Effects of cadmium on total phenolic compounds and flavonoids in *Euglena gracilis*

Efectos de cadmio en compuestos fenolicos totales y flavonoides de *Euglena gracilis*

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ABSTRACT

In the present study the production of phenolic acids and flavonoid compounds by *E. gracilis* exposed to two cadmium concentrations (0.02 and 0.14 mM) was evaluated using high-performance liquid chromatography (HPLC). The results showed that *E. gracilis* exposed to 0.02 mM Cd^{+2} increased significantly the total content of phenolic compounds (798.46 ± 12.61 µg GA/g) and total flavonoids (241.34 ± 47.63 µg QE/g) with respect to the control (137.34 ± 19.80 µg QE/g DW and 549.00 ± 8.57 µg GA/g DW, respectively). However, no significant increase in the total content of phenolic compounds (568.54 ± 17.42 µg GA/g DW) and total flavonoids (141.11 ± 9.36 µg QE/g DW) were observed in *E. gracilis* exposed to 0.14 mM Cd^{+2}. Further research is necessary to determine the specific role of flavonoids in *E. gracilis* exposed to high concentrations of Cd^{+2}.

Keywords: Heavy metals, *Euglena gracilis*, phenolic compounds, pollution

RESUMEN

En el presente estudio la producción de compuestos fenolicos y flavonoides producidos por *E. gracilis* expuesto a dos concentraciones de cadmio fue evaluado usando cromatografía liquida de alta precisión (HPLC). Los resultados mostraron que 0.02 mM de Cd^{+2} incrementaba significativamente el contenido total de compuestos fenolicos (798.46 ± 12.61 µg GA/g) y flavonoides (241.34 ± 47.63 µg QE/g) con respecto al control (137.34 ± 19.80 µg QE/g DW y 549.00 ± 8.57 µg GA/g DW) respectivamente. Sin embargo, incrementos no significativos en el contenido de compuestos fenolicos (568.54 ± 17.42 µg GA/g DW) y flavonoides totales (141.11 ± 9.36 µg QE/g DW) fueron observados en *E. gracilis* expuestas a 0.14 mM de Cd^{+2}. Futuros estudios son necesarios para determinar la función especifica de los flavonoides en *E. gracilis* expuesto a altas concentraciones de Cd^{+2}.

Palabras clave: metales pesados, *Euglena gracilis*, compuestos fenolicos, contaminación

INTRODUCTION

Cadmium is not essential for living organisms. This heavy metal may be toxic at very low concentrations and have a direct negative effect on various biochemical processes in aquatic organisms (Gonzalez-Mendoza & Zapata-Perez 2008). The occurrence of this metal in urban aquatic ecosystems is mainly due to soil applications of commercial fertilizers, sewage sludge and industrial processes (Adams *et al*. 2004, Romero-Puertas *et al*. 2007). The presence of cadmium in the environment can cause negative effects in human health due to its high potential to enter and accumulate in the food chain (Erdoğrul & Erbili 2007, Mishra *et al*. 2008). Cadmium toxicity in plants can cause inactivation of photosynthesis, formation of free radical and reactive oxygen species, which result in oxidative stress (Gonzalez-Mendoza *et al*. 2007, Kovacil *et al*. 2009). The use of microbial technology to remove specific substances, such as heavy metals, has been extensively applied to the treatment of water (Rahman *et al*. 2011). *Euglena gracilis* is a freshwater unicellular flagellate found in aquatic habitats. Due to its ecological and commercial importance, this flagellate has been extensively used in the last few decades to study the effects of heavy metals in the environment

(Cervantes-Garcia *et al*. 2011, Morales-Calderon *et al*. 2012). Studies on cadmium uptake and accumulation have shown that chloroplasts and vacuoles of *E. gracilis* may play a major role in the mechanisms of cadmium-resistance. Additionally, *E. gracilis* has the ability to remove cadmium under anaerobic conditions, which might be advantageous for metal removal from sediments of polluted water bodies or bioreactors, where O_2 concentration is particularly low (Santiago-Martinez *et al*. 2015).

Therefore, the studies on the biochemical mechanisms involved in cadmium accumulation and inactivation in cadmium-hyper-accumulating organisms such as *E. gracilis* are of great interest for bioremediation purposes (Cervantes-Garcia *et al*. 2011).

