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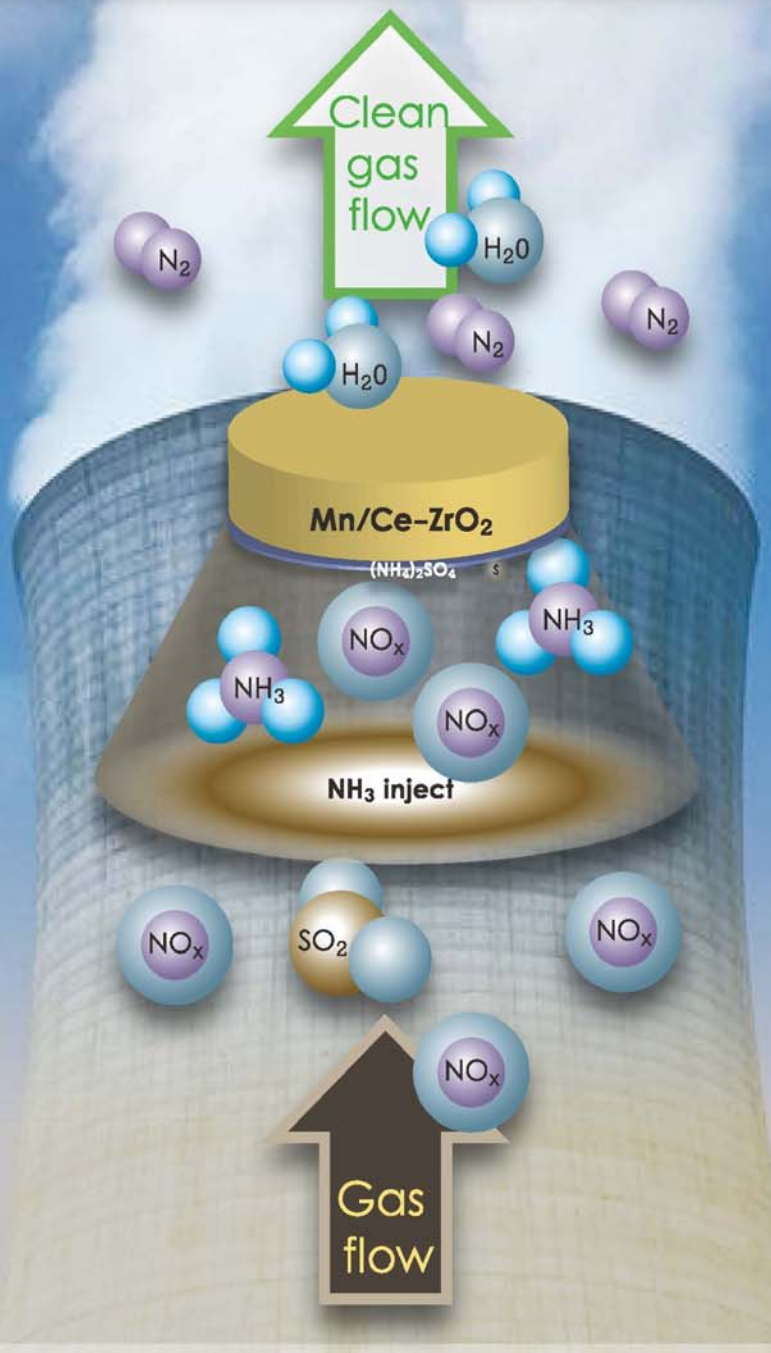

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CONTENTS

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Allelopathic effects of gallic acid from *Aegiceras corniculatum* on *Cyclotella caspia*

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Abstract

High abundance of algae and eutrophication were observed in mangrove wetlands and these were estimated to be associated with root exudates of some specific mangrove plants to a certain extent. Root exudates form allelopathic effects from mangroves. The main secondary metabolites of *Aegiceras corniculatum* had been detected to be organic phenolic acids. Gallic acid had been isolated and identified from *A. corniculatum*. The half-maximal inhibitory concentration of gallic acid on alge *Cyclotella caspia* was tested as 15.46 mg/L. The effects on algal cell morphology were mainly shown as elongated cells, with no apparent cell inclusions, such as oil droplets, chloroplast. At a dose of 2 mg/L, gallic acid had a stimulative effect on the specific growth rate of algae on day 3. The contents of malondialdehyde, superoxide dismutase, soluble carbohydrates and chlorophyll *a* in algal cells showed an overall "low promotion and high suppression". Our results could provide preliminary and valuable reference on the complex influences of mangroves on microecology and microbial communities in the rhizosphere system.

Key words: mangrove; root exdutes; microalgae; allelopathy; phenolic acids; gallic acid
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Introduction

Allelochemicals are a class of substances that plants release into their environment; these allelochemicals may have beneficial or adverse effects on other plants or microorganisms. The phenomenon of impact of allelochemicals on other plants or microorganisms is known as allelopathy. A variety of allelochemicals have been detected; the common allelochemicals are low-molecular weight organic acids, long-chain fatty acids, phenolic acids, terpenoids, and so on, and are characterized by functional characteristics, such as specificity, selectivity, complexity, diversity, etc. Allelochemicals are basically secondary metabolites of plant compounds. Phenolic acid is one such common secondary metabolite. Most phenolic acids, which are characterized by low molecular weight and simple structure, have strong allelopathic activities. So far, allelopathy has generally explored the species of allelochemicals, their mechanisms of production and secretion, isolation and identification of allelochemicals, their degree of allelopathy, and so on. Mangroves are evergreen, broad-leaved, forest plant communities that grow in tropical, subtropical coastal and estuarine intertidal zones and are predominantly represented by the Rhizophoraceae mangrove plant species. Mangrove ecosystems exist in a damp environment, with species of microorganisms and plankton, and play a significant and leading role in material recycling, energy flow, and biotic control in the forest; these activities are mostly associated with the root exudates of mangroves. Root exudates comprise the secondary metabolites of plants and form the basis for the allelopathic effect of mangrove plants.

