Allelopathic effect of rhubarb extracts on the growth of *Microcystis aeruginosa*

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ABSTRACT

With its advantages of ecological safety, environmental affinity, and high selectivity, allelopathic technology has been widely developed for algae inhibition. However, obtaining effective allelochemicals and realizing their mechanism are difficult. In this paper, a Chinese herbal medicine, namely, *Rheum palmatum* L. (Chinese rhubarb), was utilized as a source of allelopathic substances for the first time. Four units of rhubarb organic extracts were collected to study the inhibition of growth, photosynthesis, proteins, and algal toxin of *Microcystis aeruginosa*. Results showed that the ethyl acetate, n-butanol, and aqueous phases of the rhubarb extracts have notable inhibitory effects. After a 16-day treatment, the four extracts reduced *M. aeruginosa* by 64.1%, 59.3%, 61.9%, and 7.2% with disruption of algal photosynthesis and protein synthesis and reduction of algal toxin.

Key words | allelochemicals, inhibition, *Microcystis aeruginosa*, rhubarb

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HIGHLIGHTS

- The growth of *Microcystis aeruginosa* can be effectively inhibited by rhubarb extract.
- The content of algal toxin in water is effectively reduced.
- The active ingredients of rhubarb extract are aloe-emodin, emodin, chrysophanol, physcion, and rhein.

INTRODUCTION

Cyanobacterial bloom is an ecological disaster caused by eutrophication of water and has gained increased attention in recent years (Chen *et al.* 2003). It may lead to odor, poor water quality, and other problems for rivers and lakes. Furthermore, it may be harmful to animal and human health (Briand *et al.* 2008; Zhang *et al.* 2011). During a bloom, several species, such as *Radiocystis feernandoi*, *Synechococcus*, and *Microcystis*, may produce algal toxins when exposed to external stress (Merwe 2015). When ingested by humans through the food chain, they may cause hepatitis or even death (Ball *et al.* 2001).

At present, physical, chemical, and biological technologies are frequently used for cyanobacterial bloom treatment (Hua *et al.* 2018). Although physical methods, such as salvaging machinery and shading, can effectively treat the bloom, they easily cause secondary pollution because cyanobacteria are difficult to dehydrate and transport (Lürling & Faassen 2012). Chemical methods, such as oxidants, herbicides, and heavy metal algicides, are harmful to the environment. Algae may be directly or indirectly inhibited by biological methods, such as fish, bacteria, and aquatic plants, which may help rebuild ecological equilibrium. However, managing this method is complicated and time-consuming (Van *et al.* 2002). In recent years, inhibition by allelochemicals has been developed as a new technology and provides a new concept for algae inhibition using efficient and safe methods (Qin *et al.* 2006; Qu & Fan 2010; Tang *et al.* 2012).

Allelopathic algae inhibition has the advantages of ecological safety, environmental affinity, and high selectivity (Vyvyan 2002). Previous studies isolated certain allelochemicals, such as free steroids, steroids and terpenoids, simple phenols, benzoic acid and its derivatives, and cinnamic acid and its derivatives, and demonstrated their inhibition activities (Sun & Li 2018). Currently, allelopathic technology is still in the exploratory stage. Therefore, finding allelopathic materials with enhanced algae inhibitory effect, reduced environmental impact, and increased concentration with effective allelopathic

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materials has become the key limitation of this technology. Traditional Chinese medicine is a unique resource of abundant allelochemicals and is characterized by universality and low toxicity. Extracting specific ingredients from Chinese herbal medicine can provide a new source for allelopathic research and allelochemicals (Rice 1984).

The rhizome of *Rheum palmatum* L. (Chinese rhubarb; family Polygonaceae) has several effects, such as alleviating fever, detoxifying, improving blood circulation, and removing blood stasis (Guo & Liu 2006). The rhubarb has broad-spectrum antibacterial and antiviral effects and a strong inhibitory effect on dysentery bacillus, typhoid bacteria, and cholera bacteria (Mao 2005). Thus, it has a potential role in algae inhibition.

In this study, rhubarb was used as the source of allelochemicals. The algae-inhibiting effect of four extracts of rhubarb was compared. The mechanism of algae inhibition in relation to algae growth, photosynthesis, protein content, and algal toxin was studied by determining OD680 (optical density measured at 680 nm), chlorophyll *a*, carotenoid, phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE), malondialdehyde (MDA), soluble proteins, and microcystins (MCs). Key allelochemicals were analyzed by using high-performance liquid chromatography (HPLC) and the chemical mechanism of allelopathic inhibition was further investigated.