Even though the biochemical mechanisms of heavy metal tolerance or inactivation in *E. gracilis* have been previously studied, the influence of cadmium in the production of phenolic compounds has been scarcely evaluated. Therefore, the aim of this study is to evaluate the effects of different concentrations of cadmium in the production of phenolic compounds and flavonoids in *E. gracilis*.

MATERIALS AND METHODS

Culture of *E. gracilis*

Cells of *E. gracilis* Klebs (strain Z) were cultured axenically in organic *E. gracilis* medium (EGM) and incubated at 28 °C with fluorescent white light during 14/10 h light-dark period for 5 days. Aliquots of 10^5 cells ml^{-1} (5 days after previous subculture) were inoculated into 100 ml of EGM in 250 ml grass flasks. For cadmium toxicity assays the culture medium was supplied with two concentrations of $CdCl_2$ (0.02 and 0.14 mM). These doses were selected according to previous studies reported by Santiago-Martinez *et al*. (2015) who evaluated the doses-effect of different concentration of Cd (0.02 to 0.3 mM) in *E. gracilis*. Cadmium-free medium was used for the control. These experiments were carried out in triplicate. For the bioassays, static cell culture was carried out at 28±1 °C, 14/10 light-dark cycles. After 3 days of incubation, the cultures were collected by centrifugation and washed with a metal free medium.

Then, three replicates of 100 mg of biomass previously lyophilized were placed in a tube with 1.5 mL of methanol (80%), grinded at 4 °C and centrifuged at 6000 *g* for 10 min. Samples of supernatant were taken and used in the evaluation of phenol and flavonoids´ contents.

Determination of total phenol content and flavonoids

The content of total phenolic compounds and flavonoids were determined by the Folin-Ciocalteau and aluminum chloride colorimetric methods (Quettier *et al*. 2000, Nuñez-Ramirez *et al*. 2011). Total phenol values were expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), through the calibration curve with gallic acid, ranging from 0 to 300 µg/ml. The content of flavonoids was calculated as quercetin from a calibration curve. The calibration curve was prepared with quercetin solutions, at concentrations from 10 to 100 mg ml^{-1} in methanol.

Identification of phenolic acids and flavonoids by HPLC

The extractions of phenolic acids from samples of *E. gracilis* for HPLC analysis were prepared according to Cervantes-Garcia *et al*. (2012). Separation of phenolic acids was carried out by gradient elution high performance liquid chromatography (GEHPLC) based in previous studies (Sanchez-Estrada *et al*. 2009). The HPLC system consisted of quaternary pump model 9012, equipped with an UV detector model 9050, a Prodigy 5u ODS3 100A (Phenomenex, CA, USA) column (250 mm length, 4.6 mm of internal diameter and 5 µm particle size) with a C-18 guard column. The detector was set to 280 nm and flow rate to 1 ml/min (Varian Inc., Co. Palo Alto, CA, USA).

The solutions of the phenolic acid mixture were prepared with 0.01 g of each phenolic acid and mixed with a solution of 1:1 ammonium acetate buffer 200 mm pH 5.4 and methanol. The efficiency of the GEHPLC methodology for separation of phenolic acids and flavonoids was tested by preparing a solution containing the following commercially available standards: gallic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, sinapic acid, o-coumaric acid, naringenin, kaempferol, quercetin, rutin, naringin and cinnamic acid (Sigma-Aldrich Chemical Co. St. Louis, MO, USA).

Statistical analysis

The data are presented as mean values. Statistical analysis was carried out by one way ANOVA for repeated measures followed by Tukey´s posthoc test. Differences were considered significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Our result showed that the increase in the production of flavonoid and phenolic compounds by *E. gracilis* was not dependent on the concentration of cadmium used in the culture medium. Exposure of *E. gracilis* to 0.02 mM Cd^{+2} increased significantly the total content of flavonoids (241.34 ± 47.63 µg QE/g DW) and phenols (798.46 ± 12.91 µg GA/g DW) with respect to control (137.04 ± 19.80 µg QE/g DW and 549 ± 8.57 µg GA/g DW, respectively). In contrast, when *E.gracilis* was exposed to 0.14 mM Cd^{+2} no

significant increases in the total flavonoids (141.11 ± 9.36 µg QE/g DW) and phenolic compounds (568.54 ± 17.42 µg GA/g DW) were observed (Table 1).