In recent decades, there have been advances in research into allelopathic inhibition on algae. There is extensive domestic and international research data on allelopathy in the published literature. Allelopathy of the algicidal compound N-phenyl-2-naphthylamine was found exudates from the root of *Eichhornia crassipes* (Sun et al., 1993). Phenolic acid allelochemicals secreted by *Myriophyllum aquaticum* have been reported to effectively inhibit the alkaline phosphatase activity of algal cells and, therefore, influence the growth of algae (Gross et al., 1996). In addition, *Myriophyllum spicatum* has been reported to produce ellagic acid, gallic acid, vicinal, triphenol, and protocatechuic acid and, thereby, inhibit the growth of *Microcystis aeruginosa* (Nakai et al., 2000). Amide and

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cyanoethyl acetic acid substances isolated and identified from *E. crassipes* were shown to inhibit growth in *Scenedesmus obliquus* (Jin et al., 2003). Allelochemical ethyl-2-methyl acetoacetate was extracted and identified from wetland plant reed, and had notable inhibitory effects on, *M. aeruginosa*, *Chlorella pyrenoidosa*, *S. obliquus*, and other similar algae (Li and Hu, 2005). Further, Yang et al. (2005) has shown that certain types of phenolic acids, such as, vanillin, gallic acid, catechin, and others, have an obvious inhibitory effect on the growth of *Tamar alexander*. Ding et al. (2007) found that diphenol and gallic acid had growth-inhibitory effects on *M. aeruginosa*.

Research has shown that the *Kandelia candel* root secretes low-molecular weight organic acids such as formic acid, butyric acid, malic acid, citric acid, lactic acid, and others (Lu and Yan, 2007). The main secondary metabolites of *Aegiceras corniculatum* are triterpene, steroidal, flavonoids, hydroxyl benzoquinone, phenolic acids, and other organic compounds. Xu and Long (2009) and Zhang et al. (2005) isolated and identified gallic acid as an allelochemical released by *A. corniculatum*.

Gallic acid is a phenolic acid that is a secondary metabolite in a variety of plant roots and stems. With the chemical formula is $C_6H_2(OH)_3COOH$, and called 3,4,5-trihydroxy benzoic acid, it has a molecular weight of 170.12 and is easily water soluble.

In this study, we aimed to determine the effects of mangroves on algal growth by screening the mangrove species and significantly affected algal species by conducting laboratory experiments (Li et al., 2010; Huang et al., 2011). Different concentrations of gallic acid were used to study the effect of stress on the cultivation of algae and periodic observations of algal density and morphology, determination of concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), soluble carbohydrates (SC), chlorophyll *a* (Chl-*a*), and other parameters to explore the effects and mechanisms of mangrove plant root exudates on algal growth and physiology. The results could potentially enrich the basic ecological research data on the relationship between mangroves and algae, and could provide valuable reference on the complex influences of mangroves on micro-ecology and microbial communities in rhizosphere system.

1 Materials and methods

1.1 Cultivatation of *A. corniculatum* seedlings

A. corniculatum seedlings, with annual growth and average height of 30–40 cm, were collected from the Shenzhen Mangrove Nursery, China. Rhizosphere bags were prepared with a height of 10 cm and with a diameter of 5 cm using 500 mesh nylon net (30 μm). The roots were washed and mounted into the bag that was filled with 70 g of rhizosphere soil. Then, one bag was placed in an individual plastic basin (0.50 m × 0.35 m × 0.26 m) with soil depth of 18 cm and water flooding of ca. 2 cm, and cultivated in the experimental greenhouse.

1.2 Isolation, purification and cultivation of alga

Based on the field surveys on the mangrove wetlands for several years, it was defined that *Cyclotella* is the most common genus in a whole year and its dominance was as high as 82% to 97%, so *Cyclotella* sp. was identified as the target species. By separation, purification and culturing for approximately 3 months, the pure cells could be obtained (Liu et al., 2006; Li et al., 2008).

1.3 Half-maximal inhibitory concentration (IC_{50})

A liquor of 1 g/L gallic acid was prepared using 0.22 μm syringe filter sterilization under aseptic conditions. The concentration gradients were specified as 0, 2, 4, 6, 10, and 20 mg/L to be carried out with three replicates. The gallic acid mother liquor and the culture medium were mixed in the bottles, and the total volume was made up to 100 mL; then, the mixture was inoculated with 5 mL of the logarithmic growth-phase algal solution. Chl-*a* content on day 6 was selected as the basis for calculating inhibition efficiency (R, %) as Eq. (1):

$$R = (1 - \frac{C}{C_0}) \times 100\% \quad (1)$$

where, C_0 (mg/L) was the Chl-*a* contents of control, C (mg/L) was the Chl-*a* contents of gallic acids added.

With the gallic acid concentrations plotted along the abscissa and inhibition rates along the ordinate, an inhibition curve was obtained. Further calculations were used to identify the gallic acid concentration at which *C. caspia* growth was inhibited to 50%, namely IC_{50}.

