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First report of the anti-cyanobacterial activity of the invasive weed *Oxalis pes-caprae* L. against *Microcystis aeruginosa* growth

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ABSRTACT

This work aims to explore the anti-cyanobacterial potentialities of the weed *Oxalis pes-caprae* L. on *Microcystis aeruginosa* growth. In the current study, the aqueous extract of the aerial parts of *Oxalis pes-caprae* L. (AEOP) was tested to assess its activity on *M. aeruginosa* growth in an experimental bioassay. The anti-cyanobacterial effect of AEOP against *M. aeruginosa* was assessed in a batch culture experiment where several morphological and physiological indicators, and inhibition parameters were assessed. To reveal the potentially allelochemicals, phenolic compounds were analyzed in AEOP. Furthermore, the results from the bioassay demonstrated that AEOP inhibit the growth of *M. aeruginosa* in a concentration dependent way. *Microcystis* cell densities were significantly reduced during the bioassay period at the different tested concentrations (0.25, 0.5, 0.75, and 1 mg/mL). Under both highest concentration of AEOP (0.75 and 1 mg/mL), the inhibitory rate (IR) reaches 63% and 74 only after 4 d of experimentation, respectively. The highest IR (86%) was achieved on 10 d at the highest concentration (1 mg/mL). Additionally, during the 12-d experimental period, all four-treatment groups (0.25–1 mg/mL) demonstrated a significant decrease in the content of chlorophyll-a and carotenoids compared to the control. Overall, the obtained results demonstrate the anti-cyanobacterial effect of AEOP to control *Microcystis* growth. Moreover, the invasive weed *Oxalis pes-caprae* L. might be proposed as a potential ecofriendly alternative algaecide to control *Microcystis* blooms in the eutrophic water bodies.

Keywords: *Microcystis aeruginosa*; Blooms; Cyanobacterial inhibition; Algaecide; Terrestrial invasive plant; Morphological; Physiological alterations; Green approach

1. Introduction

Harmful cyanobacterial blooms (CyanoHABs) have become a serious problem for drinking water sources and recreational purposes worldwide. *Microcystis* spp. are the cyanobacterial species most involved in CyanoHABs [1]. *Microcystis* blooms are often toxic and produce hepatotoxins (Microcystins) that contaminate drinking water and cause adverse effects on the health of several living organisms [2].

To control the proliferation of toxic cyanobacteria in situ and/or in water treatment plants, diverse solutions have been used such as artificial mixing and thermal destratification

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[3,4], filtration, ultraviolet, ultrasound techniques [5,6], and coagulation and flocculation chemicals [7,8]. However, these conventional techniques generate secondary pollution, which directly or indirectly affects the health of both ecosystems and humans [9–11].

Therefore, plant-based alternatives as green approaches to control *Microcystis aeruginosa* growth have widely used macrophytes [12,13] and medicinal plants [14–17]. Recently, the use of the allelopathic potentialities of invasive plants in bioassays to control harmful algal blooms has received the interest of scientists as a two-fold innovative solution; first one, to solve the problem of invasive plant biomasses; and thus to eliminate the proliferation of toxic cyanobacteria by a natural agent [13,18,19]. However, the use of invasive alien plants remains little limited; despite the benefits that can offer in eradicating *Microcystis* blooms [13,19].

Otherwise, in terrestrial ecosystems, the phenomenon of invasive plants is one of the major emerging problems, often in agroecosystems where they cause crop damage. The excessive biomasses generated by invasive plants constitute a challenge for the agroecosystem managers [20]. Turning a supposedly "useless" biomass towards an economic and/or ecological valorization could constitute a real alternative [18].

