Research Article

Mouhcine Ajdi, Younes Gaga, Amine Assouguem*, Mohammed Kara, Safaa Benmessaoud, Riaz Ullah, Essam A. Ali, Azra Skender, Jyoti Singh, Jamila Bahhou

Study of the phytotoxicity of margines on *Pistia stratiotes* L.

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Abstract: The olive trituration activity is one of the most important industrial activities in Fez, Morocco. These effluents are highly loaded with organic, inorganic, and phenolic compounds without any preliminary treatment that affects water quality. In this sense, the waters of Oued Fez are deteriorating due to the discharge of wastewater from oil mills and the excessive proliferation of *Pistia stratiotes* L., an invasive macrophyte that represents a significant stress to the aquatic ecosystem, eutrophication, and a reduction in biodiversity. This ecological situation has prompted us to carry out phytotoxicity bioassays based on the detection of the lethal concentration of *P. stratiotes* in the wastewater from the olive oil mill to evaluate the sensitivity and tolerance of macrophytes to the different concentrations of total polyphenols (TPP) present in the olive mill wastewater. To estimate their impact on the environment. Fresh whole plants of *P. stratiotes* were exposed to varying concentrations of olive oil mill wastewater with a series of TPP concentrations (0–30 mg/l) for 1 week in the natural environment. The results also show that *P. stratiotes* is able to grow rapidly in culture with 20 mg/l of TPP; the highest growth of wet weight of *P. stratiotes* occurred at 20 mg/l treatment with an average of 13 g wet weight increase. At 0 mg/l, treatment is known to cause very slow growth with an average of 2 g. Olive oil mill wastewater was toxic to the plant at concentrations higher than 30 mg/l, and the phytotoxic effect was manifested by retardation of growth, detachment of roots, wilting, and chlorosis of leaves. This indicates that polyphenols have great potential to inhibit the proliferation of *P. stratiotes* in aquatic environments.

Keywords: olive mill wastewater, *Pistia stratiotes* L., eutrophication, phytotoxicity, Fez river

*** Corresponding author: Amine Assouguem,** Laboratory of Functional Ecology and Environment, Faculty of Sciences and Technology, Sidi Mohamed Ben Abdellah University, P.O. Box 2202 Imouzzer Street, Fez 30000, Morocco; Department of Plant Protection and Environment, National School of Agriculture, Km.10, Route Haj Kaddour, B.P.S/40, Meknes 50001, Morocco, e-mail: Assougam@gmail.com
Mouhcine Ajdi, Younes Gaga, Jamila Bahhou: Laboratory of Biotechnology, Conservation and Valorisation of Natural Resources (LBCVNR), Faculty of Sciences Dhar El Mehraz, Sidi Mohamed Ben Abdallah University, BP 1796 Atlas, Fez 30000, Morocco
Mohammed Kara: Laboratory of Biotechnology, Conservation and Valorisation of Natural Resources (LBCVNR), Faculty of Sciences Dhar El Mehraz, Sidi Mohamed Ben Abdallah University, BP 1796 Atlas, Fez 30000, Morocco
Safaa Benmessaoud: Laboratory of Biotechnology, Conservation and Valorisation of Natural Resources (LBCVNR), Faculty of Sciences Dhar El Mehraz, Sidi Mohamed Ben Abdallah University, BP 1796 Atlas, Fez 30000, Morocco; SAM Laboratory, Department of Agricultural and Environmental Engineering, Higher School of Technology Sidi Bennour, Chouaib Doukkali University, Av. des Facultés, El Haouzia, El Jadida 24123, Morocco
Riaz Ullah: Department of Pharmacognosy, College of Pharmacy, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
Essam A. Ali: Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
Azra Skender: Department of Agriculture, Biotechnical Faculty, University of Bihac, 77000 Bihać, Bosnia and Herzegovina
Jyoti Singh: Department of Food Technology, Lovely Professional University, Phagwara, Punjab, India, e-mail: jyotisingh9377@gmail.com

1 Introduction

The world today is threatened by different sources of environmental pollution, whether they are industrial discharges, agricultural discharges, or domestic discharges. All of this is due to population growth and industrialization [1,2].

