

# *Chlorella minutissima* grown free and in alginate beads and cytotoxicity of algal extracts on L929 cells

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## Abstract

**The study of natural substances produced by several marine organisms led to the discovery of biologically active compounds, several of them have been utilized as drugs, pigments, insecticides, etc. or to synthesize biologically active molecules. The growth of the phytoplanktonic alga *Chlorella minutissima* (Chlorophyceae), free-living and immobilized in alginate beads, and the cytotoxicity of algal crude extracts on fibroblasts of the continuous cell line L929 are here reported. Algae have been cultured in Walne medium; L929 cells have been maintained in DMEM medium in standard conditions. Cultures of free-growing *Chlorella minutissima* showed an exponential phase of 10-12 days and a stationary phase from 12<sup>th</sup> day onwards, with a maximum cell density of  $27 \times 10^6$  cells/ml. Algae maintained in alginate beads showed maximum growth ( $46.5 \times 10^6$  cell./ml) after 20 days. The cytotoxicity of algal crude extracts was evaluated by Trypan blue dye exclusion. On the basis of preliminary data and of the NOEC value, the extracts of 10 to  $200 \times 10^6$  algal cells have been tested on fibroblasts. The calculation of  $IC_{50}$  showed that 50% mortality of L929 cells can be obtained with extracts from  $69.23 \times 10^6$  algae (lower and upper confidence limits 65.21 and  $73.49 \times 10^6$  cells respectively). Treated cells showed evident morphological changes and approximately 27% of them showed picnotic nucleus; furthermore, in approximately 8% the occurrence of additional nuclei was emphasized.**

## Introduction

Natural products are active principles of pharmacological interest; in particular, several substances of marine origin have demonstrated significant activity as antiviral, antiinflammatory and antitumoral agents (Newman et al., 2003) and many of which have found different pharmacological application. Also marine bacteria, fungi and microalgae have been recently indicated to be an important source of bioactive compounds (Fenical and Jensen, 1993) and phytoplanktonic organisms of the genus *Chlorella* are known to produce active chemicals (Noda et al., 1996). In this study the growth parameters of *Chlorella minutissima* have been studied and crude extracts of this alga have been tested for cytotoxicity on L929 mouse fibroblasts.

## Materials and methods

*Chlorella minutissima* cells have been maintained in appropriate culture medium according to Pane et al. (1998). The cultures have been maintained at  $18 \pm 0,5^\circ\text{C}$  with a light-dark cycle 12-12 hours (Heraeus BK 6160). Algal growth was estimated through counting by haemocytometer Thoma. L929 fibroblasts have been maintained in MEM medium, added with 1% penicillin-streptomycin, 1% glutamine and 5% bovine fetal serum (FBS) (Euroclone), in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  (Cellstar). Cytotoxicity tests on L929 cells were carried out using aqueous crude extracts of *Chlorella minutissima*. For the quantification of the extract the density (cells/ml) of *Chlorella minutissima* was considered. In toxicity tests four doses (A:  $25 \times 10^6$ ; B:  $50 \times 10^6$ ; C:  $100 \times 10^6$ ; D:  $200 \times 10^6$  cells/ml) were tested on  $5 \times 10^5$  L929 cells in medium without FBS. Cell mortality after 24 hour treatment was estimated through Trypan blue dye exclusion test; the  $IC_{50}$  value was calculated through the Trimmed Spearman-Kärber method. Preliminary observations of nuclear alterations through the DAPI test were estimated.

## Results

The cultures of *Chlorella minutissima* have shown an exponential phase of 10-12 days and a stationary phase

starting from the 12<sup>th</sup> day, with the maximum cellular density ( $27 \times 10^6$  cells/ml). Crude extracts of *Chlorella minutissima* have had remarkable effects on cultured L929 cells (fig. 1), which showed with a  $IC_{50}$  value of  $69.23 \times 10^6$  algal cells, with confidence limits of 65.21 and  $73.49 \times 10^6$ ; in particular 100% mortality was observed with the dose D ( $200 \times 10^6$  cells/ml). Treated cells showed evident morphological alterations: approximately 27% of them showed picnotic nucleus and in approximately 8% the presence of nuclei in excess (fig. 2) was evidenced.

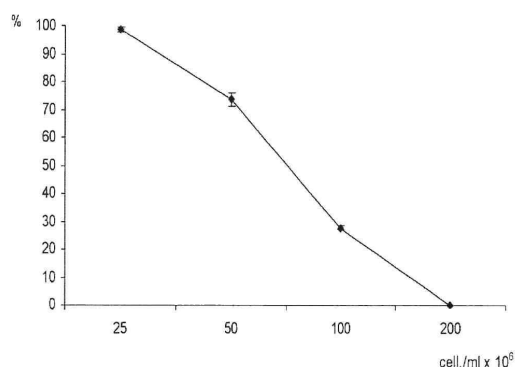


Fig. 1 - Percent survival of L929 cells after treatment with the crude extract of *Chlorella minutissima*.

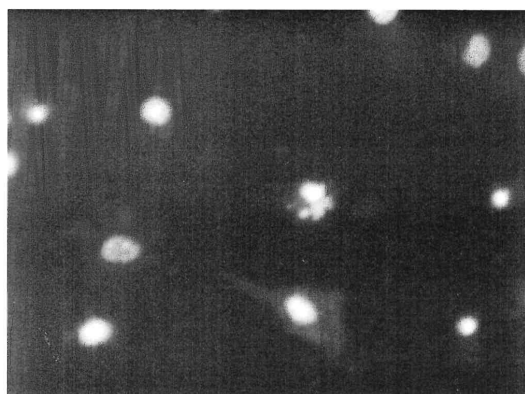


Fig. 2 - L929 cells treated with the crude extract of *Chlorella minutissima* and stained with DAPI.

## Discussion

In this study the easy maintaining of *Chlorella minutissima* cultures in the laboratory with high biomass yield at 18°C has been confirmed; this can support the need of a sufficient cell amount to carry out toxicity tests. It is known that *Chlorella* spp. is considered also in the human nutrition, thanks to its high protein, lipid, chlorophyll, carotenoid, vitamin, mineral and pigment content (Kai, 1991). *Chlorella* can furnish also a valid nutritional supplement in some pathologies (Merchant and

Andre, 2001) and, if used as alimentary integrator, it seems to attenuate the oxidative stress increasing the anti-oxidant processes (Miranda et al., 2001).

Derivatives of the chlorophyll extracted from *Chlorella* spp. moreover seem to inhibit dioxin absorption at the gastrointestinal level and to increase dioxin excretion of rats (Morita et al. 2001). Algae of the genus *Chlorella* are also used in aquaculture to feed fish larvae (Wikfors and Ohno, 2001). However, during this study crude extracts of *Chlorella minutissima* have shown an evident cytotoxic effect on L929 cells at doses of  $50 \times 10^6$  cells/ml onwards. The occurrence, even though preliminary, of nuclear alterations in treated cells with some evidence of apoptotic phenomena could make to suppose a possible, even if bland, genotoxic activity of the crude extract of *Chlorella minutissima*.

Such aspect needs however further searches and aimed tests. From other observations still in course (unpublished data), the activity of *Chlorella minutissima* could be comparable to that demonstrated for some marine unicellular algae that were seen to effect a strong anti-proliferative activity on cultured cells (Carbonnelle et al., 1999) and a strong action on the embryogenesis of marine copepods (Ianora et al., 1999).

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