Bermuda buttercup (*Oxalis pes-caprae* L.) is a perennial herb, which belongs to the Oxalidaceae family. It is indigenous of South Africa, now found in numerous countries worldwide [21], and commonly spread in Mediterranean region as one of the most invasive weeds [22,23]. *O. pes-caprae* L. is often invaded in disturbed and agricultural areas and can occur in all soil types [24] due to its high competition potential, which using the release of allelochemicals as the main invasion factor [25–27]. Furthermore, *O. pes-caprae* L. is widely recognized as an undesirable invasive species in agricultural production, especially in olive groves [28] and citrus orchards [29]. Moreover, its allelopathic effects on various plants has been demonstrated in bioassays, in particular on tomato, oat, lettuce [25], and on *Trifolium repens* [30].

Plant allelochemicals are considered as the source of green algaecides because both of their biodegradability and efficiency in the inhibition of neighbor plant growth [31]. The phytochemical characterization of *Oxalis pes-caprae* aqueous and organic extracts indicated that the main chemical compounds of this plant are luteolin and apigenin flavonoid derivatives [32], and several common phenolic acids, phenols and their derivatives [18,26]. The most of these bioactive compounds belong to the common allelochemicals and were known by their antioxidant and antimicrobial activities [18,32]. However, no indication in the literature has reported its allelopathic potential in the biocontrol of harmful algae.

This work aims to present, for the first time, the anti-cyanobacterial potentialities of the weed *Oxalis pes-caprae* L. on *M. aeruginosa* growth. in an experimental bioassay.

2. Material and methods

2.1. Biological materials

M. aeruginosa was isolated from the eutrophic Lalla Takerkoust Reservoir, Morocco, (31°21′36″ N; 8°7′48″ W) in August 2020, and was grown in laboratory cultures at 25°C ± 1°C under light intensity of 70 μE/m²·S, with a light/dark cycle of 15 h/9 h.

O. pes-caprae L. was collected in May 2021 from a private garden in Khouribga city (32°52′51″ N; 6°54′22″ W). Aerial parts were rinsed several times with distilled water to remove debris, dried away from sunlight at ambient temperature (25°C), and then crashed into powder prior to extraction.

2.2. Preparation of plant extracts

The aqueous extraction of the aerial plants was carried out according to the method described by Chen et al. [33], with small modifications. Briefly, 10 g of dried biomass powder leaves were placed in 100 mL distilled water under agitation (45°C; 48 h). The macerate was then autoclaved and kept at 4°C as aqueous extract.

2.3. Quantification of total phenolic and total flavonoids in extracts

Total phenolics (TPs) concentration was determined with the Folin–Ciocâlteu method [34]. Total flavonoids (TFs) content concentration were determined by the method described by Kim et al. [35].

2.4. Anti-cyanobacterial bioassay

5 groups of Erlenmeyer flasks (500 mL) containing Z8 medium to a final volume (300 mL) were used to contain 5 concentrations (0 (control), 0.25, 0.5, 0.75, 1 V/V%) of the aerial parts of *Oxalis pes-caprae* L. (AEOP) which are equivalents to 0, 0.25, 0.5, 0.75, 1 mg·DW/mL, respectively. Each flask was inoculated by a volume of *M. aeruginosa*, in exponential growth phase, to make an initial density (0.73×10^6 cell/mL). Flasks were incubated in a culture room at 25°C ± 1°C, illuminated in 15 h/9 h light-dark cycle with fluorescent tubes (70 μE/m²·S) within 12 d. All the experiments were conducted in triplicate. *Microcystis* growth under different treatments was quantified using Malassez hemocytometer.

2.5. Inhibition parameters

The effects of the AEOP on *Microcystis* growth were expressed using three parameters: inhibitory rate (IR), the half-maximal inhibitory concentration (IC50) and the IC90. IR of *Microcystis* growth was determined according to the following Eq. (1): IR (%) = $N_0 - N/N_0 \times 100$; where N_0 and N (cells/mL) are the cell density in the treatment and control cultures, respectively. IC50 and IC90 were calculated based on the concentration range (X) used according to the inhibition rates (Y) recorded at the end of the experiment. The calculation equation derived from the drawing graph is as follows: $Y = 96.8X - 0.8$.

