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Affecting of aquatic vascular plant *Lemna minor* by cisplatin revealed by voltammetry

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Abstract

Within the context of application of platinum derivates based effective cytostatics, we can suppose that these risk metals can get into aquatic ecosystems where they can show biologic availability for food chain. In the present work we report on investigation of affecting of duckweed (*Lemna minor*) by various doses of cisplatin (0, 5, 10, 20, 40, 80 and 160 μM) for 4 days. The toxic influence of cisplatin was evaluated on the basis of growth inhibition expressed as number of leaves, growth rate, and total amount of biomass. The result value of $96hEC_{50}$, calculated from growth inhibition with comparison of growth rates, was 6.93 μM. Moreover we aimed on determination of cisplatin content using differential pulse voltammetry. The highest content of cisplatin (320 ng g^{-1} of fresh weight) was determined in plants treated by 80 μM at the second day of treatment. Plants protect themselves against heavy metals by means of synthesis of cysteine-rich peptides such as glutathione and phytochelatins. Thus thiol determination in the treated plants by means of Brdicka reaction followed. The marked increase in thiol concentration detected is associated with defence reaction of the plant against stress caused by cisplatin.

Keywords: Adsorptive transfer stripping technique; Catalytic signal; Environment monitoring; Water pollution; Platinum based compounds

1. Introduction

Due to increasing population around the world, coming changes in climate and limited sources of drinking water, wasting of water supplies has become a concern. Recently, acute environmental load assessment focuses increasingly also on platinum metals, namely platinum (Pt), palladium (Pd) and rhodium (Rh), which enter to the environment from automobile catalytic converters and waste the water supplies [1–5]. Moreover it could be assumed that other source of environment contamination comes from hospitals' waste waters, where treatment of cancer patients take place using platinum based cytostatics. The first platinum based cytostatic drug — cisplatin, is still one of the most frequently used ones [6].

Vascular plants of duckweed family (Lemnaceae) belong to the group of bioindicators of ecotoxicological changes, mainly in aquatic system. Two species of *Lemna* genus are commonly used in toxicological tests: *Lemna minor* and *Lemna gibba* [7]. These species, which make a common part of aquatic ecosystems, have been used from the thirties of last century as suitable test organisms to define the first herbicides effects on the plants in aquatic system [7–9]. A number of investigations based

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on duckweeds ability to accumulate metals have been published, because this property makes them suitable for water quality monitoring [7,10–12].

In the present work we report on investigation of affecting of duckweed (*L. minor*) by various doses of cisplatin. Mainly, we were interested in the issues if the plants could be utilized as bioindicators of such pollution and if they could be used for remediation of cisplatin polluted water supplies. For the solving of these tasks we attempted various electrochemical methods.

2. Experimentals

2.1. Reagents

Water of ACS purity and other chemicals used were purchased from Sigma Aldrich (USA) unless noted otherwise. The chemotherapeutic drug of cisplatin was synthesized and provided by Pliva-Lachema (Brno, Czech Republic). Stock standard solutions of cisplatin (500 μg ml^{-1}) were prepared with sodium chloride solution (0.75 M, pH 5.0) and stored in the dark at −20 °C [15]. Working standard solutions were prepared daily by dilution of the stock solutions in the phosphate buffer (100 mM).

2.2. Apparatus

2.2.1. pH measurements

The pH value was measured using WTW InoLab Level 3 with terminal Level 3 (MultiLab Pilot, Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2.2. Electrochemical measurements

Electrochemical measurements were performed with the AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The hanging mercury drop electrode (HMDE) with the drop area of 0.4 mm^2 was the working electrode, the Ag/AgCl/3M KCl electrode serve as reference electrode and the graphite electrode as auxiliary ones. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. All experiments were carried out at room temperature unless noted otherwise. For smoothing and baseline correction [13], the software GPES 4.4 supplied by EcoChemie was employed.

