ALGICIDAL EFFECTS OF *ACHILLEA AGERATUM* L. AND *ORIGANUM COMPACTUM* BENTH. PLANT EXTRACTS ON GROWTH OF *MICROCYSTIS AERUGINOSA*

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Abstract. Leaf aqueous (LA) extracts of medicinal plants *Achillea ageratum* L. and *Origanum compactum* Benth. were tested to explore their potential algicidal effects on *Microcystis aeruginosa.* The growth of *M. aeruginosa* and cell morphology changes in response to LA extracts was investigated. The concentrations of total phenols, total flavonoids, and total tannins in LA extracts were analyzed to reveal the potential allelochemical compounds. The results demonstrate that both *A. ageratum* and *O. compactum* LA extracts inhibit *M. aeruginosa* growth in a concentration-dependent way. For both LA extracts, the highest inhibition rate exceeded 80% since the fifth day of exposure. The induction of inhibition effects was translated by a decrease in photosynthetic pigments (chlorophyll-a and carotenoids) and with other morphological modifications. Our results illustrate that both *A. ageratum* and *O. Compactum* LA extracts can offer an effective solution for control of *Microcystis* blooms, and these are recommended in the restoration of aquatic environments contaminated by these types of algal blooms.
Keywords: *Achillea ageratum*; *Origanum compactum*; *algicidal effects; M. aeruginosa; inhibition; algal control*

Introduction

Harmful Cyanobacterial blooms in eutrophic aquatic ecosystems are a frequent phenomenon worldwide. *Microcystis* are the most common bloom-forming cyanobacteria (Mangilia et al., 2010; Douma et al., 2016). These are able to produce hepatotoxins (microcystins) (Wiegand and Pflugmacher, 2005; Douma et al., 2017). Consequently, they are responsible for the intoxication of many organisms and pose adverse effects on water quality and agricultural products destined for animal and human consumption (Mangilia et al., 2010).

The widely used conventional methods to reduce algal biomass are centrifugation, filtration, dissolved air flotation, and flocculation (Liu et al., 2013). These measures involve high costs and may induce secondary pollution. Biological control has emerged as an economic and ecofriendly alternative (Park et al., 2009). Laboratory experiments involving several aquatic plants such as *Myriophyllum spicatum*, *Najas minor* (Zhang et al., 2014), *Stratiotes aloides*, and *Sagittaria trifolia* (Li et al., 2016) have demonstrated inhibitory effects on cyanobacterial biomasses. On the other hand, the algicidal properties of terrestrial plants received less research attention, except for rice hull (Park et al., 2009), barley straw (Ball et al., 2001), mandarin skin and banana peel (Chen et

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al., 2012), and *Ailanthus altissima* tree bark (Meng et al., 2015). In addition, the application of terrestrial medicinal plants for the biological control of invasive or harmful algal blooms remains very limited. However, several species have shown positive bioactivity and demonstrated antioxidant, antibacterial, antifungal, antiviral, and anti-insecticidal potentials (El Bouzidi et al., 2011; Dutra et al., 2016). Yi et al. (2011) have reported the algicidal activities of several Chinese medicinal plant species, including *Salvia miltiorrhiza*, *Acorus tatarinowii*, *Polygonumcuspidatum*, *Phellodendrona murense*, and *Crataegus pinnatifida*. Ye et al. (2014) showed that aqueous extracts of *Phellodendri chinensis* and *Scutellaria baicalensis* have a potential to control *Microcystis* growth.

According to phytochemical characterization, the most important bioactive constituents of medicinal plants are phenolic compounds, fatty acids, and alkaloids (Yang et al., 2015). Among all polyphenols, flavonoids and tannins exhibited an intensive inhibitory effect on *M. aeruginosa* (Chen et al., 2012).

Medicinal flora is very common in the Mediterranean area. Countries such as Morocco have a vast biodiversity of medicinal plants, which have the feasibility to be cultured (El Bouzidi et al., 2011). Among these plants, *A. ageratum* and *O. compactum* are of particular interest due to their pharmacological properties and bioactive products they contain (El Babili et al., 2011; Zhang et al., 2014).

A. ageratum is an *Asteraceae* medicinal shrub. It has demonstrated antibacterial (De la Puerta et al., 1996), antifungal (El Bouzidi et al., 2011), anti-inflammatory, analgesic, and antipyretic activities (Gómez et al., 1999). *O. compactum* is a Lamiaceae herbaceous plant native to the Mediterranean region. Previous pharmacological studies have reported their antioxidant and antimalarial activities (El Babili et al., 2011), and also antimicrobial properties against many bacteria and fungi that cause human infections (Charai et al., 1996).

