

Inhibitory action of ginkgolic acid against pathogenic fungi and characterisation of its inhibitory activities on *Nigrospora oryzae*

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ABSTRACT

Ginkgolic acid is a natural plant antifungal substance with important biological activities, present in the testa of *Ginkgo biloba*. This research aims to analyse the antifungal action of ginkgolic acid against pathogenic fungi. Ginkgolic acid was analyzed by HPLC and FTIR, and the *in vitro* inhibitory activities of ginkgolic acid against pathogenic fungi has been investigated. Results showed that ginkgolic acid was a small hydrophobic molecule composed of five ginkgolic acids and their derivatives, with the alkyl and phenolic hydroxyl groups being located on the skeleton of the aromatic ring. Among *Nigrospora oryzae* (*N. oryzae*), *Alternaria alternata* (*A. alternata*) and *Penicillium expansum* (*P. expansum*), ginkgolic acid showed the strongest inhibition against *N. oryzae*, and the inhibition rate of ginkgolic acid at 5 mg · mL⁻¹ reached 65.82%. Ginkgolic acid lysed the cell membrane of *N. oryzae*, causing the leaking of the mycelial protein and enhancement of the permeability of the cell membrane, which caused the electrical conductivity of the liquid medium to increase. In addition, it also decreased the activities of superoxide dismutase and catalase in the mycelium of *N. oryzae*, thereby weakening the cell's protective function by damaging the integrity of the biomembrane. Furthermore, ginkgolic acid can effectively protect the leaves of *Epipremnum aureum* from infection by *N. oryzae*. It was concluded that ginkgolic acid effectively inhibited the activity of *N. oryzae*.

Keywords: antifungal activity, cell membrane permeability, *Epipremnum aureum*, growth inhibition, *in vitro*

Abbreviations: *A. alternata*, *Alternaria alternata*; CAT, catalase; *E. aureum*, *Epipremnum aureum*; *N. oryzae*, *Nigrospora oryzae*; *P. expansum*, *Penicillium expansum*; SOD, superoxide dismutase.

INTRODUCTION

Ginkgo biloba has been widely planted since the 1990s as an important economic tree with medicinal value. As early as Ancient China, there are records of using ginkgo nuts as medicine. At present, *G. biloba* extract has become one of the most popular herbal supplements in the world.

The testa is the fleshy part that wraps around the hard shell of ginkgo (*G. biloba*) seeds, and it contains many active ingredients, such as biflavone, alkyl phenol and phenolic acids, sterols and steroids, palmitone, catechol,

amino acids, sugars and so on (Sun et al., 2012). These active ingredients have the functions of capturing free radicals and anti-platelet activation factors, in addition to promoting blood circulation and brain metabolism (Yang et al., 2004a, 2004b).

Ginkgolic acid is a kind of natural plant antifungal substance found in the testa of *G. biloba* with important bioactivity (Hua et al., 2017). It contains hydroginkgolic acid, hydroginkgolonic acid, ginkgoneolic acid and so on (Ndjoko et al., 2000). It has many effects, such as

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anti-oxidation, anti-inflammation, anti-tumour, anti-allergy, antibacteria, in addition to prevention and control of diseases and pests. Ginkgolic acid (C15:1 and C17:1) at $5 \mu\text{g} \cdot \text{mL}^{-1}$ and *G. biloba* extract at $100 \mu\text{g} \cdot \text{mL}^{-1}$ significantly inhibited *Escherichia coli* biofilm formation on the surfaces of polystyrene and glass, and on nylon membranes (Lee et al., 2014).

Nigrospora oryzae is a well-known saprotroph on many plant species and has been mentioned as a pathogen on many hosts (Wright et al., 2008). A survey of the literature revealed that infection due to *N. oryzae* could cause leaf spot, blight and blight symptoms, in addition to seriously harming the growth of plants (Abass et al., 2013). *N. oryzae* has caused a great deal of damage to agricultural production. *N. oryzae* infection has been found in commercial tea plantations in Darjeeling, India, which reduced the yield and quality of tea (Dutta et al., 2015). In addition, *N. oryzae* has been reported as a pathogen of sesame leaf blight in Pakistan (Rehman et al., 2021).

Epipremnum aureum is an herbaceous evergreen with a strong ability to purify air and has been widely used as an important foliage plant for indoor greening (Aydogan and Montoya, 2011). Unfortunately, it is susceptible to multiple pathogens, such as *Colletotrichum gloeosporioides*, *Cylindrocladium colhounii*, *N. oryzae* and so on; it develops leaf spot, root rot or other diseases in the growing process.

Ginkgolic acid has been proven to inhibit the growth and reproduction of a variety of pathogenic microorganisms, but the pathway and mechanism of this effect have been seldom reported. In this work, the inhibitory activities of ginkgolic acid against *N. oryzae* was investigated and its inhibitory mechanism was studied. At the same time, protection of *E. aureum* plant leaves infected by *N. oryzae* through the action of ginkgolic acid was also investigated.

MATERIALS AND METHODS

Microorganisms and chemicals

N. oryzae, *Alternaria alternata* and *Penicillium expansum* were stored in the Microbiological Culture Collection Centre of Dalian Polytechnic University, China. These pathogenic fungi were maintained on potato dextrose agar (PDA: extract of boiled potatoes, 5 g; anhydrous glucose, 20 g; agar, 20 g; and distilled water, 1,000 mL) stored at 4°C and activated by inoculating on a PDA plate and cultured at 28°C for 4 d before the experiment.

The chemicals used in the culture medium were of reagent grade; other chemicals were of analytical grade. The kits (superoxide dismutase [SOD], A001-3; catalase [CAT], A007-1) used to measure SOD and CAT were purchased from the Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China. Ginkgolic acid standard was purchased from Shanghai Tauto Biotech Co., Shanghai, China.

