed chromatins with some mitochondrial organelles around it (Figure 5). Chloroplasts were found with normal distribution around starch grains (arrow). The treated cells showed some disorganization of cell components. The treated cells with pendimethalin (20.0 ppb) revealed marked concentric cell wall system (arrow) with compacted intracellular organelles (Figure 6). It was observed heavy distributed starch grains (SG) (destroyed to small size). Moreover, there was a lack of mitochondrial organelles and heavy dense of grana chloroplast (Ch). Also, it was observed starch-pyrenoid complex (SPC) and marked vacuoles surrounded by black-colored particles (BCP) (Figure 6). Regarding nano-form of pendimethalin (19.0 ppb), treated cells revealed compacted intracellular organelles with differential shape sizes (Figure 7). It was noted heavy distribution of starch grains and fatty bodies (fb). In most cells, it was observed destroyed and less chloroplast (Ch) and marked fatty bodies (fb). It was marked a back-colored particles (BCP) and regular cell membrane (arrow) (Figure 7). In high magnification, it was observed nucleus (N) with less dense chromatin, irregular membrane and divided nuclei. Some less dense mitochondrial organelles (arrow) were noted (Figure 7).
Cytotoxicity, Oxidative Stress and Biochemical Alterations Induced by Traditional and Nanoform of Pendimethalin in Freshwater Alga Chlorella vulgaris

Noaman et al.

Discussion

The extensive use of the herbicides in agricultural sector might adversely affect algal flora. In current study, we investigate the effect of dinitroaniline herbicide, pendimethalin and its nano-form on freshwater alga *C. vulgaris* and evaluate cytotoxic potential effects and biochemical alterations which induced after 96 h exposure to this herbicide in sub-lethal concentrations.

The present study showed that, nano-form of the examined herbicide was more toxic than the traditional form, that may be due to its ability to penetrate the cells faster than the traditional form as a result of the small size. Generally, the potential toxic effect of the substances to organism is dependent on two trials: penetration through biological membrane and interaction with the site of action [36]. Pendimethalin and its nano form exhibited high toxic effect on *C. vulgaris*, because pendimethalin acts by inhibiting cell division and cell elongation. It can directly influence growth and reproduction of microalgae. Toxicity of pendimethalin has been reported for numerous microalgal species by [37] from open literature they reported that, 96 h-EC$_{50}$ values of pendimethalin for *C. pyrenoidosa* 394 μg L$^{-1}$, *C. vulgaris* 281μg L$^{-1}$, *Scenedesmus obliquus* 490 μg L$^{-1}$ and *Raphidocelis subcapitata* 179 μg L$^{-1}$. In the current study, 96 h-EC$_{50}$ value of traditional pendimethalin for *C. vulgaris* is 20 μg L$^{-1}$, but is 19 μg L$^{-1}$ value for its nano-form.

The examined herbicide showed high toxic effect on tested alga. So, quantification of the ecological risk, probabilistic analysis is useful. Subsequently, the predicted environmental concentration (PEC) and environmental risk assessments for the pesticide registrations vary in each country and region e.g. in Japan, the United States and the European Union (EU). As stated by regulation on ecological criteria (EC) No 66/2010 on the hazard statements or risk phrases specified in accordance with directive 67/548/EC. The risk phrases or hazard statements refer to impacted or affects forms of substance on tested organism. So, the list of R-phrases is stated as follows: R50, R50/53, R51/53, R52/53 and R53, respectively in specialized criteria for aquatic organisms independent on acute toxicity value. This is a method for evaluating risk as an exceedance probability of environmental concentration to toxicity of aquatic organisms by considering the uncertainty of toxicity and pesticide exposure. For example, Japan’s pesticide registration criteria based on ecological risk assessment are set by Japan’s Ministry of Environment under the pesticide regulation law [38]. Under the risk assessment scheme, acute toxicity tests are conducted for fish (*Cyprinus carpio*), daphnids (*Daphnia magna*), and alga (*P. subcapitata*) and then the acute EC$_{50}$ or LC$_{50}$ divided by an uncertainty factor that considers the species sensitivity difference (default 10, but depends on the data for fish and crustaceans and 1 for algae). Regarding EU, the data requirements, assessment methods and acceptable standards depend on acute (short-term) tests from two specific fish, *D. magna* and green algae.

The exposed algal cells to contaminants may suffer serious morphological and biochemical alterations. Colorimetric technique which is used to quantify the photosynthetic pigments led to significant decline of Chlorophyll $a$ and $b$ content in *C. vulgaris* cells which exposed to the examined herbicide. The content of pigments decreased with increasing concentrations. These findings displayed the ability of algal cells to synthesize Chlorophyll decreased in accordance with that stated by [39]. It was reported in previous studies that, cells of microalgae altered their photoautotrophic metabolism under the stress induced by herbicide to a heterotrophic metabolism [40, 41]. Pigments deficiency can be caused by photobleaching or by inhibition of their biosynthesis [42-44].

