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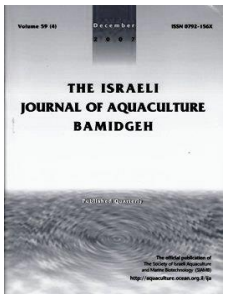

Inhibition of *Anabaena flos-aquae* by *Galla chinensis*

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Keywords: Chinese herbal medicine; *Galla chinensis*; *Anabaena flos-aquae*; inhibitory effects

Abstract

Galla chinensis is a traditional herbal medicine in China with detoxification, bacteriostatic, and anti-inflammatory properties. In this study, we exposed *Anabaena flos-aquae* to different concentrations of *Galla chinensis* aqueous extracts. As the exposure concentrations and duration increased, chlorophyll *a* of *Anabaena flos-aquae* was significantly reduced. After 72 h of exposure, protein content, MDA content, and superoxide dismutase activity in *Anabaena flos-aquae* gradually decreased with increasing concentrations of *Galla chinensis* aqueous extracts. Electron microscopy for ultrastructural analysis showed that compared to the control group, as the concentration of *Galla chinensis* increased, the *Anabaena flos-aquae* cell walls exhibited plasmolysis with blurred basic structures in the cytoplasm, deformation of algal cells, collapse, and rupture of cell walls, and most cells died. *Galla chinensis* can effectively inhibit the growth of *Anabaena flos-aquae* and exhibits significant allelopathy.

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Introduction

Anabaena flos-aquae is one of the most common cyanobacteria causing summer algal blooms and frequently occurs in many lakes, rivers, and reservoirs. *Anabaena flos-aquae* produces not only algal toxins but also strong odors and severely affects water quality (Wan and Shen, 2011; Wu et al., 2012). *Anabaena flos-aquae* has been shown to produce 6 types of anatoxins, which are all neurotoxins, mainly hepatotoxins and anatoxins. These toxins typically act on the livers of animals to cause death. Studies showed that intraperitoneal injection or oral inoculation of algal toxins to fishes can rapidly result in death within 3 h. As Anabaena causes severe damage to the aquatic environment, studies of methods for inhibiting Anabaena are needed (Cai et al., 2009). In addition, this kind of algae is common, easily cultured, and sensitive to toxicity, so it has very important experimental value (Zhang et al., 2005; Yue et al., 2006).

Galla chinensis is a traditional Chinese herbal medicine. It is the gall formed by the aphid of Pemphigidae on its host plant, *R.chinensis* Mill, *R. potaninii Maxim* of *Rhus,* Anacardiaceae. *Galla chinensis* has detoxification, bacteriostatic, and anti-inflammatory effects (Nie et al.,2015). This medicine is commonly used to treat bacterial diseases in aquatic animals (Liu et al.,2009). The active ingredients of *Galla chinensis* contain tannins and gallic acid. The gallic acid is also known as 3,4,5-trihydroxybenzoic acid and is a phenolic acid with allelopathic properties which can inhibit the growth of Anabaena (Zhang et al., 2007; Zhang et al.2015; Zou et al.,2012). *Galla chinensis* tannins are hydrolyzed tannins, which produce gallnut acid and sugar (Chen et al., 2010). Therefore, G*alla chinensis* has good prospects for algal inhibition.

In this study, we examined the effects of *Galla chinensis* aqueous extract on the inhibition rate of *A. flos-aquae*, soluble protein, malondialdehyde (MDA), and superoxide dismutase (SOD) content, algal cell membrane permeability, and morphological and ultrastructural changes in *Anabaena flos-aquae* by electron microscopy to determine the inhibition effects of *Galla chinensis* aqueous extract on *A. flos-aquae*.

This research will provide scientific evidence for developing an effective natural inhibitor of cyanobacteria blooms and reduce aquatic organism diseases.

Materials and Methods

Algaculture

A. flos-aquae was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences and cultured in BG11 culture medium. The algae were cultured in an illuminated incubator at 30±1℃ with 12-h/12-h light-dark cycles and a light intensity of 2000±200lux until the logarithmic growth phase. The cultures were shaken regularly twice per day, and their positions were randomly switched to reduce any effects related to minor changes in light density in an illuminated incubator and were randomly adjusted (Gao et al., 2015).