The margines cause an ecological imbalance for the Mediterranean countries, the main producers of olive oil. The production of these countries represents 94% of the world's production. In Fez, the annual production of margins is estimated at 250,000 m^3 [3].

The discharge of liquid effluents from oil mills directly into running water without any prior treatment leads to pollution of anthropic origin. The margins present a danger to the environment because, in addition to their acidic pH 4, the pollutant load of the margins is very high in terms of chemical oxygen demand (COD), biological oxygen demand (BOD), polyphenolic compounds, and suspended solids (SS) [4,5].

Various scientists have reported the pollution load generated by the margins [6–8]. However, the margins have fertilizing power, and the high excess of nutrients has, as a consequence, led to the development of the process of eutrophication [9]. In this sense, the waters of Oued Sebou experienced acute degradation during the olive season following the stop of the wastewater treatment plant in the city of Fez caused by a malfunction of biological reactors after receiving concentrations exceeding 20 mg/l of total polyphenols (TPP) from the direct discharges of oil mills in the sewage network of the city. Thus, comes the added risk of excessive proliferation of a floating perennial macrophyte if it escapes from its current host (Wadi Fez), as detected in Wadi Fez in 2012 [10,11].

Pistia stratiotes L. presents significant stress for the aquatic ecosystem: reduction of the oxygen rate, the strong release of H_2S, an increase in the evaporation of the water surface, and the reduction of biodiversity due to the formation of a thick tapestry covering the surface of the colonized environment [12–14]. This plant multiplies easily by various modes: vegetative thanks to its very numerous rejections (fragments of the plant) or by stolons's giving rise to new plants, as well as by its seeds [15,16].

Many studies have proven that *P. stratiotes* L. is often used for water quality monitoring and pollution detection. As crude oil is toxic to *P. stratiotes* at all concentrations from 0 to 100 ppm [17–19] and hexazinone, which is an herbicide, negatively affects the growth and development of *P. stratiotes* L., equivalent concentrations of 0.111 and 0.333 mg/l were toxic to the plant from the 7th day of the test [20]. *P. stratiotes* L. has a huge potential for pollution control. The plant has shown high tolerance to cadmium up to 20 mg/l [21,22].

The aim of this study is to eradicate *P. stratiotes* L. using margines rich in TPP after determining the threshold and lethal duration of *P. stratiotes* L. in relation to TPP in margines, which is derived from the extraction of olive oil and poses serious pollution problems due to its high concentration of organic matter and polyphenols [23].

2 Materials and methods

2.1 Study site

The experiment is carried out in the city of Fez, Morocco, characterized by a dry continental climate with an average temperature that varies between 4.5°C in January and 35.7°C in July and an interannual average rainfall of 430 mm, which favors the proliferation of *P. stratiotes*. (*P. stratiotes* L. was detected for the first time in February 2012.)

2.1.1 Origine of the margines

SOHNA is an olive oil company, NASMA, whose activities include the production of olive oil and the bottling of table oil. It is located in the industrial zone of Dokkarat Fez. It uses a continuous extraction system with two phases.

2.1.2 Collection of samples of *P. stratiotes*

Whole plants of *P. stratiotes* L. were collected downstream of the Oued Fez, which represents the main artery of the hydrographic network of the basin. It is located between parallels 33°30 and 34°08 N and between meridians 4°54 and 5°09 W, the rif in the north, the Middle Atlasian Causse in the south, the Fez-Taza corridor in the east, and the Saiss plain in the west. This watercourse appears at the surface of Ras El Ma, where it is fed upstream by the waters of Oued Chkou, including the wadis of Ain Chkef and Ain Smen, Oued El Himmer, and the wadis EL Mehraz and Boufekrane, which join the Oued Fez at the entrance of the medina. It has a SW-NE direction to join the Oued Sebou [24].

Before the experiment, the collected plants were washed with distilled water in the laboratory to eliminate all other species to have a monospecific culture of *P. stratiotes* L., and then, the plants were selected by their size and weight. The collected plants were put in pots of 3 l, of which 2 l were useful, containing spring water drawn from the medium of collection of the plant for a period of acclimatization of 48 h before being exposed to the margins.