2.6. Morphological modification

During the experiment, *Microcystis* morphology was observed using an optical microscope with a camera (Motic BA210). Several morphological criteria (cell diameter, form

and condensation of colonies, pigmentation, and vacuoles density) were elucidated.

2.7. Pigments determination

The concentrations of chlorophyll-a and carotenoids were measured by spectrophotometry according to Lichtenthaler and Wellburn [36]. They extracted with ethanol 95% at 4°C for 48 h, and then determined using a spectrophotometer (TOMOS V-1100) at 470, 649, and 665 nm. The following formulas were used to calculate the concentrations (μg/mL): [Chlorophyll-a] = 13.95xDO665 – 6.88xDO649; [Carotenoids] = [(1,000xDO470) – (2.05·Chl-a)]/229.

2.8. Statistical analysis

Data with three replicates were statistically analyzed by two-way analysis of variance (ANOVA two-way) with Tukey's test to assess differences between exposure concentrations and control at $p = 0.05$. Correlation coefficients were calculated between cellular density and TPs and TFs, concentrations in the end of experimentation.

3. Results

3.1. Inhibitory effect on growth of M. aeruginosa

Fig. 1 shows the concentration-dependent inhibition of *M. aeruginosa* growth by the AEOP. In control group, the cell densities remained between 0.73×10^6 and 154.2×10^6 cell/mL as un optimum value at 10-d. In contrast, *Microcystis* cell densities were significantly reduced ($p < 0.05$) during the bioassay period at the different tested concentrations (0.25, 0.5, 0.75, and 1 mg/L).

The inhibition rate (IR) appeared to be dose-dependent, with an overall IRs exceeding globally 52% after 2 d at the three tested concentrations (0.5, 0.75 and 1 mg/mL) (Table 1). Under both highest concentration of AEOP (0.75 and 1 mg/mL), the IR reach 63% and 74 only after 4 d of experimentation, respectively. The highest IR (86%) was achieved on 10 d at the highest concentration (1 mg/mL).

Thus, the bioassay results were expressed in terms of the inhibitory concentrations. Both the IC50 and IC90 were calculated. In the end of the experimentation, the IC50 and IC90 mentioned two values 0.52 and 0.94 mg/mL, respectively (Fig. 2).

3.2. Effects on morphological changes in M. aeruginosa

To elucidate the morphological changes in *M. aeruginosa* cultures under treatments, a series of pictures were taken (Fig. 3). In control groups, it can be seen that the *M. aeruginosa* cells were clearly structured with regular surfaces. For these last, cell forms were rounded and pigmented, with cell diameter between (2.1–2.7 μm) in the end of the treatment period (Fig. 3. C, C.I). However, under high concentrations (0.75 and 1 mg/mL), *M. aeruginosa*

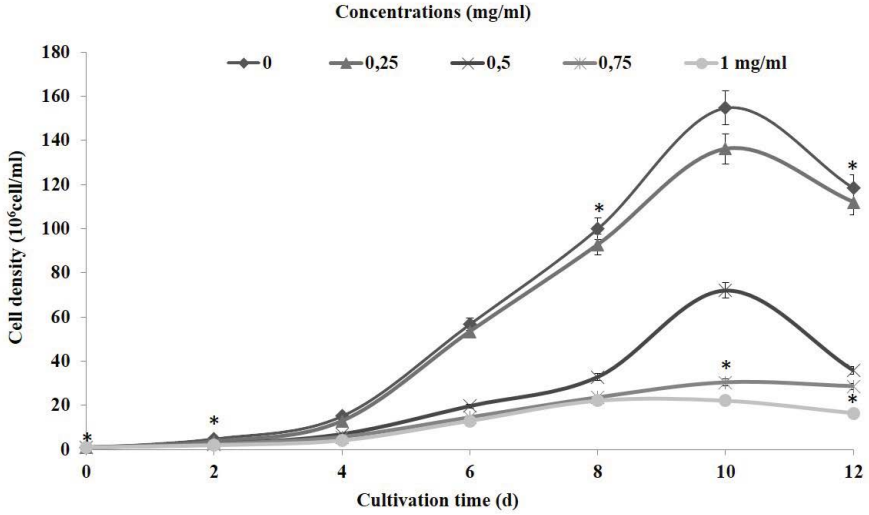

Fig. 1. Effects of the different concentrations of AEOP on *Microcystis aeruginosa* growth. Errors bars represent the standard deviation ($n = 3$). *<0.05 indicate significant differences compared to the untreated culture (ANOVA two-way).

