ALGAE GROWTH INHIBITION BY AQUEOUS EXTRACTS FROM *ALTERNANTHERA PHILOXEROIDES* AND UNDERLYING MECHANISMS

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Abstract. Using *Microcystis aeruginosa* as a research receptor, the inhibitory effects of 0.3-1.1 g/L root, stem and leaf extracts from *Alternanthera philoxeroides* on algae growth and their underlying mechanisms were studied. The biomass, oxygen radical (O_2^-), malondialdehyde (MDA), nucleic acid, microcystin and polysaccharide contents were determined and cell morphological and structural variations were observed. Results showed that the extracts from *A. philoxeroides* significantly inhibited the growth of *M. aeruginosa* in a concentration-dependent manner. In particular, root extract had the strongest inhibition effect, followed by stem and leaf extract. The growth of more than 80% of all *M. aeruginosa* was inhibited after 96 h exposure to 1.1 g/L root extract. Besides, the damage degree of cells treated with root extract after 72 h was shown by the scanning electron microscope. With the accumulation of O_2^-, the contents of all physiological indicators increased with higher extract concentrations, except for MDA content, which decreased first after 24 h culture at 0.3 g/L treatment. In conclusion, *A. philoxeroides* had a certain ecological control capacity on *M. aeruginosa* growth by aggravating oxidative stress, destroying membrane permeability, changing cell structure and finally inducing plasmatorrhexis, but the specific action of allelochemicals it contained remained to be further explored.

Keywords: *harmful algal blooms, allelopathy, growth inhibition, peroxidation reaction, microcystin, polysaccharide*

Introduction

In recent years, intense agricultural production and living activities had led to a dramatic increase of N and P levels in water, which has caused severe eutrophication and frequent harmful algal blooms (HABs) (Lapointe et al., 2015). Among these, the most common is the occurrence of cyanobacteria cells over propagation whose dominant species include *Microcysis*, *Oscillatoria*, *Anabaenopsis*, *Cylindrospermopsis* etc. (Kaur et al., 2019). The end result not only seriously affects the biodiversity of aquatic ecosystem and the development of aquaculture, but also endangers human health (Carmichael and Boyer, 2015).

At present, the use of various techniques to restrain algal growth has been widely developed and applied. Physical and chemical processes mainly include filtration, ultrasonic treatment, ball clay flocculation and algaecide (copper sulfate and hydrogen peroxide), etc. (Greenfield et al., 2014; Han et al., 2019). Bioflocculation, algicidal

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bacteria, viruses, plant-derived compounds, fish and zooplankton are considered as cost-effective, safe, ecologically healthy emerging biotechnologies for the control of algal blooms (Mecina et al., 2017; Pal et al., 2020), especially the effects of allelochemicals identified from plants on algae have attached much attention (Herrera et al., 2019).

Plants can release chemicals into the biological community to promote or inhibit the growth and development of themselves and other surrounding organisms directly or indirectly (Uddin et al., 2017). Allelopathic chemicals have been sought out in an array of plants, like *Phragmitescommunis*, *Eichhornia crassipes* and *Pistia stratiotes* Linn. Besides, allelochemicals are secondary metabolites of biosynthesis and are easily decomposed. According to their different properties and synthetic pathways, they can be divided into phenolic acids, terpenoids, alkaloids, etc. (Zhao et al., 2019). Results have shown that phenolic acids (Zhang et al., 2010), linoleic acids (Ni et al., 2015), flavonoids and tannins (Tazart et al., 2019) can strongly restrain the growth of *M. aeruginosa*, and may become promising alternatives to algae control.

M. aeruginosa is a typical cyanobacterium with a global distribution range. The large area coverage of *M. aeruginosa* in eutrophication freshwater ecosystems tends to cause lack of oxygen in water and inhibit photosynthesis of aquatic plants (Rzymski et al., 2020). What is more severe is that it can produce and release microcystins (monocyclic seven-peptide compounds synthesized from multifunctional protein complex) into the environment, and then gradually accumulate along with the food chain, which mainly induce various liver disease as a threat to animal and human life by inhibiting the function of protein phosphatases 1 and 2A (Mecina et al., 2019). So it is essential and representative to explore superior strategies for managing the outbreak of *M. aeruginosa*.

A. philoxeroides is a pernicious invasive weed in China, first appeared in South America, and as an amphibious plant, the extraction and release of allelochemicals may provide more opportunities for algae treatments, which has aroused our interests (Prabakaran et al., 2019). Whenever it invades a strange environment, *A. philoxeroides* occupies the ecological niche quickly and becomes the dominant species in the community, leading to the decline of biodiversity and the destruction of ecological balance (Portela et al., 2020). It has been reported that *A. philoxeroides* has certain edible and medicinal value in Southeast Asia, but it is generally harmful in most areas with a poor utilization (Masoodi et al., 2013). The reasonable application of *A. philoxeroides* to algae elimination may weaken algae breed potential and improve its own usage rate at the same time to achieve mutual benefit. Many studies have confirmed the allelopathy of *A. philoxeroides* on other plants (*Zoysia matrella*, *Medicago sativa*, *Cichoriumintybus*, and *Avena sativa*), ethyl propionate has been found to be one of the main allelochemicals in root extract of *A. philoxeroides* due to its higher content, in addition, the common effect substances may also include diethyl phthalate, dibutyl phthalate etc. (Huang et al., 2017), and some experiments have begun to take notice of its inhibition effect on *M. aeruginosa*. The discussion was mainly focused on the effects on photosynthesis and antioxidant activities, and there was no relatively systematic research in the aspect of inhibition and stress response of algae. Moreover, the employ of plant-derived compounds has both positive and negative effects, the extraction of some plant-derived compounds involves organic solvents and poses environmental risks, and overuse may affect the biological survival of the original habitat. So it is needed to further evaluate the amount of input in practical application (Suzuki et al., 2020).

