ASSESSMENT OF THE POTENTIALLY ALGICIDAL EFFECTS OF *THYMUS SATUREIOIDES COSS.* AND *ARTEMISIA HERBA ALBA* L. AGAINST *MICROCYSTIS AERUGINOSA*

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(Received 18th Sep 2017; accepted 15th Jan 2018)

Abstract. In search of an ecofriendly algaecide, aqueous extracts of two medicinal plants, *Thymus satureioides* Coss. and *Artemisia herba alba* L., were assessed for antialgal activity against *Microcystis aeruginosa.* An experiment was designed using five treatments (1%, 0.75%, 0.5%,0.25% and 0.1%). The growth of *M. aeruginosa*, morphological modifications, and photosynthetic pigments (chlorophyll a and carotenoids) on exposure to the extracts were explored. Also, phytochemical parameters in the extracts were analyzed to reveal the potential allelochemical compounds.
The results showed that both *T. satureioides* and *A. herba alba* extracts inhibited the growth of *M. aeruginosa* in a concentration-dependent way. After 8 days of treatment, the highest inhibition rates reached were 95%, 93% and 88.58%, and for *T. satureioides* and *A. herba alba* aqueous extracts, respectively. Chlorophyll a and carotenoid concentrations in cultures decreased especially in the 1% treatment group. Several morphological changes were observed in the treatment group compared to the controls. It was concluded that *M. aeruginosa* growth was suppressed by the potentially allelochemical compounds and probably by other allelochemical substances in aqueous extracts. Our results illustrated that both *T. satureioides and A. herba alba* extracts are able to control *Microcystis* blooms, and these may be recommended as a remedy for contamination of water bodies by harmful blooms.
Keywords: *Microcystis aeruginosa, Thymus satureioides Coss., Artemisia herba alba L., algicides, morphological and physiological changes, inhibitory effect*

Introduction

Cyanobacterial blooms are a major environmental problem in water bodies worldwide (Xiao et al., 2010). Their frequent appearance has been considered a consequence of eutrophication (Codd and Bell, 1985). *Microcystis spp.* are the most common bloom-forming cyanobacteria in eutrophic freshwaters (Douma et al., 2016; 2017). A break-out of *Microcystis* blooms releases cyanotoxins which can impair water quality, reduce productivity, decrease biodiversity, as well as causes severe illnesses to animals and humans (Rondel et al., 2008).

To control the adverse effects of cyanobacterial blooms, several strategies have been developed, often including the application of physical and chemical agents (Jeong et al., 2000). None of these methods can specifically control harmful algal blooms without causing secondary pollution or affecting aquatic organisms (Zhang et al., 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1601_903912

Therefore, there is an urgent need to develop safe algaecides for controlling these harmful algal blooms.

Recent researches have exploited plants as an alternative to chemical agents (Chen et al., 2012; Zhou et al., 2014; Meng et al., 2015; Li et al., 2016). Numerous studies have reported the inhibition of cyanobacterial biomasses by several aquatic plants such as *Potamogeton maackianus* (Wu et al., 2007), *Myriophyllum spicatum* (Zhu et al., 2010), *Sagittariatrifolia* (Li et al., 2016), and *Eichhornia crassipes* (Zhou et al., 2014). To explore the potential of terrestrial plant extracts for controlling cyanobacteria provide a new guide for developing algicide (Shao et al., 2013).

Terrestrial medicinal plants have shown positive bioactivity and have demonstrated antibacterial, antifungal, antiviral, and insecticidal properties. The most important bioactive constituents of plants are phenolic compounds (Chen et al., 2012; Zhang et al., 2013). However, there are few reports on the use of medicinal plants to control *M. aeruginosa*, notably *Achillea ageratum L* and *Origanum compactum Benth* (Tebaa et al., 2017*); Portulaca oleracea* (Wang et al., 2016); *Ailanthus altissima* (Meng et al., 2015); *Shaddock Peel, Pomegranate Peel* (Wang et al., 2015).

