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Growth inhibition and oxidative damage of *Microcystis aeruginosa* induced by crude extract of *Sagittaria trifolia* tubers

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ABSTRACT

Aquatic macrophytes are considered to be promising in controlling harmful cyanobacterial blooms. In this research, an aqueous extract of *Sagittaria trifolia* tubers was prepared to study its inhibitory effect on *Microcystis aeruginosa* in the laboratory. Several physiological indices of *M. aeruginosa*, in response to the environmental stress, were analyzed. Results showed that *S. trifolia* tuber aqueous extract significantly inhibited the growth of *M. aeruginosa* in a concentration-dependent way. The highest inhibition rate reached 90% after 6 day treatment. The *Chlorophyll-a* concentration of *M. aeruginosa* cells decreased from 343.1 to 314.2 μg/L in the treatment group. The activities of superoxide dismutase and peroxidase and the content of reduced glutathione in *M. aeruginosa* cells initially increased as a response to the oxidative stress posed by *S. trifolia* tuber aqueous extract, but then decreased as time prolonged. The lipid peroxidation damage of the cyanobacterial cell membranes was reflected by the malondialdehyde level, which was notably higher in the treatment group compared with the controls. It was concluded that the oxidative damage of *M. aeruginosa* induced by *S. trifolia* tuber aqueous extract might be one of the mechanisms for the inhibitory effects.

Introduction

Outbreaks of cyanobacterial blooms have been indeed increasing in frequency and geographical distribution in the last decades, mostly due to climate changes (Paerl, 2009; Paerl, 2012). Large-scale cyanobacterial blooms degrade water quality and pose serious threats to aquatic organisms and even human health (Paerl et al., 2001). *Microcystis aeruginosa* is one of the representative species of bloom-forming cyanobacterium that occur in freshwater cyanobacterial blooms. Microcystins, a kind of cyanotoxin produced by toxic strains of *M. aeruginosa*, can be very harmful to the human liver through the food chain due to its hepatotoxicity (Mankiewicz et al., 2003). Therefore, it is of great importance to suppress/inhibit the growth of *M. aeruginosa* in eutrophic waters.

Compared with physical methods (*e.g.*, ultraviolet irradiation (Sakai et al., 2007)) or chemical methods (*e.g.*, nitrite (Chen et al., 2011)), the utilization of biological treatment in cyanobacterium control is a relatively cost-effective and environment-friendly approach. Research using aquatic plants and their allelopathic

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effects in *M. aeruginosa* control has been extensively carried on in recent years (Chang et al., 2012; Zhang et al., 2009). *Myriophyllum spicatum* was shown to be one of the most effective macrophyte species, secreting allelochemicals such as pyrogallic acid and gallic acid to inhibit the growth of cyanobacteria (Nakai et al., 2000; Zhu et al., 2010). Chen et al. (2012) investigated the effects of eight species of aquatic macrophytes on *M. aeruginosa* growth and demonstrated that leaves of *Nymphacea tetragona*, *Typha orientalis*, *Nelumbo nucifera* and *Iris wilsonii* were the most potent tissues to inhibit its growth.

The main experimental approaches for studying how aquatic macrophytes affect the growth of phytoplankton can be concluded as follows: coexistence experiments, plant homogenates or extracts, culture filtrates, active compounds extracted from the culture filtrate, and dialysis bag experiments (Gross et al., 2007). Previous studies found that coexistence with *Lemna japonica* (Jang et al., 2007), exudates from *Stratiotes aloides* (Mulderij et al., 2005), decoction of *Radix Astragali* (Yan et al., 2011), essential oils from *Ceratophyllum demersum* and *Vallisneria spiralis* (Xian et al., 2006), and culture water of *Myriophyllum aquaticum* (Wu et al., 2008) all showed inhibitory effects on the growth of *M. aeruginosa*. In addition, allelochemicals from aquatic macrophytes, such as N,N-dimethyl-3-amino-methylindole (gramine) (Hong et al., 2009) and ethyl 2-methyl acetoacetate (EMA) isolated from *Phragmites communis* (Li and Hu 2005; Hong et al., 2008a), have been reported to be useful alternatives to inhibit the growth of *M. aeruginosa*. All those studies mentioned above suggested that aquatic macrophytes might have the ability to control cyanobacterial growth through allelopathy. In addition, other effects may also play a role, such as competing with harmful cyanobacteria for light and nutrients.