MATERIALS AND METHODS

Extraction of allelochemical components from rhubarb organic extracts

Rhubarb was cleaned with ultrapure water, dried, and pulverized to approximately 0.3 mm with a pulverizer (FW100, Tianjin Taisite Instrument Co., Ltd). It was transferred to a beaker, 70% ethanol at room temperature was added, and then it was extracted for 48 h. After vacuum filtration, the solvent was removed by a rotary evaporator (100 rpm, 45 °C), and liquid–liquid extraction was carried out to obtain four separate components, namely, A (n-hexane phase), B (ethyl acetate phase), C (n-butanol phase), and D (aqueous phase). They were collected up to a volume of 100 mL with dimethyl sulfoxide (DMSO). The concentrate was filtered under sterile conditions through a 0.22 μm filter and stored in a refrigerator at 4 °C.

Culture of *Microcystis aeruginosa*

Microcystis aeruginosa (FACHB-905) was purchased from the freshwater algae species bank of the Institute of Hydrobiology, Chinese Academy of Sciences. It was cultured in BG11 medium, inoculated in a sterile medium, and cultured statically. The temperature was controlled at 25 ± 0.5 °C; light intensity was retained at approximately 2,400 lx with a light:dark ratio of 12:12. The solution was shaken 3–4 times every day until it reached a logarithmic phase after 3 weeks, then stored for further use.

Growth density

The initial concentration of the algae solution was about 10^7 cells mL^{-1}. The crude plant extracts (1 mL) were added to the *M. aeruginosa* culture medium (100 mL) separately. The same volume of DMSO solution was added to the control group with three parallel samples set up for each group. The OD680 value of the algae solution was recorded using an ultraviolet-visible luminometer (UV-2700) and measured every 48 h.

SEM

M. aeruginosa cultures were exposed to the four extracts for 10 days, and the microstructural changes of cells were taken at 30 kV using scanning electron microscopy (SEM, SU1510).

Photosynthesis and protein

Chlorophyll *a* and carotenoid were examined according to Wang *et al.* (2010), and extractions were measured at 665, 649, and 470 nm by using a UV spectrophotometer (UV2700, Shimadzu, Japan); PC, APC, and PE were examined according to Padgett & Krogmann (1987), and extractions were measured at 650, 620, and 565 nm.

Soluble protein content was determined according to the Bradford method (Bradford 1976) using bovine serum albumin as a standard. The MDA content was determined by thiobarbital acid colorimetry (Shiu & Lee. 2005).

Microcystins

Each sample was filtered through a 0.45 μm filter membrane then analyzed for MCs. The algal toxin content was determined by a national standard method using Shimadzu fast liquid chromatography (DGU-20A, Shimadzu, Japan) (Lawton *et al.* 1994; Ikawa *et al.* 1999; Ramanan *et al.* 2000).

Statistical analysis

Three parallel samples were established during the experiments, and the Origin 9.0 software was used for drawing. The inhibition rate (IR) was according to the formula:

$$IR = [1 - (E_n/C_n)] \times 100\% \quad (1)$$

where E_n and C_n represent the data from experimental and control groups, respectively.

RESULTS

Effect of rhubarb extract on the growth of *M. aeruginosa*

The growth of algae could be reflected by its OD value. Figure 1(a) and 1(b) depict the effect of rhubarb extracts on *M. aeruginosa*.

As shown in Figure 1(a) and 1(b), rhubarb extracts have stronger inhibitory effects on the growth of *M. aeruginosa* compared with the blank group. Extracts B, C, D, and E reduced *M. aeruginosa* by 7.2%, 64.1%, 59.3%, and 61.9%, respectively. The n-hexane phase has a slightly significant inhibitory effect on the growth of *M. aeruginosa* at shorter periods, whereas growth was significantly inhibited at 16 d. The ethyl acetate, n-butanol, and aqueous phases of rhubarb were predicted to contain more allelopathic inhibitory components, whereas n-hexane contains less allelochemicals.

Phytochemical studies show that the main components of rhubarb in traditional Chinese medicine are bismuth, stilbene glycosides, chromones, and tannins (Mao 2005). The presence of these allelochemicals may be the main reason for the growth inhibition on *M. aeruginosa* by rhubarb. The specific compounds in rhubarb extract help inhibit algae growth. Therefore, rhubarb extract should be further isolated and identified in future studies.

SEM and effect on MDA in *M. aeruginosa*

In recent years, SEM has proven to be an indispensable tool for visual estimation of cell damage (Ma *et al.* 2012). Changes in the microstructural morphology of healthy and damaged cells are shown in Figure 2(a).

