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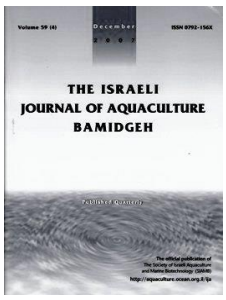

Control of *Microcystis aeruginosa* with Galla chinensis

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Key words: product of Chinese forests; Chinese medicine; Galla chinensis (nutgall); inhibitory effects; *Microcystis aeruginosa*

Abstract

Galla chinensis (nutgall), used in traditional Chinese medicine, is a product extracted from nutgall trees in Chinese forests. In this study, *Microcystis aeruginosa*, a cyanobacterium, was exposed to different concentrations of nutgall extract obtained from Galla chinensis. With increased exposure concentration, and time, the cell density of *M. aeruginosa* was significantly reduced. After 72 h of exposure, the chlorophyll a content of *M. aeruginosa* significantly decreased; superoxide dismutase (SOD) activity increased at a low nutgall concentration but decreased at higher concentrations. Cellular ultrastructure was observed by electron microscopy. With increased nutgall concentrations, *M. aeruginosa* cell walls became wrinkled, collapsed, and ruptured. The photosynthetic thylakoid lamellae were fractured and dispersed, fewer phycobilisomes attached to the photosynthetic thylakoid membrane and were dispersed into the cytoplasm, and the number of lipid particles and cyanophycin granules increased. A large void appeared in the nuclear area and most of the algal cells died. In conclusion, these observations indicate that Galla chinensis has a significant allelopathic effect, and can effectively inhibit the growth of *M. aeruginosa.*

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Introduction

Microcystis aeruginosa, a cyanobacterium, is the dominant algal species of lakes, reservoirs, and other bodies of water in which cyanobacteria blooms occur in China (Zhang et al., 2007). *M. aeruginosa* has a high rate of bloom outbreak, which produces a cyclic heptapeptide microcystin (Carbis et al., 1994; Xu et al., 2010). This microcystin seriously damages aquatic animal livers and accumulates in their gonads (Chen and Xie, 2005). As a result of blooms in places such as Taihu (China), Patos Lagoon estuary (southern Brazil) and other places, a large number of aquatic organisms have died, water quality has seriously deteriorated (Matthiensen et al., 2000; Zou et al., 2010), aquatic ecosystem landscape has been destroyed, fishery production has been affected, and human health has been threatened via the food chain.

Allelopathy, first proposed by Molisch in 1937, describes the biochemical interactions between plants, including interactions with microorganisms. Allelopathy was defined by Rice (1984) as "a biological phenomenon by which an organism produces biochemicals that influence the growth of other organisms." According to dosing trials, Nakai et al. (1999) demonstrated that aquatic plants inhibit the growth of algae by the continuous release of allelopathic substances. In many countries, including Scotland, Canada, Australia, the USA, Sweden, and South Africa, barley straw in water successfully reduced the excessive growth of algae. Moreover, terrestrial plants have been used in extensive agricultural applications for anthelmintic properties, sterilization, and algae removal (Zhou et al., 2012). Currently in China, allelopathic traditional substances, such as Coptis chinensis (Zhang et al., 2010) are being investigated.

Galla chinensis, a traditional Chinese medicine, is a product extracted from galls produced by Chinese forest nutgall trees. It originates from abnormal growth of *Rhus chinensis* Mill and *Rhus potaninii* Maxim leaf tissue in response to infestation with parasitic aphids (family Pemphigidae). It has been used for the treatment of a wide variety of diseases for thousands of years due to its antibacterial, antiviral, anticaries and antioxidative activity. In this study, the inhibitory and allelopathic effects of nutgall extract on the growth of *M. aeruginosa* were investigated. The inhibition rate of nutgall extract on *M. aeruginosa* growth, chlorophyll a, and superoxide dismutase (SOD) levels were determined. In addition, M. aeruginosa morphology and ultrastructural changes were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). This study was undertaken to provide scientific evidence for developing an effective natural inhibitor of cyanobacterial blooms, and reduction of aquatic organism diseases. We believe that these findings will provide an environmentally friendly method for water purification and ecological management of water eutrophication.