1.4 Effects of gallic acid on algae growth and physiology

The concentration of the primary sample was made up to 1 g/L gallic acid under aseptic conditions using 0.22 μm syringe filter sterilization. The concentration gradient was set to 0, 2, 5, 10, 20, and 30 mg/L for the experiments and three replicates of each treatment were carried out. The gallic acid primary sample and medium were added, and the total volume was made up to 200 mL; then, 20 mL of algal solution in the logarithmic growth phase was incubated. A 2-mL sample was drawn and used to observe the algal density; this was considered the initial (day 0) algal density. Every three days, the algal liquid was observed and algal growth changes were photographed. The algal solution was sampled and tested to determine MDA content, SOD activity, and SC and Chl-*a* content on day 6 and 12.

1.4.1 Effects on algal specific growth rate and morphology

Under aseptic conditions, 1 mL of uniform algal liquid was sampled. With the counting data recorded on day 0, the

specific growth rate μ (day^{-1}) was determined using the base algal number, as described by Eq. (2).

$$\mu = \frac{\ln(\frac{X_t}{X_0})}{t} \quad (2)$$

where, X_t (ind/mL) denoted the algal cell number on day t, X_0 (ind/mL) denotes algal cell number on day 0. At the same time, cell morphology was photographed using 10 × 40 magnification display.

1.4.2 Algal physiological index

The thiobarbituric acid method was used for the determination of MDA (Shanghai Institute of Plant Physiology, Chinese Academy of Sciences, 1999). Algal cells were obtained by centrifugation, and ground into a homogenate by adding 5 mL of 80% TCA and a small quantity of quartz sand. The mixture was centrifuged for 10 min at 4000 r/min; then 2 mL of supernatant was collected and added to 2 mL of 0.6% TBA and placed in a boiling water bath extraction for 10 min. The mixture was again centrifuged for 15 min at 3000 r/min after cooling. The supernatant was collected and its volume and absorbance value were measured, with 0.6% TBA solution as the blank for the determination processes. MDA content ($C_{\text{MDA-cont.}}$, μmol/10^9 cells) was determined according to Eqs. (3) and (4).

$$C_{\text{MDA-conc}} = 6.45(A_{532} - A_{600}) - 0.56A_{450} \quad (3)$$

$$C_{\text{MDA-cont}} = \frac{C_{\text{MDA-conc}} \times 100 \times V_1}{V_2 \times N} \quad (4)$$

where, $C_{\text{MDA-conc}}$ (μmol/L) is the MDA concentration, A is the absorbance value at different wavelength; V_1(mL) is the total volume of extraction, V_2 (mL) is the volume of algal liquid, N is the algal density, in 10^4 individual/mL (ind/mL).

A photochemical reduction method with nitro blue tetrazolium was used for the determination of SOD activity (Hao et al., 2004). For SOD enzyme coarse liquid extraction, algal cells were obtained by centrifugation, ground in a glass bowl to a homogenate with the addition of 0.05 mmol/L phosphate buffer solution of 10 mL (pH 7.8, including 1% polyvinylpyrrolidone) and a small amount of quartz sand; this mixture was centrifuged for 15 min at 4°C and 10,000 r/min; the supernatant obtained was considered a crude enzyme solution and refrigerated at 0°C.

Chromogenic reaction mixture was prepared using two 10 mL glass tubes. One for the blank, shaded by double black cardboard sleeve, and the other for the test reaction for 15 min under a 4000 Lx daylight lamp at the same time.

To measure SOD activity, OD value is determined in 560 nm of each tube. A unit of enzyme activity (U) was determined by inhibiting nitro blue tetrazolium photochemical reduction of 50%, and the total SOD activity (SOD, U/10^5 cells) was calculated as indicated in Eq. (5).

$$\text{SOD} = \frac{(A_{\text{control}} - A_{\text{e}}) \times V_{\text{T}} \times 10}{0.5 \times V_3 \times A_{\text{control}} \times V_4 \times N} \quad (5)$$

where, A_{control} is the absorbance of control, A_{e} is the absorbance of the sample tubes, V_{T} (mL) is the total extract volume, V_3 (mL) is the volume of the enzyme solution used in the determination, V_4 (mL) is the volume of the centrifuged algal liquid.

The SC content was determined by the anthrone-sulphuric acid colorimetric method (Li, 2007). For preparation of standard curve, the glucose standard solution, the anthrone reagent and H_2SO_4 were used to make the standard curve, as shown in **Table 1**. The sample was mixed uniformly immediately after addition of the glucose standard solution (100 μg/mL) and water for each tube; the anthrone reagent (1 mg/mL) was added and the mixture was placed in a boiling water bath and heated for 7 min, and then rapidly cooled in an ice bath. After cooling to room temperature, the optical absorption value at 630 nm was measured with first tube, which was the blank. The concentrations of SC in the samples (μg) in 2–7 solution tubes were plotted as the abscissa and the absorbance (OD_{630}) as the ordinate, to obtain a standard curve of sugar content and the OD_{630} value.

Algal liquid samples were centrifuged to obtain algal cells. Setting the constant volume to 25 mL by using distilled water, 2 mL of sample was drawn into a colorimetric tube to measure the light absorption value at 630 nm, in accordance with the procedure for the preparation of the standard curve. Then, the values were checked against the standard curve for determination of SC.

Calculation of SC content (C_{SC}, mg/10^7 cells) was shown in Eq. (6):

$$C_{\text{SC}} = \frac{c \times V_5}{V_6 \times V_7 \times N} \quad (6)$$

where, c (μg) is the amount of reducing sugar checked against a standard curve, V_5 (mL) is the total volume of extracted liquid, V_6 (mL) is the volume of extraction added

Table 1 Standard curve for the determination of soluble carbohydrate content

Reagent	1	2	3	4	5	6	7
Glucose standard solution (100 μg/mL)	0	0.1	0.2	0.3	0.4	0.6	0.8
H_2O (mL)	2.0	1.9	1.8	1.7	1.6	1.4	1.2
Anthrone reagent (1 mg/mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H_2SO_4 (98%)	5	5	5	5	5	5	5

in the colour systems, V_7 (mL) is the volume of the algal liquid.