The present data indicated that, an increase in Carotenoids content of algal cells was observed with increasing concentration of the herbicide. As documented in the literature, Carotenoids content increase plays an important role in antioxidant activity of photosynthetic membranes that protects Chlorophyll. It has defense properties, involving in the photosynthetic membranes protecting against photo-oxidation and in peroxide radical’s neutralization, preventing lipid membranes of chloroplasts and Chlorophyll degradation [45]. This concept may be explained, where Carotenoids have higher tolerance to herbicides action than Chlorophylls and
provides an indicator of the protective role of this pigment against oxidative stress induced by the herbicides on the algal cells. In addition, Carotenoids are considered potent quenchers of ROS, particularly singlet oxygen [46, 47]. These changes in the functioning of *C. vulgaris* photosynthetic apparatus affect the whole complex of metabolic transformations. Chlorophyll α initially is destructed under unfavorable conditions, whereas Carotenoids are more stable. The last is important protectors for green pigment and non-enzymatic antioxidant components of cells [48]. These results are in accordance with that reported by [49], where they recorded a reduction in Chlorophyll content in *C. vulgaris* cells exposed to 20 mg L\(^{-1}\) of herbicide, topramezone and a decrease in Carotenoids content of the algal cells was also obtained. A slight increase in Carotenoid content of biomass after 48 h exposure was observed.

The examined herbicide reduced the cellular protein content. This reduction is attributed to different factors. For example, decrease in protein content may be due to the deficiency of protein synthesis or increase in the rate of its degradation of amino acids, which may be fed to tricarboxylic acid (T-CA) cycle through aminotransferases probably to cope up with high energy demands in order to meet the stress conditions [50]. Also, it may be due to inhibition of aromatic amino acid synthesis, which lead to the inhibition of nucleic acid metabolism, and protein synthesis. The alterations in protein synthesis in the algae grow under stress could be due to changes in gene expression [51]. Another factor may be due to higher protease activity and decrease in carbon and nitrogen assimilation under stress conditions [52]. The present data are in accordance with that obtained by [53] who demonstrated a decrease in total protein content of *Anabaena* cells exposed to insecticide trichlorofon. Also, [54] who demonstrated that, 38% of protein was reduced in *Oscillatoria limnetica* after exposure to 20 mg L\(^{-1}\) of topramezone.

The present data showed significant reduction in protein content for pendimethalin greater than the nano-form. This phenomenon may be due to variation in chemical components of each or used additives. The variables were achieved in FTIR patterns of them. Pendimethalin showed strongly overlapped peak at 3440 cm\(^{-1}\), but slightly similar peak in the nano-form pattern (Figure 1B).

In the present study, the treatments increased carbohydrate content greater than the control (untreated). These data are in accordance with that obtained by [53], where carbohydrate content in *Anabaena* cells increased as a result of trichlorfon treatment. Similarly, it increased in *P. botryoides* after exposure to pendimethalin [55]. In addition, *Anabaena variabilis* showed significant increase in the carbohydrate content following malathion exposure: 25, 50, 75 and 100 μg ml\(^{-1}\) [56].

Increase profile of carbohydrate contents in the exposed alga to herbicide may be explained as an increase in sugar content is an adaptive measure aimed to survival under toxicant stress condition. It generally known that, when protein synthesis suppressed by various factors, algal cells depending on genotype are transformed to synthesize either carbohydrate or lipids [57]. This unbalanced cell composition could be due to disturbances in nitrogen metabolism and photosynthetic activity [55].

The present study showed MDA levels induced from the two treatments greater than the control. As documented in the literature, MDA is a good signature of oxidative stress increases with increasing concentrations of the herbicides. Our results are in accordance with that obtained by [58], where diuron did not cause any significant change in the level of lipid peroxidation on alga *Scenedesmus opolensis*. However, in the presence of methylviologen and glufosinat the degree of effect increased more than two times as compared with control. This concept indicates these herbicides induce membrane damage and impair transmembrane transport processes by causing structural changes in lipid bilayer. In another study by Manikar et al. [56], when *A. variabilis* cultures were exposed to 25, 50, 75 and 100 μg ml\(^{-1}\) of malathion, MDA significantly increased to 63, 86, 115 and 152%, respectively, compared with the untreated control. Regarding *C. vulgaris*, herbicide topramezone significantly induced increase in MDA level [49].

Generally, lipid peroxidation induction in the algal cells is an outcome of the chemicals trigger oxidative stress by producing ROS e.g. O\(_2\)\(^-\), O\(^.-\) and H\(_2\)O\(_2\). The excessive ROS is not completely cleared by the algal cells and eventually produced cell damage. Cell membranes are made of unsaturated phospholipids and are susceptible to oxygen radical attack resulting in MDA accumulation [59-61].

