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Original Article

# Chemical constituents and biological activity of *Euglena gracilis* extracts

*Analía Tolivia* [a,*], *Visitación Conforti* [a,b], *Osvaldo Córdoba* [c], *Luján Flores* [c,d]

[a] *Instituto de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina*
[c] *Cátedra de Química Biológica II, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Argentina*
[d] *Cátedra de Farmacognosia, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Argentina*

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ABSTRACT

*Background:* Euglena gracilis is a microalgae with a wide range of nutritional requirements, suggesting the existence of diverse physiological patterns. The aim of this work is to carry out a study about secondary metabolites biosynthesis on two strains of *E. gracilis* cultured *in vitro*.

*Methods:* Extracts from a *Euglena gracilis* (Klebs) commercial strain and a wild type isolated from an urban polluted river (MAT) were screened for preliminary identification of chemical constituents. Both strains were studied in their photosynthetic and bleached forms, on their exponential and stationary growth phases. Chromatographic analysis of pigments, lipids, and flavonoids were performed. Besides antioxidant, growth inhibition, and toxic activity were tested *in vitro*.

*Results:* The phytochemical analysis of extracts indicated the presence of steroids in all samples, cardenolids and triterpenes in the exponential growth phase. With the exception of the photosynthetic MAT strain, tannins were present in all the other on exponential phase samples and flavonoids were only observed in the stationary phase of both photosynthetic strains. Chromatographic profiles show that chlorophyll content decreased while carotenoids content increased in the stationary phase of both photosynthetic strains, and reveal the presence of flavonols derived from quercetin. In concordance with the presence of polyphenols, the fractions with the highest polarity showed antioxidant activity against DPPH• and growth inhibition activity *in vitro* even in the absence of paramylon, previously reported to have antitumoral properties.

*Conclusion:* This work constitutes the first report about polyphenol production in Euglenoids, which allows us a first assessment of the potential of *E. gracilis* as a source of bioactive products.



* *Corresponding author*. Intendente Güiraldes 2160, Ciudad Universitaria, Pab. II, C1428EGA Buenos Aires, Argentina. Tel.: +54 11 4576 3300.
E-mail addresses: atolivia@bg.fcen.uba.ar, analiatolivia@gmail.com (A. Tolivia).
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## 1. Introduction

Although most pharmacognostic studies focus on plants, other types of organisms are also regarded as pharmacognostically interesting. *Euglena gracilis* is a microalgae member of the Euglenoids, that can grow autotrophically, heterotrophically or myxotrophically that it has been extensively studied,[1,2] mainly on primary metabolites production,[3–5] but little is known about secondary metabolites biosynthesis. The most startling findings about this species concern to 4α-methylsterols, detected in trace amounts.[6,7] *E. gracilis* has a wide range of nutritional requirements, suggesting the existence of diverse physiological patterns, generating different metabolites and/or variation in the proportion they are biosynthesised. The aim of this work is to carry out a preliminary study on two strains of *E. gracilis* cultured *in vitro*, both in their photosynthetic and bleached forms, on their exponential and stationary growth phase. The *Euglena* reserve polysaccharide paramylon has been previously shown to have general antitumoral properties and reduce the negative effects of stressors.[8,9] Since paramylon precipitates in ethanol, our work explores the antioxidant and antitumoral *in vitro* effect of the extracts in its absence.

## 2. Methods

### 2.1. Culture conditions

Two *E. gracilis* strains were used: a commercial (UTEX-753) and a wild type strain (MAT) isolated from Matanza River.[10] Studies were performed on the photosynthetic (ph) strains and their bleached (b) counterparts, obtained by treatment with streptomycin. The cultures were grown in a growth chamber at 24 ± 1 °C, with 12:12 cool-white fluorescent light (150 μE $m^{-2}$ $s^{-1}$ irradiance) in EGM medium.[11] Cells were quantified with Neubauer's chambers and biomass was obtained via centrifugation at 4 °C after 72 h (exponential phase, -EX) and 144 h of growth (stationary phase, -ST). Biomass was washed four times with distilled water at 4 °C, and then dried by lyophilisation.