In the Mediterranean area, medicinal plants are endemic (Bellakhdar, 1997). *T. satureioides* and *A. herba alba* are two well-known Moroccan medicinal plants (Ismaili et al., 2004; Bezza et al., 2010). *Thymus* is a Lamiaceae herbaceous species. It is a well-known aromatic perennial herb used extensively throughout the Mediterranean basin (Ismaili et al., 2004). A wide range of biological and pharmacological properties, including antiseptic, anthelmintic, and anti-inflammatory activities, have been reported for these species (Elhabazi et al., 2008; Ichrak et al., 2011). Several reports have demonstrated their strong antimicrobial, insecticidal, antifungal, antiviral, and antioxidant properties due to the rich content of phenolic compounds such as thymol and carvacrol (Jamali et al., 2013).

A. herba alba, belonging to the family Asteraseae, has been used by the local population as a medicinal plant as well as a flavoring additive in tea and coffee (Bezza et al., 2010). It has been extensively used to treat stomach disorders, hepatitis, and certain poisoning cases, as well as an antitumor, antispasmodic, antiseptic, antigenotoxic, antidiabetic, and antibacterial agent (Bezza et al., 2010; Mighri et al., 2010).

Phytochemical components and secondary metabolites such as flavonoids and others phenolic compounds present in these make them attractive for medicinal uses (Mohamed et al., 2010). However, little information is available on the use of medicinal plants for the treatment of bloom-forming cyanobacterium *M. aeruginosa* (Ni et al., 2011; 2012, Tebaa et al., 2017).

The aim of this study was to assess the potentially algicidal effects of *T. satureioides* and *A. herba alba* plants against *M. aeruginosa* with special attention to their photosynthetic pigments and allelochemicals compounds. To the best of our knowledge, this is the first report to study the impact of these two Moroccan specimens on *Microcystis spp.*

Materials and methods

Algal and floral materials

M. aeruginosa was isolated from the Moroccan eutrophic reservoir Lalla Takerkoust (31°21′36″ N; 8°7′48″ W) in October 2015. The isolated strain was grown in Z8 medium at 26±2°C under light intensity of 4000 $lx.m^{-2}$ s^{-1}, with a light/dark cycle of

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15 h/9 h. The cyanobacteria were cultivated to an exponential growth phase (1–2 × 10^6 cells/ml). The two medicinal plants *T. satureioides* and *A. herba alba* were collected in May 2016 from two locations, Oukaimden and Tahanaout (High Atlas, Marrakesh area). Specimens were botanically identified, confirmed, and deposited in the Herbarium Mark (Faculty of Sciences Semlalia, Cadi Ayyad University of Marrakesh). The aerial plant parts were rinsed several times with distilled water to remove debris and epiphytic microbes. Leaves were separated and conserved for further use.

Preparation of aqueous extracts

The aqueous extraction of plants was carried out according to the method described by Ball et al. (2001), slightly modified by Li et al. (2016). Briefly, 10g of leaves were cut into tiny pieces and placed in 100 ml distilled water under agitation (37°C; 48 h). The macerate was filtered through a filter paper (Whatman GF/C, 0.22 μm). Then the filtrate was adjusted with distilled water to 100 ml and kept at 4°C as aqueous extract.

Algal bioassay

Experimental cultures were carried out in Erlenmeyer flask 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 (1–2 × 106 cells/ml). Microcystis cultures were exposed to different concentrations (0% [control], 0.1%, 0.25%, 0.5%, 0.75%, 1%) of aqueous extract. The cultures were incubated in a culture room at 26±2°C, illuminated in 15 h/9 h light-dark cycle with fluorescent tubes (4000 $lx.m^{-2}$ s^{-1}). All experiments were carried out in triplicates.

Biomass estimation

M. aeruginosa growth under treatment was quantified through a daily sampling (every 24 h) of a constant volume of culture (2 ml each replicate) using Malassez hemocytometer. Growth inhibitory rate (IR) of *M. aeruginosa* under different concentrations was determined according to *Equation 1:*

$$\mathrm{IR}(\%) = (\mathrm{N0} - \mathrm{N} / \mathrm{N0} \times 100, \qquad \text{(Eq.1)}$$

where N0 and N (cells/ml) are cell density in treatment and control cultures, respectively.

