The decoction of *Radix Astragali* inhibits the growth of *Microcystis aeruginosa*

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**A B S T R A C T**

Many measures have been developed to control the harmful algal blooms that are potentially threatening potable waters. The pilot experiments showed that the unfiltered and the sterile-filtered decoctions of *Radix Astragali* inhibited the growth of *Microcystis aeruginosa*. The inhibitory effect diminished in natural pond conditions after 68 days, due to photo-degradation of the flavonoids from *Radix Astragali* that appear to be responsible for the action on *M. aeruginosa*. Four phases (assemblage, conglomeration, cell membrane destruction and decomposition) can be characterized in the process of cell death with increasing decoction dose. The quantum yields and electron transport rates of photosynthesis system II of *M. aeruginosa* cells markedly decreased during contact with the decoction, resulting in the disruption of *M. aeruginosa* photosynthesis. The results indicate that the application of *Radix Astragali* decoction for the inhibition of *M. aeruginosa* growth is feasible when the dose is less than 20 ml L⁻¹.

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**1. Introduction**

Harmful algal bloom (HAB), often cyanobacterial, in freshwater, is becoming a major environmental problem worldwide. It significantly affects the normal use of water resources, disrupting drinking water supplies and potentially decreasing the biodiversity (Paerl et al., 2001). In addition, cyanobacterial blooms usually associated with toxigenic strains are common features of eutrophic water systems all over the world (Havens, 2007; Paerl and Huisman, 2008). Cyanotoxins, including cylindrospermopsins, microcystins, nodularins and saxitoxins, are generally water-soluble substances (Paerl et al., 2001; Sivonen and Jones, 1999), thus aquatic biota may be at risk of poisoning in water containing the toxic compounds. Moreover, the transfer of these toxins through food chains may reach humans (Bourne et al., 2006). Therefore, control and elimination of cyanobacterial blooms have become significant issues.

At present, several measures are used to control the HABs. These include physical methods: e.g., mechanical algaecide (Conservancy, 2007) and capping (Eek et al., 2007); chemical ones: e.g., ozonation, bluestone, potassium permanganate, algaecide or anti-algal flocculants (Hullebusch et al., 2002; Wu et al., 2005) and biological ones: e.g., introducing fish, cultivating aquatic plants and, using algaecidal bacteria and biopond-wetland system (Shi et al., 2006; Wu et al., 2010a). Chemical methods have been widely adopted to control HABs as a frontline attack on this increasingly urgent environmental concern (Murphy et al., 1999).

However, many chemical algaecides have the potential to change environmental conditions and even harm aquatic organisms. Chemical treatments like hydrogen peroxide, aluminum sulphate or ferric sulphate change pH-value or salinity (Momani et al., 2008; Wu et al., 2010b), which may threaten the life in the lake. Lime could increase the concentration of ammonia in water to a level toxic to water organisms (Murphy et al., 1999). In addition, carcinogenic substances like trihalomethanes (THMs) form in the presence of organic compounds, such as humic and fulvic acids during the chlorination of drinking water (Price, 1969). Thus, there is a need to find an environmentally benign chemical algaecide with high efficiency.

*Astragalus* species are perennial legumes, which grow mainly in Shanxi, Inner Mongolia, Hebei, Gansu and other provinces in Northern China. The root (known as *Radix Astragali*) is one of the most familiar and inexpensive Chinese traditional medicines, which has been widely used within the traditional Chinese system for centuries (Mao et al., 2006). *Astragalus mongholicus* and *A. membranaceus* are the two species most often prescribed. The medicinal uses of *Radix Astragali* (Chinese name: Huangqi) include treating general debility and chronic illnesses, as well as, improving immunity and the overall vitality of patients. Recently, *Radix Astragali* was shown to have a wide range of...
immunopotentiating effects and has proven efficacious as an adjunct cancer therapy (Block and Mead, 2003). The main components of Radix Astragali are triterpenoid saponins, polysaccharides, flavonoids and amylase (Jasbi et al., 2002; Li et al., 2007). Some of these compounds such as the flavonoids are structurally related to reported anti-bacterials or allelochemicals (Ohara and Ohira, 2003; Weir et al., 2003) and nitrification inhibitors (DeLuca et al., 2002).