### 2.2. Extraction and chemical analyses

A general extraction was performed in all dried samples obtained with ethanol 96° and fractionated by pH changes, and partitioned with different polarity solvents (Fig. 1). The four fractions obtained were analysed with standard screening tests to detect the principal secondary metabolites. From residues of the ethanol extractions lipids were extracted with chloroform–methanol (2:1).[12]

### 2.3. Chromatographic profiles

Flavonoids were analysed using planar chromatography with two different mobile phases (BAW: *n*-butanol–acetic acid–water, 4:1:5; Forestal: acetic acid–conc. HCl–water, 30:3:10).

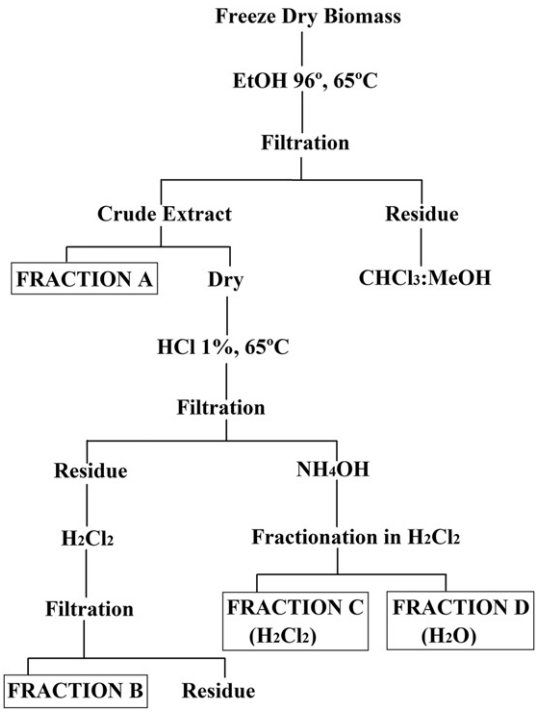


**Fig. 1 – General extraction.**

For lipids, a one-dimensional system was used on Silica gel G60 impregnated with ammonium sulphate, with benzene–acetone–water (30:91:8) as mobile phase.[13]

Pigments were determined from the soluble fractions in dichloromethane in Silica gel G60-calcium carbonate (2:1) with petroleum ether–acetone–i-propanol (35.5:14:0.5) used as mobile phase.[14] Furthermore, the second exhaustive extraction of pigments was performed using acetone and $MgCO_3$ to avoid the accidental formation of chlorophyll metabolites. The extracts were centrifuged at 670 × *g*, dried under vacuum and resuspended in 500 μl of acetone. The extracts where analysed by HPLC-RP-DAD.[15] The pigments were identified by co-chromatography with appropriate standards during elution, and by comparing their absorption spectra with reference standards. Standards and extracts were run through a C18 column, using a solution of acetonitrile: water (90:10) as mobile phase, at 1 ml/min flow rate and readings were taken at 436 nm.

**Table 1 – Biomass production efficiency.**

| Strain | Nutritional condition | Growth phase | g Liofilized/10 l culture | Cells/ml |
|---|---|---|---|---|
| UTEX | ph | EX | 1.34–1.67 | $4.00\times10^5$–$4.97\times10^5$ |
| | | ST | 1.92–1.94 | $5.71\times10^5$–$5.77\times10^5$ |
| | b | EX | 0.89–1.01 | $2.65\times10^5$–$3.01\times10^5$ |
| | | ST | 1.19–1.20 | $3.55\times10^5$–$3.58\times10^5$ |
| MAT | ph | EX | 1.37–1.60 | $4.07\times10^5$–$4.75\times10^5$ |
| | | ST | 1.91–1.97 | $5.84\times10^5$–$5.87\times10^5$ |
| | b | EX | 1.03–1.16 | $3.08\times10^5$–$3.46\times10^5$ |
| | | ST | 1.24–1.26 | $3.70\times10^5$–$3.76\times10^5$ |

