Tannic Acid Content in the Cultured Diatom *Skeletonema costatum* (Greville) Cleve 1873, Isolated from the Romanian Black Sea Waters *Oana Vlas, Laura Bucur, Marius Făgăraș*	"Cercetări Marine" Issue no. 53 Pages 63- 69	2023
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TANNIC ACID CONTENT IN THE CULTURED DIATOM *SKELETONEMA COSTATUM* (GREVILLE) CLEVE 1873, ISOLATED FROM THE ROMANIAN BLACK SEA WATERS

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ABSTRACT

Skeletonema costatum is a dominant species in the spring phytoplankton composition of the Romanian Black Sea coastal waters. The species was isolated during a spring bloom and maintained in laboratory conditions for the past 10 years. The biomass was obtained by culturing the species in growth medium for diatoms under continuous light and shaking for four days. The biomass was harvested by vacuum filtration and lyophilized. The tannic acid content was determined by the HPLC method in the methanolic extract of *S. costatum* biomass. The tannic acid content was 0.46% (4.6 mg tannic acid/g biomass). Also, the chromatogram showed the presence of other separate, but unidentified organic compounds, which may contribute to its therapeutic potential. Our results justify further research to highlight *S. costatum* antioxidant and antimicrobial activity.

Keywords: *S. costatum*, polyphenols, biotechnologies, tannic acid

AIMS AND BACKGROUND

The microalgae are a natural resource of bioactive compounds such as vitamins, essential amino-acids, polyunsaturated fatty acids, minerals, carotenoids, enzymes, and fibres (Matos, 2017). Due to their potential, the microalgae became one of the most promising and innovating nutritional and pharmaceutical resources. Many pharmaceutical studies highlighted that the oxidative stress and the free radicals increased quantities are connected to chronic diseases, including cancer, aging and neurodegenerative illness such as Alzheimer and Parkinson, and cardiovascular disease, such as atherosclerosis. The phenolic compounds are secondary metabolites found in plants with the potential to prevent many degenerative diseases by eliminating these radicals (Jerez-Martel *et al*., 2017). Some researchers focused on analyzing the polyphenols concentrations of *Chlorella* strains under stress

conditions. Other authors concluded that the high nitrogen concentrations increase the polyphenols because nitrogen is an important component of proteins and nucleic acids, vital macromolecules for development (Aremu *et al.*, 2016). Also, in the diatom's *Phaeodactylum tricornutum* cell was determined a higher phenolic compounds content before exposure to a higher concentration of metals (iron and cupper), which might reflect those compounds contribution against iron and cupper toxicity (Rico *et al*., 2013). In other study, the phenolic compounds were identified in the biomass of many chlorophytes (*Ankistrodesmus* sp., *Spirogyra* sp.), euglenoids (*Euglena cantabrica*) and cyanobacteria (*Nostoc commune*), but also it was highlighted the lack of those compounds in other cyanobacteria species, such as *Nostoc* sp., *Nodularia spumigena*, *Leptolyngbya protospira*, *Phormidiochaete* sp. and *Arthrospira platensis* (Jerez-Martel *et al*., 2017).

The present study aims to identify a new source of tannic acid and to quantify this polyphenol in the biomass of the *S. costatum* strain isolated from the Romanian Black Sea waters.

EXPERIMENTAL

Culture maintenance and growth conditions

Monospecific cultures of *S. costatum* (Fig. 1) are maintained in growth medium for diatoms prepared according to ISO 10253:2016, in Erlenmeyer flasks (250 ml) at 20°C (Culcea, 2017). The gas exchange and the cells suspension are stimulated by shaking the cultures at 70 rotations/minute. The light is provided by fluorescent neon set at approximately 4500-5000 lx and a photoperiod of 14:10 light to dark. Subcultures are made every 3-5 days, during exponential growth phase, in freshly prepared growth medium. The glassware is cleaned and sterilized at 180°C for 3 hours using a hot air laboratory oven. The stoppers (cellulose) are autoclaved at 121°C for 20 minutes (Vlas *et al*., 2020).