Hence, in our experiment, *M. aeruginosa* was selected as the research object, and the allelopathy effects on *M. aeruginosa* were investigated with different concentrations of

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A. philoxeroides extracts, from the following aspects: (a) the influence of *A. philoxeroides* extracts on the biomass of *M. aeruginosa*; (b) impacts of *A. philoxeroides* on physiological and biochemical characteristics of *M. aeruginosa*, oxygen radical, MDA, nucleic acid, microcystin and polysaccharide content were detected; (c) morphology and structure changes of *M. aeruginosa* under *A. philoxeroides* disposition (microscopic observation). We intend to develop a novel environment-friendly alga-growth inhibitor. We also want to supply scientific and theoretical basis for turning this invasive plant into a resource.

Materials and methods

Test materials

M. aeruginosa was purchased from the freshwater algae seed bank of the institute of aquatic biology, Chinese Academy of Science (PCC7806). The alga was cultivated in a climate chamber until they were in their logarithmic growth phase. *A. philoxeroides* used in the experiment was collected at the Zheshan campus lawn of Anhui Normal University, Wuhu, in eastern China (31°34′N, 118°38′E).

Methods

Algae cultivation

100 mL of *M. aeruginosa* stock was cultivated with the BG-11 culture medium at 25 ± 1 ℃, 4,000 lx, and 12 h: 12 h day light photoperiod for ten days. Fresh culture medium was supplemented and the culture container was shaken 3-4 times per day until *M. aeruginosa* was in its logarithmic growth phase.

The composition of BG-11 medium included $NaNO_3$ 1.5 g/L, K_2HPO_4 40 mg/L, $MgSO_4$ 75 mg/L, $CaCl_2 \cdot 2H_2O$ 36 mg/L, Ammonium ferric citrate 6 mg/L, EDTA-2Na 1 mg/L, Na_2CO_3 20 mg/L, A5 + Co (mother liquor) 1 mg/L. Among them, mother liquor formula was $MnCl_2 \cdot 4H_2O$ 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ 0.22 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g/L, $CuSO_4 \cdot 5H_2O$ 0.079 g/L, $Co(NO_3)_2 \cdot 6H_2O$ 0.049 g/L.

Extracts preparation

Fresh *A. philoxeroides* plant was collected and was first rinsed with tap water, then washed three times with distilled water. Roots, stems and leaves of *A. philoxeroides* were sampled separately and dried (15-30 min at 105 ℃, and then dried to constant weight at 60-70 ℃). Each plant part was grounded with a grinder until sufficient amount was available for the experiment. The powder of 40 g was soaked in 200 mL distilled water at a ratio of 1:5 (W/V) for 48 h, and then qualitative filter paper and quantitative filter paper were used for double filtration. Final concentration of the filtered solution was considered as 200 g/L. The concentrations of aqueous extracts were adjusted to 0.3, 0.5, 0.7, 0.9, 1.1 g/L by dilution with distilled water and stored at 4 ℃ prior to experiment.

Algae growth inhibition test

100 mL of *M. aeruginosa* was transferred to 250 mL sterilized conical flask. Initial concentration of these algae was 1.43×10^6 cells/mL. Different concentrations of the root, stem and leaf extracts (0, 0.3, 0.5, 0.7, 0.9, 1.1 g/L) were then added to conical flasks containing *M. aeruginosa*. Since only a small amount of the extract was added to

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each conical flask, there was little effect on algal density, so extract volume was not considered. The test was replicated three times. The number of algal cells was counted with a blood cell counter once every 24 h and recorded to 96 h.

The growth inhibition rate of *M. aeruginosa* by the extracts of *A. philoxeroides* was calculated as the following formula:

$$IR\ (\%)=(1-{}^{N1}/_{N0})\times 100\% \quad \text{(Eq.1)}$$

where *N1* represents the algae density in the treatment group at day *N*, while *N0* represents the algae density in the control group at day *N*. Units of both *N1* and *N0* are cell counts/mL.

Determination of physiological parameters of M. aeruginosa

Nucleic acid, MDA and O_2^- were determined at 24, 72 and 120 h after cultivation. 12 mL algae solution was collected and centrifuged at 4,000 r/min for 10 min. The supernatant was used to measure the content of nuclear acid using UV-Vis spectrophotometer (UV-3802, Unico, USA) at 260 nm (Sun et al., 2004). The sediment was dissolved in 1 mL phosphate buffer saline (PBS) (pH 7.0), for three times of repeated freeze-thaw at -80 ℃. After centrifugation at 12,000 r/min for 15 min, the supernatant was the enzyme solution of MDA. 2 mL enzyme solution (control plus 2 mL distilled water) was added to 2 mL 10% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA). The mixture was placed in a boiling water bath at 100 ℃ for 15 min, cooled rapidly and then placed in a centrifuge at 4,000 r/min for 10 min. The supernatant was taken and the absorbance was measured at 450, 532 and 600 nm respectively. MDA (umol/g) = 6.45 × (A532 - A600) - 0.56 × A450. In the determination of O_2^- content, 2 mL supernatant of algal liquid was taken with the same centrifugation at 4,000 r/min for 10 min, and 0.4 mL 10 mmol/L hydroxylamine hydrochloride was added. After mixing, the supernatant was placed in a water bath at 25 ℃ for 20 min. Then 2 mL α-naphthylamine (7 mmol/L) and 2 mL p-aminobenzene sulfonic acid (17 mmol/L) were added, mixed and the reaction was carried out at 25 ℃ for 25 min. The same volume of butyl alcohol was used, shaken thoroughly and the reaction mixture was set aside for layering. The upper butyl alcohol was taken to measure the light absorption value at 530 nm.