2.2.2.1. Differential pulse voltammetry of cisplatin. The electrochemical determination of cisplatin was done according to Vrana and Brabec [14] with modifications [15]. The supporting electrolyte (sodium chloride 0.75 M pH 5.0) was purchased from Sigma Aldrich in ACS purity. DPV parameters were as follows: the initial potential of −1.0 V, the end potential −1.75 V, the modulation time 0.057 s, the time interval 0.2 s, the step potential 1.05 mV, the modulation amplitude of 25 mV. The pH of solution analysed was measured using pX module in connection with pH-electrode (SenTix-H, pH 0–14/3 M KCl). For more details see in Ref. [15].

2.2.2.2. Adsorptive transfer stripping technique differential pulse voltammetry Brdicka reaction of thiols. The plant samples have been analysed by adsorptive transfer stripping technique in connection with differential pulse voltammetry — Brdicka reaction [16]. Principle of the adsorptive transfer stripping technique (AdTS) is based on the strong adsorbing of the target molecule on the electrode surface at an open electrode circuit [17]. The electrode is washed in a rinsing buffer. The electrode is further transferred to the supporting electrolyte and measured. The Brdicka supporting electrolyte containing 1 mM Co $(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq)+NH_4Cl, pH=9.6) was used; surface-active agent was not added. The electrolyte was changed after five analyses. AdTS DPV Brdicka reaction parameters were as follows: the initial potential of −0.6 V, the end potential −1.6 V, the modulation time 0.057 s, the time interval 0.2 s, the step potential of 1.05 mV, the modulation amplitude of 250 mV, E_{ads}=0 V, the temperature 4 °C.

2.3. Procedures

2.3.1. Experimental design

Modified method of OECD 221 *Lemna* sp. Growth Inhibition Test was used for duckweed test. In comparison to this method, the total time of the test was shortened from 7 days (168 h) to 4 days (96 h). Freshwater plant Lesser Duckweed *L. minor* cultivated in a laboratory to age of 2 weeks was used for testing. *L. minor* colonies in the phase of two or three leaves were cultivated in glass beakers of the volume of 150 ml filled with 100 ml of medium. Synthetically prepared nutrient solution of SIS (Swedish Standards Institute) diluted with distilled water was used as control. Beakers with test plants were covered with food foil wrapper and kept under fluorescent lamps at non-stop lighting of 6.500–10.000 lx and temperature of 24±2 °C. Static method without change of medium was chosen. Plants condition and number of leaves were controlled and recorded every 24 h at particular concentrations. Light magnifier was used for observing the plants. The effect of tested substance on final amount of biomass was evaluated on the basis of fresh biomass weight.

The plants harvested from particular testing beakers were washed off on the plastic sieve with distilled water, 0.2 M EDTA, and distilled water. After short drying on the filter paper, the plants were weighted. Cisplatin was tested in the concentration range of 5, 10, 20, 40, 80 and 160 μM. Four repetitions were made for every concentration and control. After every 24 h, the fresh biomass was weighted in one of the four replications. The results of particular tests were evaluated on the basis of the determination of growth inhibition with comparison of the growth rate (I_μ), on the basis of the determination of inhibition with comparison of the areas under growth curves (I_A), and on the basis of total biomass. Calculating of these values resulted from changes of the leaves number founded during four days exposition to every concentration and at every control. The result value of 96hEC_{50} was described in Results and discussion section.