Damage in the electron transfer system can result in the formation of reactive oxygen species (ROS), such as superoxide radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), and may then cause peroxidation damage to both plasmalemma and intracellular membranes, finally leading to cell dysfunction and death (Scandalios 1993; Thannickal and Fanburg 2000). Under normal conditions, cells have specific antioxidant protective processes to combat the danger posed by ROS to a certain extent (Mallick and Mohn 2000) so that living cells can maintain a dynamic equilibrium between ROS generation and removal. But excessive radicals, if not eliminated in a timely fashion, may finally lead to cell damage and death. It was reported that allelochemicals from plants could induce ROS production and then lead to oxidant damage in *M. aeruginosa* cells (Hong et al., 2008b; Wang et al., 2011; Zhang et al., 2011a).

Sagittaria trifolia (also called *Arrowhead* due to the shape of its leaves) is one of the main emergent macrophytes and is widely spread in most parts of China. The edible tubers of *S. trifolia* have long been used as vegetables and traditional Chinese medicines in China. Works have reported that *Sagittaria* can absorb nitrogen and phosphorus in eutrophic water and show good effects in water purification (Li et al., 2009). However, to our knowledge, there is little information available about the inhibitory effects of *S. trifolia* on cyanobacterial growth. The use of plant extracts has been considered to be one of the most common experimental approaches for phytoplankton growth inhibition by macrophytes (Hilt and Gross 2008). The research method in the current study was designed after several kinds of pre-experiments involving *S. trifolia* leaf aqueous extract, *S. trifolia* root aqueous extract, and *S. trifolia* planting water. According to the comparison of experimental results, we found that the *S. trifolia* tuber aqueous extract was the most effective material for use in inhibitory experiments. Owing to their large biomass and widespread occurrence, the tuber of *S. trifolia* was chosen as the active inhibition material in our experiment and its aqueous extract was prepared. The purpose of our present work is to investigate the inhibitory effect of *S. trifolia* tuber aqueous extract on *M. aeruginosa* and to assess the extract-induced oxidant damage on *M. aeruginosa* cells by measuring several indices, including superoxide dismutase (SOD) activity, peroxidase (POD) activity, glutathione (GSH) content and malondialdehyde (MDA) level, to elucidate the potential anti- cyanobacterial mechanism.

1. Materials and methods

1.1. Materials and culture conditions

The tubers of *S. trifolia* were purchased from a farm in Huai'an City, Jiangsu Province, and stored in plastic buckets with moist soil at room temperature (about 20°C) before extraction. The cyanobacterium species *M. aeruginosa* was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Then it was cultured in autoclaved MA medium containing (in mg/L): $NaNO_3$ 50, KNO_3 100, $Ca(NO_3)_2{\cdot}4H_2O$ 50, Na_2SO_4 40, $MgCl_2{\cdot}6H_2O$ 50, β-sodium glycerophosphate 100, Na_2EDTA 5, $FeCl_3{\cdot}6H_2O$ 0.5, $MnCl_2{\cdot}4H_2O$ 5, $ZnCl_2{\cdot}7H_2O$ 5, $CoCl_2{\cdot}6H_2O$ 5, $Na_2MoO_4 \cdot 2H_2O$ 0.8, H_3BO_3 20, Bicine 500, under an illumination intensity of 4,000 lx and a light/dark regime of 12:12 hr at 25°C. The culture flasks were placed in a shaking incubator (Digital temperature water bath thermostatic oscillator, Changzhou Putian Instrument Manufacturing Co., Ltd., China) and shaken for 3 times during the light cultural period per day, each time lasting about 1 min.

1.2. Preparation of *S. trifolia* tuber aqueous extract

Selected *S. trifolia* tubers were washed in flowing water and then rinsed by ultrapure water three times, to remove debris and attached microorganisms as much as possible. The cleaned tubers were dried in a drying oven at 50°C to constant weight. The dehydrated samples were then cut into small pieces, powdered and mixed with ultrapure water (1:40 *w/v*). After boiling in covered flasks for 40 min (60°C, 40 min), the solution was filtered with filter paper to remove tuber residues. All the flasks containing the *S. trifolia* tuber aqueous extract were sterilized at 121°C for 20 min. The filtrate was utilized as the cyanobacteria-inhibiting aqueous extract in our experiment and kept at 4°C before use.