The goals of this study were to: (1) document the effect of Radix Astragali decoction (crude extract) on the growth (inhibition or stimulation) of M. aeruginosa and explain any effects; and (2) assess the effect of Radix Astragali crude extract on microbial activities.

2. Materials and methods

2.1. Preparation of Radix Astragali decoction

Decoction (crude extract) of Radix Astragali (A. mongholicus) was prepared by placing 3.0 g dry root (chopped into 0.5 cm lengths) into 210 mL reverse-osmosis (RO) purified water in a new gallipot with a lid at 70 ± 5 °C for 2 h before cooling to room temperature (25–30 °C). The decoction was then simply kept at room temperature for 2 days before being used for bioassays.

2.2. Laboratory bioassays of uni-algal cultures

The sterilized water used for cyanobacterial cultivation in the present study was prepared according the method of Jin and Dong (2003), with pH at 7.5 and salinity at 30%. Microcystis aeruginosa (M. aeruginosa), representing the most widely distributed cyanobacterium causing harmful algal blooms in freshwater worldwide, was selected for bioassays. The organism was grown in BG-11 medium (Rippka and Herdman, 1992) at 25 ± 1 °C under illumination at 3000 lx with a 12-h light: 12-h dark cycle in illuminating incubators with shaker (150 rpm). The M. aeruginosa was cultivated to the exponential growth phase for use. During the experiment, 1 mL M. aeruginosa broth was placed in 250 mL disinfectant flasks containing fresh BG-11 medium with the total experimental volume being adjusted to 200 mL. Achieving an initial concentration of chlorophyll-a (~670 μg L⁻¹) as a proxy for M. aeruginosa population approximately 670 μg L⁻¹ of chlorophyll-a. These cyanobacterial cultures were defined as “monoculture”, serving as the controls throughout the experiment. Samples were collected at 24 h intervals and monitored for the growth of the cyanobacteria by determining chlorophyll-a (Chl-a).

Bioassays were performed in sterile 250 mL flasks with fresh BG-11 medium. 1 mL M. aeruginosa culture was inoculated and the total experimental volume was adjusted to 200 mL. The unfiltered decoction and the sterile-filtered (pore size 0.22 μm) decoction of Radix Astragali were added to separate flasks, such that the initial cyanobacterial concentrations gave approximately 700 μg L⁻¹ of Chl-a, and then placed under the same conditions as the monoculture. To test if illumination levels affected the compounds responsible for inhibiting M. aeruginosa growth, a 250 mL glass bottle with unfiltered decoction of Radix Astragali was placed in an incubator with strong illumination (8000 lx) for 7 days, and then used in the bioassay above.

To test the effect (and dose dependence) of decoction of Radix Astragali on the photosynthesis of M. aeruginosa cells, different amounts of unfiltered decoctions were added to flasks containing BG-11 medium. Pure cultures of M. aeruginosa at log phase were inoculated into the media in triplicate, each in a 150 mL container with 100 mL of the medium. The flasks were incubated at 28 ± 1 °C and a light intensity of 2500 lx under a 12-h light: 12-h dark cycle. At regular intervals, the effective quantum yields and growth were readily photo-degraded.

2.3. Pond application of Radix Astragali decoction

Two ponds in Jinning, Kunming, China were monitored to examine the effects of Radix Astragali decoction on algal biomass. The ponds measured 1050 m² with average depths of 1.3 and 1.35 m, respectively. Both ponds were eutrophic during the experimental period (from April to July, 2008), exhibited high nutrient levels (total nitrogen and phosphorus ranged in concentration from 3.50 to 12.60 mg L⁻¹ and from 0.34 to 1.56 mg L⁻¹, respectively).

At the beginning of the experiment, one pond received an insufflation of Radix Astragali decoction (Radix Astragali-water = 1:70 (v/v)) equivalent to 1.3 g m⁻³ and the other, used as control, was not treated. Three water samples were collected from each pond from the water surface to a depth of 25 cm, approximately 2 m from shore and analyzed for Chl-a.