Materials and Methods

M. aeruginosa was supplied by the Institute of Hydrobiology, Chinese Academy of Science. The dehydrated products of Galla chinensis were purchased from a Chinese pharmacy. Algae were cultured in 1,000 mL conical flasks containing 220 mL BG11 medium at 25°C ± 1°C under a 12-h light:dark cycle with a light density of 2,000 ± 200 Lux. The initial algae concentration was 1×10^6 cells/mL. The flasks were shaken 3 times per day, and their positions were randomly switched to reduce any effects related to minor changes in light intensity.

Preparation of nutgall extract solution. Galla chinensis powder (5.0 g; 80 mesh) was mixed with 100 mL of distilled water in a 250 mL volumetric flask. After 30 min of ultrasonic extraction, 100 mL of distilled water was added to adjust the volume. Immersion extraction occurred overnight in the dark. The volume was then adjusted to 250 mL, and the solution was well mixed and stored at 4℃ in the refrigerator until use.

Assay of growth inhibition. Nutgall extract solutions were formulated into 4 different concentrations with 3 replicates per concentration, as follows: 0 g/L (control; C), 0.009 g/L, 0.046 g/L, and 0.181 g/L. After filtering with 0.45 μm microporous membranes, nutgall extract solutions were added to the culture medium containing exponentially growing *M. aeruginosa*. Algal cell numbers were counted using a hemocytometer

observed under a microscope (16 × 10). Samples were removed from the cultures at 0 h, 24 h, 48 h, and 72 h for each extract concentration to measure the algal cell density.

Inhibitory rates (IR) were calculated based on the defined formula:

IR = (No - Ns)/No × 100%,

where IR is inhibitory rate (%), No is algal cell density in the C group, and Ns is algal cell density in the treatment group (Chai et al., 2010).

Assays for chlorophyll a content and SOD activity. According to a previously described method (Chai et al. 2010), cultures were exposed for 72 h to different concentrations of nutgall extract solution (0 g/L, 0.009 g/L, 0.046 g/L, and 0.182 g/L) to determine the chlorophyll a content. SOD activity was measured by the nitro blue tetrazolium photochemical reduction method, as described previously (Stewert and Bewley, 1980).

Scanning Electron Microscopy (SEM) of algal cells. C group and treated algal cell samples were enriched after 72 h exposure. The enriched samples were fixed with 2.5% glutaraldehyde for 24 h before incubating at room temperature and then were fixed with 1% osmium tetroxide for 1 h. Samples were then washed three times with phosphate buffer solution (pH = 7.4). Each sample was then dehydrated with increasing concentrations of ethanol, i.e., 30%, 50%, 75%, 95%, and 100%, for 15 min per concentration. After dehydration, samples were dried for 2 h using a critical point drying apparatus (Leica EM CPD030). Dried samples were coated using an ion-sputtering instrument (Hitachi E-1045) and then observed and imaged using a Field Emission Scanning Electron Microscope (Hitachi SU8010).

Transmission Electron Microscopy (TEM) of algal cells. Procedures of algal cell enrichment, fixation, and dehydration were the same as for SEM. Dehydrated samples were embedded in Resin Epon815 at 60°C for 2 h. The embedded block was sliced with an Ultramicrotome (Leica EM UC7). After staining with uranyl acetate-lead citrate, the slices were observed and imaged with a TEM (Hitachi HT7700).

Statistics. Cultures in every assay were sampled 3 times, and replicate values were averaged. The results are expressed as arithmetic mean ± standard deviation (SD). The data were statistically analyzed by SPSS 17.0 Software. $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

Results

Effect of Galla chinensis on M. aeruginosa cell density. Algal cell density was determined at different concentrations of nutgall extract at 24 h exposure intervals (0 h, 24 h, 48 h, and 72 h), showing decreased cell density with increased nutgall concentrations and increased exposure time (Table 1).

