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Production of Phenolics and Flavonoids Compounds in *Euglena gracilis* under Copper Stress

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In the present study the identification of phenolics acids and flavonoids compounds from *E. gracilis* exposed at two copper sulfate ($CuSO_4$) concentrations (0.4 and 3 mM) in the culture medium was evaluated using high-performance liquid chromatography (HPLC). The results showed that *E. gracilis* treated with 0.4 mM Cu^{+2}, increased significantly, the total phenolics content (718.86 ± 12.61 μg GA/g) and total flavonoids (361.46 ± 51.93 μg QE/g) with respect to control (137.04 ± 19.80 μg QE/g DW and 669 ± 0.57 μg GA/g DW respectively). However, a non-significant increased of total phenolics content (667.2 ± 6.29 μg GA/g DW) and total flavonoids (150.17 ± 24.23 μg QE/g DW) were observed in *E. gracilis* exposed to 0.8 mM Cu^{+2} with respect to control. The HPLC analysis showed that the exposure of *E. gracilis* at high doses (0.8 mM Cu^{+2}) stimulated the presence of ferulic and chlorogenic acids with respect to control. In contrast, the presence of flavonoids quercetin, and kaempferol were mainly observed in *E. gracilis* treated with 0.4 mM Cu^{+2}. Finally, the present study clearly showed change in the phenolics and flavonoids in *E. gracilis* exposed to copper. Therefore, the present data could suggest that feluric, chlorogenic acids, quercetin and kaempferol production are involved in Cu^{+2} detoxification.

Key words: Copper, Flavonoids, Phenolics acids, *Euglena gracilis,* HPLC.

Heavy metals are common pollutants in urban aquatic ecosystems, arising from effluent discharges, urban and agricultural runoff and solid waste dumping. The metals most often elevated in contaminated water bodies, and thus of greatest ecotoxicological concern, are zinc (Zn), copper (Cu) and lead (Pb) due to their effect to human health and prevalence in estuarine sediments. The occurrence of toxic metals in urban aquatic ecosystems adversely affects the lives of local people since they utilize this water for daily requirements. These heavy metals can be incorporated into food chain and their levels can increase through biological magnification (Cardwell *et al.,* 2002; Mishra *et al.,* 2008). Numerous metals are essential for living organisms at very low concentrations, but at high concentrations most are toxic and have a direct and adverse influence on various physiological and biochemical processes. In this sense the copper is an essential metal that participates in growth, metabolism and enzyme activities (Ke *et al.,* 2007). Although, the copper excess may stimulate the formation of free radical and reactive oxygen species, perhaps resulting in oxidative stress in the organism (Sanchez-Viveros *et al.,*

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2010). In this sense, the effect of copper is of interest, as this element has become a widespread contaminant due to its use as an algaecide and a fungicide in agriculture. On the other hand, *Euglena gracilis* is a freshwater unicellular flagellate found in many aquatic habitats. This flagellate has a fast growth and can be cultured easily and economically both under autotrophic and heterotrophic conditions and due to their ecological and commercial importance, considerable research on the effect of heavy metal pollutants in this organism has been conducted over the last few decades (Rodriguez-Zavala *et al.,* 2007; Morales-Calderon *et al.,* 2012).

To cope with heavy metal toxicity, *E. gracilis* has developed a variety of mechanisms that include the accumulation and production of phytochelatin conjugates, glutathione, proline and biofilm formation(Rodriguez-Zavala *et al.,* 2007; Cervantes-Garcia *et al.,* 2011; Morales-Calderon *et al.,* 2012). These characteristics make *Euglena* a model organism for use in ecotoxicological evaluation of the aquatic environment (Azizullah *et al.,* 2011). However, even though the biochemical mechanisms of heavy metal resistance have been studied in *E. gracilis*, the influence of potentially toxic elements, such as Cu^{+2}, in the phenolic compounds production has been scarcely studied. Given the previous, this study has the overall goal of elucidating the effects of different Cu^{+2} concentrations on phenolic production in *Euglena gracilis.*

MATERIALS AND METHODS

Cultivation and exposure to copper

Stock cultures of *Euglena gracilis* Klebs (strain Z) was grown axenically, using 14/10 h light-dark cycles under fluorescent white light (60–70 µmol quanta m^{-2} s^{-1}) at 26 °C, on organic *Euglena gracilis* (EGM) at pH: 5.5. Aliquots of 10^5 cells ml^{-1} at five days (logarithmic growth phase) were inoculated into 100 ml of EGM in 250 ml grass flasks. The cultures were supplied with different concentrations of $CuSO_4$ (0, 0.4 and 0.8 mM, respectively) and a metal free medium was used as a control. The experiment was carried out by triplicate under the same growing conditions, on static cultures containing at 26±1 °C, 14/10 light-dark cycles. All glassware was cleaned with dilute nitric acid (10%) and rinsed several times with Milli-Q water before experiments. At the end of the incubation period (3 days), the cultures were filtered and washed several times with metal free medium; at least three replicates for each sample and controls were lyophilized and stored until further use.