2.2 Margine's physico-chemical characterization

The characterization of the margines was based on the study of the following parameters: pH, SS, COD, electrical conductivity, and TPP [25]. The pH, dissolved oxygen, and conductivity measurements were done by a HACH HQ40d multimeter. SS are determined by filtration on membranes with a pore diameter of 0.45 µm. The SS content is determined by the difference in weight of the filter before and after filtration and drying in an oven at 105°C for 24 h. The realization of a dilution is necessary because of the overloading of certain margins in matters of suspension, which causes the clogging of the filters [26].

The determination of COD is performed by the potassium dichromate method. The principle of this method is based on the boiling oxidation (150°C for 2 h) of the reducing materials by an excess of potassium dichromate in an acid medium (H_2SO_4) in the presence of silver sulfate as a catalyst and mercury sulfate as a chloride complexing agent. At the end of the reaction, the COD is evaluated by taking a sample suitably diluted before the oxidation. The optical density of the sample is obtained by spectrophotometry at a wavelength of 620 nm. The COD values are measured with a UV/visible spectrophotometer of the HACH DR3900 brand (France).

The extraction of TPP from raw margines is a liquid–liquid extraction based on the principle of solubility in organic solvents. The choice of the solvent depends on the nature of the compounds to be extracted and their solubility in the solvent. The phenolic compounds contained in the margines are extracted according to the method described by De Marco et al. [27].

The content of phenolic compounds in the different extracts obtained from the crude margines was estimated by the method of Folin–Ciocalteau [28,29]. The quantification of phenolic compounds was made according to a linear calibration curve of the form $y = ax$ carried out using gallic acid as a reference. The results will therefore be expressed in gallic acid equivalents. The number of TPP is calculated from the regression equation of the calibration range established with gallic acid (0–200 µg/ml) and expressed in milligrams of gallic acid equivalent. An estimation of the TPP of the same sample is performed by LCK 345 HACH kits to confirm the results obtained by the colorimetric method with a measurement range of 0.05–5.0 mg/l.

2.3 Tolerance of *P. stratiotes* L. to polyphenols

After the acclimatization period, the aquatic plants were exposed to different concentrations of polyphenols for 1 week. Dilutions of 0.5, 10, 15, 20, 25, and 30 mg/l were prepared from a stock solution of raw margines with an initial concentration of polyphenols of about 888 mg/l and spring water with three replicates for each concentration (Table 1). The concentrations were chosen on the basis of a preliminary study posing macrophytes at a series of polyphenol concentrations from 5 to 50 mg/l to study their influence on *P. stratiotes* L.

To test plant growth in various treated soils, chlorophyll content was assessed as a physiological descriptor combining soil toxicity and potential nutrient deficiency [30].

The leaves of the plant are put in contact with acetone 80%, which will damage them mechanically and destroy the membranes of the cells to obtain a chlorophyll solution.

The dosage of chlorophylls *a* and *b* is carried out on the acetone extract by measurement with the spectrophotometer by making measurements of optical densities at different wavelengths (645, 652, and 663 nm). A reference tank contains pure acetone diluted to 80% (to regulate the zero of the spectrophotometer) [31].

3 Results

3.1 Physico-chemical characterization of margines

The physico-chemical characteristics obtained from spring water samples are raw margines (Table 2) and diluted margines (Table 3).

3.2 Growth monitoring of *P. stratiotes* L.

We selected seven plants of *P. stratiotes* L. of the same weight, 61 g per plant, for weight monitoring. For the control plant, its growth gained 2 g in 6 days, or 63 g at the end of the experiment (Figure 1).

Table 1: Dilutions of margines

Phenol (mg/l)	Margine volume (ml)	Volume of spring water (ml)
0	0 ± 0.001	2,000 ± 0.001
5	11.26 ± 0.001	1988.74 ± 0.001
10	22.52 ± 0.001	1977.48 ± 0.001
15	33.78 ± 0.001	1966.22 ± 0.001
20	45.05 ± 0.001	1954.95 ± 0.001
25	56.31 ± 0.001	1943.69 ± 0.001
30	67.57 ± 0.001	1932.43 ± 0.001

Table 2: Physico-chemical characteristics of mineral water and raw margines

	pH	Conductivity	Dissolved O_2	COD	MES
Spring water	7.25 ± 0.01	1,865 ± 0.2	5.87 ± 0.05	2.29 ± 0.25	0.007 ± 0.003
Raw margines	4.85 ± 0.01	12,466 ± 0.2	0.3 ± 0.05	14,758 ± 0.25	16.5 ± 0.003

MES: suspended matter; COD: chemical oxygen demand.