Table 1. Effect of Galla chinensis on *Microcystis aeruginosa* cell density

Galla chinensis	*Exposure time (h), Algal density (×10⁶ cells/mL)*			
Concentration (g/L)	*0 h*	*24 h*	*48 h*	*72 h*
CK	1.235 ± 0.022	1.519 ± 0.001aA	1.834 ± 0.093aA	2.141 ± 0.097aA
0.009	1.235 ± 0.022	1.363 ± 0.163abA	1.540 ± 0.249abAB	1.731 ± 0.137bAB
0.046	1.235 ± 0.022	1.308 ± 0.175abA	1.288 ± 0.201bAB	1.301 ± 0.277cBC
0.181	1.235 ± 0.022	1.221 ± 0.001bA	1.155 ± 0.033bB	1.053 ± 0.047cC

Values are represented as means ± SD. Values with different lowercase (abc) letters indicate significant differences, $P < 0.05$; values with different uppercase (ABC) letters indicate highly significant differences, $P < 0.01$.

At 24 h and 0.181 g/L nutgall concentration, *M. aeruginosa* cell density was 1.221 × 10^6 cells/mL, which was significantly lower than the C group ($P < 0.05$), and IR was 19.6%. At 48 h and 0.046 g/L and 0.181 g/L nutgall concentrations, *M. aeruginosa* cell density was 1.288 × 10^6 cells/mL and 1.155 × 10^6 cells/mL, differences were significant ($P < 0.05$) and highly significant ($P < 0.01$) from C, and IR values were 29.8% and 37.0%, respectively. At 72 h and 0.009 g/L nutgall concentration, *M. aeruginosa* cell density was 1.731 × 10^6 cells/mL, which was significantly lower than the C group (P <

0.05), and IR was 19.1%. At 0.046 g/L and 0.181 g/L nutgall concentrations, *M. aeruginosa* cell densities were 1.301 × 10^6 cells/mL and 1.053 × 10^6 cells/mL, and significantly lower than the C group ($P < 0.01$), and IR values that were 39.2% and 50.8%, respectively. As a result, the 72-h semi-efficiency concentration (EC_{50}) of Galla chinensis on *M. aeruginosa* was calculated as 0.148 g/L, using the probability unit method.

Effect of Galla chinensis on M. aeruginosa chlorophyll content. Chlorophyll content was determined for algal cultures exposed for 72 h to different concentrations of nutgall extract (Fig. 1).

Chlorophyll content per liter of algal solution was negatively correlated with the concentration of Galla chinensis. Specifically, chlorophyll a content significantly decreased, compared with the C group ($P < 0.05$), upon exposure to a low nutgall concentration (0.009 g/L). Whereas, with increased concentrations of 0.046 g/L and 0.181 g/L, the chlorophyll content was less than 60% of that in the C group ($P < 0.01$). These results were consistent with the color changes of the algal solutions during the assay, which changed from green to yellow to brown. Based on the microscopic observation, algal cells exhibited reduced suspension ability and became lighter and more yellow, which correlated positively with the concentration of Galla chinensis.

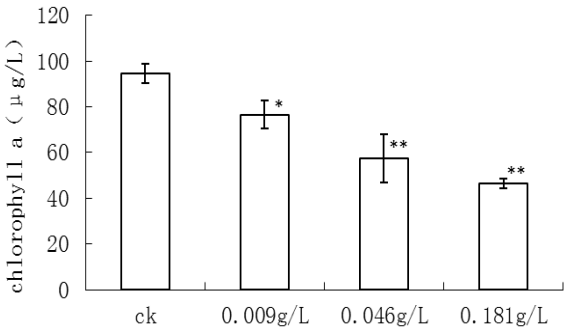


Fig.1 Effect of different concentrations of Galla chinensis on *Microcystis aeruginosa* chlorophyll a content

Effect of Galla chinensis on M. aeruginosa SOD activity. SOD activity was measured for algal cultures exposed for 72 h to different concentrations of nutgall extract (Fig. 2).

SOD are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. SOD is the only enzyme with oxygen radicals as substrates (Sarvajeet et al, 2010); thus, SOD can promptly remove excess cellular superoxide anions during stress, thereby inhibiting peroxidation of membrane lipids. SOD enzymes protect cells from oxidation. *M. aeruginosa* cells exposed to a low nutgall concentration (0.009 g/L) showed increased SOD activity which was highly significant ($P < 0.01$) compared with C (Fig. 2). On the other hand, with increased nutgall concentrations of 0.046 g/L and 0.181 g/L, SOD activity decreased to around 40% of that in C ($P < 0.01$), which suggests that algal cells were substantially damaged by such high concentrations.