2.4. Analysis and statistics

The samples were filtered (Whatman, GF/C, pore size 0.45 μm) and stabilized with MgCO₃ for chlorophyll analysis. All residues were immediately placed on ice and quickly transferred to −20 °C until analysis (< one month). Chlorophyll-a (Chl-a) was collected using the acetone extraction method. 0.1–0.3 L of lake water was filtered through a Whatman GF/C filter. The residue on the filter was dissolved in water, resulting in 10 mL aqueous solution. After overnight extraction into acetone (90% v/v), the absorbance of this solution was measured at 663 and 750 nm in 1-cm light path length glass cuvettes (Golterman and Clymo, 1969).

All extraction procedures and samples were performed in triplicate. Statistical analysis (One-way ANOVA) was executed using SPSS (version 12.0). The level of statistical significance was accepted when p < 0.05.

3. Results

3.1. Effects of doses of Radix Astragali on growth of M. aeruginosa

Frequent visual evaluation indicated that the M. aeruginosa in monoculture (control) grew substantially during the two-week incubation period. The concentration of Chl-a in controls increased from 693.2 to 1347.2 μg L⁻¹ within 48 h while the removal efficiencies of M. aeruginosa in treatments with 2, 4, 6 and 8 mL of decoction were 55%, 74%, 91% and 95%, respectively (Fig. 1). The concentration of Chl-a in the control was significantly different from that of the treated culture after 48 h (p < 0.05). This result is indicative of growth inhibition by Radix Astragali decoction.

Growth of M. aeruginosa was significantly (p < 0.05) inhibited within 48 h by both, sterile-filtered and unfiltered Radix Astragali decoction in doses ranging from 10 to 40 mL L⁻¹ (Fig. 1). The rates of inhibition of M. aeruginosa growth were dose-dependent, with the inhibition by sterile filtered decoction not significantly different from that of unfiltered decoction. This indicated that any microorganisms present in the unfiltered decoction did not significantly affect the inhibitory action of Radix Astragali on the growth of M. aeruginosa.

Furthermore, the growth of M. aeruginosa was unaffected by the unfiltered decoction that had received strong illumination. This implied that the compounds responsible for inhibiting M. aeruginosa growth were readily photo-degraded.

**Fig. 1.** Mean cyanobacterial biomass (expressed as chlorophyll-a concentration) inhibited when the M. aeruginosa cells were in contact with the Radix Astragali decoction 48 h. Replicate cultures were maintained in BG-11 media, augmented with unfiltered Radix Astragali decoction, or sterile-filtered Radix Astragali decoction. Error bars represent standard deviation. *Treatment different from control with P<0.05. **Treatment different from control with P<0.01.**
3.2. Effects of Radix Astragali contact times on growth of M. aeruginosa

Both the unfiltered and sterile-filtered decoctions of Radix Astragali inhibited the growth of M. aeruginosa, as shown by the decrease of Chl-a concentration in the media (Fig. 2). Microscopic visual evaluation showed that M. aeruginosa cells were beginning to die (become yellow) and be deposited after incubation for 6 h. Death and deposition of M. aeruginosa cells increased with the incubation time. Statistical analysis showed that there was an exponential decay relationship between the Chl-a concentration and the contact time of Radix Astragali decoction with M. aeruginosa, which indicates that the inhibitory effect on M. aeruginosa growth was also time-dependent.

3.3. Changes in M. aeruginosa stressed by Radix Astragali decoction

To understand the change in M. aeruginosa, the cellular microstructure was observed when the cells had been in contact with the decoction for 24 h. Fig. 3 shows that the M. aeruginosa cells grew normally in a control (monoculture) situation, resulting in a symmetrical distribution of the intact M. aeruginosa cells. When the dose of the unfiltered decoction increased from 10 to 20 ml L⁻¹, the M. aeruginosa cells began to converge and aggregate. As the dose increased to 30 ml L⁻¹, the M. aeruginosa cell membranes began to break down, and the cells had substantially decomposed when the dose reached 40 ml L⁻¹. These observations show that the unfiltered Radix Astragali decoction had negatively affected the structure of M. aeruginosa community when the dose was lower than 20 ml L⁻¹ and destroyed the cell membrane of M. aeruginosa when the dose was higher than 30 ml L⁻¹.