1.3. Experimental design

To study the inhibitory effects of different concentrations of *S. trifolia* aqueous extract on the growth of *M. aeruginosa*, conical flasks (500 mL) with cotton plugs were prepared and divided into 6 experimental groups. Each group of flasks contained 200 mL MA medium with different proportions of ultrapure water and S. *trifolia* tuber aqueous extract. The content of aqueous extract in the culture medium in each group was 0%, 10%, 30%, 50%, 70% and 100% (V/V), respectively. The group without aqueous extract (0%) served as the control sample. The pH in each flask was adjusted to 8.3 to 8.5. Each flask was inoculated with *M. aeruginosa* culture in the exponential growth phase to give an initial algal density of about 1.2–1.5 × 10-^{6} cells mL^{-1} under an aseptic environment. To obtain the growth curve of *M. aeruginosa* under treatment of different concentrations of *S. trifolia* tuber aqueous extract, the cyanobacterial cells were counted every day using a light microscope (BA310-T, Motic China Group Co., Ltd., China) with a hemocytometer, from 0 to 6 days. According to Fig. 1, we could see that the relative inhibitory effect was most remarkable at the 50% concentration. The concentration of 50% aqueous extract of *S. trifolia* tubers was adopted for its prominent inhibitory effect. Samples were collected from both control and 50% concentration treatment groups at 0, 12, 24, 36, 48, 72, and 120 hr, to determine the physiological responses of *M. aeruginosa* cells and analyze the possible mechanism of the inhibitory effect. All the experiments were conducted in triplicate. The cultures were incubated under the conditions described in Section 1.1.

1.4. Preparation of enzyme extracts

Cyanobacterial cells were harvested by centrifugation at 4000 ×*g* for 25 min. After removing the supernatant, 2 mL of phosphate-buffer (0.2 mol, pH 7.4) was added into the centrifuge tubes to re-suspend the cells. Then the cells were disrupted and homogenized by an ultrasonic cell pulverizer (SCIENTZ-IID, Ningbo Scientz Biotechnology Co., Ltd., China) at 300 W for a total time of 5 min (ultrasonic time: 2 sec, rest time: 8 sec) in an ice bath. The homogenate was then centrifuged at 4000 ×*g* for 15 min at 4°C. The supernatant was preserved as a cell-free enzyme extract and used for the following assays.

1.5. Physiological assays of *M. aeruginosa* cells

The Chl-*a* concentration, SOD activity, POD activity, GSH content and MDA level in *M. aeruginosa* cells were investigated in this research. The SOD activity, POD activity and GSH contents were tested using assay kits purchased from Nanjing Jiancheng Bioengineering Institute, China, following the manufacturer's instructions (Fan et al., 2011; Luo et al., 2011). SOD activity and GSH content were determined by Enzyme-linked Immunosorbent Assay (ELISA). For POD activity measurement, the absorbance was read on a spectrophotometer.

Chlorophyll-*a* concentration was measured spectrophotometrically (Wang et al., 2010), and calculated referring to the method described by Lichtenthaler and Wellburn (1983). Samples were centrifuged first and the cyanobacterial clusters were then extracted in the dark with 95% ethanol at 4°C for 48 hr. After extraction, the samples were centrifuged for 30 min at 2500 ×*g* and the supernatants were collected for measurement at the absorbance of 665 and 649 nm. 95% ethanol was used as the blank solution. Chl-*a* concentration (mg/L) was calculated according to the following Eq. (1):

$$\text{Chl-}a = 13.95 \times OD_{665} - 6.88 \times OD_{649}. \quad (1)$$

Lipid peroxidation was reflected by the MDA level and determined according to the work by Li et al. (2011). Samples were collected and centrifuged at 4000 ×*g* for 20 min. The cell pellets were then homogenized with 2 mL 10% (*w*/*v*) trichloroacetic acid (TCA) and centrifuged at 12,000 ×*g* for 10 min at 4°C. After centrifugation, 2 mL of the supernatant was mixed with 2 mL of 0.6% thiobarbituric acid (in 10% TCA) and heated in boiling water for 15 min. The reaction was stopped by transferring the reaction tubes into an ice bath. Following cooling, the samples were then centrifuged at 12,000 ×*g* for 10 min. The absorbance of the supernatant was measured at 532, 600 and 450 nm, taking a mixture of 2 mL ultrapure water and 2 mL 0.6% TBA as reference. The MDA level (μmol/L) was calculated according to Eq. (2):

$$\text{MDA} = 6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}. \quad (2)$$