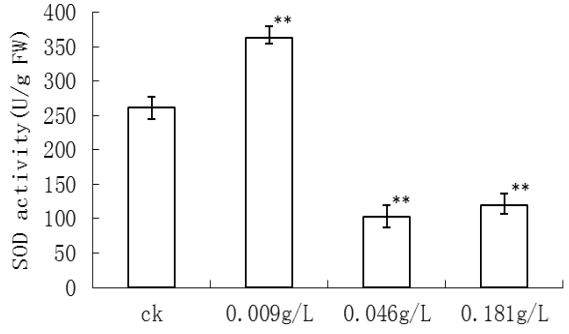
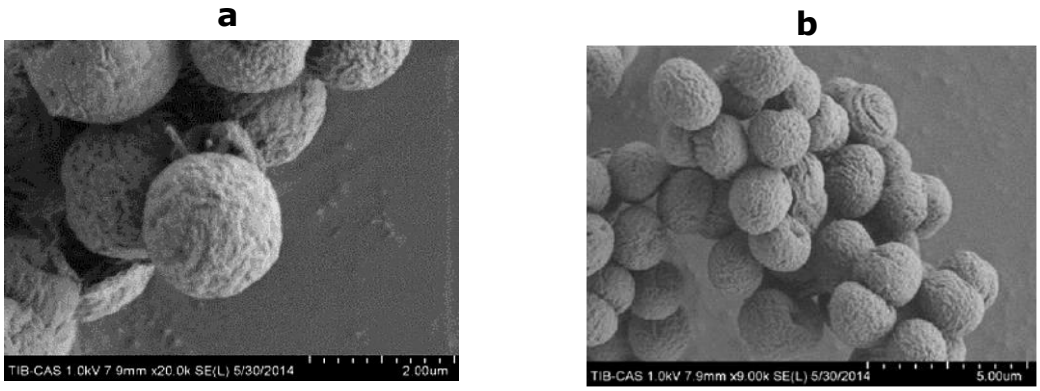
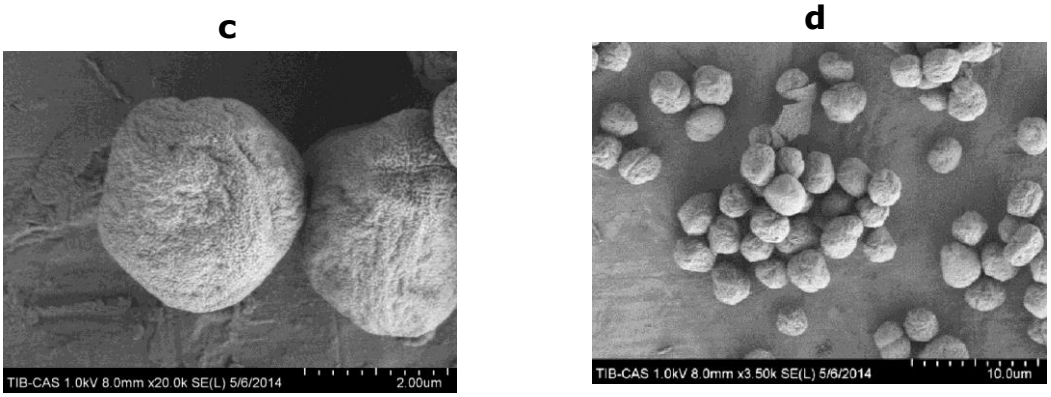