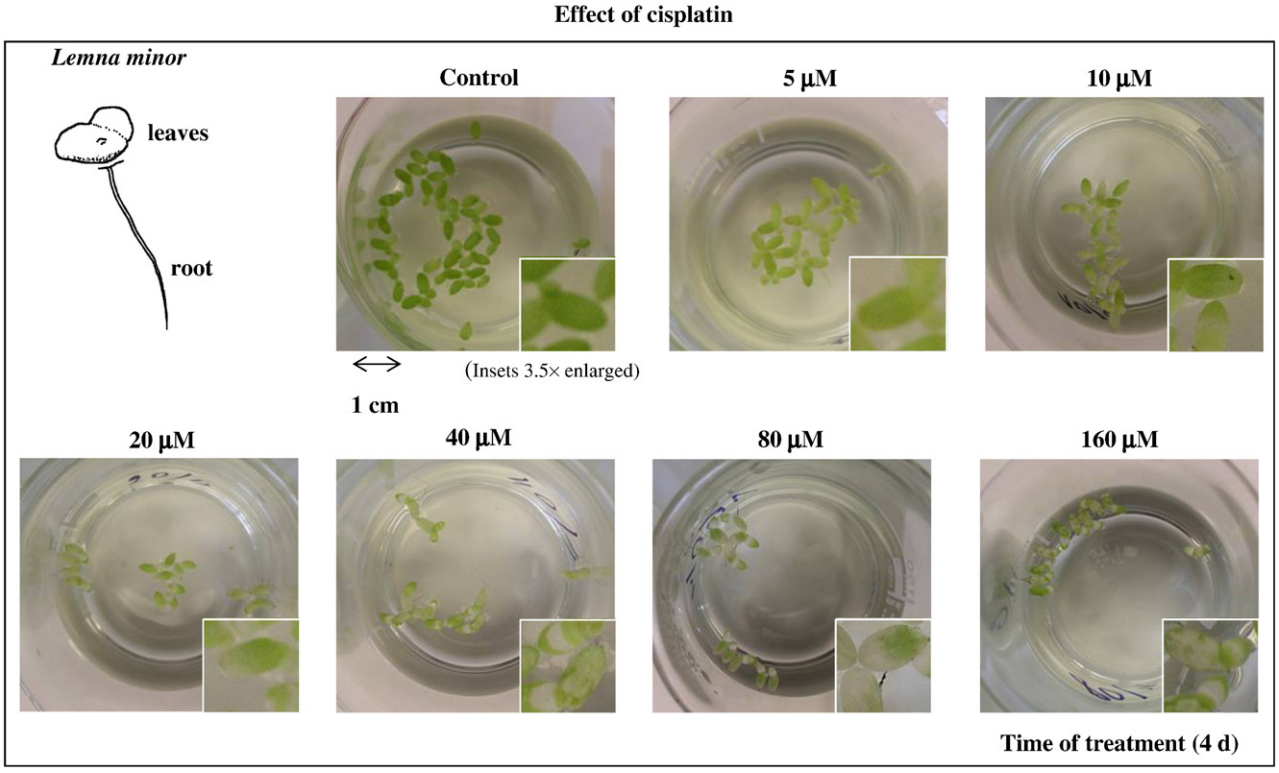

Fig. 1. Illustration of dependence of growth, shape and colouring of *Lemna minor* on cisplatin concentration (0, 5, 10, 20, 40, 80 and 160 µM) after 4 days of cultivation in cisplatin containing water. Insets are 3.5 × enlarged. Other details see in the Experimentals section.

2.3.2. Preparation of plant tissues prior to analysis

Duckweed tissues (approximately 0.2 g) were frozen by liquid nitrogen in a test-tube to disrupt the cells. Frozen tissues were spread in mortar and then exactly 1000 µl of 0.2 M phosphate buffer (pH 7.2) was added to the obtained powder. The mixture was homogenised by shaking on Vortex-2 Genie (Scientific Industries, New York, USA) at 4 °C for time of 30 min. The homogenate was centrifuged (14 000 *g*) for 30 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). The supernatant was filtered