Preparation of Galla chinensis extract solution

Galla chinensis powder (5.0g; 80mesh) was mixed with distilled water. After 30 min of ultrasonic extraction, the volume was adjusted, and immersion extraction occurred overnight in the dark. The solution was shaken well and stored at 4℃ in the refrigerator for use.

Assay of growth inhibition

Galla chinensis extract solutions were formulated into 4 different concentrations with 3 replicates per concentration, as follows: 0mg/L (control; C), 9.1mg/L, 45.5mg/L, and 181.8mg/L. After filtering with 0.45μm microporous membranes, *G. chinensis* extract solutions were added in the culture medium containing exponentially growing *Anabaena flos-aquae*. Samples were removed from the cultures at 0h, 24h, 48h, and 72h for each extract concentration to measure chlorophyll *a* content in *A. flos-aquae.* (Chai et al. 2010) The content of chlorophyll *a* in each liter of algal solution was substituted for the cell biomass in algal solution.

The effects of *G. chinensis* on *A. flos-aquae* growth were described using growth inhibition rates (*IR)*, which were calculated using equation (2).

$$IR = (No-Ns)/No \times 100\% \quad (2)$$

where *IR* is the growth inhibition rate, *No* is the chlorophyll *a* content in the algal solution of the control group, and *Ns* is the chlorophyll *a* content in the algal solution of the treatment group.

Assays for protein content, malondialdehyde (MDA)content, SOD activity and cell membrane permeability in A. flos-aquae

According to previously described method, cultures were exposed for 72h to different concentrations of *G. chinensis* extract solution (0, 9.1, 45.5, 181.8 mg/L) to determine the protein content, MDA, SOD, cell membrane permeability.

The protein content (soluble protein content was determined by Coomassie brilliant blue G-250) (Ni et al.2017) malondialdehyde (MDA) content (thiobarbituric acid (TBA) colorimetric method) (Qing et al.2018), SOD activity (SOD activity was determined by nitrotetrazolium chloride (NBT) photoreduction method) (Stewert and Bewley, 1980) and cell membrane permeability (OD264 determination method) (Xie et al.1986) were determined.

Scanning Electron Microscopy (SEM) of algal cells

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 buffer solution (PH=7.4). Each sample was then dehydrated with gradient concentrations of ethanol, i.e., 30%, 50%, 75%, 95%, and 100%, for 15 min per gradient. After dehydration, samples were dried for 2 h using a critical point drying apparatus (Leica EM CPD030). Dried samples were coated by an ion-sputtering instrument (Hitachi E-1045) and then observed and imaged using a Field Emission Scanning Electron Microscopy (Hitachi SU8010).

Transmission Electron Microscopy (TEM) of algal cells

Steps of algal cell enrichment, fixation, and dehydration were the same as for SEM. Dehydrated samples were embedded in Resin Epon815 at 60℃ 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 Transmission Electron Microscope (Hitachi HT7700).

Statistics

Cultures in every assay were sampled 3 times, and replicate values were averaged. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to analyze data by one-way analysis of variance (ANOVA), least significant difference, Duncan (D) pairwise comparison, and univariate analysis. A difference of $P<0.05$ was considered significant and $P<0.01$ was considered extremely significant.