Figure 2(a1) shows that the surfaces of healthy cells with diameters of about 3 μm are smooth and nearly spherical, and particles are evenly distributed. *M. aeruginosa* treated

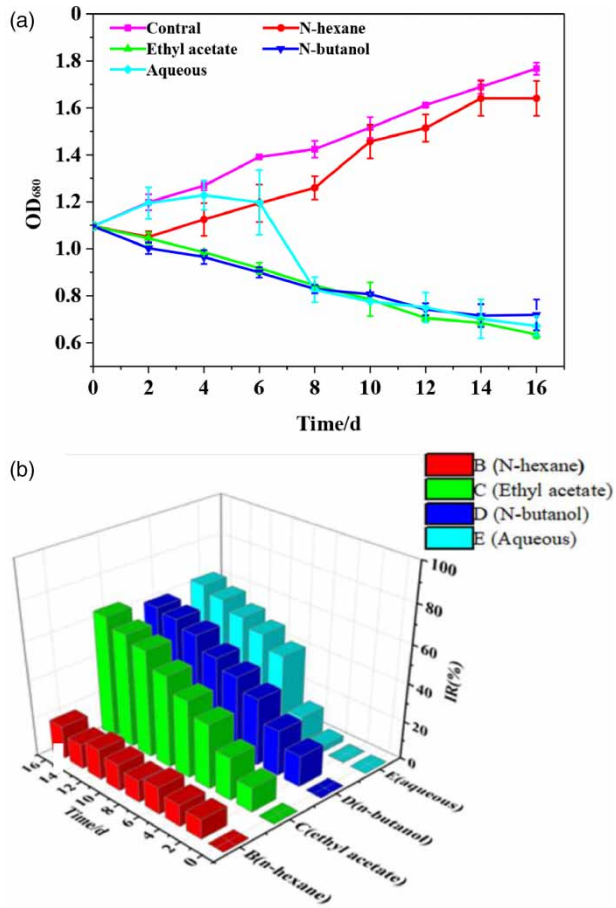

Figure 1 | Effect of rhubarb extracts on growth of *M. aeruginosa*.

with the n-hexane phase displays micro-cavities with little predicted damage (Figure 2(a2)). Microcystis cells treated with the ethyl acetate, n-butanol, and aqueous phases were incomplete; intracellular matter was released, and hollow spaces formed on the cell surfaces, while most of them were clearly destroyed (Figure 2(a3)–2(a5)). This result may have occurred because the algae-inhibiting component destroys the integrity of the cell membrane and causes the internal matter to overflow (Xu 1993). Shen *et al.* (2011) believed that allelochemicals may cause membrane structure damage and function loss, which in turn destroy chlorophyll and enzyme structure, thereby affecting the photosynthesis and enzyme activities of algal cells and ultimately leading to cell death.

MDA is the product of cellular lipid peroxidation, which is typically utilized as a marker of cellular physiological stress and oxidative damage. It is often used to reflect the degree of damage to the cell membrane structure (Hua *et al.* 2018). The changes in the MDA content of *M. aeruginosa* are shown in Figure 2(b). The MDA content

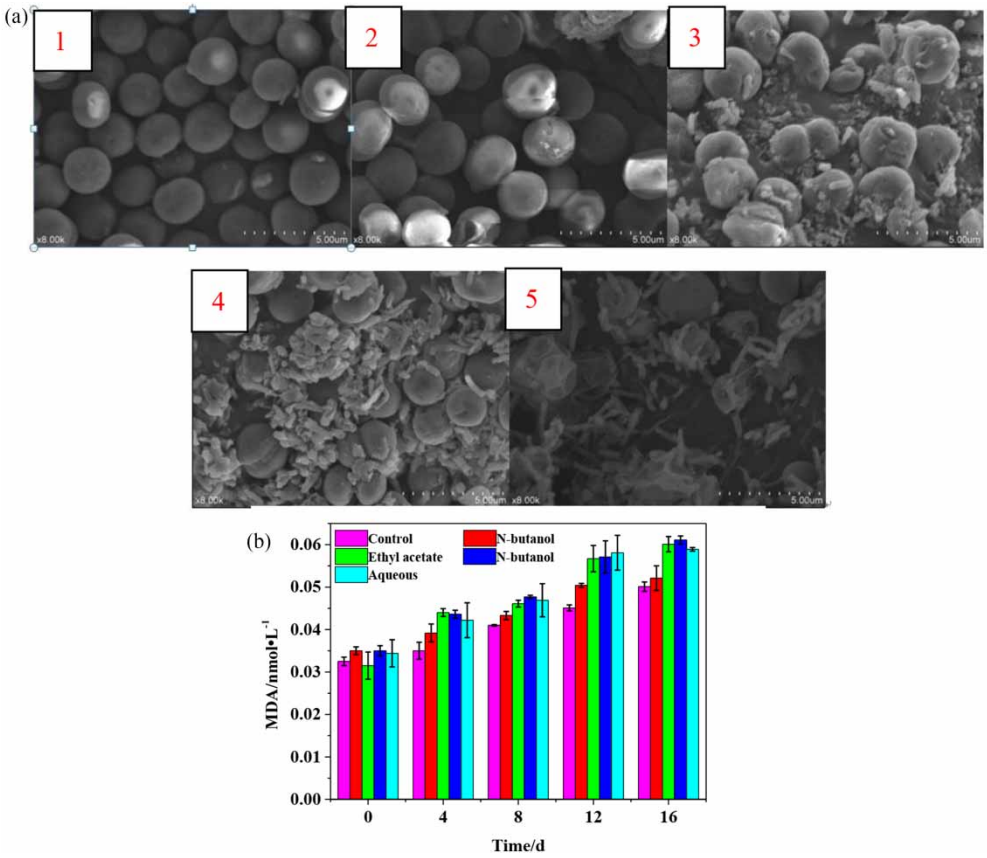

Figure 2 | (a) SEM images of *M. aeruginosa* after treatment by different rhubarb extracts. (1) Control, (2) n-hexane, (3) ethyl acetate, (4) n-butanol, (5) aqueous phases. (b) Effects of rhubarb extracts on MDA in *M. aeruginosa*.