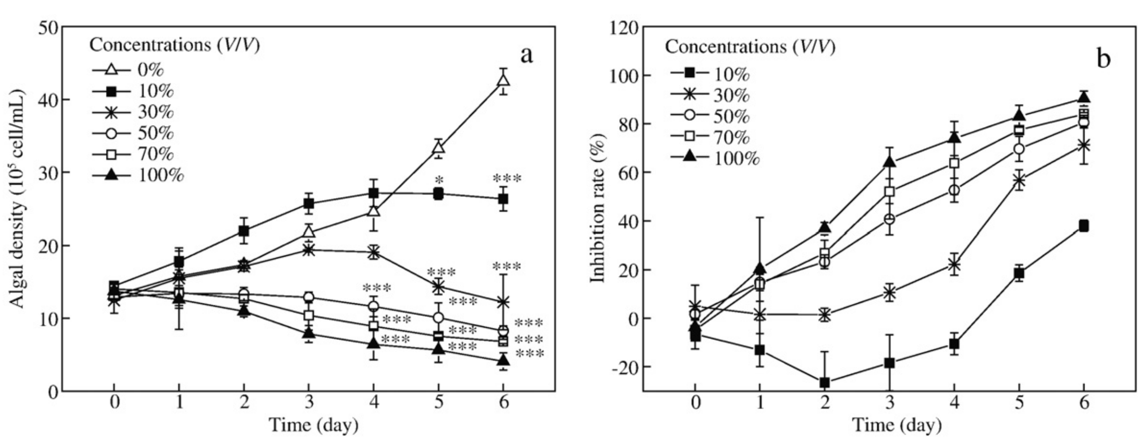

Fig. 1 – Effects of different concentrations of *Sagittaria trifolia* tuber aqueous extract on the growth of *Microcystis aeruginosa*. (a) Growth curve, (b) inhibition curve. Data are means ± SD (*n* = 3). $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate significant differences compared with the corresponding controls.

1.6. Statistical analysis

The inhibition rate (IR) of cyanobacterial growth under treatment of different concentrations of aqueous extract was calculated according to the following Eq. (3):

$$IR = (N_o - N_s)/N_o \times 100\% \quad (3)$$

where N_o and N_s (cell/mL) represent cell density in the control and treatment groups, respectively.

The data were expressed as the mean ± standard deviation (SD). The statistical significance between the control group and treatment group was confirmed using one-way analysis of variance (ANOVA) followed by a Tukey test (Wang et al., 2010). All the tests were carried out using SPSS 19.0. $p < 0.05$ was considered to indicate a significant difference. Figures were generated using Origin 8.0 software.

2. Results

*2.1. Inhibitory effect of **S. trifolia** tuber aqueous extract on growth of **M. aeruginosa***

From Fig. 1a, it can be seen that *S. trifolia* tuber aqueous extract at all the five concentrations (10%, 30%, 50%, 70%, 100%, V/V) inhibited the growth of *M. aeruginosa* after 6 days of cultivation compared with the control group ($p < 0.001$) and exhibited a concentration-dependent trend. The cell densities in the 10% concentration group showed an increase at the early stage but were suppressed in the subsequent exposure time. No increase of cyanobacterial density was observed after the 4th day in all treatment groups. As a whole, the IR increased with the increase of aqueous extract concentration (Fig. 1b). After treatment for 6 days, the maximum IR reached 90.4% in the 100% concentration group, while the lowest IR was 37.9% under the treatment of 10% *S. trifolia* aqueous extract. Furthermore, by visual evaluation it was evident that the growth of *M. aeruginosa* increased with time and the green color of the culture medium gradually deepened in the control groups; while the culture medium became transparent with yellow sediment at the bottom after 6 days in all treatment groups. Almost 100% inhibition was observed at 100% *S. trifolia* tuber aqueous extract.

*2.2. Effects of **S. trifolia** tuber aqueous extract on pH value in culture medium and Chlorophyll-a concentration of **M. aeruginosa***

The pH level increased in both treatment and control groups (Fig. 2). For the treatment group, it ranged from an initial value of 8.36 at 0 hr to the highest level of 9.48 at 120 hr, which was faster than the change in the control group from 8.45 to 9.23. The differences between the two groups became apparent after 36 hr ($p < 0.01$).

Measurements showed that *M. aeruginosa* cells in the control group were significantly increased during the 120-hr experiment, which showed a significant difference compared with the corresponding treatment group, and the Chl-*a* concentration increased from 331.11 to 523.58 μg/L. In treatment samples, the Chl-*a* concentration decreased from 343.14 to 289.25 μg/L within 120 hr (Fig. 3). The two groups did not differ much before 72 hr ($p > 0.05$) but significant differences were observed after that time ($p < 0.001$).