**Table 2 – Extraction efficiency obtained of different fractions analysed (fraction D data not shown due to the formation of a salt that prevented its calculation).**

| Strain | Growth Phase | Efficiency (%) | | | |
|---|---|---|---|---|---|
| | | Fraction A | Fraction B | Fraction C | Fraction D |
| UTEX-ph | EX | 18.8–21.6 | 10.9–12.4 | 1.5–1.52 | – |
| | ST | 31.4–31.5 | 7.2–9.5 | 1.6–2.3 | – |
| MAT-ph | EX | 26.7–30.8 | 5.8–9.5 | 0.9–1.4 | – |
| | ST | 26.4–27.7 | 8.1–11.3 | 1.1 | – |
| UTEX-b | EX | 25.5–26.8 | 9.5–10.8 | 1.6–1.9 | – |
| | ST | 21.4–26.6 | 10.2–11 | 4.9–5.2 | – |
| MAT-b | EX | 11.9–12.9 | 4.1–4.3 | 0.8–2.6 | – |
| | ST | 20–24 | 7.1–8.3 | 0.5–0.9 | – |

### 2.4. *Biological activity studies*

The scavenging activity on diphenyl-2-picryl hydrazyl (DPPH) radicals of ethanolic and dichloromethane fractions (A and B respectively, Fig. 1) was assayed. The radical scavenging activities expressed as percentage inhibition of DPPH• were calculated.[16] The $SC_{50}$ values were calculated by linear regression.[17]

Only high polar extracts (Fraction A) were analysed by the wheat rootlet growth inhibition bioassay (*Triticum sativum*)[18] since assay requires the sample to be soluble in water. Vinblastine sulphate was used as a positive control.

The toxicity of the extracts was monitored by the brine shrimp lethality test.[19]

## 3. Results

### 3.1. *Biomass productivity and phytochemical study*

The efficiency of biomass production and the four fractions obtained is shown in Tables 1 and 2. The phytochemical screening showed in all samples the presence of carbohydrates of low molecular weight, lipids, and steroids. Cardenolids were only present in the exponential phase samples, and triterpenes only in the exponential phase samples of the bleached strains. With the exception of the MAT (ph), tannins were present in the exponential phase of all the other samples. In contrast, flavonoids were only detected in the stationary phase samples of photosynthetic strains (Table 3).

### 3.2. *Chromatographic profile*

The presence in all photosynthetic samples of chlorophylls *a*, *b*; β, β-carotenes; diadinoxanthin and neoxanthin was verified by TLC. The second analysis performed by RP-HPLC-DAD allowed yields between 33% (UTEX-h-ST) and 68.8% (MAT-ph-ST). Table 4 shows for each pigment detected the retention times (RT), the real absorption maxima in the elution solvent, and the extraction yields. The bleached strains showed complete pigment loss.

The photosynthetic strains showed differences between them and between the different growth phases analysed. During the exponential growth phase chlorophylls a, a' and b' predominated, being chlorophyll a the major pigment (40.53% in UTEX and 46.49% in MAT). In the exponential phase of the MAT strain the minor carotenoids and xantophylls pigments β-cryptoxanthin, antheraxanthin, micronone-like were identified, and four other compounds were detected but unidentified; none of these were detected on the UTEX strain.

In the stationary phase chlorophylls a, a' and b were detected in both strains. Chlorophyll b was the major chlorophyll in the UTEX strain (23.48%), while, as in the exponential phase, chlorophyll a was the major one for the MAT strain. Both strains showed carotenoids and xantophylls pigments in the stationary growth phase: violaxanthin in similar proportions in both strains (8.10% for UTEX and 8.12% for MAT), α-cryptoxanthin at higher proportion in UTEX (3.96%) than in MAT (2.99%), neoxanthin and microxanthin were found in the UTEX strain only (5.03% and 3.96% respectively), and fucoxantol was only found in MAT (4.59%).