Preparation of ginkgolic acid

Ginkgo fruits (*G. biloba* L. cv. 'Luanguofoshou') were harvested at a mature stage from a special garden base in Dalian, Liaoning, China (longitude: $121^\circ 57'$, latitude: $38^\circ 95'$). All rotten or visibly damaged fruits were removed to obtain seeds; then, the testae were air-dried at room temperature. We then washed the *G. biloba* testa, dried it to a constant weight, crushed it and sifted it. Crude ginkgolic acid was extracted according to the method reported by Hausen (1998). Briefly, the powder was reflux-extracted with 80% ethanol (solid-liquid ratio of 1:20) at 80°C for 3 h. Then, the mixture was centrifuged; the supernatant was concentrated and freeze-dried to obtain crude ginkgolic acid.

Crude ginkgolic acid was purified using AB-8 macroporous resin and silica gel column chromatography, followed by analysis using high-performance liquid chromatography (HPLC). In brief, ginkgolic acid standard and purified ginkgolic acid (5 mg) were dissolved separately in anhydrous methanol (1 mL) and filtered through a $0.2 \mu\text{m}$ syringe filter. The ginkgolic acid was analysed by HPLC (2695E; Waters, Massachusetts, USA) using a C18 chromatographic column ($4.6 \text{ mm} \times 250 \text{ mm}$, $5 \mu\text{m}$) (Agilent, California, USA), a method described by Lei et al. (2014) with slight modifications. Various concentrations of ginkgolic acid were prepared before the following experiments.

Infrared spectroscopy

The purified ginkgolic acid sample was mixed with KBr under vacuum, ground and pressed into tablets, and the infrared spectrum was scanned in the range of $4,000\text{--}400 \text{ cm}^{-1}$.

Fungistatic rate measurement

Fungistatic rate was measured by radial growth assay with slight modifications (Tan et al., 2017). A medium containing mycelium with a diameter of 5 mm was taken from the same circumference of the edge of the pre-cultured colony by a puncher. Various concentrations of ginkgolic acid were added to the medium ($2 \text{ mL} \cdot 100 \text{ mL}^{-1}$ medium). Then, the colony was cultured in PDA medium with ginkgolic acid ($0 \text{ mg} \cdot \text{mL}^{-1}$, $2.5 \text{ mg} \cdot \text{mL}^{-1}$, $5 \text{ mg} \cdot \text{mL}^{-1}$, $10 \text{ mg} \cdot \text{mL}^{-1}$) at 30°C ; ginkgolic acid showed an inhibitory effect in this range. Ginkgolic acid was dissolved in 80% ethanol; so the trial with 80% ethanol instead of ginkgolic acid was used as a control. When the colony in the control group grew close to the plate wall, the diameter of a single colony was measured, and the fungistatic rate was calculated according to the following equation:

$$\text{Fungistatic rate (\%)} = \left[(D_1 - D_2) / D_1 \right] \times 100\%, \quad (1)$$

where D_1 was the increment of colony diameter in the control group; D_2 was the increment of colony diameter in the treatment group.

Morphological observation of the cell membrane of *N. oryzae*

The effect of ginkgolic acid on the cell membrane of *N. oryzae* was investigated using an optical microscope. *N. oryzae* was inoculated in potato dextrose liquid medium with or without ginkgolic acid treatment. It was then placed under an optical microscope (10 × 40) for observation.

Pre-culture of *N. oryzae*

N. oryzae was inoculated in potato dextrose liquid medium and cultured at 28 °C for 3 d on a rotary shaker at 150×g. Mycelium (3 g; wet weight) was obtained from the above mixture using a sterile inoculator, dried with sterile filter paper and transferred into 100 mL potato dextrose liquid medium. After sub-culturing at 28 °C with shaking at 150×g for 3 d, ginkgolic acid was added and *N. oryzae* was cultured in the presence of ginkgolic acid (5 mg · mL⁻¹) for subsequent experiments. Ethanol (80%) was used in the trial as a control instead of ginkgolic acid.

Effect of ginkgolic acid on the membrane permeability of *N. oryzae*

The mycelium of *N. oryzae* was inoculated into 100 mL liquid medium, to which 5 mg · mL⁻¹ ginkgolic acid solution was added. *N. oryzae* was cultured in shake flasks (150×g) at 28 °C for 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 30 h, 48 h and 55 h in the media supplemented with ginkgolic acid. The conductivity of the medium was measured using a conductivity meter (DDS-307A; Yoke Instrument Co., Shanghai, China) to analyse the changing trend of cell membrane permeability (Xu et al., 2003).

Effect of ginkgolic acid on the mycelial protein content of *N. oryzae*

At 0 h, 3 h, 6 h, 9 h, 12 h, 24 h and 48 h, respectively, 1 g of mycelium was obtained from the mixed culture in which ginkgolic acid was added. The mycelium was thoroughly ground in a mortar containing a small amount of quartz sand and phosphate buffer saline (PBS), and the mixture was collected and put into a centrifuge tube. After centrifugation at a speed of 4,000×g for 10 min, the supernatant was transferred into a colorimetric tube whose specification was 10 mL, diluted with PBS to the specified scale and mixed well.

We then put 1 mL of the diluted solution into a test tube and added 4 mL of Coomassie brilliant blue G-250 reagent. The mixture was allowed to react for 2 min and then the absorbance was measured at 595 nm (Asryants et al., 1985).

Effect of ginkgolic acid on the SOD and CAT of *N. oryzae*

Mycelium (1 g) was obtained from the liquid medium of *N. oryzae* treated with ginkgolic acid for 0 h, 3 h, 6 h, 9 h, 12 h, 24 h and 48 h. After the mycelium was thoroughly ground, the activities of SOD and CAT were

measured using the enzyme-linked immunosorbent assay (ELISA) test kit.