Results

Effects of Galla chinensis aqueous extract on growth of A. flos-aquae

Anabaena flos-aquae were treated with different concentrations of *Galla chinensis* aqueous extracts for 24, 48, 72h. Table 1 shows the changes in the chlorophyll *a* content in the *A. flos-aquae* algal solutions. As *Galla chinensis* concentration and treatment duration increased, chlorophyll *a* content in the algal solution decreased. In Group 1 (CK), the chlorophyll *a* content in the algal solution continuously increased from 134.9μg/L at 0 h to 190.0μg/L after 72 h. In the various treatment groups (9.1, 45.5, 181.8 mg/L), as *G. chinensis* aqueous extracts increased, chlorophyll *a* content in the algal solution decreased. At 24 h and 181.8mg/L *G. chinensis* concentration, chlorophyll *a* content in *A. flos-aquae* solution was 133.4μg/L, which was significantly lower than the C group ($P<0.05$), and IR was 13.1%. At 48 h and 45.5mg/L *G. chinensis* concentration, chlorophyll *a* content in *A. flos-aquae* solution was 151.1μg/L, which was significantly lower than the C group ($P<0.05$), and IR was 8.6%. At 48 h and 181.8mg/L *G. chinensis* concentration, chlorophyll *a* content in *A. flos-aquae* solution was132.4μg/L, which was significantly lower than the C group ($P<0.01$), and IR was 19.9%. At 72 h and 181.8mg/L *G. chinensis* concentration, chlorophyll *a* content in *A. flos-aquae* solution was 129.9μg/L, which was extremely significantly lower than the C group ($P<0.01$), and IR was 31.6%.

Table 1. Effects of different concentrations and different treatment durations of *Galla chinensis* aqueous extract on growth of Anabaena flos-aquae

Chinese gall treatment concentration (mg/L)	*Different treatment durations (h), chlorophyll a content (μg/L)*			
	0 h	24 h	48 h	72 h
Ck	134.9±2.5	153.4±2.1^{aA}	165.3±4.8^{aA}	190.0±4.2^{aA}
9.1	134.9±2.5	149.7±3.3^{aA}	161.2±3.1^{abA}	183.4±9.5^{aA}
45.5	134.9±2.5	144.2±5.3^{abA}	151.1±3.4^{bA}	173.0±6.3^{aA}
181.8	134.9±2.5	133.4±6.7^{bA}	132.4±4.3^{cB}	129.9±2.5^{bB}

Different lowercase letters indicate that differences were significant (P<0.05); different uppercase letters indicate that the differences were extremely significant (P<0.01).

Effects of Galla chinensis aqueous extract on protein content in A. flos-aquae

Protein content was determined for algal cultures exposed for 72 h to different concentrations of *G. chinensis* extract (Figure 1). Protein content per litre of algal solution was negatively correlated with the concentration of *G. chinensis.* At 72h and 45.5mg/L *G. chinensis* concentration, protein content in *A. flos-aquae* solution was significantly lower than the C group (P<0.05). At 72h and 181.8mg/L *G.chinensis* concentration, protein content in *A. flos-aquae* solution was extremely significantly lower than the C group (P<0.01).

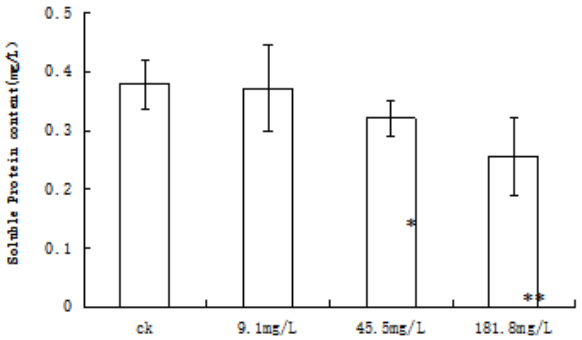


Fig. 1. Effects of *Galla chinensis* on soluble protein content in *Anabaena flos-aquae*

Effects of Galla chinensis aqueous extracts on malondialdehyde (MDA) content in A. flos-aquae

MDA is a product of lipids peroxidation and its formation and cumulative amount can be used as a marker of structural damage to the cell membrane and stress in *M. aeruginosa* (Wang et al., 2004). Figure 2 shows the changes in MDA content in *A. flos-aquae* algal solutions following exposure to different concentrations of *Galla chinensis* aqueous extracts for 72h. As *Galla chinensis* aqueous extract concentrations increased, MDA content first increased and then decreased. MDA content in the 9.1mg/L group was significantly increased (*P*<0.05). When the *Galla chinensis* concentration increased to 181.8mg/L, MDA content was extremely significantly decreased (P<0.01).