Pigment quantification

Chlorophyll-a and carotenoid concentrations were extracted in the dark with 95% ethanol at 4°C for 48 h, and measured using a UV spectrophotometer (Carré 50 Scan) at 470, 649 and 665 nm. Pigment quantification was calculated according to Lichthentaler and Wellburn (1983). The following formulas (*Eq. 2* and *3*) were used to calculate the concentrations:

$$\text{Chlorophyll-a} = 13.95 \times \mathrm{OD665} - 6.88 \times \mathrm{OD649} \qquad \text{(Eq.2)}$$

$$\text{Carotenoids} = \left(\left[\left(1000 \times \mathrm{OD470}\right) - \left(2.05\ \text{chlorophyll-a}\right)\right]\right) / 2 \qquad \text{(Eq.3)}$$

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Determination of Total phenolic (TPs) , Total flavonoids (TFs), Total tannins (TTs)

TP concentration was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The total phenolic compounds were expressed as µg gallic acid equivalents per milliliter of aqueous extract.

TF concentration was determined by the method described by Kim et al. (2003). Measurements were calibrated to a standard curve of prepared catechin (Fluka) and the results were expressed as µg catechin equivalents per milliliter of aqueous extract. TT content was determined using the Folin-Denis test described by Salunkh et al. (1990), with slight modification. This method quantifies both condensed and hydrolysable tannins. A calibration curve was obtained using a tannic acid solution and the results were expressed as µg tannic acid equivalent per milliliter of aqueous extract.

Statistical analysis

The statistical significance between control and treatment groups was confirmed by analysis of variance (ANOVA) using SPSS V20.0 Windows 2010. Two-way ANOVA and Tukey's test were used to test differences between exposure concentrations and control at $p = 0.05$.

Results

Inhibitory effects on M. aeruginosa

As shown in *Figure 1 A-B* and *Table 1*, both *T. satureioides* and *A. herba alba* extracts exhibited a marked inhibitory effect on *M. aeruginosa.* In contrast to the control group, the cell densities of *M. aeruginosa* at all three concentrations (0.5%, 0.75% and 1%) for *T. satureioides* and *A. herba alba* extracts, respectively, were significantly reduced during the 8-day test period ($p < 0.05$). Moreover, the inhibition rates were dose-dependent within 8 days. For both *T. satureioides coss* and *A. herba alba* extracts, the maximum inhibition of cell growth (94.46%, 94.60%, and 95.93%, and 88.58%, respectively) was achieved at 0.5%, 0.75% and 1%, and 1% concentrations, respectively. The results suggest that *T. satureioides* showed a strong inhibitory effect on algal cells, and the effective doses ranged from 0.5% to 1% (*Fig. 1 C-D*).

Table 1. *Total phenolic (TPs) , Total flavonoids (TFs), Total tannins (TTs) amounts in A. herba alba and T. satureioides LA extracts; and correlations between all amounts and IRs of the three high concentration (0.5, 0.75, 1%) after 8 days of exposure*

	PT[1]	**FT[2]**	**TT[3]**
Plant	***T. satureioides***		
Concentrations	285 ± 34.82	25.83 ±4	0.032± 0.002
Coefficient of correlation	-0.044	-0.458	**0.941**
Plant	***A. herba alba***		
Concentrations	290±19.8	37.61±0.66	0.024±0.005
Coefficient of correlation	-0.999	**0.536**	-0.215