increased substantially with the increase in exposure time, especially in the ethyl acetate, n-butanol, and aqueous phases of rhubarb.

Effects on chlorophyll *a* and carotenoids in *M. aeruginosa*

Chlorophyll *a*, carotenoid, and phycobiliprotein contents could be used to reflect the physiological responses of *M. aeruginosa* to allelochemicals (Hong *et al.* 2009). Chlorophyll *a* and carotenoid contents are indispensable indicators for photosynthetic rate determination and play an important role in energy capture and transfer during photosynthesis (Zhang *et al.* 2013). Figure 3(a) and 3(b) show that the four extracts have an inhibitory effect on the expression of chlorophyll *a* in algae cells compared with the control. The inhibition rate of chlorophyll *a* was 21.2% with the n-hexane phase on the 16th day; the chlorophyll *a* in the algae solution with ethyl acetate, n-butanol, and aqueous phases showed substantial decreases compared with the control group. On the 16th day, the inhibition rate of chlorophyll *a* reached 59.9%, 61.8%, and 69.5%, respectively.

The four extracts have certain inhibitory effects on the expression of carotenoids (Figure 3(c) and 3(d)). Although the carotenoids with the n-hexane phase showed an upward trend, the growth rate was considerably lower than that of the control group. On the 16th day, the inhibition rate for carotenoids was 21.1%. When the ethyl acetate, n-butanol, and aqueous phases were added to the algae liquid, the inhibition rates of carotenoid increased to about 47.1%, 49.9%, and 67.4%, respectively.

Effect on phycobiliproteins in *M. aeruginosa*

Figure 4(a)–4(f) depict the effects of rhubarb extracts on PC, APC, and PE. Unlike the blank control group, the four organic extracts of rhubarb have inhibitory effects on the expression of phycobiliproteins in *M. aeruginosa*, wherein the inhibition rate of PC with n-hexane, ethyl acetate, n-butanol, and aqueous phases was 19.4%, 84.1%, 88.4%, and