At 72 h, 20 mL algae solution was collected and centrifuged at 4,000 r/min for 15 min, the supernatant was transferred to Eppendorf tube and saved at -20 ℃ for the determination of extracellular polysaccharides and microcystins in *M. aeruginosa*. The sediment was transferred with 1 mL PBS (pH 7.2-7.4), repeated freeze-thaw was carried out at -20 ℃ for 3 times, then the supernatant was taken and stored at -20 ℃ for determination of intracellular polysaccharides and microcystins in *M. aeruginosa*. Both microcystins and polysaccharides were analyzed by enzyme-linked immunosorbent assay (ELISA) kit (96T/48T, Shanghai Xinyu Biotechnology Co., Ltd), and the specific operation method was referred to the kit instructions.

Observation of cell superficial structure of M. aeruginosa

The algae cells treated with root extracts of different concentrations for 72 h were collected, fixed with 2.5% glutaraldehyde for 24 h and 1% osmium acid for 1 h, and then washed with PBS (pH 7.4) for 3 times. The ethanol gradients of 15%, 30%, 50%, 75%, 95% and 100% were used to elute for 15 min respectively. After the supernatant

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was discarded, 10 mL 1:1 mixture of isoamyl acetate and ethanol was added and soaked for 10-20 min. The sediment left by centrifugation was further soaked and shaken in pure isoamyl acetate for 20 min, and then observed and photographed under a scanning electron microscope (JSM-6390LV, JEOL) after coating.

Data analysis

The obtained data were expressed in terms of mean value ± SE (standard error), and SPSS 19.0 analysis software was used to analyze the differences between the control group and treatment group for one-way ANOVA followed by a T-test. $P < 0.05$ represents statistical significance, and $P < 0.01$ represents a greater significant difference.

Results and analyses

Effects of A. philoxeroides extracts on M. aeruginosa growth

When *M. aeruginosa* was exposed to the extracts of root, stem and leaf from *A. philoxeroides* for 24 h, 48 h, 72 h and 96 h, the growth inhibition rate of algal cells increased with increasing concentration of extracts (*Table 1*). In particular, when *M. aeruginosa* was treated for 24 h by ≥ 0.5 g/L root extract, ≥ 0.7 g/L stem extract and ≥ 0.5 g/L leaf extract from *A. philoxeroides*, significant differences generated compared with lower concentration treatments ($P < 0.05$). Also, during the entire experimental process, the growth inhibition rate of each treatment group showed an obvious trend of increasing with culture time. After 96 h exposure, the optimal concentration of root extract with the highest growth inhibition was 0.9 g/L, the IR was close to 80.48%, while the optimal concentrations of stem and leaf extracts were both 1.1 g/L, and the IR was 55.79% and 45.64%, respectively.

Table 1. *The effect of A. philoxeroides extracts on M. aeruginosa growth*

Treatment	Growth inhibition rate (%)				
	Concentration (g/L)	24 h	48 h	72 h	96 h
Root extract	0.3	25.22 ± 1.21a	43.61 ± 1.78a	55.72 ± 2.09a	64.42 ± 1.13a
	0.5	28.11 ± 1.84b	56.45 ± 0.65b	57.46 ± 1.04b	70.05 ± 2.36a
	0.7	34.62 ± 1.47c	63.37 ± 0.55c	66.53 ± 1.35c	74.62 ± 1.76b
	0.9	51.57 ± 1.58d	73.07 ± 1.19d	74.75 ± 0.33d	80.48 ± 1.13c
	1.1	51.57 ± 0.87d	72.95 ± 0.63d	74.70 ± 0.32d	80.34 ± 1.18c
Stem extract	0.3	29.96 ± 3.32a	23.67 ± 0.45a	32.33 ± 1.01a	43.61 ± 0.97a
	0.5	30.60 ± 0.72a	23.67 ± 1.06a	35.19 ± 0.20b	47.53 ± 0.73b
	0.7	42.97 ± 4.87b	38.98 ± 0.45b	45.57 ± 1.21c	52.15 ± 0.52c
	0.9	42.81 ± 0.91b	45.66 ± 1.82c	49.07 ± 1.06d	54.91 ± 0.56d
	1.1	51.08 ± 1.05c	48.31 ± 0.96d	51.66 ± 0.62e	55.79 ± 1.67d
Leaf extract	0.3	13.33 ± 6.06a	10.03 ± 2.16a	20.58 ± 1.05a	31.61 ± 0.83a
	0.5	25.65 ± 2.64b	30.90 ± 0.45b	32.27 ± 0.31b	35.44 ± 0.92b
	0.7	38.88 ± 1.24c	34.22 ± 0.48c	36.46 ± 0.45c	38.81 ± 1.18c
	0.9	42.01 ± 0.37c	40.66 ± 1.88d	41.53 ± 0.77d	42.96 ± 1.18d
	1.1	42.25 ± 1.24c	44.04 ± 0.28e	44.78 ± 0.22e	45.64 ± 0.62e

Data in the table are mean value (n = 3) ± SE. Different letters in the same column indicate significant difference among treatments of different concentrations of extracts from the same plant part at $P < 0.05$, while the same letters indicate no significant difference within the treatment (plant organs)

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Effects of A. philoxeroides extracts on nucleic acid contents in M. aeruginosa

As shown in *Table 2*, a series of diluted extracts from various organs of *A. philoxeroides* had different effects on the nucleic acid release of *M. aeruginosa*. Among them, after 24 h, 72 h and 120 h culture, the nucleic acid content of the treated group was significantly higher than that of the control group when the concentration of root extract was ≥ 0.3 g/L, stem extract was ≥ 0.5 g/L, ≥ 0.3 g/L, ≥ 0.5 g/L, and leaf extract was ≥ 0.7 g/L, ≥ 0.5 g/L, ≥ 0.5 g/L ($P < 0.05$). And nucleic acid content was the highest after 120 h treatment when the concentration of root, stem and leaf extracts was 1.1 g/L, which was increased by 96.94%, 62.14% and 51.82% respectively compared to that in the control group. *M. aeruginosa* cells exhibited the most nucleic acid release in the root extract treatment group, followed by the stem and leaf extract treatment groups with the same concentration.