Table 3: pH and conductivity of different concentrations of TPP and salt water

Concentration TPP mg/l	pH	Cond. (µS/cm)
0	7.85 ± 0.01	1,865 ± 0.2
5	7.66 ± 0.01	2,117 ± 0.2
10	7.44 ± 0.01	2,239 ± 0.2
15	6.83 ± 0.01	2,334 ± 0.2
20	6.39 ± 0.01	2,403 ± 0.2
25	5.90 ± 0.01	2,501 ± 0.2
30	5.50 ± 0.01	2,670 ± 0.2
Salt water	8.08 ± 0.01	20,550 ± 0.2

The weight monitoring of *P. stratiotes* L. at different dilutions of TPP showed a progressive weight gain during the first 4 days, going from 4 to 13 g for all dilutions (5, 10, 15, and 20 mg/l of TPP) against 1 g of weight gain for the control and 2 g for the 25 mg/l dilution. This growth curve is reversed on the 6th day with a decrease in plant weight of 1 g for the concentrations of 5 and 10 mg/l against 3 g for the dose of 15 mg/l.

On the other hand, at 30 mg/l, we notice a drop in weight from the first 48 h, from 61 to 51 g at the end of the experiment (Figure 2).

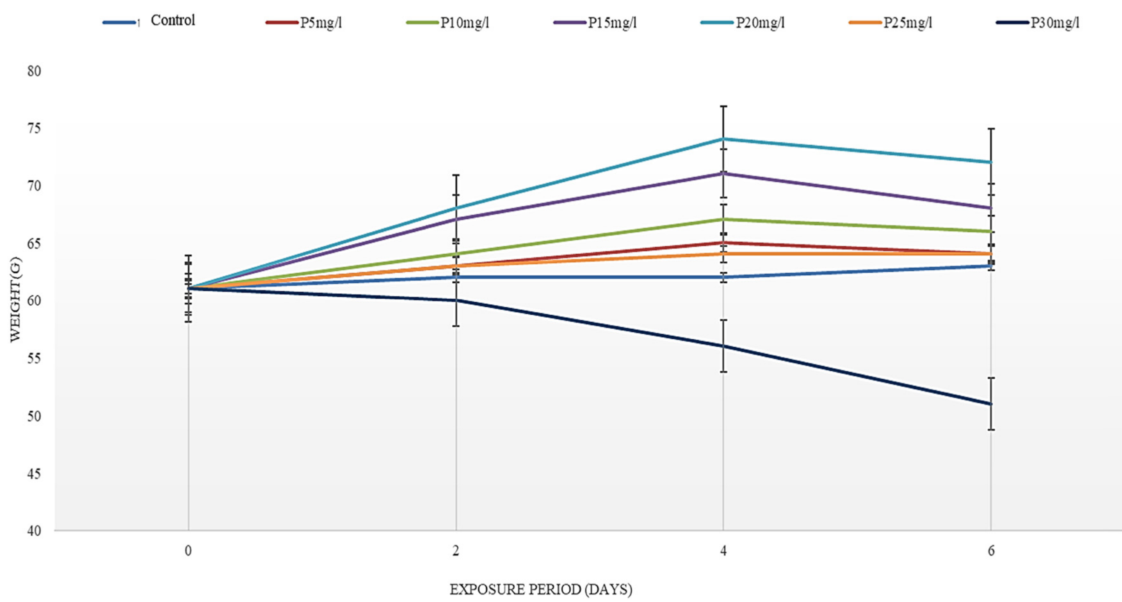

Figure 1: Weight monitoring of *P. stratiotes* at different concentrations of TPP for 6 days (mean ± SD, n = 3).