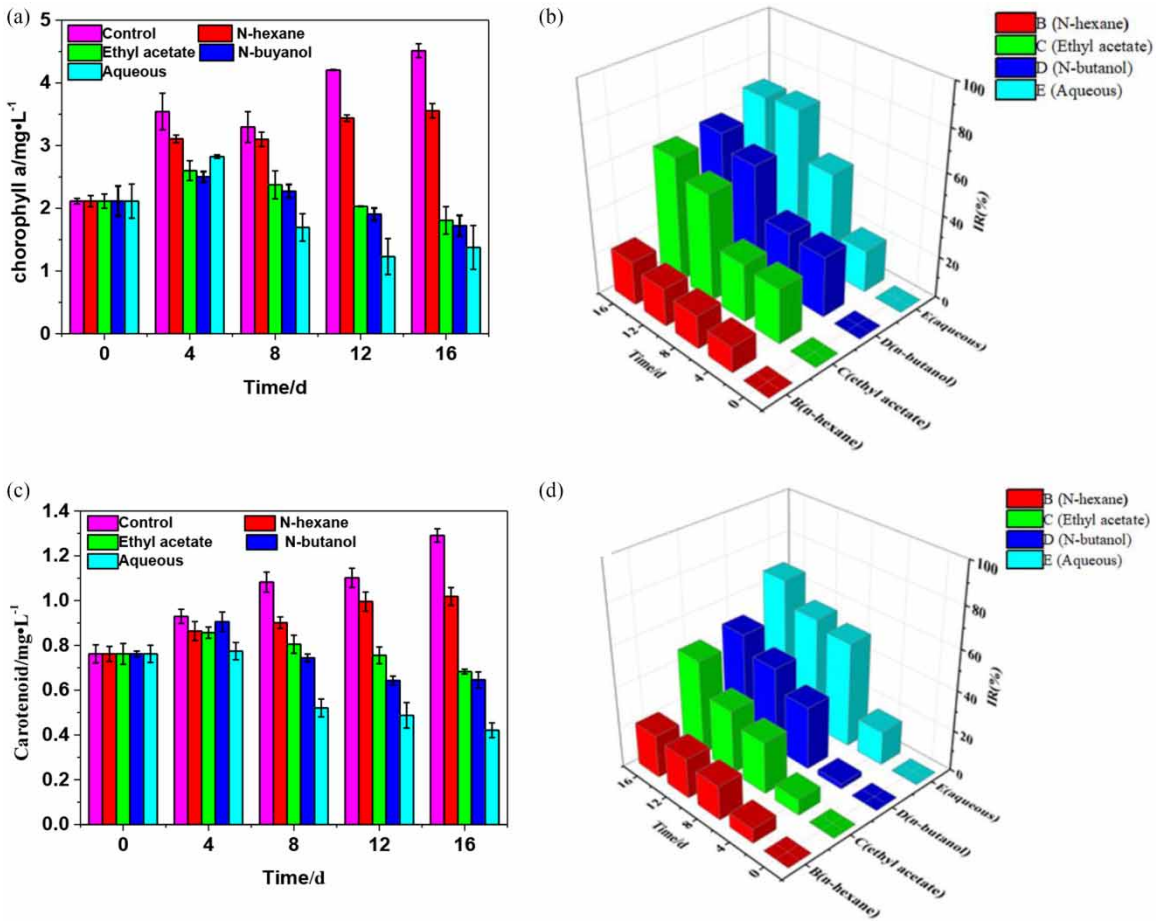

Figure 3 | Effects of rhubarb extracts on chlorophyll *a* and carotenoid in *M. aeruginosa*.

88.4% on the 16th day, respectively (Figure 4(a) and 4(b)). The inhibition rates of allophyte proteins were 9.8%, 60.5%, 77.1%, and 87.3%, respectively (Figure 4(c) and 4(d)). The inhibition rates of PE were 42.4%, 54.3%, 68.9%, and 65.5% (Figure 4(e) and 4(f)). Results show that the ethyl acetate, n-butanol, and aqueous phases have good inhibitory effects on phycobiliproteins.

Effects on soluble proteins within *M. aeruginosa*

The inhibitory effects on soluble protein with organic extracts of rhubarb are shown in Figure 5. The soluble protein content can reflect the metabolic activity of algal cells (Zhang *et al.* 2013). As shown in Figure 5, all four rhubarb extracts have significant inhibitory effects on the synthesis of soluble protein of *M. aeruginosa*. Compared with the control, the soluble protein within *M. aeruginosa* cells with the organic extracts of rhubarb clearly decreased. The content of soluble protein with the n-hexane phase increased, and the inhibition rate increased slightly to 36.9% on the 16th day. However, this rate is much lower than that of the control group. The three other extracts showed a downward trend, and the inhibition rate reached 91.3%, 90.1%, and 89.9% on the 16th day, respectively. Results indicated that rhubarb extract inhibits protein synthesis, which further leads to the abnormal physiological metabolism of *M. aeruginosa* and accelerates the death of algal cells (Lu *et al.* 2014).

Effect on MCs

When algae cells are destroyed, toxins may be released from the algae cells, especially toxins such as MCs (Wu *et al.* 2010; Pei *et al.* 2014). The inhibition and oxidation of microcystin-LR (MC-LR) are important for allelopathic technology. The effect of rhubarb extract on MC-LR is shown in Figure 6. The extracts showed stronger inhibitory effects on MCs compared with the control group, where the n-hexane phase first