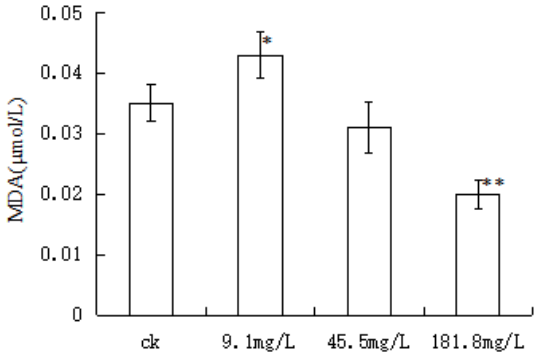


Fig. 2. Effects of *Galla chinensis* aqueous extracts on malondialdehyde content in *Anabaena flos-aquae*

Effects of Galla chinensis aqueous extracts on superoxide dismutase (SOD) activity in A. flos-aquae

SOD is a protective enzyme that is vitally important in the cells and catalyzes the disproportionate superoxide anions (O_2^-) produced in stressful conditions and prevents membrane lipid peroxidation chain reactions. Figure 3 shows the changes in SOD activity in *A. flos-aquae* algal solutions after exposure to different concentrations of *Galla chinensis* aqueous extracts for 72 h. When *A. flos-aquae* was exposed to *Galla chinensis* aqueous extracts, the SOD activity of the low concentration group (9.1mg/L) was not significantly different to the control group. As *Galla chinensis* concentration increased to 45.5mg/L, SOD activity was significantly lower than in the control group ($P<0.05$). When the *Galla chinensis* concentration was increased to 181.8mg/L, SOD activity decreased to 40% of that in the control group ($P<0.01$), showing that *A. flos-aquae* algal cells exposed to this concentration experienced physical damage.

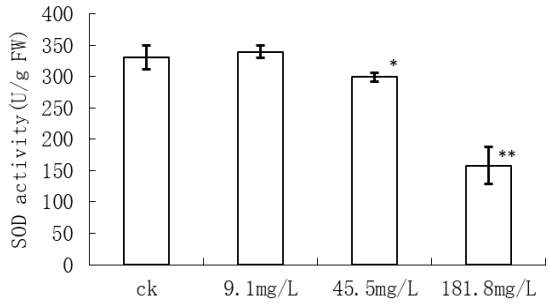


Fig. 3. Effects of *Galla chinensis* l on SOD activity in *Anabaena flos-aquae*

Effects of Galla chinensis aqueous extract on cell membrane permeability in A. flos-aquae

Cell membrane permeability changes when algal cells are under stress. Figure 4 shows the changes in *A. flos-aquae* cell membrane permeability after exposure to different concentrations of *Galla chinensis* aqueous extracts for 72 h. Different *Galla chinensis* concentrations significantly affected *A. flos-aquae* cell membrane permeability, which was positively correlated with the *Galla chinensis* concentration. At a low exposure concentration (9.1mg/L), cell membrane permeability increased slightly compared to the control group. As *Galla chinensis* concentration increased to 45.5 g/L, cell membrane permeability increased significantly compared to the control group ($P<0.05$). When the concentration was 181.8mg/L, cell membrane permeability extremely significantly increased ($P<0.01$).

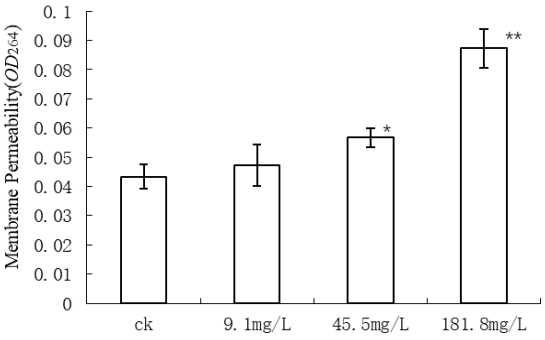


Fig. 4. Effects of *Galla chinensis* on *Anabaena flos-aquae* cell *membrane* permeability