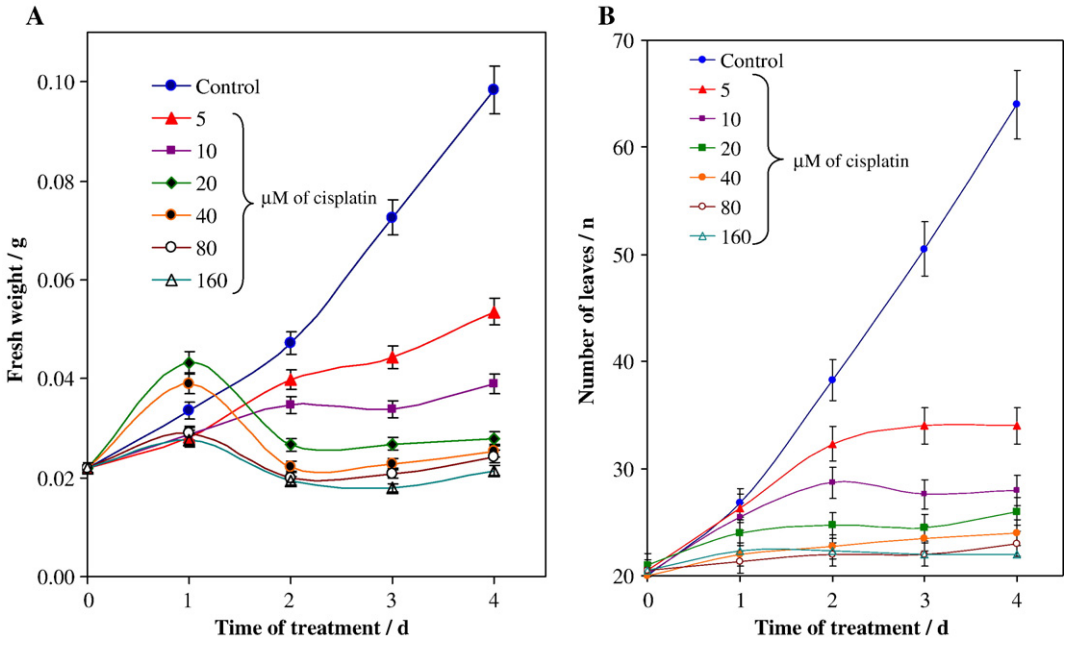

Fig. 2. Growth curves of *Lemna minor* treated with different cisplatin doses expressed as fresh weight (A) and number of leaves (B). Other details see in the Experimentals section.

through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA) prior to measurements of cisplatin or total concentration of thiols. For other experimental details see Ref. [18].

3. Results and discussion

The duckweed has many properties, which make it suitable for laboratory testing of toxicity of various compounds and for assessment of pollution of water ecosystems. A paper, where metal phytotoxicity was evaluated with the use of *L. minor* and the green algae *Selenastrum capricornutum*, has been published. Based on these results the duckweed showed higher sensitivity than the algae [19]. On the other hand, the effect of cisplatin on duckweed has not been investigated yet.

3.1. Influence of cisplatin on basic growth characteristic of L. minor

We attempted to investigate the effect of cisplatin on duckweed (*L. minor*). The plants were treated by 0, 5, 10, 20, 40, 80 and 160 μM of cisplatin for four days (Fig. 1). The basic characteristic as growth curve, length of roots, numbers of leaves and observable microscopic changes have been observed during the treatment (Fig. 2A). The decrease in growth varied from 50% at 5 μM to 80% at 160 μM in comparison to control during the fourth day of treatment (Fig. 2A). On the other hand, we observed slight increase in fresh weight at variants treated by 5–80 μM of cisplatin at the very beginning of the treatment (first day). The increase probably associate with higher metabolic activity of plants as can be observable at other plant species such as maize and spruce embryos [18,20,21].