Fig.2 Effect of Galla chinensis on *Microcystis aeruginosa* SOD activity

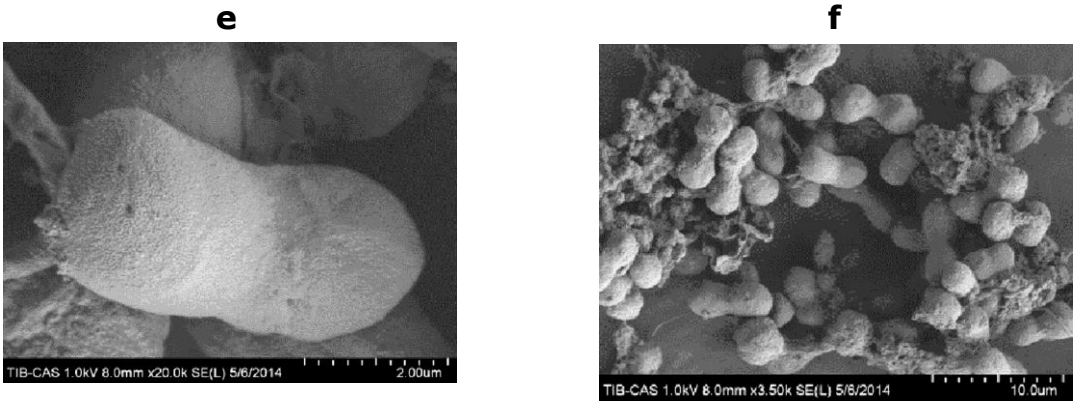
Effect of Galla chinensis on M. aeruginosa ultrastructure, observed by SEM. Untreated *M. aeruginosa* cells were distinct and individual with complete cell morphology and were mostly densely clumped. Untreated cells were also nearly spherical or elliptical. Many cells divided and appeared as two-segment bodies with obvious constriction (Figs. 3a, 3b).



In contrast, treated *M. aeruginosa* cells showed external structural damage, which was exacerbated with increasing nutgall concentrations. Algal cells at a low nutgall concentration (0.009 g/L) exhibited slight wrinkles or local projections on the surface, and cells were arranged loosely and did not aggregate normally (Figs. 3c, 3d).



At 0.046 g/L exposure, some algal cells developed holes in their walls, which discharged the intracellular material, and some dividing cells appeared as the two-joint bodies and suffered deformities during the division process (Figs. 3e, 3f).



When nutgall concentration was high (0.181 g/L), the overall structure of algal cells showed very serious injury (Figs. 3g, 3h). Most of the algal cell walls collapsed, which also led to discharge of the intracellular material. Further, the holes on the algal cell walls expanded until the entire cell completely disintegrated.

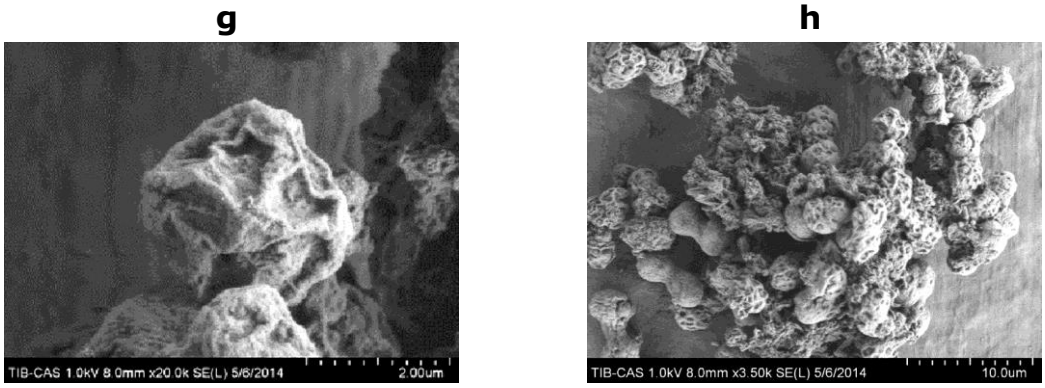
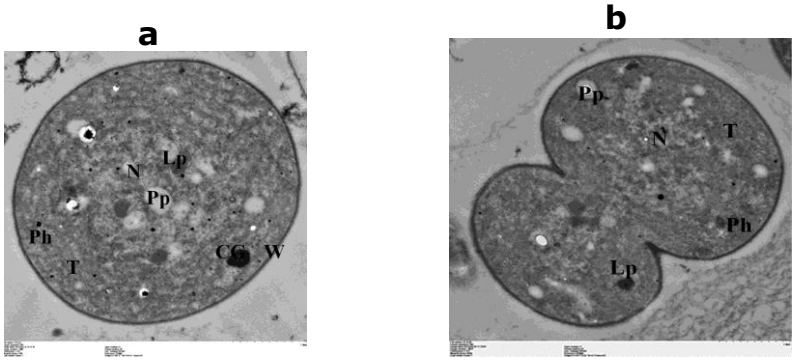
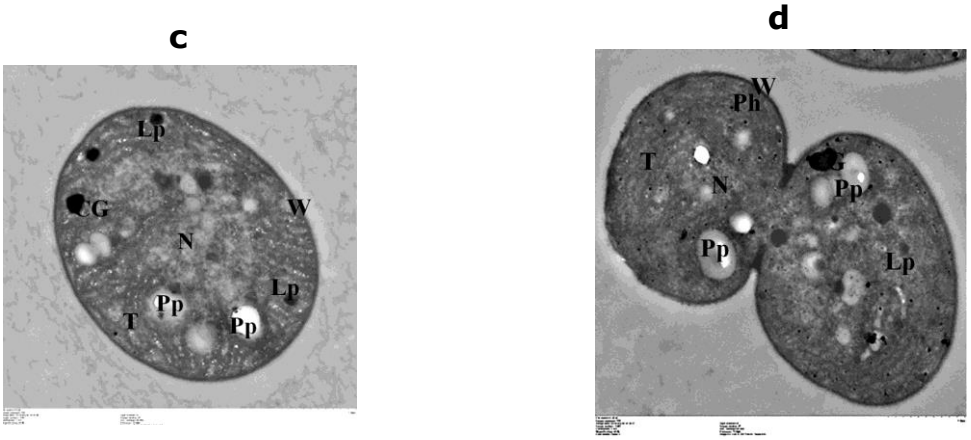