[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

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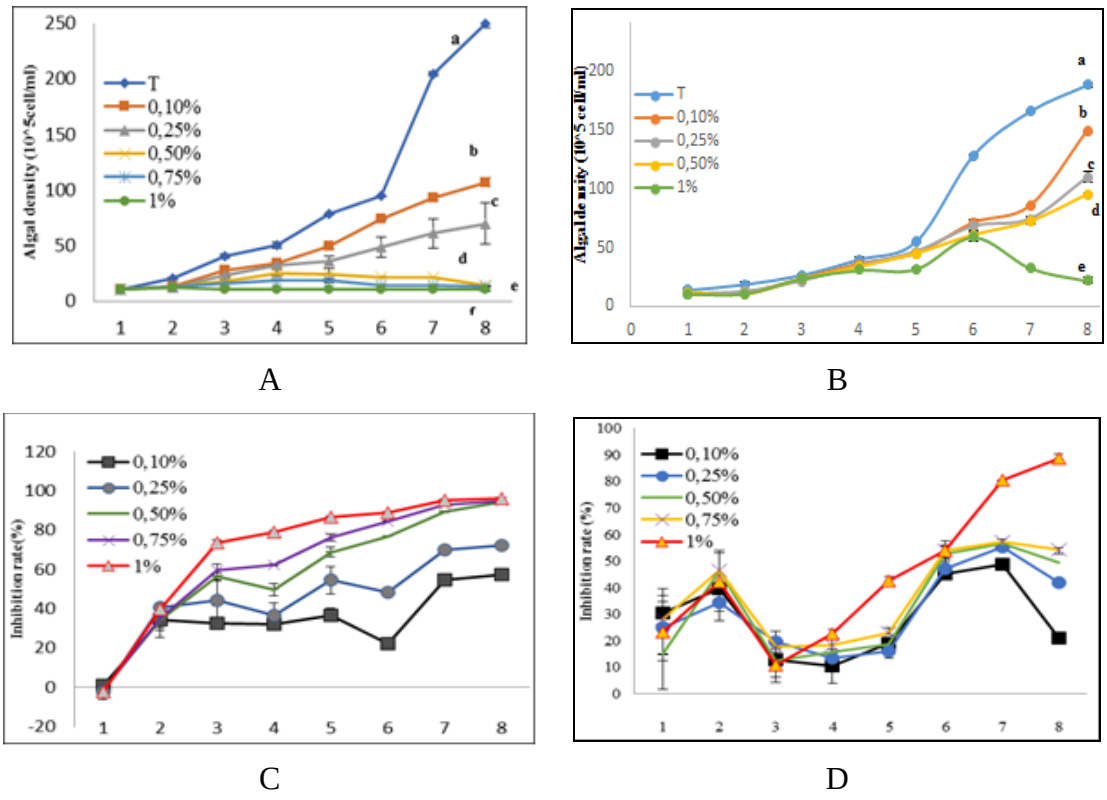

Figure 1. *Inhibitory effect of aqueous extracts of T. satureioides and A. herba alba on M. aeruginosa growth. A and B: density curves of T. satureioides, A. herba alba, respectively. C and D: IR inhibition rate curves of T. satureioides, A. herba alba, respectively. The values are means ± standard deviations calculated from different repetitions (n = 3). The value of p < 0.05 was considered significant. Letters a to f: groups not sharing the same letter are significantly different means*

Effects on photosynthetic pigments

The levels of two photosynthetic pigments (chlorophyll-a and carotenoids) were used as physiological indicators of the inhibition of *Microcystis*. In the control, the levels of both pigments increased as a function of tested concentrations (*Fig. 2*). Whereas in treatments, these globally decreased with the increase of concentrations. This demonstrated the significant difference between the treatment and control groups ($p < 0.05$) (*Fig. 2*). The levels of pigments appeared to be strongly inhibited at 95.93% and 88.58% concentrations of *T. satureioides* and *A. herba alba*, respectively.

Inhibitory mechanism of plant extracts on M. aeruginosa

According to the values of TPs, TFs, and TTs shown in *Table 1*, the concentrations in *T. satureioides* are higher than those in *A. herba alba*. As well, a significant correlation in IR was obtained between three significant concentrations (0.5–1%) with regard to TT concentrations (coef > 0.8) for *T. satureioides* sand with regard to TF concentrations (coef > 0.5) for *A. herba alba*.