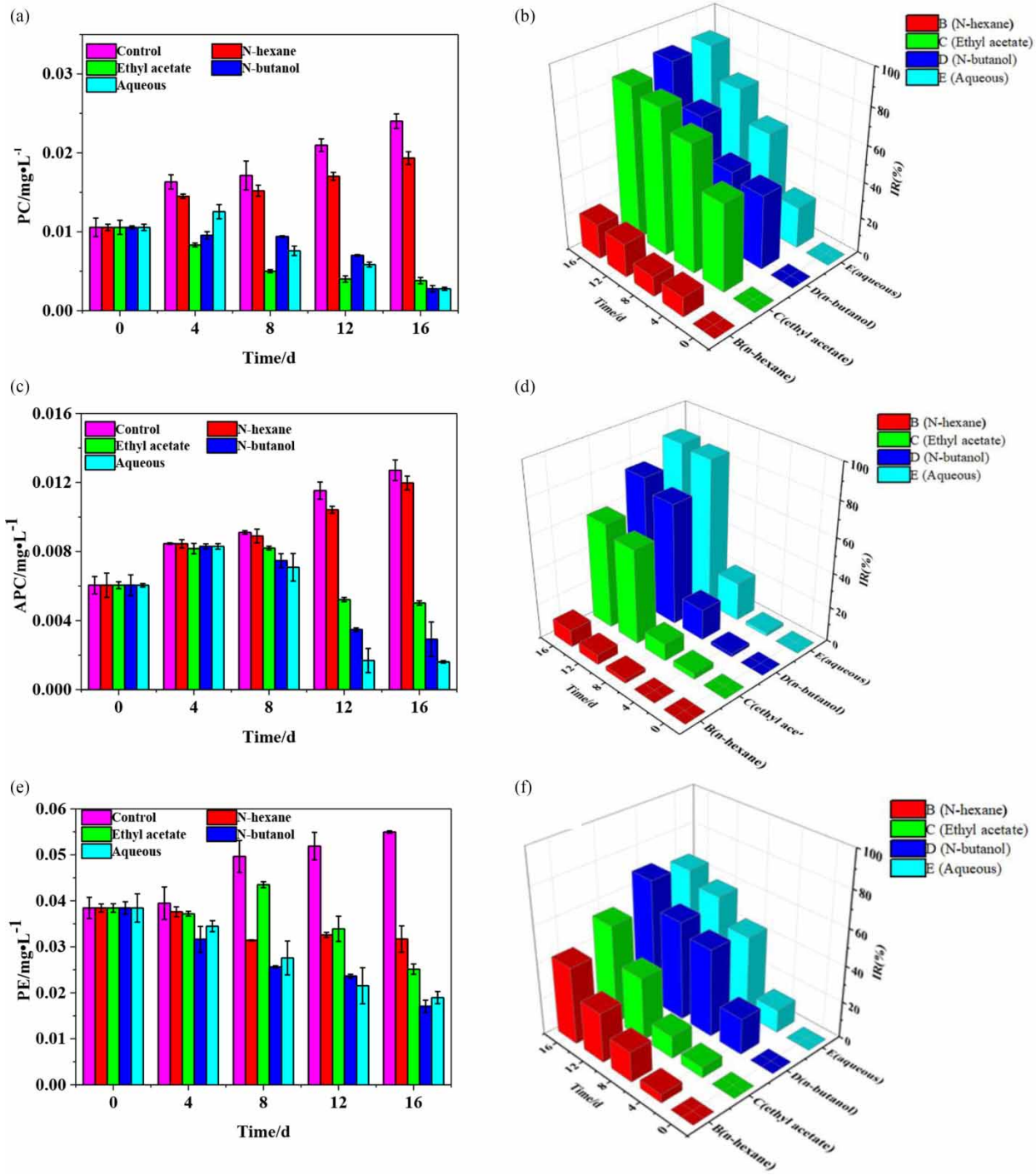

Figure 4 | Effects of rhubarb extracts on phycobiliprotein in *M. aeruginosa*.

exhibited a decreasing then increasing trend. The MC inhibition was 25.8% on the 16th day. After the ethyl acetate phase was added, the algal toxin content was below the detection limit, whereas the inhibition rate reached 100% on the fourth day. The inhibition rates with the n-butanol and aqueous phases reached 100% on the 16th day. The reduction of algal toxin reflects the number of algal cells, where we predict that the rhubarb extracts are rich in oxidizing substances, which can oxidize and decompose algal toxins.

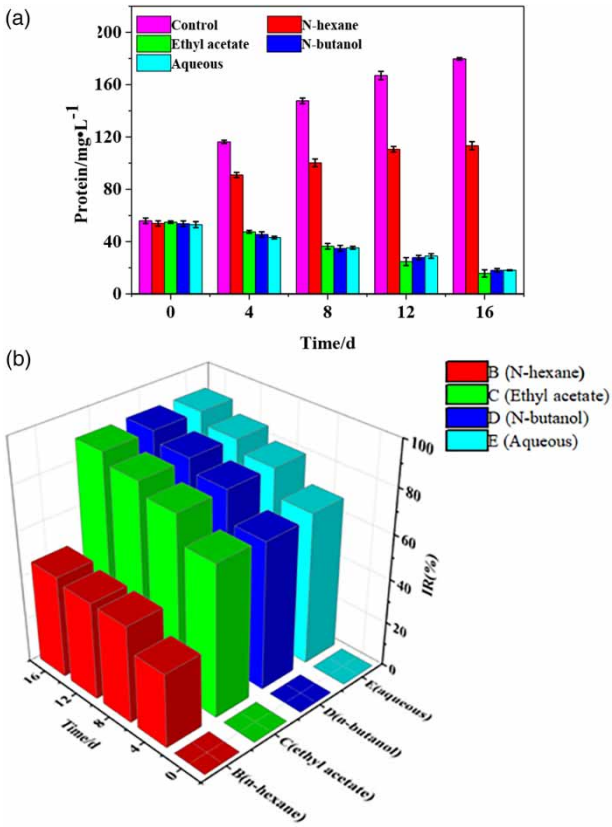

Figure 5 | Effects of rhubarb extracts on soluble protein content in *M. aeruginosa*.