Table 2. *Effects of A. philoxeroides extracts on nucleic acid content of M. aeruginosa*

Treatment	Nucleic acid content (OD_{260})			
	Concentration (g/L)	24 h	72 h	120 h
Control	0	0.0773 ± 0.0031a	0.0823 ± 0.0025a	0.0872 ± 0.0031a
Root extract	0.3	0.0850 ± 0.0026b	0.1177 ± 0.0015b	0.1307 ± 0.0015b
	0.5	0.0857 ± 0.0040b	0.1233 ± 0.0021c	0.1363 ± 0.0025c
	0.7	0.0913 ± 0.0012c	0.1143 ± 0.0040d	0.1580 ± 0.0026d
	0.9	0.1323 ± 0.0021d	0.1600 ± 0.0020e	0.1680 ± 0.0020e
	1.1	0.1303 ± 0.0025d	0.1613 ± 0.0021e	0.1717 ± 0.0021e
Stem extract	0.3	0.0770 ± 0.0017a	0.0870 ± 0.0021b	0.1037 ± 0.0021a
	0.5	0.0883 ± 0.0021b	0.1023 ± 0.0025c	0.1157 ± 0.0012b
	0.7	0.0913 ± 0.0031b	0.1117 ± 0.0031d	0.1270 ± 0.0030c
	0.9	0.0917 ± 0.0035b	0.1210 ± 0.0026e	0.1370 ± 0.0020d
	1.1	0.1000 ± 0.0010c	0.1277 ± 0.0021f	0.1413 ± 0.0015e
Leaf extract	0.3	0.0753 ± 0.0014a	0.0847 ± 0.0015a	0.1010 ± 0.0030ab
	0.5	0.0797 ± 0.0012ab	0.0927 ± 0.0025b	0.1083 ± 0.0025b
	0.7	0.0833 ± 0.0012b	0.1037 ± 0.0025c	0.1173 ± 0.0021c
	0.9	0.0893 ± 0.0015c	0.1163 ± 0.0021d	0.1297 ± 0.0071d
	1.1	0.0940 ± 0.0014d	0.1183 ± 0.0012d	0.1323 ± 0.0035d

Data in the table are mean value (n = 3) ± SE. Different letters in the same column indicate significant difference among treatments of different concentrations of extracts from the same plant part at $P < 0.05$, while the same letters indicate no significant difference within the treatment (plant organs)

Effect of A. philoxeroides extracts on O_2^- contents in M. aeruginosa

After the treatment of *M. Aeruginosa* with root, stem and leaf extracts of different concentrations for 24 h, 72 h and 120 h, the O_2^- content in the treatment group increased with the rise of extract concentrations, and with the extension of culture time, the O_2^- content in algal cells of the treatment group reached a significant higher level than the control group at a lower extract concentration (*Fig. 1*). Of which, under 120 h exposure, significant differences appeared at the concentration ranges of root extract ≥ 0.3 g/L, stem extract ≥ 0.3 g/L and leaf extract ≥ 0.5 g/L ($P < 0.05$). O_2^- content attained the highest value when the extracts of root, stem and leaf was 1.1 g/L, which was 2.67, 1.96

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and 1.83 times higher than the control group, respectively. Besides, according to our results, root extract had the most powerful effect on the induction of O_2^- in *M. aeruginosa*, followed by stem and leaf extract, respectively.