The aim of this study was to assess the algicidal effects of *A. ageratum* and *O. compactum* on toxic bloom-forming *Microcystis aeruginosa*. The experiments focused on (i) growth inhibition, (ii) morphological and physiological changes, and (iii) characterization of total polyphenols (TPs), total flavonoids (TFs), and total tannins (TTs) as potential allelochemicals. To our knowledge, this is the first report of the algicidal effects of these two medicinal plants.

Materials and methods

Materials

M. aeruginosa was isolated from the eutrophic Lalla Takerkoust reservoir (31°21'36" N; 8°7'48" W), Morocco, in October 2015. Using Z8 medium, *Microcystis* was cultured in laboratory at 26±2°C under light intensity of 65 $\mu Em^{-2}S^{-1}$, with a light/dark cycle of 15h/9h. The aerial parts of *A. ageratum* and *O. compactum*, collected in April 2016 from two locations (Demnat and Ourika in High Atlas of Marrakesh area, respectively), were washed under tap water and then rinsed several times with distilled water to remove debris and epiphytic microbes.

Preparation of LA extracts

LA extracts of *A. ageratum* and *O. Compactum* were prepared according to the methods described by Ball et al. (2001), slightly modified by Li et al. (2016). Briefly,

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10 g was cut into small pieces in 100 ml distilled water under agitation (37°C; 40 min). The solution was filtered through a glass fiber paper (Whatman GF/C, 0.22 µm). Then, the filtrate was adjusted with sterilized distilled water to 100 ml and maintained at 4°C as an aqueous extract.

Determination of TPs, TFs, and TTs in LA extracts

The concentration of TPs was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Briefly, 0.5 ml of the extract was added to 0.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) in water, and then 0.5 ml sodium carbonate solution (20% w/v) was added. The mixture was left for 1 h at room temperature and absorbance was measured at 760 nm.

The concentration of TFs was determined using the method described by Kim et al. (2013). Briefly, 500 µl of aqueous extract was mixed with 500 µl distilled water. Then 150 µl sodium nitrite solution (5%) was added, followed by 150 µl aluminum chloride solution (10%) after 5 min. Test tubes were incubated for 11 min at ambient temperature, and then 500 µl sodium hydroxide (1 M) was added. The mixture was vortexed and absorbance was determined at 510 nm.

The concentration of TTs was determined using the Folin-Denis test described by Salunkhe et al. (1990). Briefly, 1 ml of the aqueous extract was added to 75 ml distilled water, and then 5 ml Folin Denis reactif (Sigma-Aldrich) solution and 10 ml sodium carbonate solution were added. The mixture was vortexed and absorbance was determined after 30 mn at 760 nm.

Experimental design

Six concentrations (0% control, 0.1%, 0.25%, 0.5%, 0.75%, 1%; V/V) of *A. ageratum and O. compactum* LA extracts were prepared in six groups of Erlenmeyer flasks (500 mL) containing Z8 medium to a final volume (300 ml). Each flask was inoculated by a volume of *M. aeruginosa* in exponential growth phase to make an initial density of 1–2 × 10^6 cell/ml. The cultures were incubated at 26±2°C, illuminated in 15h/9h light-dark cycle with fluorescent tubes (65 µ$Em^{-2}S^{-1}$) for 8 days. All the experiments were conducted in triplicates.

Biomass estimation

The growth of *Microcystis aeruginosa* under different treatment concentrations was quantified every day using a Malassez hemocytometer. Inhibitory rate (IR) of *M. aeruginosa* growth was determined according to the following equation: IR (%) = (N_0–N/N_0×100, where N_0 and N (cells/ml) are cell density in treatment and control cultures, respectively.

Pigments determination

Chlorophyll-a and carotenoid pigments were extracted with 95% ethanol at 4°C for 48 h and then determined using a UV spectrophotometer (Carré 50 Scan) at 470, 649, 665 nm. Pigments were calculated according to the Lichtentaler and Wellburn method (1983). The following formulas were used to calculate their concentrations:

$$\text{Chlorophyll-a} = 13.95 \times OD665 – 6.88 \times OD649;$$

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$$\text{Carotenoids} = [(1000 \times OD470) - (2.05 \times \text{chlorophyll-a})] / 2$$

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS v20.0 (Windows 2010). One-way ANOVA and Tukey's test were used to test differences between exposure concentrations and control at $p = 0.05$. Correlation coefficients were calculated between cellular density and concentrations of PTs, FTs, and TTs.