The enzyme activity of SOD is calculated as the amount of enzyme that inhibits the auto-oxidation rate of hydroquinone up to 50% per minute in 1 mL of the reaction liquid. The unit enzyme activity of CAT is defined as the amount of 1 μmol H₂O₂ decomposed per gram of sample per second.

Inhibitory activity of ginkgolic acid on *E. aureum* infected by *N. oryzae*

E. aureum (Family: Araceae) with green leaves (>6 cm in diameter) was cultivated as an ornamental plant, which was planted in the plantation of the Dalian Polytechnic University for >3 years.

Spore suspensions were prepared by scraping spores from PDA cultures of *N. oryzae* using a sterile inoculator and suspending them in sterile distilled water. Spore concentrations were adjusted to 10⁶ colony-forming units (CFU) · mL⁻¹ assisted by the haemocytometer.

Leaves of similar size and no obvious mechanical damage were selected from healthy *E. aureum* and disinfected with 0.1% sodium hypochlorite for 1 min, washed with sterile water and cultured at room temperature (20 °C).

A sterilised scalpel was used to make a uniform wound (1 mm × 1 mm) in the same position on each blade. The wound site was inoculated with 10 μL sterile distilled water or *N. oryzae* spore suspension (10⁶ CFU · mL⁻¹). After 3 d of incubation at room temperature, 20 μL sterile distilled water or ginkgolic acid (5 mg · mL⁻¹) was inoculated at the wound site. Leaves were cultured at room temperature for 20 d after being treated as described.

Data analysis

In this paper, all experiments were performed in triplicate. The obtained data were subjected to one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS, version 16.0, Chicago, USA); *p* < 0.05 was regarded as statistically significant.

RESULTS

HPLC analysis of ginkgolic acid

The HPLC spectrum of ginkgolic acid was analysed. The results revealed that the purity of ginkgolic acid was 69.18% and it was composed of five ginkgolic acids, that is, C13:0, C15:1, C17:2, C15:0 and C17:1, and their derivatives. The peak area of purified ginkgolic acid was 710.74 mAU*s. The content of C15:1 was the highest, accounting for 38.53% of the purified substance and 55.70% of the total purified ginkgolic acid (Figure 1).

Infrared spectroscopy

The infrared spectrum of ginkgolic acid was determined by the KBr tablet method; the results are shown in Figure 2, and the main characteristic peaks are assigned in Table 1. It can be seen from the spectrum that there

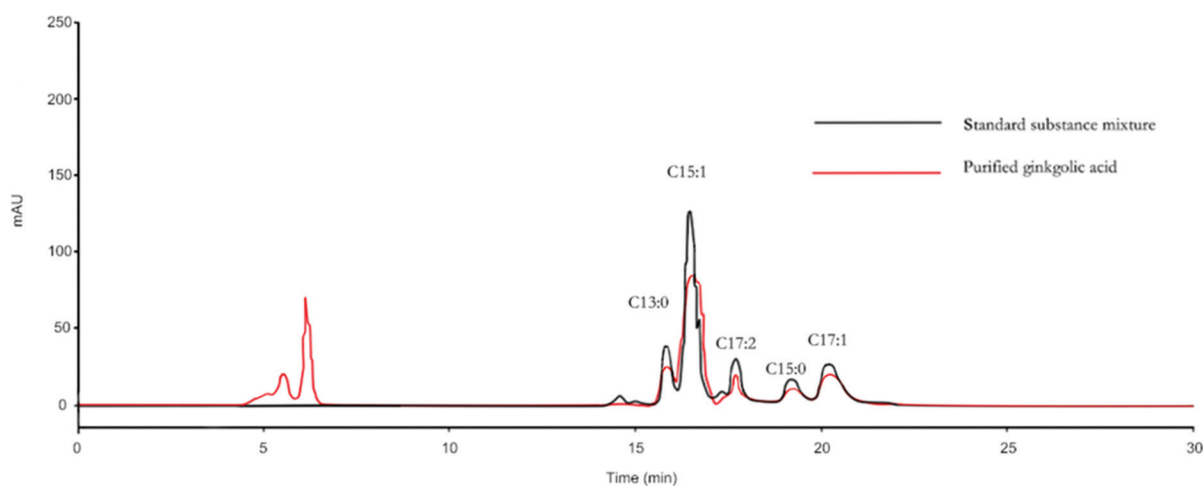


Figure 1. HPLC chromatogram of the standard substance mixture and purified ginkgolic acid. Standard samples are indicated by the black line and by the red line for purified ginkgolic acid by the red line. HPLC, high-performance liquid chromatography.