The algal cells were collected by using a 3.0 μm glass fibre membrane to filter the algal liquid. The membrane was put into a 10 mL tube with 5 mL of 95% ethanol for extraction in darkness for 2 hr, and then centrifuged; the supernatant was obtained and made up to a constant volume of 10 mL. With 95% ethanol as the control, photometric density (OD) was determined and the Chl-*a* content ($C_{\text{Chl-}a}$, mg/L) was calculated by Eq. (7):

$$C_{\text{Chl-}a} = \frac{(13.95 \times A_{665} - 6.88 \times A_{649}) \times V_8}{V_9} \quad (7)$$

where, V_8 (mL) is the final volume of extract and V_9 (mL) is the volume of the algal liquid.

1.5 Experiment equipments and reagents

Equipments for the experiments included a light incubator (LRH-400-G, Thaihong Medical Equipment Limited Company, Shaoguan, China), spectrophotometer (W2450, Shimadzu, Japan), microscope (Olympus CX21 with Canon PC1252 camera, Japan), high-speed refrigerated centrifuge (D-78532, Hettich, Germany).

The main reagents for the reaction include anthrone, TCA, thiobarbituric acid, riboflavin and nitro blue tetrazolium, all from Acros Organics, Belgium. All reagents are were of analytical grade and used without further purification or processing.

1.6 Data analysis

The experimental data were assimilated using Excel and analysed with SPSS17.0; Origin 8.0 was used for the data mapping.

2 Results

2.1 Separation, purification and cultivation of algal species

Pure algal species obtained after isolation was identified as *Cyclotella caspia* Qi 1997, as shown in **Fig. 1** (Guo and Qian, 2003). Morphologically normal (control) cells were filled with chloroplasts, had a regular short cylindrical shape, and were yellow-green in color. The length of the algal apical axis was 9.80 μm from the valve view and of the pervalvar axis was 12.25 μm from the girdle view. The most special of the cell is that the girdle view could be near square.

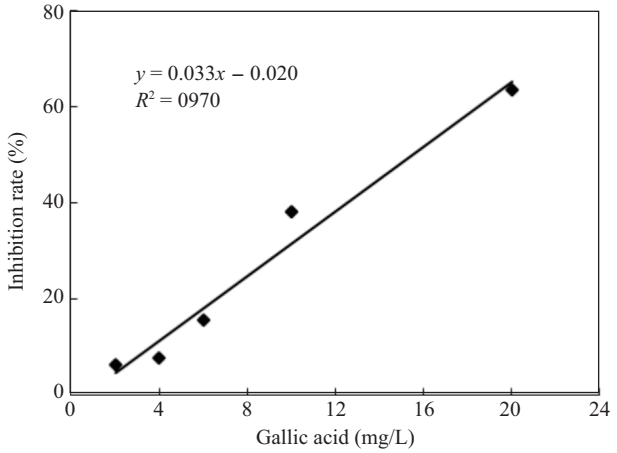

Fig. 2 Correlation relationships between the contents of gallic acid and the inhibition rate.

2.2 Half-maximal inhibitory concentration of gallic acid on *C. caspia*

The rates of inhibition at gallic acid concentrations of 2, 4, 6, 10, and 20 mg/L were 6.34%, 7.68%, 15.55%, 38.14%, and 63.40%, respectively (**Fig. 2**). For correlational analysis, values of gallic acid concentration were plotted on the abscissa and the rate of inhibition on the ordinate. A linear regression equation was obtained as $y = 0.0337x - 0.0209$, $R^2 = 0.9707$; in the F test, $F = 99.52$, $F_{0.01}(1, 3) = 34.12$; therefore, as $F > F_{0.01}$, the regression equation was considered to be accurate. From the regression equation, the concentration at which algal growth was inhibited by 50% (IC_{50}) was calculated as 15.46 mg/L.

2.3 Effects of gallic acid on specific growth rate of algae

Details of the specific growth rate of *C. caspia* under the influence of gallic acid are shown in **Table 2**. Overall, the specific growth rate decreased with increase in gallic acid concentration. However, at a low concentration (2 mg/L) of gallic acid, the maximum specific growth rate on day 3

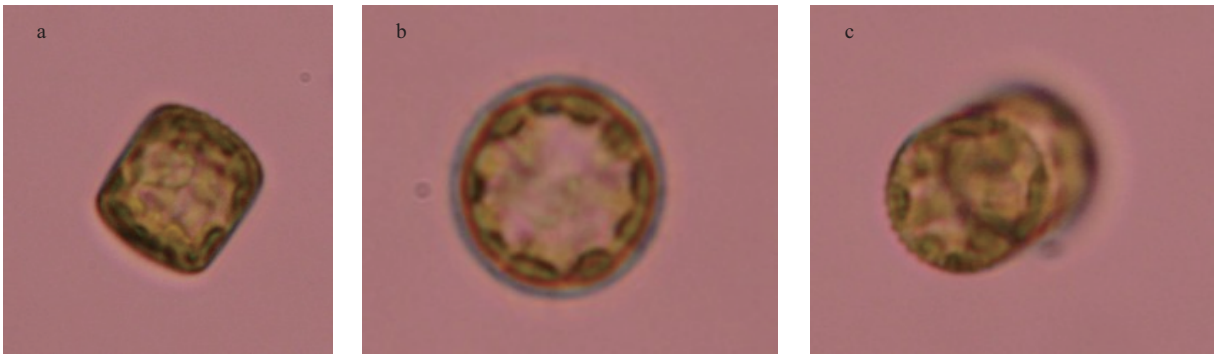

Fig. 1 Pure algae species of *Cyclotella caspia*. (a) girdle view; (b) valve view; (c) girdle view and valve view.