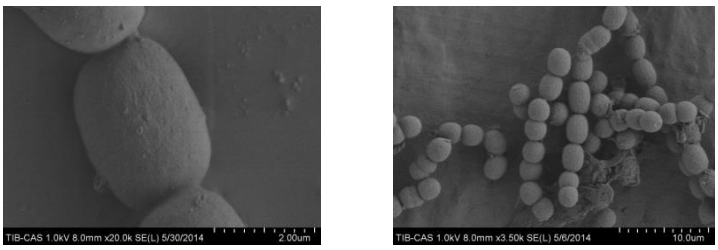
Scanning electron microscopy analysis of A. flos-aquae cells

Scanning electron microscopy analysis revealed that *A. flos-aquae* cells that were not exposed to *Galla chinensis* (Figure 5 a and b) showed single filaments which clumped together to form colonies. Algal filaments were of equal width or had ends that were thin and showed straight and irregular spiral curvature. Single cells were saturated and waist

drum-shaped, while the cell wall surface was smooth, and its structure was intact. No damage was observed.

Fig. 5. Effects of *Galla chinensis* on *Anabaena flos-aquae* cell ultrastructure as observed by scanning electron microscopy

a **b**



Anabaena flos-aquae cells exposed to *Galla chinensis* showed increasing external structural damage to cell morphology as the concentration was increased. Algal cells (Figure 5 c and d) in the low concentration group (9.1mg/L) showed slight wrinkling, but most cells exhibited intact morphology and minor morphological differences. In the 45.5mg/L group (Figure 5 e and f), some algal cells showed wrinkling and shrinkage, and the cell wall surfaces appeared rough. In the high concentration group (181.8mg/L) (Figure 5 g and h), the algal cells showed morphological changes with rough and deformed cell walls or patchy flakes.

c **d**

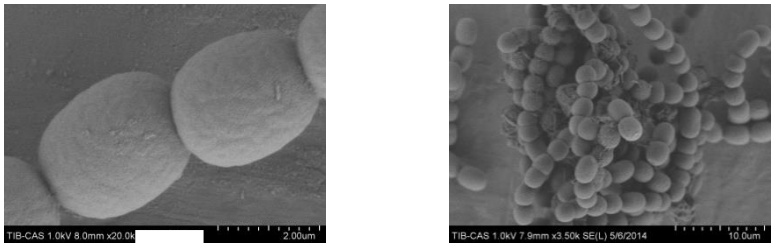


Fig. 5. a b

(CK group)

e **f**

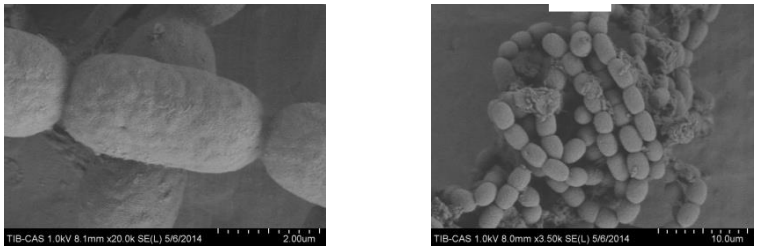


Fig. 5. c d

(9.1mg/L treatment group)

g **h**

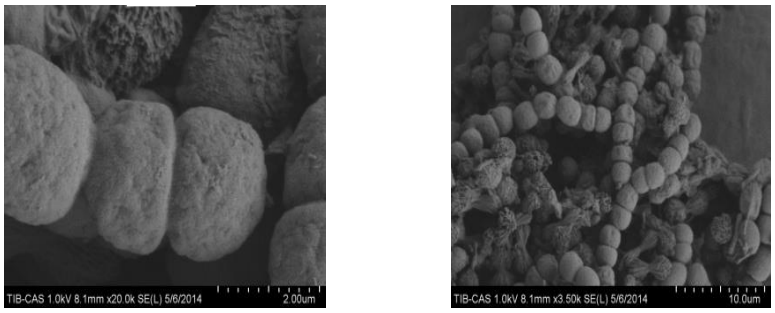


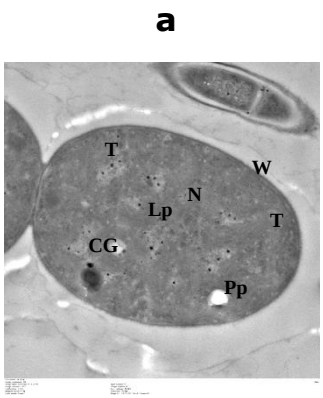
Fig. 5. e f

(45.5mg/L treatment group)