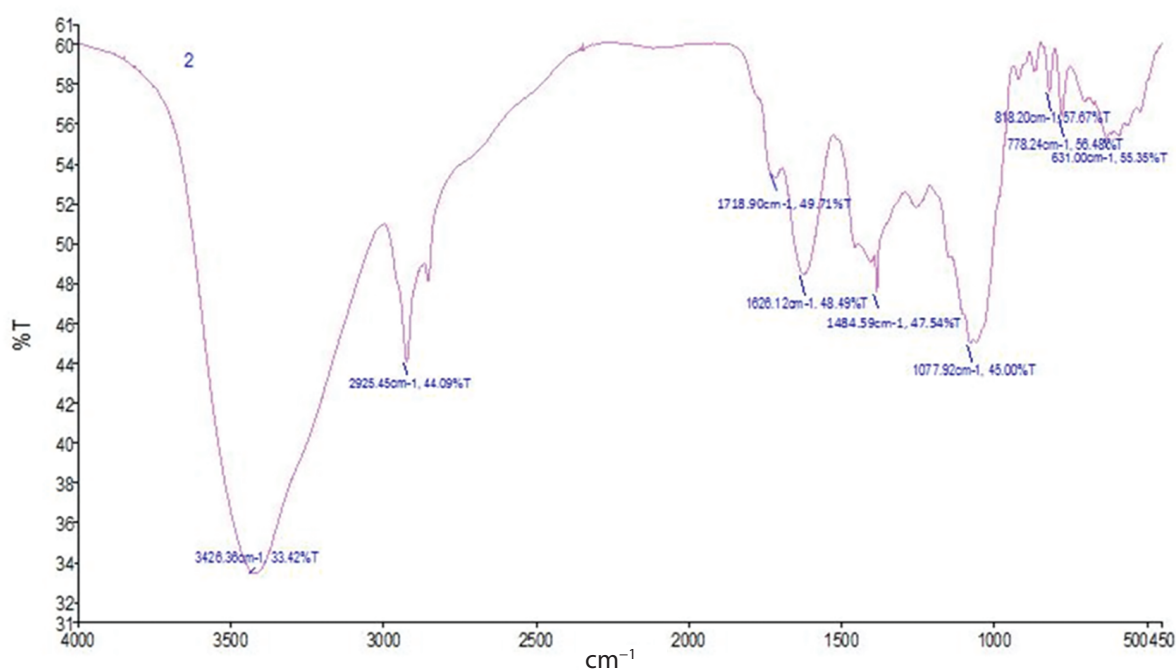


Figure 2. Infrared spectrum of ginkgolic acid.

were multiple absorption peaks at $3,426.36\text{ cm}^{-1}$, $2,925.45\text{ cm}^{-1}$, $1,718.90\text{ cm}^{-1}$, $1,626.12\text{ cm}^{-1}$, $1,484.59\text{ cm}^{-1}$, $1,077.92\text{ cm}^{-1}$, 818.20 cm^{-1} and 778.24 cm^{-1} .

The inhibitory effect of ginkgolic acid on three pathogenic fungi

The experiment results showed that various concentrations of ginkgolic acid had different inhibitory effects on the pathogens. With an increase in concentration, ginkgolic acid showed a similar inhibitory trend to *N. oryzae* and *A. alternata*; the inhibition was the strongest at the concentration of $5\text{ mg} \cdot \text{mL}^{-1}$, and the inhibition rates against *N. oryzae* and *A. alternata* reached

65.82% and 56.96%, respectively. In addition, it exhibited the highest inhibitory activity against *N. oryzae* and no significant inhibitory effect on *P. expansum* (Table 2). So, further research on the effect of ginkgolic acid against *N. oryzae* was conducted in the study.

Therefore, according to the results from Figure 3, the inhibitory action of ginkgolic acid at a concentration of $5\text{ mg} \cdot \text{mL}^{-1}$ against *N. oryzae* was studied.

Effects of ginkgolic acid on N. oryzae cell membrane

The untreated *N. oryzae* showed an intact cell membrane structure when observed under an optical microscope

Table 1. The wavenumber of ginkgolic acid.

Wavenumber, cm ⁻¹	Main characteristic peak assignment	The group
3,426.36	O–H stretching vibration	–OH
2,925.45	C–H stretching vibration	–CH ₃
1,718.90	C=O stretching vibration	–C=O
1,626.12	Aromatic ring –C=C stretching vibration	Benzene ring
1,484.59	Aromatic ring –C=C stretching vibration	Benzene ring
1,077.92	C–O stretching vibration	–C–OH
818.20	Aromatic ring =C–H plane bending vibration	–C–H
778.24	Aromatic ring =C–H plane bending vibration	–C–H

Table 2. Inhibition of fungi by ginkgolic acid.

Species	Control	Concentration of ginkgolic acid		
		2.5 mg · mL ⁻¹	5 mg · mL ⁻¹	10 mg · mL ⁻¹
		Fungistatic rate, %		
<i>Nigrospora oryzae</i>	0.792 ± 0.023	62.032 ± 0.056	65.822 ± 0.032	65.291 ± 0.028
<i>Penicillium expansum</i>	2.883 ± 0.021	2.891 ± 0.048	3.041 ± 0.043	3.044 ± 0.031
<i>Alternaria alternata</i>	1.271 ± 0.019	49.374 ± 0.052	56.963 ± 0.029	55.563 ± 0.052

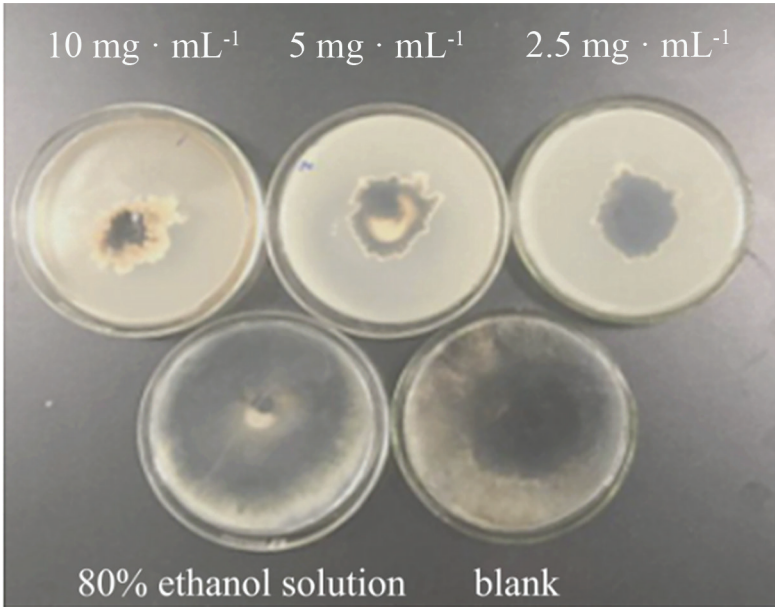


Figure 3. Growth status of *Nigrospora oryzae* on PDA medium with 80% ethanol and different concentrations of ginkgolic acid (0 mg · mL⁻¹, 2.5 mg · mL⁻¹, 5 mg · mL⁻¹ and 10 mg · mL⁻¹). PDA, potato dextrose agar.