Table 2 Effects of gallic acid on specific growth rate of *Cyclotella caspia*

Gallic acid (mg)	Specific growth rate of *C. caspia* (day^{-1})				
	Day 3	Day 6	Day 9	Day 12	Day 15
0	0.62	0.40	0.31	0.27	0.24
2	0.64	0.38	0.30	0.26	0.22
5	0.58	0.36	0.28	0.24	0.22
10	0.49	0.36	0.28	0.24	0.22
20	0.51	0.32	0.28	0.24	0.20
30	0.34	0.23	0.22	0.19	0.17

showed an increasing trend as compared with the control, but the rate decreased after day 6. High concentrations (≥ 5 mg/L) of gallic acid have been associated with declining growth trends from day 3, illustrating the inhibition of algal cell growth.

2.4 Effects of gallic acid on algal morphology

On microscopic observation, cell morphology appeared to have become deformed under the influence of gallic acid; the changes were mainly marked with elongated cells, lighter color of chlorophyll, fewer oil droplets and chloroplast, and fuzzy cell inclusions. In the 5 mg/L treatment group on day 12, more numbers of oil droplets appeared; however, in the 30 mg/L group on day 9, there were more oil droplets, as shown in **Fig. 3**. With increase in gallic acid concentration and the extended duration of culture, algal cell numbers were observed to have increased trend. Chlorosis of algal cells appeared at high gallic acid concentrations of 20 and 30 mg/L on day 12, and had a lethal effect on algal cells.

2.5 Effects of gallic acid on algal physiology

Effects of different concentrations of gallic acid on algal MDA contents, algal SOD activity, algal SC contents, and algal Chl-*a* contents are shown in **Fig. 4**.

As shown in **Fig. 4A**, there is an increasing trend in MDA content, and it reached a maximum value at gallic acid concentration of 30 mg/L. On day 6, at gallic acid concentration of 2, 5, 10, 20, and 30 mg/L, the MDA concentration was 1.39, 2.27, 2.19, 3.93, and 8.01 times that of the control, respectively, and, thus, showed significant differences compared with the control. The MDA content on day 12 was less than that on day 6. At a gallic acid concentration of 5 mg/L, MDA content in all the treatment groups was higher than that of the control, and was 1.09, 1.67, 3.10, and 6.79 times that of the control; however, a significant difference was observed only for gallic acid concentrations of 20 and 30 mg/L.

In **Fig. 4B**, with gallic acid concentrations of 5, 10, 20, and 30 mg/L, enzyme activities of SOD were increased by 12.48%, 14.63%, 17.40%, and 18.35%, respectively, as compared with the control on day 6, but the difference was not statistically significant. On day 12, the enzyme activity was the highest. At gallic acid concentrations of 5 and 30 mg/L, SOD activity reached 1.00 and 0.82 $U/10^5$ cells and was 4.36 and 2.54 times that of the control, respectively; however, the enzyme activities at 10 and 20 mg/L gallic acid were less than that at 5 and 30 mg/L.

Figure 4C shows changes in SC content in *C. caspia* under the influence of gallic acid. On day 6, SC content increased in response to stress at gallic acid concentrations 2, 5, 10, 20, of 30 mg/L, and SC content was 1.15, 1.11, 1.45, 1.86, and 3.46 times, respectively, compared with the control, and showed significant difference at gallic acid concentrations ⩾ 5 mg/L. With increase in the rate of algal growth and metabolism, algal SC content in the control group was normal or decreased slightly; however, the SC contents in algal cells decreased obviously in gallic acid treatment groups. At a gallic acid concentration of 30 mg/L, SC content on day 12 was only 16.71% that of the value on day 6, indicating that gallic acid had affected the normal metabolic process in algal cells.

Figure 4D shows changes in Chl-*a* content of *C. caspia* under the influence of gallic acid. Chl-*a* contents showed decreasing trends and consistency with changes in algal density. On day 6 of algal growth at gallic acid concentrations of 5, 10, and 20 mg/L, Chl-*a* contents decreased to 21.98%–61.66% that of the control and the difference was statistically significant. The minimum Chl-*a* value appeared at a gallic acid concentration of 30 mg/L, as 0.063 mg/L and decreased to 78.66% that of the control, indicating remarkable differences. Change in Chl-*a* content on day 12 was almost the same as that on day 6. With increasing concentrations, of the decrease in Chl-*a* contents became more marked as compared with the control.

3 Discussion

3.1 Effects of gallic acid on algae with the relationship of gallic acid structure

It was hypothesized that, as with phenolic hydroxyl group structure of phenolic acids compounds, when there is a transition, metal ions are generated and induce autoxidation (Furukawa et al., 2003). Everall and Lees (1997) showed that the inhibitory effect of stalk was associated with the phenolic acids compounds, especially with the synergistic effect of phenol oxidation. Nakai et al. (2001) pointed out that the autoxidation of phenolic acids, such as catechol, phloroglucinol, pyrogallol and gallic acid was responsible for growth inhibition of *M. aeruginosa*.