Determination of total phenol content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Nuñez-Ramirez *et al.,*2011). In brief, 100 mg of biomass dried were placed in an eppendorf tube, with 1.5 ml of methanol (80%), grinded at 4 °C and centrifuged at 14000 × g for 15 min. Reaction mixture consisted of mixing 1ml of the extract added with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 ml, 1 M) were then added. After 30 min, the phenols were determined by colorimetry at 765 nm using a spectrophotometer (GENESYS™ 20). Total phenol values are 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^{-1}.

Determination of total flavonoids

The content of flavonoids of the samples was evaluated using spectrophotometric method (Quettier *et al.,* 2000). Briefly, aliquots of 1ml of samples were mixed with solution A (0.3 ml of $NaN0_2$ and 4 ml of double distilled water). The samples were incubated for 5 min at room temperature. After incubation the samples were mixed with 0.3 ml of $AlCl_3$ (10%) solution dissolved in methanol and incubated for 1 min. The samples with $AlCl_3$ solution were mixed with Solution B (2 ml of NaOH 1M and 2.4 ml of double distilled water). Finally, the absorbance of the reaction mixture was measured at 415 nm against a methanol blank on a spectrophotometer (GENESYS™ 20) and quercetin as a standard. Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 10 to 100 µg ml^{-1} in methanol.

Extraction of phenolic acids for HPLC analysis

In brief 0.1 g of lyophilized biomass was mixed with a methanol solution containing 2 g/l of 2,3-ter-butyl-4-hydroxyanisol (BHT) and 10% acetic acid solution in a proportion of 85:15 (v/v).

The mixture was sonicated for 30 min and

brought to a 10 ml with HPLC grade water. The slurry was passed through a 0.2 µm Millipore filter (Millipore Co., MA, USA) and the filtrate was kept in a microfuge tube at -20 C and covered with aluminium foil to maintain phenolic acid solution away from light.

Phenolic acids and flavonoids separation and identification

Phenolic acids separation was carried out by gradient elution high performance liquid chromatography (GEHPLC) with an HPLC 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 of 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). A linear gradient elution was optimized with a solution of 200 mm acetate buffer pH 5.4 (A) and methanol (B) based in previous work (Sanchez-Estrada *et al.,* 2009). The efficiency of the GEHPLC methodology for phenolic acids and flavonoids separation described above was tested by preparing a solution containing the following commercially available standards: gallic, protocatechuic, hydroxybenzoic, vanillic, chlorogenic, caffeic, ferulic, p-coumaric, sinapic, o-coumaric, naringenin, kaempferol, quercetin, rutin, naringin and cinnamic acids (Sigma-Aldrich Chemical Co. St. Louis, MO, USA). The solution of the phenolic acid mixture was prepared with 0.01 g of each phenolic acid and added a solution made by mixing 1:1 ammonium acetate buffer 200 mm pH 5.4 and methanol. Solution was filtered through a 0.2 µm membrane (Millipore, MA, USA) before 20 µl were injected into the HPLC equipment.

Statistical analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS V.13 (SPSS Inc., Chicago, USA).

One way analysis of variance (ANOVA) and the Duncan's New Multiple-range test were used to determine the differences among the means. *P* values < 0.05 were regarded to be significant.

RESULTS

The results showed that *E. gracilis* treated with 0.4 mM Cu^{+2}, increased significantly, the total phenolics content (718.86 ± 12.61 µg GA/g) and total flavonoids (361.46 ± 51.93 µg QE/g) with respect to control (137.04 ± 19.80 µg QE/g DW and 669 ± 0.57 µg GA/g DW respectively). However, a non-significant increased of total phenolics content (667.2 ± 6.29 µg GA/g DW) and total flavonoids (150.17 ± 24.23 µg QE/g DW) were observed in *E. gracilis* exposed to 0.8 mM Cu^{+2} with respect to control (Fig. 1 a, b). These results indicated that the increase of flavonoids and phenolics compounds were not dependent of the concentration of Copper used in this experiment.