(Figure 4A). However, after being treated with ginkgolic acid, a blurred cell membrane and intracellular material leakage were observed under the optical microscope (Figure 4B).

Effect of ginkgolic acid on the electrical conductivity of *N. oryzae*

The extracellular conductivity was measured to evaluate the cell membrane permeability of *N. oryzae*. The effect of ginkgolic acid on the electrical conductivity of *N. oryzae* is shown in Figure 5A. After adding ginkgolic acid, the conductivity of the sample increased significantly between 0 h and 2 h, decreased slightly

after 6 h and then stabilised at a high level. Electrical conductivity of the control group showed a general downward trend; although it rose during the period of 0–4 h and reached the peak at the fourth hour, it never exceeded that of the treatment group.

Effect of ginkgolic acid on the mycelial protein content of *N. oryzae*

For the treatment group, the protein content in the mycelia fell significantly between 0 h and 12 h and then remained stable at an extremely low level. However, in the control group, the protein content in *N. oryzae* increased slightly within 6 h of co-culture, and there

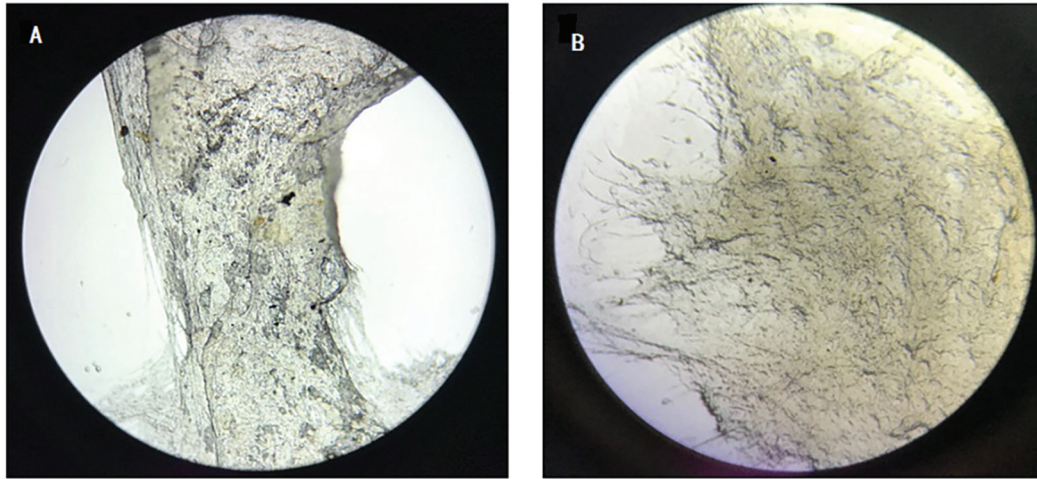


Figure 4. Optical microscopic images (10×40) of cell membranes of *Nigrospora oryzae* cultured (A) without and (B) with ginkgolic acid.