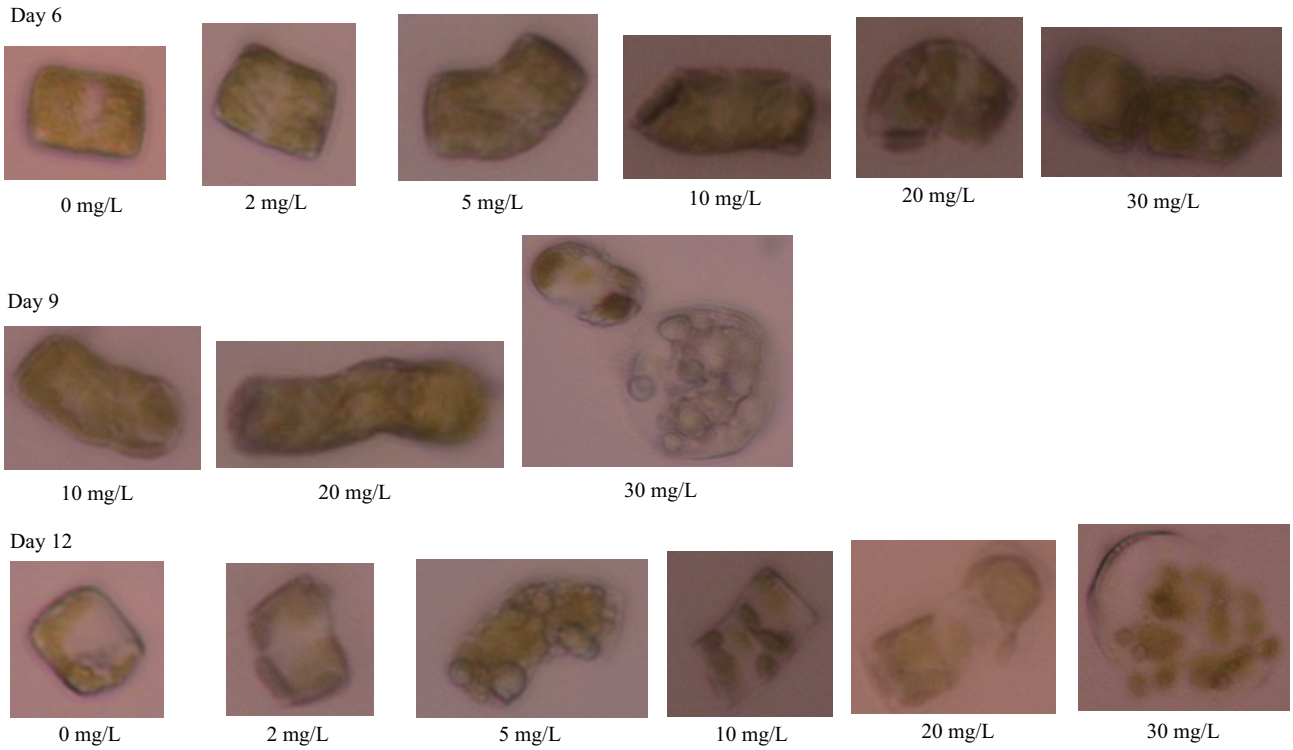

Fig. 3 Effects of different concentrations of gallic acid on cell morphology of *Cyclotella caspia* (10 × 40).

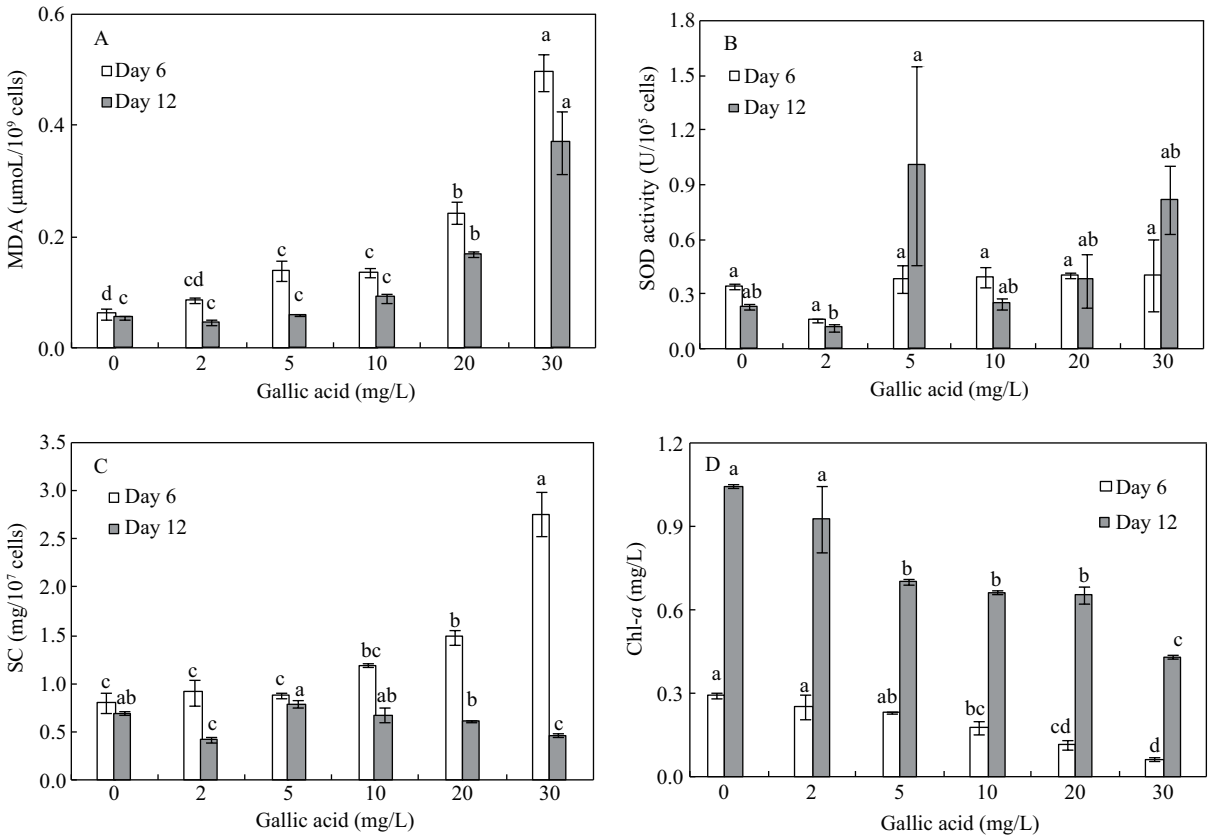

Fig. 4 Effects of gallic acid on algal MDA contents (A), SOD activity (B), SC contents (C), and Chl-*a* contents (D) in *Cyclotella caspia*. Different letters indicate significant differences.