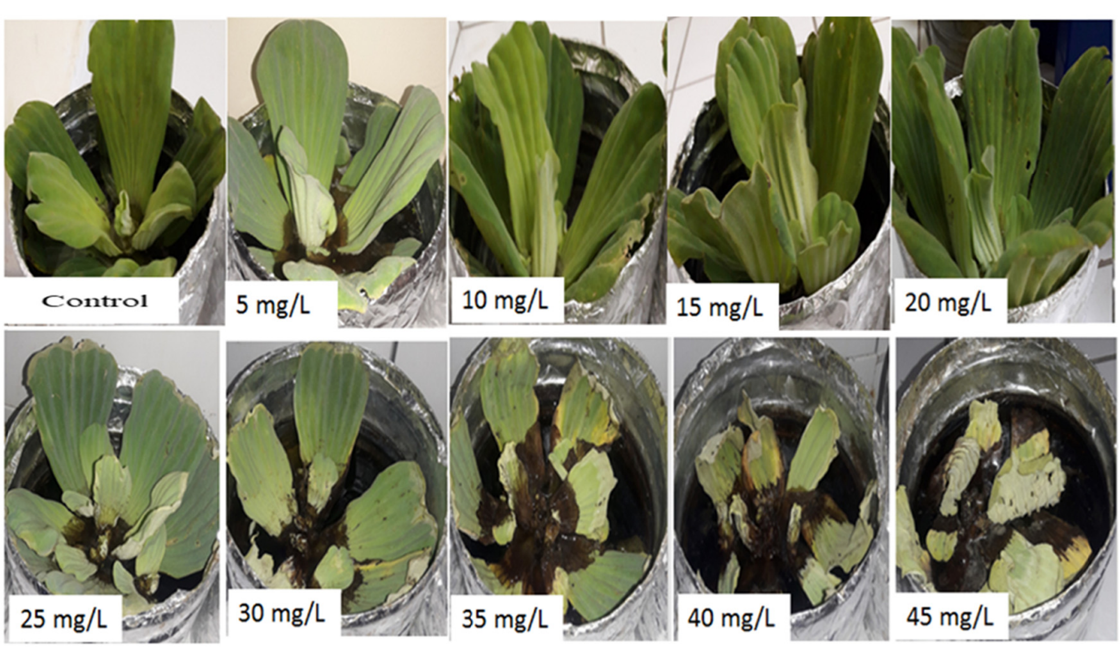

Figure 2: *P. stratiotes* L. exposed to different dilutions of TPP after 4 days of testing.

After 4 days of treatment with different concentrations of TPP, the optimal growth is favored at 20 mg/l of TPP, with a growth at the level of the roots reaching 30 cm for the concentration of 20 mg/l; on the contrary, the phytotoxic effect was manifested for all concentrations superior to 25 mg/l of TPP by the detachment of the totality of the fine roots (Figure 3).

TPP, being secondary plant compounds, are thought to influence nutrient cycling by affecting the degradation of organic matter, mineralization rates, nitrogen availability, and humus formation. Since *P. stratiotes* L. is exposed to high concentrations of TPP, this has affected its growth and physiology. To withstand these conditions, the plant has developed a mechanism based on the detachment of the entire fine root.

The determination of chlorophylls *a*, *b*, and *a* + *b* after completion of the experiment (6th day) showed a drop in the concentration of chlorophylls *a*, *b*, and *a* + *b* for all dilutions tested compared to the control, with the lowest values at 5 mg/l and the highest value at 30 mg/l (Figure 4).

4 Discussion

The margines are effluents that are very rich in fertilizing elements. They contain appreciable quantities of mineral nutrients, especially phosphorus and nitrogen, and are an excellent substrate for the development of microflora, which favors the improvement of the physico-chemical properties of soils [32–34].

A study was conducted on the presence of *P. stratiotes* L. in one of the eutrophic lakes in Nigeria. The weed removed 83.1% of BOD, 93.3% of ammonia nitrogen, and 75.0% of phosphorus [35–38].

It also produces a biomass of about 2,375 kg/ha. *P. stratiotes* has an enormous capacity to absorb nitrates (NO_3^+), ammonium (NH_4^+) [39], mercury (Hg) [40], cadmium (Cd) [41], and chromium (Cr) [42].