Table 1
Inhibitory effects expressed as inhibitory rate (%) of AEOP on *Microcystis aeruginosa* growth

Treatments (mg/mL)	0	2	4	6	8	10	12
0.25	0	29 ± 0.13	16 ± 0.06	6 ± 0.07	7 ± 0.01	12 ± 0.93	6 ± 0.01
0.5	0	52 ± 0.07	55 ± 0.28	65 ± 0.11	67 ± 0.08	53 ± 0.26	70 ± 1.35
0.75	0	53 ± 0.16	63 ± 0.15	75 ± 0.04	76 ± 0.04	80 ± 0.07	76 ± 0.15
1	0	63 ± 0.21	74 ± 0.06	77 ± 0.16	78 ± 0.24	86 ± 0.02	86 ± 0.03

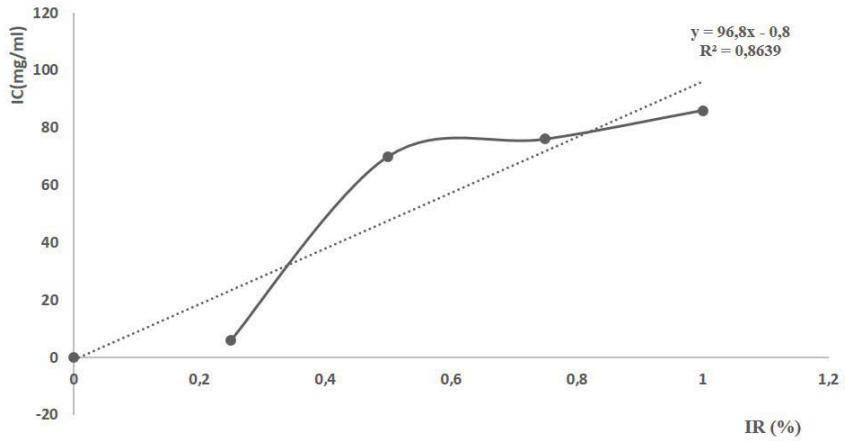

Fig. 2. Inhibitory concentrations recorded according to the inhibition rates during the bioassay.