Other studies on the inhibitory effects of acids, phenols, phenolic acids, and other substances on *M. aeruginosa*, found that acids did not have an inhibitory effect on *M. aeruginosa*, whereas the inhibitory effects of phenols and phenolic acids were associated with their autoxidation; the strength of the effect was closely linked with the number of hydroxyl groups and their relative position on benzene rings (Nakai et al., 2001). The autoxidative capacity of phenolic acids is the main mechanism modulating its allelopathy, with wide applicability, and may be closely related to its degree of growth inhibition of algae (Sun et al., 2010). The *f*/2 medium used in the present study contained specific concentrations of Cu^{2+} and Fe^{3+}, and could meet the conditions of phenolic acid autoxidization; this led to the hypothesis that the growth-inhibitory effects of gallic acid on *C. caspia* were associated with the autooxidation of gallic acid.

3.2 Effects of gallic acid on algal growth

3.2.1 Half-maximal inhibitory concentration

Yang et al. (2005) found that gallic acid concentrations of 4, 20, and 40 mg/L had algal growth-inhibitory effects on *T. alexander* in an obvious dose-dependent manner; the growth of algae was completely inhibited at the concentration of 40 mg/L. Although the IC_{50} of gallic acid on *M. aeruginosa* was 1.73 mg/L, at gallic acid concentration of 2 mg/L, the rate of inhibition of *M. aeruginosa* reached 72.5% (Ding et al., 2007). The *p*-hydroxybenzoic acid at a concentration of 0.6 mmol/L (0.083 mg/L) can induce growth-inhibitory effects on *Anabaena flosaquae* and *Chlorella pyrenoidosa* (Zhang et al., 2007); concentrations > 1.6 mmol/L can significantly inhibit the algal growth of *T. alexander* and *Karenia mikimitoi* (Sun et al., 2010). This study found that IC_{50} of gallic acid on *C. caspia* was 15.46 mg/L, indicating that different allelochemicals have different allelopathic effects on different algal species. The effect of *p*-hydroxybenzoic acid is stronger than that of gallic acid. This could be because, being a diatom, *C. caspia* has a silica shell; therefore, its IC_{50} was high and, after 12 days of treatment with concentrations of 20 and 30 mg/L, algae began to show lethal effects. *M. aeruginosa* was quite sensitive to gallic acid, with a sensitivity that was approximately 10 times that of *C. caspia*. In this research, the authors proposed that *C. caspia*, being a single-celled diatom, is more suited for toxicity testing.

3.2.2 Algal cell structures

The overall effect of gallic acid on *C. caspia* was inhibition, and the high concentration was proportionately related to growth inhibition and lower density of algal growth. At 2 mg/L on day 3, gallica acid had a promoting role, demonstrating a "low promotion and high suppression" phenomenon, similar to most allelochemicals. In an actual mangrove forest, gallic acid concentrations much lower than 2 mg/L and, therefore, mangroves will promote the growth of algae to a certain degree.

Allelopathic substances will/can damage cell structures. Allelochemicals secreted by reeds can increase the appearance of intracellular starch grains, increase in volume and number, loss of nuclei, and disintegration of cellular organelles such as mitochondria in *S. obliquus* (Men et al., 2006). Under the influence of N-phenyl-2-naphthylamine, plasmolysis, smaller nuclei, change in chloroplast structure and damage to cell structures was seen in *Chlorella* spp. (Qian et al., 2009). The reed allelochemical ethyl-2-methyl acetoacetate had effects on the composition and content of cell membrane phospholipid fatty acids in *C. pyrenoidosa* and *M. aeruginosa*, and studies found that the concentration of unsaturated fatty acids in cell membrane of the two algae species had increased, indicative of cell membrane destruction in these two algal species (Li et al., 2007).

The effects of gallic acid on *C. caspia* were mainly expressed by elongated cells in cell morphology, although there was no appearance of cell inclusions, such as starch grains, oil droplets, chloroplast, etc., which may have been because gallic acid caused greater damage on internal cell structure; the appearance of oil droplets is an important phenomenon in the destruction of cell membrane structure. More oil droplets appeared in the 5 mg/L treatment group on day 12 and 30 mg/L group on day 9, indicating that the 5 mg/L group on day 12 demonstrated a greater effect on the cell membrane, whereas a significant effect for the 30 mg/L group was observed on day 9. This led to the conclusion that the effects of gallic acid on algal morphology were dose dependent.

3.3 Algal physiology

Allelopathic substances can alter selective permeability of cell membrane and affect the membrane liquidity. When the cell membrane damaged, it may cause membrane lipid peroxidation and generate a peroxidation byproduct, MDA; which can be used as an indicator of cell membrane injury. Phenolic acid autoxidation can induce production of H_2O_2 and quinone, H_2O_2 can directly cause lipid peroxidation in cells (Furukawa, 2003). The inhibitory phenomenon of phenolic acids on algae was characterized by lipid peroxidation of the cell membrane (Zhang et al., 2007).