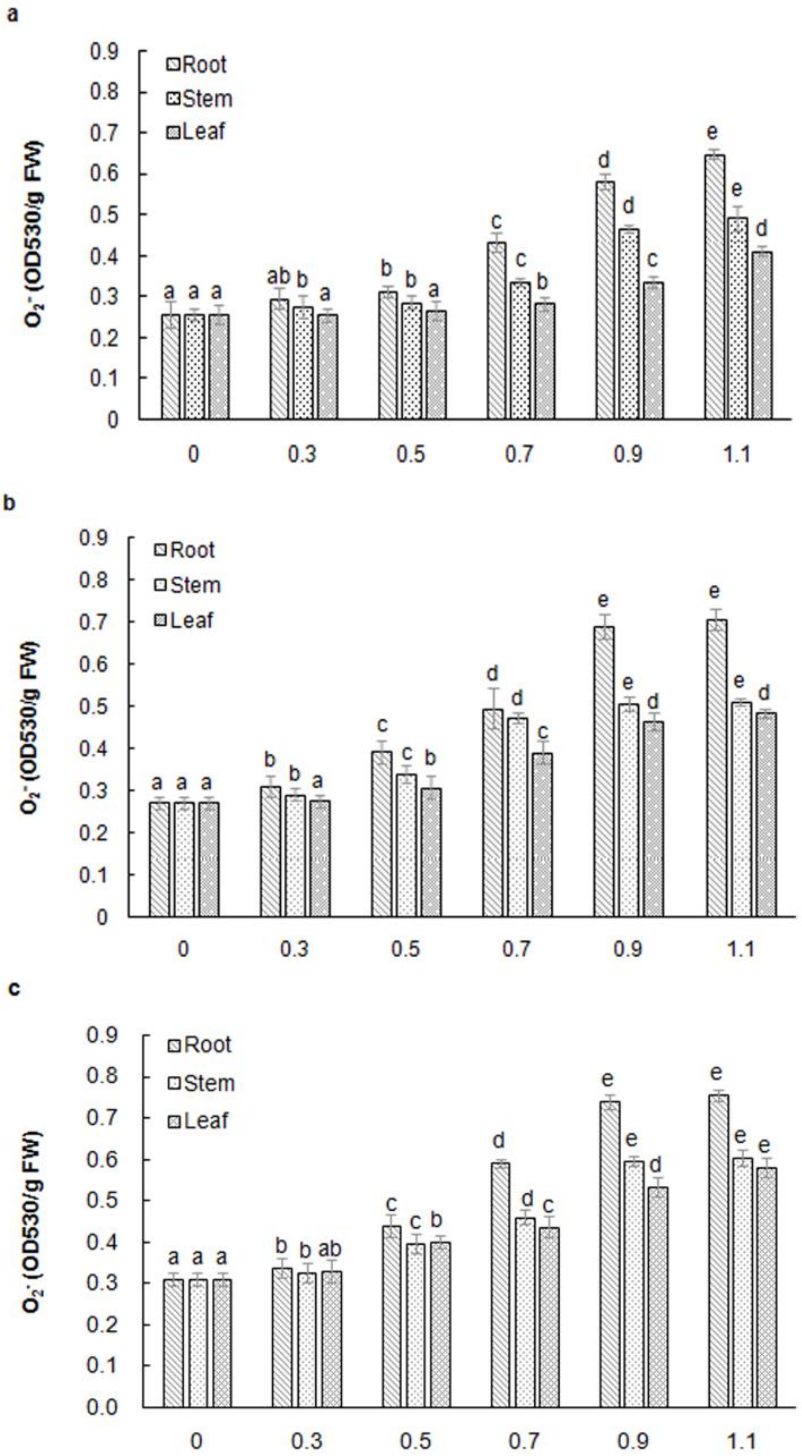

Figure 1. *O_2^- contents in M. aeruginosa exposed to different concentrations of A. philoxeroides extracts for 24 h (a), 72 h (b) and 120 h (c). Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

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Effect of A. philoxeroides extracts on MDA contents in M. aeruginosa

The MDA content of *M. aeruginosa* treated with extracts from *A. philoxeroides* for 24 h, 72 h and 120 h overall increased with increasing concentration of extracts. In particular, after 24 h exposure, MDA content decreased initially (from 0 to 0.3 g/L) in all treatments and was significantly increased when *M. aeruginosa* cells were treated by ≥ 0.5 g/L root, stem extracts and ≥ 0.7 g/L leaf extract ($P < 0.05$), finally reached the peak at 1.1 g/L (*Fig. 2a*). However, after 120 h exposure, MDA content in the treatment group was significantly higher than that in the control group from the beginning with 0.3 g/L extracts ($P < 0.05$) and elevated continually (*Fig. 2c*). In addition, beneath high concentration for a long time, the allelopathy of three extracts from *A. philoxeroides* showed the following sequence: root > stem > leaf, the effect of root extract on MDA contents in *M. aeruginosa* was the most obvious.

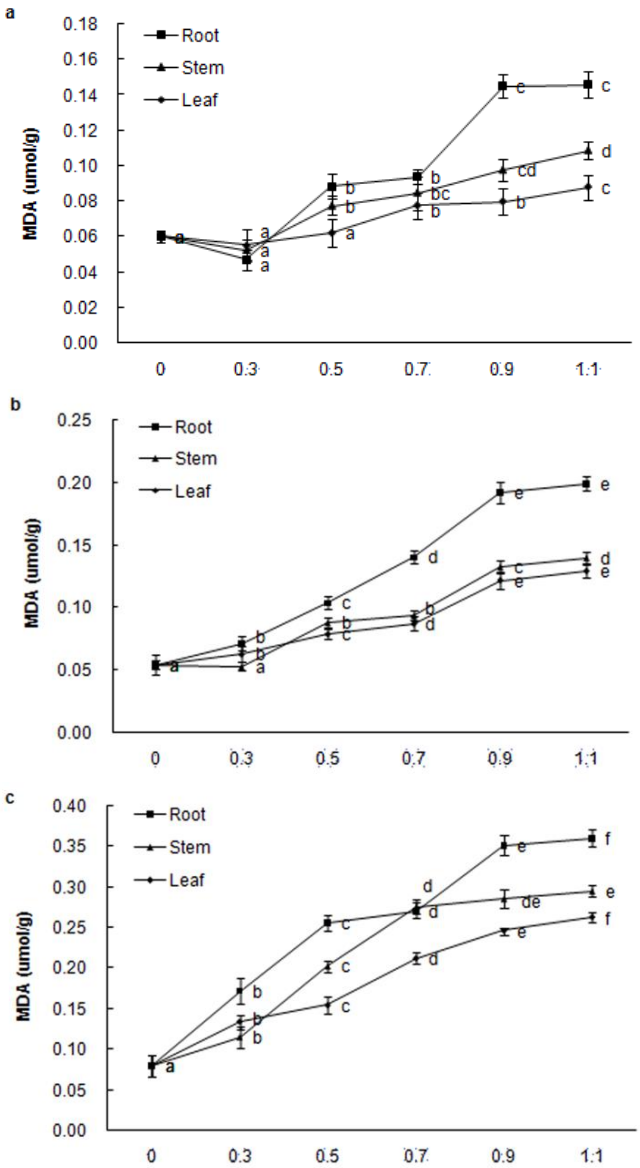

Figure 2. *MDA content in M. aeruginosa cells exposed to different concentrations of A. philoxeroides extracts for 24 h (a), 72 h (b), and 120 h (c). Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

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Effects of A. philoxeroides on intracellular and extracellular microcystin contents in M. aeruginosa