Fig. 3 Effect of Galla chinensis on *Microcystis aeruginosa* ultrastructure, observed by SEM.

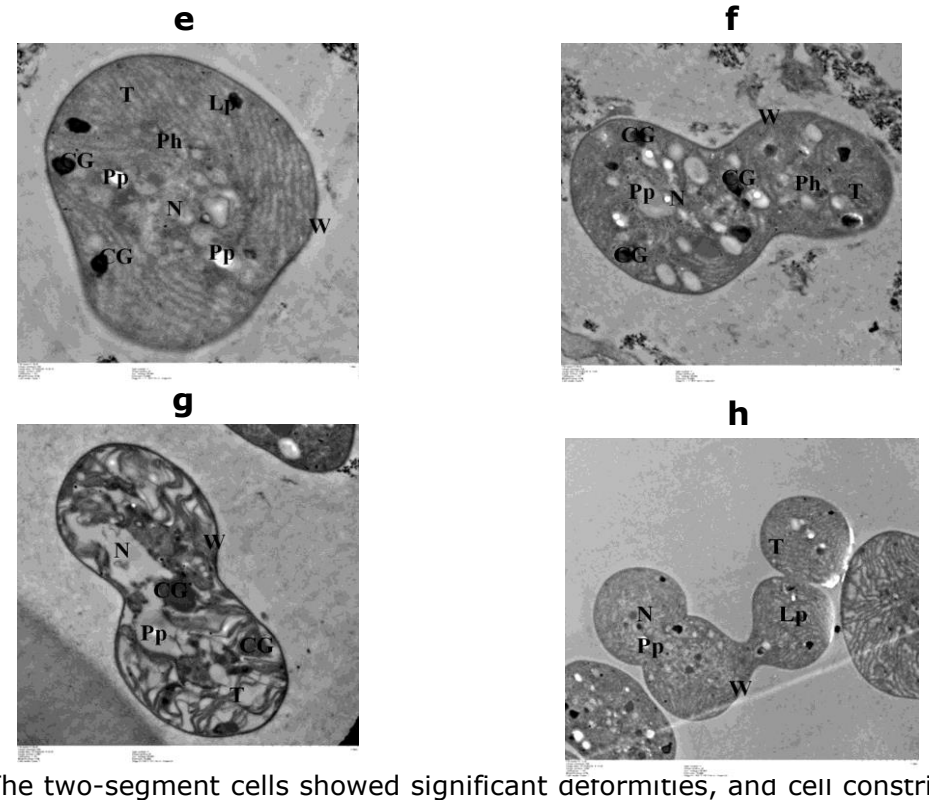
Effect of Galla chinensis on Microcystis aeruginosa ultrastructure, observed by TEM. Untreated *M. aeruginosa* cells were enclosed by cell walls and membranes (Figs. 4a, 4b), and cells had dense cytoplasm, an obvious central nucleus, abundant photosynthetic thylakoid lamellar structures, and a high density of phycobilisome sac structures, seen as small particles which were uniformly attached to the surface of flat sac thylakoid structures. There were a few polyhedral lipid particles and polyphosphate bodies distributed in the cytoplasm. Dividing algal cells appeared as two-segment bodies with obvious constriction, and edges that connected the two cells. (Fig. 4b).