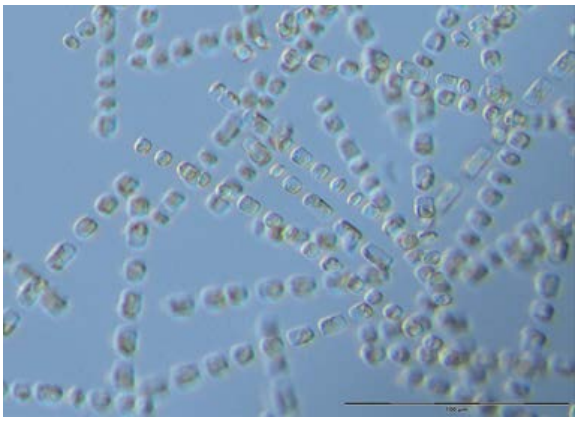

Fig. 1. Microscopic image of *Skeletonema costatum* grown in laboratory cultures (Olympus IX73)

Biomass production, harvesting and drying

To obtain the biomass needed for the tannic acid analysis, cells from a culture in exponential growth phase were inoculated in an Erlenmeyer flask, with 2 L of growth medium (ISO 10253:2016). The biomass production was stimulated by incubating the culture at 7-8000 lx, continuous light and 50 rotations/minute.

After 4 days, the biomass was harvested using a vacuum filtration system and four 0.45 µm Millipore filters (Fig. 2). Then, the filters were placed in a freeze dryer container and weighted. It was weighted also another freeze dryer container with an empty filter. Afterwards, both containers were kept in the refrigerator until the next day.

Fig. 2. Biomass harvesting by vacuum filtration

After freeze drying by LABCONCO (vacuum 0.033 Torr, collector -80°C), the containers were weighted again. Then, the lyophilized biomass was transferred from the container to a petri dish and sealed with paraffin (Fig. 3).

Fig. 3. Biomass harvesting and drying (a. Filters prepared for weighing before freeze drying; b. Biomass freeze drying; c. Filters weighing after freeze drying; d. *S. costatum* dried biomass

Estimation of density and growth rate

1 mL samples were taken from the culture with a micropipette after inoculation and before harvesting (4 days after inoculation). The samples were counted immediately using a hemocytometer and an inverted microscope. Density was estimated according to Karlson *et al*., 2010: density (cells/mL) = counted cells x 10^4/number of squares counted.

The growth rate was calculated according to ISO 10253:2016: $\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0}$, where: t_0 = start time; t_L = is the time of completion; N_0 = initial cell density; N_L = is the cell density measured at time t_L.

HPLC analysis

Identification and determination of tannic acid content was performed by HPLC method, with the following characteristics: PerkinElmer FLEXAR High Performance Liquid Chromatograph (HPLC), binary pump, PDA plus detector, thermostat column compartment, degassing system, autosampler.
The standard substance was tannic acid solubilized in methanol, in the following dilutions: 10, 20, 30, 40, 50, 100 µg/mL, which were used for the calibration curve. The method is reproducible with $r^2 = 0.99659$ (Fig. 4).

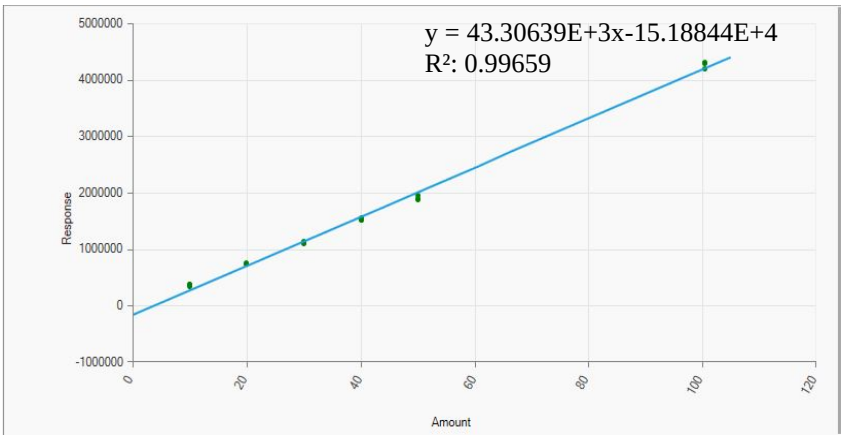

Fig. 4. Tannic acid calibration curve

The methanolic extract was obtained by refluxing 0.15 g of dried biomass with 20 mL of methanol for 30 minutes. After filtration, it was completed to 20 mL with the same solvent.

RESULTS AND DISCUSSION

The culture initial density was $11.25 \cdot 10^3$ cells/mL. Before harvesting, the cultured turned brown, homogenous, without aggregates, typical to an exponential growth phase culture (Fig. 5). The culture reached $673 \cdot 10^3$ cells/mL after 4 days, with a growth rate of 1.02/day.