The release of microcystins in *M. aeruginosa* at different concentrations of extracts from *A. philoxeroides* showed a similar growth situation as above (*Fig. 3*). In general, the intracellular and extracellular content of microcystins in the treatment group was significantly higher than that in the control group when *M. aeruginosa* cells were treated by ≥ 0.3 g/L root extract, ≥ 0.5 g/L stem extract and ≥ 0.5 g/L leaf extract, or ≥ 0.3 g/L of all kinds of extracts respectively ($P < 0.05$). 1.1 g/L of root, stem and leaf extracts had the most adverse stimulation, that is intracellular (by 57.70%, 23.00% and 13.05%) and extracellular (by 62.82%, 47.50% and 38.65%) microcystins had the largest increase compared to the control. Under different treatments, the extracellular content of microcystins released by *M. aeruginosa* cells was always lower than that in the intracellular. And according to the consequences, it could be intuitively reflected that the restrain to algal cells from strong to weak was root, stem and leaf extracts.

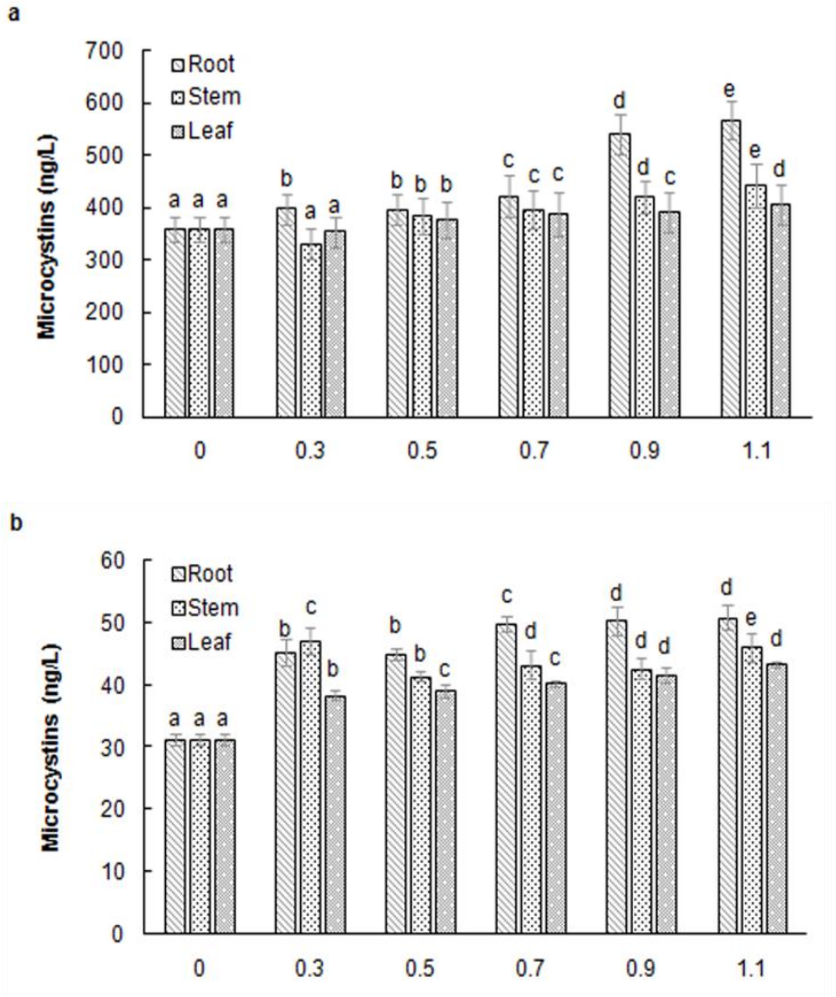

Figure 3. *Effects of extracts from A. philoxeroides on intracellular (a) and extracellular (b) microcystin contents in M. aeruginosa. Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

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Effects of A. philoxeroides on intra- and extra-cellular polysaccharide contents in M. aeruginosa

The results showed that both intra- and extra-cellular contents of polysaccharides in *M. aeruginosa* increased with increasing concentration of extracts, and the content of extracellular polysaccharides was relatively high (*Fig. 4*). After 72 h exposure of *M. aeruginosa* to ≥ 0.3 g/L of root, stem and leaf extracts, the intracellular content of polysaccharides in the treatment group was significantly higher than that in the control group ($P < 0.05$). While extracellular content of polysaccharides achieved significant differences when *M. aeruginosa* cells were exposed to ≥ 0.3 g/L root extract, ≥ 0.3 g/L stem extract and ≥ 0.7 g/L leaf extract ($P < 0.05$). The action strength of the extracts from *A. philoxeroides* was root > stem > leaf, and 1.1 g/L had the greatest influence on the production of polysaccharides in *M. philoxeroides*.