Results

Effects on growth of M. aeruginosa

The inhibitory effects of both *A. ageratum* and *O. compactum* LA extracts, at different concentrations, on *M. aeruginosa* cultures are shown in *Figure 1A* and *B*. In contrast to the control group, the cell biomass of *M. aeruginosa* in all five tested concentrations (0.1%, 0.25%, 0.5%, 0.75%, and 1%; V/V) was significantly reduced during the 8-day test period ($p<0.05$). The reduction was more remarkable for the three highest concentrations, especially during the last days (*Fig. 1A-B*). Under *A. ageratum* and *O. compactum* LA extracts, *Microcystis* growth appeared to have strongly reduced at 0.5–1% and 1% concentrations, respectively. At these concentrations, the inhibitory rates exceeded 80% since the fifth day and reached more than 90% during the last day (*Table 1*).

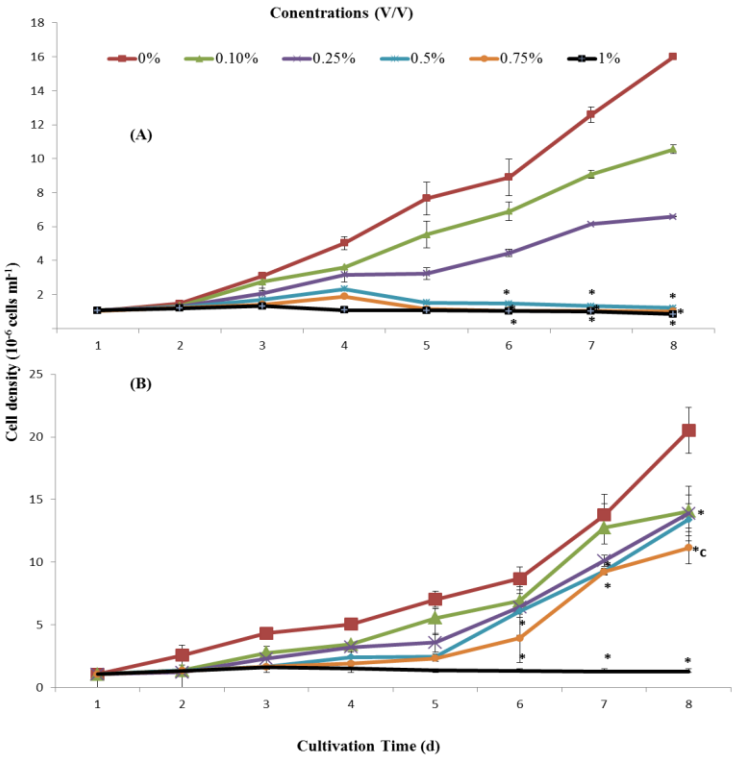

Figure 1. *Effects of different concentrations of A. ageratum (A) and O. compactum (B) LA extracts on Microcystis aeruginosa growth. Errors bars represent standard deviation (n=3). *p<0.05 indicates significant difference compared to controls.*

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Table 1. *Inhibitory effects of A. ageratum and O. Compactum aqueous extracts on M. aeruginosa growth.*

	PT [1]	FT [2]	TT [3]
plant	*A. ageratum*		
Concentrations	79,19 ± 8,37	28,17 ± 5,5	0,08± 0 ,005
Coefficient of correlation	-0,044	**0,518**	-0,803
plant	*O. compactum*		
Concentrations	69,03±5,99	23,64±13,69	0,03±0,005
Coefficient of correlation	**0,994**	-0,285	**0,86**

([1]): µg gallic acid equivalent ml^{-1}aqeous extract, ([2]): µg catechin equivalent/ml aqueous extract, ([3]): µg tannic acid equivalents ml^{-1}aqeous extract

Morphological changes in M. aeruginosa

The morphological changes in *M. aeruginosa* cultures under treatments were photographed and presented in *Fig. 2*. In the control group, *M. aeruginosa* colonies were clearly structured with regular surfaces. Their cell forms were rounded, clearly vacuoled, and pigmented, with cell diameter 2.8–3.5 µm by the end of treatment period. However, under high concentrations (0.75% and 1%), *M. aeruginosa* colonies lost their regular form, becoming floating aggregates (2.5–3.4 µm cell diameter) during the first days of culture. Then, for a heterogeneous culture with planktonic cells (1.1–1.5 µm) and sediment mass with anaform cells which the observed cells were destroyed and shrinking cell surface, especially, in the end of the test period (*Fig. 2*).