The same effect as described above was observable, if we counted the number of leaves. The number of leaves of control variant of the duckweed was about 60 at the end of the experiment, but the number of leaves of treated variants was within 20 to 30 according to dose of cisplatin (Fig. 2B). In addition, we observed marked affecting of growth of roots by cisplatin, which decreased with increasing cisplatin concentration. It clearly follows from the results obtained that hallmarks of growth depression appeared at all of the variants investigated. Appearing of chlorosis on leaves can be also observed. The concentration of cisplatin increased, the chlorosis appeared markedly. Particularly, plants treated by higher concentration of cisplatin than 40 μM poorly grown and lost more than 40% of chlorophyll in comparison to control (Fig. 1). The result value of 96hEC_{50}, calculated from growth inhibition with comparison of growth rates, was 6.93 μM (confidence interval of 95%=6.51–7.34). The value of EC_{50}, calculated from growth

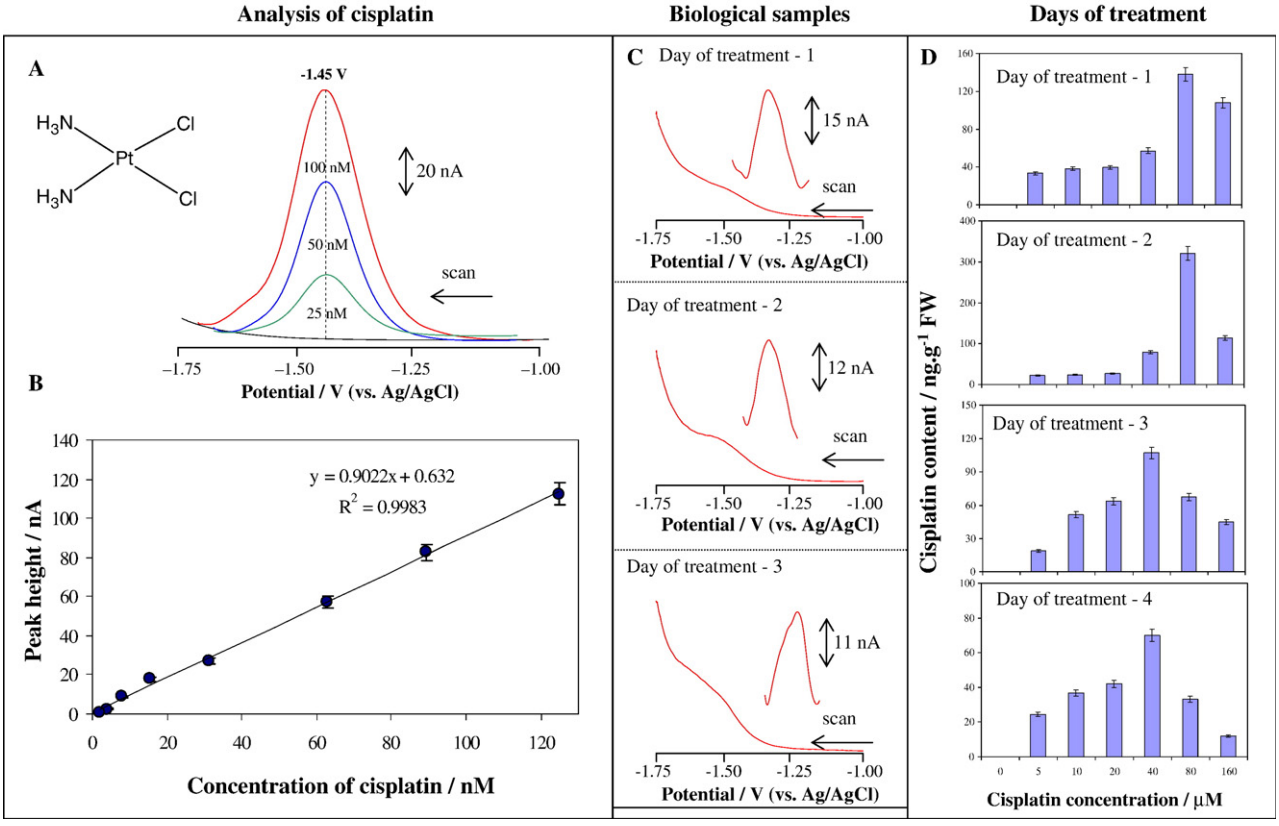

Fig. 3. *Analysis of cisplatin*. Typical DP voltammogram of cisplatin (25, 50 and 100 nM) measured in the presence of sodium chloride (0.75 M, pH 5.0) (A); inset: formula of cisplatin. Dependence of height of cisplatin signal on its concentration in the range from 0 to 125 nM (B). DP voltammograms of analysis of extracts obtained in the first, second and third day of treatment at cisplatin dose of 160 μM (C); insets: peaks after baseline correction. Changes of cisplatin content with increasing time of treatment and cisplatin dose (0, 5, 10, 20, 40, 80 and 160 μM) (D). Volume of homogenate (5–20 μl). DPV parameters were as follows: the initial potential of −1.0 V, the end potential −1.75 V, the modulation time 0.057 s, the time interval 0.2 s, the step potential 1.05 mV, the modulation amplitude of 25 mV. Other details see in Fig. 2 and in the Experimentals section.

inhibition with comparison of areas under growth curves, was <5 μM. The experiment showed that cisplatin shows toxic effect on *L. minor* and influences its growth significantly. The following step in our experiments was to detect concentration of cisplatin in tissues of treated plants.

3.2. Electrochemical analysis of cisplatin