Under the influence of gallic acid, MDA contents in *C. caspia* on day 6 were increased, indicating that gallic acid concentrations of 2–30 mg/L caused membrane peroxidation damage to the algal cells, particularly at a concentration of 30 mg/L. MDA contents on day 12 were lower than that on day 6 in all treatment groups, indicating that the high concentration had caused greater damage to the cell membrane such that cells were unable to produce more MDA. It could be that gallic acid alters membrane permselectivity, induces membrane damage, and eventually entering the cell, causing changes in cytoplasmic osmotic pressure, thus disturbing cellular metabolism. The

higher the concentration of gallic acid, the greater is the disturbance and the higher content of MDA, eventually leading to the disruption of the cell membrane and the cell-killing effect.

SOD is a type of metallic enzyme extensively present in organisms that converts superoxide anions (O_2^-) to H_2O_2 and O_2 by disproportionate reaction of catalysis, and it reduces or prevents damage resulting from excessive superoxide free radical anion produced during metabolic processes. SOD is the only enzyme that uses free radicals as a substrate, and plays a very important role in the maintenance of dynamic balance in metabolism within the organism.

Benzenetriol can increase intracellular SOD activity in *M. aeruginosa* (Shao et al., 2009). *E. crassipes* secretions can stimulate SOD activity in *Scenedesmus* sp. algae cell, causing it to increase first and later decrease (Tang et al., 2000). The phenomenon of inhibition of phenolic acids on algae occurs as a result of increase in intracellular $O_2^-\cdot$ free radicals, and the activity of SOD increases or decreases (Hua et al., 2008). Phenolic acid autoxidation can induce production of H_2O_2 and quinone; quinones are potential peroxides and can generate reactive oxygen species ROS through a redox cycle posing a hazard to cell growth (Bors et al., 2000).

SOD enzyme activity at gallic acid concentration of 2 mg/L decreased in comparison with the control, indicating that 2 mg/L gallic acid did not produce an inhibitory effect on algae. At gallic acid concentrations of 5–30 mg/L, SOD activity increased slightly on day 6 indicating that gallic acid did not have a greater effect on algal growth on day 6. However, SOD enzyme activity increased dramatically in the 5 mg/L gallic acid group on day 12, indicating that the cincetration produced significant inhibitory effects on algae (could also be seen from cell morphology). SOD enzyme activities at 10 and 20 mg/L on day 12 decreased compared with that on day 6 indicating that 10 and 20 mg/L gallic acid had a lethal effect on algae, and the SOD enzyme had no ability to repair itself. However, SOD enzyme activity increased at 30 mg/L and on day 12 making these results difficult to interpret. SOD enzyme activity could better reflect the effects of gallic acid on algal growth stress and physiological status.

Algal cells can adapt to environmental changes by rapid synthesis of osmoregulatory substances, such as polyol or its derivatives, sugar or polysaccharides and certain amino acids, such that the adjustment of cell osmotic pressure occurs in a timely manner. SC is one of the main mechanisms for osmotic adjustment in algae, and has a protective effect on lipid membranes. Under salt stress, the SC content in seaweed decreases with increase in salinity; this is because the key enzyme activity associated with the synthesis of sucrose will decrease during high salinity stress (Touchette, 2007). Under the action of gallic acid, the SC contents in each treatment group were all increased on day 6 in comparison to the control groups, indicating that, in the initial stage, algal cells could alleviate membrane lipid peroxidation by osmotic adjustment. Thereafter, cell growth and metabolism became inhibited, and SC content decreased on day 12; therefore, after long-term exposure to stress, algae did not have the capacity to sustain stress and, eventually, a lethal effect appeared.

Allelochemicals can disrupt and even collapse mitochondrial structure. Mitochondria are closely related to respiration, and an adverse impact on cell respiration would occur secondary to mitochondrial damage. The phenomenon of the inhibition by phenolic acids causes decrease of algal growth and Chl-*a* content (Zhang et al., 2007; Hua et al., 2008). Different from the other parameters, under the action of gallic acid, although the Chl-*a* contents were all decreased as compared with controls, the Chl-*a* contents on day 12 showed a steep increase as compared to values on day 6. It revealed that, on one hand, although in general gallic acid showed an inhibitory effect as compared with controls, in fact, gallic acid had a certain role in promotion of chloroplast at a concentration of 30 mg/L gallic acid and on day 12, when chloroplast still retained metabolic activities. On the other hand, Chloroplast had strongest tolerance, and penetration of gallic acid did not have a significant impact on chloroplast, except to stimulate algal cells to produce more chloroplast to cope with stress. Chloroplast is an important cell component to combat stress and maintain algal growth.

4 Conclusions

The effect of gallic acid on algae has a direct relationship with its structure; gallic acid has three hydroxyl groups and belongs to the class of phenolic acids, its autoxidation could adversely impact cell growth. The IC_{50} of gallic acid on *C. caspia* was 15.46 mg/L. The effects of gallic acid on cell inner structure were associated with concentration and duration of action. Effects on cell morphology of gallic acid were represented by elongated cells, absence of cell inclusions such as starch grains, oil droplets and chloroplast. Gallic acid led to cell membrane damage and decreased MDA content on day 12. The increased amplitude of SOD enzyme activity was greatest at 5 mg/L gallic acid on day 12. At the first 6 days, algal cells could alleviate membrane lipid peroxidation by increasing SC content and osmotic adjustment. It had been concluded that chloroplast is an important cell organelle that helps to combat stress and maintain algal growth.

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