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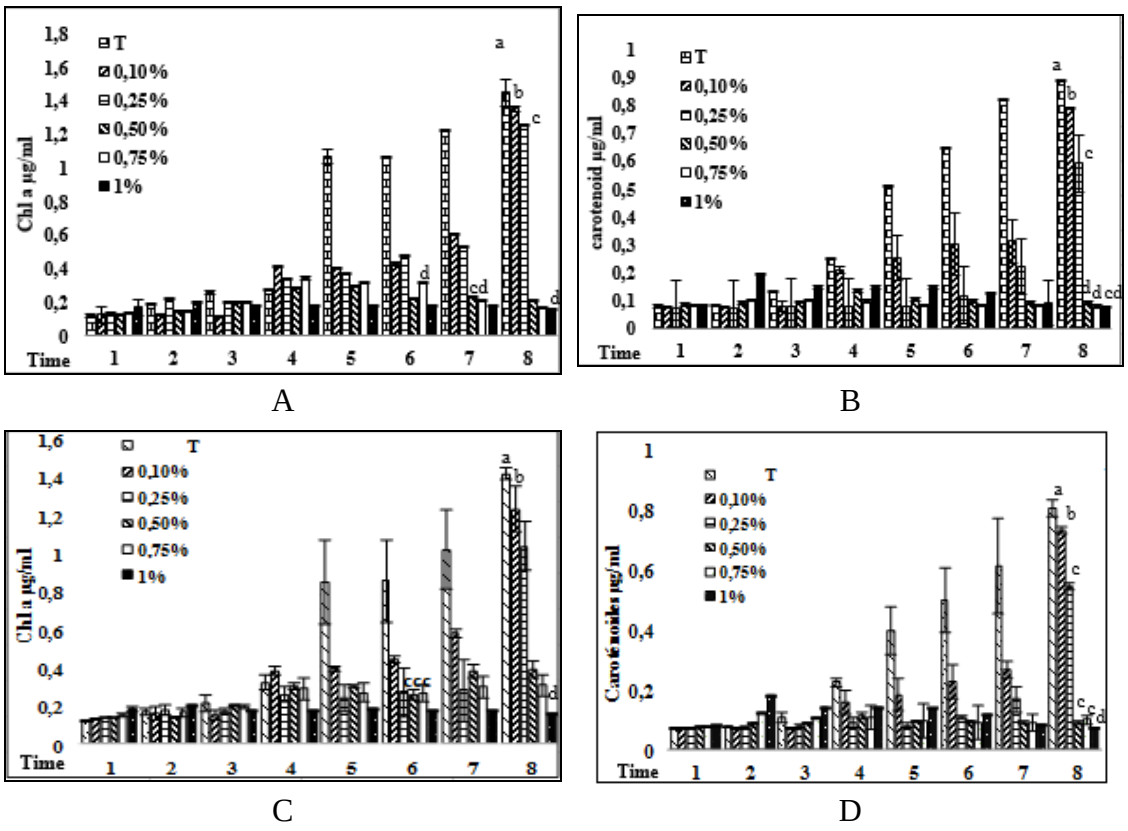

Figure 2. *Effect of aqueous extracts on photosynthetic pigments of M. aeruginosa. A and C: Contents of chlorophyll a of T. satureioide and A. herba alba, respectively. B and D: Carotenoid contents of T. satureioides and A. herba alba, respectively*

Discussion

According to this study, both *A. herba alba* and *T. saturoides* aqueous extracts had potential algicidal effects on *Microcystis* growth. They significantly reduced the biomass and photosynthetic pigments of *M. aeruginosa.*

After 7 days of treatment at 1% concentration, the maximum IRs of 95%, 93%, and 88.58% were reached using *T. satureioides* and *A. herba alba* aqueous extracts, respectively. Meng et al.(2015) demonstrated that *A. altissima* extracts showed an IR of 90% against *M. aeruginosa* while completely destroying the organelle responsible for photosynthesis in algal cells.

In a study by Li et al. (2016), *S. trifolia* tuber aqueous extract demonstrated the highest inhibition rate of 70% on *M. aeruginosa* after 6 days of treatment. Ye et al. (2014) studied several Chinese herb aqueous extracts on *M. aeruginosa* and obtained the maximum IRs in the range of 51–98% after 10 days. Besides, the lowest concentrations of aqueous extracts had only negligible inhibition effect on *M. aeruginosa* (Ye et al., 2014; Li et al., 2016). Similar trends were reported by Xiao et al. (2010).

On the other hand, *T. saturoides* at a concentration of 0.5% was sufficient to elicit a strong inhibition on the growth of *M. aeruginosa*. The difference between the present results and that of Nakai et al. (1999) might be explained by the importance of aerobic decomposition to generate more inhibitors for algal suppression (Li et al., 2016).

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For *A. herba alba*, only the concentration of 1% was significantly inhibitory compared to the controls, which suggests that the potentially bioactive substances inhibited the growth of *M. aeruginosa*. At all other concentrations (0.1%, 0.25%, 0.5%, 0.75%), the growth retardation of *M. aeruginosa* was dose-dependent.

Furthermore, by microscopic observations, the presence of green color signified the abundant growth of *M. aeruginosa* in control cultures, whereas under treatments, the cultures became transparent with yellow sediment at the bottom after 6 days of treatment with *A. herba alba* (1%) and *T. saturoides* (0.5%,0.75% and 1%) aqueous extracts (*Fig. 3*).