Electroanalytical techniques belong to preferred technique in the field of cisplatin analysis due to their low operating costs and very low detection limits for platinum and platinum based compounds [22,23]. Recently, we have reported on sensitive determination of cisplatin [15]. We have found out that the most suitable supporting electrolyte determination of cisplatin was 0.75 M NaCl, pH 5.0. Typical DP voltammograms of cisplatin (25, 50 and 100 nM) measured in the presence of 0.75 M NaCl (pH 5.0) is shown in Fig. 3A. Signals corresponding to cisplatin observed at potential of −1.45 V were well developed and detectable. We construed dependence of cisplatin peak height on its concentration within the range from 1.95 to 125 nM (Fig. 3B). The height of the peak was linearly proportional to concentration of cisplatin. The dependence obtained was strictly linear ($y=0.9022x+0.632$, $R^2=0.9983$, R.S.D. 2.2%). Based on the results obtained detection limit for cisplatin expressed as $3S/N$ was estimated as 95 pM. The detection limit was calculated according to Long [24], whereas N was expressed as standard deviation of noise determined in the signal domain. The other approaches of for estimation of detection limits were reported by Lavagnini et al. [25].

3.2.1. Changes in cisplatin level in duckweed

Content of a "free" heavy metal, that means metal, which is not bound by any compounds, has a crucial biological importance, because these metal ions affect biochemical pathways directly [20,26,27]. Utilizing of the electrochemical techniques for determination of content of "free" heavy metal is very suitable. Thus, we used technique described above and obtained well reproducible peaks of cisplatin after baseline correction (Fig. 3C). Peaks of cisplatin shifted to more positive potential during analysis of the duckweed samples. It clearly follows from the results obtained that the technique can be used to detect cisplatin in duckweed treated by cisplatin (Fig. 3C).

The content of cisplatin increased with increasing time of treatment and dose of cisplatin up to second day of cultivation and dose of 80 μM, then, the content decreased (Fig. 3D). The highest content of cisplatin (320 ng g^{-1} FW) was determined in plants treated by 80 μM at the second day of treatment. The decrease of cisplatin content at the third and fourth day of cultivation relates with affecting of DNA replication and increase synthesis of thiols [26]. Under affecting of duckweed by dose of cisplatin 160 μM, the content of cisplatin was highest at the first day of treatment and then decreased. This different behaviour could be associated with toxicity of cisplatin and with attempting of plants to protect themselves through preventing of uptake of the cisplatin. To evaluate our assumption that treated plants protect themselves through synthesis of thiols, determination of thiols followed.