The above explains our results and the stimulation of growth by the margines at low concentrations of polyphenols inferior to or equal to 20 mg/l of TPP, which is fixed as the threshold of resistance of *P. stratiotes* L. concluded by this study (gain of 13 g in 4 days). *P. stratiotes* L. tolerates and uses the margines as fertilizer. At 25 mg/l, the macrophyte was able to maintain its development capacity, although some toxicity symptoms appeared, leading to root detachment and plant death. At 30 mg/l, the appearance of toxicity signs included the detachment of fine roots, chlorosis, and wilting.

Plants are constantly subjected to environmental variations. These changes can cause stress that alters cellular homeostasis through the production of reactive forms of oxygen. The phytotoxic accumulation of these different oxygen radicals can lead to the death of the plant [43,44].

Chlorophyll is the basis of plant photosynthesis and is involved in the absorption, transmission, and conversion of light energy, which reduces the toxicity of TPP for plants.

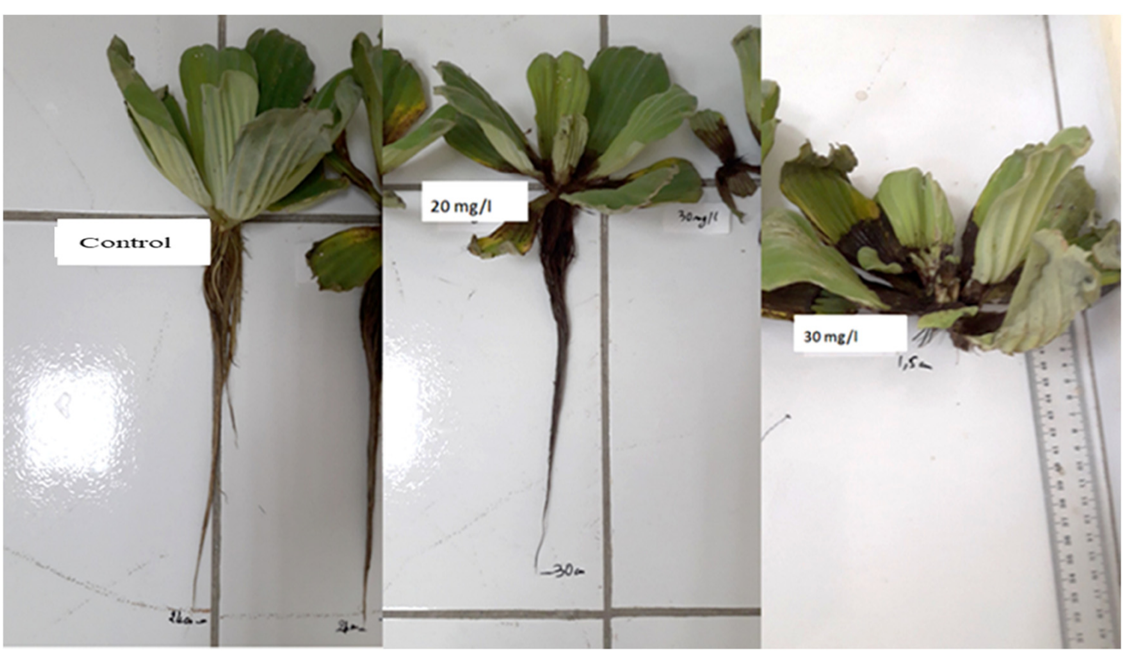

Figure 3: Effects of TPP of margines on the roots of *P. stratiotes* L. after 4 days of testing.