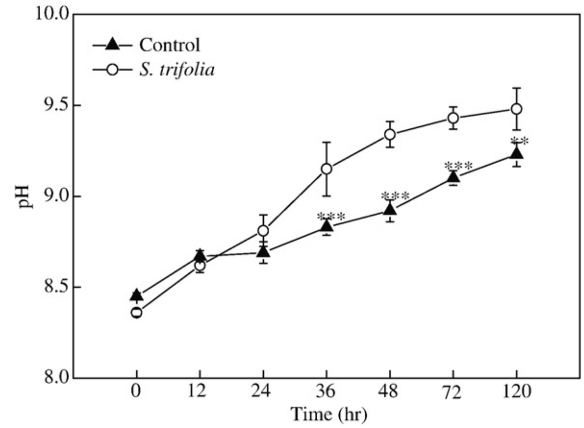

Fig. 2 – pH value in culture medium of *Microcystis aeruginosa*. Control stands for the group with no addition of *Sagittaria trifolia* tuber aqueous extract. *Sagittaria trifolia* stands for the treatment group with 50% concentration of the *Sagittaria trifolia* tuber aqueous extract. Data are means ± SD (*n* = 3). ***p* < 0.01 and ****p* < 0.001 indicate significant differences compared with the corresponding controls.

*2.3. Effects of **S. trifolia** tuber aqueous extract on physiological changes in **M. aeruginosa***

2.3.1. Effects of S. trifolia tuber aqueous extract on MDA level in Microcystis aeruginosa

MDA is the end product of lipid peroxidation in organisms and is frequently used as an indicator of lipid peroxidation

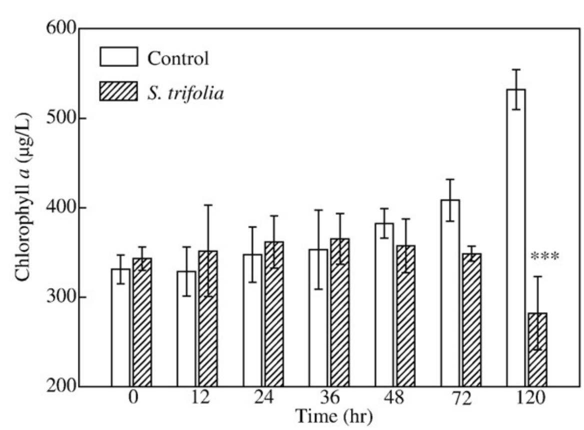

Fig. 3 – Chlorophyll-*a* concentration of *Microcystis aeruginosa* cells in control group and treatment group with 50% concentration of the *Sagittaria trifolia* tuber aqueous extract. Data are means ± SD (*n* = 3). ****p* < 0.001 indicate significant differences compared with the corresponding controls.

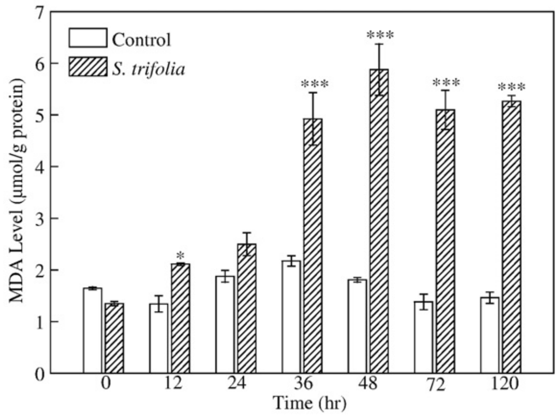

Fig. 4 – MDA level of *Microcystis aeruginosa* cells in control group and treatment group with 50% of the *Sagittaria trifolia* tuber aqueous extract. Data are means ± SD (n = 3). *$p < 0.05$ and ***$p < 0.001$ indicate significant differences compared with the corresponding controls.

(Seljeskog et al., 2006). As shown in Fig. 4, the MDA level in *M. aeruginosa* cells exposed to 50% concentration of the aqueous extract exhibited a significant increase, while the MDA level in the control remained unchanged over time. The differences between treated and untreated cyanobacterial cells were apparent from 36 hr on ($p < 0.001$). The maximal MDA value was 5.88 μmol g/protein at 48 hr, which was 2.7 times that in the control group.