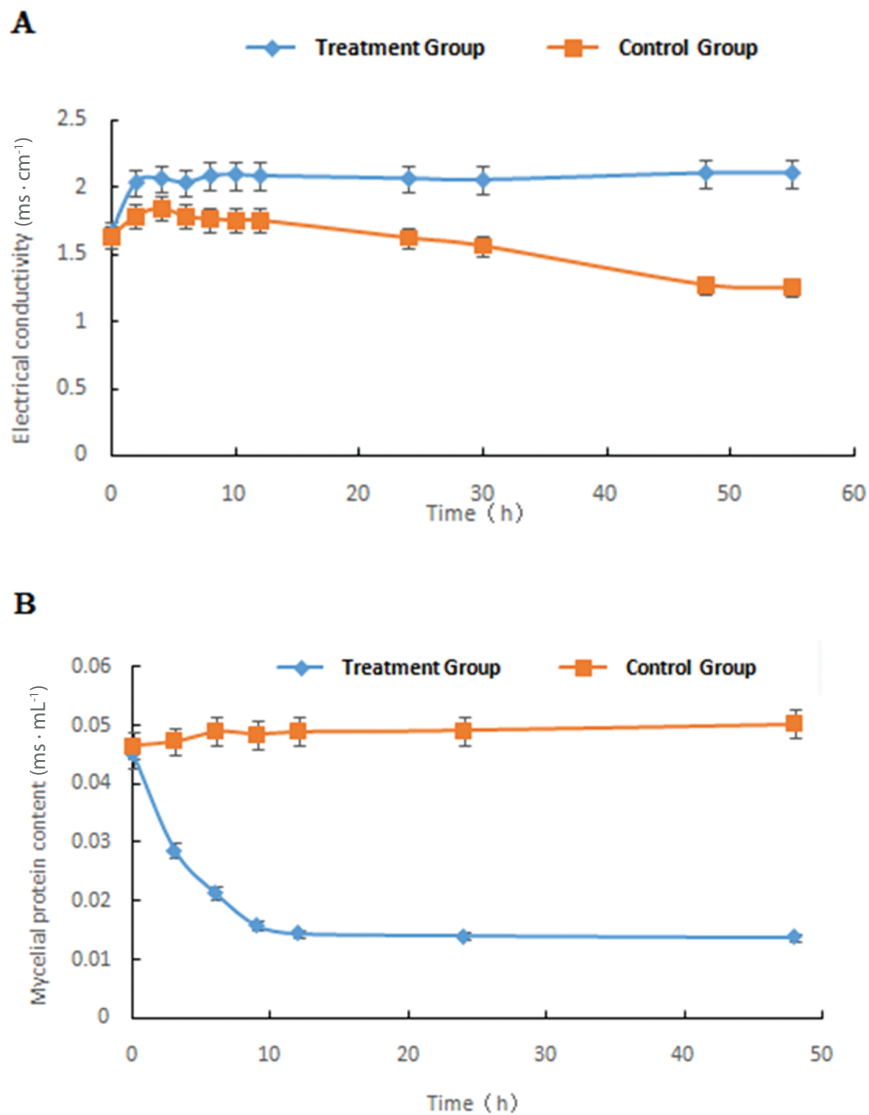


Figure 5. Effect of ginkgolic acid on electrical conductivity (A) and mycelial protein (B) of *Nigrospora oryzae*. Vertical lines represent standard errors of three replicates. Statistical analysis was performed using *t*-test at $p < 0.05$.

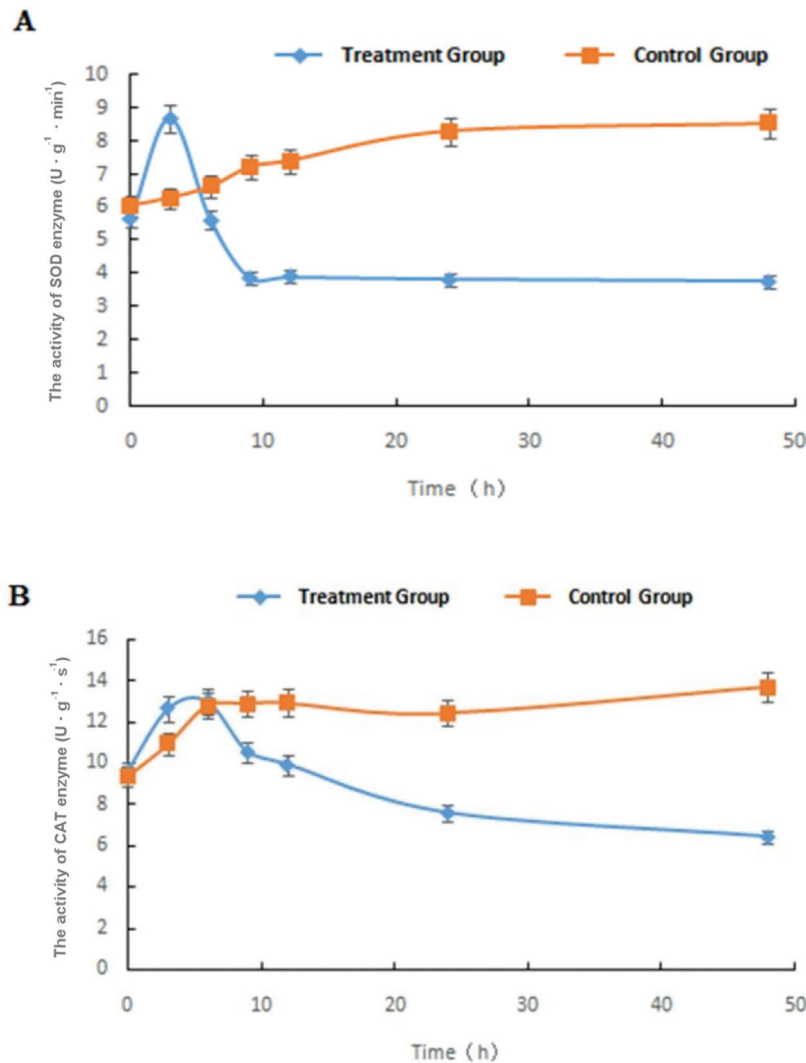


Figure 6. Effect of ginkgolic acid on the activities of (A) SOD and (B) CAT in *Nigrospora oryzae*. Each value is the mean of three experiments. Bars represent the standard error of the mean. Statistical analysis was performed using *t*-test at $p < 0.05$. CAT, catalase; SOD, superoxide dismutase.

was no significant change after 6 h. In addition, the protein content in the treatment group was much lower than that in the control group (Figure 5B).

Effect of ginkgolic acid on the activity of SOD and CAT in *N. oryzae*

As shown in Figure 6A, the activity of SOD in the treatment group first rose sharply from 0 h to 3 h, then declined rapidly until 10 h and finally, it remained stable. In the control group, the overall trend of enzyme activity was up slightly. After treatment for 6 h, SOD activity in the treatment group was significantly lower than that in the control group.

Effect of ginkgolic acid on the activity of CAT in *N. oryzae* is shown in Figure 6B. CAT activity increased significantly from 0 h to 3 h and peaked at the sixth hour, then decreased rapidly between 6 h and 9 h and finally dropped slowly. In the control group, CAT almost remained at the same level after rising, and CAT activity

was significantly higher than that in the treatment group after 6 h.

Inhibition of *N. oryzae* growth on the leaves of *E. aureum* by ginkgolic acid

Leaves of *E. aureum* were infected by *N. oryzae* and cultured at room temperature for 20 d (Figure 7). The results showed that ginkgolic acid slowed down the disease symptoms. The degree of disease on the leaves is shown in Figure 8. After infection with *N. oryzae* spore suspension, leaves treated with sterile distilled water had sub-circular brown spots around the wounds with black dotted grains around the spot margins, while leaves treated with ginkgolic acid showed significantly less morbidity and no black dotted grains around the spot margins. Similarly, after mechanical damage, leaves treated with sterile distilled water showed slight lesions around the wound. On the other hand, the wound healed well after treatment with ginkgolic acid.