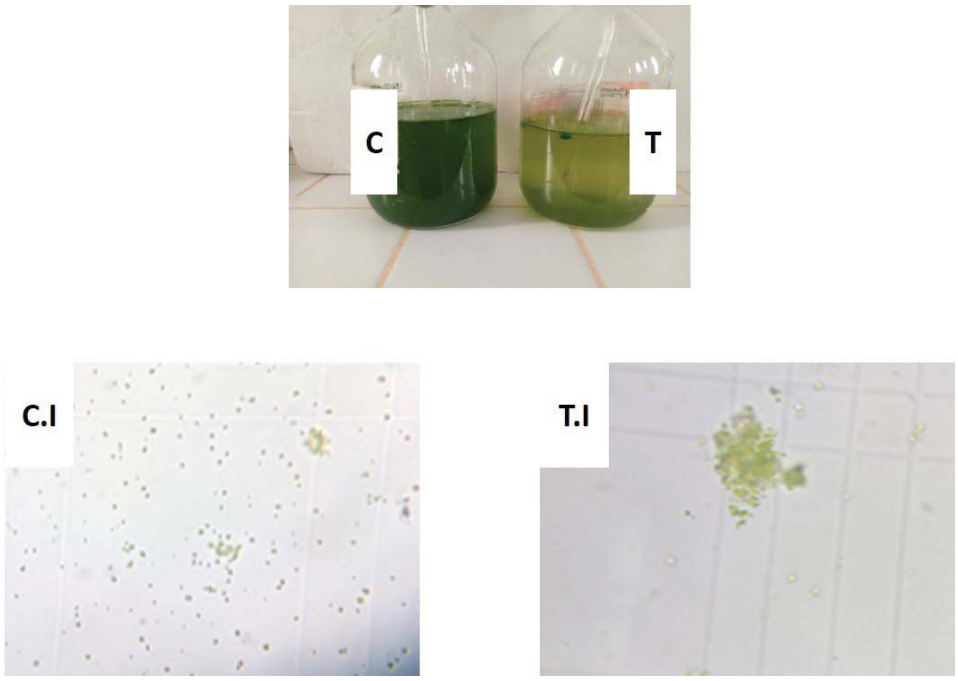

Fig. 3. Visual and microscopic observations of *Microcystis aeruginosa* cells in the control (C, C.I) and treatment groups (T, T.I) (1 mg/mL) of *Oxalis pes-caprae* L. aqueous extracts (Gr. x 40), with sedimented cells, completely devacuolated and decomposed.

cells lose their standard and regular form to a cell clusters (1.5–1.8 µm cell diameter), forming sediment aggregates, with uniform, destroyed and shrinking, especially, in the end of the test period (Fig. 3. T, T.I). These morphological changes are accompanied by the coagulation and sedimentation of cyanobacterial cells, especially after 4 d of exposure, with yellowing colors.

3.3. Effects on photosynthetic pigments

In order to assess the physiological modification, two photosynthetic pigments were measured (Chl-a and carotenoids) as physiological indicators of *Microcystis* growth in the bioassay. During the 12 d experimental period, all four-treatment groups (0.25–1 mg/mL) demonstrated a significant decrease ($p < 0.05$) in the content of Chl-a and carotenoids compared to the control. With the increase in extract concentrations, the pigment contents appear to be strongly inhibited.

After 8 d of bioassay, Chl-a and carotenoid contents at both the highest concentration treatment (0.75–1 mg/mL) decreased by 53% and 50%, respectively (Fig. 4).

3.4. Phytochemical characterization

The results of the phytochemical characterization are shown in Table 2 AEOP exhibited important values on TPs, TFs. As well, a high significant correlations have been well obtained between the IRs of the three high concentration (0.5%–1%) and TPs, and TFs concentrations (0.95 and 0.93), respectively.