Transmission electron microscopy observations of Anabaena flos-aquae cells

In normal *A. flos-aquae* algal cells (Figure 6 a and b), the cells were surrounded by a cell wall and membrane, the cytoplasm was thick, a central nuclear region was evident, and the thylakoid photosynthetic lamellae structure was abundant. High-electron density phycobilisomes are small granules that uniformly attach to the surface of the flat, sack structure in the thylakoids. The cytoplasm contains small numbers of polyhedral bodies, lipid particles, and polyphosphate bodies. (Figure 6 b) shows an algal cell that is dividing, exhibiting a dyad, with evident constriction, and the edges of the two connected cells are clear.

Fig.6. Effects of *Galla chinensis* on *Anabaena flos-aquae* cell ultrastructure observed by transmission electron microscopy



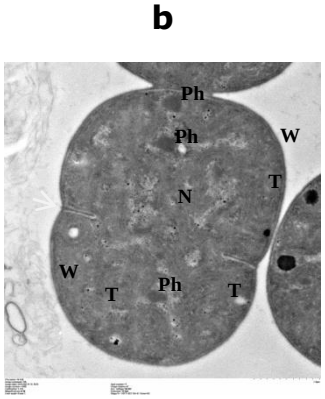
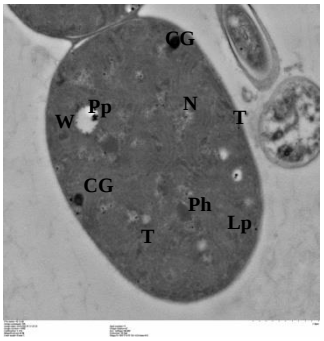


Fig.6. a b

(CK group)

Compared to the control group, algal cells treated with *Galla chinensis* showed significant changes in their ultrastructure and injury increased with increasing treatment concentrations. In the 9.1mg/L treatment group (Figure 6 c and d), changes in the morphological structure of *A. flos-aquae* cells were not significant. In the 45.5m g/L treatment group (Figure 6 e, f, g, h), the thylakoids were scattered, some thylakoids were ruptured, and the number of phycobilisomes attached to the thylakoid lamellae was reduced and scattered in the cytoplasm. There was an increase in lipid particles and cyanophycin granules in the cytoplasm. The nucleoplasm was diffused in all directions and some gaps appeared in the nuclear region. In the 181.8m g/L treatment group (Figure 6 i, j, k, l), the nucleoplasm of algal cells further diffused in all directions and gaps in the nuclear region were further enlarged. The thylakoids and phycobilisomes were further reduced in size. The cell wall was basically intact, with some plasmolysis. The basic structures in the cytoplasm appeared blurred, deformation of algal cells occurred, cells walls collapsed and ruptured, and most cells died.



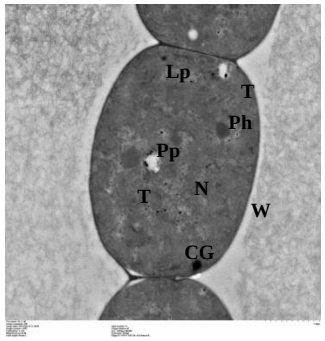


Fig.6. c d

(9.1mg/L treatment group)