2.3.2. Effects of S. trifolia tuber aqueous extract on enzymatic antioxidant activities in M. aeruginosa

To investigate whether the cellular oxidative defense system was activated, the SOD and POD activities were examined (Fig. 5a, b). Results showed that the differences in the enzyme activities between control and treatment groups were significant and visible after 24 hr in the case of POD and after 36 hr in the case of SOD. Differences between control and treatment groups were especially apparent from 36 to 120 hr ($p < 0.05$). SOD activities in cyanobacterial cells in both treatment and control groups increased on the first day. From 24 hr on, SOD activity in the treatment group became obviously higher than that in the control group and reached its peak of 956.5 U mg/prot at 36 hr, but decreased dramatically in the following time (Fig. 5a). Changes of POD activity showed a similar pattern as SOD activity. The value changed from 136.1 U mg/prot at 0 hr to 239.6 U mg/prot at 48 hr, but finally decreased to 162.7 U mg/prot at 120 hr (Fig. 5b) in the treatment group.

2.3.3. Effects of S. trifolia tuber aqueous extract on GSH contents in M. aeruginosa

Fig. 6 shows the variations of GSH contents in *M. aeruginosa* cells when exposed to a 50% concentration of *S. trifolia* tuber aqueous extract, from which we can see the obvious change in the treatment group in contrast with the control group. GSH content exhibited no significant difference in both groups in the first day but was then was stimulated markedly in the treatment group and reached its peak at 72 hr ($p < 0.001$), at a 2.52-fold level relative to the controls.

3. Discussion

The growth inhibition of *M. aeruginosa* and physiological responses of cyanobacteria vary under different kinds of stress. The difference is generated not only by species with different sensibilities to stressors, but also by the pretreatment methods, including co-culture of *M. aeruginosa* and macrophytes, addition of aqueous or organic solvent extract of active fractions of plants to the culture medium of the cyanobacteria, or direct addition of autoclaved tissues of plants to the culture medium, *etc.* As described by Chen et al. (2012), pretreatments, including autoclaving and mechanical grinding, were necessary to release more cyanobacterial inhibitor in their experiment. We dealt with *S. trifolia* tubers similarly in this study.

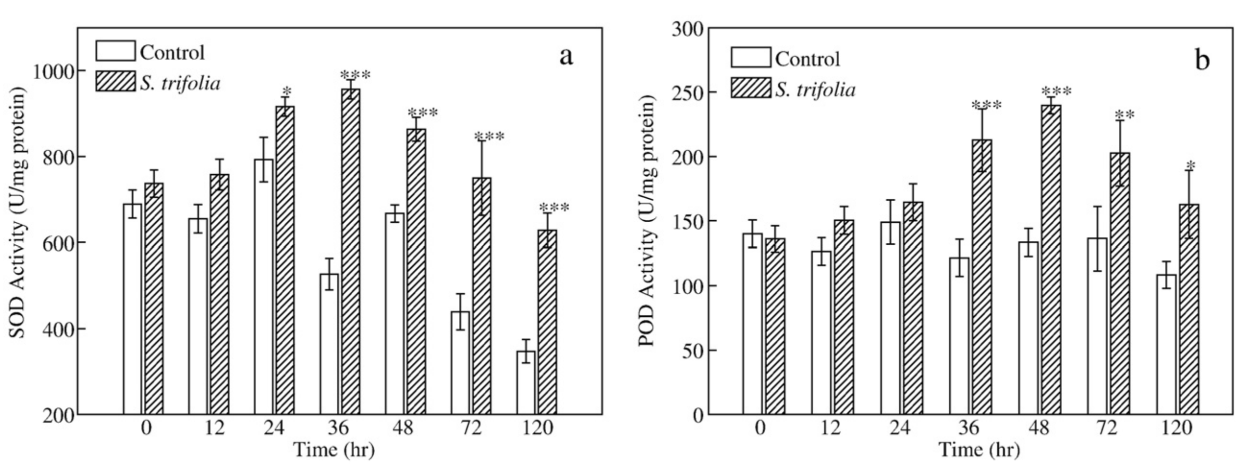

Fig. 5 – Activities of SOD (a) and POD (b) of *Microcystis aeruginosa* cells in control group and treatment group with 50% concentration of the *Sagittaria trifolia* tuber aqueous extract. Data are means ± SD (n = 3). * $p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ indicate significant differences compared with the corresponding controls. SOD: superoxide dismutase; POD: peroxidase.