The organisms have a group of antioxidant enzymes and antioxidant substances that protect them against the potential damaging effects of ROS. The activity of one or more of these enzymes in generally increased when the plants are exposed to stressful conditions [62, 63].

The present data are in accordance with that were concerning in increase of SOD activity in *O. limnetica*. Glyphosate stress may have stimulated the generation of ROS which were reduced by the raised levels of these enzymes and helped the algal cells to tolerate the herbicide stress [64, 65]. Similarly, increased activities of SOD, CAT, and APX in *P. boryanum, Aulosa reniformis*, *A. variabilis* and *Nostoc muscorum* were induced under test of endosulfan [52, 66]. Regarding unicellular green alga, glufosinate and paraquat increased SOD activity at 0.5 mM by 3-4 times over than the control cultures of *C. vulgaris* [67, 68]. Similar results were stated by [69] who found that, enzymatic defense increased when glyphosate was present in the growth medium of alga *C. kessleri*. In another investigation, significant increases were occurred to three antioxidant enzymes in cyanobacteria after exposure to series of concentrations of malathion [56]. Stress responses of *A. cylindrical* to sub-lethal concentrations (0.8-2.0 mM) of herbicide bentazon resulted in increased activity of SOD after 72 h exposure [54].

Many plants and animals including micro-organisms accumulate small organic compounds called osmolytes to protect them from various stresses. In the current study, we obtained that osmolytes such as proline and sucrose are enhanced by the examined herbicide. It was observed that, this herbicide exerted toxic effect on the algal cells and increased...
the levels of these components compared with the control.

The present findings indicate that, proline content gradually increased with increasing concentrations of the examined herbicide. These results are in accordance with that demonstrated by [70], where proline and other amino acids contents expressed on cell dry weight basis in C. vulgaris showed an increase at various doses of herbicide, diuron. Similar finding was obtained by [56], where proline was significantly increased at concentrations: 25, 50, 75 and 100 μg ml⁻¹ of malathion compared to the untreated control in A. variabilis.

As stated in literature, proline is not only one of the essential amino acids, but is also an important antioxidant molecule involved in the response to a variety of environmental stresses and is suggested as a signal or regulatory molecule that can activate multiple physiological and molecular responses [71].

Sucrose content also gradually increased with increasing the examined herbicide concentrations compared to the control. Different stress situations directly or indirectly cause accumulation of ROS. It has generally been considered an adaptive response to stress conditions in the higher plants [72]. Sugar metabolism and carbon skeletons are essential to the synthesis of numerous compounds that are involved in anti-oxidative protection.

Ultrastructural investigation provides a good tool to evaluate cytotoxic effects of the chemicals on the organisms. The disruption of the algal cell components, especially the membrane induces inhibition of photosynthesis and finally results in the increasing proportion of death [73].

The present findings display significant alterations in the cellular components of C. vulgaris compared with the untreated cells. Stress of pendimethalin and its nano-form focused on destruction of mitochondrial organelle and alteration in fatty bodies and starch grains synthesis. These concepts are in accordance with that obtained by [74] who found that, some algal cells of Chlorella genus had their cell wall damaged after the treatment with pharmaceutical, chloramphenicol. However, S. obliquus wall damaged after the treatment with pharmaceutical, genus had their cell membrane induces inhibition of photosynthesis and finally results in the increasing proportion of death [73].

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As noted by Environmental Protection Agency (EPA), nano-pesticides are more effective targeting of the pests, and use of smaller quantities of the pesticide. For example, the major use of nano-emulsion is to increase the apparent solubility of poorly soluble a.i, while limiting the concentration of surfactant present in the formulation [85]. It can improve efficacy of herbicides, resulting in greater production of crops with an ecofriendly way. However, there are lack studies concern risk of nano-products. So, nano-pesticides may be employed to a largely insufficient for reliable risk assessment before used [11]. Regarding these concepts, the present findings may provide suitable approach for the ecotoxicity of the prepared nano-herbicide on an aquatic model (C. vulgaris).

From all findings, the biosafety procedures generally, must
be considered on non-target species including algae and others before decision of nano pesticide practices. The current study introduces herbicide pendimethalin as a model. Also, the use of the bio-based surfactants like plant oils and polymers aid up the eco-friendly properties of the nano pesticide. Moreover, other considerations must be followed for risk assessment of nanomaterials as well as new analytical methodologies which are used to characterize the levels of nano pesticides over time in regulatory fate [11].

**Conclusion**

The present study realized the desirable targets, where the prepared nano-emulsion was characterized in good items for nonformula. Nano-herbicide was more potent toxic on algal cell population than its traditional form. The examined components, osmolytes and ultrastructural changes in algal cell population than its traditional form. The examined for nonformula. Nano-herbicide was more potent toxic on non-target species including algae and others. Ecological risk concern non-target organisms may be considered for decision-making, before regulatory of nano-herbicide practices. Also, new approaches are used to characterize the nano-materials in the environment.

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**Conflict of Interest**

The authors declared no conflict of interest.

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