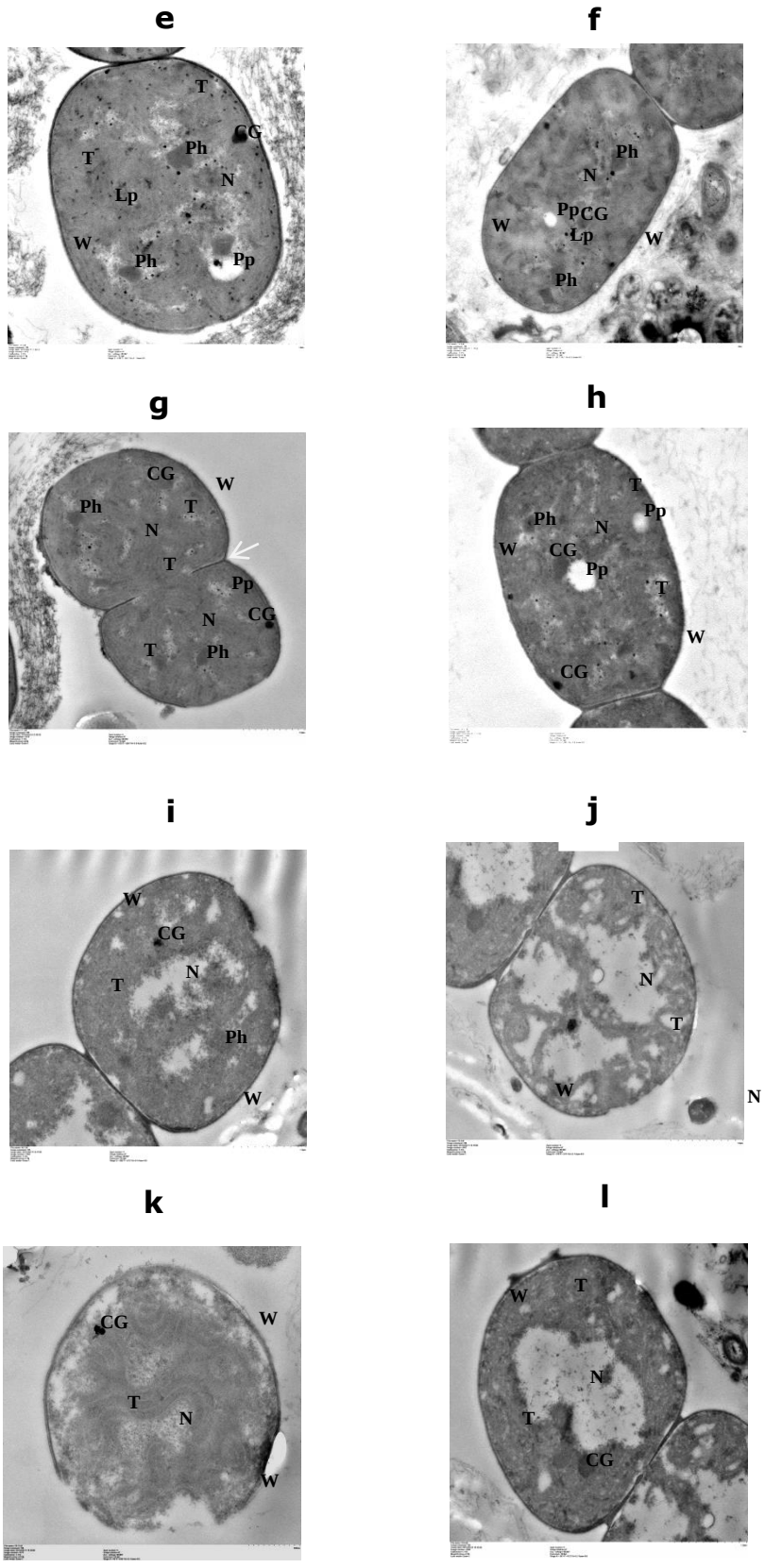


Fig. 6.

e f g h

(45.5mg/L treatment group)

Fig. 6.

i j k l

(181.8mg/L treatment group)

W: cell wall; T: thylakoid; N: nucleoid; Lp: lipid body; Ph: polyhedral body; Pp: polyphosphate body; CG: cyanophycin granules.