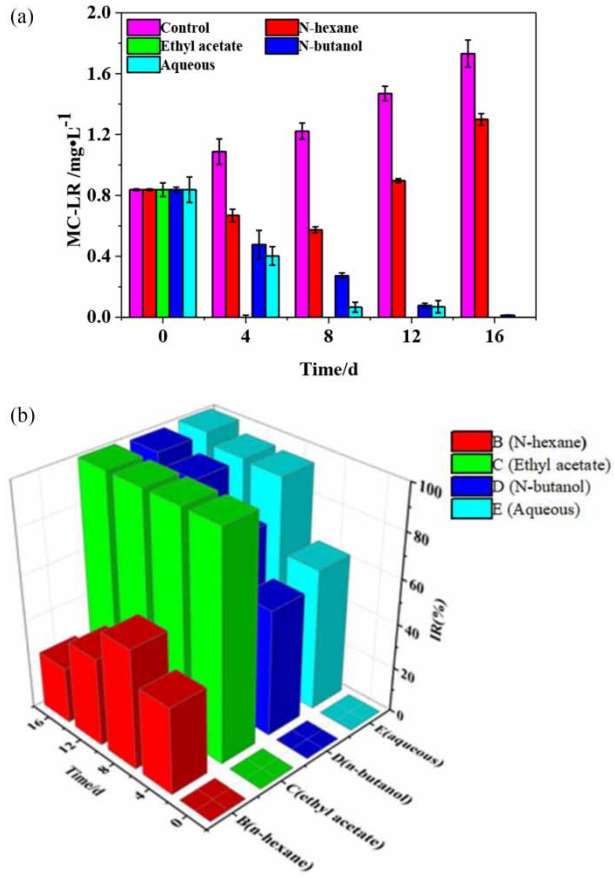

Figure 6 | Effects of rhubarb extracts on MC-LR in *M. aeruginosa*.

Identifying effective allelochemicals

The key components of organic extracts of rhubarb were identified by HPLC (Figure 7). Aloe-emodin, emodin, chrysophanol, rhein, and rheochrysidin were detected in the ethyl acetate, n-butanol, and aqueous phases. We predict that these five anthraquinone derivatives may be the key components that inhibited *M. aeruginosa*. Moreover, the ethyl acetate phase contained a large amount of anthrone derivatives, anthracenes and anthrones, and other organic acids, such as tannins and phenylbutanone glycosides. Similar compounds were detected in the n-butanol phase, with several volatile organic acids. A number of the combined glucosides, such as aloe-emodin, emodin, rhein, and chrysophanol, composed the aqueous phase.

DISCUSSION

Cyanobacteria are single-celled organisms without a nucleus. However, genetic matter is present in the center of the cell, usually in the form of granules or reticulates. Chromatin and pigment are evenly distributed in cytoplasm. Allelochemicals mainly lead to photosynthetic system deterioration (Hong *et al.* 2009), cell membrane structure cleavage (Shao *et al.* 2009), decreased enzyme activity (Zhang *et al.* 2010), deterioration of the antioxidant system (Zhang *et al.* 2014), abnormal gene expression, and inhibition or direct killing of cell division (Qian *et al.* 2009), thereby inhibiting the growth of cyanobacteria.

Rhubarb is rich in anthraquinones (mainly rhein, aloe-emodin, emodin, and chrysophanol), which inhibit many bacteria (Pratt 1942). Thus, rhubarb is a good allelopathic material. According to the changes in OD and cell surfaces as depicted by the SEM images in this research, we speculate that the main allelopathic components in rhubarb may rupture cell membranes. Li & Hu (2007) found that 2-methylacetamidine acetate, which was isolated from reeds, can break cell membranes completely, thereby leading to K^+, Ca^{2+}, and Mg^{2+} leakages. Thus, algae are finally inhibited. Phenolic acid allelochemicals act on the cyanobacterial cell membrane, which causes the peroxidation of membrane lipids, interferes with ester metabolism, and

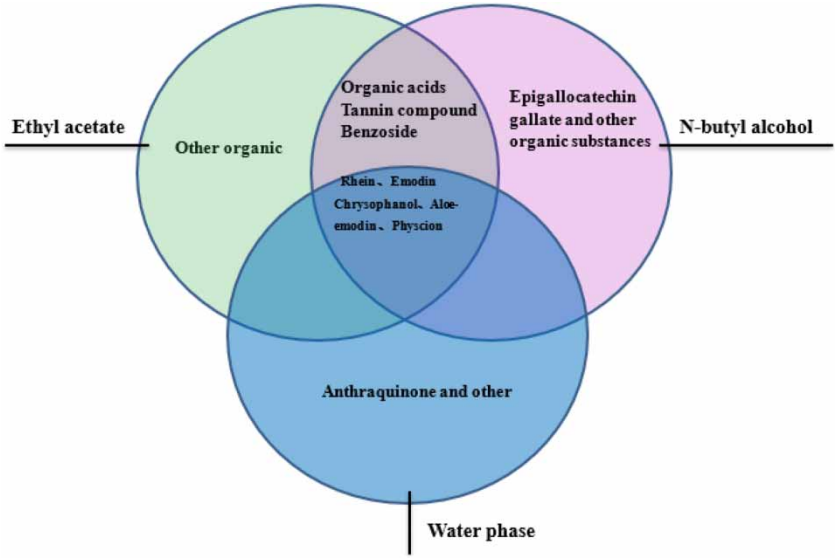

Figure 7 | Key components of rhubarb allelochemicals identified by HPLC.