The lipids chromatographic analysis allowed corroborate the presence of mono- and di-galactosyl di-acilglycerides,

**Table 3 – Phytochemical group test in MAT and UTEX extracts. ph: photosynthetic; b:bleached; EX: exponential phase; ST: stationary phase.**

| Chemical group | Fraction | UTEX ph | | MAT ph | | UTEX b | | MAT b | |
|---|---|---|---|---|---|---|---|---|---|
| | | EX | ST | EX | ST | EX | ST | EX | ST |
| Flavonoids | A | – | + | ± | + | – | – | – | – |
| Tannins | A | + | – | – | – | + | – | + | – |
| Lipids | A | + | + | + | + | + | + | + | + |
| Carbohydrates | A | + | + | + | + | + | + | + | + |
| Anthraquinones | B | – | – | – | – | – | – | – | – |
| Steroids | B | + | ++ | ++ | +++ | + | ++ | ± | ++ |
| Triterpenes | B | – | – | – | – | + | – | + | – |
| Cardenolides | C | + | – | + | – | + | – | + | – |
| Steroids | C | – | – | – | – | – | – | – | – |
| Triterpenes | C | – | – | – | + | – | – | – | – |
| Alkaloids | C | – | – | – | – | – | – | – | – |
| Leucoanthocyanins | C | – | – | – | – | – | – | – | – |
| Alkaloids $NH4^+$ | D | – | – | – | – | – | – | – | – |

**Table 4 – Pigments detected by RP-HPLC-DAD in MAT and UTEX photosynthetics. EX: exponential phase; ST: stationary phase.**

| Strain-growth phase | Extraction efficiency (%) | TR (min) | $\lambda_{max}$ (nm) in eluent | Pigment | Percentage |
|---|---|---|---|---|---|
| UTEX-EX | 60.2 | 15 | 410, 508, 538, 608, 664 | Unidentified | 31.12 |
| | | 33.50 | 467, 603, 648 | Chlorophyll b′ | 8.40 |
| | | 39.00 | 431, 619, 664 | Chlorophyll a | 40.53 |
| | | 40.00 | 390, 415, 430, 619, 664 | Chlorophyll a′ | 10.72 |
| | | 49.50 | 408, 504, 535, 606, 664 | Unidentified | 9.24 |
| UTEX-ST | 57 | 5.30 | 402, 498, 516, 615, 665 | Unidentified | 7.80 |
| | | 6.10 | 4.10, 467, 502, 535, 608, 666 | Unidentified | 4.79 |
| | | 7.10 | 409, 464, 504, 535, 608, 665 | Neoxanthin impure. | 5.03 |
| | | 8.80 | 421, 445, 474, 663 | Violaxanthin impure. | 8.10 |
| | | 10.00 | 410, 456, 507, 538, 609, 665 | Unidentified | 5.68 |
| | | 10.5 | 428, 456 | Microxanthin | 3.72 |
| | | 12.00 | 452, 479 | α-Cryptoxanthin impure | 3.96 |
| | | 23.10 | 455, 583, 632 | Unidentified | 6.98 |
| | | 24.50 | 465, 598, 648 | Unidentified | 1.83 |
| | | 30.00 | 420, 614, 649 | Chlorophyll b impure | 23.48 |
| | | 33.00 | 337, 386, 415, 432, 616, 663 | Unidentified | 18.39 |
| | | 34.90 | 390, 415, 430, 617, 664 | Chlorophyll a | 5.14 |
| | | 36.50 | 433, 626, 666 | Chlorophyll a impure | 1.24 |
| | | 46.30 | 412, 451, 476, 527, 604, 663 | Chlorophyll a′ impure | 2.31 |
| | | 54.80 | 409, 507, 537, 606, 665 | Unidentified | 1.54 |
| MAT-EX | 68.4 | 14.80 | 410, 476, 508, 537, 602, 665 | Unidentified | 9.84 |
| | | 16.00 | 445, 475 | Antheraxanthin & micronone-like | 5.62 |
| | | 18.50 | 428, 456 | β-Cryptoxanthin | 6.47 |
| | | 33.00 | 467, 600, 649 | Chlorophyll b′ | 9.56 |
| | | 39.00 | 386, 415, 430, 617, 664 | Chlorophyll a | 46.49 |
| | | 41.00 | 385, 411, 430, 619, 664 | Chlorophyll a′ | 11.72 |
| | | 50.00 | 409, 536, 606, 661 | Unidentified | 3.84 |
| | | 52.50 | 409, 507, 536, 606, 664 | Unidentified | 3.19 |
| | | 58.50 | 409, 505, 537, 606, 664 | Unidentified | 3.28 |
| MAT-ST | 68.8 | 7.50 | 409, 465, 507, 536, 608, 665 | Unidentified | 12.50 |
| | | 10.00 | 414, 445, 475, 537, 609, 665 | Violaxanthin impure | 8.12 |
| | | 10.50 | 410, 508, 538, 609, 665 | Unidentified | 7.80 |
| | | 12.50 | 428, 456 | Fucoxanthol impure | 4.59 |
| | | 14.10 | 428, 451, 478 | α-Cryptoxanthin like. | 2.99 |
| | | 29.50 | 467, 602, 649 | Chlorophyll b impure. | 7.05 |
| | | 32.00 | 420, 614, 660 | Unidentified | 1.60 |
| | | 34.50 | 337, 385, 414, 430, 617, 664 | Unidentified | 40.81 |
| | | 36.00 | 381, 415, 430, 617, 664 | Chlorophyll a | 11.22 |
| | | 47.00 | 408, 504, 535, 606, 664 | Chlorophyll a′ impure | 3.31 |