To investigate how the photosynthetic mechanism of M. aeruginosa was affected by unfiltered decoction of Radix Astragali, the effective quantum yield and electron transport rate were determined. Our results show that both processes markedly decreased during the application of unfiltered decoction of Radix Astragali (Fig. 4). This indicates that the photosynthesis system II of M. aeruginosa was destroyed by the unfiltered decoction.

In addition, the concentrations of decoction (from 0.1 to 0.6 mg L⁻¹) on M. aeruginosa growth inhibition were much lower than those of the above bioassay experiment (from 10 to 40 mg L⁻¹), which indicates that the death of M. aeruginosa was mainly due to photosynthesis system II dysfunction when the decoction was applied.

3.4. Field application of Radix Astragali decoction

The treated pond had significantly lower average algal biomass than the control (p < 0.05) during the experimental period. Average algal biomass (determined via Chl-a) in the treated pond declined rapidly after the unfiltered Radix Astragali decoction was applied.
4. Discussion

The inhibitory effect of the unfiltered *Radix Astragali* decoction on *M. aeruginosa* growth was not significantly different from that of the sterile-filtered (0.22 μm) treatment, which implied that the inhibition of *M. aeruginosa* growth should be attributed to the compounds extracted from *Astragalus* root rather than to a biotic effect. In the field experiment, growth of *M. aeruginosa* in the pond treated with unfiltered *Radix Astragali* decoction had recovered after 68 days, indicating that the compounds responsible for the inhibition of *M. aeruginosa* growth decomposed quite readily under natural conditions in waterways during this time.

The experimental area belongs to a subtropical area at high altitude (~1890 m) and is characterized by strong radiant intensity, where illumination for between 30–40% of sunshine times is higher than the 3000 lx that was used in the pilot experiment (Zhang et al., 2002). As a result, the photo-degradation of the compounds responsible for inhibiting *M. aeruginosa* growth was accelerated under the strong radiant conditions.

The specific compound(s) in the decoction responsible for the inhibitory effects is/are still unknown. However, information from the literature provides some clues for further work on this subject. It is known that *Radix Astragali* contains three kinds of medically bioactive compounds that are structurally similar to those that inhibit plant germination and growth (Mao et al., 2006). Phenylpropanoids, lignans and terpenoids, ubiquitous in plant materials, inhibit the growth of alfalfa radicles (Ohira and Yatagai, 1994) and germination of *Pinus laricio* (Muscolo et al., 2001). Flavonoids, are known to inhibit the ATPase activity of plasma membranes isolated from oat (Avena sp.) roots (Balke, 1985). Triterpenoid saponins also inhibit germination and growth of alfalfa, and the inhibitory effects are structure-dependent (Ohara and Ohira, 2003). Although *M. aeruginosa* was not used as a test species in these previously published reports, it appears that there are structural similarities between the medically bioactive compounds and the easily photodegradable compounds occurring in *Astragalus* root.

Among these three classes of compounds, flavonoids are quite readily photo-degraded. In living plants, they are relatively resistant to heat, oxygen, dryness, and moderate degrees of acidity but can be modified by light (Kühnau, 1976; Passamonti et al., 2009). Photostability of the flavonoid molecule depends on the nature of the hydroxyl group attached to C-3 of ring C. The presence of this hydroxyl group results in low photostability of the molecule (Smith et al., 2000). Furthermore, flavonoids exhibit a wide range of biological effects, including antibacterial, and antiviral activities (Hanasaki et al., 1994). Indeed, the cyanobacterium *M. aeruginosa* is a kind of phytoplankton-bacteria. Many articles in the literature, including the following, have reported that compounds, such as vanillin, gallic acid, catechins (Yang et al., 2005) and flavonol glycosides (Aliotta et al., 2005), inhibit the growth of cyanobacteria (*M. aeruginosa*) and/or *Alexandrium tamarense*. Thus, it is likely that flavonoids in *Radix Astragali* are responsible for the inhibition of growth of *M. aeruginosa*.