3.3. Analysis of thiols

Plants protect themselves against heavy metals by means of synthesis of cysteine-rich peptides such as glutathione and phytochelatins [28]. Chromatographic techniques coupled with different detectors have been intensively used for the determination of these thiols [18,21,29–31]. These techniques suffer from difficulties associated with high cost. In contrast to this, stationary electrochemical methods used for determination of plant thiols are an attractive alternative method for electroactive species detection, because of its inherent advantages of simplicity, ease of miniaturization, high sensitivity and relatively low cost [26,32–36]. Thus, we utilized electroanalytical method called Brdicka reaction to determine total content of plant thiols in tissues of treated duckweed.

3.3.1. Concentration of thiols in duckweed tissues treated with cisplatin

Homogenates of duckweed tissues were 10× diluted prior to electrochemical analysis with ACS water. We decided to use Cat2 signals for quantification of thiols (other details see in [16]). Concentration of thiols changed with increasing time of treatment and dose of cisplatin. Up to second day of the treatment concentration of thiols was low and decreased with increasing dose of cisplatin. It could be assumed that cisplatin interacts with DNA and, thus, influences proteome-synthetic apparatus of a cell (Fig. 4). This assumption relates with the increasing content of cisplatin at beginning of the experiment (Fig. 3). From the third day of the treatment marked metabolic change appeared, which means that content of free cisplatin decreased, whereas thiol concentration increased (1500–2500 μg g^{-1} FW, Fig. 4). The marked increase in thiol concentration is associated with protective

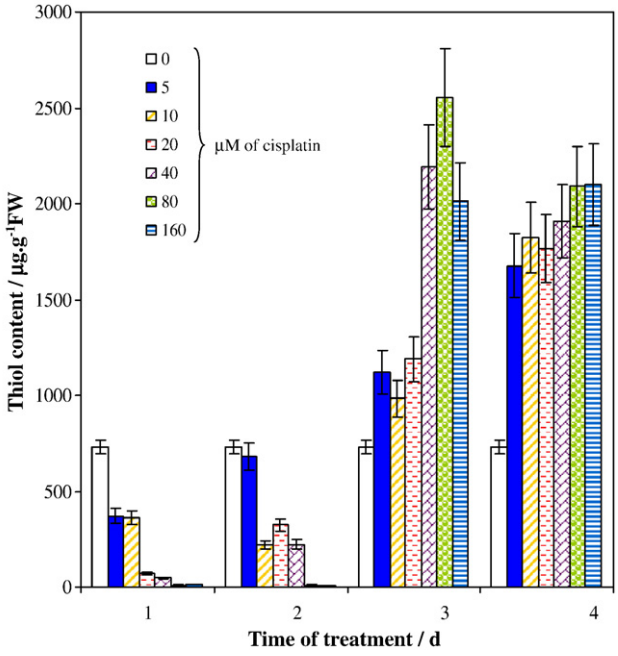

Fig. 4. Influence of different doses of cisplatin on thiols concentration. The results were obtained by means of Brdicka reaction ($n=5$). Other details see in the Experimentals section.

reaction of the plant against stress caused by metabolically active compounds. The results obtained show the ability of the plant to trigger protective pathway. The similar changes have been observed at different plant model systems such as maize, sugar beet and spruce embryos [20,21,29].

4. Conclusion

Prior to assessment of water pollution by heavy metals new bioindicators of such polluting need to be evaluated. In the present work, we tested the influence of cisplatin on duckweed with respect to utilize this plant specie as bioindicator of cisplatin pollution. We showed that the lowest dose of cisplatin resulted in both visible and non-visible (metabolic) changes. Based on these results we assume that duckweed can be utilized as the bioindicator.

Moreover, the other aim of this work was to investigate the possibility of using of duckweed for remediation of cisplatin polluted water supplies (mainly wastewater from hospitals). For these purposes we determined total content of thiols as a marker of ability of a plant withstands heavy metal stress. The cisplatin treatment resulted in enhancing of thiol synthesis more than three times in comparison with control plants (Fig. 4). This phenomenon encourages us to propose this plant as suitable for phytoremediation purposes.

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