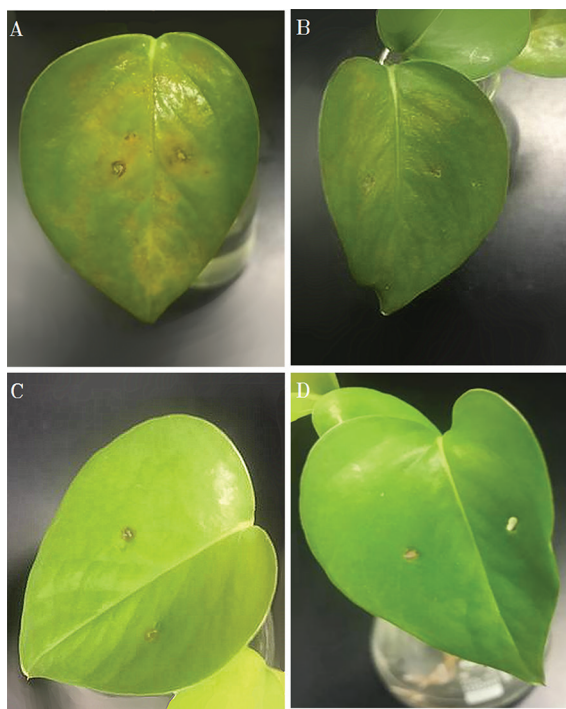


Figure 7. Protection of leaves by ginkgolic acid. (A) treatment with sterile distilled water after *Nigrospora oryzae* inoculation; (B) treatment with ginkgolic acid after *N. oryzae* inoculation; (C) treatment with sterile distilled water after inoculation with sterile distilled water; (D) treatment with ginkgolic acid after inoculation with sterile distilled water. The concentration of spore suspension used for inoculation was 10^6 CFU $\cdot$ mL $^{-1}$, and the concentration of ginkgolic acid was 5 mg $\cdot$ mL $^{-1}$. CFU, colony-forming units.

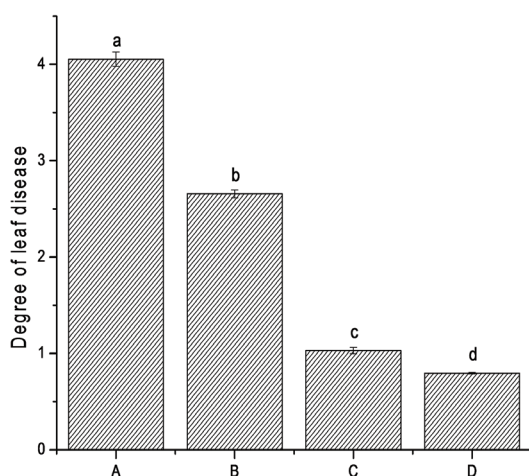


Figure 8. Degree of leaf disease: (A) treatment with sterile distilled water after *Nigrospora oryzae* inoculation; (B) treatment with ginkgolic acid after *N. oryzae* inoculation; (C) treatment with sterile distilled water after inoculation with sterile distilled water; (D) treatment with ginkgolic acid after inoculation with sterile distilled water. The concentration of spore suspension used for inoculation was 10^6 CFU $\cdot$ mL $^{-1}$, and the concentration of ginkgolic acid was 5 mg $\cdot$ mL $^{-1}$. CFU, colony-forming units.

DISCUSSION

AB-8 macroporous resin has been widely used in the purification of active substances in plants due to its excellent selectivity in the adsorption of some weak polar organic molecules in the solution system (Gao et al., 2013; Zhao et al., 2013). Silica gel chromatography can separate substances according to their polarity and has been used in the isolation of a large number of compounds (Zhao et al., 2016). It has been reported that among the constituents in the alcohol extracts of the testa of *G. biloba*, the main weak polar compounds with bioactivity are ginkgolic acid, ginkgolic flavonoid and ginkgolide (Zhao, 2013; Wang and Zhang, 2019). Ginkgolides have no obvious inhibitory effect on fungi, and ginkgolic flavonoids can only inhibit the growth of fungi at a very high concentration (≥ 70 mg $\cdot$ mL $^{-1}$); so they do not have an impact on this experiment (Fan, 2017).

From the spectrum chart, it can be seen that there are absorbers at 1,626.12 cm $^{-1}$ and 1,484.59 cm $^{-1}$, which are characteristic peaks of the vibration of the aromatic ring skeleton. Combined with the absorption peaks at 818.20 cm $^{-1}$ and 778.24 cm $^{-1}$ in the fingerprint region, it can be basically determined that there is an aromatic ring in the molecule. The absorption peaks at 2,925.45 cm $^{-1}$ prove the presence of alkyl groups in the molecule. The absorption peak at 3,426.36 cm $^{-1}$ proves the existence of phenolic hydroxyl groups in the molecule. The absorption peak at 1,718.90 cm $^{-1}$ proves the existence of carbonyl groups. However, most of the functional groups generally move to low wavenumbers, which is caused by the conjugation effect of (i) the carbonyl group in the carboxyl group directly connected to the benzene ring and (ii) the molecular hydrogen bond (Swamy et al., 2007).

Ginkgolic acid has been verified to have inhibitory activities on Gram-positive bacteria (Yang et al., 2004a, 2004b). In addition, a literature survey revealed that ginkgolic acid exhibited an inhibitory effect on many common pathogenic fungi infecting vegetables (Lin et al., 2010). Similarly, in the current study, ginkgolic acid inhibited the growth and reproduction of *N. oryzae*, *A. alternata* and *P. expansum* to different degrees. The radial growth assay is a commonly used method to test the inhibition of fungal substances (Cruz et al., 2019).