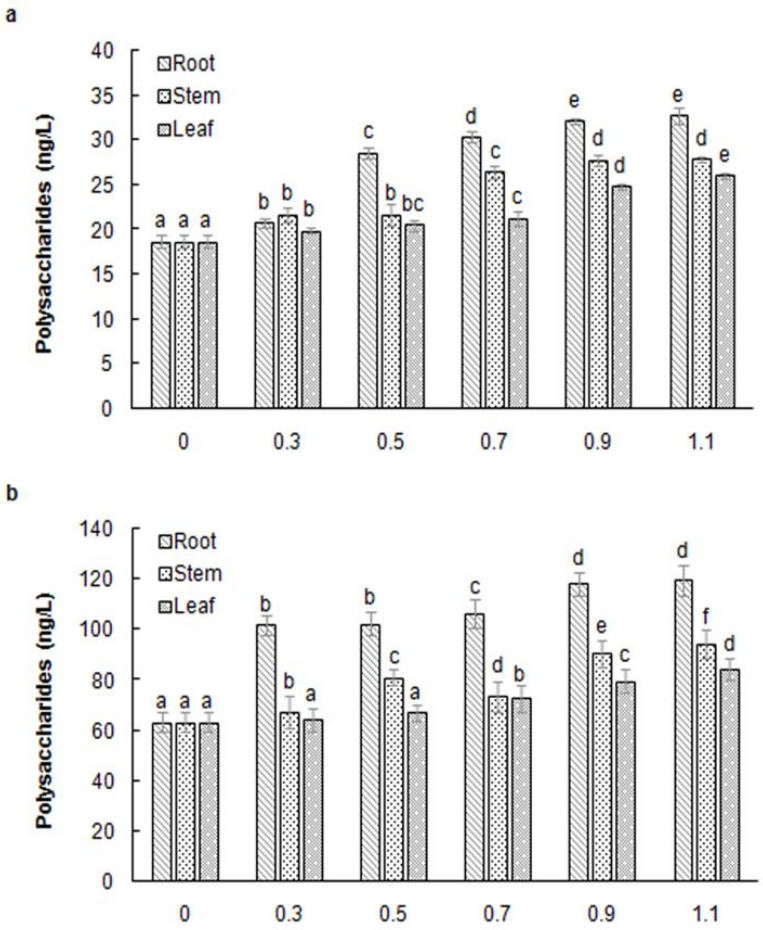

Figure 4. *Effects of extracts from A. philoxeroides on intracellular (a) and extracellular (b) polysaccharide contents in M. aeruginosa. Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

Effect of A. philoxeroides on cell superficial structure of M. aeruginosa

It was observed by scanning electron microscope that the *M. aeruginosa* cells in the control group remained their integrity with a round and smooth appearance, while the

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surface structure of *M. aeruginosa* cells in the treatment group was severely damaged after being exposed to root extract from *A. philoxeroides* for 72 h (*Fig. 5*). With the increasing concentrations of root extract, the morphology of *M. aeruginosa* cells changed gradually, cell shrank, cell wall ruptured and intracellular material flowed out until the cell disintegrated. The 1.1 g/L of root extract was the most destructive on the structure of *M. aeruginosa* cells.

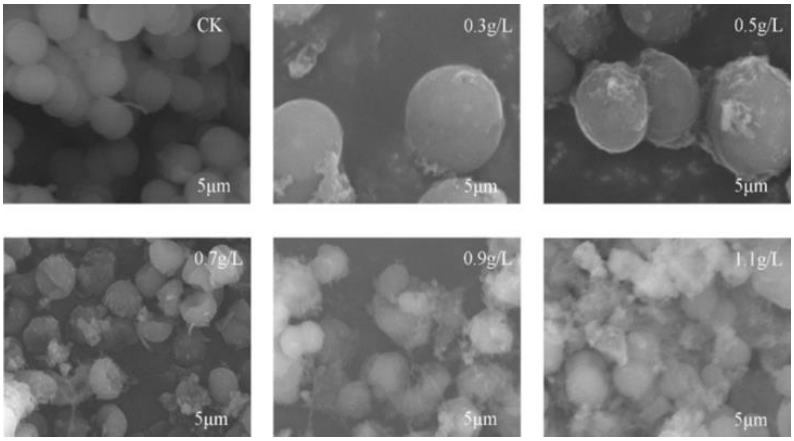

Figure 5. *Scanning electron microscope images of cell morphology and structure of M. aeruginosa damaged by root extract (0-1.1 g/L) from A. philoxeroides (× 5000)*

Discussion

Recent research has found that the growth of *M. aeruginosa* was inhibited by the root, stem and leaf extracts from *A. philoxeroides*. In general, the inhibition intensity was weak at low concentrations and increased at high concentrations, that is the growth of algae was greatly restricted. The same inhibitory trend was also found in the study of Li et al. (2016) about *Sagittaria trifolia* tubers extract influence on *M. aeruginosa*. After being treated with 0.9 g/L root extract for 96 h, the growth inhibition rate of *M. aeruginosa* cells was 80.48% (*Table 1*), which showed that *M. aeruginosa* cells could hardly survive in this environment. Scanning electron microscopy (SEM) displayed that the algae cells were damaged in varying degrees with the increase of the concentration of root extract for 72 h (*Fig. 5*), which means the extracts of *A. philoxeroides* could inhibit cell growth by destroying the morphological structure of *M. aeruginosa* cells, promoting cell lysis and finally leading to cell death. As one type of small molecules, nucleic acid is normally enclosed in the cell. Sometimes, on account of adverse stimulus, the cell membrane cannot maintain the relative stability of the internal structure and function of cells, and control the transport of substances inside and outside the cells due to the loss of selective permeability, thus nucleic acid is released (Shi et al., 2018). With the increase of extracts concentration and culture time, the increase of nucleic acid content further verified the damage degree of algal cells (*Table 2*).

In order to survive in an unfavorable living environment, organisms have evolved a set of strategies to outwit their adversaries. One of them is the antioxidant enzyme system (García et al., 2016). Under normal circumstances, the process of cell metabolism to complete all kinds of life activities can produce reactive oxygen species (ROS). Due to the antioxidant enzymes, cells can effectively fight against the negative impacts of reactive oxygen species and maintain the intracellular ROS level in

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equilibrium (Pereira et al., 2018). Nevertheless, when the cells are subjected to adverse external stimulation (ultraviolet-B, heavy metal and allelochemicals), the original balanced state is broken, and excessive reactive oxygen exists, leading to peroxidation damage of the cells (Zhang and Benoit., 2019).