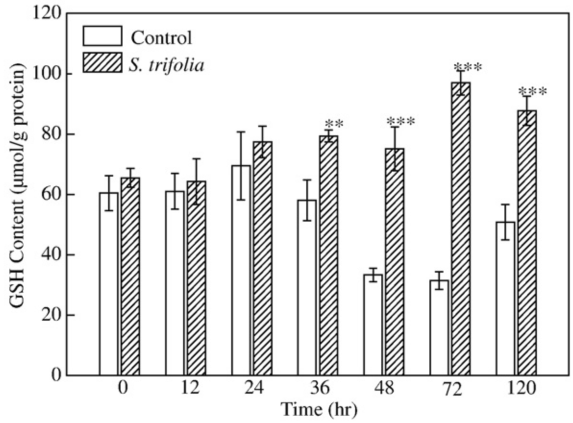

Fig. 6 – GSH content of *Microcystis aeruginosa* cells in control group and treatment group with 50% concentration of the *Sagittaria trifolia* tuber aqueous extract. Data are means ± SD (*n* = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences compared with the corresponding controls.

From the quantitative determination of cyanobacterial growth, we can infer that the growth of *M. aeruginosa* was inhibited efficiently by *S. trifolia* tuber aqueous extract. The relationship between the concentration of aqueous extract and the growth of *M. aeruginosa* is displayed in Fig. 1. The less diluted the aqueous extract, the more pronounced the inhibitory effect. Similar variation trends were presented by Xiao et al. (2010) and Żak et al. (2012). However, we observed that *S. trifolia* tuber aqueous extract at concentration 10% slightly stimulated the growth of *M. aeruginosa* during the first three days when compared to the control. Special attention should be addressed to this stimulatory effect when dealing with macrophytes and allelochemicals that affect *M. aeruginosa* growth, since the stimulatory effect disappeared in the 4th day of exposure and the cyanobacterial growth was inhibited after that time in our experiment.

The pH of control and treatment groups was related to the cultural environment (Fig. 1). The pH of the treatment group was evidence of the inhibitory effect, revealing that a harsh environment was caused by the *S. trifolia* tuber aqueous extract. It can also be found that the pH had a negative correlation with cell density in the treatment group. The variation of pH in the treatment group was an important issue which could affect the growth of *M. aeruginosa*.

Chl-*a* is a major pigment of microalgal photosynthetic systems (Yang et al., 2012). Chl-*a* concentration can be used to reflect cyanobacterial biomass indirectly (Lawton et al., 1999). As shown in Fig. 3, the Chl-*a* concentration of *M. aeruginosa* kept rising in the control group, but was suppressed in the 50% concentration group. Although Chl-*a* was less sensitive than cell density in the treatment group, the change of Chl-*a* still demonstrated that the growth and reproduction of *M. aeruginosa* were restrained by *S. trifolia* tuber aqueous extract.

ROS can oxidize biomembranes to cause membrane lipid peroxidation and result in the formation of MDA (Yang et al., 2013). Therefore, the MDA level is an important index that indirectly reflects the degree of cellular damage. The results demonstrated that there was an acute increase in MDA level, which indicated that the polyunsaturated acids in cell membranes were attacked by oxygen radicals, and that the cyanobacterial cells were subjected to serious oxidative stress (Fig. 4). However, the reaction slowed down at the latter part of time and the cyanobacterial cells gradually died. *Thalia dealbata* root aqueous extract (Zhang et al., 2011b) and Nanaomycin A methyl ester (Feng et al., 2013) were found to cause damage in the structure of *M. aeruginosa* cell membranes, and the MDA content showed a rising trend, which was similar to that found in our research.