sulpholipids, phosphatidylethanolamine, phosphatidylcholine and sterol glycosides (only in pigmented strains).

The chromatographic profile of flavonoids shows the existence of flavonols, in particular those derived from quercetin.

### 3.3. *Biological activity*

Antiradical activity was detected in higher polarity fractions (A) with $SC_{50} = 147.7$ μg/ml and 157.2 μg/ml (MAT-ph-ST and UTEX-ph EX respectively) and slightly polar fractions (B) with

**Table 5 – Antioxidant activity of MAT and UTEX photosynthetic (-ph) and bleached (-b) strains. A: ethanolic fraction; B: dichloronethane fraction; EX: exponential phase; ST: stationary phase; % SC: percentage of scavenger capacity; $SC_{50}$: concentration of antioxidant necessary to remove 50% of the free radicals.**

| Fraction | Phase | MAT | | UTEX | |
|---|---|---|---|---|---|
| | | $SC_{50}$ ph (μg/ml) | $SC_{50}$ b (μg/ml) | $SC_{50}$ ph (μg/ml) | $SC_{50}$ b (μg/ml) |
| A | EX | 654.3 | 1453.6 | 157.2 | 454.5 |
| | ST | 147.7 | 1117.2 | 240.1 | 2150.7 |
| B | EX | 233.6 | 746.8 | 641.0 | 754.2 |
| | ST | 179.3 | 555.8 | 238.4 | 123.4 |