We found in this study, O_2^- transformation in *M. aeruginosa* could be affected by *A. philoxeroides* extracts, especially root extract (at 1.1 g/L) with a strong competence. The O_2^- content in the treatment group was significantly higher than that of the control group after exceeding a certain concentration range and constantly elevated with the extension of culture time (*Fig. 1*). Huang et al. (2013) also showed the accumulation of intracellular reactive oxygen species in the study of allelopathy of *Solidago canadensis* L. against *M. aeruginosa*, and pointed out that the response mainly depended on the allelopathic substance released by the plants rather than the plant species. Furthermore, induced accumulation of reactive oxygen species in cells may cause damage to photosynthetic pigment, protein, DNA and lipid (Apel and Hirt, 2004). Algal cell membranes are composed of unsaturated phospholipids and are susceptible to ROS, MDA as one of the products of lipid decomposition, it is usually used to mark the process of membrane lipid peroxidation, and the changes in its concentration can reflect the damage of cell membrane and the ability of cells to resist harmful external interference (Davey et al., 2005). The results indicated that MDA content in *M. aeruginosa* increased gradually, which coincided with the increase of O_2^- content, but after 24 h treatment with the root, stem and leaf extracts of 0.3 g/L, MDA content decreased slightly compared with the control group (*Fig. 2*). Under the stimulation of short time and low concentration of the extracts, stress response occurred in algal cells thus membrane permeability and enzyme activities heightened, which could promote cells to absorb the nutrients contained in the extracts and temporarily maintain cells growth (Yuan et al., 2020). In addition, a small amount of ROS was removed by antioxidant enzymes, and the content of MDA was also decreased. It was needed noting that severe membrane lipid peroxidation would lead to a continuous increase of MDA content as the effect of extracts exceeded the tolerance threshold of algal cells (Chen et al., 2019).

The formation of microcystins (MCs) and polysaccharides (PSs) is thought to be a way for algal cells to maintain their growth and a defense mechanism to fight against adverse external factors. MCs are secondary metabolites produced by *M. aeruginosa*, which are usually trapped in living cells, ROS can transmit signals to cause oxidative stress in algal cells, thus promote the combination of MCs with some specific proteins to regulate self-synthesis and release process (Tsai, 2015). Wu et al. (2013) found that allelochemical extracts of *Pistia stratiotes* had influences on *M aeruginosa*, during the whole culture period, high concentration extract had no significant role in the release of extracellular MC contents while the intracellular MC contents increased in a concentration-dependent manner and finally remained stable. Hou et al. (2019) pointed out that the allelopathic inhibition of juglone on *M. aeruginosa* resulted in the increase of both intracellular and extracellular MC contents with the rise of juglone concentration. Our experiment showed the same results with Hou et al. (2019) that intracellular and extracellular MC contents increased in a dose-dependent manner after 72 h treatment (*Fig. 3*). The main reason was that membrane lipid peroxidation promoted the production of MCs, and the extracellular MC contents were released gradually with the metabolic activities and life process of the cells. The content changes display a positively interactive effect between intracellular and extracellular MC

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contents (Chen et al., 2015). It was worth saying that the production and release of MCs could be used as an important indicator to evaluate the treatment of algal blooms by allelochemicals. In our study, although the contents of MCs increased gradually in the test, the extracellular MC contents was far less than the intracellular. Especially at high concentration of root extract (0.9 g/L 96 h), the inhibition rate of algal cells was more than 80%, that is the cell survival rate was low, but most MCs still existed in the cells. In the follow-up research, it is necessary to further isolate and identify allelochemicals that play a major inhibitory role in *A. philoxeroides* and estimate the usage amount of *A. philoxeroides* in order to better evaluate the ecological safety of its application as an algicide.

Polysaccharides (PSs) are a kind of macromolecular substances with high activity. It has been reported that various biological factors (predation, competition) and non-biological factors (nutrition, temperature, light) can affect the production and release of PSs from *M. aeruginosa* (Zhu et al., 2014). The presence of PSs can not only promote algal cells to form large coenobium so as to protect cells from invasion, but also had the ability to remove excess ROS in cells to relieve oxidative stress reaction (EI-Sheekh et al., 2012). It was found in our study that both intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPSs) contents increased with the increasing concentration of extracts from different plant parts of *A. philoxeroides* after 72 h (*Fig. 4*). And the change of the PSs was consistent with that of MCs contents, indicating that algal cells could produce MCs and PSs, and improve the yield with the increase of stress intensity for self-protection in the face of awful environment. Both MCs and PSs may be regulated by cell peroxidation caused by oxygen radicals, and a certain amount of MCs could activate PSs synthesis genes (Mohamed, 2008). For the reason why the content of EPSs was much higher than that of IPSs, some studies have mentioned that EPSs were produced by IPSs and then secreted to the outside of the cells, and there was a significant positive correlation between them, particularly, under terrible environmental conditions, cells would try to release more PSs outside (Liu et al., 2020).

Conclusions

Our current study revealed that the root, stem and leaf extracts of *A. philoxeroides* could inhibit the growth of *M. aeruginosa*. It was also found that root extract had the strongest inhibitory effect, followed by stem and leaf, which may be related to the specific allelochemicals contained in different plant parts of *A. philoxeroides*. With the extension of culture time and the increase of extracts concentration, the superfluous generation of O_2^- led to cell damage and the contents of MDA and nucleic acid increased. At the same time, *M. aeruginosa* cells could resist harmful irritation by releasing microcystins and polysaccharides in a dependent manner with the concentration of the extracts.

Nonetheless, the contents discussed in this study are still insufficient. The important allelochemicals in the extracts of *A. philoxeroides* and more internal mechanisms limiting the growth of *M. aeruginosa* will be the focus of future research. Meanwhile, measuring the pros and cons of ecological application of plant aqueous extracts remains the key, and a large number of studies under natural conditions are requisite.

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