The responses of antioxidants in *M. aeruginosa* cells to oxidative stress have been presented in many studies (Ana et al., 2011; Sana et al., 2007; Shao et al., 2009; Zhang et al., 2013). Several common physiological indices were determined in our study relating to toxic responses. These physiological indices were crucial for elucidating the possible mechanism of the inhibitory effect caused by *S. trifolia* tubers. SOD and POD are important enzymatic antioxidants in organisms. Once O_2^-, a precursor of ROS (Hong et al., 2008b), is produced, SOD could play an important role in eliminating O_2^- to reduce cell damage. It catalyzes the dismutation of O_2^- and results in the production of hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Monk et al., 1989). POD catalyzes the reaction in which H_2O_2 can be converted into harmless H_2O molecules (Weir et al., 2004). Excess ROS can lead to severe cell injury, and even death, so it is of great importance to maintain the normal action of the cellular antioxidant defense system. In some cases, allelochemicals were reported to upregulate SOD and POD activity at lower concentrations, while downregulating those at higher concentrations compared with the control (Zhang et al., 2011a; Yang et al., 2012). In our present study, SOD activity in *M. aeruginosa* cells represented a trend from an increase to a decrease (Fig. 5a) when the cells were exposed to a 50% concentration of the *S. trifolia* tuber aqueous extract. Generally, when *M. aeruginosa* cells were subject to oxidative stress, which was caused by the production and reaction of ROS, more SOD was produced to scavenge O_2^- as a cellular detoxification response. In the earlier stage of our experiment, the enhancement of the SOD and POD activity of *M. aeruginosa* indicated that the antioxidant defense system was activated under the environmental stress, and the two enzymes had played a role in ROS elimination. When the exposure time reached 48 hr, SOD activity decreased notably, which may reflect the degradation of the detoxification process in cyanobacterial cells. The decrease of SOD activity was probably related to the excessive production of O_2^- over time and depletion of SOD production. As a result, SOD in algal cells could not efficiently catalyze the continuous production of O_2^- over time, and cell damage occurred. The change of POD activity could also be explained similarly. GSH is an important non-enzymatic antioxidant which is not only linked to the detoxification of H_2O_2 in the ascorbate–glutathione cycle (AGC), but also plays a role in protecting proteins against denaturation (Noctor et al., 2002). The increase of GSH content in our study not only indicated that the production of H_2O_2 was increased, but also implied that GSH took part in the reaction of the antioxidant defense system against the stress during early exposure. However, the GSH content also decreased markedly after 72 hr due to the continuous accumulation of ROS (Fig. 6).

Overall, *S. trifolia* tuber aqueous extract can exert pressure on the antioxidant defense system and lead to cell damage in *M. aeruginosa*. However, it should be noted that our experiments were conducted under optimal laboratory conditions (*e.g.*, light and nutrients), so we do not know if the inhibitory effect of *S. trifolia* tuber aqueous extract on *M. aeruginosa* would be affected by other macrophytes and phytoplankton under field conditions. Therefore, more related studies in the aquatic ecosystem, including the stability of the extract and interaction between *S. trifolia* tuber aqueous extract, *M. aeruginosa* and other aquatic biota, *etc.*, should be studied systematically in future research to determine whether *S. trifolia* tubers could be used in practical cyanobacterial inhibition or not. In terms of practical application, the question as to whether this method is environment-friendly and could be effectively used in the real world, such as in inland rivers, lakes and ponds in parks, and reservoirs, which we hope, is also of interest in our future studies. What is more, the material that caused the inhibitory effect on *M. aeruginosa* in our report is just a crude extract of the *S. trifolia* tubers. The identity of the active compounds that really caused the inhibitory effect also needs to be studied. It may be more effective if the compounds could be extracted and applied in controlling the cyanobacterial bloom of *M. aeruginosa*. Notwithstanding the inadequacies mentioned above, our study did investigate the effects of *S. trifolia* tuber aqueous extract on the growth and the antioxidant defense system of *M. aeruginosa*, and we inferred that the lipid peroxidation and oxidative damage induced by *S. trifolia* tuber aqueous extract may be an important mechanism involved in the inhibitory effect on *M. aeruginosa* growth.

4. Conclusions

Our study showed that the inhibitory effect of *S. trifolia* tuber aqueous extract on the growth of *M. aeruginosa* was obvious compared to the controls. The cyanobacterial cells turned yellow and deposited gradually under exposure to *S. trifolia* tuber aqueous extract. The changes of Chl-*a* concentration, MDA level and the antioxidant defense system (from activation to exhaustion) demonstrated in terms of physiology that *M. aeruginosa* cells were subjected to oxidative damage, which was probably caused by the allelopathic compounds from the *S. trifolia* tuber aqueous extract.

However it should be noted that the result of this research is limited and more detailed works are needed in further studies. We need to know whether or not *S. trifolia* tuber aqueous extract inhibited the growth of *M. aeruginosa* by other mechanisms besides lipid peroxidation and oxidative damage. Thus, more detailed potential mechanisms of the inhibitory effect, such as effects on the process of photosynthesis and respiration of the cyanobacteria, should be further studied.

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