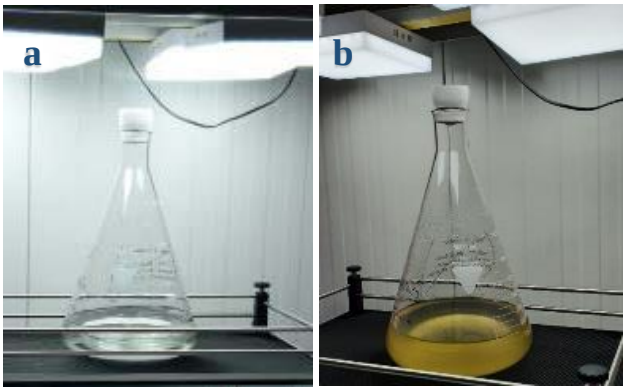

Fig. 5. Visual aspect of the culture during lag phase (a) and exponential growth phase (b)

1.44 g wet biomass were harvested by 0.45 µm Millipore filters vacuum filtration, respectively, 0.15 g of dried biomass by vacuum freeze drying (Fig. 6).

2 L	***S. costatum* concentrated culture**
⇩	Harvested by vacuum filtration system
1.44 g	**Wet biomass**
⇩	Freeze drying Temperature: -80°C Pressure: 0.033 Torr
0.15 g	**Dried biomass**

Fig. 6. Harvesting scheme

The identification and quantification of tannic acid was performed based on the standard substance for which the retention time was determined at RT = 1.15 min ± 0.05 (Fig. 7).

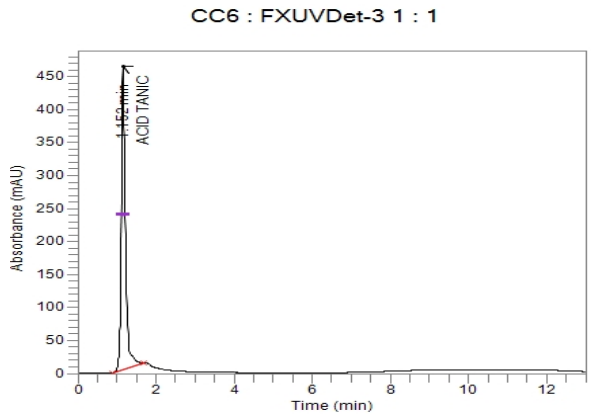

Fig. 7. Tannic acid chromatogram at 100 ppm

The test sample had a peak at the retention time RT = 1.18 min identified based on the standard substance as tannic acid (Fig. 8). The tannic acid content determined by the HPLC method in the methanolic extract of *S. costatum* was 0.46% (4.6 mg tannic acid/g biomass).

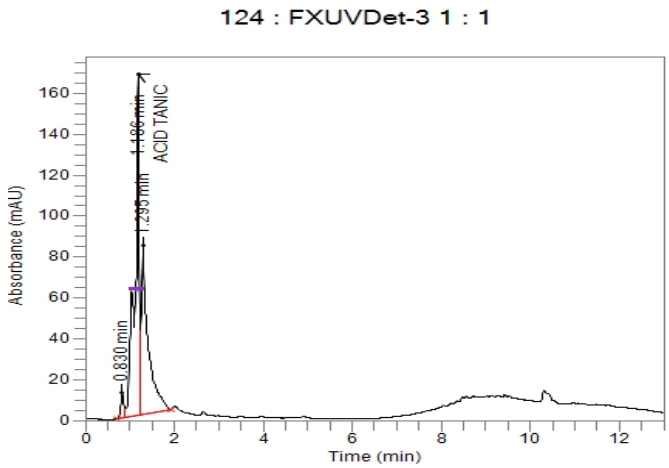

Fig. 8. *S. costatum* solution chromatogram

CONCLUSIONS

The presence of tannic acid in the methanolic extract (4.6 mg tannic acid/g biomass) justifies further research with biological studies to highlight the antioxidant and antimicrobial activity of the diatom *S. costatum*. Considering the potential of tannic-acid-based materials with a wide scope of functions and applications (Chen *et al.*, 2022) and the presence of other separate but unidentified organic compounds showed by our analysis, the therapeutic potential of *S. costatum* biomass seems promising. A step forward would be to improve the method by comparing the tannic acid content in the biomass harvested during different growth phases and by exposing the species to conditions that increase the polyphenols concentration.

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