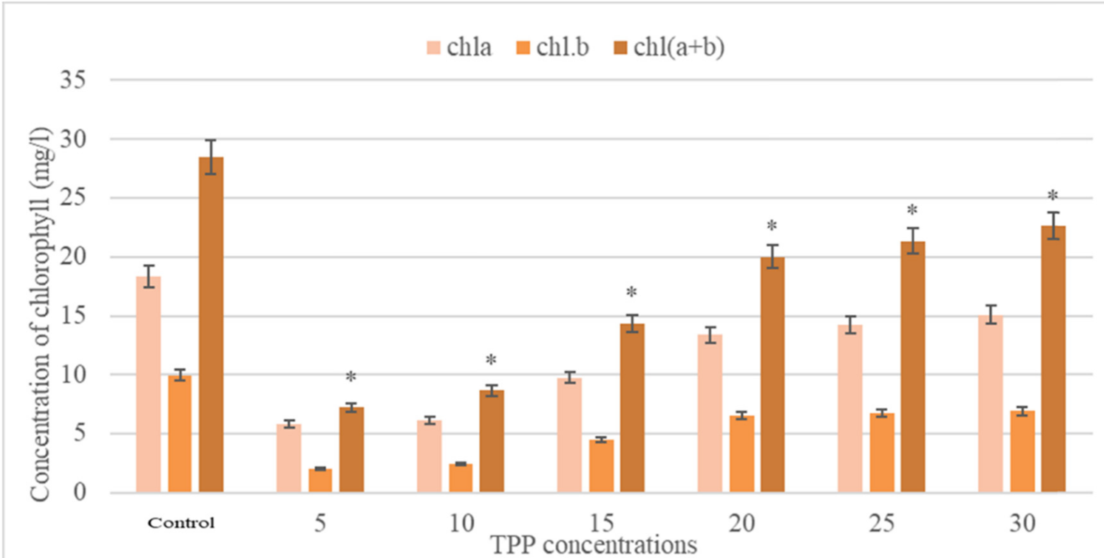

Figure 4: Concentration of chlorophylls *a*, *b*, and *a* + *b* after completion of the experiment (6th day) The superscript asterisk indicates the significant difference between the means of the treated and control plants in the same-colored histograms (mean ± SD, $n = 3$).

Our results show inhibitory effects on biomass production and chlorophyll synthesis due to phytotoxicity.

The chlorophyll secretion in *P. stratiotes* L. leaves decreased significantly under the treatments. This is consistent with the symptoms observed, which include chlorosis and decomposition of the leaves and reduced root growth [45–47]. These results follow on from a study on the remediation potential of *P. stratiotes* L. paper mill wastewater in terms of uptake and elimination of ten trace elements [48,49].

The above can explain the mortality of *P. stratiotes* L. in the face of the increase in the TPP host environment of the margine, which leads to a stress on the plant leading to its death. However, the increasing value of chlorophylls *a*, *b*, and *a* + *b*. The increase in TPP concentrations in the host environment is not consistent with the state of stress in the plant, which remains unexplained for the time being. Physiological responses to NaCl salinity were studied in two floating aquatic macrophytes, *P. stratiotes* L. and *Salvinia molesta* L., by Upadhyay. *P. stratiotes* tolerates life in a 200 mM saline environment [50].

These results can be explained either by the substitution of Mg^{2+}, which inhibited chlorophyll biosynthesis, TPP, which disrupted chlorophyll biosynthesis, or heavy metal ions, which inhibited protochlorophyll activity [51–53].

The toxicity of margines can be due not only to TPP but also to NaCl [54,55], and the concentration of the latter in margines depends on the system of crushing olives. For our samples, the concentration ranges from 0 to 30 mg/l [16]. The conductivity remains normal compared to the conductivity of salt water; therefore, the phytotoxicity in our study is due only to the polyphenols in margines [50].

5 Conclusion

To stop the excessive proliferation of *P. stratiotes* L., an invasive macrophyte represents a major stress for the aquatic ecosystem, and to make the most of the margins, effluents are highly charged with phenolic compounds and come from the olive-pressing activity. We carried out phytotoxicity tests on *P. stratiotes* L. using TPP present in the margins at different concentrations. The results showed that *P. stratiotes* L. is tolerant to TPP at concentrations of 20 mg/l or less and uses margins as a fertilizer. At 25 mg/l, *P. stratiotes* L. began to show symptoms of toxicity. At 30 mg/l, the appearance of strong signs of toxicity included root detachment, chlorosis, and wilting leading to plant death.

These results indicate that polyphenols have great potential to inhibit the proliferation of *P. stratiotes* in aquatic environments. Other studies are underway to better understand the mechanisms underlying the control of invasive plants by TPP and to make this product an effective phytotoxicant.

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Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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