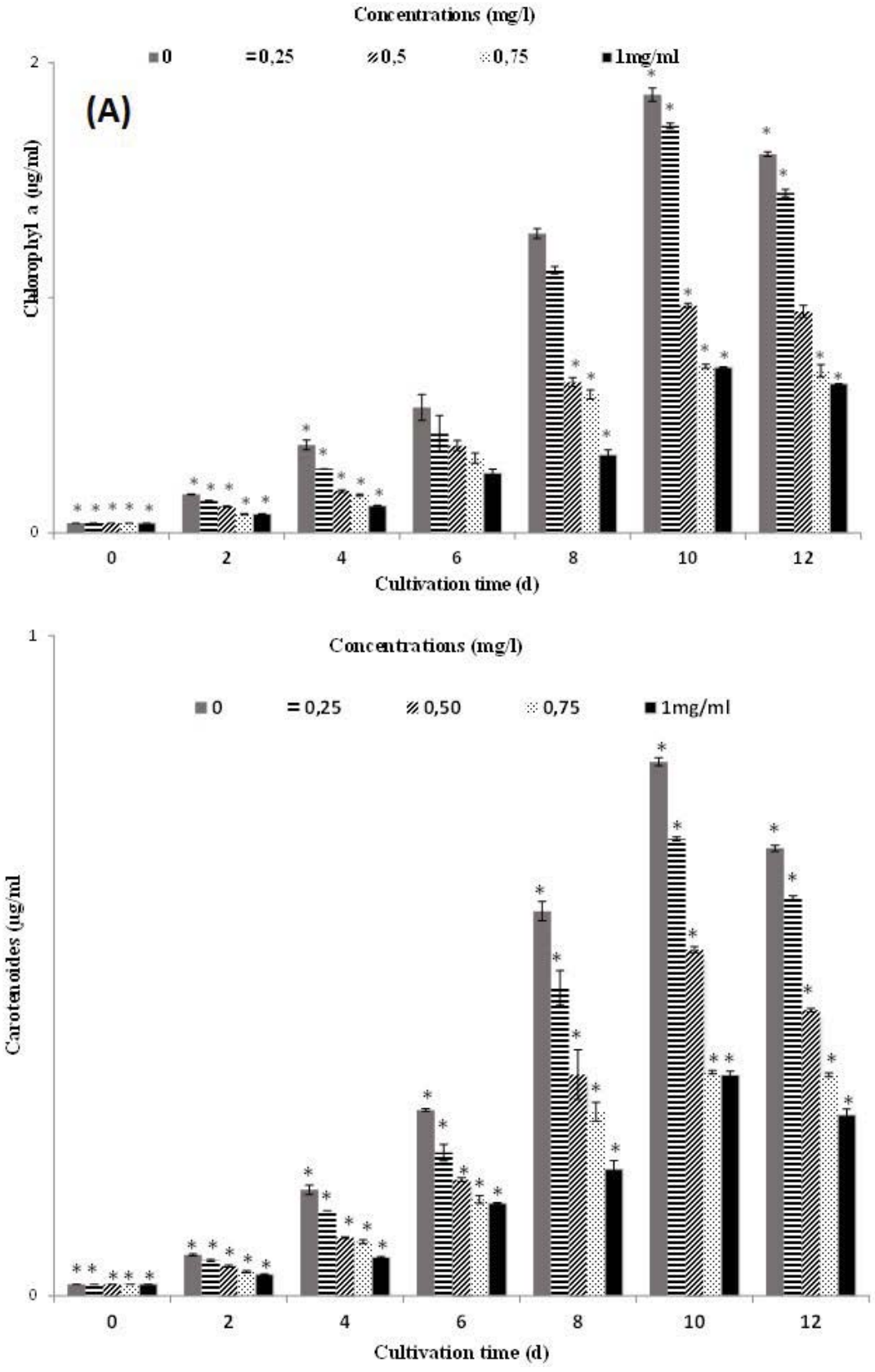

Fig. 4. Effects of AEOP on Chl-a (A) and carotenoids (B) in *Microcystis aeruginosa* cultures, respectively. Each value is the mean ± SD of three replicates, a, $p < 0.05$ indicate significant differences compared to the untreated culture (ANOVA two-way).

Table 2
Total phenolic, total flavonoids, amounts in AEOP and correlations between all amounts and inhibition rates of the three high concentration (0.5%, 0.75%, and 1%) after 10 d of exposure

	TP[a]	TF[b]
Concentrations	803 ± 0.03	3,690 ± 0.49
Coefficient of correlation	0.95	0.93

[a]µg gallic acid equivalent/mL aqueous extract;
[b]µg catechin equivalent/mL aqueous extract.

4. Discussion

This study is the first report of the anti-cyanobacterial activity of *O. pes-caprae* L. on *M. aeruginosa*. As is obtained, AEOP act negatively on the *M. aeruginosa* growth where the inhibitory effect appeared dose dependent (Fig. 1). The highest inhibition rate (IR) exceeds 74% on day 4 of experimentation; and it was achieved (86%) on 10-d under the highest concentrations of extract (1 mg/mL) (Table 1). Thus, the inhibition rates are confirmed by very powerful IC50 and IC90 values (0.52 and 0.94 mg/mL), respectively (Fig. 2 and Table 1). This strong inhibition demonstrated the high anti-cyanobacterial potential of AEOP on *M. aeruginosa*. The obtained results remain globally similar to those observed in other previous works that studied certain plants: *Ailanthus altissima* (66.3%–91.8%) on 5 d [37], *Thalia dealbata* (92.7%) on 7 d [38], *Nymphaea tetragona*, *Typha orientalis*, *Nelumbo nucifera* and *Iris wilsonii*, (75%–82%) during 19 d [33]. Tebaa et al. [15–17] showed that the growth of *M. aeruginosa* was effectively inhibited by aqueous extracts, with strongest inhibition rates for *Thymus satureioides*, *Achillea ageratum*, *Artemisia herba-alba*, *Origanum compactum* (IR values between 88% and 95%) after 8 d.