Detection of phenols and flavonoids compound in *E. gracilis* treated with copper

In the present study, the data showed that the exposure of *E. gracilis* to Cu^{+2} stimulated the ability of this microorganism to produced phenols and flavonoids. In the Fig. 2 and 3, illustrates the separation of a standard HPLC system: phenolic acids and flavonoids, respectively. The results showed that *E. gracilis* from cultures without metal (0 mM Cu^{+2}), present the presence of three phenolics compounds (Fig. 4 a). However, in *E. gracilis* treated with low doses of Cu^{+2} only gallic

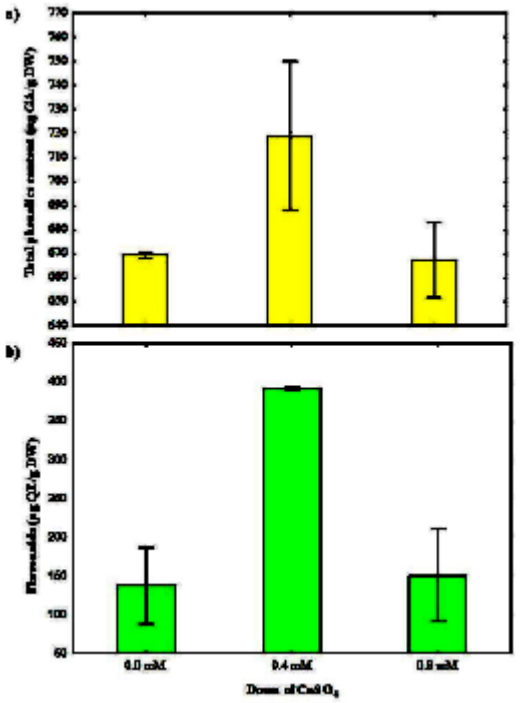


Fig. 1. Total phenolic content (a) and total flavonoids (b) from *Euglena gracilis* exposed to Cu^{+2}. (n = 3; values are expressed such mean ± S.D.)

and caffeic acids were observed (Fig. 4 b). In contrast, the exposure of *E. gracilis* at high doses (0.8 mM Cu^{+2}) stimulated the presence of two different phenolics compounds (ferulic and chlorogenic acids) with respect to control (Fig. 4 c). On the other hand, the presence of flavonoids quercetin, and kaempferol were mainly observed in *E. gracilis* treated with 0.4 mM Cu^{+2} (Fig. 5 a,b) with respect to control.

At difference, *E. gracilis* cells exposed to 0.8 mM Cu^{+2} showed only the presence of kaempferol (Fig. 5c).

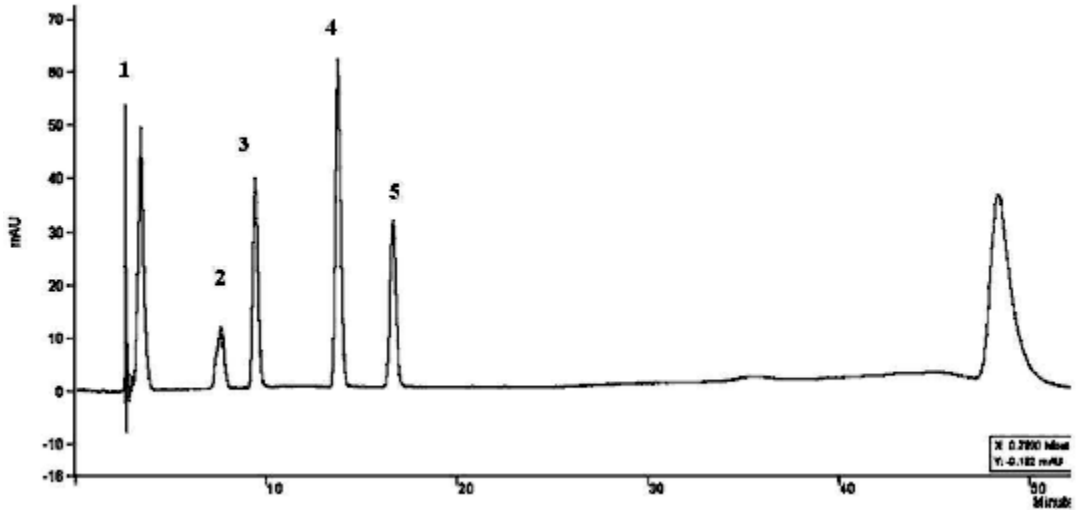


Fig. 2. Typical HPLC chromatogram of phenolic acids standards at 280 nm. Peaks: (1) gallic acid, (2) chlorogenic acid, (3) caffeic acid, (4) *p*-coumaric acid, (5) ferulic acid