**Table 6 – Wheat rootlet growth inhibition bioassay results of MAT and UTEX extracts; ph: photosinthetic, b: bleached heterotrophic; EX: exponential phase; ST: stationary phase; E: stimulates the growth.**

| Strain | Growth phase | Concentration (mg/ml) | Average | Inhibition (%) |
|---|---|---|---|---|
| UTEX-ph | ST | 0.05 | 1.9 | 33.9 |
| | | 0.1 | 0.8 | 70.9 |
| | EX | 0.05 | 1.1 | 60.7 |
| | | 0.1 | 2.4 | E |
| MAT-ph | ST | 0.05 | 1.5 | 48.7 |
| | | 0.1 | 2.4 | E |
| | EX | 0.05 | 2.1 | 29.1 |
| | | 0.1 | 1.6 | 45.3 |
| UTEX-b | ST | 0.05 | 2.4 | 17.9 |
| | | 0.1 | 1.7 | 41.9 |
| | EX | 0.05 | 2.3 | 20.2 |
| | | 0.1 | 3.3 | E |
| MAT-b | ST | 0.05 | 0.1 | 95.5 |
| | | 0.1 | 2.6 | E |
| | EX | 0.05 | 2.1 | 28.2 |
| | | 0.1 | 1.2 | 57.3 |
| Water | | – | 2.9 | 0 |
| Vinblastine | | 0.02 | 1.24 | 57.6 |

$SC_{50}$ = 123.4 μg/ml and 179.3 μg/ml (UTEX-b ST and MAT-ph ST respectively, Table 5). Table 6 summarises the results obtained by the wheat rootlet growth inhibition bioassay. The strains showed considerable concentration-related growth inhibition in stationary phases of UTEX (-ph 33.9% and 70.9%; -b 17.9% and 41.9%), and in the exponential phases of MAT (-ph 29.1% and 45.3%; -b 28.2% and 57.3%). In contrast, some of the concentrations assayed stimulated growth (stationary phase in MAT and exponential phase in UTEX). Finally, none of the extracts negatively affected *Artemia salina*.

## 4. Discussion

Several authors have described pigment variation in *Euglena*. We can observe a decrease in chlorophyll content and an increase in carotenoids in both strains during the stationary phase compared to the exponential growth phase. These relationships suggest that carotenoids may be involved in the formation of chlorophylls. Studies indicate that the same porphyrin-like molecule may influence the synthesis of both pigments.

In this study we show in *E. gracilis* the biosynthesis of flavonoids and tannins, generally regarded to be bioactive and having free radical scavenging properties.[20] There are some reports about flavonoids in algae,[21] but this is the first work to report this chemical group in Euglenoids. When nutrients become scarce, *E. gracilis* cells enter into a non-growth phase known as stationary phase and develop a multiple-stress resistance response. The presence of flavonoids in the stationary phase may be associated to that response.

Differences were also observed in the distribution of chemical groups found between the photosynthetic strains, particularly regarding polyphenols. The flavonoids in UTEX were only found in the stationary phase, whereas MAT seems to produce them also in the exponential phase. Another group of phenols, the tannins, were only found in UTEX in the exponential phase; these were not detected in any of the growth phases of MAT. The screening methodology does not include quantification, but is widely used as qualitative method to study new source of natural products.[22] For microalgae, particularly for *E. gracilis*, there is no information on this matter. Antioxidant production in *Euglena* has been previously reported in different strains, especially in relation to the presence of vitamin E and C and ß-Carotene.[23] Nevertheless, the antioxidant activity of *E. gracilis* had not been related to the polyphenols (and other polar compounds). In concordance with the presence of polyphenols, our study shows that the fractions of major polarity have the highest scavenging activity.

At an initial stage, the antitumour activity may be inferred by simple bioassays such as the growth inhibition of wheat seeds. Antitumoral activity has been previously mentioned in *Euglena*,[8] but was related to paramylon. In this study we show evidence of antitumoral activity with extracts that lack paramylon, since paramylon stays in the residue (Fraction A). The wheat rootlet growth inhibition assay results suggest that phenols may be responsible for the growth inhibition effect, but we cannot be conclusive since some of the concentrations assayed stimulated growth. The primary biological activity test carried out complement the chemical screening and allows a first assessment of the potential of *E. gracilis* as a source of bioactive products.

## Conflicts of interest

All authors have none to declare.

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