Compared with the C group, ultrastructures of the nutgall-treated algal cells were significantly altered. The damage was exacerbated with increased nutgall concentration. At 0.009 g/L nutgall concentration (Figs. 4c, 4d), the thylakoids became fragmented and some were broken. There were fewer phycobilisomes attached to the thylakoid lamellae, but they were dispersed in the cytoplasm. In addition, lipid particles and cyanophycin particles increased in the cytoplasm. The nucleoplasm began to spread, and the nuclear areas appeared partly empty. The dividing algal cells, which appeared as two-segment bodies with obvious constriction, had partially protruding cell walls, and the edges connecting the two cells were quite clear (Fig. 4d).



At a concentration of 0.046 g/L (Figs. 4e, f, g, h), the nucleoplasm of the algal cells spread further, the empty fields in the nuclear areas expanded, and thylakoids and phycobilisomes decreased further. Cell walls basically remained intact however, plasmolysis was apparent, and infrastructure in the cytoplasm was not clear. Treated, dividing algal cells are shown in Figs. 4f,g.



The two-segment cells showed significant deformities, and cell constrictions were not obvious; moreover, a few triplet teratocytes were visible (Fig. 4h). At the concentration of 0.181 g/L (Figs. 4 i, j, k, l), the algal cells were deformed. The cell walls collapsed and ruptured, and plasmolysis was clearly visible. The cytoplasm infrastructure was not evident, large voids were visible in the nuclear areas, and most of the algal cells died.

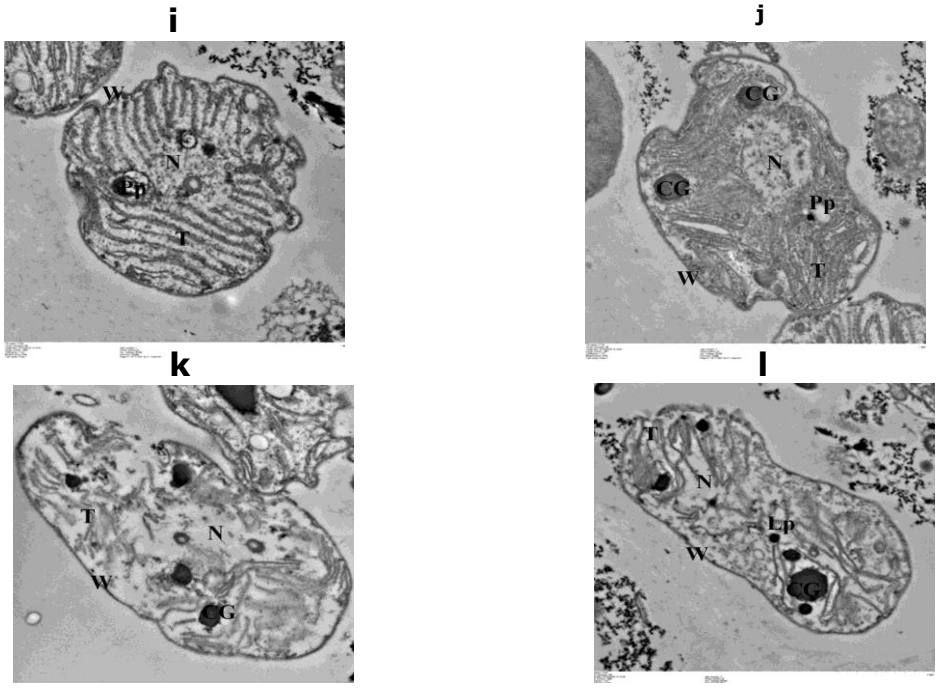


Fig.4 Effect of Galla chinensis on *Microcystis aeruginosa* ultrastructure, observed by TEM.
W: cell wall; T: thylakoid; N: nucleoid; Lp: lipid body; Ph: polyhedral body; Pp: polyphosphate body; CG: cyanophycin granules.