No matter what kind of compounds are responsible for the growth inhibition of *M. aeruginosa*, the processes of dysfunction and dying of cells stressed by *Radix Astragali* decoction can be divided into four ‘phases’. For lower doses (≤ 20 ml L⁻¹) of decoction, the process is “assemblage”, in which the *M. aeruginosa* cells begin to converge. As the dose of decoction increased (from 20 to 30 ml L⁻¹), the *M. aeruginosa* cells began to clump together; this is the “agglomeration” phase. With an even higher dose (from 30 to 40 ml L⁻¹), the third phase of “cell membrane destruction” was apparent. The cell membranes of *M. aeruginosa* started to break down as the dose of decoction continued to increase (≥ 40 ml L⁻¹), resulting in the increased penetration capacity of some mineral materials and the destruction of cytoplasmic transportation. As the dose of decoction and contact time increased, many of *M. aeruginosa* cell membranes disappeared. As a result, the cells died and then decomposed, in a phase designated as “decomposition”.

It is well known that toxins may be released from algal blooms and especially those produced by *Microcystis* species when the cell wall is destroyed (Momani et al., 2008; Wu et al., 2010a). In this study, when the dose of decoction was less than 20 ml L⁻¹, the *M. aeruginosa* cell wall structure was still intact, implying that no toxins was released from the *M. aeruginosa* cells. Thus, it is recommended that the dose of decoction used in a practical application should be less than 20 ml L⁻¹.

In the main, it appears the death of *M. aeruginosa* cells was caused by the dysfunction of photosystem two (PS II). This is because PS II, being the first link in the chain of photosynthesis, captures photons and uses the energy to extract electrons from water molecules. When components of the decoction entered PS II, the shuttle of electrons to form a quinone terminal electron acceptor so necessary for the oxidation of water was disrupted. This means that photosynthesis is no longer possible, thus leading to cell death. Additionally, microscopic observation indicated intact cell walls after death of *M. aeruginosa* caused by low doses of the decoction. This further indicates that the death of *M. aeruginosa* cells was not due to direct cell wall damage by unfiltered decoction of *Radix Astragali*.

Previous reports have shown that the use of *Radix Astragali* is bio-safe (Ma and Fu, 2002; Yang et al., 2009). Results of toxicity experiments using *Radix Astragali* suggest that regular use of the root is safe. For example, *Radix Astragali* decoction injected into the rat abdomen daily, continuing for one month, at a dose of 0.5 g kg⁻¹ d⁻¹ was found to have no significantly different effects on the weights or food-intakes of the experimental rats compared with the controls, and the experimental animals displayed no signs of toxicity (Ma and Fu, 2002). The chronic toxicity of lyophilized powder of *Radix Astragali* extract in rats and dogs was studied. There were no significant differences found in body-weight, food-intake, hematological and hemato-biochemical parameters, and organ coefficient between the test groups and the controls, and that safe doses for rat and dog were 39.90 and 19.95 g kg⁻¹, respectively (Yang et al., 2009). Overall, it is likely
that the use of Radix Astragali root decoction to inhibit *M. aeruginosa* growth is environment-friendly, but further study needs to be done in the aquatic ecosystem before large-scale field application is pursued.

5. Conclusions

This work shows that, the decoction of *Radix Astragali* is a highly acceptable material that can be used to control harmful algal blooms in centralized drinking waters as an emergency measure. At low doses of the decoction (less than 20 ml L$^{-1}$), no toxic materials are released from the algal cells. Microscope data show there are four readily identifiable phases, namely, “assembly”, “agglomeration”, “cell membrane destruction” and “decomposition” defining the death process of *M. Aeruginosa*. The process by which the active molecules in the decoction penetrated the *M. aeruginosa* cell wall was gradual (diffusion). In addition, the rapid decrease of effective quantum yields and electron transport rate of photosynthesis system II in *M. aeruginosa* cells indicate that the failure of photosynthesis system II was responsible for the death of *M. aeruginosa*.

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