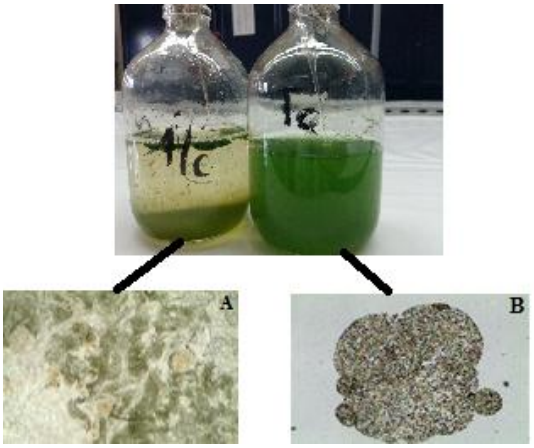

Figure 3. *Microscopic observation of the behavior of M. aeruginosa in the concentration groups 1% of T. satureioides aqueous extracts. A: (Gr. x 40) with sedimented cells, completely devacuolated and decomposed. B control group: (Gr. x 40); with M. aeruginosa in normal growth*

The results indicate that the extracts had a potential to accelerate *M. aeruginosa* cell lysis. These observations are in agreement with previous studies that investigated the cell morphology variations under treatments (Meng et al., 2015; Li et al., 2016). In our study, the photosynthetic pigments of M. *aeruginosa* were destroyed by aqueous extracts.

The antialgal allelochemicals reported in literature include fatty acids, polyphenols, terpenoids and polyethers. Previous studies have found that *Myriophyllum spicatum* releases four polyphenols and fatty acids that have an inhibitory effect on algae (Nakai et al., 2000).

In this study, tannins and flavonoids as potential allelochemicals made significant contribution to algal inhibition. These describe a group of phenolic compounds with a wide variety of allelochemical actions. Potential synergy between tannins and flavonoids may account for the maximum inhibition 95.93% ± 0.49 noted in *T. satureioides.*

Tannins have a toxic activity against filamentous fungi, yeasts, and bacteria, the antimicrobial activity of tannins could be due to their ability to form complexes with transport proteins (Scalbert, 1991). The inhibition of algal growth can be attributed to allelochemicals, including tannic acids. But because the correlation coefficient was less than 0.5 for *T. saturoides*, it is assumed that the phenolic compounds are one type among other secondary metabolites that cause algal inhibition (Chen et al., 2012).

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When investigating the antialgal activity of leaves of aquatic species *Iris wilsoni* on *Microcystis* sp., Whittaker and Feeny (1971) have shown that flavonoids, terpenoids, steroids, alkaloids, and organic cyanides are among allelochemicals to side polyphenolic compounds.

Chlorophyll-a and carotenoids are major pigments in microalgal photosynthetic systems (Yang et al., 2012). Their decrease shows the disturbance of photosynthesis affecting the growth and reproduction of *M. aeruginosa* (Li et al., 2016).

Allelopathic compounds behave like natural algicides; they often have multiple sites of action and various effects on the target organism (Ni et al., 2012). Some allelochemicals act by inhibiting photosynthesis, slowing down the growth of *M. aeruginosa.* They also attack the reactive oxygen species (ROS) on cell membranes by degrading unsaturated phospholipids and consequently increasing their permeability, leading to a disorder in cell organization (Meng et al., 2015).

The results of this study show that the use of these two plant extracts could bring great results in the biological control of harmful algae in aquatic ecosystems. However, further works will be needed to verify the application of these extracts in lakes for HAB inhibition. Also, it should be noted that the specific compounds responsible for such effects should be isolated and identified to explore the mechanism of inhibition in future studies and for field applications.

Conclusion

The present work demonstrated the inhibitory effect of aqueous extracts of *T. satureioides and A. herba alba* on *M. aeruginosa* growth. In addition to their antimicrobial, anti-inflammatory, analgesic, and antipyretic potentialities, these two medicinal plants can be used for ecofriendly restoration of aquatic environments contaminated by *Microcystis* blooms. Other future approaches will be necessary to optimize the allelochemical agents, as well as the conditions of interactions with other potential components, especially other pathogens, in the aquatic ecosystem.

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DOI: http://dx.doi.org/10.15666/aeer/1601_903912