Discussion

Tannins are the main active ingredient of Galla chinensis (Chen et al., 2010). Gallotannins have nearly 20% gallic acid, and 7% methyl gallate. Gallotannins consist of a mixture of glucose acyl, which is surrounded by several gallic acid units, and further gallic acid units can be attached through depside bonding of additional galloyl residues. Tannins are polyphenolic compounds which are soluble in water and are extracted from Galla chinensis. Tannins can be hydrolysed to produce gallic acid. Gallic acids are phenolic acids with strong allelopathic properties (Gu et al., 2013) affecting plant growth. In this study, we illustrated that 72 h exposure to 0.009 g/L nutgall extract solution can significantly reduce *M. aeruginosa* cell density, and that the 72 h (EC_{50}) was 0.148 g/L. The inhibitory effects of nutgall extract on *M. aeruginosa* may be due to gallic acid produced by tannin hydrolysis.

M. aeruginosa is a prokaryotic cyanobacterium. Generally, in Chinese traditional medicine, Galla chinensis is regarded as antibacterial and antiviral, and it is used for the prevention of dental caries, burn treatment, and prevention of fish diseases. Consequently, we inferred that the inhibitory effect of Galla chinensis on *M. aeruginosa* may be associated with its antibacterial effect.

M. aeruginosa chlorophyll levels were consistent with the changes in algal cell density, in which the chlorophyll a content significantly decreased with increased concentrations of nutgall extract. These results are consistent with those of Hu et al. (2012), who studied the effect of berberine sulphate on chlorophyll a levels in *M. aeruginosa* cells.

When *M. aeruginosa* cells were treated with Galla chinensis extracts, SOD activity significantly increased at the low concentration (0.009 g/L). On the one hand this result indicates that stress caused by Galla chinensis changed the metabolic balance of reactive oxygen species (ROS) in algae and increased ROS accumulation. As a result, defense mechanisms, including SOD and ROS scavenging activity, were strengthened, protecting algae from oxidative damage. On the other hand, when the nutgall concentration reached 0.046 g/L and 0.181 g/L, SOD activity significantly decreased. This result suggests that SOD can play a protective role only in low nutgall concentrations, but high concentrations may directly or indirectly inhibit SOD synthesis or alter its molecular structure, reducing SOD levels and/or activity. These results are consistent with the effects of *Hydrilla verticillata* on *M. aeruginosa*, (Wang et al. 2004).

Based on observations from SEM and TEM, with increasing nutgall concentrations, the extent of external morphological and internal structural damage on *M. aeruginosa* gradually intensified. SEM showed that the cell walls became wrinkled, collapsed, and ruptured; two-segment cell deformities appeared in dividing algal cells. On the other hand, TEM showed that the photosynthetic thylakoid lamellae were fractured and dispersed. Fewer phycobilisomes were attached to the photosynthetic thylakoid membrane, and these were dispersed in the cytoplasm. Increased lipid particles were also observed. This result has been demonstrated in thylakoid rupture (Zhang, 2010). Large numbers of cyanophycin granules emerge from *M. aeruginosa* cells subjected to environmental stress (Zhang, 2010). Furthermore, dividing algal cells showed deformities such as two-segment bodies or triplets, which suggest that the cell division process was affected.

Galla chinensis may inhibit the growth and recovery of *M. aeruginosa*, because of specific allelopathic substances in the extract. Findings from this study show that Galla chinensis can be used to inhibit algal blooms but the detailed inhibition mechanisms should be further clarified. The Chinese herb Galla chinensis has been used as medicine to treat different aquatic animal diseases for many years in China (Dong et al., 2014; Jeney et al., 2009). Galla chinensis is low in toxicity and has the potential to control algal blooms thereby aiding fish farmers to eliminate or alleviate the problems they cause in fish ponds. Since it is environmentally friendly it can replace traditional treatment with $CuSO_4$.

Acknowledgements

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