Discussion

The active ingredients of *Galla chinensis* are Chinese gall tannins and gallic acid. Gallic acid is also known as 3,4,5-trihydroxybenzoic acid, is a phenolic acid with allelopathic effects, and can inhibit the growth of *Anabaena* (Zhang et al., 2007).

In this study, the inhibition rate of *Galla chinensis* aqueous extracts on *A. flos-aquae* increased with increasing concentration, showing an increasing inhibition rate. Additionally, as treatment duration increased, the inhibition rate also increased. The inhibitory effect of *galla chinensis* aqueous extract on *Anabaena flos-aquae* may be related to the main effective component of *Galla chinensis*, galla chinensis acid.

Organisms from the genus *Anabaena* are prokaryote nitrogen-fixing cyanobacteria. *Galla chinensis* is a traditional herbal medicine in China with detoxification, bacteriostatic, and anti-inflammatory effects. This medicine is commonly used to treat bacterial diseases in aquatic animals (Zu et al., 2007). The inhibitory effect of *Galla chinensis* on *Anabaena flos-aquae* is probably similar to bacteriostasis of *Galla chinensis.*

Variations in chlorophyll content in *A. flos-aquae* cells were consistent with the changes in the color of the algal solution, i.e. the color of algal solutions changed from green to yellow, and to yellow-brown. As the concentration of *Galla chinensis* aqueous extracts increased, chlorophyll *a* content significantly decreased. This result is consistent with that of Zhang et al., (2007) who examined the effects of phenolic acids on chlorophyll *a* content in *A. flos-aquae* cells.

The protein content of *A. flos-aquae* gradually decreased with increasing *Galla chinensis* aqueous extract concentrations. This was consistent with the results of a study on the effects of cerium on soluble protein content in *A. flos-aquae* cells (Lv et al., 2012).

When *A. flos-aquae* experiences stress due to *Galla chinensis*, SOD activity increased slightly at low concentrations (9.1mg/L) of *Galla chinensis* aqueous extract. This shows that *Galla chinensis* stress alters the metabolic equilibrium of reactive oxygen species in the algae, increasing the accumulation of reactive oxygen species. As a result, the defense function in the cells is strengthened, and SOD activity in the defense system is increased to remove the reactive oxygen species, thus preventing injury to the algae. When the *Galla chinensis* concentrations reached 0.046g/l and 0.181g/l, SOD activity decreased significantly. Thus, SOD only elicits its protective effects at low concentrations of *Galla chinensis*, but excessively high *Galla chinensis* concentrations directly or indirectly disrupt SOD synthesis or the enzyme structure, decreasing its levels or activity. This is consistent with the results on the effects of algicidal metabolic products on SOD activity in *A. flos-aquae* (Huang et al. ,2009).

Scanning electron microscopy and transmission electron microscopy observations showed that the level of damage to external morphology and internal structures in *A. flos-aquae* gradually increased as *Galla chinensis* concentration increased. Under scanning electron microscopy, algal cells showed wrinkling and shrinkage, cell wall surfaces appeared rough, and cell morphology was altered. Transmission electron microscopy observations of the internal structure of algal cells showed that thylakoids were scattered. Some of thylakoids were ruptured, and the number of phycobilisomes attached to the thylakoid lamellae was reduced and scattered in the cytoplasm. Lipid particles and cyanophycin granules in the cytoplasm increased. An increase in lipid granules is associated with thylakoid rupture, as this is the end result of degradation of the thylakoid membrane. The appearance of large numbers of cyanophycin granules suggests that *A. flos-aquae* experienced environmental stress. This caused the nucleoplasm to diffuse in all directions and gaps appeared in the nuclear region.

Conclusion

Galla chinensis can irreversibly inhibit the growth of *Anabaena flos-aquae* and this inhibitory effect is due to the allelopathic gallic acid in *Galla chinensis*. This requires further study. The results of this experiment aimed to provide a theoretical basis for the treatment of cyanobacteria algal bloom using Chinese herbal medicine.

Acknowledgements

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