Effects on pigments as physiological indicators

For both *A. ageratum* and *O. compactum*, two photosynthetic pigments were measured (chlorophyll-a and carotenoids) that would act as physiological indicators. For the control, the chlorophyll-a and carotenoids concentrations were dose-dependent (*Fig. 3*). These decreased negatively with the augmentation of extract concentrations. For *O. compactum*, a significant difference in concentrations between the treatment and control groups was observed ($p<0.05$) (*Fig. 3A2–B2*). The pigments appeared to be strongly inhibited at 1% concentration since the sixth day. For *A. ageratum*, the concentrations 0.5%, 0.75%, and 1% seemed to be clearly inhibitory since the fifth day (*Fig. 3A1–B1*).

Phytochemical characterization

A. ageratum demonstrated higher values of TPs, TFs, and TTs compared to *O. compactum*. In addition, a significant correlation between the IRs of three high

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concentrations (0%, 5%, 1%) and TFs concentrations (coef>0,5) for *A. ageratum* , and between those of TPs and TTs concentrations (coef> 0,8) for *O. compactum* (*Table 2*).

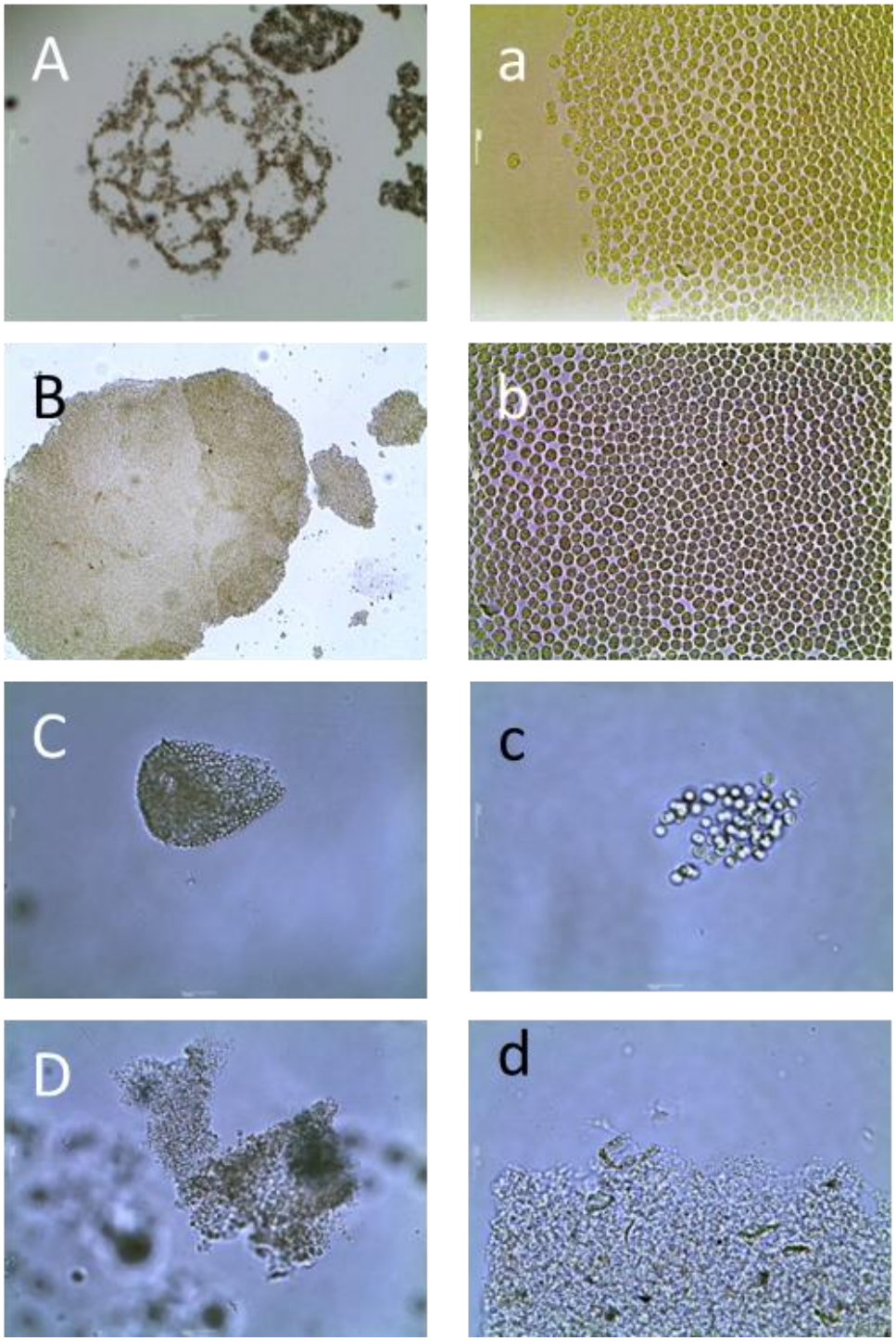

Figure 2. *Modification in M. aeruginosa colonies (A–D)(Gr. ×40) and cells (a–d)(Gr. ×1000) under treatments. (A) M. aeruginosa in normal growth (control), (B) formation of flotants and aggregate colonies, (C) colonies less vacuolated and breaking down, (D) sediment cells, completely devacuolated and decomposed.*

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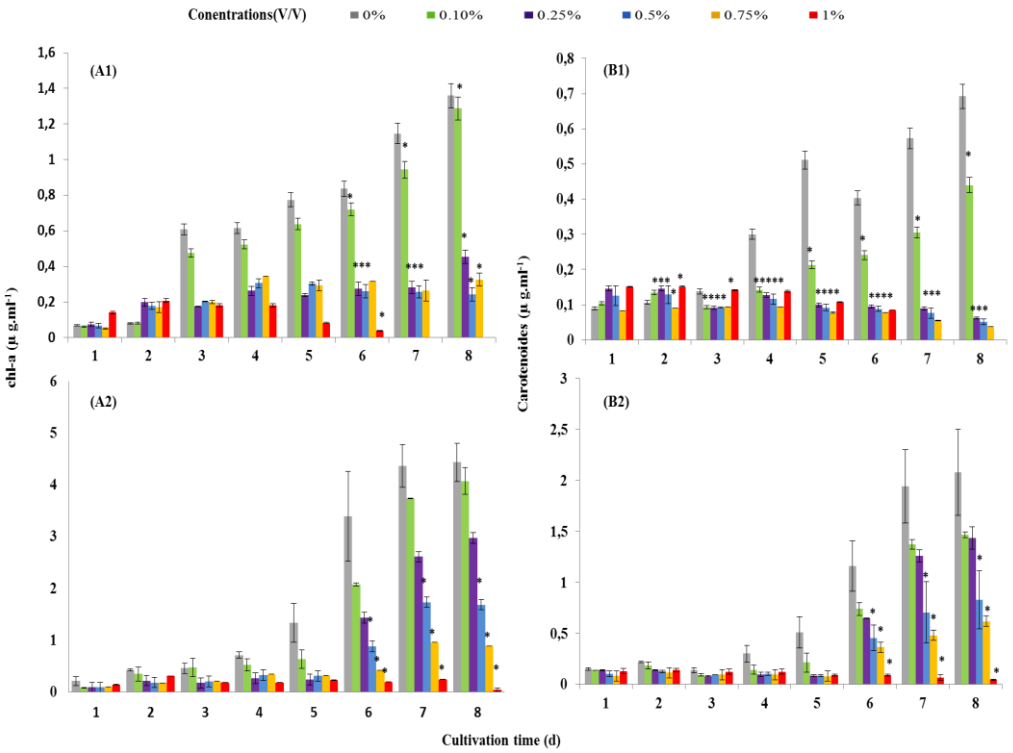

Figure 3. *Effects of A. ageratum (A1, B1) and O. compactum (A2, B2) LA extracts on chlorophyll-a and carotenoids in M. aeruginosa cultures. Data are means + SD (n=3). *p <0.05 indicates significant differences compared to controls.*

Table 2. *TP, TF, and TT amounts in different A. ageratum and O. compactum LA extracts; and correlations between all amounts and IRs of three high concentrations (0.5%, 0.75 %, 1%) after 8 days of exposure.*