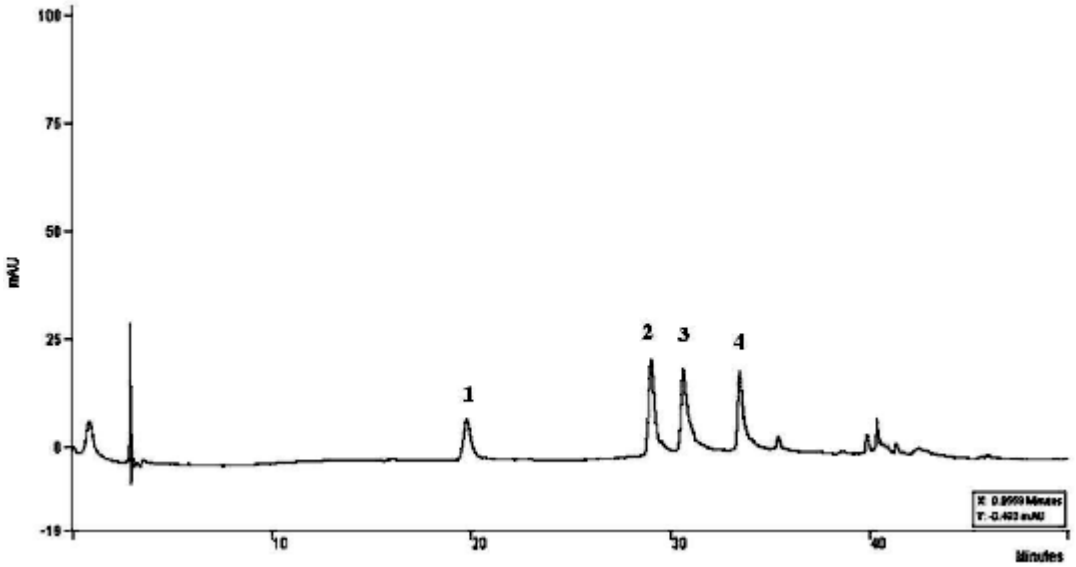


Fig. 3. Typical HPLC chromatogram of flavonoids compounds standards at 280 nm. Peaks: (1) rutin, (2) naringenin, (3) quercetin, (4) Kaempferol