These results show that exposure of *E. gracilis* to low concentration of Cd^{+2} (0.02 mM) stimulated the production of phenols and flavonoids. Surprisingly, no effects were observed when *E. gracilis* was exposed to high concentration of Cd^{+2} (0.14 mM). The response to low concentrations of cadmium may be the result of the presence of antioxidant metabolites such as proline or glutathione in *E. gracilis*, which help in the reduction of stress caused by Cd^{+2} (Cervantes-Garcia *et al*. 2011). In this sense, the phenol and flavonoid may play an important role in the tolerance of *E. gracilis* at defined ranges of cadmium concentrations.

Table 1. Total flavonoids and phenolic contents of *Euglena gracilis* exposed to Cadmium.

Tabla 1. Contenidos totales de flavonoides y fenoles de *Euglena gracilis* expuestas a Cadmio.

Cadmium doses (mM)	Total phenolics contents (µg GA/g DW)	Flavonoids contents (µg QE/g DW)
0.0	549.00 ± 8.57[a]	137.34 ± 19.80[a]
0.02	798.46 ± 12.91[b]	241.34 ± 47.63[b]
0.14	568.54 ± 17.42[a]	141.11 ± 9.36[a]

Values correspond to mean ± standard error (n=3). Within each column different capital letter indicate significant difference according to Tukey test ($p<0.05$)

An alternative hypothesis to explain the lack of increase in phenolic and flavonoid compounds in *E. gracilis* exposed to 0.14 mM Cd^{+2} could be attributed to the impairment of antioxidative system responses due to the exposure to a high concentration of Cd^{+2}, in such a way that this organism is not able to synthesize new phenolic and flavonoid compounds. Similar results has been reported in *Spartina densiflora* and *Erica andevalensis*, which are not able to counteract the effects of exposure to high concentration of Cd^{+2}. In these cases, exposure to low concentrations of Cd^{+2} caused the synthesis of antioxidant metabolites, such as ascorbic acid and glutathione compounds (Márquez-Garcia *et al*., 2012, Dominguez-Garcia *et al*., 2010).The HPLC analysis showed the presence of three phenolic compounds (gallic acid, caffeic acid, and *p*-coumaric acid) when *E. gracilis* was culture n medium containing no Cd^{+2} (Fig. 1a). In contrast, presence of gallic acid and *p*-coumaric acid was observed when *E. gracilis* was exposed to 0.02 mM Cd^{+2} (Fig. 1b). The exposure of *E. gracilis* to high concentrations of Cd^{+2} (0.14 mM) showed the presence of only gallic acid (Fig. 1c).

Analysis of flavonoid contents by HPLC showed that only naringenin was identified in *E. gracilis* exposed to Cd^{+2} (0.02 or 0.14 mM) and in the control (Fig. 2 a, b and c). Tolerance of *E. gracilis* to Cd^{+2} is related to mechanisms that involve the synthesis of short-chain of phytochelatins, glutathione and the activity of antioxidant enzymes (Castro-Guerrero *et al*. 2008, Garcia-Garcia *et al*. 2014). However, recent studies has reported that flavonoid and phenolic compounds could act as alternative antioxidants when antioxidative enzymes and glutathione are affect by heavy metals (Kovacik *et al*. 2009, Sanchez-Viveros *et al*. 2010). Recent work from our laboratory showed that phenolic and flavonoid compounds