	Cultivation Time (d)	1	2	3	4	5	6	7	8
	Concentrations (%)	Inhibitory rate (%)							
A. ageratum	0.1	-1	9	11	28	28	23	28	34
	0.25	-1	12	34	37	58	50	51	59
	0.5	1	15	46	**54**	80	84	89	92
	0.75	0	16	**55**	62	85	88	92	94
	1	-1	19	**57**	79	86	88	92	95
O. compactum	0.1	-1	48	36	32	21	20	7	31
	0.25	-1	53	47	36	49	26	27	32
	0.5	1	48	62	52	65	30	33	35
	0.75	0	48	62	62	67	55	33	46
	1	-1	49	62	70	80	85	91	94

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Discussion

Both *A. ageratum* and *O. compactum* LA extracts acted negatively on *M. aeruginosa* growth (*Fig. 1*). This inhibitory effect appeared to be dose-dependent.

A. ageratum showed high IRs with three high concentrations (0.5–1%) compared to *O. compactum* (only 1%). For these last concentrations, since the fifth day, a marked IRs were observed for *A. ageratum and O. compactum*, exceeding 90% at the end of the experiment for each plant. This finding can be used for the optimization of extracts for a recommended treatment, not envisaging a complete reduction of *M. aeruginosa* biomass.

The maximum IRs of high concentrations remain similar to those observed in previous works. *Sagittaria trifolia* aqueous extract showed inhibition on *M. aeruginosa* growth with IRs exceeding 70% after 6 days (Li et al., 2016). Ye et al. (2014) studied the effects of several Chinese herbal aqueous extracts on *M. aeruginosa* and found that the maximum IRs were 51–98% after 10 days. Thus, the inhibitory effect of our aqueous extracts is similar to that reported in previous works (Chen et al., 2012; Zhang et al., 2014; Meng et al., 2015; Li et al., 2016).

The inhibition of photosynthesis is a prominent mode of action for many allelopathically active compounds (Gross, 2009). The effects of plant extract on chlorophyll-a and carotenoids are shown in *Figure 3*. Some studies have shown the negative effects of the extracts on chlorophyll-a (Meng et al., 2015). The disturbance in photosynthesis thus affects the growth and reproduction of *M. aeruginosa* (Li et al., 2016).

The phytochemical characterization of the aqueous extracts shows important amounts of TPs, TFs, and TTs (*Table 2*). FTs, and PTs and TTs contribute to the inhibitory action of *A. ageratum* and *O. compactum*, respectively. These results agree with previous works showing the effects of PTs, FTs, and TTs on *M. aeruginosa* (Yan et al., 2011; Chen et al., 2012). Furthermore, other compounds have been identified during the characterization of *O. compactum* (carvacol and thymol; El Babili et al., 2011), and *A. ageratum* (artemisyl acetate; El Bouzidi et al., 2011), which contribute to the algicidal process.

As concluded by previous studies, the allelochemical products inhibit cell growth by attacking the reactive oxygen species on cell membranes, degrading the unsaturated phospholipids, and consequently increasing their permeability, leading to a disruption of cell organization (Meng et al., 2015). Under optimum stress, the perturbations of the antioxidant defense system lead to the inhibition of photosynthesis and oxygen evolution through interactions with the components of PS II (Einhellig, 1995) and finally slowing down cell death.

In our study, growth inhibition was well demonstrated by morphological changes in treatment groups (*Fig. 2*). In addition, the cells in treatment groups decreased in size and lost a significant volume of vacuoles (*Fig. 2*). Some have completely destroyed and sedimented; others resisted by attempting to escape through a floating agglomeration. The aggregation trend of cells in high concentrations of extracts appeared to be a response to stress conditions. The growth inhibition accompanied by photosynthetic pigments and morphological changes in *M. aeruginosa* are indicators of physiological alterations under a stress environment (Yan et al., 2014; Meng et al., 2015; Li et al., 2016).

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Conclusions

The present work demonstrates the inhibitory effect of LA extracts of *A. ageratum and O. compactum* on *M. aeruginosa* growth. In addition to their antimicrobial, anti-inflammatory, analgesic, and antipyretic potentialities; and according to our results, these two medicinal plants can be recommended as an ecofreindly solution to restore the aquatic environments contaminated by *M. aeruginosa* blooms. Other future approaches will be necessary to specify and optimize the allelochemical agents, as well as the conditions of interactions with other potential components, especially others pathogens microbes, in the aquatic ecosystem.

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Conflict of Interest. The authors declare no conflict of interest.

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