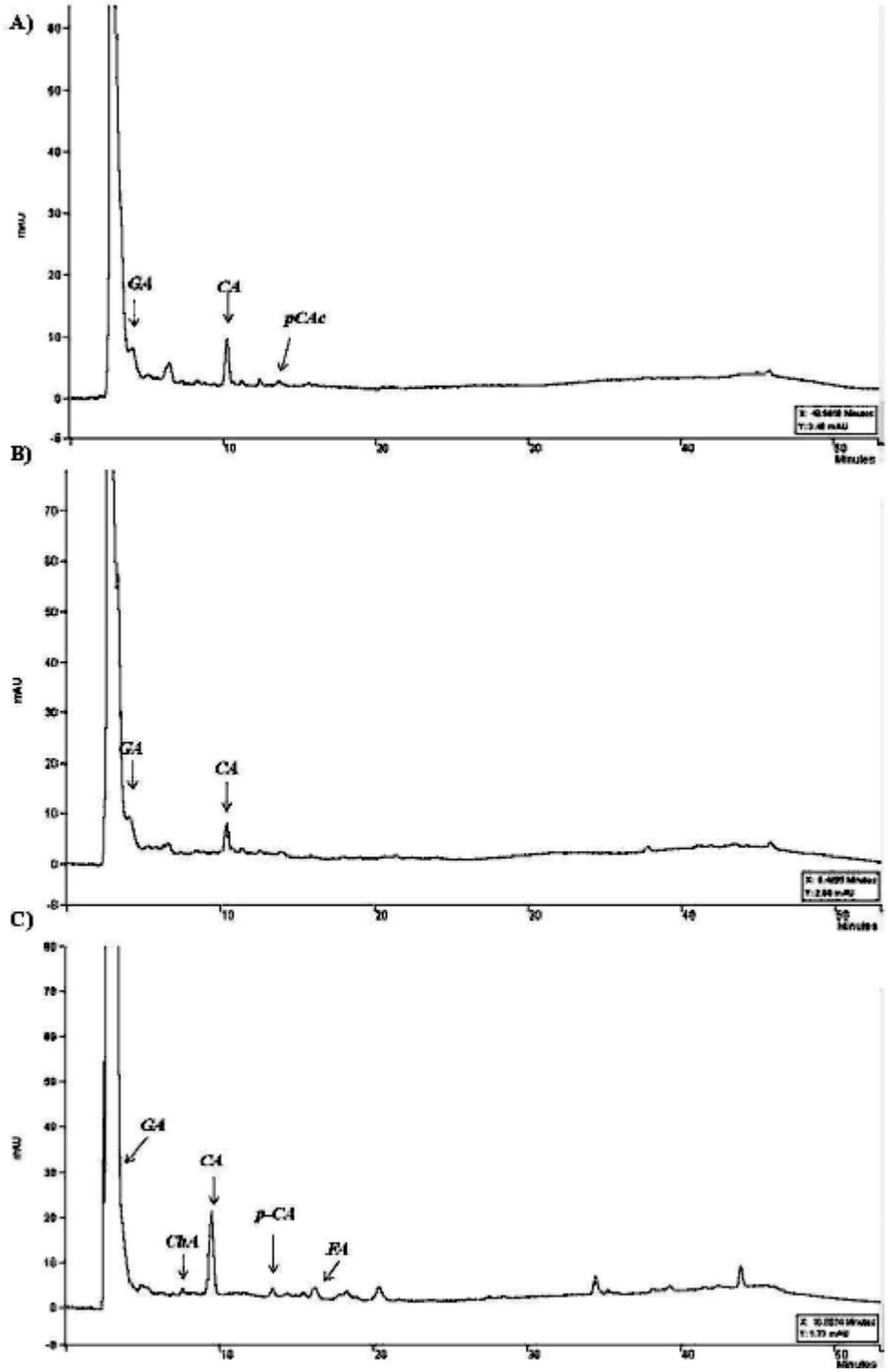


Fig. 4. Differences in the phenolic compound of hydrolysed extracts of *Euglena gracilis:* (A) control, (B) and (C) treated with 0.4 Cu^{+2} and 0.8 mM Cu^{+2} respectively. Peaks: *GA*, gallic acid; *CA*, caffeic acid; *pCA*, *p*-coumaric acid;; *ChA*, chlorogenic acid; *FA*, ferulic acid