inhibits esterase activity. As a result, membrane integrity is reduced and causes cell pyknosis. For these reasons, the growth of cyanobacteria was inhibited (Hua & Chen 2008), which is consistent with the result of this study. MDA contents increased with four organic extracts of rhubarb, which indicates that the rhubarb extract caused lipid oxidation of the cell membrane, resulting in decreased protein content, cleavage, and overflow of internal dissolved matter, which then resulted in algae inhibition.

Studies have shown that most allelochemicals affect the algal photosynthetic system (Hong *et al.* 2008). Cinnamic acid can reduce the oxygen consumption of algae and simultaneously transfer electrons to other pathways, such as non-cytochromes. Allelochemicals can also inhibit the activity of adenosine triphosphatase in algal mitochondria, thereby inhibiting photosynthesis and respiration of algal cells (Peñuelas *et al.* 1996). In this paper, chlorophyll *a* and carotenoids were inhibited by the four rhubarb extracts. The ethyl acetate, n-butanol, and aqueous phases had significant effects on the photosynthesis of *M. aeruginosa* cells. The phycobiliproteins of *M. aeruginosa*, namely, PC, APC, and PE, can transfer absorbed energy to the photosynthetic system II of algae. These three phycobiliproteins are the main functional groups of cyanobacteria photosynthesis (Sun *et al.* 2015). Thus, a decrease in phycobiliproteins may reduce the ability to capture and absorb light and further lower the efficiency of photosynthesis (Hu *et al.* 2017). As a result, the photosynthetic efficiency of *Microcystis* cells and of electron transport rate would notably decline under treatment (Lu *et al.* 2014).

Protein is not only one of the key components of organisms; it also makes up enzymes that take part in biochemical metabolism (Zhang *et al.* 2013). The soluble protein content indicates the metabolic activity of cells. Artemisinin can inhibit the synthesis of protein in *M. aeruginosa*, which affects biochemical reactions at normal levels and causes metabolic dysfunction (Ni *et al.* 2012). In this study, the decrease in protein content may be due to the inhibitory action of the rhubarb extract on protein synthesis, which resulted in normal physiological and metabolic disorder of algae cells. This hypothesis is consistent with those of previous studies (Ni *et al.* 2012; Zhang *et al.* 2013) and may be one of the mechanisms of algal inhibition.

MCs are synthesized and present in cyanobacteria and released into water after death or breakdown (Figueiredo *et al.* 2004). The MC yields of the four extract groups were significantly lower than those of the control group. The ethyl acetate extract showed the lowest output, reaching below the detection limit. On the fourth day, the algal toxin content decreased to a level below the detection limit. The algal toxin in the n-butanol and aqueous phases fell below the detection limit on the 16th day as well. This finding indicates that the rhubarb extract inhibits the synthesis of algal toxins indirectly while inhibiting the growth of *M. aeruginosa* and decomposing MCs after release.

The key allergic components in rhubarb are anthraquinone derivatives, such as aloe-emodin, chrysophanol, rhein, and emodin, all of which are small molecular compounds that can pass through cell membranes and have broad-spectrum antibacterial and antiviral properties. Their antibacterial

property is achieved by blocking the synthesis of proteins and nucleic acids of bacteria (Mao 2005). Rhubarb is rich in terpenoids (mainly rhein, emodin, aloe-emodin, and chrysophanol), which inhibit a variety of bacteria through antibacterial mechanisms and inhibition of the mitochondrial respiratory chain. Bacterial growth is inhibited by mechanisms such as electron delivery; inhibition of respiration; oxidation of amino acids, sugar, and protein metabolism intermediates; inhibition of the end result of nucleic acid; and protein synthesis. Emodin, rhein, and aloe-emodin have the strongest effects and are good allelopathic materials.

In summary, this study finds that rhubarb has a rich variety of oxidizing substances, which can inhibit the cyanobacterial cell membrane, photosynthetic system, protein synthesis, and metabolic functions. Moreover, rhubarb can control the number of cyanobacteria through its remarkable inhibitory effect.

CONCLUSION

Experiments show that Chinese rhubarb extract has a potential role in inhibiting the growth of *M. aeruginosa*. The ethyl acetate, n-butanol, and aqueous phases displayed notable inhibitory effects, which lead to the destruction of algae photosynthesis and protein synthesis, and algal toxin reduction. The key allelochemicals may be aloe-emodin, emodin, chrysophanol, rhein, and physcion, which could provide a new resource for allelopathic technology.

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