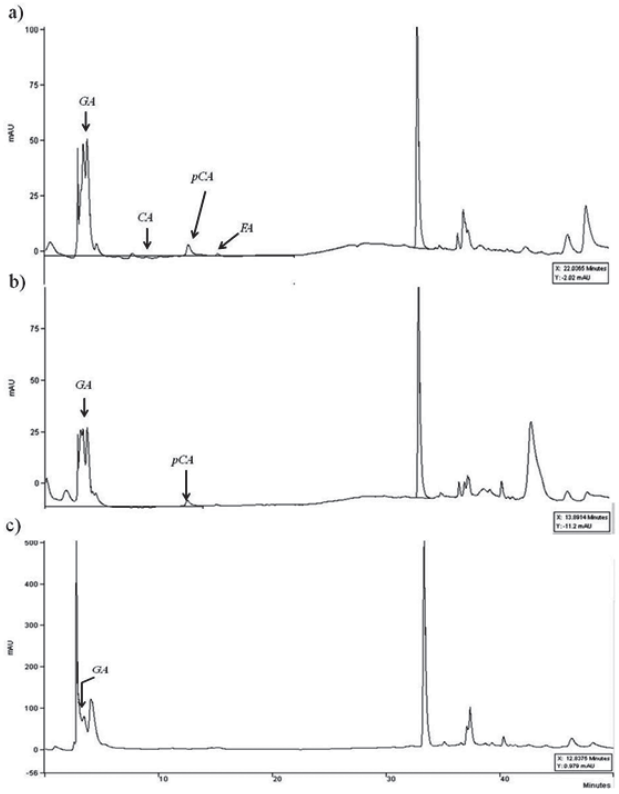

Figure 1. Differences in phenolic compounds of hydrolysed extracts of *Euglena gracilis:* (a) control, (b) and (c) exposed to 0.02 and 0.14 mM Cd^{+2}, respectively. Peaks: *GA*, gallic acid; *CA*, caffeic acid; *pCA*, *p*-coumaric acid;*FA*, ferulic acid.

Figura 1. Diferencias en los compuestos fenólicos de extractos hidrolizados de *Euglena gracilis*: (a) control, (b) y (c) expuestos a 0,02 y 0,14 mM Cd + 2, respectivamente. Peaks: *GA*, ácido gálico; *CA*, ácido cafeico; *pCA*, ácido *p*-cumárico; *FA*, ácido felúrico.

can act as nonenzymatic antioxidant and protect *E. gracilis* against oxidative stress when exposed to copper. However, in the present study our results suggest that production of phenolic and flavonoid compounds by *E. gracilis* do not represent a mechanism to Cd^{+2} tolerance. These results are not in agreement with previous work by Bai *et al*. (2004) and Dai *et al*. (2012) that suggest antioxidant and metal chelating properties of phenolic and flavonoid compounds in plants exposed to cadmium.

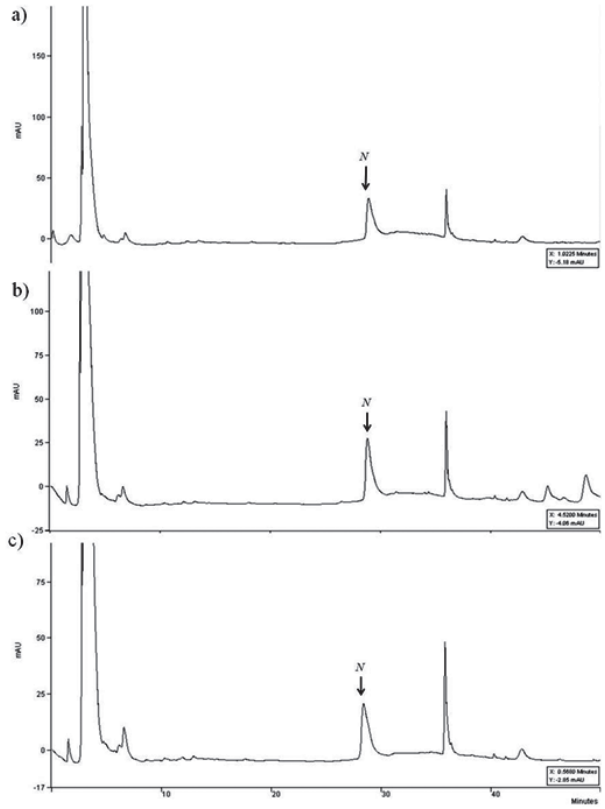

Figure 2. Differences in flavonoid compounds of hydrolysed extracts of *Euglena gracilis:* (a) control, (b) and (c) exposed to 0.02 and 0.14 mM Cd^{+2}, respectively. Peaks: *N*, narigenin

Figura 2. Diferencias en los compuestos flavonoides de extractos hidrolizados de *Euglena gracilis*: (a) control, (b) y (c) expuestos a 0,02 y 0,14 mM Cd + 2, respectivamente. Peaks: N, naringenina

CONCLUSION

In the present study the exposure of *E. gracilis* to Cd^{+2} showed that the production of phenolic compounds may participate in heavy metal tolerance. Further research is necessary to study the specific contribution of flavonoids in the exposure of *E. gracilis* to high concentration of Cd^{+2}.

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