Moreover, in our experimental study, the growth inhibition is amply supported by the decrease of the two photosynthetic pigments (Chl-a and carotenoids), as well as by morphological changes in treatments groups (Figs. 3 and 4). The growth inhibition accompanied by photosynthetic pigments reduction and morphological changes of *M. aeruginosa*, were mainly indicators of physiological alterations occurring in a stressful environment. Additionally, some studies specifically demonstrated the negative effect of the extracts on Chl-a content [15,16,39]. Their decrease demonstrates the disturbance of the photosynthesis, which affect the growth and reproduction of *M. aeruginosa* [40].

This inhibitory effect could be related to the potential allelochemicals released by *O. pes-caprae* L. In various works devoted to the research of the algicidal and allelopathic potentialities of plants, plant-derived polyphenolics were the most common allelochemicals in HABs control [17,39,41]. The phytochemical investigation of extracts allowed the identification of several compounds. They are mainly flavonoids, glycosides, terpenoids, saponins and several phenolic acids [33,40,42].

The Phytochemical characterization of *Oxalis pes-caprae* aqueous and organic extracts indicated that the main chemical compounds of this plant are luteolin and apigenin flavonoid derivatives [43], and several common phenolic acids and phenols and their derivatives such as the coumaric acid, cinnamic acid, benzoic acid, methoxyphenol and hydroxyethyl phenol [18,26,44,45]. The most of these bioactive compounds belong to the common allelochemicals and were known by their antioxidant and antimicrobial activities [18,32].

From he obtained results, the relatively high values of TPs and TFs seem to play a greater role in the inhibitory activity (Table 2). These results agreed with previous works showing the effect of TPs and TFs, in the *M. aeruginosa* inhibition [39,46,47].

In various previous studies, there is ample evidence that the allelochemical compounds inhibited the growth of the cell by altering both the physiological state and cellular structure [46,48]. Phenolic acids exhibit cell-permeability features because of their amphiphilic and lipophilic nature [49]. According to Wang et al. [50], p-coumaric acid and ferulic acid disrupted the cell membrane integrity of *M. aeruginosa*. Furthermore, reactive oxygen species (ROS) act on cell membranes during stressful situations by degrading unsaturated phospholipids, which increases the permeability of the membranes [37]. Thus, the perturbations of antioxidant defence system cause the inhibition of photosynthesis and oxygen evolution due to interactions with components of PS II [51], which ultimately induce the cell death [38].

5. Conclusion

This study demonstrates the AEOP plant's ability to suppress the growth of *M. aeruginosa*. This effect is dose-dependent. The highest inhibition rate (IR) exceeds 74% on day 4 of experimentation; and it was achieved (86%) on 10 d under the highest concentrations of extract (1 mg/mL). Thus, the inhibition rates are confirmed by very powerful IC50 and IC90 values (0.52 and 0.94 mg/mL), respectively. Furthermore, during the 12-d experimental period, all four-treatment groups (0.25–1 mg/mL) demonstrated a significant decrease in the content of chlorophyll-a and carotenoids compared to the control. TPs, TFs, characterized might be the main responsible allelochemicals.

Consequently, oxalis plant can be recommended in the treatment of the waters contaminated by *M. aeruginosa* blooms. Other approaches in the future will be required to identify the dominant and specific allelochemicals, as well as to study its potential effects on the aquatic ecosystems in its different dimensions.

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Conflict of interests

The authors declare no conflict of interests.

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