During the growth phase, microorganisms use nutrients, such as mineral salt, in the culture medium. In general, the conductivity of the medium will decline with the extension of microbial culture time. However, when a microbe is destroyed, the permeability of the cell membrane will increase, and small molecules such as inorganic salts in the cells leak out, resulting in an increase in the conductivity of the liquid medium. Therefore, the change of cell membrane permeability can be evaluated by the conductivity in the culture medium (Shen et al., 2015). As shown in Figure 5A, the conductivity of the treatment group with ginkgolic acid increased significantly from 0 h to 2 h, indicating

that ginkgolic acid damaged *N. oryzae* and increased its membrane permeability. The reason why conductivity declined slightly at the sixth hour was that the activity of CAT reached its peak at this time point (as described later on). It caused the stress reaction in *N. oryzae* and protected the damaged cell membrane. And then the conductivity tended to be stable. It indicated that under the action of ginkgolic acid, the growth metabolism of *N. oryzae* became slow or stopped, resulting in no change of the conductivity, and no utilisation of inorganic salt ions and other nutrients in the culture medium (Pavlin et al., 2005).

The stability of the cell membrane is mainly related to the hydrophobic interaction. After the hydrophobic core in the membrane structure is destroyed, the polarity of this region will be enhanced, and the osmotic balance of polar molecules will be disturbed, leading to the loss of membrane integrity. Previous reports have demonstrated that some compounds that inhibit microbial growth, such as antimicrobial peptides, interact with the hydrophobic groups in the lipid bilayer of the pathogen's cell membrane to exert their antibacterial activity (Oren et al., 1997; Yan et al., 2003). Ginkgolic acid is a small hydrophobic molecule. In this study, the cell membrane of *N. oryzae* treated with ginkgolic acid was ruptured. Our hypothesis is that ginkgolic acid penetrated the cell wall of *N. oryzae* and interacted with the hydrophobic groups on the cell membrane, thereby damaging the cell membrane structure. So the interaction between ginkgolic acid and hydrophobic groups will be studied further.

The intracellular protein content of the treatment group decreased, implying that ginkgolic acid could cause a large amount of protein leakage out of the mycelia or directly lead to mycelial protein degeneration. Furthermore, ethanol, a common solution used for disinfection because it dehydrates microbial proteins, had no obvious effect on the mycelial protein content of *N. oryzae* at low doses. After treatment for 12 h, the change in protein content tended to be stable. It could be inferred as mycelium protein denaturation or mass leakage, leading to the death of the fungus. Studies have shown that antifungal substances acting on the cell membrane can cause protein leakage in the mycelium (Kumari et al., 1994; Zhang et al., 2011). According to the above results, we deduced that the inhibitory effect of ginkgolic acid on *N. oryzae* was related to the destruction of the cell membrane, resulting in osmotic imbalance, leading to the leakage of cell components and, ultimately, to cell death.

SOD catalyses the dismutation of superoxide anion free radicals, thus eliminating harmful substances in the metabolic process and protecting the microorganism from damage (Powers et al., 2008; Zhang et al., 2011). From 0 h to 3 h, *N. oryzae* in the treatment group responded to the stress of ginkgolic acid and increased the activity of SOD to protect the biome. After 3 h, the enzyme activity began to decline.

According to the results shown in Figure 6A, it can be inferred that ginkgolic acid destroyed the biomembrane integrity, increased its permeability and caused the leaking out of a large amount of proteins and other substances from the cell. This resulted in the weakened resistance to external adversity and the weakened activity of SOD in *N. oryzae* (Ron, 2002).

CAT can eliminate H_2O_2 , which is generated in a disproportion reaction, and eventually produces H_2O and O_2 , thus protecting cells from damage. It is one of the key enzymes of the biological defence system (Ortiz et al., 2013).

At the beginning, in order to protect microorganisms under stress, the activity of CAT showed an upward trend and reached its peak at the sixth hour. Subsequently, because of the serious cell damage in the treatment group, the CAT activity of *N. oryzae* in the treatment group began to decrease. The loss of enzyme activity resulted in the situation that H_2O_2 produced in the disproportionation reaction could not be completely and effectively eliminated. It proved that ginkgolic acid had an inhibitory effect on *N. oryzae*. However, low doses of ethanol could not decrease the activity of CAT or effectively inhibit the growth of *N. oryzae*.

Ginkgolic acid effectively reduced damage in leaves infected by *N. oryzae*. So, ginkgolic acid can protect the leaves from infection by *N. oryzae* and facilitate the growth of plants. The inhibitory trend of ginkgolic acid on *N. oryzae* was similar in both leaf and agar plate. The difference in details is due to the conditions on the leaf being different from those on the agar plate (Zhao et al., 2012). Similarly, it was reported by Zhao (2013) that the extracts of *G. biloba* testa affected cabbage seed germination, promoting growth at low concentrations and inhibiting growth at high concentrations.

CONCLUSION

In this study, the antifungal action of ginkgolic acid on *N. oryzae*, *A. alternata* and *P. expansum* was investigated under *in vitro* conditions. Ginkgolic acid showed different inhibitory effects on these three pathogens and had the strongest inhibitory activities on *N. oryzae*. Ginkgolic acid increased membrane permeability by destroying the cell membrane of *N. oryzae*, which then led to leakage of mycelial proteins and other contents and, ultimately, *N. oryzae* cell death. Ginkgolic acid also reduced the activity of SOD and CAT in *N. oryzae*, thereby slowing the fungal infection. Moreover, ginkgolic acid showed a protective effect on the leaves of *E. aureum* after infection by *N. oryzae*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work. J.L. performed the experiments, prepared the original draft of the manuscript and revised the manuscript. J.C. analysed the data statistically and helped in writing the manuscript. S.Y. supervised the experiments and helped in writing, reviewing and editing. Y.D. supervised the experiments. S.G. performed the experiments, collected data and applied statistical analysis. N.F. performed the experiments, collected data and applied statistical analysis.

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