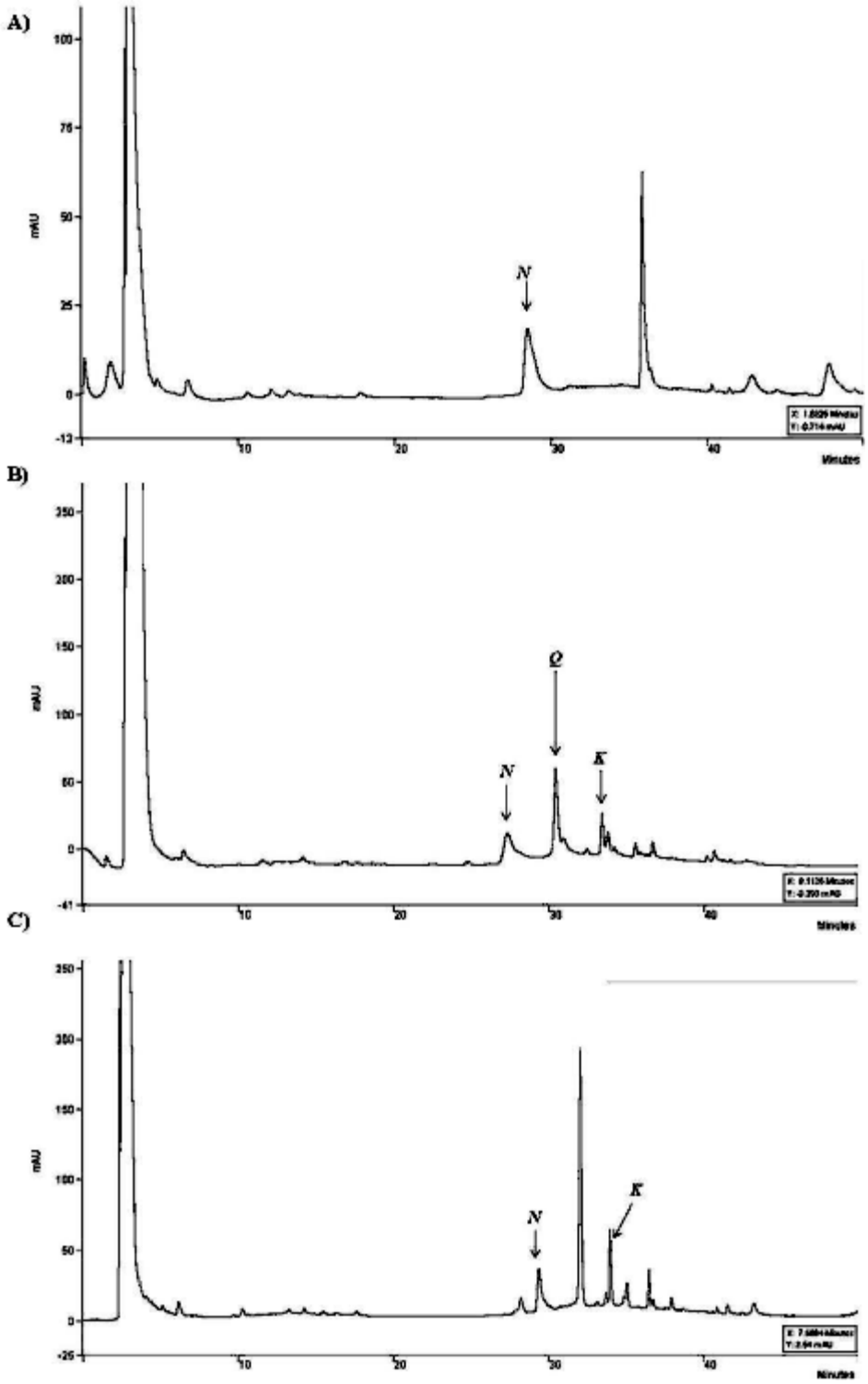


Fig. 5. Differences in the flavonoids compound of hydrolysed extracts of *Euglena gracilis:* (A) control, (B) and (C) treated with 0.4 Cu^{+2} and 0.8 mM Cu^{+2} respectively. Peaks: *N*, naringenin; *CA*, Q, quercetin; K, kaempferol

DISCUSSION

Previous studies have reported that the heavy metals toxicity in the organism can be results of mechanism such as: a) presence of reactive oxygen species by autoxidation and Fenton reaction caused mainly for transition metals; b) blocking of essential functional groups in molecules by non-redox-reactive heavy metals for example cadmium; and c) displacement of essential metal ions from molecules, which occurs with different types of heavy metals (Keilig and Ludwig-Müller, 2009). On the other hand, researchers have reported decreased of antioxidative enzymes (*eg*., glutathione reductase) and depletion of Glutathione (GSH) in several organism under the impact of heavy metals (Mazid *et al.,* 2011). Therefore, alternative antioxidants, such as flavonoids and phenolics compounds may be produced.

In the present study, the flavonoids and phenolics compounds present in *E. gracilis* were affects by the different Cu^{+2} concentrations used in the experiment. Consequently, the increases of total phenolics (ferulic and chlorogenic acids) and flavonoids (quercetin and kaempferol) detected in *E. gracilis* suggest that these compounds could be considered a protection mechanism in *E. gracilis* when is exposed to Copper. In this way, recent reports from Cervantes-Garcia *et al.* (2011) showed that phenolic compounds and proline increased the antioxidants activity in *E. gracilis* exposed to lower concentrations of Cu^{+2}. This is possible because the antioxidant activity of phenolic compounds is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Gonzalez-Mendoza *et al.,* 2009).

Therefore the increased total phenolics and flavonoids might contribute to protect cells from ion-induced oxidative damage by chelating the ions, thereby reducing their toxicity on cytoplasmic structures and detoxification of H_2O_2 (Dai *et al.,* 2006; Kováèik *et al.,* 2010). On the other hand, two main total phenolics (ferulic and chlorogenic acids) and flavonoids (quercetin and kaempferol) were detected in our present study, and further research is needed to study the specific flavonoids and total phenolics generated by Cu^{+2} stress. Finally is important elucidate the role that flavonoids or total phenolics playing in *E. gracilis* adaptation to Cu^{+2} stress.

CONCLUSION

In conclusion, the present study clearly showed that the exposure of *Euglena gracilis* to Cu^{+2} changes the phenolics and flavonoids production content in the cells. Therefore, the present data suggest that ferulic, chlorogenic acids, quercetin and kaempferol production are involved in Cu^{+2} detoxification.

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